# STUDIES IN HUMAN GENETICS AND CYTOGENETICS

by

Grant Robert SUTHERLAND, B.Sc., M.Sc.(Melb.), Ph.D.(Edinburgh)

Doctor of Science
University of Edinburgh 1983

TABLE OF CONTENTS

# I DECLARATION

I declare that the publications contained in this thesis have not been previously submitted for any higher degree except where clearly indicated. The work contained in the Publications was carried out by me or under my immediate supervision except where indicated under the Explanations re Joint Authorship and in the Acknowledgements to each publication.

Grant Robert Sutherland

Adelaide, June 1983

## II ABSTRACT

This thesis consists of 85 publications, of which 21 have been submitted for other degrees and are only included for completeness, leaving 64 assessable works. These fall into four broad categories, population cytogenetics, clinical genetics and cytogenetics, studies on amniotic fluid and prenatal diagnosis, and studies of heritable fragile sites on human chromosomes.

The section on population cytogenetics includes most of the Australian studies on XYY males, epidemiological studies on Down syndrome in Australia and studies on the cytogenetics of paediatric necropsies. The clinical cytogenetics section mainly contains clinical case reports, which include a description of one of the first recognised insertional translocations in man, an important paper on trisomy 9 and one of the first discussions of genetic counselling of pericentric inversion carriers. This section also includes papers on gene mapping, alpha-1-antitrypsin phenotypes in chromosome abnormalities with descriptions of a new alpha-1-antitrypsin allele and studies on sister chromatid exchange in various groups of individuals with the documentation of an increase in this phenomenon in patients with multiple sclerosis.

The section on prenatal diagnosis includes studies of the enzymology of amniotic fluid and cultured amniotic fluid cells, the discovery of rapidly adhering cells in amniotic fluid and documentation of their increased numbers in amniotic fluid surrounding fetuses with neural tube defects, and studies of chromosomal mosaicism in cultured amniotic fluid cells.

The most important section of this thesis is the final one on studies of heritable fragile sites on human chromosomes. This section documents the discovery of the tissue culture requirements for the expression of fragile sites in lymphocyte culture, the finding of several new folate sensitive fragile sites and the co-discovery of the BrdU requiring fragile site at 10q25. Contributions to establishing fragile X-linked mental retardation as the second commonest genetic cause of mental retardation after Down syndrome, and population cytogenetic data for fragile sites are presented. Genetic linkage studies with fragile sites have established that a fragile site is coded for at the locus of the fragile site. Micronucleus studies have suggested that the folate sensitive fragile sites might be special examples of chromosome damage due to deprivation of DNA precursors.

## III THE SUBMISSION

This submission includes the major published works of the candidate. Published abstracts of papers presented to conferences have not been included nor have minor comments published as letters to editors. Two sections of the submission contain papers included in theses submitted for the degrees of M.Sc. in the University of Melbourne and Ph.D. in the University of Edinburgh. These papers are included for completeness but are not an assessable part of this submission. There are thus 64 papers which form the examinable portion of this thesis. They are divided into four sections but there is considerable overlap and assignment of some papers to any particular section is arbitrary.

The four sections are :

1. Population Cytogenetics
2. Clinical Genetics and Cytogenetics
3. Studies of Amniotic Fluid and Prenatal Diagnosis
4. Fragile Sites on Human Chromosomes

# IV LIST OF PUBLICATIONS

## A. Population Cytogenetics

(1) XYY males in a Melbourne prison. *Lancet,* (1968), *i,* 150
Wiener, S., Sutherland, G.R., Bartholomew, A.A., Hudson, B.

(2) A normal XYY man. *Lancet,* (1968), *ii,* 1352.
Wiener, S., Sutherland, G.R.

(3) A murderer with 47,XYY and an additional autosomal abnormality.
*Aust. N.Z. J. Criminol.,* (1969), *2,* 20.
Wiener, S., Sutherland, G.R., Bartholomew, A.A.

(4) A defence of insanity and the extra Y chromosome: R v Hannell.
*Aust. N.Z. J. Criminol.,* (1969), *2,* 29.
Bartholomew, A.A., Sutherland, G.R.

(5) Alcoholism, drug dependency and sex chromosome abnormalities.
*Med. J. Aust.,* (1969), *2,* 440. Bartholomew, A.A., Sutherland, G.R.

(6) Chromosome survey in a security ward: Total ascertainment.
*Aust. N.Z. J. Criminol.,* (1970), *3,* 99. Sutherland, G.R.
Bartholomew, A.A.

(7)* Chromosome abnormalities in newborn babies. *Aust. J. Ment. Ret.,*
(1970), *1,* 77. Sutherland, G.R.

(8) Chromosome survey in a mental deficiency security ward:
Total ascertainment. *Aust. N.Z. J. Criminol.,* (1971), *4,* 82.
Sutherland, G.R., Bartholomew, A.A.

9 Chromosome studies in a mental deficiency hospital:
Total ascertainment. *Aust. J. Ment. Ret.,* (1971), *1,* 246.
Sutherland, G.R., Wiener, S.

10 XYY males in Victoria. *Med. J. Aust.*, (1972) *1*, 1249. Sutherland, G.R., Wiener, S., Bartholomew, A.A., Fitzgerald, M.G.

11 Cytogenetics of 271 Mongols. *Aust. Paediat. J.*, (1972), *8*, 90. Sutherland, G.R., Wiener, S.

12 Chromosome abnormality and perinatal death. *Lancet*, (1974), *i*, 752. Sutherland, G.R., Bauld, R., Bain, A.D.

13 Chromosome studies in investigation of stillbirths and neonatal deaths. *Arch. Dis. Child.*, (1974) *49*, 782. Bauld, R., Sutherland, G.R., Bain, A.D.

14 Cytogenetic survey of a hospital for the mentally retarded. *Hum. Genet.*, (1976), *34*, 231. Sutherland, G.R., Murch, A.R., Gardiner, A.J., Carter, R.F., Wiseman, C.

15 Chromosome studies at the paediatric necropsy. *Ann. Hum. Genet. (Lond.)*, (1978), *42*, 173. Sutherland, G.R. Carter, R.F., Bauld, R., Smith, I.I., Bain, A.D.

16 Down's syndrome in South Australia. *Med. J. Aust.*, (1979), *2*, Sutherland, G.R., Clisby, S.R., Bloor, G., Carter, R.F.

17 Distribution of $\alpha_1$-antitrypsin (PI) phenotypes in chromosome abnormalities. *Hum. Genet.*, (1981), *57*, 176. Mulley, J.C. Sutherland, G.R.

18 Cytogenetic studies : An essential part of the paediatric necropsy. *J. Clin. Path.*, (1983), *36*, 140. Sutherland, G.R., Carter, R.F.

B. Clinical Genetics and Cytogenetics

19 Six cases of the Cri-du-chat syndrome. *Aust. J. Ment. Ret.*, (1971), *1*, 239. Flynn, H., Carmichael, A., Sutherland, G.R.

20 A male with karyotype 46,XX. *Ann. Génét.*, (1972), *15*, 187. Sutherland, G.R. Wiener, S., Bartholomew, A.A.

21 Difficulty in showing mosaicism in the mother of three mongols. *Arch. Dis. Child.*, (1972), *47*, 970. Sutherland, G.R. Fitzgerald, M.G., Danks, D.M.

22 Familial insertional translocation. *Lancet*, (1972), *ii*, 231. Grace, E., Sutherland, G.R., Bain, A.D.

23 Partial trisomy of 7q resulting from a familial translocation. *Ann. Génét.*, (1973), *16*, 51. Grace, E., Sutherland, G.R. Stark, G.D., Bain, A.D.

24 A girl with Wolf-Hirschhorn syndrome and mosaicism, 46,XX/46,XX,4p-. *J. Ment. Defic. Res.*, (1974), *18*, 79. Judge, C., Garson, O.M., Pitt, D.B., Sutherland, G.R.

25 Partial and complete trisomy 9: Delineation of a trisomy 9 syndrome. *Hum. Genet.*, (1976), *32*, 133. Sutherland, G.R. Carter, R.F., Morris, L.L.

26 Familial pericentric inversion of chromosome 19, inv(19)(p13q13) with a note on genetic counselling of pericentric inversion carriers. *Clin. Genet.*, (1976), *10*, 54. Sutherland, G.R., Gardiner, A.J., Carter, R.F.

27 46,XX/46,XX,r(2)(p25q37) mosaicism: Clinical and cytogenetic studies. *Ann. Génét.*, (1978), *21*, 164. Sutherland, G.R., Carter, R.F.

28 46,XY females. *Rec. Adel. Child. Hosp.*, (1978), *2*, 73. Sutherland, G.R., Binns, G.F., Carter, R.F., Dow, R.

29 Picture of the Month. Fetal Turner Syndrome. *Am. J. Dis. Child.*, (1978), *132*, 417. Sutherland, G.R., Rogers, J.G.

30 Genetic counselling for neurological disorders. *Aust. Fam. Physician*, (1979), *8*, 1265. Sutherland, G.R.

31 Increased sister chromatid exchange in multiple sclerosis. *New Engl. J. Med.*, (1980), *303*, 1126. Sutherland, G.R., Baker, E., Seshadri, R.S., Black, A.

32 Additions to the exclusion map of man. *Ann. Genet.*, (1980), *23*, 198. Mulley, J.C., Bryant, G.D., Sutherland, G.R.

33 Sister chromatid exchange in aplastic anaemia. *Cancer Genet. Cytogenet.*, (1981), *3*, 81. Seshadri, R., Baker, E., Leonard, P., Sutherland, G.R., Morley, A.A.

34 Two unusual G-band variants of the short arm of chromosome 9. *Clin. Genet.*, (1981), *19*, 331. Sutherland, G.R., Eyre, H.

35 A large kindred with an inv(3)(p25q23): Clinical, cytogenetic and genetic marker studies. *Ann. Génét.*, (1981), *24*, 202. Sutherland, G.R., Mulley, J.C., Goldblatt, E.

36 Sister-chromatid exchange (SCE) analysis in mothers exposed to DNA-damaging agents and their newborn infants. *Mutation Res.*, (1982), *97*, 139. Seshadri, R., Baker, E., Sutherland, G.R.

37 Further exclusions by deletion mapping. *Ann. Génét.*, (1982), *25*, 152. Mulley, J.C., Sutherland, G.R.

38 SCE, X-radiation sensitivity and mutation rate in multiple sclerosis. *Mutation Res.*, (1983), in press. Seshadri, R.S., Sutherland, G.R., Baker, E., Kutlaca, R., Wigmore, D., Morely, A.A.

39 A new allele of $\alpha_1$-antitrypsin. PI.NADELAIDE. *Hum. Genet.*, (1983), *63*, 73. Mulley, J.C., Cox, D.W., Sutherland, G.R.

C. Studies of Amniotic Fluid and Prenatal Diagnosis

[40]* The role of amniocentesis in genetic counselling. *Aust. J. Ment. Ret.*, (1972), *2*, 85. Sutherland, G.R.

[41] Culture of cells from the urine of newborn children. *Nature*, (1972), *239*, 231. Sutherland, G.R., Bain, A.D.

42 Lysosomal enzymes in amniotic fluid. *Clin. Chim. Acta*, (1972), *39*, 275. Butterworth, J., Sutherland, G.R., Bain, A.D., McCrae, W.M.

[43] Metaphase chromosomes from neonatal urine. *Humangenetik*, (1973), *17*, 273. Sutherland, G.R., Grace, E., Bain, A.D.

[44] Lysosomal enzymes of cultured amniotic fluid cells. *Clin. Chim. Acta*, (1973), *44*, 295. Butterworth, J., Sutherland, G.R., Broadhead, D.M., Bain, A.D.

[45] Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. 1. α-Glucosidase and β-Glucosidase. *Life Sciences*, (1973), *13*, 713. Butterworth, J., Sutherland, G.R., Broadhead, D.M., Bain, A.D.

[46] Antenatal diagnosis of inborn errors of metabolism: Tissue culture aspects. *Humangenetik*, (1973), *20*, 251. Sutherland, G.R., Bain, A.D.

[47] The antenatal diagnosis of trisomy 18. *Clinical Genetics*, (1974), *5*, 110. Gordon, G., Sutherland, G.R., Bauld, R., Bain, A.D.

[48] Lysosomal enzyme variations in thirteen cell strains cultured from one amniotic fluid. *Clin. Chim. Acta,* (1974), *52,* 211. Sutherland, G.R., Butterworth, J., Broadhead, D.M., Bain, A.D.

49 Lysosomal enzymes of amniotic fluid in relation to gestational age. *Am. J. Obstet. Gynec.,* (1974), *119,* 821. Butterworth, J., Broadhead, D.M., Sutherland, G.R., Bain, A.D.

[50] Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. II. α-Galactosidase, β-Galactosidase and α-Arabinosidase. *Clin. Genet.,* (1974), *5,* 351. Sutherland, G.R., Butterworth, J., Broadhead, D.M., Bain, A.D.

[51] Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. III. β-Glucuronidase, N-acetyl-β-D-glucosaminidase, α-Mannosidase and acid phosphatase. *Clin. Genet.,* (1974), *5,* 356. Butterworth, J., Sutherland, G.R., Broadhead, D.M., Bain, A.D.

[52] Effect of serum concentration, type of culture medium and pH on lysosomal enzyme activity of cultured human amniotic fluid cells. *Clin. Chim. Acta,* (1974), *53,* 239. Butterworth, J., Sutherland, G.R., Broadhead, D.M., Bain, A.D.

[53] Observations of human amniotic fluid cell strains in serial culture. *J. med. Genet.,* (1974), *11,* 190. Sutherland, G.R., Bauld, R., Bain, A.D.

54 Amniotic fluid macrophages and the antenatal diagnosis of anencephaly and spina bifida. *J. med. Genet.,* (1975), *12,* 135. Sutherland, G.R., Brock, D.J.H., Scrimgeour, J.B.

55 Chromosomal mosaicism in amniotic fluid cell cultures. *Clin. Genet.*, (1975), *7*, 400. Sutherland, G.R., Bowser-Riley, S.M., Bain, A.D.

56 Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. IV. % N-acetyl-β-D-glucosaminidase. *Clin. Genet.*, (1976), *9*, 505. Butterworth, J., Sutherland, G.R., Guy, G.J., Bowser-Riley, S., Bain, A.D.

57 Amniotic-fluid alpha-fetoprotein in Turner syndrome. *Lancet*, (1977), *i*, 649. Sutherland, G.R., Holt, D., Rogers, J.G.

58 * Prenatal diagnosis of chromosome abnormalities. *Rec. Adel. Child. Hosp.*, (1977), *1*, 260. Sutherland, G.R.

D. Fragile Sites on Human Chromosomes

59 Fragile sites on human chromosomes: Demonstration of their dependance on the type of tissue culture medium. *Science,* (1977), *197,* 265. Sutherland, G.R.

60 Marker X chromosomes and mental retardation. *New Engl. J. Med.,* (1977), *296,* 1415. Sutherland, G.R.

61 Carrier detection in X-linked mental retardation. *Med. J. Aust.,* (1978), *2,* 624. Sutherland, G.R.

62 Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. *Am. J. Hum. Genet.,* (1979), *31,* 125. Sutherland, G.R.

63 Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects and cytogenetics. *Am. J. Hum. Genet.,* (1979), *31,* 136. Sutherland, G.R.

64 X-linked mental retardation with macro-orchidism and the fragile site at Xq27 or 28. *Hum. Genet.,* (1979), *48,* 117. Sutherland, G.R., Ashforth, P.L.C.

65 Heritable fragile sites on human chromosomes. III. Detection of fra(X)(q27) in males with X-linked mental retardation and their female relatives. *Hum. Genet.,* (1979), *53,* 23. Sutherland, G.R.

66 Heritable fragile sites on human chromosomes. IV. Silver staining. *Hum. Genet.,* (1979), *53,* 29. Sutherland, G.R., Leonard, P.

67 Heritable fragile sites on human chromosomes.
V. A new class of fragile site requiring BrdU for expression. *Am. J. Hum. Genet.*, (1980), *32*, 542. Sutherland, G.R., Baker, E., Seshadri, R.S.

68 Familial X-linked mental retardation with an X chromosome abnormality and macro-orchidism. *J. med. Genet.*, (1980), *17*, 73. Sutherland, G.R., Judge, C.G., Wiener, S.

69 Heritable fragile sites on human chromosomes.
VI. Characterisation of the fragile site at 12q13. *Hum. Genet.*, (1981), *57*, 217. Sutherland, G.R., Hinton, L.

70 Heritable fragile sites on human chromosomes.
VII. Children homozygous for the BrdU requiring fra(10)(q25) are phenotypically normal. *Am. J. Hum. Genet.*, (1981), *33*, 946. Sutherland, G.R.

71 Heritable fragile sites on human chromosomes.
VIII. Preliminary population cytogenetic data on the folic acid sensitive fragile sites. *Am. J. Hum. Genet.*, (1982), *34*, 452. Sutherland, G.R.

72 Heritable fragile sites on human chromosomes.
IX. Population cytogenetics and segregation analysis of the BrdU requiring fragile site at 10q25. *Am. J. Hum. Genet.*, (1982), *34*, 753. Sutherland, G.R.

73 * The autosomal fragile sites. *Karyogram*, (1982), *8*, 32. Sutherland, G.R.

74 * The fragile X chromosome. *Int. Rev. Cytol.*, (1983), *81*, 103. Sutherland, G.R.

75 Genetic length of a human chromosomal segment measured by recombination between two fragile sites. *Science*, (1982), *217*, 373. Sutherland, G.R., Baker, E., Mulley, J.C.

76 The fragile X chromosome: Current methods. *Am. J. Med. Genet.*, (1982), *11*, 489. Hecht, F., Jacky, P.B., Sutherland, G.R.

77 Heritable fragile sites on human chromosomes. X. New folate sensitive fragile sites : 6p23, 9p21, 9q32, 11q23. *Am. J. Hum. Genet.*, (1983), *35*, 432. Sutherland, G.R., Jacky, P.B., Baker, E., Manuel, A.

78 * Fragile Chromosomes. In: Butterworth, C.E. (Editor), Nutritional Factors in the Induction and Maintenance of Malignancy. Academic Press, New York, (1983), in press. Sutherland, G.R.

79 Heritable fragile sites and micronucleus formation. *Ann. Genet.*, (1983), *26*, 5. Beek, B., Jacky, P.B., Sutherland, G.R.

80 Fragile sites on chromosomes: A model for the study of spontaneous chromosome breakage? *Science*, (1983), *220*, 69. Jacky, P.B., Beek, B., Sutherland, G.R.

81 Regional localisation for HLA by recombination with a fragile site at 6p23. *Am. J. Hum. Genet.*, (1983), in press. Mulley, J.C., Hay, J., Sheffield, L.J., Sutherland, G.R.

82 A search for linkage in families with fragile sites. *Hum. Genet.*, (1983), in press. Mulley, J.C., Nicholls, C., Sutherland, G.R.

83 Thymidylate synthetase inhibitors and fragile site expression in lymphocytes. *Am. J. Hum. Genet.*, (1983), in press. Jacky, P.B., Sutherland, G.R.

84 Protease inhibitor (PI) phenotype of individuals with chromosomal fragile sites. *Ann. Genet.*, (1983), in press. Mulley, J.C., Sutherland, G.R.

85 Heritable fragile sites on human chromosomes. XI. Factors affecting expression of fragile sites at 10q25, 16q22 and 17p12. *Am. J. Hum. Genet.*, (1983), in press. Sutherland, G.R., Jacky, P.B., Baker, E.

Footnote

* Denotes Review Article

( ) Previously submitted for the degree of M.Sc.

[ ] Previously formed part of Ph.D. thesis.

# V SYNOPSIS OF PUBLISHED WORK

## A. Population Cytogenetics

Much of this section was the basis of my M.Sc. thesis and as such is not formally assessable as part of this submission. I have a continual interest in the contribution of chromosome abnormalities to mental handicap and my studies of whole institutional populations (9,14) form a significant contribution in this field. Most other similar studies have involved selection of particular patient groups.

My early work on XYY males (10) was the only major study of this condition carried out in Australia.

I have an on-going interest in Down syndrome (11) as the commonest chromosome abnormality in man, and my epidemiological work on this disorder (16) was the first to be carried out in Australia since the early studies of Collman and Stoller (see 16 for references to their work), although it was being carried out concurrently with another Australian study of which I was unaware at the time (Mulcahy, 1979).

Perhaps the most important part of the work on population cytogenetics has been that on paediatric autopsies (12,13,15,18). Only two other series of paediatric autopsies have been studied chromosomally (Machin, 1974; Kuleshov, 1976).

The paper on $\alpha_1$-antitrypsin phenotypes in chromosome abnormalities (17) is a major work on this topic which remains controversial (Fagerhol and Cox, 1981).

## B. Clinical Genetics and Cytogenetics

Some of this section relates to clinical case reports and is not now of great scientific interest, although it usually did contribute at the time of publication. Several of the reports are still of interest and include a description of one of the first recognised insertional translocations (22,23), an important paper on trisomy 9 (25), and one of the first discussions of genetic counselling of pericentric inversion carriers (26).

My collaboration with Mr. J.C. Mulley has produced several publications in the area of gene assignment to chromosomes (32,35,37) and constitutes a significant proportion of the available data on exclusion mapping. We also discovered a new allele for $\alpha_1$-antitrypsin (39) and examined the distribution of $\alpha_1$-antitrypsin phenotypes in fragile site carriers (84).

Collaboration with Dr. R.S. Seshadri has resulted in several papers on sister chromatid exchange (31,33,36,38) including the first report of an increase in this phenomenon in multiple sclerosis (31).

C. Studies of Amniotic Fluid and Prenatal Diagnosis

Much of this work was carried out as part of my Ph.D. project in this University, and some of the papers in this section for assessment have resulted from my collaboration during the period with Dr. J. Butterworth on the enzymology of amniotic fluid and the cells from it in tissue culture (42,49,56).

During my doctoral studies I made two new findings, the first was that urine cells can be grown in tissue culture (41) and the second that amniotic fluid from pregnancies in which the fetus has a neural tube defect have greatly increased numbers of cells which rapidly adhere to the substrate in tissue culture flasks. I, perhaps erroneously, called these cells macrophages; they have now become known as 'rapidly adhering cells' although there is good evidence that some of them are macrophages, many are most likely cells of neural origin. This work has now been greatly extended by Gosden (see Gosden and Brock, 1978, for details) and immunohistochemical studies are helping to delineate the nature of the cells involved (Cremer et al., 1981). The documentation of this work (54) on rapidly adhering cells and other articles on aspects of prenatal diagnosis (55-60) are for assessment as is a brief letter (57) recording an observation which went much of the way towards settling controversy about α-fetoprotein levels in the amniotic fluid surrounding fetuses with Turner syndrome.

My detailed documentation of mosaicism in amniotic fluid cell cultures (55) helped in the early understanding of what remains a difficult problem to interpret and deal with.

D. Fragile Sites on Human Chromosomes

This topic has absorbed most of my research efforts over the last seven years and is by far the most important section of this thesis.

This work stemmed from my discovery of the tissue culture requirements for expression of fragile sites. The preliminary results of this work were presented to the Genetical Society in London (Sutherland, 1977) in November 1976 and subsequently published in *Science* (59).

My preliminary observations were followed by detailed studies of the tissue culture requirements for fragile site expression in lymphocytes, with some work on other cell types, and formed the basis of two papers which remain the major works on fragile sites (62,63). This early work dealt mainly with what have now become known as the folate sensitive fragile sites. Later studies on the use of thymidylate synthetase inhibitors (83) documented for the first time the use of FCdR to induce the folate sensitive fragile sites. The folate insensitive fragile sites have now been the subject of an extensive study (85) that has documented a range of compounds which will induce the fragile sites at 16q22 and 17p12.

Much more work has been done on fragile sites including the documentation of five further folic acid sensitive fragile sites (69,77), the discovery of the BrdU requiring fragile site at 10q25 (67) and a study of its population cytogenetics and segregation (72). The importance of the fragile site at Xq27

as a diagnostic marker for one form of X-linked mental retardation and some of the clinical and cytogenetic features of this syndrome have been documented (64,66,68). A possible role for the folate sensitive autosomal fragile sites in the aetiology of mental retardation has been suggested from work on population cytogenetics (71) and efforts are continuing to confirm or refute this suggestion. Linkage studies with fragile sites (75,81) have shown that control of expression of the fragile site is probably located at the fragile site and (82) provided a considerable amount of exclusion data for other genes from areas near fragile sites.

My collaboration with Dr. Beek in the application of the techniques used in studies of mutagenesis to the study of fragile sites is in its early stages. However, the two publications (79,80) which have ensued suggest that the biochemical knowledge gained about chromosome breakage at fragile sites might be relevant to an understanding of other forms of apparently non-specific chromosome breakage.

The work on fragile sites has been summarised in three invited review articles. The first on the autosomal fragile sites (73) was published in *Karyogram,* the official publication of the (American) Association of Cytogenetic Technologists, and is not a fully referenced paper in accord with the format of that journal. The second (74) is mainly concerned with the fragile X but views this chromosome in its context as one of the folate sensitive fragile sites. The third (78) is the written version of a paper I was invited to present at the

Second Annual Bristol-Myers symposium on Nutrition Research entitled 'Nutritional Factors in the Induction and Maintenance of Malignancy' in Washington in December 1982.

The work on fragile sites has been the subject of numerous editorial comments (Hecht and Kaiser-McCaw, 1979, 1980; Gerald, 1980, 1981; Turner and Opitz, 1980; Lancet 1981; Hecht et al., 1981) and has been instrumental in opening up a whole new area of cytogenetics. Virtually every diagnostic cytogenetics laboratory has changed, or will soon need to change, its way of performing chromosome studies to ensure that the folate sensitive fragile sites are detected.

References

Cremer, M., Schachner, M., Cremer, T., Schmidt, W., Voigtlander, T.: Demonstration of astrocytes in cutlured amniotic fluid cells of three cases with neural tube defect. *Hum. Genet., 56,* 365-370, 1981.

Fagerhol, M.K., Cox, D.W.: The PI polymorphism: Genetic, biochemical and clinical aspects of human $\alpha_1$-antitrypsin. *Adv. Hum. Genet., 11,* 1-62, 1981.

Gerald, P.S.: Editorial : X-linked mental retardation and an X-chromosome marker. *New Engl. J. Med., 303,* 696, 1980.

Gerald, P.S.: Commentary : X-linked mental retardation and the fragile-X syndrome. *Pediatrics, 68,* 595, 1981.

Gosden, L., Brock, D.J.H.: Combined use of alphafetoprotein and amniotic fluid cell morphology in early prenatal diagnosis of fetal abnormalities. *J. med. Genet., 15,* 262-270, 1978.

Hecht, F., Kaiser-McCaw, B.: Editorial : The importance of being a fragile site. *Am. J. Hum. Genet., 21,* 223-225, 1979.

Hecht, F., Kaiser-McCaw, B.: Editorial : Inducing fragile sites to express themselves. *Am. J. Hum. Genet., 32,* 626, 1980.

Hecht, F., Glover, T.W., Kaiser-Hecht, B.: Commentary : Fragile sites on chromosomes. *Pediatrics, 69,* 121-123, 1982.

Kuleshov, N.P.: Chromosome anomalies of infants dying during the perinatal period and premature newborn. *Humangenetik, 31,* 151-160, 1976.

Lancet: Editorial : X-linked mental retardation. *Lancet, i,* 1086-1087, 1981.

Machin, G.A.: Chromosome abnormality and perinatal death. *Lancet, i,* 549-551, 1974.

Manuel, A., Sutherland, G.R., Molesworth, J.: A new fragile site at 9p21. Presented at Fifth Ann. Sci. Meeting, Hum. Genet. Soc. Australasia, Canberra, May 1981.

Mulcahy, M.T.: Down's syndrome in Western Australia : Cytogenetics and incidence. *Hum. Genet., 48,* 67-72, 1979.

Sutherland, G.R.: Heritable fragile sites on human chromosomes. *Heredity, 38,* 271, 1977 (Abstract).

Turner, G., Opitz, J.M.: Editorial comment : X-linked mental retardation. *Am. J. Med. Genet., 7,* 407-415, 1980.

## VI EXPLANATIONS RE JOINT AUTHORSHIP

Publications 1-8 formed part of the candidate's M.Sc. thesis in the University of Melbourne and publications 40,41,43-48,50-53 were appended to the candidate's Ph.D. thesis in the University of Edinburgh. Since these two groups of papers cannot form an assessable part of this submission, and are only included for the sake of completeness, no explanations of their authorship are given. Unless otherwise stated, all cytogenetics, tissue culture and family studies were carried out by me. I offer the following comments regarding papers jointly written:

9 This work was done by me, at my own instigation, and the paper was written by me. Dr. S. Wiener was a sessional consultant physician in charge of the laboratory.

10 This is a compilation of all the studies on XYY males which had been done in Victoria to the time of publication. For Dr. S. Wiener see 9;
Dr. A.A. Bartholomew was consultant forensic psychiatrist who provided access to and details of the individuals studied. Ms. M.G. Fitzgerald was a cytogeneticist at the Royal Children's Hospital, Melbourne, who studied the two XYY males found there. The paper was written by me.

11 See 9. S.W. corrected the proofs.

12,13 This work was done in part by me and the remainder by my research assistant, Ms. R. Bauld. Dr. A.D. Bain was consultant pathologist in charge of the department, the autopsies were carried out by him or under his supervision. I wrote the paper.

14 This work was mostly done by me, the concept was mine, the paper was written by me. Mr. A.R. Murch did some of the cytogenetics, Drs. A.J. Gardiner and C. Wiseman provided clinical information about the patients. Dr. R.F. Carter was the consultant pathologist in charge of my unit.

15,18 See 12,13. Dr. I.I. Smith was a pathologist in Edinburgh who performed some of the autopsies.

16 This project was a joint one involving all authors. I wrote the paper. Dr. S.R. Clisby is a medical practitioner and Mr. G. Bloor a social worker with Intellectually Retarded Services.

17 This was a joint project. Mr. J.C. Mulley is a member of the staff of my unit who did the PI typing. The paper was jointly written.

19 This was a joint project. A. Carmichael was a medical student spending a period in the area of genetics in mental retardation partly under my supervision. Dr. H. Flynn was a medical practitioner who wrote the paper.

20 I wrote the paper. See 10.

21 I wrote this paper. Ms. M.G. Fitzgerald did some of the cytogenetics. The patient had been under the management of Professor D.M. Danks who provided considerable advice and help during this study.

22,23 Mrs. E. Grace was a cytogeneticist who worked under my supervision, she did most of the work and wrote the papers on Dr. G.D. Stark's patient. Dr. A.D. Bain was consultant pathologist in charge of the department.

24 I did most of the cytogenetics and dermatoglyphics but had little direct involvement in the publication.

25 I wrote the paper, Dr. R.F. Carter did the autopsies, Dr. L.L. Morris did the radiology.

26 I wrote the paper. Dr. A.J. Gardiner provided clinical details of the patient. Dr. R.F. Carter was pathologist in charge of the unit.

27 See 26. I wrote the paper.

28 I wrote the paper. Drs. G.F. Binns and R.F. Carter did the histology, Dr. R. Dow, gynaecologist, provided clinical details.

29 I prepared this article. Dr. J.G. Rogers was involved in the prenatal diagnosis.

31 I wrote the paper. Mrs. E. Baker is my senior technician and research assistant who did most of the technical work. Dr. A. Black, neurologist, provided samples from the patients.
Dr. R.S. Seshadri was a full collaborator and did the statistics.

32 For Mr. J.C. Mulley, see 17. G.D. Bryant did the blood groups. This was a joint project between J.C.M. and G.R.S., the paper was jointly written.

33 Some of the technical work was done under my supervision, my involvement in the publication was minimal.

34 I wrote the paper. Mrs. H. Eyre is a technician in my unit who did some of the cytogenetics under my supervision.

35 I wrote the paper. Mr. J.C. Mulley performed the genetic marker studies. Dr. E. Goldblatt, cardiologist, was in charge of the patient.

36 A joint project by Dr. R.S. Seshadri and G.R.S.
For Mrs. E. Baker see 31. The paper was jointly written by R.R.S. and G.R.S.

37 See 32.

38 This was a joint project involving G.R.S., R.R.S. and A.A.M. The SCE was carried out by E.B. under my supervision. R.K. and D.W. were staff in the laboratory of R.R.S. and A.A.M. The paper was jointly written by R.R.S. and G.R.S.

39 For J.C.M. see 17. D.W.C. controls a reference laboratory for the confirmation of new PI alleles and provided this service. The paper was jointly written by J.C.M. and G.R.S.

42,49,56 This series of papers were from a joint project by Dr. J. Butterwoth, biochemist, and G.R.S. All were jointly written. Other authors include Dr. A.D. Bain, pathologist in charge of the department, and Mr. D.M. Broadhead, research assistant to J.B.

54 I wrote this paper. Dr. D.J.H. Brock measured the alphafetoprotein levels, Dr. J.B. Scrimgeour performed the amniocenteses.

55 I wrote this paper. Mrs. S.M. Bowser-Riley was a cytogeneticist working under my supervision who did some of the cytogenetics. Dr. A.D. Bain, see 42.

57 I wrote this letter. Ms. D. Holt measured the alphafetoprotein, Dr. J.G. Rogers was involved in the prenatal diagnosis.

64 I wrote this paper. Dr. P.L.C. Ashforth measured the testicles.

66 I wrote this paper. Mr. P. Leonard was a cytogeneticist working under my supervision who did most of the silver staining.

67 I wrote this paper. For Mrs. E. Baker see 31. This discovery was made during a joint project (36) of Dr. R.S. Seshadri and G.R.S.

68 I wrote this letter and did the testicular measurements. The patients were under the control of Dr. C.G. Judge and their chromosomes had been studied in the laboratory of Dr. S. Wiener.

69 I wrote this paper. Mrs. L. Hinton was a technician in my unit who did some of the cytogenetics under my supervision.

75 I wrote this paper. For Mrs. E. Baker see 31. Mr. J.C. Mulley assisted with the linkage analysis.

76 Dr. F. Hecht wrote the paper, Dr. P.B. Jacky, now on my staff, organised the workshop. I helped in the organisation of the workshop, commented upon the manuscript and provided the figure.

77 I wrote the paper. Dr. P.B. Jacky and Mrs. E. Baker did some of the cytogenetics, Ms. A. Manuel did some of the family study on the patient with fra(9)(p21) and provided blood samples from this patient.

79 This was a joint project involving all authors. Dr. B. Beek was a senior geneticist from West Germany who spent a short sabbatical period in my laboratory, the paper was jointly written by B.B. and G.R.S.

80 As for 84, except that I wrote the paper and P.B.J. did most of the micronucleus scoring.

81 Paper jointly written by J.C.M. and G.R.S. Dr. J. Hay did the HLA typing, Dr. L.J. Sheffield referred the index case.

82 A joint project. Paper largely written by J.C.M. Catherine Nicholls was our graduate research assistant who determined many of the genetic markers under the supervision of J.C.M.

83 A joint project, the paper was jointly written.

84 A joint paper, the paper was jointly written.

85 A joint paper, I wrote the paper.

## VII ACKNOWLEDGEMENTS

Most of those who have assisted me in many ways have been acknowledged in the papers submitted. In particular, I would like to thank Dr. A. Douglas Bain who was Director of the Department of Edinburgh in which I carried out my Ph.D. studies and other work contained in this thesis. Dr. Bain provided me with much encouragement and support at an important stage of my scientific development. Dr. R.F. Carter, as Director of the Department in which my present Unit functions, has provided much support and encouraged my research efforts. Without the support from Dr. Carter my work on fragile sites on chromosomes would not have advanced to the extent it has. Special thanks are due to the research and technical assistants who have helped with various aspects of this work, particularly Mrs. Rhona Bauld in Edinburgh and Mrs. Elizabeth Baker in Adelaide.

Financial support for my work came initially from the Mental Health Authority in Victoria, then the Royal Hospital for Sick Children, Edinburgh, and finally The Adelaide Children's Hospital.
In Adelaide further support has been received from the National Health and Medical Research Council of Australia, the Channel 10 Children's Medical Research Foundation, the National Multiple Sclerosis Society, The Adelaide Children's Hospital Research Trust and the Anti-Cancer Foundation of the Universities of South Australia.

# VIII PUBLICATIONS

*Letter to the Editor*
*reprinted from* THE LANCET, *January* 20, 1968, *p.* 150

## XYY MALES IN A MELBOURNE PRISON

SIR,—An unusually high proportion of males with antisocial or criminal behaviour have an XYY chromosome complement.[1–3] These XYY males tend to be tall and dull.

We have lately begun a study of the prisoners in H.M. Prison Pentridge, a multipurpose prison in Melbourne. Its daily population of about 1200 inmates includes individuals convicted or remanded for various crimes and offences ranging from murder to drunkenness and vagrancy. Some of the inmates are high-grade mental defectives or psychotics who at their trial were found unfit to plead or were found not guilty on the grounds of " insanity ". The leucocytes of 34 prisoners, 69–82½ in. (175–210 cm.) tall, have so far been cultured and karyotyped. Of these 34 prisoners, 30 had a normal chromosome constitution, 3 had 47 chromosomes with an XYY chromosome complement, and 1 was an XYY/XYYY mosaic. The clinical diagnosis in all 4 XYY subjects was psychopathy: 1 had committed murder,[4] and another had been found unfit to plead to the same crime; no abnormality in the external sex organs was detectable in any of the 4, and 2 had children. Other findings in these 4 men are shown in the accompanying table. These results strongly support the concept that an extra Y chromosome is associated with antisocial or criminal behaviour which leads to confinement in an institution.

The incidence of XYY in the general population seems to be less than 0·2%,[1] and it is also low [5] amongst those who are mentally ill. In 30 mentally retarded men without criminal record who were 72 in. (183 cm.) and taller, and in more than 300 retarded boys, we have found none with an XYY chromosome complement. In the original report by Jacobs et al.[1] there was a significant increase in height of males with two Y chromosomes. Statistical analysis [6] suggests that an extra Y

1. Jacobs, P. A., Brunton, M., Melville, M. M., Brittain, R. P., McClemont, W. F. *Nature, Lond.* 1965, **208**, 1351.
2. Casey, M. D., Segall, C. J., Street, D. R. K., Blank, C. E. *ibid.* 1966, **209**, 641.
3. Price, W. H., Strong, J. A., Whatmore, P. B., McClemont, W. F. *Lancet*, 1966, i, 565.
4. Burns, C. The Tait Case. Melbourne, 1962.
5. Casey, M. D., Blank, C. E., Street, D. R. K., Segall, L. J., McDougall, J. H., McGrath, P. J., Skinner, J. L. *Lancet*, 1966, ii, 859.
6. Hunter, H. *ibid.* 1966, i, 984.

CLINICAL AND LABORATORY FINDINGS OF 4 PSYCHOPATHIC MALES WITH SUPERNUMERARY Y CHROMOSOMES

| Indices | Case no. | | | |
|---|---|---|---|---|
| | 3 | 5 | 17 | 19 |
| Genotype | XYY | XYY | XYY/XYYY | XYY |
| Year of birth | 1935 | 1947 | 1924 | 1916 |
| Height | 82·5 in. (210 cm.) | 70 in. (178 cm.) | 70 in. (178 cm.) | 71·5 in. (182 cm.) |
| Biacromial diameter | 17 in. (43 cm.) | 15½ in. (39 cm.) | 15½ in. (39 cm.) | 16 in. (41 cm.) |
| Intercristal diameter | 13 in. (33 cm.) | 10½ in. (27 cm.) | 11½ in. (29 cm.) | 12½ in. (32 cm.) |
| Maternal age at birth (yr.) | 46 | 29 | 29 | 32 |
| Paternal age at birth (yr.) | 43 | 32 | 33 | 38 |
| Birth-rank | 8 | 2 | 4 | 4 |
| Fertility | 1 girl | Nil | Nil | 1 child (? sex) |
| I.Q. by Wechsler adult scale (verbal/performance/full-scale) | 97/106/101 | 65/85/71 | 94/95/94 | 79/92/84 |
| Plasma-testosterone (μg. per 100 ml.) | 0·65 | 0·66 | .. | .. |
| Crime | Attempted murder | Murder | Murder | Larceny |

but not an extra X chromosome is associated with excessive height. However, in the 9 subjects described by Price et al.,[3] 4 were under 72 in. (183 cm.) and 1 was only 67 in. (170 cm.) high. Our tallest subject had a sister who was even taller (86 in., 218 cm.), and a grandfather alleged to have been over 72 in. (183 cm.). Further studies on the stature of parents and siblings of XYY subjects are obviously desirable.

Most XYY boys have average intelligence.[7] The number of XYY subjects with lowered intelligence was not statistically different from that of similar patients who had no extra chromosome.[8] 2 of our 4 XYY subjects had normal intelligence. So far no XYY male has been described with a superior intelligence. The possibly deleterious effect of an extra Y chromosome on intelligence may be age-dependent, becoming more pronounced with increasing age when the associated personality disturbance is severe. The electroencephalogram (E.E.G.) was considered abnormal in 3 of our XYY subjects, but, since the incidence of abnormal E.E.G. (often non-specific) findings in our prison population is not known, the prediction [9] that a high frequency of abnormal E.E.G.s will be found in XYY subjects remains unproven.

7. Balodimos, M. C., Lisco, H., Irwin, I., Merrill, W., Dingman, J. F. *J. clin. Endocr. Metab.* 1966, **26**, 443.
8. Price, W. H., Whatmore, P. B. *Br. med. J.* 1967, i, 533.
9. Forssman, H., Hambert, G. *Lancet*, 1966, ii, 282.

We were unable to confirm the increase of plasma-testosterone reported in 1 case.[10]

A retarded boy with an XYYY genotype has been described previously,[11] but case no. 17 (see table) seems to be the first reported of an XYY/XYYY mosaic; the details of his karyotype will be published later.

Chromosome Laboratory,
St. Nicholas Hospital, Melbourne.
SAUL WIENER
GRANT SUTHERLAND.

Psychiatric Clinic,
H.M. Prison Pentridge, Melbourne.
ALLEN A. BARTHOLOMEW.

Department of Medicine,
Monash University, Melbourne.
BRYAN HUDSON.

10. Welch, J. P., Borgaonkar, D. S., Herr, H. M. *Nature, Lond.* 1967, **214**, 500.
11. Townes, P. L., Ziegler, N. A., Lenhard, L. W. *Lancet*, 1965, i, 1041.

Printed in
Great Britain

The Lancet Office,
7 Adam Street, Adelphi, London W.C.2

*Letter to the Editor*
*reprinted from* THE LANCET, *December* 21, 1968, *p.* 1352

## A NORMAL XYY MAN

SIR,—All previously reported XYY men have been found either in penal or mental institutions or because of a personal or family history suggesting a chromosomal abnormality. We report here a case discovered by chance.

A 41-year-old man donated a sample of blood required for testing a new batch of tissue-culture medium. He was the first volunteer we approached, and after successful culture his chromosomes were incidentally counted. He is the only child of a father and mother who were 26 and 19 years old respectively when he was born. His mother had severe puerperal sepsis and during her illness, according to his father, the infant was fed on " boiled water ". During the " depression " years food was scarce, and he developed rheumatic fever and later poliomyelitis, which severely interrupted his schooling. He joined the army in 1948 and worked as a medical orderly until he was honourably discharged 18 months later. During the next 15 years, he had several semiskilled labouring jobs. For the past 3 years, he has been employed at this hospital.

The man has always been a teetotaller and has never smoked. According to his father, he was a placid and obedient child. He has never been in conflict with the Law, has financially supported needy members of his family, and is exceptionally fond of his parents, with whom he lives. He is of cheerful disposition and mild temperament, has no apparent behavioural disturbance, and has never required psychiatric advice. Shyness and fear of poverty seem to have prevented him from getting married.

The subject was found to be 182 cm. tall, and weighed 76 kg. His arm-span was 190·5 cm., and the biacromial and bitrochanteric distances were 40·5 cm. and 32·5 cm., respectively. He had a highly arched V-shaped palate and, in addition to his permanent cuspids, he had retained deciduous upper cuspids. The little fingers were abnormally short but his digital and palmar creases were normal. His trunk had a rash caused by tinea versicolor. Genitalia and body hair were of normal

development. His heart and lungs were clinically clear and his blood-pressure was 130/70 mm. Hg, with a regular pulse of 80 per minute. Electrocardiogram was normal with a PR interval of 0·14 second. Electroencephalogram showed non-specific abnormalities (excess low-voltage theta and delta activities). Vision for red and green was normal. His I.Q. (Wechsler) was 97 in both verbal and performance tests. Culture of his leucocytes showed a karyotype of 47, XYY. The karyotype of his father was 46, XY.

Recently, Court Brown et al.[1] have described a 34-year-old XO/XYY mosaic of normal intelligence and free from behavioural disturbance. This case and the present one show the difficulty of characterising XYY males, but it is evident that they may lead a normal life. Reliable data on the incidence of the XYY genotype in the population are indeed required.[2]

Chromosome Laboratory,
St. Nicholas Hospital,
Melbourne.

SAUL WIENER
GRANT SUTHERLAND.

1. Court Brown, W. M., Price, W. H., Jacobs, P. A. *Br. med. J.* 1968, ii, 325.
2. Leff, J. P., Scott, P. D. *Lancet*, 1968, i, 645.

Printed in Great Britain

The Lancet Office,
7 Adam Street, Adelphi, London W.C.2

# A Murderer with 47,XYY and an Additional Autosomal Abnormality

S. WIENER, G. SUTHERLAND and ALLEN A. BARTHOLOMEW

*Reprinted from The Australian and New Zealand Journal of Criminology*

# *A Murderer with 47,XYY and an Additional Autosomal Abnormality*

S. WIENER*, G. SUTHERLAND** AND ALLEN A. BARTHOLOMEW***

CASE reports are now accumulating relating to "patients" found to have the karyotype 47,XYY[1]. Many of these reports, although from selected samples, have tended to demonstrate a relationship between the extra Y chromosome and criminality or "criminal propensities"[2].

The case presently described is of interest from two points of view. In the first place the individual was charged with murder (1961) and convicted of that crime after his defence of insanity had been rejected by the jury. He was sentenced to death, a mandatory sentence in the State of Victoria, and after an appeal to the State Full Court[2a] and an application for leave to appeal to the High Court of Australia had been rejected the death sentence was confirmed by the Govenment of the day. This led to an application for leave to appeal to the Judicial Committee of the Privy Council in London which was refused. After this, somewhat unusual litigation took place in the Victorian courts in their lunacy jurisdiction[3] and later further efforts were made in the original criminal jurisdiction[4]. The matter ended when the Government commuted the death sentence and the prisoner was certified to a security mental hospital. The overall story has been well set out in general terms by Burns[5].

After a short stay in the security mental hospital the prisoner was returned to prison to continue serving his sentence — natural life imprisonment. In 1967 we began a karyotype survey of our prison population and this prisoner was provisionally reported to be an XYY/XYYY mosaic[6]. Further study has led to the conclusion that his karyotype is 47,XYY, Dp+.

---

*Consultant Pathologist, M.D. (Melb.), PhD. (Melb.), M.R.A.C.P.
**B.Sc. (Melb.), Geneticist. Both of the Chromosome Laboratory, St. Nicholas Hospital, Mental Health Department of Victoria.
***M.B. (Lond.), D.P.M. (Lond.). Psychiatrist Superintendent, H.M. Prison Pentridge, Melbourne.

1. Nielsen, J. (1968) "The XYY Syndrome in a Mental Hospital" Brit.J.Criminol. 8 186; Court Brown, W.M., Price, W.H. and Jacobs, P.A. (1968) "Further Information on the Identity of 47,XYY Males" Brit.Med.J. ii 325.
2. Although this is so at least two cases have been reported of "normal" males with the XYY chromosome constitution; Court Brown *et al. Ibid;* Wiener, S. and Sutherland, G. (1968) "A Normal XYY Man" Lancet ii 1352.

2a. *R v Tait* (1963) V.R. 520.

3. See Feltham, J.D. (1964) "The Common Law and the Execution of Insane Criminals" 4 Melbourne University Law Review 434; *Re Tait* (1963) V.R. 532.
4. *Tait v R* (1963) V.R. 547.
5. Burns, C. (1962) The Tait Case. Melbourne University Press.
6. Wiener, S., Sutherland, G., Bartholomew, A.A., and Hudson, B. (1968) "XYY Males in a Melbourne Prison" Lancet i 150.

The presentation here will set out firstly the clinical history of the prisoner in some detail, secondly the chromosomal picture will be given and finally the matter will be discussed from two points of view. These two aspects will be the finding of a chromosomal abnormality in its own right and secondly the importance of the abnormal finding in this case where the individual might very well have been hanged in 1962; five years or so before we were in a position to determine that he had an abnormal chromosome constitution.

*Clinical History*

The prisoner was born in Scotland in December 1924 and he was the fourth of five children. Nothing is known of his early history other than his father was a publican given to heavy drinking and aggressive behaviour. The prisoner attended an elementary school until aged about 9 years when he sustained injuries following a fall of some 40 feet. The major injury was a dislocation of the left knee. He was also knocked unconscious for an unknown period and, in all, was detained in hospital for a period of about six months. After this he was sent to a special school for physically and mentally handicapped children. This was largely due to the fact that he was on crutches and had missed a considerable amount of schooling. This reason was given to the Crown psychiatrist by the prisoner's estranged wife who was "certainly not very friendly" toward her husband and the defence endeavoured to maintain that the need for special schooling was indicative of mental backwardness as a child.

In his book, Burns comments that[7] "as a child (the prisoner) was fey; as a young man childish" and gives a description given by one of the prisoner's sisters that in his younger days

> He wasn't silly or anything like that. He was just slow and very childish. He was always bringing home stray animals. It used to keep Mum poor taking them to the vet. He loved reading comics. Even in the Navy in the war he read comics. If he read a sad comic or heard a sad play on the wireless he would cry. He wasn't one for the girls. He loved to go to the pictures or a dance now and then. But he never hung around with corner boys. He was a big child. Bertie (the family's name for him) could never make a decision — he always used to ask us about simple things, such as whether he should go to the pictures.

After leaving the special school at the age of about 14 years he worked as a messenger boy for a short period until beginning an apprenticeship as a motor mechanic. In 1943 (when aged 18 years) he joined the Royal Navy as a stoker and served both in the Mediterranean and off Singapore. Whilst in the navy he began to drink heavily and develop homosexual tendencies. Indeed, during his time in the service he admitted to "a certain amount of buggery".

He was demobilized in 1947 and married a Lithuanian girl the following year. The girl's brother was in Australia and persuaded them to emigrate. They came to Australia in 1952 and almost as soon as they arrived the marriage began to break up, which it did finally in 1954,

---

7. Burns, C. *op. cit.* p.24.

due largely to the husband's heavy drinking. However, Burns[8] comments that apart from the drinking "there is some evidence that his sexual abnormalities were becoming more pronounced and that at least once, perhaps twice, he had violently assaulted his wife and threatened to kill her". After his wife left him he began to drink even more heavily.

Between 1954 and 1959 he wandered round the continent earning money from casual labour, getting into minor trouble with the law and drinking heavily. During this period his sexual deviance became more pronounced and after a period of having heterosexual relations with aboriginal women he abandoned such behaviour and found satisfaction in transvestism and sado-masochism. As Burns puts it[9]

> His sexual satisfaction came from wearing or handling women's underclothes, which he either bought or stole, from making compulsive sexual assaults on young women, attempting roughly to handle their breasts against their will and from inflicting pain on himself by inserting sharp instruments in his anal canal while masturbating.

On August 6th, 1959, after heavy drinking he assaulted a seventy-year-old woman and as a result was charged with robbery with assault (there is some evidence that, apart from taking money, he indulged his sexual drive by handling the woman's breasts), was convicted and sentenced to three years imprisonment being ordered to serve two years before becoming eligible for parole. He was seen by a psychiatrist at the start of his sentence and it was simply noted that he was a heavy drinker, possibly an alcoholic; that he was a social isolate; that he had sexual problems of some five years' standing including transvestism; and that he had requested treatment. At no time over the following two years was he in receipt of treatment.

He was released from prison on 27th July, 1961, after having served some 22 months of his sentence. He was on parole and had a special condition attached that he should abstain from alcohol. Between the date of his release and the murder (8th August, 1961) he was seen by his parole officer on six occasions and on the 7th August was seen by a psychiatrist. When seen by the psychiatrist (10.30 a.m.) the parolee was sober, agreed that he had been drinking since his discharge from prison but denied he was an alcoholic and refused to consider any suggestions of possible treatment.

The following day, being out of work, he drank quite heavily and consumed at least half a bottle of whisky and "a few beers". He wanted more to drink but had no money. He remembered a vicarage from previous contact and so went there and broke in. Once inside he rummaged around looking for money when the vicar's aged mother disturbed him. In the prisoner's words, "I did my block," and punched her. He then, the order of events is not known, removed her clothing and dressed himself in her undergarments and placed a torch in her vagina. He denied any recollection of the latter behaviour.

When seen after the murder (22nd August, 1961) he showed little anxiety and simply said, "I'm in a real mess now". He was investigated with both psychological tests and electro-encephalographic recording.

The gist of the psychologist's report was that the prisoner had an

---

8. *Ibid* p.24.
9. *Ibid* p.25.

average intelligence the IQ (WAIS full score) being 94. Other tests (TAT and Rorschach) suggested that there was a "neurotic adjustment" but that there was no other evidence of gross mental illness.

The electro-encephalogram was reported upon (by the late Dr. A. King) as "is not suggestive of organic cerebral disorder. It reflects his personality but does not show organic disease".

Because of the apparent late onset of his sexual deviance and because of his alcoholic nomadic way of life a blood test for syphilis was done but found to be negative.

Finally, additional physical measurements were taken (height, biacromial and intercristal diameters) and these have been set out elsewhere[10].

Whilst in prison under sentence of death the prisoner remained essentially mentally stable and only very occasionally demonstrated any evidence of becoming "psychotic". This period was exceedingly stressful to him as his execution had a date set on some four occasions, the date being changed as a result of the various appeals taking place in the courts[11]. He was finally "certified" to a mental hospital under the provisions of the *Mental Health Act* (*Vict.*) 1959, s.52. However, this step was taken after his sentence had been commuted whilst an appeal was being considered in the High Court of Australia. When announcing that the death sentence had been commuted by the Government the Premier, Mr. (now Sir Henry) Bolte, said that events had culminated in a stay of execution for a further indefinite period by order of the High Court. This had followed frequent postponements in the immediate past. "The consequent effect on the mental condition of the prisoner has finally resulted in the issue of certificates under the new *Mental Health Act* that his mental health had been substantially impaired"[12]. It is important to recognize that the prisoner was not certified as "insane" but as suffering from "a psychiatric or other illness which substantially impairs mental health"[13] This act of certification cannot be construed as indicating that the prisoner had been "insane" since at least August 1961[14].

The prisoner remained in a security mental hospital for a little less than three months. He returned to H.M. Prison Pentridge on the 18th January, 1963[15]. Since that time it would appear that he has remained in "normal" mental health and he has certainly not required any specific treatment — psychopharmacotherapy or psychotherapy.

*Cytogenetics*

*Method*: Blood for chromosome studies was collected by venepuncture (15 ml in a heparinized syringe) and transported to the laboratory. About 4-5 hours elapsed before the leucocytes were separated for culture. The leucocytes were added to a tissue culture medium of T199 with 20% foetal calf serum at a concentration of 7 x $10^5$ per 1 ml of medium. The cultures

10. Wiener *et al.* (1968) *op. cit.*
11. Howard, C. (1963) "Time and the Judicial Process" 37 Aust.L.J. 39; Idem, "An Australian Letter: The Principle of Fair Trial" (1963) Crim.L.R. 603.
12. Burns, C. *op. cit.* p.141.
13. Mental Health Act (Victoria) 1959, sec.3. It should be noted that this Act came into operation on November 1st 1962; 8 hours before the prisoner was to be executed on the final date set.
14. See correspondence (1963) Crim.L.R. 870.
15. The Age (Melbourne) 21st January 1963.

were harvested according to the method of Moorhead et al.[15a] and stained with Giemsa. Skin cultures were prepared and harvested by the method described by Ferguson[16].

*Results*: Three leucocyte cultures and one skin fibroblast culture[17] were undertaken and the results are set out in Table 1. The results of

TABLE 1 *Results of the Chromosome Counts*

| | Number of Chromosomes | | | | | |
|---|---|---|---|---|---|---|
| | 45 | 46 | 47 | 48 | 49 | Total |
| Leucocyte 1 | 2 | 3 | 78 | 17 | — | 100 |
| Leucocyte 2 | 2 | 3 | 48 | 6 | — | 59 |
| Leucocyte 3 | 1 | 5 | 91 | 2 | 1 | 100 |
| Skin Fibroblast | — | — | 73 | 3 | — | 80 |

the first two leucocyte cultures were rejected due to a high increase in chromatid breaks and aneuploid cells. The final leucocyte culture and the skin culture gave a consistent karyotype of 47,XYY, Dp+ (See Figures 1 and 2)

FIG. 1. *Note the extra Y chromosome and the Dp+ chromosome which is arrowed.*

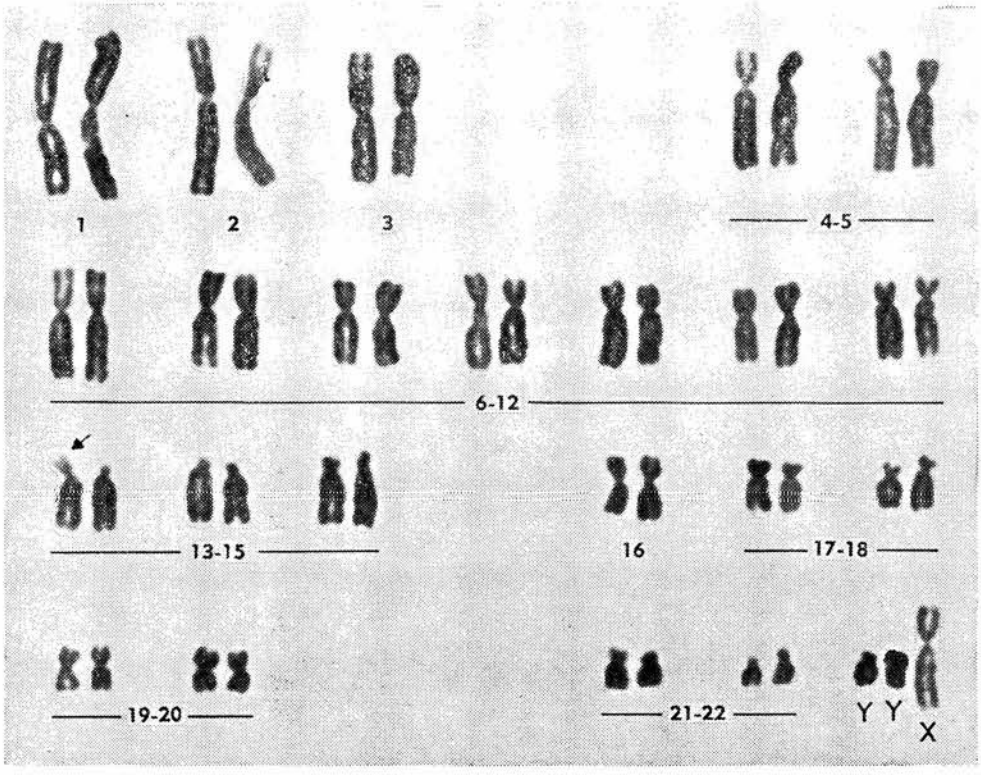
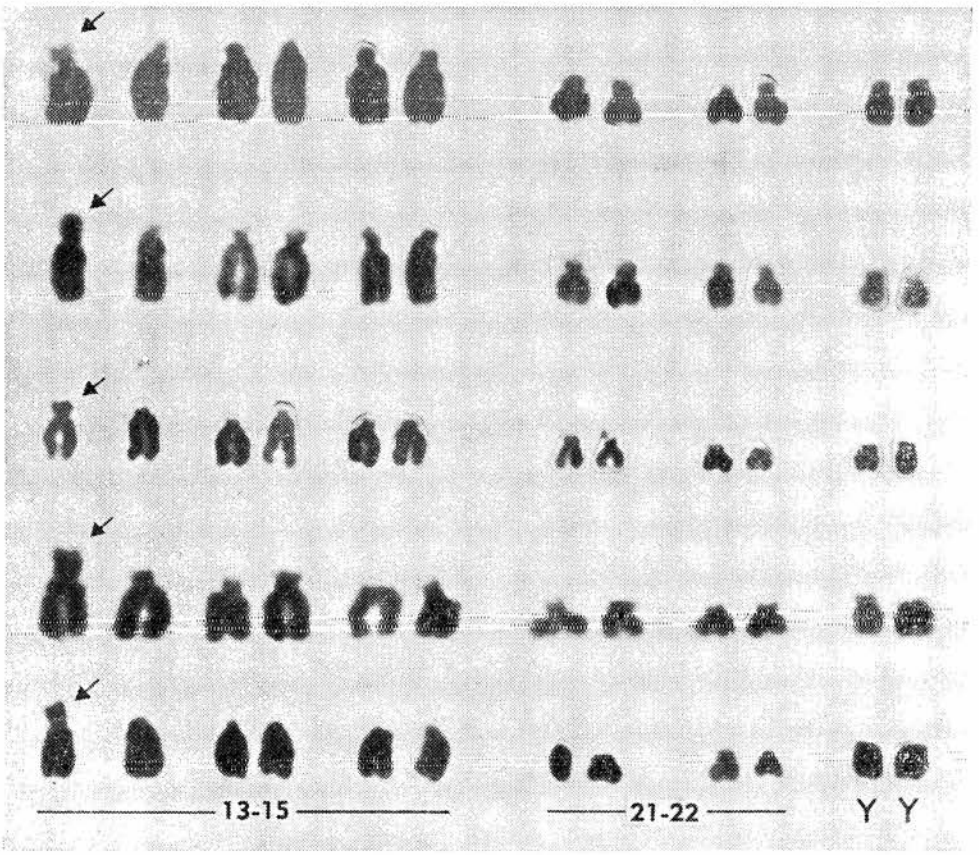


FIG. 2. *Chromosomes from groups D and G showing the appearance of the Dp+ chromosome (arrowed) and the Y chromosomes from a further five cells.*




with random differences in the aneuploid cells. Satellites were not detected on the Dp+ chromosome although it was occasionally in "satellite associations" with both D and G — group chromosomes.

*Comment on Cytogenetic Findings*: The reasons for the increased breakage and aneuploidy in the first two leucocyte cultures is uncertain, however similar results were encountered in cultures from other subjects in the same batch of medium. It was therefore suspected that some toxic factor in the medium was responsible. The prisoner was not on any medication at the time of the first culture but did have a barium meal just prior to the second.

---

FOOTNOTES TO PREVIOUS PAGE

15a. Moorhead, P.S., Nowell, P.C., Willman, W.J., Battips, D.M. and Hungerford, D.A. (1960) "Chromosome Preparations of Leucocytes Cultured from Human Peripheral Blood" Exp.Cell.Res. 20 613.

16. Ferguson, J. (1962) "Chromosome Studies of Human Cells in Tissue Culture" Med.J.Aust. i 40.

17. We should like to thank Miss J. Ferguson of the Commonwealth Serum Laboratories for preparing the skin fibroblast culture.

Aberrant D — group chromosomes of the type presented here in which the origin of the extra material in the short arm is unknown, appear to have a low incidence in the normal population. Hungerford and Chandra[18] reported finding a Dp+ chromosome in a normal girl and subsequently in her father and paternal uncle. In a study of 756 individuals Court Brown *et al.*[19] found four cases; three of these were clinically normal and one had primary amenorrhoea. However, the same chromosome was found in four presumably normal members of her family. Ferrier *et al.*[20] studied an XO/XY/XYY male pseudohermaphrodite whose father carried a Dp+ chromosome with no apparent clinical abnormality.

It is therefore probable that in some individuals Dp+ chromosomes may be present in the absence of any detectable clinical abnormality and, as Ferrier *et al.*[21] suggests, may be "part of asymptomatic variation found in the general population". Nevertheless, we do not know that the Dp+ chromosome in association with the extra Y chromosome is without clinical effect.

*Discussion*

Two matters appear to be important in this case. The first matter concerns the significance of the extra Y chromosome in relation to crime. If one accepts that the incidence of the XYY chromosome constitution in the general population be less than 0.2%[22] then the finding of a greater incidence of this karyotype in a delinquent or criminal population tends to lead to the conclusion that the extra Y chromosome may well be a factor in some cases of anti-sociality. However, the position is very far from clear and there is no doubt that at the present time unduly exaggerated claims are being made. An example of this is the statement made by Mackay[23] that

> studies have indicated that approximately 50% of tall (over 6ft.) males with criminal tendencies have the XYY constitution.

Although the murderer described in this article did have the XYY chromosome constitution (plus the additional autosomal abnormality) we are not attempting in any way to set out an immediate causal relationship between the karyotype and the anti-social behaviour. A fair comment regarding the XYY constitution and anti-social behaviour has been made by Court Brown *et al.* who wrote that[24]

---

18. Hungerford, D.A. and Chandra, H.S. (1966) "Further Studies of a Kindred Having an Aberrant Autosome (13-15) Without Apparent Phenotypic Effect" J. Genet. **59** 239.
19. Court Brown, W.M., Buckton, K.E., Jacobs, P.A,, Tough, I.M., Kuenssberg, E.V. and Knox, J.D.E. (1966) "Chromosome Studies on Adults" Eugenics Laboratory Memoirs XLII. The Galton Laboratory, University College London. Cambridge University Press, London/NewYork.
20. Ferrier, P.E., Ferrier, S.A., Scharer, K.O., Genton, N., Hedinger, C. and Klein, D. (1967) "Multiple Chromosome Aberrations: XO/XY/XYY mosaicism and a Translocation in the Same Family" Helv.paedial. Acta. **22** 516.
21. *Ibid.*
22. Jacobs, P.A., Brunton, M., Melville M.M. and McClemont, W.F. (1965) "Aggressive Behaviour, Mental Subnormality and the XYY Male" Nature (Lond.). **208** 1351; Court Brown, W.M. (1968). "Chromosomes and Crime" New Scientist 31st October, p.235.
23. Mackay, E.V. (1967) "The Clinical Significance of Disorders of the Sex Chromosomes" Med.J.Aust. ii 552.
24. Court Brown, W.M., Price W.H. and Jacobs, P.A. (1968) "The XYY Male" Brit.med.J iv 513.

> the only conclusion that can be reached at present about a male with extra Y chromosome is that, by comparison with an XY male, he incurs some increased risk of developing a psychopathic personality. There are no data which permit a quantitative evaluation of this increase; it may be small or it may be considerable.
> There is, however, no evidence which indicates that an XYY male is inexorably bound to develop anti-social and criminal traits . . .

It is considered unlikely that the XYY chromosome constitution would be important in relation to the raising of a defence of insanity to any criminal charge[25], although the finding might be useful in a defence based upon diminished responsibility. It has been clearly stated[26] that "Criminal responsibility is assessed on evidence of a man's state of mind", the test being of a cognitive nature, and not on possible aetiological factors. There is therefore no reason to suppose that the prisoner concerned in this article would have been any more successful in his insanity defence even if, in 1961, he and his legal advisers had been aware of his chromosome constitution.

Whilst it may be true that the XYY finding may not be of great interest in terms of criminal responsibility it is likely that

> ethical problems are likely to be much more serious in relation to the wider social consequences of the diagnosis. In the criminal field, difficulty will not be in the court, but will occur when the parole board has to try to assess the significance of an XYY constitution in a recidivist of otherwise unexceptional mentality who is due for release[27].

This brings one to the second matter which is concerned with punishment, and, in particular, hanging as a criminal sanction. Quite apart from the morality of hanging persons, of sound mind, found guilty of murder, there is the difficulty of what one means by "of sound mind". Very frequently a "normal murderer", who might be a perpetrator of a *crime passionel,* will have his death sentence commuted because the murder was "normal" or "understandable". It is likely that the more "abnormal" or "not understandable" murder will be committed by a more psychiatrically disturbed individual. In other words, if the "normal" murderer has his sentence commuted and the "abnormal" murderer also has his sentence commuted on the basis of his abnormality then there is virtually no need to retain hanging as a criminal sanction. It is a sobering thought to reflect that if this prisoner had been known to have had the described chromosomal abnormalities in 1961-2 he would possibly have had his death sentence quickly commuted. It may well be with hanging that any one executed might later be found to have suffered from a disease or disorder which, had it been recognized prior to execution, would have constituted a ground for clemency. The case of *Sodeman*[28] is relevant here in that

---

25. See Bartholomew, A.A. (1968) "The Extra Y Chromosome and Criminal Behaviour" (Editorial Annotation) Aust.N.Z.J.Psychiat. **2** 6; "Medico-Legal" in Brit.med.J. (1968) iv 389; Fox, R. (1969) "XYY Chromosomes and Crime" Aust.N.Z.J. Criminol. **2**, 1, 5, and Bartholomew, A.A., and Sutherland, G. (1969) "A Defence of Insanity and the Extra Y Chromosome: R v Hannell" Aust.N.Z.J. Criminol. **2**, 1, 29.

26. "Medico-Legal" *op. cit.*

27. Gibbens, T.C.N. (1968) "Genetics and Ethics" New Scientist 31st October, p.236.

28. *R v. Sodeman* (1936) V.L.R. 99; (1936) 55C.L.R. 192. See also Morris, N. and Howard, C (1964) "Studies in Criminal Law" Clarendon Press. p.49.

at his post mortem after his execution it was noted that he suffered "from brain damage so extensive that the doctors wondered how he had survived and functioned at all at the time he committed the murders"[29]. One wonders, if capital punishment be continued, how many who are hanged would not perhaps have later been found to have suffered from some significant abnormality.

It is certain that genetic research has done little more than get started and it is also very likely that in the months and years to come further abnormalities will be recognized, abnormalities effecting behaviour and possibly correlating with anti-sociality, perhaps aggressive and criminal anti-sociality. Without going the whole way in terms of genetically determined behaviour, one may note the comment of Parsons[30] that with mice

> investigations have shown a correlation between strains for alcohol preference and an enzyme breaking down alcohol, namely liver alcohol dehydrogenase (A.D.H.) . . . The mouse results do indicate, however, that a behavioural trait, the desire to drink alcohol, may have some biochemical basis . . . Recent work is showing more and more that there is at least some genetic and biochemical basis to human behaviour.

On this basis it may well be that this murderer may not have been a fully responsible person in relation to his general anti-sociality (his XYY constitution?) or, at another level, his alcoholism. It is becoming ever more accepted that alcoholism is a disease, without any concern as to its possible genetic basis, and in the United States there is a trend in the direction of accepting the alcoholic as a sick person and certainly not responsible for his condition[31], and presumably not responsible for many of the acts which stem from intoxication in such a person. One may simply summarize the position by saying that we are far too ignorant at this time to say with any meaningful certainty "This man is 'normal' " or even "responsible". Inevitably to hang any person is to take away the life of an individual who may well be abnormal in terms of tomorrow's knowledge.

---

29. Burns, C. *op. cit.*
30. Parsons, P.A. (1967) "Genes, Behaviour and Biology" La Trobe University Inaugural Lecture. F.W. Cheshire, Melbourne.
31. Editorial (1968) "Alcohol and Crime" Aust.N.Z.J. Criminol. **1** 65.

# A Defence of Insanity and the Extra Y Chromosome: R v Hannell

Allen A. Bartholomew and G. Sutherland

*Reprinted from The Australian and New Zealand Journal of Criminology*

# *A Defence of Insanity and the Extra Y Chromosome: R v Hannell*

ALLEN A. BARTHOLOMEW*

G. SUTHERLAND**

IN a recent trial of a man charged with murder, heard in the Supreme Court of Victoria (*R v Hannell,* unreported), considerable interest was aroused, and much inaccurate comment, in relation to the basis of the defence raised: the defence of insanity. During the course of the psychiatric evidence given for the defence it was stated that the accused man had a chromosome constitution of 47,XYY and this led to such headlines as "Killer with odd cells is insane"[1], "Not guilty for killer with criminal trait"[2] and "Jury clears XYY man: Insanity"[3]. These headlines, and the texts they headed, give the impression that the accused successfully raised the defence of insanity on the basis of his chromosomal abnormality. This is not a true representation of the position.

Although an aspect of this trial has been commented upon briefly in the *Journal* as an Editorial[4], it is thought that the whole case, clinical features, genetic findings and the evidence offered to the court, should be presented in full.

*Clinical Features*

*Clinical History*: He was born in Victoria one of twin boys, both having an older sister and later two younger brothers. He was the second of the twins to be born and his mother thinks that he suffered some birth injury. Further, the mother commented, "He's always been a problem child," and stated that he was sick for the first two years of his life. From the Royal Children's Hospital the early clinical history was that he had seven admissions over the first seventeen months of his life. His first admission was for prematurity (birth weight 4lb 3ozs), a transient systolic murmur, vomiting and diarrhoea, and sclerema. His second admission was for the scleremátous condition. The third admission was for asthmatic bronchitis

* M.B. (Lond), D.P.M. (Lond), M.A.N.Z.C.P., Psychiatrist Superintendent, H.M. Prison Pentridge, Melbourne.
** B.Sc. (Melb), Geneticist, Chromosome Laboratory, St. Nicholas Hospital, Melbourne.

1. Herald (Melbourne) 9th October, 1968.
2. Australian (Melbourne) 10th October, 1968.
3. Sun (Melbourne) 10th October, 1968.
4. Editorial (1968) "The Defence of Insanity and XYY" Aust.N.Z.J. Criminol. **1**, 4, 199.

and a hypochromic anaemia (haemoglobin 37%) which required a blood transfusion. The fourth occasion of hospitalization was for bilateral otitis media requiring bilateral paracentesis, and a condition of gastro-enteritis. The fifth occasion was for further investigation of the anaemia (haemoglobin 48%) and another blood transfusion, whilst the sixth and seventh admissions were for a right basal pneumonia and a subsequent persistent cough.

Against this hospital background his mother continued: "He has always been much slower than his twin, he was slow at everything. You wouldn't think they were twins. He was twenty months before he walked." He was considered very backward at school and left when aged 14 years and in the 5th grade. When he left he could barely read and write and had little knowledge or understanding of simple arithmetic.

Since leaving school he has had "a large number of jobs", most of them casual work and labouring tasks. His social activities included an interest in swimming and horse-riding but he tended to do these alone as "he wasn't one for making friends, particularly girl friends". He very rarely drank alcohol but was a heavy smoker. His only previous offence was "Loitering for homosexual purposes"; he was fined in the adult court.

The relationship of H with his family was that he got on "extra well" with his father and "very well" with his twin and younger brothers. He had an indifferent relationship with his mother who separated from her husband when H was about 15 years of age. It is said the separation took place due to the excessive drinking of the mother. After the separation H lived with the mother for various periods of time but at intervals he would "suddenly leave the home and go off and live somewhere else".

*The Alleged Offence*: The killing of the victim (his aged landlady) took place on Easter Sunday, 26th March, 1967. The general background would appear to have been that two weeks or so prior to Easter, H had gone hop-picking with his mother but that he suddenly returned to Melbourne without saying anything to her. A possible explanation for this somewhat precipitous return is that H's father had promised to have him over Easter but had gone away: later both parents felt that this had caused H to be lonely over the Easter period.

The killing is best described as a virtually motiveless attack on a 78 year-old lady by stabbing. Owing to the near inability of the prisoner to tell an accurate, connected or consistent story, one cannot be certain of the exact sequence of events, but a likely reconstruction is that he was lodging with the victim; that he went out of the house with a knife and cut the lead from the outside television antenna to the sets in the house (he says, "I don't know" why); he then knocked on both her front door and window, and when admitted, asked if her television set was working properly; when the landlady answered "no" he offered to do something about it and the offer was accepted; on leaving the landlady's flat he stated, "I sort of tripped on my heel and pushed her and she fell down," after which he stabbed her — "I don't know why I did it". After this he ran away and caught a train to Werribee (some 30 miles away) where he visited an aunt and found his mother there. He remained the night returning to his room on Easter Monday at about mid-day. Some 12 hours later (12.50 a.m. on the Tuesday) he telephoned the police having earlier broken some glass beside the landlady's front door in order to get in and turn her TV set either down or off. When the police wireless patrol arrived he

told them a story of having heard a burglar breaking glass and running away. The police reassured him and promised to return later. When they did return they found the body of the old lady. H initially persisted in his story (with variations) of a burglar but then suddenly broke down and admitted the killing.

*Examinations in Custody*: H was first seen in H.M. Prison Pentridge on 4th April, 1967, when it was known that he was accused of the murder of his landlady. It was immediately apparent that he was intellectually very dull and only able to give a very poor account of his background in general and the events surrounding the killing. Subsequent examinations made it clear that he had little idea regarding what he was alleged to have done and even less idea or understanding of what was involved in a trial. As a result he was tested by the psychologist (Mr. E. Plumridge) who reported:

> Intellectual Assessment: The W.A.I.S.
> Verbal I.Q. = 65
> Performance I.Q. 83 Full Scale I.Q. = 71
> The full scale score and the quality of test responses is consistent overall with borderline defective intelligence.
>
> Rorschach: There were but eight scoreable responses of the simple usual kind. The responses as such were reality based. The formal quality of responses were consistent with below average intelligence.

The clinical impression plus the formal testing led to the Crown being informed in July 1967 that H was considered "On a very fine balance of probabilities . . . to have been legally responsible for his actions at the time of the alleged offence" and that "he is also a borderline case regarding 'fitness to plead' ". He appeared in the Supreme Court in July 1967 when the issue of fitness to plead was raised (*vide infra*). He was found unfit to plead and was returned to H.M. Prison Pentridge.

On his return to the prison he became involved in a karyotype survey and was thus found to have a chromosome constitution of 47,XYY. This lead to an investigation of the karyotypes of the father and twin brother.

*Cytogenetics*: Blood for chromosome studies was collected by venepuncture (15 ml in a heparinized syringe) and transported to the laboratory. About 4-5 hours elapsed before the leucocytes were separated for culture. The leucocytes were added to a tissue culture medium of T199 with 20% foetal calf serum at a concentration of 7 x $10^5$ per 1 ml of medium. The cultures were harvested according to the method of Moorhead *et al.*[5] and stained with Giemsa.

*Results*: The chromosome counts of the prisoner, his twin brother and the father are shown in Table 1 on the next page. The karyotype of the prisoner was 47,XYY (See Figure 1, also on the next page). Both the non-identical twin and the father had the normal male karyotype of 46,XY.

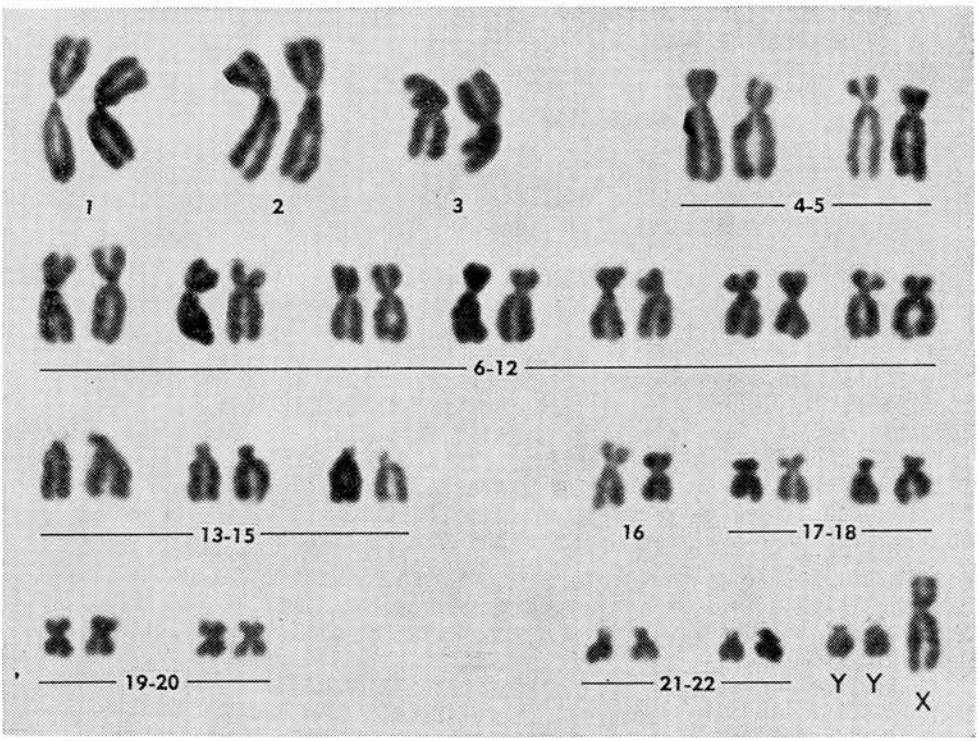
---

5. Moorhouse, P.S., Nowell, P.C., Mellman, W.J., Battips, D.M., and Hungerford, D.A. (1960) "Chromosome Preparations of Leucocytes Cultured from Human Peripheral Blood" Exp.Cell Res. **20,** 613.

TABLE 1. *Chromosome Counts of Prisoner, Twin Brother and Father.*

| Subject | 46 | 47 | Total |
|---|---|---|---|
| Prisoner (H) | 1 | 19 | 20 |
| Twin brother | 20 | — | 20 |
| Father | 20 | — | 20 |

FIGURE 1. *Karyotype showing the extra Y chromosome.*




*Electro-encephalographic Investigation*: This was carried out for two reasons. The first was as a routine investigation, particularly in those found to have chromosomal abnormalities. The second reason was that when the prisoner was first seen he had some historical evidence of periodic clouding of consciousness and since then he has clearly had episodes of motiveless aggressive outbursts with no later recollection.

The report on the electro-encephalographic recording concluded with the comment:

> The record is abnormal in the presence of the background slow activity and the somewhat more marked slow activity in the right temporal region. This is consistent with a diagnosis of mental deficiency,

although it is unusual to find abnormal records other than with the most severe types of deficiency. The focal slow activity suggests that certainly one temporal region is likely to be malfunctioning. Whether this is an epileptic type of disorder must be a clinical decision and the record offers no evidence to help make this (Report by Dr. N. Gordon).

It was concluded finally, on clinical grounds, that H did suffer from a temporal lobe epilepsy and that he might very well have suffered from such a "fit" at the time he stabbed the old lady.

As a result of these further investigations it was reported to the Crown that the earlier opinion (*vide supra*) had been modified so that it was now thought the prisoner was "on a very fine balance" legally irresponsible at the time of the alleged offence. It was also indicated that a trial might well take place although his "fitness to plead" might well change on the day of a trial due to the increase in stress.

*Physical Findings*: Physical examination demonstrated no significant abnormalities. His genitalia were quite normal, he had no acne and had no old scarring from acne. Some physical measurements were recorded and these have been reported elsewhere[6].

*The Court Hearings*

On the 28th July, 1967, H appeared in the Supreme Court of Victoria, in Melbourne (Starke J.), and the preliminary issue of the fitness of the prisoner to plead and to stand his trial was raised pursuant to the provisions of Section 393 of the *Crimes Act,* [1958]. The defence did not contest the Crown's case that the prisoner was unfit to plead and the basis of that case was the evidence of a psychiatrist that H was a mental defective (verbal I.Q. on the WAIS being 65) and that he satisfied the criteria of "unfitness" as set out in *R v Presser,* [1958] V.R. 45. H was found unfit to plead and thus insane within the meaning of Section 393 and was therefore ordered by the court to be kept in strict custody until the Governor's pleasure be known.

H was returned to H.M.Prison Pentridge and remained there until he was again presented before the Supreme Court in Melbourne (Monahan J.) on the 8th October, 1968. On this occasion the issue of fitness to plead was not canvassed before a jury but both counsel assured the trial judge that they were both satisfied that the accused man was, at that time, fit to plead and stand his trial.

As the defence raised was one of insanity, counsel for the accused made a number of admissions on behalf of his client and the Crown case was further expedited by reason of little or no cross-examination of the Crown witnesses. The Crown case closed at about 4 p.m. on the first day and counsel for the defence then indicated that the accused would stand mute and a psychiatrist called as the only witness.

During the few remaining minutes of the first day the psychiatrist was qualified and he indicated to the court the various matters taken into consideration and relied upon in forming his conclusions concerning the psychiatric status of the accused and his responsibility at the time of the

6. Wiener, S., Sutherland, G., Bartholomew, A.A., and Hudson, B. (1968) "XYY Males in a Melbourne Prison" Lancet i 150.

alleged offence. At this stage it was given in evidence that electro-encephalographic examination revealed an abnormal recording with a particular focus in the right temporal lobe — a focal abnormality described as an epileptic focus. It was also stated at this time that a "blood test" was done which revealed an abnormal chromosome constitution, 47,XYY rather than the normal 46,XY, a chromosome constitution which would be found in every cell in his body[7] including the brain. At this point the court adjourned until the following day. And at this same point the medical evidence had not been other than descriptive. There had been no attempt to relate the electro-encephalogram and the chromosome abnormality to the matter of responsibility at the time of the alleged offence.

The following morning H was again examined prior to his being placed in the dock, and the view was expressed that it was doubtful whether he was any longer fit to stand his trial. In the event, the psychiatrist, who was continuing his evidence, was asked a question about the intelligence of the accused. Then the following question was asked and answered:

> Q. These three matters, that is, the E.E.G., the blood test and the general intelligence level, taken together with the general history that you have been able to gather, have you come to some conclusion in respect of the condition of this accused so far as the legal definition of insanity is concerned?
> A. Yes. I consider now that at the time of the alleged offence he was suffering from a mental disease or disorder productive of a defective reason, such that I am sure he did not know that what he was doing was wrong, or at least he could not argue about the wrongness of his act with a reasonable degree of composure.

A further four short questions followed by three general questions in cross-examination ended the medical evidence.

The prosecutor then, in the absence of the jury, indicated to the trial judge that there was doubt about the capacity of the prisoner being fit to continue standing his trial after the first five or ten minutes of the day's hearing. The prosecutor put the matter thus:

> it was thought advisable just to conclude sufficient evidence to found a decision on the lines that both the Crown and the defence desire and then to draw this matter to Your Honor's attention in case Your Honor felt you should investigate this to see whether the trial should continue . . . What we had in mind, Your Honor, is that, having in the first five minutes got the doctor's opinion, perhaps Your Honor might see fit to ask the jury if they want to hear any more.

The trial judge was favourably disposed to this view and remarked

> I will do that, but I should have to give them (the jury) a sort of abbreviated charge, wouldn't I? I would have to warn them that I can only receive one verdict, for instance.

Whilst the trial judge considered that there was merit in the suggestion put to him he, in turn, made the suggestion that before he gave his abbreviated charge, the medical witness should be recalled and give evidence

---

7. It is recognised that the cells in the testes that have undergone reduction division will not contain 47,XYY but the comment was made in general terms.

of his opinion given at the earlier hearing in July 1967 regarding H's unfitness to plead. This was done and the judge then charged the jury.

The charge took the form of explaining the problem of H being possibly unable to continue standing his trial. The judge then said:

> in your absence, the learned prosecutor has suggested to me — and I think it is a proper suggestion that he has made — that I might consult you in regard to this matter and ask you whether you think you could reach a verdict at this stage without further ado. Now the only verdict that I could receive in this case at this stage would be one of not guilty. It is inconceivable that I could receive a verdict of guilty without the case proceeding right to the bitter end. It is equally inconceivable that in this case I could receive a verdict of not guilty *simpliciter,* that is, that the prisoner should be allowed to go free, having regard to the evidence that you have heard . . . Now if you felt at this stage that you had heard enough to enable you to make up your minds that this man committed the act that caused this woman's death, but that he was not criminally responsible for that act because at the time that he did the act he was suffering from such a defect or disorder or disturbance of his mind due to a mental disease as to be unable fully to appreciate the nature and quality of the act that he did, or that he was unable, for that same reason, to appreciate the wrongness of what he was doing, then you may say, at this stage, that you are satisfied he is not guilty of murder because he was insane at the time of the act that is relied upon by the Crown in presenting him here on a charge of murder.

The judge went on to speak of the notion of insanity in the criminal law, and continued his charge:

> you have heard . . . today that this man is just a borderline mental defective. His intelligence quotient is far, far below the average minimum that is regarded as normal. He is less than normal mentally in regard to his intelligence and the doctor has further expressed the opinion that at the time this man — if he did this act, and I do not suppose you have any doubt that he did do it — this man at the time that he did this act was not capable of reasoning with some degree of calmness and composure to consider the rightness or wrongness of the act that he was about to do or perform.

The charge ended with the statement that if a jury should find an accused not guilty on the ground of insanity then Section 420 of the *Crimes Act,* 1958, provides for such a person to be kept in strict custody until the Governor's pleasure is known.

*Discussion*

Two matters are of interest in this case. Firstly, there is the matter of the prisoner being a further example of a male with the karyotype 47,XYY. Secondly there is the importance, or lack of importance, of the 47,XYY karyotype in a criminal trial where the defence of insanity is raised.

As far as the social and medical background of this prisoner is concerned one may only say that he was from the first a mentally retarded individual and that he was always a problem including a history of one

prior conviction, as an adult, for loitering for homosexual purposes. His only skin lesion has been that of sclerema neonatorum and he has no history of acne. One may also note that he is not an alcoholic, or even a problem drinker. Another feature of interest is that he is not unduly tall being only 5' 10" (178cms). In other words, of the triad of features commonly associated with the 47,XYY syndrome H is mentally dull but not particularly tall and with little, from the historical point of view, that may be termed "criminal propensity".

As has been recorded, H has a twin brother who has a normal male karyotype, 46,XY. This karyotype finding only confirms clinical impression; the two twin brothers look different and behave differently, the other twin being of average intelligence.

When one turns to the criminal law and the defence of insanity, and particularly in regard to the trial of H, the first matter to note was the abrupt ending of the trial. This sudden termination of the case meant, as has been indicated, that the medical evidence was not fully presented and not evaluated through the agency of cross-examination. Indeed, the psychiatric evidence, lasting in all perhaps 20 minutes, was little more than a statement of three findings and an assertion of legal insanity, and thus non-responsibility, at the time of the alleged offence. The three findings, mental deficiency, an abnormal electro-encephalogram and the karyotype were described with equal emphasis and only the aspect of mental deficiency was indicated clearly as reducing understanding and thus perhaps produce a state of legal non-responsibility. Although the electro-encephalographic record was described as abnormal with a temporal lobe focus, no evidence was given that at the time of the alleged offence the prisoner very possibly suffered from some epileptic episode. Similarly, although the chromosome constitution was described as abnormal no evidence was given as to any possible significance. Again, because of the swift end to the trial, there was no detailed charge by the judge to the jury, and the only clinical feature the judge commented upon was the mental deficiency. This part of the charge has already been quoted. There was not one word about the electro-encephalogram or the chromosomes.

It is for reasons such as these that one may not contend that the verdict was based on the "extra Y chromosome". One may not even say that the "extra Y chromosome" played any part at all in the verdict. It is, in fact, quite possible that the jury would have returned the same verdict on the basis of his mental deficiency alone, his abnormal electro-encephalograph and his epilepsy alone, or both, without there being any mention of his genetic make up.

Quite apart from this particular case and trial, it must be recognized that the karyotype 47,XYY is a recent finding and is still a rare finding. Fox[7a] writes that the first case was reported in 1961 and that there are still less than a 100 reported cases. Then again, it is very difficult to evaluate our present findings for, as Court Brown *et al* have written,[8]

> the bulk of our information on XYYs is based on the examination of adults found from the surveying of groups of men which by definition consists of men the great majority of whom, if not all, have criminal

---

7a. Fox, R.G. (1969) "XYY Chromosomes and Crime" Aust.N.Z.J.Criminol. **2**, 1, 5.

8. Court Brown, W.M., Price, W.H., and Jacobs, P.A. (1968) "The XYY Male" Brit.med.J. iv 513

> records. The second feature is our lack of knowledge of the incidence of XYY males at birth or in the general adult population of men.

Whilst it may be that the possession of the extra Y chromosome does lead to a greater likelihood of developing a psychopathic personality and becoming involved with anti-social behaviour there is, nevertheless, no reason to suppose that the extra Y chromosome must inevitably lead to such a result. At least two cases have been described recently of "normal" males with the XYY constitution, one a mosaic. Court Brown *et al*[9] has described a case, a mosaic 45,X/47, XYY, or possibly 45,X/47, XYY/46XY, synoptically as follows:

> Normal schooling, leaving at 15 years. Worked as errand boy, then apprentice electrician, and now as trained electrician. No behavioural disturbances. Tends to faint easily. E.E.G. normal. Married with two normal sons both having a 46,XY chromosome complement. Average intelligence.

As the authors comment, such a case raises the general question of "to what extent the presence of a second (and maybe a third) cell line may modify the effects of a 47,XYY line.

In Melbourne, Wiener and Sutherland[10] have given a description of a further "normal" 47,XYY male: a local 41-year-old, single, hospital worker; always considered a placid obedient child; rheumatic fever and poliomyelitis as a child which interfered with schooling; joined army in 1948 and honourably discharged 18 months later; various semi-skilled labouring; never in conflict with law; teetotal; exceptionally fond of his parents with whom he lives; 182cms. tall; non-specific E.E.G. abnormalities IQ 97 on both verbal and performance tests.

As Court Brown *et al*[11] have written in regard to behaviour and XYY sex chromosomes

> our own experience in Edinburgh suggests a spectrum of behaviour ranging from the apparently normal through those with a mild personality defect to those who are severe psychopaths.

If this be a fair comment, and there is no reason to doubt that it is, then an extra Y chromosome cannot, in our present state of knowledge, be accepted as a basis for legal insanity leading to a lack of criminal responsibility. The best that may be said at this time is that

> Studies showing the incidence of chromosome abnormalities among offenders and the mentally ill may provide supporting evidence of the likelihood of mental abnormality in the case of an individual who has a chromosome abnormality[12].

The important word here is "supporting"; but the test for insanity remains a cognitive test and is most certainly not concerned with various aetiological matters. High blood pressure and cerebral arteriosclerosis may produce a mental state satisfying the requirements of the law on insanity (See *R v Kemp* (1956) 40 C.A.R. 121) but an increased blood pressure or arterial disease *per se* is not sufficient. So also is the case with a chromosomal abnormality.

9. Court Brown, W.M., Price, W.H., and Jacobs, P.A. (1968) "Further Information on the Identity of 47,XYY males" Brit.med.J. ii 325.
10. Wiener, S., and Sutherland, G. (1968) "A Normal XYY Man" Lancet ii 1352.
11. Court Brown, W.M., Price, W.H, and Jacobs, P.A. (1968) "The XYY Male Brit.med.J. iv 513.
12. "Medico-Legal" (1968) "Abnormal Chromosome" Brit.med.J. iv 398.

Reprinted from *The Medical Journal of Australia*, 1969, 2: 440 (August 30)

# ALCOHOLISM, DRUG DEPENDENCY AND SEX CHROMOSOME ABNORMALITIES[1]

ALLEN A. BARTHOLOMEW, M.B., B.S., D.P.M., AND G. SUTHERLAND, B.SC.

*Department of Mental Health, Victoria*

**A selected sample of 143 prisoners was investigated for the presence of sex chromosome abnormalities. The incidence of abnormal sex chromosome findings in this criminal population was very low, and any such abnormalities are simply interesting findings. However, there is likely to be an increased incidence of sex chromosome abnormalities in a population of alcoholics.**

THE finding of a 47,XYY karyotype in a well-known criminal, Tait (Wiener *et alii*, 1968; Wiener *et alii*, 1969), and in the highly publicized case of Hannell (Wiener *et alii*, 1968; Bartholomew and Sutherland, 1969), as well as in two further cases of less notoriety (Wiener *et alii*, 1968), led to the notion that, whilst perhaps tallness and a degree of mental retardation might be correlates of the 47,XYY chromosome constitution, the most meaningful correlation might be that of psychopathic personality (Bartholomew, 1968*a*).

If such a proposition be accepted then it is clearly worthwhile looking at a population of "alcoholics" and "drug-dependent" individuals (Bartholomew, 1968*b*). Such persons are not infrequently diagnosed as "psychopaths" and they often have a long history of behaviour disorder that has not responded to a variety of treatment techniques; indeed their abuse of alcohol and/or drugs may date back many years. This comment may not be true of all "alcoholics" but would seem to be true of very many who are seen in a prison setting. Apart from these theoretical considerations it is of interest that Nielsen (1968) describes three patients with 47,XYY chromosome constitutions found in a "state hospital and a forensic psychiatric ward" who were above 180 cm (about 5 ft 11 in.) tall. All three patients might well be termed "psychopathic", all had a full scale IQ of between 92 and 100 and, in addition, all three had "an alcoholic problem". On the other hand, in the review of the XYY problem by Price and Whatmore (1967), it is apparent that of their nine subjects, eight had IQs of between 60 and 80 and, due to their early hospitalization, did not have a chance of demonstrating alcoholism or drug dependence; the mean age of their admission to the State hospital was 20 years and the mean age of their first committal to a hospital was 15 years. In a survey of a further 15 males who had either a 47,XYY complement or were mosaics in whom this cell line predominated (Court Brown *et alii*, 1968*a*), only one of a total of 10 patients aged over 20 years (a 46,XY/47,XYY) had any note regarding alcoholism.

[1] Read at the Fourth Summer School of Alcohol Studies held at St Vincent's Hospital, Melbourne, January 30, 1969.

The problem of the sex chromosomes and alcoholism should not be restricted to the 47,XYY constitution. Nielsen (1964) found four cases of delinquency and three of severe alcoholism in ten cases of Klinefelter's syndrome in a mental hospital in Sweden, whilst Hunter (1968) found two cases of alcoholism in 17 cases of 47,XXY. Fox (1969) possibly puts the matter fairly when he writes:

> There have been some strong suggestions that this defect (47,XXY) is linked with anti-social behaviour, especially alcoholism and homosexuality, but as yet few findings of significance have been reported.

Apart from the interest in the relationship between the sex chromosomes and alcoholism, the opportunity of carrying out this investigation in a prison setting is additionally worthwhile when one recalls the extraordinary claim of Mackay (1967), namely, that "studies have indicated that approximately 50% of tall (over 6 ft) males with criminal tendencies have the XYY constitution".

## METHOD

All that need be said under this heading is that our sample of 143 prisoners is in no way random as regards the prison population. Initially we began looking for subjects who were tall and preferably of dull intelligence. Gradually we thought of a possible correlation between XYY and psychopathy and, later, with our interest in alcoholism and drug abuse (dependency), included alcoholics and those convicted and imprisoned for drunkenness and disorderliness.

The technique adopted for karyotyping was as follows: blood was collected by venepuncture (15 ml in a heparinized syringe) and transported to the laboratory. About 4 to 5 hours elapsed before the leucocytes were separated for culture. The leucocytes were added to a tissue culture medium of T199 with 20% fœtal calf serum at a concentration of $7 \times 10^5$ per millilitre of medium. The cultures were harvested according to the method of Moorhead *et alii* (1960).

## RESULTS

In all, 143 prisoners were karyotyped and eight (5·6%) were recognized as having sex chromosome abnormalities. Of these, four were XYY, one was XYY plus an autosomal abnormality (Wiener *et alii*, 1969), two were XXY, and one was XXY/XY mosaic. Data relating to these eight cases are presented in Table 1.

These cases may be considered as five XYY (3·5%) and three XXY (2%) constitutions in a total of 143 subjects. The figure of 3·5% for XYY patients is very similar to the incidence found by Price and Whatmore

TABLE 1
*Data of Eight Cases Demonstrating Sex Chromosome Abnormalities*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Genotype | XYY[1] | XYY | XYY | XYY | XYY | XXY/XY | XXY | XXY |
| Age (years) (on 1.1.69) | 45 | 22 | 34 | 53 | 39 | 37 | 21 | 42 |
| Height (inches) | 70 | 70 | 82·5 | 71·5 | 73·5 | 64·5 | 69 | 71 |
| Offence (" present ") | Murder | Murder[2] | Attempted murder | Larceny, perjury, breaking and entering, Assault with a weapon | Larceny | Gross indecency | Shop-breaking, malicious damage, Resisting arrest with a firearm | Buggery |
| IQ (full-scale WAIS) | 94 | 71 | 101 | 84[3] | 65 | 105 | 75 | Not tested (passed "Intermediate") |
| Previous imprisonment | Several | None | Very many | Very many | Several | None | Two | None |
| Maudsley personality inventory (extraversion/neuroticism) | 21/12 | 18/10 | 22/6 | 14/14 | — | 10/15 | — | 13/12 |
| Alcoholic/drug dependent | + | − | − | + | − | + | + | + |

[1] Plus a further autosomal abnormality.
[2] Found " not guilty on the grounds of insanity ".
[3] An unreliable figure; the prisoner tested quite differently at different times.

(1967) in Carstairs, but it must be appreciated that while some of our 143 men were selected in terms of height, none of them were considered suitable for transfer to an institution for the mentally defective or mentally ill.

Because of our interest in alcoholism, we divided the population of 143 into two groups: (i) non-alcoholics (including non-drug takers), of which there were 47, and (ii) alcoholics (including drug takers), of which there were 96. The alcoholics we further divided into: (i) those sentenced for being "drunk and disorderly" (36 cases), and (ii) "serious offenders" considered as alcoholic (60 cases) (see Bartholomew, 1968*c*).

## Non-Alcoholics

Among the 47 subjects considered as non-alcoholic (and non-drug abusers) there were three with sex chromosome abnormalities, and in each case the karyotype was 47,XYY (see Table 1). We say no more about this group.

## Alcoholics

### *The "Drunk and Disorderly"*

There were 36 cases in this group. The mean age was 46 years (range, 23 to 72 years) and the mean number of previous imprisonments was 46. The distribution in terms of height was as follows: nine were under 70 in., 20 were between 70 and 72 in. and seven had a height in excess of 6 ft. The problem of assessing the IQ in such a group is considerable and, although Raven's Matrices were used, only 20 in the group either could, or would, attempt the test, and only nine finished it. It is thought that at least two-thirds of the group had an IQ of between 90 and 110, or had had such an intelligence before their deterioration through drink and general neglect. No men in this group admitted taking any drugs other than ethyl alcohol and methylated spirits. The psychiatric diagnoses of these men would be various but it is likely that the majority would be classed as "psychopaths" or "inadequate personalities". This was the diagnosis given for 17 of the men whose hospital records were available.

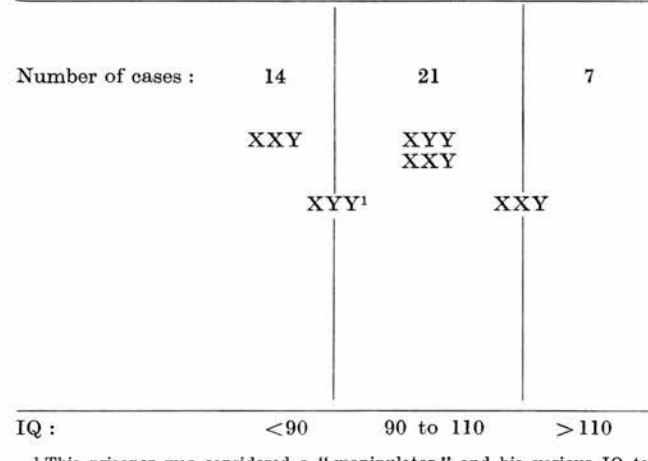
There was not one case of sex chromosome abnormality in this group.

### *The "Serious Offender" Alcoholic*

In this group of 60 there were five patients with sex chromosome abnormalities (8·3%). It was thought worthwhile dividing the 60 prisoners into three subgroups: (i) alcoholics *simpliciter* (42 cases), (ii) alcoholics also taking, or having taken, drugs (14 cases), and (iii) those who were drug dependent, taking little or no alcohol (4 cases).

*Alcoholics Simpliciter.*—This subgroup of 42 patients had a mean age of 29 years, with a range of 17 to 53 years. Previous imprisonments averaged approximately eight per

FIGURE 1: Distribution of the "serious offenders" considered as alcoholics *simpliciter* in terms of IQ, and of the five subjects with sex chromosome abnormalities.




[1] This prisoner was considered a " manipulator " and his various IQ test results unreliable. His IQ was variously assessed as between 65 and 100 in different hospitals and at different times.

man and many of the prisoners appeared to have evidence of officially recognized delinquency in their childhood. The IQ ranged from about 65 to 115, with a mean value of 93. The distribution of the subgroup in terms of the IQ is shown in Figure 1, which also indicates the position of the five cases in which there was sex chromosome abnormality. The distribution of the subgroup in relation to height is shown in Figure 2. It is worth noting that of the 13 prisoners whose height was in excess of 6 ft,

eight had an IQ of less than 90: in other words, they might well have been thought to be likely to possess the additional Y chromosome.

*Alcoholics who were Also Drug Abusers.*—For convenience we have considered the 14 alcoholic prisoners who were taking or had taken other drugs with the very small group of four prisoners who were drug dependent (and who frowned upon the taking of alcohol). In this group of 18 there were no cases of sex chromosome abnormality.

FIGURE 2: Distribution of the "serious offenders" considered as alcoholics *simpliciter* in terms of height, and of the five subjects with sex chromosome abnormalities.

| | <5 ft 10 in. | 5 ft 10 in. to 6 ft 0 in. | >6 ft 0 in. |
|---|---|---|---|
| Number of cases : | 10 | 19 | 13 |
| | XXY<br>XXY | XYY<br>XYY<br>XXY | |

These 18 prisoners were nearly identical with the alcoholics *simpliciter* in terms of age distribution, previous imprisonments, height and intelligence. They were, however, distinguished to some extent by a greater "degree" or severity of psychopathy, by greater social disorganization, more mental hospital admissions, more suicide attempts and, of course, by the taking of drugs.

It is difficult to assess histories relating to the taking of drugs, and one is aware that some of the stories may be considerably exaggerated or even invented. All 18 prisoners who alleged they took drugs claimed that they had smoked "pot", often regularly and over a reasonably long period of time. Five of the 14 alcohol-drug takers and all four pure drug takers claimed to have taken "trips" with LSD, six of them more than twice and four of them in excess of three times. Two of these individuals also claimed to have been treated by psychiatrists with LSD or equivalent drugs. Twelve of the prisoners, including the four pure drug takers, claimed to have taken, on occasions, morphine, heroin and cocaine. All 18 claimed to have taken both barbiturates and amphetamines over a long period of time and in considerable quantities. These drugs were taken either by mouth or by intravenous injection.

All four of the pure drug takers were of dull average intelligence, all were between 5 ft 10 in. and 6 ft tall, all had a number of previous imprisonments, all had had a disturbed childhood (two were expelled from school), and three were considered homosexual, one of these being a homosexual prostitute.

## DISCUSSION

It has to be admitted that this paper presents negative findings, in that the incidence of abnormal sex chromosome findings in a criminal population is not great; indeed, it is very small. Our figure for the incidence of such abnormalities in a prison population is in the vicinity of 5%, with a slightly higher figure in the case of "alcoholics" who are "serious offenders". It should be noted that among our "serious offenders" there were 13 whose height was in excess of 6 ft, so that if Mackay (1967) was correct in his comment we should have found at least six cases of 47,XYY which would, alone, give a 10% incidence of sex chromosome abnormalities. In fact, the two patients with 47,XYY constitution were both under 6 ft in height. Thus the comment of Mackay is not supported by these findings.

A matter which should be stressed is that the extra Y chromosome has been given great publicity in relation to criminal behaviour, and psychopathy in general. However, the findings presented here do suggest that the extra X (Klinefelter's syndrome) is to be considered just as carefully as the extra Y chromosome. Our results support the comment of Court Brown *et alii* (1968*b*) when they wrote:

> To call an extra Y chromosome the "criminal" chromosome as has been done by the popular news media seems odd, when no such notoriety is accorded the extra X chromosome in XXY males, for there are reasons to believe that they also incur some added risk of behavioural disturbances.

On the other hand it should be recorded that in some 170 consecutive male alcoholics investigated in the alcoholism out-patient clinic at St Vincent's Hospital, Melbourne, not one chromatin-positive patient was found (Garson, 1969).

Another feature of interest in our results is that the five prisoners found to be XYY were by no means, as a group, the most severe psychopaths that one sees in a forensic psychiatric practice. Further, as has been shown (Table 1), the four prisoners able to do the test tended to score quite highly on extraversion and low on neuroticism as assessed on the Maudsley Personality Inventory (Bartholomew, 1963).

This very preliminary piece of research indicates that sex chromosome abnormalities cannot be seen as anything very much more than interesting findings in this population and that such abnormalities may correlate with personality difficulties and behaviour disorders, but cannot be seen as being a causal factor in either criminality or alcoholism, including drug dependency. One needs to remember the cases now being described of "normal" men who have a 47,XYY constitution or a 47,XYY mosaic (Wiener and Sutherland, 1968; Court Brown *et alii*, 1968*a*). However, there would seem little doubt that there is likely to be an increased incidence of sex chromosome abnormalities in a population of alcoholics. If the present estimates of an incidence in the general male population of 0·2% for the 47,XXY constitution and less than 0·2% for the XYY karyotype are accepted, then incidence figures in a deviant population are likely to be greater, perhaps especially in those with a low and low average intelligence.

## ADDENDUM

Since the original paper was written, a further 21 males have been karyotyped and this has resulted in the identification of a prisoner with a 47,XXY karyotype and, a prisoner with 47,XYY karyotype. The XXY individual is a male aged 31 years, and 69 in. tall. He was convicted of arson, has a full-scale Wechsler's adult intelligence scale (WAIS) rating of 95, has one previous conviction (for buggery), and is considered to be an alcoholic. The XYY individual is a youth aged 17 years who is 71 in. tall, was convicted of buggery, has a full-scale WAIS rating of 85, has no previous convictions, and is not considered to be an alcoholic or drug-dependent person. He is considered to be a confirmed homosexual and was earlier diagnosed as a schizophrenic who was successfully treated. This second patient has been under the care of Dr L. Chatz, whom we thank for the information made available to us.

## REFERENCES


BARTHOLOMEW, A. A. (1963), "Some Comparative Australian Data for the Maudsley Personality Inventory", *Aust. J. Psychol.*, 15: 46.

BARTHOLOMEW, A. A. (1968*a*), "The Extra Y Chromosome and Criminal Behaviour", Editorial annotation, *Aust. N.Z. J. Psychiat.*, 2: 6.

BARTHOLOMEW, A. A. (1968*b*), "The Psychological and Psychiatric Aspects of Drug Addiction", MED. J. AUST., 2: 1068.

BARTHOLOMEW, A. A. (1968*c*), "Alcoholism and Crime", *Aust. N.Z. J. Criminol.*, 1: 70.

BARTHOLOMEW, A. A., and SUTHERLAND, G. (1969), "A Defence of Insanity and the Extra Y Chromosome: R. versus Hannell", *Aust. N.Z. J. Criminol.*, 2: 29.

COURT BROWN, W. M., PRICE, W. H., and JACOBS, P. A. (1968*a*), "Further Information on the Identity of 47,XYY Males", *Brit. med. J.*, 2: 325.

COURT BROWN, W. M., PRICE, W. H., and JACOBS, P. A. (1968*b*), "The XYY Male", *Brit. med. J.*, 4: 513.

FOX, R. G. (1969), "XYY Chromosomes and Crime", *Aust N.Z. J. Criminol.*, 2: 5.

GARSON, O. M. (1969), A comment made from the floor at the Fourth Summer School of Alcohol Studies, St Vincent's Hospital, University of Melbourne, January 30, 1969.

HUNTER, H. (1968), "Klinefelter's Syndrome and Delinquency", *Brit. J. Criminol.*, 8: 203.

MACKAY, E. V. (1967), "Disorders of the Sex Chromosomes", MED. J. AUST., 2: 552.

MOORHEAD, P. S., NOWELL, P. C., MELLMAN, W. J., BATTIPS, D. M., and HUNGERFORD, D. A. (1960), "Chromosome Preparations of Leucocytes Cultured from Human Peripheral Blood", *Exp. Cell Res.*, 20: 613.

NIELSEN, J. (1964), "Klinefelter's Syndrome and Behaviour", *Lancet*, 2: 587.

NIELSEN, J. (1968), "The XYY Syndrome in a Mental Hospital", *Brit. J. Criminol.*, 8: 186.

PRICE, W. H., and WHATMORE, P. B. (1967), "Behaviour Disorders and Patterns of Crime Among XYY Males Identified at a Maximum Security Hospital", *Brit. med. J.*, 1: 533.

WIENER, S., SUTHERLAND, G., BARTHOLOMEW, A. A., and HUDSON, B. (1968), "XYY Males in a Melbourne Prison", *Lancet*, 1: 150.

WIENER, S., and SUTHERLAND, G. (1968), "A Normal XYY Man", *Lancet*, 2: 1352.

WIENER, S., SUTHERLAND, G., and BARTHOLOMEW, A. A. (1969), "A Murderer with 47,XYY and an Additional Autosomal Abnormality", *Aust. N.Z. J. Criminol.*, 2: 20.


AUSTRALASIAN MEDICAL PUBLISHING COMPANY LIMITED
71-79 Arundel Street, Glebe, Sydney, N.S.W., 2037

# Chromosome Survey in a Security Ward: Total Ascertainment

G. SUTHERLAND and A. A. BARTHOLOMEW

*Reprinted from the Australian and New Zealand Journal of Criminology.*

# *Chromosome Survey in a Security Ward: Total Ascertainment*

G. SUTHERLAND* and ALLEN A. BARTHOLOMEW**

THE notion of the increased incidence of the additional Y chromosome in a population of antisocial individuals, particularly amongst those who tend to be tall and dull, is becoming generally accepted. To these features might be added the diagnosis of psychopathic personality (Bartholomew, 1968). However, whilst the number of case reports of XYY individuals is increasing, there are few reported surveys of total ward or hospital populations. Price and Whatmore (1967) found two cases of XYY in the population of 119 in the West Wing of the Carstairs Security Hospital which was composed of mentally ill patients who "exhibited dangerous, violent or criminal propensities".

We have recently carried out a karyotype survey in the maximum security ward for the male mentally ill (including some who are mentally retarded) in the State of Victoria. The ward is attached to a general mental hospital (Aradale). The ward accommodates 50 patients; during the time of the study 55 patients were investigated.

*Method*

The technique of karyotyping has been described elsewhere (Wiener et al., 1969).

*Results*

Karyotype: Three abnormal karyotypes were detected. Two were 47, XYY and the third was 46,XY, 16q+.

The Population: This had a mean age of 36.2 years (range 19-60 years) and a mean duration of intermittent or continuous mental hospitalization of 17 years (range 1-34 years). The patients were held under a number of different sections of the *Mental Health Act,* 1959; the majority (51) being "certified" under some section of the Act.

Diagnosis: On the basis of a study of the clinical records we have categorised the population under three headings:

| | |
|---|---|
| Schizophrenic | 41 |
| Psychopathic Personality | 11 |
| Chronic Brain Syndrome and Epilepsy | 3 |

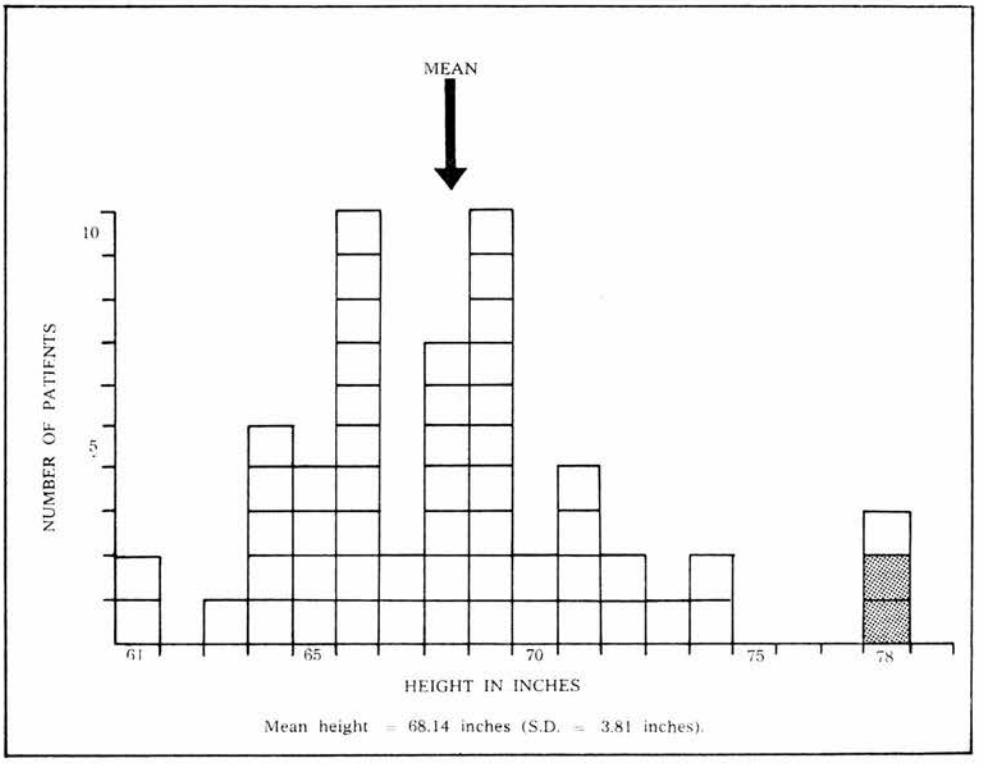
A number of patients in all groups were considered to be "mildly mentally retarded" but this was only a clinical impression.

---

* B.Sc., Geneticist, St. Nicholas Hospital, Melbourne.
** M.B., B.S., D.P.M., M.A.N.Z.C.P., Psychiatrist Superintendent, H.M. Prison, Pentridge, Melbourne.

**Height:** The height distribution of this population is shown in Figure 1.

FIGURE 1. *The distribution of height in the population with the two cases of 47,XYY being hatched.*




**Clinical Features:** These, as they relate to the two cases of 47, XYY, are set out in Table 1.

TABLE 1. *Clinical information concerning the two patients with the 47,XYY karyotype.*

| | A | B |
|---|---|---|
| Age | 25 years | 42 years |
| Height | 78 inches | 78 inches |
| Intelligence | WAIS (F.S.) 88 | "Dull" |
| Diagnosis | Schizophrenic | Psychopath and borderline defective |
| Age of first hospitalization | 14 years | 31 years |
| Age started present hospitalization | 21 years | 34 years |
| Previous criminal record | None | A number of property offences |

*Discussion*

The incidence of two cases of 47,XYY in a population of 55 patients is larger than that found by Price and Whatmore (1967) in their West Wing.

However, it is difficult, if not impossble, to compare such institutions. In Victoria, for example, men found not guilty on the grounds of insanity or found unfit to plead are not automatically sent to security hospital wards (Bartholomew and Sutherland, 1969a), wards where there is also no clear separation between "mentally subnormal patients" and those suffering from "mental illness".

Although family studies were not possible, the aberrant chromosome in the patient with 46,XY, 16q+ was considered to be a normal variant (Court Brown *et al.*, 1966); the patient was 68 inches tall and diagnosed as suffering from schizophrenia.

Our results support the correlation between the extra Y chromosome and height but any correlation with psychopathy cannot be deduced due to the small sample size.

It is of interest that no case of 47,XXY was found in this study as in a karyotype survey of prisoners we found four such cases in 164 prisoners (Bartholomew and Sutherland, 1969b).

ACKNOWLEDGEMENTS

WE should like to thank Dr. Saul Wiener for his interest and encouragement during this study and for his critical reading of our manuscript. We thank Mr. Ian Barker for technical assistance and Dr. P. Woods, the Psychiatrist Superintendent of Aradale Hospital, for his permission to investigate the patients.

REFERENCES

Bartholomew, A. A. (1968) The Extra Y Chromosome and Criminal Behaviour, Editorial Annotation. Aust.N.Z.J.Psychiat. **2** 6

Bartholomew, A. A., and Sutherland, G. (1969a) A Defence of Insanity and the Extra Y Chromosome: *R v Hannell*. Aust.N.Z.J.Criminol. **2** 29

Bartholomew, A. A., and Sutherland, G. (1969b) Alcoholism, Drug Dependency and Sex Chromosome Abnormalities. Med.J.Aust. ii 440

Court Brown, W. M., Buckton, K. E., Jacobs, P. A., Tough, I. M., Kuenssberg, E. V., and Knox, J. D. E. (1966) Chromosome Studies on Adults. Eugenics Laboratory Memoirs XLII. Cambridge University Press, London.

Price, W. H., and Whatmore, P. B. (1967) Behaviour Disorders and Pattern of Crime Among XYY Males Identified at a Maximum Security Hospital. Brit.med.J. i 533.

Wiener, S., Sutherland, G., and Bartholomew, A. A. (1969) A Murderer with 47,XYY and an Additional Autosomal Abnormality. Aust.N.Z.J.Criminol. **2** 20.

*Aust. J. Ment. Ret.*
(1970) 1: 77-81

# CHROMOSOME ABNORMALITIES IN NEWBORN BABIES

GRANT R. SUTHERLAND

**Reprinted from**
**The Australian Journal of Mental Retardation**
**Vol. 1, No. 3, September 1970, Pages 77-81**

*Aust. J. Ment. Ret.*
(1970) 1: 77-81

# CHROMOSOME ABNORMALITIES IN NEWBORN BABIES

GRANT R. SUTHERLAND

SUMMARY

The incidence of chromosome abnormalities in the population is discussed with emphasis on current work being done on the screening of newborn babies. Sampling techniques and sample size are discussed. Findings from surveys so far available are summarized and some of their possible implications regarding the Australian population are considered.

Since the associations between chromosomal abnormality and clinical syndromes of maldevelopment were first described in 1959 (Lejeune *et al.*, Jacobs and Strong., Ford *et al.*, Jacobs *et al.*) there has been increasing interest in the epidemiology of all the chromosome abnormalities in both institutional and the general populations. With the knowledge that chromosomal and developmental abnormalities need not be causally related (Carr, 1963, Pitt *et al.*, 1964) it has become increasingly desirable for data on the incidence of chromosomal abnormalities in the population at birth to be obtained. This data is essential to determine the significance of chromosome abnormalities detected in individuals and for comparison with the frequency of such abnormalities as may be found in highly selected groups.

## TECHNIQUES

There are three main techniques which may be used for the detection of individuals with chromosome abnormalities. Each has its advantages and limitations.

1. Recognition of a clinical syndrome which results directly from a chromosome abnormality. This is very useful for the well defined syndromes such as the autosomal trisomies, since very large numbers at risk can be surveyed by routine clinical examination; doubtful and atypical cases may however be missed. Hence any estimates of frequency from this approach will always be minimum estimates. The rare and more obscure syndromes will probably not be diagnosed in the neonatal period.

2. Sex chromatin studies can identify most cases involving numerical abnormalities of the X chromosome, and much less reliably, some structural abnormalities. Sex chromatin determination can be performed relatively easily on the buccal mucosal cells, or, if done at birth, on amniotic membrane cells (Mikamo, 1968). A serious limitation of this technique is that most cases of mosaicism will be missed; this is especially so since the incidence of mosaicism in Turner's Syndrome is probably higher than in any other chromosome disorder (Hecht and MacFarlane, 1969). The recently reported technique for identifying the Y chromosome in interphase nuclei (Pearson *et al.*, 1970) may enable large populations to be screened for Y chromosome abnormalities using buccal mucosal or amniotic membrane cell methods. The sensitivity and reliability of this technique must await further study.

3. Chromosome analysis. This is the only technique capable of detecting all chromosome abnormalities. However, full chromosome analysis is an expensive and time consuming procedure, involving the microscopy of about 30 cells per case with photographic analysis of a few of these cells and the preparation of a karyotype. Even then a few cases of mosaicism with small numbers of non-modal cells will be missed. In a survey of selected newborns, Walzer *et al.* (1969) showed that this technique can be abbreviated to the study of only two cells with very little loss of sen-

Received 10th July, 1970.
1. (Mr.) Cytogeneticist, Chromosome Laboratory, St. Nicholas Hospital, Carlton South, Victoria, Australia, 3053.

**TABLE 1**

**Frequency of Sex Chromatin Anomalies in Neonates**
(Court Brown, 1968)

| Anomaly | Incidence |
|---|---|
| Chromatin positive males | 1.7/1000 males |
| Chromatin negative females | .3/1000 females |
| Doubly chromatin positive females | 1.1/1000 females |

sitivity of the method. Most, if not all mosaics would escape detection using this method, but it is the best technique available for screening work, and will probably remain so until computer analysis is fully developed and a full chromosome analysis can be performed on each case.

## PROGRESS

From clinical recognition of a syndrome, much data is available for the most common autosomal abnormality, mongolism, 47,21+[2] As early as 1933, before the chromosomal basis of this syndrome was confirmed, Jenkins estimated its frequency as 1 in 636. This differs little from the incidence of 1 in 688 which was found by Collman & Stoller (1962) in a survey of all live births in Victoria from 1942 until 1957. This is the most comprehensive study yet published, and by far the most reliable estimate, since the number at risk was 780,168, from which 1134 mongols were ascertained. The frequencies of Edwards' (47,E+) and Patau's (47,D+) syndromes have been estimated by Taylor (1968) as 1 in 6766 and 1 in 7602 respectively, however, due to their rarity and the numbers involved, these figures must be accepted as very approximate. Probably the next most common autosomal syndrome is the 'cri-du-chat' (46, Bp−) which has an estimated incidence of between 1 in 50,000 and 1 in 100,000 (Polani, 1970). The other autosomal syndromes such as 18p−, 18q−, 13q−, Dr, etc., are all extremely rare, not many cases of each having been described, and any estimates of their frequency must await further study.

The sex chromatin anomalies and their frequencies are listed in Table 1. Chromosome analysis following detection (Court Brown, 1969), reveals that of the chromatin positive males, 1.3/1000 have straight Klinefelter's Syndrome, 47,XXY, and .4/1000 are mosaics, mainly 46,XY/47XXY; males with more than two X chromosomes are very rare. Almost all chromatin negative females have Turner's Syndrome, 45,X. The doubly chromatin positive females are mainly 47,XXX (1.0/1000) the remainder being mosaics; females with more than three X chromosomes are extremely rare.

Chromosome analysis of large numbers of newborns has commenced only fairly recently, and so far results for the major chromosome abnormalities are available for 1015 females and 6784 males. The main reason for this sex ratio is that it is largely interest in Y chromosome abnormalities which has prompted these studies. It is also probable that information obtained from the males about translocations and the minor chromosome anomalies (Dp+, Gp+, 17ps, etc) will also be valid for females. In fact, abbreviated chromosome analysis of females may prove unrewarding since it is probably no more sensitive than sex chromatin determination in detecting chromosome anomalies limited to females. Of the 1015 fe-

2. Chromosome complements are expressed the Chicago notation (Chicago Conference, 1966). However when both sexes are involved the sex chromosome complement is omitted, e.g., for mongols of both sexes 47,21+ rather than 47,XX (or XY),21+. In some cases sex chromosome abnormalities may be referred to only by the sex chromosome complement, e.g., for Klinefelter's Syndrome XXY instead of 47,XXY.

**TABLE 2**

**Results of Chromosome Screening of Newborn Males**

| Series | Nos. studied | Abnormalities | | | | |
|---|---|---|---|---|---|---|
| | | XYY | XXY | Mongols | Trans-locations | Others |
| Sergovich et al., (1969) | 1066 | 4 | 1 | — | 1 | — |
| Lubs and Ruddle (1969) | 2222 | 3 | 4 | 2 | 2 | 1 |
| Ratcliffe et al., (1970) | 3496 | 5 | 3 | 5 | 6 | 1 |
| TOTAL | 6784 | 12 | 8 | 7 | 9 | 2 |

males studied (Sergovich et al., 1969), two were mongols, one had the 'cri-du-chat' syndrome, and one was a t(DqDq) translocation carrier, the only normal infant whose chromosome abnormality would have gone undetected by clinical examination. The results for the males are shown in Table 2. Here the incidence of major abnormality is 5.6/1000.

All the XYY and most of the translocation carriers would have gone undetected by clinical examination and most of the XXY would have required sex chromatin determination for their detection.

### A NOTE ON SAMPLE SIZE AND TECHNIQUE

Probably the best method of determining the incidence of chromosomal abnormalities in newborns is the study of consecutive live births in a hospital or group of hospitals. There are, however limitations on a sample ascertained in this way. The hospital or hospitals concerned may serve only a limited section of the population. In a survey being carried out in Melbourne (Wiener et al., unpub.) most of those tested have come from the economically depressed classes and include a high proportion of immigrants. This is a difficult problem to overcome. In countries where expected normal births are domicillary, those born in hospital would again be a selected group. There is some evidence (Mikamo 1968) that there is seasonal variation in the incidence of X chromosome abnormalities; it may be wise to conduct surveys over yearly periods. The results of Walzer et al. (1969) are not included in Table 1 because of their method of sampling. They excluded all infants with significant malformation, any condition requiring special medical or nursing care, those delivered by caesarean section and those with low birth weight (less than 5 pounds). In view of the report by Chen et al. (1970) of an association between low birth weight and chromosome abnormality in mentally retarded patients, they may have selected out many cases of chromosome abnormality.

Sample size is of course important if the results are to be meaningful. If the frequency of XYY males from the above data is considered, the problem can be illustrated. The observed frequency is 12 in 6784, which is about .18%, however the 95% confidence limits range from .08% to .28%. The question which immediately arises is how many neonates will have to be screened to give reliable incidence figures? It can be shown that for 95% confidence limits (Hoel, 1960, p. 153) the number necessary $= \frac{3.8416\ (1-p)p}{e^2}$ where p is the estimated frequency of occurrence and e is the permissible error. To be able to state, with 95% confidence, that the frequency of XYY males lies between 1.5 and 2/1000 it is necessary to examine 107,377 newborn males.

### DISCUSSION

Many more newborns must be screened before even the relative frequencies of the various chromosome abnormalities can be established. On theoretical grounds it has been suggested that XYY should be less frequent than XXY (Court Brown, 1968), however if the above figures are considered then XYY is the most frequently observed chromosome abnormality in males, followed by XXY, and mongolism, the rarer syndromes being lead by Edwards' and Patau's. The position of translocation carriers in this incidence ranking is uncertain. Court Brown et al. (1966) found about .5% of adults were heterozygous for a structural rearrangement. In the 7799 newborns studied so far, there have been only 10 with translocations, and at least two of these had Patau's Syndrome. Hence the observed incidence of structural rearrangements in apparently normal infants is not more than 8 in 7799, which is significantly different from .5%.

It is of interest to apply the incidence figures to the Australian population. In 1967 there were 229,296 live births in Australia (Year Book Australia, 1969). Of these it is expected that there were about 330 mongols, 210 XYY males, 150 XXY males and about 3 cases each of Edwards' and Patau's Syndromes. There would have been about 35 45,X Turner's Syndrome cases and about 120 XXX females. Many of those with a chromosome anomaly have a higher mortality rate than normal children. Edwards' and Patau's Syndrome cases usually do not reach 6 months of age, mongols have a higher infant mortality rate than normals, and those who live to adulthood have a lower life expectancy than either normals or other non-mongol retardates. Turner's syndrome is often associated with congenital heart disease which increases the mortality rate. There is some evidence that XXY males have a slightly increased neonatal mortality, but this has not been determined with certainty (Court Brown, 1969). From the history of childhood illness of many XYY males who

have been detected in adulthood, there is the possibility that they may be selected against, but to what degree, if any, must await further study.

A major question is what happens to those who survive? The majority of the autosomal trisomies either die in early childhood or spend their lives as residents of institutions for the mentally defective. The Turner's Syndrome cases who survive have normal mental development and probably normal physical development until puberty, when they may present as endocrine problems, due to failure of puberty or short stature, where they may be correctly diagnosed for the first time, or as infertility problems if their chromosomal status escapes detection until after marriage. Those individuals who have an extra X chromosome (XXY, XXX) have a tendency to be mildly mentally retarded and to have behaviour problems which may bring them into conflict with the law. The frequency of XXY males in hospitals for the mentally subnormal is 7.6/1000 males and of XXX females is 5.6/1000 females (Court Brown, 1968). Bartholomew and Sutherland (1969) found 4 XXY males in 164 prisoners. However not all those with an extra X chromosome are retarded or behave abnormally. Some of the XXY males may be detected by partial failure of puberty or at infertility clinics after marriage. Some of the XXX females will develop normally, both mentally and sexually, and are fertile. The relative proportions which follow each course of development are unknown.

There is even less knowledge of what happens to the XYY males. However, certain things are known; they grow taller than XY males; they are over-represented in prisons and hospitals for the criminally insane; they may develop perfectly normally (Wiener & Sutherland, 1968). They are fertile but may be hypofertile. They would be extremely difficult to give genetic advice, since most of those with abnormal children have been ascertained because of this. There is only one known case where an XYY male may have passed his abnormality to a son (Sundequist and Hellström, 1969) and in no known cases have XXY offspring been produced. It would appear that a newborn XYY male would have about a twenty fold increased chance of ending up in a prison or hospital for the criminally insane, since the frequency of XYY at birth is about .18% and in such institutions is about 3% (Court Brown, 1968).

With so much uncertainty about the development of some of these individuals with chromosome abnormalities, there arises the problem of what to do when one is identified as having such an abnormality. This problem has been discussed in a write-in symposium (Amarose & Burks, 1969) and the diversity of opinions on many of the aspects discussed, reflects the need for more information regarding the development of persons with chromosome disorders.

What may we expect to gain from the chromosome screening of newborns? Apart from the basic information regarding the frequency of the various abnormalities, there is the identification of the affected individuals. This presents an invaluable opportunity to study their development and perhaps to answer some of the questions which still remain about this. There is also the possibility of prophylatic treatment of some of these disorders. There is some evidence that hormone replacement therapy is helpful to XXY males especially if given in early adolescence, effecting an improvement of secondary sexual characteristics and possibly in having a beneficial effect on behavioural patterns (Myhre et al., 1970). There is evidence that similar therapy can help maintain secondary sexual characteristics in Turner's Syndrome and that administration of human growth hormone may lead to some increase in the height of these girls, most of whom are less than 5 feet tall (Tzagournis, 1969).

In the future it may become possible to screen all newborns for chromosomal and metabolic abnormalities and to treat these by some form of genetic engineering. It will be the information provided by the present surveys which will largely determine whether such mass screening would be economically feasible.

**ACKNOWLEDGMENT**

I thank Mr. G. C. Webb for considerable assistance with statistics, and the Mental Health Authority for permission to publish this paper.

**REFERENCES**

Amarose, A. P. and Burks, J. L. (1969) (Eds.) Disclosure of genetic information. An invitational symposium. *J. Reprod. Med.* (Lying-In), **2:** 212.

Bartholomew, A. A. and Sutherland, G. (1969). Alcoholism, drug dependency and sex chromosome abnormalities. *Med. J. Aust.*, **2:** 440.

Carr. D. H. (1963). Chromosomal abnormalities and their relation to disease. *Canad. med. Ass. J.*, **88:** 456.

Chen, A. T. L., Sergovich, F. R., McKim, J. S., Barr, M. L. and Gruber, D. (1970). Chromosome studies in full-term, low-birth-weight, mentally retarded patients. *J. Pediatrics*, **76:** 393.

Chicago Conference (1966). Standardisation in Human Cytogenetics. Birth Defects. Original Article Series II.2. New York.

Collman, R. D. and Stoller, A. (1962). A survey of mongoloid births in Victoria, Australia, 1942-1957. *Am. J. Pub. Hlth.*, **52:** 813.

Court Brown, W. M. (1968). Cytogenetic studies on humans: Some population aspects. *Proc. R. Soc. Med.*, **61:** 164.

Court Brown, W. M. (1969). Sex chromosome aneuploidy in man and its frequency with special reference to mental subnormality and criminal behaviour. *Int. Rev. Exp. Path.*, **7:** 31.

Court Brown, W. M., Buckton, K. E., Jacobs, P. A., Tough, I. M., Kuenssberg, E. V. and Knox, J. D. E. (1966). Chromosome Studies on Adults. Eugenics Laboratory Memoirs XLII. Cambridge University Press. London.

Ford, C. E., Jones, K. W., Polani, P. E., DeAlmeida, J. C. and Briggs, J. H. (1959). A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's syndrome). *Lancet* (i): 711.

Hecht, F. and MacFarlane. J. P. (1969). Mosaicism in Turner's syndrome reflects the lethality of XO. *Lancet* (ii): 1197.

Hoel, P. C. (1960). Elementary Statistics. John Wiley & Sons Inc. New York.

Jacobs, P. A., Baikie, A. G., Court Brown, W. M., MacGregor, R. N., Maclean, N. and Harnden, D. G. (1959). Evidence for the existence of the human 'super-female'. *Lancet* (ii): 423.

Jacobs, P. A. and Strong, J. A. (1959). A case of human intersexuality having a possible XXY sex determining mechanism. *Nature* (Lond.), **183:** 302.

Jenkins, R. L. (1933). Etiology of Mongolism. *Am. J. Dis. Child.*, **45:** 506.

Lejeune, J., Gautier, M. and Turpin, R. (1969). Etude des Chromosomes somatiques de neuf enfants mongolien. *C.R. Acad. Sci.* (Paris), **284:** 1721.

Lubs, H. A. and Ruddle, F. H. (1969). Personal communication to Ratcliffe *et al.* (1970).

Mikamo, K. (1968). Sex chromatin anomalies in newborn infants. A 3 year survey of fetal membranes. *Obstets. Gynec.*, **32:** 688.

Myhre, S. A., Ruvalcaba, R. H. A., Johnson, H. R., Thuline, H. C. and Kelly, V. C. (1970). The effects of testosterone treatment in Klinefeter's syndrome. *J. Pediatrics*, **76:** 267.

Pearson, P. L., Bobrow, M. and Vosa, C. G. (1970). Technique for identifying Y chromosomes in human interphase nuclei. *Nature* (Lond.), **226:** 78.

Pitt, D. B., Ferguson, J. and Baikie, A. G. (1964). Normal and abnormal sibs with a familial chromosomal translocation. *Aust. Ann. Med.*, **13:** 178.

Polani, P. E. (1970). The incidence of chromosomal malformations. *Proc. R. Soc. Med.*, **63:** 50.

Ratcliffe, S. G. Stewart, A. L., Mellville, M. M., Jacobs, P. A. and Keay, A. J. (1970). Chromosome studies on 3500 newborn infants. *Lancet* (i): 121.

Sergovich, F., Valentine, G. H., Chen, A. T. L., Kinch, R. A. H. and Smout, M. S. (1969). Chromosome aberrations in 2159 consecutive newborn babies. *New Eng. J. Med.*, **280:** 851.

Sundequist, U. and Hellström, E. (1969). Transmission of 47, XYY karyotype? *Lancet* (ii): 1367

Taylor, A. I. (1968). Autosomal trisomy syndromes: A detailed study of 27 cases of Edwards' Syndrome and 27 cases of Patau's Syndrome. *J. med. Genet.*, **5:** 227.

Tzagournis, M. (1969). Response to long term administration of human growth hormone in Turner's syndrome. *J. Am. Med. Ass.*, **210:** 2373.

Walzer, S., Breau, G. and Gerald, P. S. (1969). A chromosome survey of 2,400 normal newborn infants. *J. Pediatrics*, **74:** 438.

Wiener, S. and Sutherland. G. (1968). A normal XYY man. *Lancet* (ii): 1352.

Year Book Australia (1969). **55**, Commonwealth Government Printer, Canberra.

# Chromosome Survey in a Mental Deficiency Security Ward: Total Ascertainment

G. SUTHERLAND AND ALLEN A. BARTHOLOMEW

*Reprinted from the Australian and New Zealand Journal of Criminology.*

# *Chromosome Survey in a Mental Deficiency Security Ward: Total Ascertainment*

G. SUTHERLAND* AND ALLEN A. BARTHOLOMEW**

WE have published previously the results of a chromosome survey in a security ward for the mentally ill (Sutherland and Bartholomew, 1970), a ward not dissimilar to the west wing of the Carstairs Hospital that was investigated by Price and Whatmore (1967). We now report the results of a chromosome survey in a security ward for the male mentally retarded, a ward in a mental hospital (Aradale) that has much in common with the east wing of the Carstairs Hospital (Price and Whatmore, 1967). During the period of investigation 37[1] patients were examined and karyotyped.

*Method*

The technique of karyotyping has been described elsewhere (Wiener et al., 1969).

*Results*

*Karyotype*: Four abnormal karyotypes were found: 48,XXYY; 47,XXY; 46,XX; and 47,XY,G+. Thus, of the population of 37, three cases of sex chromosome abnormality were found, all examples of Klinefelter's syndrome; an incidence of 8.1%.

*Population*: This had a mean age of 33.2 years (range 14-58 years) and a mean mental hospitalization history of 13.5 years (range, less than 1 to 42 years). The patients were held under a number of different sections of the *Mental Health Act*, 1959; the majority (24) being "certified".

*The Diagnosis*: All the patients had been diagnosed by the mental hospital staff as mentally retarded and all had been designated as R0, R1 or R2

---

* B.Sc., Geneticist, St. Nicholas Hospital, Melbourne.

** M.B., B.S., D.P.M. (Lond.), M.A.N.Z.C.P., M.A.P.S., Psychiatrist Superintendent, H.M. Prison Pentridge. The authors thank Dr. S. Wiener for his interest and encouragement during this study, Dr. P. Wood, Psychiatrist Superintendent of Aradale Hospital, for permission to investigate the patients, and Mr. Ian Barker for technical assistance. We also thank the Mental Health Authority for permission to publish this report.

1. During the period of the investigation there were 38 patients in the ward, but in one case it proved impossible to obtain an adequate specimen of blood. The patient was 51 years of age, the diagnosis was R29E,P, he was 69 inches tall and had 26 years of hospitalization. He was a "non-certified" patient.

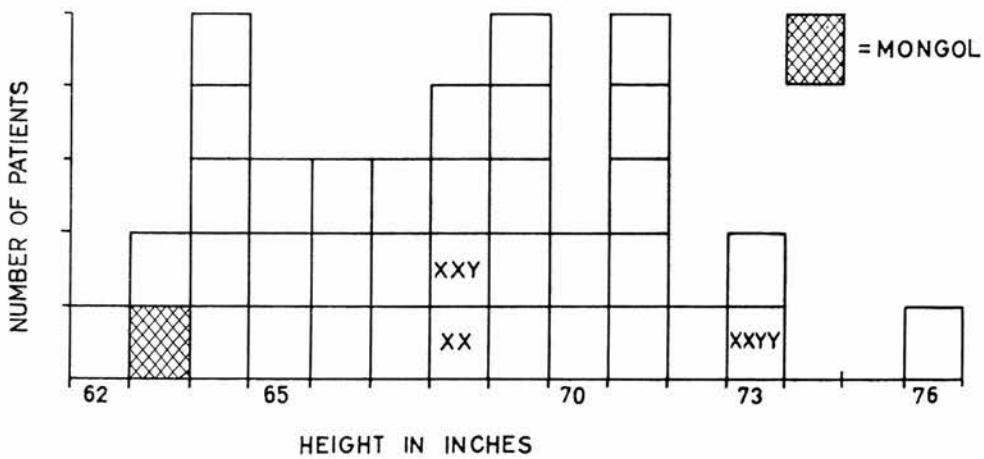
under the World Health Organization classification. Using such a system the population was categorized into:

| | |
|---|---|
| R0 (IQ = 68-85) . . . . . | 3 |
| R1 (IQ = 52-67) . . . . . | 16 |
| R2 (IQ = 36-51) . . . . . | 18 |
| | 37 |

The second digit in 31 of the cases as 9 which indicated that the mental retardation was of unspecified aetiology. Further, 13 cases included the letter B in the diagnostic formulation, which indicated "Behavioural disorder", 8 included the letter E (epilepsy), 4 included the letter P (psychosis present), and one case each had the letter A (alcoholism) and S (suicide attempt).

*Height*: The distribution of height of the population is shown in Figure 1.

FIGURE 1. *The distribution of the population in terms of height and the position of the four abnormal karyotypes.*




*Clinical Features*: These, as they relate to the three cases having abnormalities of the sex chromosomes, are set out in Table 1 (see next page). All had features consistent with the diagnosis of Klinefelter's syndrome. The patient with the karyotype 47,XY,G+ was a typical case of Down's syndrome and had been diagnosed prior to the present chromosome study.

*Discussion*

The main concern in undertaking this survey was interest in the incidence of the extra Y chromosome. Price and Whatmore (1967) found seven cases of 47,XYY in a population of 196 patients from the east wing of Carstairs Hospital. However, they found only two cases of Klinefelter's syndrome (XY/XXY/XXYY and XXY) among these patients. Hence it is of interest that we have found no cases of the extra Y chromosome, but three

TABLE 1

*Clinical information concerning the three patients with sex chromosome abnormalities.*

| | A | B | C |
|---|---|---|---|
| Karyotype | 47,XXY | 48,XXYY | 46,XX |
| Age | 55 yrs. | 35 yrs. | 19 yrs. |
| Height | 68 inches | 73 inches | 68 inches |
| Intelligence | R2 | R1 | R1 |
| Aetiology of retardation | Unspecified | Unspecified | Following trauma or physical agents |
| Age of first hospitalization | 52 yrs. | 14 yrs. | Uncertain |
| Age started present hospitalization | 52 yrs. | 31 yrs. | 19 yrs. |
| Previous criminal record. | none known | none known but known history of sexual deviance | minor delinquency. |

cases of Klinefelter's syndrome in our patient population. The difference in incidence may well not be valid in view of the small numbers involved and the difficulty that we have pointed out earlier (Sutherland and Bartholomew, 1970) in comparing such institutions.

It is of interest that the three cases of Klinefelter's syndrome had different karyotypes. The case of 48,XXYY was 73 inches tall, perhaps supporting the notion that the extra Y chromosome may lead to increased height, even in the presence of Klinefelter's syndrome (Hunter, 1969). The much rarer case of 46,XX in a male is at present under investigation and will be published in detail elsewhere. Suffice to say that the clinical picture was that of a typical case of Klinefelter's syndrome — a normal penis, hypoplastic testes and lack of facial hair.

Our three patients described here all demonstrated a psychopathic personality starting early in life and this tends to agree with the general proposition that abnormality of the sex chromosomes may well correlate with psychopathy. This has been suggested by Bartholomew (1968) in the case of the extra Y cases, while Burnand et al. (1967) have commented regarding Klinefelter's syndrome.

> (Some psychological tests) tended to show Klinefelter males to be of psychopathic type of personality with difficulties concerning aggressive impulses.

Similarly, Hunter (1969) considered that 14 of 15 cases of Klinefelter's syndrome were abnormal personalities best described as "predominantly inadequate or passive psychopaths". He also writes:

> When considering the effects of chromosome aneuploidy on the nervous system available evidence demonstrates no obvious changes in peri-

pheral nerve, spinal cord, or medullary function. Most effects appear to result from malfunction at cortical level. There is failure to learn, leading to intellectual subnormality, delinquency, and anti-social activity. Emotional control is underdeveloped resulting in immaturity of emotional response and erethism.

## REFERENCES

Bartholomew, A. A. (1968), The Extra Y Chromosome and Criminal Behaviour. Aust.N.Z.J.Psychiat. **2** 6

Burnand, G., Hunter, H., and Hoggart, K (1967), Some Psychological Test Characteristics of Klinefelter's Syndrome. Brit.J.Psychiat. **113** 1091.

Hunter, H. (1969), A Controlled Study of the Psychopathology and Physical Measurements of Klinefelter's Syndrome. Brit.J.Psychiat. **115** 443

Price, W. H., and Whatmore, P. B. (1967), Behaviour Disorders and Pattern of Crime Among XYY Males Identified at a Maximum Security Hospital. Brit.Med.J. i 533.

Sutherland, G., and Bartholomew, A. A. (1970), Chromosome Survey in a Security Ward: Total Ascertainment. Aust.N.Z.J.Criminol. **3** 99

Wiener, S., Sutherland, G., and Bartholomew, A. A. (1969), A Murderer with 47,XYY and an Additional Autosomal Abnormality. Aust.N.Z.J.Criminol. **2** 20.

*Aust. J. Ment. Ret.*
(1971) 1: 246-247

# CHROMOSOME STUDIES IN A MENTAL DEFICIENCY HOSPITAL: TOTAL ASCERTAINMENT

GRANT R. SUTHERLAND[1] and SAUL WIENER[2]

Chromosome studies on individuals suffering from mental defects are common but the screening of the total population of a mental deficiency hospital has not, to our knowledge, been reported. Such a survey was recently completed at St. Nicholas Hospital, Carlton. This is a hospital of 150 beds for mentally defective children. Most of the children are severely or profoundly retarded. The age range is from 3 months to 16 years with the majority being in the 2 to 12 years range.

## METHODS

Blood was collected from a heel or finger-prick and cultured by standard methods. Two metaphases from each patient were examined in detail and the chromosomes of a further 10 metaphases were counted by the technical staff. When a chromosome abnormality was detected at least 20 more cells were examined and a few selected for analysis after photomicrography.

## RESULTS

During the period of this study 161 children were in the hospital. Of these, 159 were available for chromosome analysis, one died and another was discharged before they could be studied. There were 89 boys and 70 girls. The results are summarised in Table 1.

### Boys

Seventy-six boys had normal male karyotypes. A 47, XY, G+ karyotype was found in 12 boys, all of whom had been clinically diagnosed as having Down's syndrome. One boy had a small extra chromosome similar in appearance to the Philadelphia chromosome; he had none of the features of Down's syndrome.

### Girls

Sixty-three girls had normal female karyotypes. Five had 47, XX, G+ karyotypes and had been recognised as cases of Down's syndrome. Another girl with Down's syndrome had 48 chromosomes. She had an extra G group chromosome and a small centric fragment with satellites on both arms, similar in appearance to a (Gp Gp) chromosome. This family had been studied before the survey began. Another girl with a karyotype of 47, XX, G+ was regarded as a possible case of trisomy 22 (Ferguson and Pitt, 1963).

## DISCUSSION

Of the 159 children studied, 13 had had chromosome studies done previously, these included the irregular mongol and the two non-mongols with abnormal karyotypes. Hence no chromosome abnormality was found in 139 non-mongol children who had not been studied previously and no irregular mongol was found amongst the 14 mongols who had not been previously studied.

The results show that, apart from Down's syndrome, chromosome abnormalities do not play a very significant role in the etiology of mental defect as seen at St. Nicholas Hospital.

No sex chromosome abnormalities were detected. For a population showing the severity of mental retardation seen here, the expected frequency of sex chromosome abnormalities is about twice that found in the newborn population (0.2%). On this basis the absence of sex chromosome abnormalities in the 159 children is in accordance with expectation.

The results of this survey contrast markedly with a total ascertainment study of a male mental deficiency security ward (Sutherland and Bartholomew, 1971) in which the patients

1. (Mr) Cytogeneticist, St. Nicholas Hospital. Present address Royal Hospital for Sick Children, Edinburgh, Scotland.
2. (Dr) Consultant Pathologist, St. Nicholas Hospital.
Address for reprints: Dr Saul Wiener, P.O. Box 105, Carlton, South Victoria, Australia, 3053.

**TABLE 1**
**Results of Chromosome Studies on 159 Children**

| | Regular Mongols | Normal | Other* | Total |
|---|---|---|---|---|
| Males | 12 (2) | 76 (4) | 1 (1) | 89 (7) |
| Females | 5 (1) | 63 (3) | 2 (2) | 70 (6) |
| Total | 17 (3) | 139 (7) | 3 (3) | 159 (13) |

* see text

Figures in parentheses are numbers in each group which had been studied prior to the start of this survey.

ranged from borderline to moderately retarded. Amongst the 38 patients studied there were three with sex chromosome abnormalities (karyotypes 46, XX male, 47, XXY and 48, XXYY) and one with Down's Syndrome.

Total ascertainment studies of mental deficiency institutions when viewed against chromosome studies of unselected newborns, will provide valuable information regarding the relationship between chromosome abnormalities and mental retardation.

**ACKNOWLEDGEMENTS**

We thank Dr. D. W. Maginn, Psychiatrist Superintendent of St. Nicholas Hospital for allowing us to study the patients under his care and Mr. Ian Barker for technical assistance. We thank the Mental Health Authority for permission to publish this paper.

**REFERENCES**

Ferguson, J., Pitt, D. B. (1963). Another child with 47 chromosomes. *Med. J. Aust.*, **1:** 545.

Sutherland, G., Bartholomew, A. A. (1971). Chromosome studies in a Mental Deficiency Security Ward: Total ascertainment. *Aust. N.Z. J. Criminol.*, **4:** (in press).

Reprinted from *The Medical Journal of Australia*, 1972, 1: 1249 (June 10)

# XYY MALES IN VICTORIA[1]

GRANT R. SUTHERLAND, M.SC.,[2] SAUL WIENER, M.D., PH.D., F.R.A.C.P.,[3]
ALLEN A. BARTHOLOMEW, M.B., M.R.C.S., D.P.M., F.A.N.Z.C.P., M.A.PS.S.[4]
AND MARGARET G. FITZGERALD, B.SC.[5]

*Melbourne*

*Med. J. Aust.*, 1972, 1: 1249-1252.

**Fifteen XYY males have been identified in Victoria over a period of ten years ending in February, 1971. Details of some have not been published to date; others have been the subject of publications, either as individuals or in small groups. This paper collates these various reports, and presents information regarding the unpublished cases. The possible effects of the extra Y chromosome on an individual are considered.**

BEFORE 1965, only a dozen XYY males had been described, most of them suffering from hypogenitalism (Balodimos *et alii*, 1966). In 1965, it was shown that there was a high incidence of XYY and XXYY males in institutional populations (Casey *et alii*, 1966; Jacobs *et alii*, 1965); these findings have since been confirmed. The report briefly summarizes the work in Victoria which has led to the identification of 15 XYY males. The cases which have not been published previously are briefly described, and, using correct case identification, those already published are now fully documented.

## METHODS AND CYTOGENETICS

The methods used in the survey work have been described elsewhere (Wiener *et alii*, 1968). In surveys, 10 cells per patient were examined, and, when possible, cultures were repeated in all cases in which abnormal karyotypes were found. The cases detected at laboratories other than the Chromosome Laboratory at St Nicholas Hospital were found during routine chromosome analyses.

## RESULTS

Institutional surveys (Table 1) have resulted in the detection of eight XYY males; another was a hospital staff member who volunteered some blood to test a batch of culture medium; and six have been identified in public hospitals in Melbourne (four of these were at the Royal Children's Hospital). With two exceptions, all have karyotypes 47,XYY. There is one 46XY/47XYY mosaic, and one subject has a structurally abnormal autosome (karyotype 47,XYY,Dp+, Wiener *et alii*, 1968).

### Clinical Findings

The 15 XYY males comprise 12 adults (a boy, aged 14 years, is included in this group), and three young children with congenital malformations who were identified at the Royal Children's Hospital. The cases not previously published include those of the following three babies.

CASE 1.—This child (M.101163) was referred for chromosome analysis at the age of three years. He was born four weeks prematurely, to a 33-year-old woman and her 33-year-old husband. The birth weight was 5 lb 7·5 oz. He was the fourth child in a family of five. When he died at the age of six years, he was 104 cm tall, and he had a head circumference of 47·5 cm. He had an unusual facies, and was severely mentally retarded. He suffered from cerebral palsy and epilepsy, and had microcephaly, scaphocephaly and hydrocephaly. Other features included mild obesity, hypoplastic genitalia and bilateral undescended testes. He was regarded as a possible example of the Praeder-Willi syndrome. Post-mortem examination confirmed that death had been due to a respiratory disorder with right pulmonary collapse and right thoracic empyema. Visceral abnormalities noted included agenesis of the right kidney and ureter, a Meckel's diverticulum, and an accessory spleen.

CASE 2.—This child (N.161270) was born after 41 weeks' gestation; the birth weight was 5 lb, the length at birth 46 cm, and the head circumference 33 cm. Pregnancy was complicated by glycosuria and by hyperemesis, for which an unknown antiemetic had been taken. The child had an odd facies and other malformations including a left phocomelia and congenital heart defects. Death occurred at the age of one month after a series of convulsive episodes. Post-mortem examination revealed the following malformations: incomplete

TABLE 1

*Results of Institutional Surveys Carried Out in Victoria in an Attempt to Identify XYY Males*[1]

| Institution | Number Surveyed | Chromosome Abnormality | |
|---|---|---|---|
| | | XYY | Other[2] |
| H.M. Prison, Pentridge .. | 266 | 5 | 5 |
| Turana .. .. .. | 78 | 1 | 1 |
| Aradale Hospital: | | | |
| J ward[3] .. .. | 55 | 2 | — |
| TC3 ward[4] .. .. | 38 | — | 4 |

[1] Prisoners from Pentridge and Turana (a youth training centre) were selected on the bases of height, criminal history and psychiatric diagnosis. Patients from Aradale were studied on a total ascertainment basis.

[2] Six patients with 47,XXY; one patient with 46,XY/47,XXY; one patient with 46,XX male; one patient with 48,XXYY; one patient with 47,XY,G+ (Down's syndrome).

[3] Sutherland and Bartholomew (1970).

[4] Sutherland and Bartholomew (1971).

[1] Read at the Third Human Cytogenetics Conference, Sydney, March 16, 1971.

[2] Cytogeneticist, St Nicholas Hospital, Carlton.

[3] Consultant Pathologist, St Nicholas Hospital, Carlton.

[4] Psychiatrist Superintendent, H.M. Prison Pentridge, Coburg.

[5] Cytogeneticist, Royal Children's Hospital, Parkville.

Address for reprints: Mr G. R. Sutherland, Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LN, Scotland, U.K.

TABLE 2

| Case Number | Age at Discovery | Height (cm) | IQ | Criminal Record | Mode of Discovery | Clinical Features | Other References[1] |
|---|---|---|---|---|---|---|---|
| A. 191035 | 33 years | 210 | 101 | Attempted murder, rape | Selected from Pentridge population | Psychopath | 2, Case 3; 6, Case 3; 7; 10, Case 3 |
| B. 190447 | 21 years | 178 | 71 | Murder | Selected from Pentridge population | Borderline defective, with epilepsy | 1; 2, Case 2; 6, Case 5; 10, Case 5 |
| C. 141224 | 44 years | 178 | 94 | Murder | Selected from Pentridge population | Psychopath, alcoholic (47,XYY,Dp+) | 2, Case 1; 3; 5; 6, Case 17; 10, Case 17; 11 |
| D. 050516 | 52 years | 182 | 84 | Larceny, perjury, assault (many offences) | Selected from Pentridge population | Psychopath, alcoholic | 2, Case 4; 6, Case 19; 10, Case 19 |
| E 031228 | 38 years | 187 | 65 | Larceny | Selected from Pentridge population | Psychopath, borderline defective | 2, Case 5; 6, Case 42 |
| F. 270252 | 16 years | 180 | 85 | Buggery | Selected from Turana population | Psychopath | 2, addendum |
| G. 070145 | 25 years | 198 | 88 | None | Total ascertainment of security ward for mentally ill | Psychopath | 8, Case A. |
| H. 290928 | 42 years | 198 | "Dull" | Larceny | Total ascertainment of security ward for mentally ill | Schizophrenic | 8, Case B. |
| I. 280147 | 20 years | 197 | 95 | None | Height, referred by endocrinologist | Varicose ulceration, neurotic | 4 |
| J. 301049 | 19 years | 196 | "Dull" | None | Height, referred by endocrinologist | Gynæcomastia, "female voice", undescended testes, neurotic | |
| K. 270956 | 12 years | 196 | "Dull" RO | Larceny | Height and behaviour problems, referred by pædiatrician | Psychopath with possible epilepsy | |
| L. 230927 | 41 years | 182 | 97 | None | Random (see text) | Normal man | 9 |
| M. 101163 | 3 years | 104[2] | 20 | None | Malformed child | Mental retardation, died 1.5.70 | |
| N. 161270 | 2 weeks | 46[3] | — | None | Malformed child | Congenital malformations, died 13.1.71 | |
| O. 261270 | 2 weeks | 48[3] | — | None | Malformed child | Congenital malformations, died 25.2.71 (mosaic XY/XYY) | |

[1] References are numbered as follows: 1, Bartholomew and Sutherland (1969*a*); 2, Bartholomew and Sutherland (1969*b*); 3, Burns (1962); 4, Cowling *et alii* (1969); 5, Feltham (1964); 6, Hudson *et alii* (1969); 7, Marcus and Richmond (1970); 8, Sutherland and Bartholomew (1970); 9, Wiener and Sutherland (1968); 10, Wiener *et alii* (1968); 11, Wiener *et alii* (1969).

[2] Age at 6 years; normal for age, 121 cm.

[3] Birth length.

transverse fissure of the right lung; incomplete oblique fissure of the left lung; and hypoplastic kidneys. Cardiac malformations demonstrated were the tetrad of Fallot, a patent ductus arteriosus, a patent foramen ovale and persistent left superior vena cava. The genitalia were normal, with the testes in the scrotum. The brain was moderately œdematous.

CASE 3.—This child (O.261270) was the third infant, and the second live-born child, of a 21-year-old women and her 25-year-old husband. The second pregnancy resulted in the birth of a macerated fœtus at term. The patient was the result of an uncomplicated pregnancy which ended, 16 days after the expected date of confinement, in a labour complicated by meconium staining of the liquor during the second stage. The child was born in poor condition and treated for meconium inhalation. The birth weight was 5 lb 9·5 oz, the length was 48 cm and the head circumference was 32·5 cm. The child was noted to have an unusual facies due to a malshaped nose and small eyes with redundant tissue of the eyelids. Other malformations included a high arched palate, bilateral undescended testes and rocker-bottom feet with large toes. On the fourth day of life there was a massive cerebral hæmorrhage, and hydrocephalus developed. Chromosome analysis revealed 46XY/47XYY mosaicism. Death from bronchopneumonia occurred at the age of two months. Post-mortem examination revealed no visceral abnormalities, with the exception of absence of the gall-bladder; the common bile duct was patent. No genital anomalies were noted at this time. There was gross hydrocephalus, the brain being markedly enlarged with distended ventricles and the cortex thinned to 2 cm.

Only two of the 12 adults have not been the subject of a publication. They are the 14-year-old boy mentioned above, and the boy whose abnormality was detected in a general hospital.

CASE 4.—The patient (K.270956) was born to a 24-year-old woman and her 22-year-old husband. He had three younger sisters and was the heaviest baby of the family. At the age of two years he may have suffered brain damage when he impaled his head (left temporo-parietal area) on a tap. He spent two weeks in hospital at four years for bronchopneumonia. At the age of eight years, his Binet IQ was 94, but at 13 years, the Wechsler Intelligence Scale for Children test placed him at the "RO" level (WHO classification of borderline retardation). At the age of 12 years, his bone age was estimated to be 15 years, and at this time he was referred for chromosome studies because of intractable behaviour problems and an excessive height, 73·25 in. At the age of 13 years, he was 76·25 in. tall, and still growing at the rate of a quarter of an inch per month. When last measured at the age of 14 years 6 months, he was 77·25 in. tall. His behaviour problems (lying, stealing, truancy) had drawn the attention of the police to him since he was eight years old. At 13·5 years, he was charged in the Children's Court with larceny involving the removal of tools from a shed. An electroencephalographic examination revealed a posterior temporal focus, but as he had been "dazed" in a car accident a few days previously, the significance of this finding is uncertain. A repeat EEG examination, although recommended, was not carried out. He does not suffer seizures.

CASE 4.—This patient (J.301049) was referred to an endocrinologist at the age of 17 years because of excessive height, gynæcomastia and a "female voice". Investigations included chromosome studies on blood and skin, and both revealed an XYY karyotype. He had undergone surgery for bilateral undescended testes at the age of 13 years. A psychiatrist's report reads: ". . . excessive sensitivity, anxiety, histrionic behaviour and homosexual traits. Poor school record." He has no criminal record.

## DISCUSSION

When the possible effects of the extra Y chromosome are under consideration, attention may be focused on several areas of development. They include physical characteristics such as height, the incidence of congenital malformations, and parameters of a psychological nature such as mental disorder, antisocial behaviour and intelligence.

The relevance of increased height was considered by Court Brown (1968), who estimated that 50% of XYY males will be taller than 6 ft. In our series, height was a factor which led to identification of all except three of the adults (L., G., and H.), although their heights were 70 in., 78 in. and 78 in. respectively. It is reasonable, therefore, to conclude that XYY males will, on the average, be taller than XY males, but the exact differences between the height distribution of XYY males and that of XY males must await prospective studies.

The presence of the extra Y chromosome in the three children with multiple congenital malformations (M., N., O.,) may be regarded as a fortuitous finding unrelated to their clinical conditions. One reason for this conclusion is that about 2,000 males have been karyotyped in Victoria because of "congenital abnormality and/or mental retardation", and only these three have been found to have an XYY sex complement. This does not differ from the incidence of 1·8 per 1,000 found in the newborn population (Sutherland, 1970). The incidence of congenital malformations among XYY males is probably not greater than among XY males.

Psychological characteristics (antisocial behaviour, mental disorder and intelligence) are harder to resolve, as they are now more difficult to measure than physical ones, can alter with time, and to some extent are interrelated. In spite of intense screening of institutional populations for XYY males, only eight of the 15 in this series were discovered in this way. Of the four adults found outside institutions, three had no criminal record and did not show the antisocial type of behaviour which would lead them to an institution. Two points are clear. First, there is an increased prevalence of XYY males in Victorian institutions of the prison or security-ward type above that in the newborn population. This differs from the results of surveys of 2,500 boys in penal institutions in Scotland (Jacobs *et alii*, 1971), in which no increase in the frequency of XYY males in the institutions studied was found. Secondly, the notion that XYY males inevitably show antisocial behaviour leading to incarceration is demonstrably false.

The frequency of personality disorder shown by XYY males may be the reason for their increased prevalence in institutions, since it may be a reflection of the tendency of persons with severe personality disorder to be committed to institutions. Bartholomew (1968) has suggested that the majority of XYY males will have some personality disorder with a tendency to psychopathy. There is some such disorder in 11 of the 12 adults in this series: seven are classed as psychopaths, two as neurotics, one as schizophrenic and one as a borderline defective with epilepsy.

The effect of the extra Y chromosome on intelligence is far from clear, but a trend towards a lower IQ than normal is noticeable. In this series, the nine adults formally tested have a mean IQ of 87 (range 65 to 101), while the other three adults are regarded as "dull". There are three possible explanations for this trend. First, it may be an artefact due from the smallness of our sample; this is unlikely, however, as most (but not all) XYY males reported in the literature are of dull normal intelligence. Secondly, IQ testing of persons with personality disorders may be unreliable. In fact, one patient, D, had his IQ assessed as between 65 and 100, in different hospitals at different times (Bartholomew and Sutherland, 1969*b*). Finally, it is possible that the extra Y chromosome exerts a lowering effect on IQ similar to its apparent elevating effect on height.

Some other possible effects of the extra Y chromosome have been reported. Price (1968) noted an increase in the *P-R* interval on the electrocardiogram; however, this finding has not been confirmed by others (e.g., Char and Borgaonkar, 1971). Penrose (1967) found the total fingerprint ridge count (TRC) of 14 XYY males to be 133·6, compared with a control value of 145 for XY males. In this series, the mean TRC of nine adults is 136·2 (range 101 to 178). Changes in the hormonal status of XYY males have been sought, but have not been conclusively demonstrated (Hudson *et alii*, 1969).

Much information has been accumulated about XYY males. It has come from the discovery of such persons through surveys of institutional populations and newborn children and the routine karyotyping of congenitally malformed children. However, a complete picture of the development of XYY males will not be obtained until those identified as neonates have been followed up for 20 years or more.

## ACKNOWLEDGEMENTS

We thank the following people for providing us with information about the patients in their care: Dr W. J. D. McKellar, Dr P. Taft, Dr C. Trood, Dr E. Turner, and Dr H. E. Williams; Dr O. M. Garson and Miss J. Ferguson for cytogenetic details on one of the patients, and Dr L. Chatz for his assistance during the survey in Turana. We thank all the cytogeneticists in Melbourne for their cooperation in this study. We thank the Mental Health Authority for permission to publish this paper.

## REFERENCES

BALODIMOS, M. C., HERMANN, L., IRWIN, I., *et alii* (1966), "XYY Karyotype in a Case of Familial Hypogonadism", *J. clin. Endocr.*, 26: 446.

BARTHOLOMEW, A. A. (1968), "The Extra Y Chromosome and Criminal Behaviour", (Editorial Annotation), *Aust. N.Z. J. Psychiat.*, 2: 6.

BARTHOLOMEW, A. A., and SUTHERLAND, G. (1969*a*), "A Defence of Insanity and the Extra Y Chromosome: R. *v.* Hannell", *Aust. N.Z. J. Criminol.*, 2: 29.

BARTHOLOMEW, A. A., and SUTHERLAND, G. (1969*b*), "Alcoholism, Drug Dependency and Sex Chromosome Abnormalities", MED. J. AUST., 2: 440.

BURNS, C. (1962), "The Tait Case", Melbourne Univ. Press.

CASEY, M. D., SEGALL, L. J., STREET, D. R., and BLANK, C. E. (1966), "Sex Chromosome Abnormalities in Two State Hospitals for Patients Requiring Special Security", *Nature (Lond.)*, 209: 641.

CHAR, F., and BORGAONKAR, D. S. (1971), "Electrocardiogram in Males with the 47,XYY Karyotype", *Lancet*, 1: 1242.

COURT BROWN, W. M. (1968), "Males with an XYY Sex Chromosome Complement", *J. med. Genet.*, 5: 341.

COWLING, D. C., RIGO, S., and MARTIN, F. I. R. (1969), "The YY Syndrome", MED. J. AUST., 2: 443.

FELTHAM, J. D. (1964), "The Common Law and the Execution of Insane Criminals", *Melb. Univ. Law Review*, 4: 434.

HUDSON, B., BURGER, H., WIENER, S., *et alii* (1969), "Plasma Testosterone and Luteinising Hormone in XYY Men", *Lancet*, 2: 699.

JACOBS, P. A., PRICE, W. H., RICHMOND, S., and RATCLIFF, R. A. W. (1971), "Chromosome Surveys in Penal Institutions and Approved Schools", *J. med. Genet.*, 8: 49.

MARCUS, A. M., and RICHMOND, G. (1970), "The XYY Syndrome, A Short Review, A Case Study and Investigatory Model", *J. forens. Sci.*, 15: 154.

PENROSE, L. S. (1967), "Finger Print Pattern and the Sex Chromosomes", *Lancet*, 1: 298.

PRICE, W. H. (1968), "The Electrocardiogram in Males with Extra Y Chromosomes", *Lancet*, 1: 1106.

SUTHERLAND, G. (1970), "Chromosome Abnormalities in Newborn Babies", *Aust. J. ment. Retard.*, 1: 77.

SUTHERLAND, G., and BARTHOLOMEW, A. A. (1970), "Chromosome Survey in a Security Ward: Total Ascertainment", *Aust. N.Z. J. Criminol.*, 3: 99.

SUTHERLAND, G., and BARTHOLOMEW, A. A. (1971), "Chromosome Survey in a Mental Deficiency Security Ward: Total Ascertainment", *Aust. N.Z. J. Criminol.*, 4: 82.

WIENER, S., and SUTHERLAND, G. (1968), "A Normal XYY Man", *Lancet*, 2: 1352.

WIENER, S., SUTHERLAND, G., and BARTHOLOMEW, A. A. (1969), "A Murderer with 47,XYY and an Additional Autosomal Abnormality", *Aust. N.Z. J. Criminol.*, 2: 20.

WIENER, S., SUTHERLAND, G., BARTHOLOMEW, A. A., and HUDSON, B. (1968), "XYY Males in a Melbourne Prison", *Lancet*, 1: 150.

AUSTRALASIAN MEDICAL PUBLISHING COMPANY LIMITED
71-79 Arundel Street, Glebe, N.S.W., 2037

*Aust. paediat. J.*
(1972) 8: 90-91

# CYTOGENICS OF 271 MONGOLS

**G. R. SUTHERLAND[1] and S. WIENER[2]**

*From the Chromosome Laboratory, St. Nicholas Hospital, Melbourne*

**SYNOPSIS**

The cytogenic findings in 271 mongols are described and discussed. The cases were ascertained by routine referral and also by total ascertainment studies of institutionalised mongols. 23 of the 271 did not have regular mongol karyotypes. It is suggested that all mongols should be referred for chromosomal studies.

Since the establishment of the St. Nicholas Hospital Chromosome Laboratory in 1963, cytogenic studies have been carried out on 271 mongols. They have been studied for two main reasons; one group was studied as the result of referral for the confirmation of a clinical diagnosis, and/or to provide information on which to base genetic advice to their parents. The second group was studied during total ascertainment surveys of three institutional populations. One case was in a security ward for the mentally defective (Sutherland and Bartholomew, 1971); 18 cases were in St. Nicholas Hospital when all inpatients were studied (Sutherland and Wiener, 1972), and 176 were studied during a survey of all mongols in the Children's Cottages, Kew, in Melbourne. One child was included in both the St. Nicholas and the Children's Cottages surveys and there were 44 whose chromosomes had been examined earlier, at the time of their referral as patients (see Table 1).

## METHODS

The standard leucocyte culture techniques were used in all cases. In those studied as patients, 20 (or more if indicated) cells were examined, whereas 2 cells per patient were examined in the total ascertainment surveys.

## RESULTS

In 23 the karyotypes were other than 47,XX (or XY),G+. These consisted of

9 mosaics with karyotypes 46,XX(or XY)/47,XX(or XY),G+

8 were t(DqGq) translocation carriers

4 were t(GqGq) translocation carriers;

1 case had extra small centric fragment in addition to the extra G-group chromosome (See Sutherland and Wiener (1972) for details of this patient), and

1 case had a Dp+ chromosome.

Family studies were not possible in this last case, but the Dp+ chromosome is considered to be a normal/variant (Lubs and Ruddle, 1971).

The method of ascertainment of those with irregular karyotypes is shown in Table II. Where possible, family studies were done on all the non-mosaic irregular cases, and the origins of the translocations are shown in Table III.

## DISCUSSION

Because only 2 cells per patient were examined in the total ascertainment studies, only mosaics with a large proportion of normal cells would have been detected. This probably accentuates the difference in the incidence of mosaicism found in the course of surveys (1 in 150) as compared with the incidence in patients referred for chromosome studies. In addition, doubtful cases of mongolism are more likely to be referred for chromosome studies than typical cases, and the incidence

Received 19 October, 1971.
1. Cytogeneticist.
2. Consultant Pathologist.

of mosaicism in the former group is expected to be greater.

The incidence of traslocations is 2.9% for t(DqGq) and 1.5% for t(GqGq). This does not differ significantly from that found in other studies. Dey (1971) found 3.1% with t(DqGq) and 0.6% with t(GqGq) in 160 Australian mongols. Richard's (1969) review of 2,466 cases of mongolism yielded equal numbers of each type of translocation (2.1%).

In our experience the majority of translocation mongols are not being detected by the rather haphazard selection of mongol patients for referral. Of the 8 t(DqGq), only one was found among the 121 routine patient referrals, whereas 6 were found in 150 new cases examined during surveys. Of the 4 with t(GqGq) there were 2 in each group. It is therefore advisable that all mongols be referred for chromosome study regardless of whether the parents seek genetic conselling. The detection in the population of translocation carriers who have an increased risk of producing mongol offspring, should be regarded as the prime reason for cytogenetic studies on all mongols. With the advent of chromosome analysis in the foetus, it is possible to give appropriate practical counsel to these translocation carriers.

**TABLE I**

**Ascertainment of 271 Cases of Mongolism**

| Ascertainment | Male | Female | Total |
|---|---|---|---|
| Kew Survey | 115* | 61 | 176* |
| St. Nicholas Survey | 12* | 6 | 18 |
| Patients not included above** | 40 | 38 | 78 |
| Totals | 167* | 105 | 272* |

*one case was in both the Kew and St. Nicholas surveys.

**includes the case found in security ward (Sutherland and Bartholomew, 1971).

**TABLE II**

**Ascertainment of 23 Irregular Cases**

| | t(DqGq) | | t(GqGq) | | Mosaic | | Other | |
|---|---|---|---|---|---|---|---|---|
| Ascertainment | Male | Female | Male | Female | Male | Female | Male | Female |
| Survey (new cases) | 4 | 2 | 2 | | | 1 | | |
| Patient referral | 2 | | 1 | 1 | 2 | 6 | | 2 |
| Total | 6 | 2 | 3 | 1 | 2 | 7 | | 2 |

**TABLE III**

**Origins of the Translocations**

| Translocation | Familial | Sporadic | Unknown |
|---|---|---|---|
| t(DqGq) | 2* | 2 | 4 |
| t(GqGq) | | 3 | 1 |

*maternal

**ACKNOWLEDGEMENTS**

We thank Drs. J. R. Ball, D. W. Maginn, and D. B. Pitt for their assistance in the total ascertainment surveys, Miss M. G. Fitzgerald for the cytogenetic details of one of the familial t(DqGq) translocations, Miss H. Prabucki and Mr. I. Barker for technical assistance during the surveys, and the Mental Health Authority for permission to publish this paper.

**REFERENCES**

Dey, Judith (1971). Survey of 500 cases of Down's Syndrome. *Aust. J. ment. Ret.*, **1:** 154-159.

Lubs, H. A. and Ruddle, F. H. (1971). Chromosome polymorphism in American negro and white populations. *Nature (Lond.)*, **233:** 134-136.

Richards, B. W. (1969) Mosaic mongolism. *J. ment. Defic. Res.*, **13:** 66-83.

Sutherland, G. R. and Bartholomew, A. A. (1971). Chromosome survey in a mental deficiency security ward: Total ascertainment. *Aust. N.Z. J. Criminol.*, **4:** 82-85.

Sutherland, G. R. and Wiener, S. (1972). Chromosome studies in a mental deficiency hospital: Total ascertainment. Aust. J. ment. Ret., (in press).

**Dr. S. Wiener,**
**Chromosome Laboratory,**
**St. Nicholas Hospital,**
**Melbourne, Australia, 3053.**

*Letter to the Editor*
*Reprinted from* THE LANCET, *April* 20, 1974, *pp.* 752

## CHROMOSOME ABNORMALITY AND PERINATAL DEATH

SIR,—We read with interest Dr Machin's report (March 30, p. 549) on the incidence of chromosome abnormalities in perinatal deaths. We have been attempting to karyotype all late abortions, stillbirths, and neonatal deaths coming to necropsy in the Edinburgh area. The results for the perinatal deaths in our series for the first year are shown in table I. The chromosome abnormalities detected included 5 cases of trisomy 18 and 1 case each of trisomy 21,

TABLE I—CHROMOSOME FINDINGS IN 213 PERINATAL DEATHS STUDIED IN THE ONE YEAR PERIOD ENDING SEPT. 30, 1973

| Status | No. | Chromosome results (no.) | Abnormal karyotypes. no. (%) |
|---|---|---|---|
| Macerated stillbirth .. | 52 | 3 | 1 (33) |
| Non-macerated stillbirth .. | 47 | 32 | 1 (3·1) |
| Neonatal death .. .. | 114 | 98 | 7 (7·0) |
| Total .. .. .. | 213 | 133 | 9 (6·8) |

TABLE II—CHROMOSOME FINDINGS ACCORDING TO CONGENITAL MALFORMATIONS

| Malformations | Chromosome results (no.) | Abnormal karyotypes. no. (%) |
|---|---|---|
| None .. .. | 81 | 3 (3·7) |
| Primarily C.N.S. .. | 28 | 0 (0) |
| Not primarily C.N.S. | 24 | 6 (25) |
| Total .. .. | 133 | 9 (6·8) |

triple-X female, unbalanced reciprocal translocation, and mosaicism for an extra C-group chromosome.

The chromosome findings according to the types of congenital malformations are shown in table II.

These findings are very similar in a number of respects to those of Dr Machin. The incidence of chromosome abnormalities in perinatal deaths is 10 times greater than that in the neonatal population in Edinburgh (1 in 140).[1] The types of chromosome abnormality are more similar to those found in liveborn infants than in abortuses. Trisomy E is the most commonly encountered chromosome abnormality. Obtaining chromosome results from macerated fetuses is difficult, but this group is worthy of study because it appears to have a high incidence of chromosome

abnormality. Most of the chromosome abnormalities are found in cases of multiple congenital malformation not primarily affecting the central nervous system. Even in cases in which there is no congenital malformation the incidence of chromosome abnormality is about 5 times greater than expected.

We support Dr Machin's contention that chromosome studies should become a routine part of the perinatal necropsy.

Full details of this study will be published elsewhere.

This work was supported in part by the Distillers Co. Ltd. grant to the University of Edinburgh.

Royal Hospital for Sick Children
and University of Edinburgh,
Edinburgh EH9 1LF.

GRANT R. SUTHERLAND
RHONA BAULD
A. DOUGLAS BAIN.

1. Ratcliffe, S. G., Keay, A. J. *Archs Dis. Childh.* 1973, **48**, 407.

Printed in
Great Britain

The Lancet Office,
7, Adam Street, Adelphi, London, W.C.2.

# CHROMOSOME STUDIES IN INVESTIGATION OF STILLBIRTHS AND NEONATAL DEATHS

BY

**RHONA BAULD, GRANT R. SUTHERLAND, and DOUGLAS BAIN**

*Reprinted from Archives of Disease in Childhood, Vol. 49, No. 10 October 1974*



LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, LONDON WC1H 9JR

*Archives of Disease in Childhood*, 1974, **49**, 782.

# Chromosome studies in investigation of stillbirths and neonatal deaths*

RHONA BAULD, GRANT R. SUTHERLAND, and A. DOUGLAS BAIN

*From the Department of Pathology, Royal Hospital for Sick Children, Edinburgh, and the University of Edinburgh*

**Bauld, R., Sutherland, G. R., and Bain, A. D. (1974).** *Archives of Disease in Childhood*, **49**, 782. **Chromosome studies in investigation of stillbirths and neonatal deaths.** Chromosome studies have been attempted on all late abortions, stillbirths, and neonates coming to necropsy in Edinburgh over a 1-year period. Results were obtained in 153 cases and of these 7·2% had chromosome abnormalities. It is suggested that chromosome studies should become a routine part of the perinatal necropsy.

With the decrease in perinatal mortality in recent years the genetic component in this problem has assumed increasing significance. There is only one report of cytogenetic studies having been carried out on a consecutive series of perinatal deaths (Machin, 1974). Chromosome abnormalities in such a population were found to be 10 times more frequent than in unselected liveborn infants. Over a period of one year ending on 30 September 1973, chromosome studies were carried out on all late abortions, stillbirths, and neonates coming to necropsy in this department.

## Materials and methods

All routine necropsies on late abortions, stillbirths, and neonatal deaths in the Edinburgh area are carried out in this department. For the purpose of this study they were categorized as follows.

*Abortions:* fetuses of more than 20 but of less than 28 weeks' gestation.

*Perinatal deaths:* stillbirths of more than 28 weeks' gestation and all liveborn infants who have not lived for more than one week, regardless of gestation. These were further divided in 3 groups, macerated stillbirths, nonmacerated stillbirths, and early neonatal deaths.

*Older neonates:* those who lived for more than one week but for less than 28 days.

Where possible, chromosome studies were carried out on all such cases; three techniques were used. (1) Heart blood lymphocyte culture which was attempted only in the case of liveborn infants who had been dead for less than 8 hours. (2) Spleen lymphocyte culture in the presence of phytohaemagglutinin using the method of Bain and Gauld (1964). (3) Fibroblast culture for which a variety of tissues are suitable. In this survey gonad and pericardium were the main tissues used. Towards the end of the survey fibroblast culture of placental amnion from macerated stillbirths was attempted (Machin, 1974). The tissue was collected aseptically at necropsy, placed in a sterile vial containing culture medium, and transported to the laboratory. Explants of tissue were set up in glass culture bottles using a chicken-plasma clot technique. After these primary cultures had been subcultured, chromosome results were obtained using standard methods. A minimum of 10 cells per case were analysed and then checked by an independent observer; a karyotype was prepared. When chromosome banding was required the technique of Grace and Bain (1972) was employed.

## Results

During this survey there were 242 consecutive necropsies in the categories selected for chromosome studies. Chromosome studies were not attempted on 42 macerated stillbirths in the early part of the survey, nor in 16 other instances for a variety of reasons (such as severe pyogenic infection).

The chromosome findings according to category are shown in Table I. A high percentage of chromosome abnormalities were found among the abortions and macerated stillbirths, but the numbers were too small to attach significance to these findings. The figures for perinatal deaths are more

Received 20 February 1974.

*Based on a paper presented to the 19th Annual Meeting of the Paediatric Pathology Society, Glasgow, October 1973.

TABLE I

*Chromosome results by category*

| Status | Total no. of necropsies | Chromosome studies attempted | Results | | Abnormal karyotypes | |
|---|---|---|---|---|---|---|
| | | | No. | % | No. | % |
| Abortion | 9 | 5 | 5 | 100 | 2 | 40 |
| Macerated stillbirths | 52 | 14 | 3 | 21 | 1 | 33 |
| Nonmacerated stillbirths | 47 | 39 | 32 | 82 | 1 | 3·1 |
| Early neonatal deaths | 114 | 108 | 98 | 91 | 7 | 7·1 |
| Older neonates | 20 | 18 | 15 | 83 | — | — |
| Total | 242 | 184 | 153 | 83 | 11 | 7·2 |

representative of the incidence of chromosome abnormalities in the population studied since the numbers are larger. It is interesting to note that no chromosome abnormalities were found in the 'older neonates'.

The tissues from which chromosome results were obtained are listed in Table II. A number of the results were obtained from blood lymphocyte culture established before death. The number of heart blood samples used in the study was small because of the time delay between death and necropsy, which was usually greater than 8 hours, generally about 2 days, and occasionally up to 5 days. Approximately one-quarter of the spleen samples yielded chromosome results. When results were obtained from these tissues, the slower fibroblast cultures were discarded. Of the 145 cases where fibroblast cultures were continued there were 31 cases where chromosome results were not obtained. This was due to bacterial contamination in 7 cases, and to primary growth failure in 24 cases. In 11 of these 24 cases the tissue was from macerated fetuses and growth failure was not unexpected. In these fetuses all the cells are dead, but placental amnion may contain viable cells which are suitable for fibroblast culture. In 13 cases there was no apparent reason for the growth failure.

TABLE II

*Tissue from which results were obtained*

| Tissue | No. attempted | No. successful |
|---|---|---|
| Blood (during life) | 10 | 10 |
| Heart blood | 2 | 2 |
| Spleen | 102 | 27 |
| Other tissue (fibroblast cuitures) | 145 | 114 |

The chromosome results according to primary necropsy findings are shown in Table III. The overall incidence of chromosome anomalies was 7%.

TABLE III

*Chromosome results in relation to primary necropsy findings*

| Primary necropsy findings | No. | Chromosome results obtained | Abnormal karyotypes | |
|---|---|---|---|---|
| | | | No. | % |
| Abortion | 9 | 5 | 2 | 40 |
| Macerated fetus without congenital malformations | 44 | 2 | — | — |
| Prematurity associated disease | 60 | 53 | 1 | 1·9 |
| Primary CNS abnormality | 43 | 29 | — | — |
| Multiple congenital abnormalities | 42 | 32 | 6 | 18·8 |
| Primary anoxia | 35 | 26 | 1 | 3·8 |
| Infection | 5 | 4 | 1 | 25·0 |
| Miscellaneous | 4 | 2 | — | — |
| Total | 242 | 153 | 11 | 7·2 |

Of 61 cases with severe congenital malformations there were 6 with chromosome abnormalities, an incidence of 10%. Of the remaining 91 cases without significant congenital malformations 4 (4%) had chromosome abnormalities. Except for the cases of Edwards's syndrome and the unbalanced translocation, the other chromosome abnormalities were not suspected either clinically or at necropsy. This includes the case of Down's syndrome, an infant of 26 weeks' gestation where the extra chromosome was identified as number 21 using G-banding. Details of the cytogenetics of the 11 abnormal cases are listed in Table IV. In all cases analysis of 40 cells was attempted in order to rule out mosaicism, but this was considered unlikely in Case 479, where the unbalanced karyotype was of paternal derivation, and only 25 cells were examined. When non-modal cells were observed the loss of chromosomes appeared to be random. Failure to examine 40 cells in other cases was due to loss of the cell strain; this was also one reason for not

TABLE IV

*Cytogenetic findings in the cases with abnormal karyotypes*

| Case no. | Category | Karyotype | Tissue | Chromosome counts | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 45 | 46 | 47 | 3N | Total |
| 246 | Early neonatal death | 47, XX, +21 | Spleen | 2 | 3 | 45 | | 50 |
| 218 | ,, | 47, XY, +18 | Fibroblast | | | 40 | | 40 |
| 472 | Macerated stillbirth | 47, XY, +18 | Fibroblast (amnion) | | 2 | 9 | | 11 |
| 351 | Early neonatal death | 47, XX, +18 | Spleen | | 1 | 39 | | 40 |
| 387 | ,, | 47, XX, +18 | Blood | | 2 | 38 | | 40 |
| 419 | ,, | 47, XX, +18 | Fibroblast | 1 | 5 | 34 | | 40 |
| 264 | Nonmacerated stillbirth | 47, XXX | Fibroblast | | | 35 | | 35 |
| 479 | Early neonatal death | 46, XY, der 13, t(7; 13) (q36; q22) pat. | Heart blood | | 25 | | | 25 |
| 461 | ,, | 46, XX/47, XX, +C | Fibroblast | | 28 | 4 | | 32 |
| 294 | Abortion | 46, XY/47, XY, +C | Fibroblast | | 49 | 6 | | 55 |
| 297 | ,, | 69, XXY | Fibroblast | | | | 50 | 50 |

TABLE V

*Seasonal incidence of chromosome abnormalities*

| | Oct | Nov | Dec | Jan | Feb | Mar | Apr | May | June | July | Aug | Sept |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chromosome results | 9 | 11 | 17 | 12 | 8 | 12 | 16 | 13 | 13 | 15 | 10 | 16 |
| Chromosome abnormalities | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | 1 | | 3 |
| Trisomy 18 | | 1 | | | | | 1 | 1 | | 1 | | 1 |

identifying the extra C group chromosomes in the two mosaics. Studies of histological sections of Case 461 showed some cells which appeared to have two sex chromatin bodies. Similar studies of Case 294 showed no evidence of sex chromatin.

The seasonal incidence of the birth of chromosomally abnormal cases is shown in Table V. These appear to be randomly distributed overall, but there does seem to be some clustering of the E trisomies.

The cases of chromosome abnormality were not uniformly distributed with respect to maternal age, as can be seen from Table VI, a higher proportion being born to older mothers. The ages of 11 mothers were not ascertained and these were distributed proportionately into the various age groups.

TABLE VI

*Distribution of cases with chromosome abnormalities according to the age of the mother*

| | Age intervals (yr) | | | | | |
|---|---|---|---|---|---|---|
| | <20 | 20–24 | 25–29 | 30–34 | 35–39 | ⩾40 |
| All karyotyped infants | 12 | 59 | 51 | 17 | 9 | 5 |
| Chromosomally abnormal | — | 3 | 4 | 1 | 1 | 2 |
| Abnormal karyotype (%) | | 5·1 | 7·8 | 5·9 | 11·1 | 40 |

## Discussion

The only reported study comparable to the present one is that of Machin (1974) who karyotyped 500 unselected perinatal deaths in the London area over a 3-year period. This series is compared with the present one in Table VII. The findings in the two series are in close agreement, especially if the two categories, abortions and older neonates, not studied by Machin (1974) are excluded. The incidence of chromosome abnormality in the Edinburgh survey would then be 6·8% compared with 5·6% for the comparable data from London. The incidence of 7·2% of chromosome abnormality in the present series is 10 times higher than the incidence of 0·71% of chromosome abnormalities detected in a series of 11,000 unselected liveborn infants also studied in Edinburgh (Ratcliffe and Keay, 1973). Hence, the only two series of chromosome studies on perinatal deaths agree that there is an excess of infants with chromosome abnormalities dying in this period.

The types of chromosome abnormality detected in perinatal deaths are compatible with survival beyond birth and are not generally of the type usually seen in early abortions (Hamerton, 1971). In both series the most frequently encountered chromosome

TABLE VII

*Comparison of the results of two chromosome surveys carried out on infants dying in the perinatal period*

| Category | Series | | | |
|---|---|---|---|---|
| | Machin (1974), London | | This study, Edinburgh | |
| | No. karyotyped | % abnormal | No. karyotyped | % abnormal |
| Late abortion | — | — | 5 | 40 |
| Macerated stillbirth | 34 | 8·8 | 3 | 33 |
| Nonmacerated stillbirths | 122 | 4·1 | 32 | 3·1 |
| Early neonatal deaths | 344 | 5·8 | 98 | 7·1 |
| Older neonates | — | — | 15 | 0 |
| Total | 500 | 5·6 | 153 | 7·2 |

abnormality was trisomy 18. In the series of Machin (1974) 8 out of 28 infants with chromosome abnormality had trisomy 18, and in the present series this abnormality accounted for 5 of the 11 cases found. Hence, the present findings support the contention (Bain, 1973; Machin, 1974) that trisomy 18 may be more common than was originally estimated by Taylor (1968).

As would be expected, most of the chromosome abnormalities were associated with congenital malformations. If the severe congenital malformations were subdivided according to whether or not they were primarily of the CNS (anencephaly, spina bifida, encephalocele, etc.), it is seen that while the overall chromosome abnormality rate in congenital malformations is 10·2% these were confined exclusively to those cases in which the malformations did not primarily involve the CNS, and in this group the incidence of chromosome abnormality was 18·9%. Machin (1974) did not divide his congenital malformations in this manner, but found that 13% of this whole group had a chromosome abnormality.

In those without recognizable malformations there were 4·4% with a chromosome abnormality and these are of particular interest since they were unsuspected and would have gone undetected but for this survey. Machin (1974) found that 2·5% of his cases without malformations had a chromosome abnormality. Hence, even in perinatal deaths where there is no congenital malformation, the incidence of chromosome abnormality is about 5 times greater than that in unselected liveborn infants.

Seasonal clustering of the chromosome abnormalities was not apparent in the present series as a whole, but 4 of the 5 cases of trisomy 18 were found in the period from April to September. This corresponds with the findings of Machin (1974) whose 8 cases with this abnormality occurred between March and July. Taylor (1968) found that there was a clustering of both D and E trisomic infants in the period from June to November. All these periods of increased incidence of trisomy 18 overlap, but they do indicate that this anomaly is less likely to occur in the winter months than at other times of the year. Others have reported seasonal variations in the incidence of chromosome abnormalities (e.g. Robinson *et al.*, 1969; Jongbloet, 1970; Nielsen, Petersen, and Therkelsen, 1973), but little data from chromosome surveys of unselected neonates has been analysed in this manner.

The increase in the incidence of chromosome abnormalities with maternal age is well recognized; nevertheless, the relation between maternal age and chromosome abnormality in the two series of studies on perinatal deaths is striking. The stillbirths and neonatal deaths from mothers younger than 20 years had about a 1% chance of having a chromosome abnormality, yet those from mothers older than 40 years had a 30 to 40% chance of having such an abnormality. Mothers in this latter group should routinely be offered antenatal diagnosis of chromosome abnormalities (Sutherland, 1972; Bain and Sutherland, 1973).

The techniques for obtaining chromosome results at post mortem have lower success rates than similar studies during life. Obtaining results from macerated fetuses is particularly difficult, though amnion culture (Machin, 1974), which was only attempted towards the end of the present series, has produced results on occasions when other cultures have failed. This is obviously the method of choice for such fetuses and it is anticipated that in future chromosome results will be obtained from a proportion of these. When suitable tissue is available from nonmacerated stillbirths or from neonates then success rates in the vicinity of 90% should be attained. The elimination of microbial contamination of cultures could increase the success

rate, but this still leaves a number of cases of unexplained primary growth failure.

Hence it has been shown, in two different centres, that chromosome abnormalities are present in 5 to 10% of perinatal deaths. In view of this it is suggested that chromosome studies should become an integral part of the perinatal necropsy. Chromosome results from dead neonates are particularly valuable when counselling parents. A normal result suggests that the chances of recurrence should be sought from the other pathological findings and an abnormal result indicates that amniocentesis in subsequent pregnancies should be strongly considered.

Some necropsies in this series were carried out by Dr. J. M. Anderson. We thank Mrs. Elizabeth Grace and Mrs. Susan Bowser-Reilly for help with cytogenetic studies, and Mrs. Eleanor Cochrane and Miss Dorothy Seymour for help with the tissue culture.

We thank the M.R.C. Clinical and Population Cytogenetics Unit for allowing us to use their results on some of the infants studied while alive. This work was supported in part by the Distillers Co. Ltd. grant to the University of Edinburgh.

REFERENCES

Bain, A. D. (1973). Chromosomal abnormalities. In *Textbook of Paediatrics*. Ed. by J. O. Forfar and G. C. Arneil. Churchill Livingstone, Edinburgh and London.

Bain, A. D., and Gauld, I. K. (1964). The use of thymus and spleen in the demonstration of chromosomes postmortem in foetuses and infants. *British Journal of Experimental Pathology*, **45**, 530.

Bain, A. D., and Sutherland, G. R. (1973). Antenatal screening for Down's syndrome. *Lancet*, **1**, 423.

Grace, E., and Bain, A. D. (1972). A simple method for chromosome banding. *Journal of Clinical Pathology*, **25**, 910.

Hamerton, J. L. (1971). *Human Cytogenetics*, Vol. 2. Academic Press, London and New York.

Jongbloet, P. H. (1970). Chromosomal aberrations and month of birth. *Lancet*, **2**, 1317.

Machin, G. A. (1974). Chromosome abnormality and perinatal death. *Lancet*, **1**, 549.

Nielsen, J., Petersen, G. B., and Therkelsen, A. J. (1973). Seasonal variation in the birth of children with aneuploid chromosome abnormalities. Report from the Danish Cytogenetic Central Register. *Humangenetik*, **19**, 67.

Ratcliffe, S. G., and Keay, A. J. (1973). Chromosome studies on 11,000 newborn infants. *Archives of Disease in Childhood*, **48**, 407.

Robinson, A., Goad, W. B., Puck, T. T., and Harris, J. S. (1969). Studies on chromosomal nondisjunction in man. III. *American Journal of Human Genetics*, **21**, 466.

Sutherland, G. R. (1972). The role of amniocentesis in genetic counselling. *Australian Journal of Mental Retardation*, **2**, 85.

Taylor, A. I. (1968). Autosomal trisomy syndromes: a detailed study of 27 cases of Edwards' syndrome and 27 cases of Patau's syndrome. *Journal of Medical Genetics*, **5**, 227.

Correspondence to Mrs. R. Bauld, Department of Pathology, Royal Hospital for Sick Children, Edinburgh EH9 1LF.

## Appendix

This appendix consists of the main pathological findings in the 11 cases with chromosome abnormalities listed in Table IV in the text.

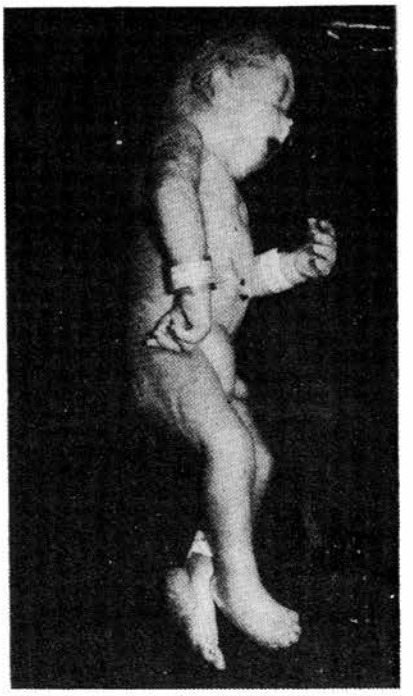


FIG. 1.—*Case 218. Note abnormal ears, flexion deformity of right fingers, short great toes, rocker-bottom feet.*

**Case 246.** Karyotype 47,XX,+21; age 1¼ hr; gestation 26 weeks; weight at necropsy 1335 g. No recognizable malformations; gross ascites and subcutaneous oedema confined to the trunk. Brain and meninges normal. Heart normal. Small intestine was narrow indicating ascites had been present for some time.

**Case 218.** Karyotype 47,XY,+18; age 31 hr; gestation 38 weeks; weight at necropsy 1880 g. External features were in keeping with a chromosome abnormality but not typical of trisomy 18. Ears abnormal. Unilateral flexion deformities of the fingers; right talipes equinovarus; left foot had a rocker-bottom appearance; great toes short. Single umbilical artery. No Simian creases. Head: anterior falx cerebri defect, adhesion of cerebral hemispheres. Heart: high interventricular septal defect. No other visceral abnormality. See Fig. 1.

**Case 472.** Karyotype 47,XY,+18; stillborn (macerated); gestation 39 weeks; weight at necropsy 1193 g. Multiple abnormalities; ears primitive, small and with only partial separation of the pinnae from the scalp. Microphthalmia, hypertelorism, microstomia, and micrognathia. Bilateral flexion deformities of fingers; dysplastic nails; no Simian creases. Exomphalos.

FIG. 2.—*Case 472. Note maceration, flexion deformity of fingers, short great toes, small penis, grossly oedematous umbilical cord.*

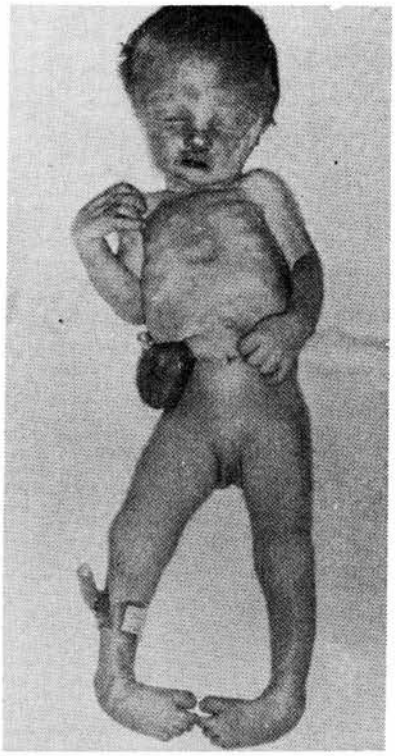


FIG. 3.—*Case 387. Note exomphalos, talipes, short great toes.*

Normal placenta with grossly oedematous umbilical cord; single umbilical artery. Small penis; undescended testes. Oedematous legs. Brain normal. Heart enlarged with transposition of the great vessels and ventricular septal defect. Malrotation of the ileum and ascending colon; absent appendix. Meckel's diverticulum with ectopic pancreatic tissue at its tip. Left hydroureter with ureteric stenosis, right double ureter. See Fig. 2.

**Case 351.** Karyotype 47,XX,+18; age 6½ hr; gestation 34 weeks; weight at necropsy 1395 g. Multiple abnormalities; odd facies with large mandible; exomphalos; right talipes equinovarus; partial syndactyly of 4th and 5th toes. Brain normal. Heart: ventricular septal defect. Single umbilical artery.

**Case 387.** Karyotype 47,XX,+18; age 48 hr; gestation 38 weeks; weight at necropsy 1249 g. Multiple external abnormalities. Odd facies. Nipples widely spaced. Exomphalos. Bilateral partial syndactyly of 3rd and 4th fingers. Flexion deformity of both thumbs, but no flexion deformity of other fingers. Bilateral single transverse palmar creases. Bilateral talipes with rocker-bottom feet; short great toes. Brain normal. Oesophageal atresia, jejunal stenosis. Heart: atrial septal defect; ventricular septal defect, mitral atresia, widely persistent ductus arteriosus. Horseshoe kidney. Hypoplastic uterus; vesicovaginal fistula. See Fig. 3.

**Case 419.** Karyotype 47,XX,+18; age 1½ hr; gestation 35 weeks; weight at necropsy 1020 g. Twin pregnancy—other twin chromosomally normal. Multiple external abnormalities. Odd facies with micrognathia; ears flattened with the helix underdeveloped. Flexion deformities of fingers and bilateral single palmar creases. Rocker-bottom feet; short great toes. Exomphalos. Head: anterior falx defect with fusion of the cerebral hemispheres in this region. Oesophageal atresia. Heart: high interventricular septal defect. Meckel's diverticulum with ectopic pancreatic tissue at its tip. Kidneys normal.

**Case 264.** Karyotype 47,XXX; stillborn; gestation 31 weeks; weight at necropsy 1512 g. No obvious external deformity. Brain and spinal cord showed normal developmental features consistent with 30–32 weeks' gestation. Lungs airless. Multiple petechial haemorrhages present over the visceral pleura and pericardium. Heart normal. No visceral or genital abnormality. Rhesus isoimmunization.

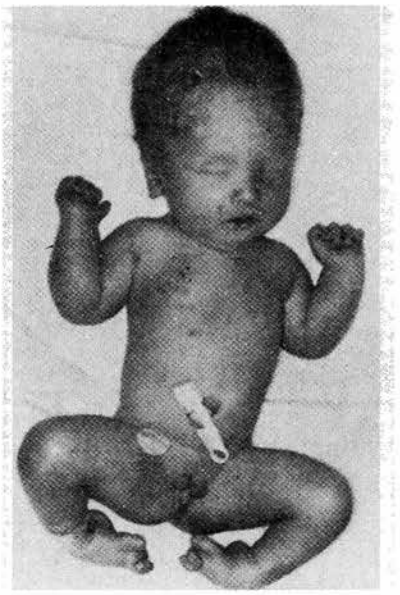


FIG. 4.—*Case 479. Note anomalous genitalia, bilateral equinovarus deformity, large head.*

**Case 294.** Karyotype 46,XY/47,XY,+C; abortion; gestation 27 weeks; weight at necropsy 1125 g. No external deformities recognized. Brain and meninges: normal, developmental features consistent with 26 weeks' gestation. Multiple petechiae present over both lungs. Heart normal. No visceral abnormalities.

**Case 461.** Karyotype 46,XX/47,XX,+C; age 58 hr; gestation 32 weeks; weight at necropsy 1580 g. Infant showed discoloured anterior abdominal wall. Epicanthic folds; micrognathia, low-set ears. Brain and meninges normal. Thoracic organs normal. Patchy infarction of the intestine from midileum distally. Large intestine severely affected. Liver extremely congested. Death due to enterocolitis.

**Case 297.** Karyotype 69,XXY; abortion; gestation 23 weeks; weight at necropsy 500 g. Male fetus with no obvious external abnormality. Brain meninges and spinal cord normally developed for 20 weeks' gestation. Thoracic organs structurally normal. Right kidney showed cystic dysplasia and was 3 times normal size; left kidney normal. No other visceral abnormality. Placenta weighed 423 g.

**Case 479.** Karyotype 46,XY, der 13, t(7; 13) (q36; q22) pat.; age 6 days; gestation 39 weeks; weight at necropsy 2578 g. Anomalous external genitalia. Severe bilateral equinovarus with syndactyly of 4th and 5th digits. Head was large; bilateral microphthalmia; cerebral holosphere with hydranencephalus. Spinal cord normal. All thoracic organs normal. No visceral abnormality except for hypoplastic kidneys. See Fig. 4.

Hum. Genet. 34, 231—245 (1976)


# Cytogenetic Survey of a Hospital for the Mentally Retarded

Grant R. Sutherland, Ashleigh R. Murch*, Andrew J. Gardiner, Rodney F. Carter, and Carole Wiseman

Cytogenetics Unit, Department of Histopathology, The Adelaide Children's Hospital (Inc.), and Strathmont Training Centre, Australia

Received July 28, 1976

*Summary.* A cytogenetic survey of all 588 patients in Strathmont Training Centre, an Australian hospital for the mentally retarded, was carried out. Abnormal karyotypes were found in 90 (15.3%) patients, of whom 73 (12.4%) had clinical Down syndrome, 12 (2.04%) other autosomal abnormalities, and 5 (0.85%) sex chromosome abnormalities.

## Introduction

Whilst chromosome studies are often performed on patients with mental retardation there have been few studies in which the total population of a hospital for the mentally retarded has been karyotyped (Sutherland and Wiener, 1971; Fujita and Fujita, 1974; Cassiman et al., 1975). Accordingly, we report here a cytogenetic survey of the whole population of Strathmont Training Centre, a large Australian hospital for the mentally retarded.

## Materials and Methods

### *The Hospital*

Strathmont Training Centre is a residential institution for the mentally retarded. Residents come from all areas of the State of South Australia. There is a waiting list for admission and priority is based on social rather than medical criteria. All admissions are informal. When the Centre first opened in 1971 a number of residents were transferred from long stay wards for the retarded within the State psychiatric hospitals, and a number of people were admitted directly from the community. Nearly all admissions at present are directly from the community with a very small number coming via general hospitals or other institutions.

Borderline retarded people are not normally admitted to the Centre, and mildly retarded people are only admitted if there are other significant factors which make admission desirable. In this latter case an attempt would be made to deal with the problem and then find the person more appropriate accommodation within the community.

### *Cytogenetics*

Capillary blood samples were collected by fingerprick for a standard microculture chromosome technique. In any cases of repeated failure using this method venous blood was collected and similarly cultured. If indicated, skin biopsies were collected for fibroblast chromosome

* *Present Address:* Department of Pathology, Sir Charles Gairdner Hospital, Western Australia 6008.

studies. A list of all inpatients on 20th February 1975 was prepared and those who had not been previously karyotyped were then studied. This report pertains then to the completed studies on all the 588 patients in the hospital on that date. A minimum of 30 cells per patient were counted and analysed microscopically and one or two were photographed and a karyotype prepared. Appropriate banding studies were employed to identify aberrant chromosomes but karyotypes apparently normal by unbanded criteria were not studied further. Parental chromosomes were studied if possible in all cases of chromosome abnormality but paternity checks were not made.

All cases with aberrant karyotypes were examined clinically. All individuals with trisomy G were examined to ensure that they did have Down syndrome. Ages and W.H.O. classification numbers (International Classification of Diseases, 1967) were extracted from patients' records to provide data on the degree of retardation and diagnostic categories.

## Results

The population of 588 consisted of 330 males and 258 females. The age distribution of the population is shown in Table 1, the mean age was 28.5 years (range 4—75 years). The diagnostic categories (International Classification of Diseases, 1967) into which the patients had been assigned are shown in Table 2, only four of the individuals with abnormal chromosomes (other than Down syndrome) had been recognised as such prior to this survey. The degree of retardation of the patients is shown in Table 3.

There were 272 males and 226 females with normal karyotypes and 90 individuals with abnormal karyotypes (Table 4). There were 73 individuals with Down syndrome, all had been recognised as such clinically and there were no patients who were thought to have Down syndrome who had normal karyotypes. There were 5 sex chromosome abnormalities, one had karyotype 46,X,+21/47,XX,+21 clinically she was not typically Down syndrome and had not been diagnosed as such. There were 12 other autosomal abnormalities, three of which [inv(19), t(13;14),t(2;10)] appeared to be euploid. Clinical and cytogenetic data on the

Table 1. Age distribution of the population according to chromosomal status

| Age (years) | Normal karyotype | Down syndrome | Other abnormality | Total |
|---|---|---|---|---|
| 0— 4 | 3 | — | — | 3 |
| 5— 9 | 30 | 8 | 2 | 40 |
| 10—14 | 79 | 14 | 1 | 94 |
| 15—19 | 74 | 9 | 2 | 85 |
| 20—24 | 65 | 4 | 4 | 73 |
| 25—29 | 62 | 4 | 3 | 69 |
| 30—34 | 38 | 6 | 1 | 45 |
| 35—39 | 24 | 6 | — | 30 |
| 40—44 | 18 | 4 | 1 | 23 |
| 45—49 | 30 | 5 | 1 | 36 |
| 50—54 | 27 | 8 | — | 35 |
| 55—59 | 19 | 3 | 1 | 23 |
| 60—64 | 16 | 2 | 1 | 19 |
| 65—69 | 8 | — | — | 8 |
| 70—74 | 5 | — | — | 5 |
| | 498 | 73 | 17 | 588 |

Table 2. Diagnostic classifications of the patients studied, as obtained from their records, showing the distribution of chromosome abnormalities not associated with Down syndrome

| | | |
|---|---|---|
| Following infections and intoxications | 60 | |
| Following trauma or physical agents | 102 | (4)[a] |
| With disorders of metabolism, growth or nutrition | 19 | |
| Associated with gross brain disease | 12 | |
| Unknown prenatal influences | 57 | (4) |
| Chromosome anomalies | 76 | (3) |
| Associated with prematurity | 15 | |
| Following major psychiatric disorder | 8 | (1) |
| Environmental deprivation | 16 | |
| Unknown | 223 | (5) |
| Total | 588 | (17) |

[a] Numbers in parentheses represent the chromosome abnormalities not associated with Down syndrome found within each group.

Table 3. Degree of mental retardation according to chromosomal status

| | | Normal karyotype | Down syndrome | Other abnormality | Total |
|---|---|---|---|---|---|
| Borderline | (IQ 68—85) | 7 | | | 7 |
| Mild | (IQ 52—67) | 65 | 9 | 2 | 76 |
| Moderate | (IQ 36—51) | 116 | 29 | 3 | 148 |
| Severe | (IQ 20—35) | 195 | 31 | 7 | 233 |
| Profound | (IQ < 20) | 115 | 4 | 5 | 124 |

Table 4. Abnormal karyotypes detected in this survey

| Karyotype | No. |
|---|---|
| 47,XXY | 2 |
| 47,XYY | 1 |
| 46,X,+21/47,XX,+21 | 1 |
| 45,X/46,X,+mar/46,XX | 1 |
| 46,XY/47,XY,+21 | 1 |
| 47,XY,+21 | 47 |
| 47,XX,+21 | 23 |
| 46,XX/47,XX,+21 | 1 |
| 46,XY,—14,t(14;21)(14qter→cen→21qter) | 1 |
| 47,XY,+mar | 3 |
| 47,XX,+mar | 1 |
| 46,XX,r(13) | 1 |
| 46,XY,r(18) | 1 |
| 46,XX,5p— | 1 |
| 46,XY,inv(19)(p13q13) | 1 |
| 46,XY,ins(2)(2pter→2q21:: ?::2p21→2qter) | 1 |
| 46,XX,der(9)t(9; ?)(p2 ?; ?) | 1 |
| 45,XX,t(13;14)(13qter→cen→14qter) | 1 |
| 46,XX,t(2;10)(q22;p13) | 1 |

Table 5. Chromosome counts on mosaics diagnosed during this survey

| Case No. | Counts | | | | | | Karyotype |
|---|---|---|---|---|---|---|---|
| | ≦44 | 45 | 46,+mar | 46 | 47 | 48 | |
| 21/69 | 2 | | | 16 | 132 | 1 | 46,X,+21/47,XX,+21 |
| 53/71 | | 10 | 33 | 17 | | | 45,X/46,X,+mar/46,XX |
| 94/71 | | | | 6 | 25 | | 46,XY/47,XY,+21 |
| 243/74 | | | | 14 | 19 | | 46,XX/47,XX,+21 |

patients with sex chromosome anomalies and autosomal anomalies other than Down syndrome are given in the appendix. Chromosome counts on the four mosaics encountered are shown in Table 5.

## Discussion

Apart from the present study there have been 3 surveys published in which the total population of a hospital for the mentally retarded has been karyotyped. The results of these surveys are summarised in Table 6: also included in this table are the results of a survey by Newton et al. (1972a,b) who karyotyped all patients older than 15 years in such a hospital. The populations of the hospitals in these surveys are similar with respect to degree of retardation but the 3 previous total population studies involved only young patients. The findings in all the series in Table 6 are similar except for that of Fujita and Fujita (1974) where Down syndrome is greatly under-represented and sex chromosome anomalies over-represented; this is probably due to the small hospital population of 59.

Table 6. Cytogenetic surveys of hospitals for the mentally retarded

| Authors | Age range | I.Q. | Down syndrome | | Other autosomal abnormalities | | Sex chromosome abnormalities | | Total No. studied |
|---|---|---|---|---|---|---|---|---|---|
| | | | No. | % | No. | % | No. | % | |
| Sutherland and Wiener (1971) | 3 months to 12 years | Mostly less than 35 | 18 | 11.3 | 2 | 1.25 | 0 | 0 | 159 |
| Newton et al. (1972a,b) | 16 to 70+ years | 78% less than 50 | 103 | 8.2 | 15 | 1.20 | 10 | 0.80 | 1255 |
| Fujita and Fujita (1974) | 6 to 18 years | All less than 66 | 1 | 1.7 | 1 | 1.7 | 2 | 3.4 | 59 |
| Cassiman et al. (1975) | All less than 18 years | All less than 50 | 111 | 13.0 | 18 | 2.10 | 3 | 0.35 | 857 |
| Present series | 4 to 75 years | 86% less than 51 | 73 | 12.4 | 12 | 2.04 | 5 | 0.85 | 588 |
| Total | | | 306 | 10.5 | 48 | 1.64 | 20 | 0.69 | 2918 |

Down syndrome is the most common chromosome anomaly encountered in hospitals for the mentally retarded, often accounting for 10—15% of the inmates. The slightly lower incidence of this anomaly in the series of Newton et al. (1972b), may be due to the acceptance by the hospital they surveyed of patients from the courts who are unlikely to have Down syndrome, and to the different age distribution of the population. The sex ratio of the Down syndrome patients in the present study of 2.17 was similar to that of 2.0 found in another large Australian hospital for the mentally retarded (Pitt et al., 1972), but intermediate between that of Cassiman et al. (1975) which was 3.6 and that of Newton et al. (1972b), which was 1.48. This excess of males with Down syndrome must reflect a greater liability of males to hospitalisation compared with females as suggested by Pitt et al. (1972). The number of cases of Down syndrome is too small to assess the relative frequencies of the various chromosomal forms but they do not appear to differ from other Australian series of karyotyped individuals with this disorder (Dey, 1970; Sutherland and Wiener, 1972).

Patients with small additional marker chromosomes form the next most common group of autosomal anomalies after Down syndrome. This group appears to be heterogeneous both cytogenetically and clinically, and in most instances the marker chromosomes remain unidentified and their etiological significance cannot be determined with certainty. Additional marker chromosomes are found in about 1 in 3000 unselected liveborn neonates (Nielsen and Sillesen, 1975) and are usually not associated with phenotypic abnormality detectable in the neonatal period. In the series summarised in Table 6 at least 13 such patients have been identified, an incidence 13 times greater than in the unselected neonates.

Jacobs (1974) has suggested that the presence of de novo structural rearrangements may be of etiological significance in mental retardation. In the present series there were 3 apparently euploid structural rearrangements, a Robertsonian translocation, a reciprocal translocation and a pericentric inversion, and of these only the reciprocal translocation, t(2;10), was shown to have arisen de novo. Even the finding of one such mutant out of 3 possible cases lends some support to Jacob's hypothesis, as apparently euploid de novo structural rearrangements are rare.

The frequency of X chromosome aneuploidy is increased amongst inmates of hospitals for the mentally retarded but this is more marked amongst patients with an IQ greater than 50 (Court Brown, 1969). Although none of the patients with X chromosome aneuploidy in the present study has an IQ greater than 50, none is severely or profoundly retarded with the exception of the one who also has an autosomal aneuploidy. The finding of one XYY male does not suggest that this condition is disproportionately represented in the population studied.

The contribution of chromosome abnormality to the etiology of mental retardation as seen in the present and similar surveys is highly significant. The series summarised in Table 6 show an incidence of chromosome abnormality of 12.8% compared with an incidence of 0.575% in unselected neonates (Nielsen and Sillesen, 1975). However, apart from Down syndrome which accounts for 10.5% of this

incidence figure, chromosome abnormality is probably responsible for only about 2% of the mental retardation as seen in the types of institutions studied.

## Appendix

*Case No. 619/75*

Karyotype 47,XXY

Age 62, weight 44.7 kg, height 162.5 cm.
Birth weight, parental ages, unknown.

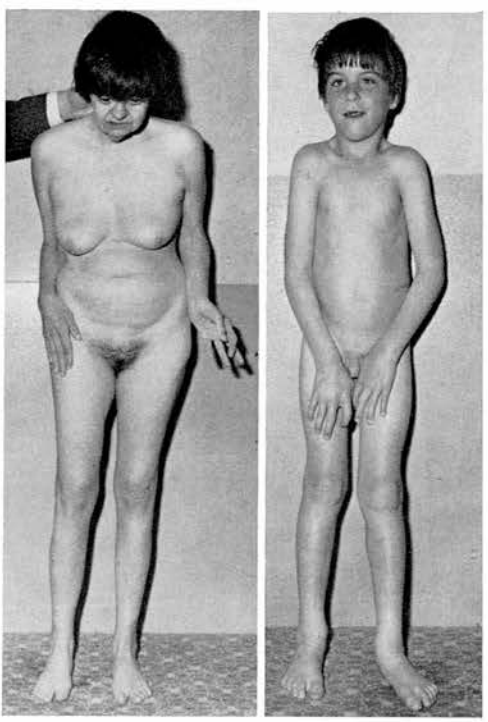
Moderately retarded emaciated old man with absent body hair, widely spaced nipples without gynaecomastia, bilateral cataracts, kyphosis, testicular atrophy and a small penis. Record of aggressive but not violent behaviour.

*Case No. 621/75*

Karyotype 47,XXY

Age 55, weight 48.1 kg, height 168 cm, head circumference 57 cm.
Birth weight, parental ages, unknown.

IQ (WAIS) 48 (full scale). Mild gynaecomastia with deeply pigmented nipples, sparse body and scalp hair, small testes, normal penis and pubic hair, bilateral pes cavus. Schizophrenic and diabetic.



1 2

Fig. 1. Case 21/69. Karyotype 46,X,+21/47,XX,+21

Fig. 2. Case 55/71. Karyotype 47,XY,+mar

*Case No. 70/75*

Karyotype 47,XYY

Age 24, weight 62.1 kg, height 187 cm, head circumference 54 cm.
Parental ages: mother 21 years, father unknown. Birth weight 1.36 kg.

IQ (Binet) 75 at 7 years of age. E.E.G. showed a focus in the posterior right temporal lobe. Subject to fits of uncontrollable rage, has a low frustration tolerance and these have lead to arrest by the police on several occasions. Clinically a large, well built man with no physical abnormality.

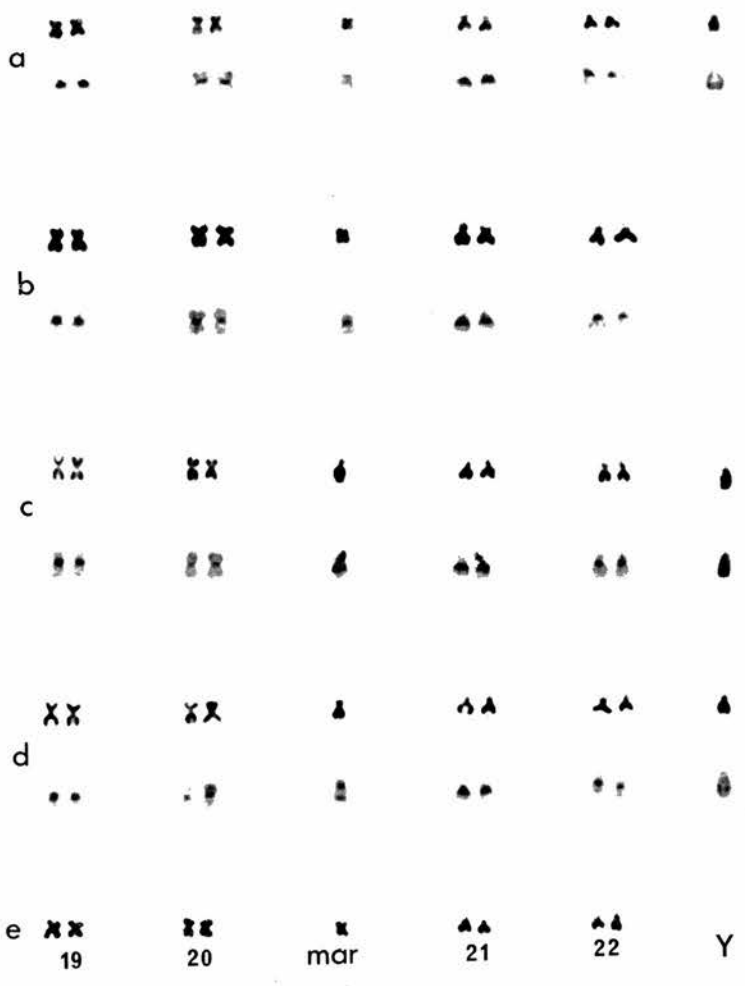



Fig. 3a—e. F- and G-group and marker chromosomes of (a) case 55/71, (b) case 415/74, (c) case 343/73, (d) case 77/74 and (e) case 53/71. Conventionally stained chromosomes are in the upper row and G-banded in the lower row, banded chromosomes are not included in (e). The markers in (c) and (d) are bisatellited, the others do not show satellites

*Case No. 21/69* (Fig. 1)

Karyotype 46,X,+21/47,XX,+21

Age 38, weight 36.6 kg, height 149 cm, head circumference 51 cm.

Parental ages unknown. Birth weight 4.20 kg.

Severely mentally retarded woman who lacks the typical appearance of Down syndrome but has many of the clinical findings: brachycephaly, epicanthic folds, Brushfield spots, mongoloid slant of the palpebral fissures, mid-facial hypoplasia. Somatic features of Turner syndrome absent but no record of menstruation in hospital records. Marked thoraco-lumbar kyphosis and a protuberant abdomen. Parental chromosomes not studied.

*Case No. 55/71* (Fig. 2)

Karyotype 47,XY,+mar (Fig. 3a)

Age 6 years, weight 20.01 kg, height 114 cm, head circumference 50 cm.

Parental ages: mother 25 years, father 42 years. Birth weight 3.60 kg.

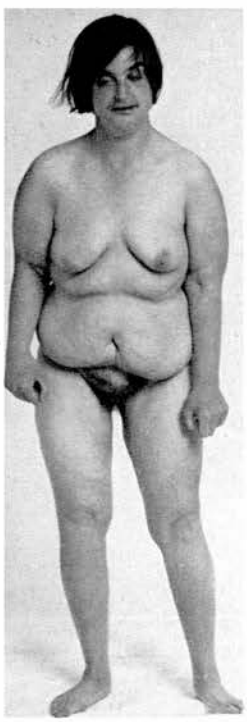
Born at term, perinatal anoxia due to mild respiratory distress syndrome. Right inguinal hernia repaired at 12 weeks of age. An E.E.G. at 3 years of age was abnormal, showing a moderate degree of non-specific cerebral dysrhythmia of non-focal type. At 4½ years Vineland Social Maturity Scale score was 2.0 years. Now a phenotypically normal boy with flat feet and mild spastic quadriplegia who suffers from epileptic seizures. Both parents have normal karyotypes.

*Case No. 415/74* (Fig. 4)

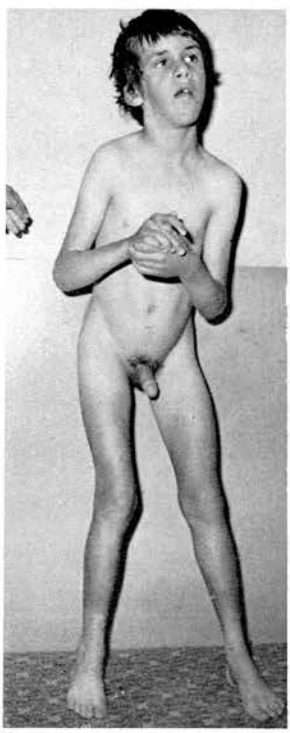
Karyotype 47,XX,+mar (Fig. 3b)

Age 23 years, weight 56 kg, height 144.5 cm, head circumference 52.5 cm.

Parental ages: mother 42 years, father 46 years. Birth weight 3.72 kg.



4



5

Fig. 4. Case 415/74. Karyotype 47,XX,+mar

Fig. 5. Case 343/73. Karyotype 47,XY,+mar

Profoundly retarded and obese with unusual fat distribution — large rolls over the elbows, posterior costal margins and lower abdominal wall. Flexion contractures of the fingers and a left simian crease. Hirsuite with synophrys. Secondary sexual characteristics normal and she menstruates. Both parents have normal karyotypes.

*Case No. 343/73* (Fig. 5)

Karyotype 47,XY,+mar (Fig. 3c)

Age 15 years, weight 31.2 kg, height 142 cm, head circumference 56 cm.

Parental ages: both 34 years. Birth weight 3.23 kg.

Anoxic at birth, inguinal hernia repair 2 years of age, uncontrolled epileptic seizures since age of 5 weeks. Severe mental retardation, prominent occiput and frontal bossing of head, left simian crease, spastic quadriplegia; all other findings normal. Mother normal karyotype, father not tested.

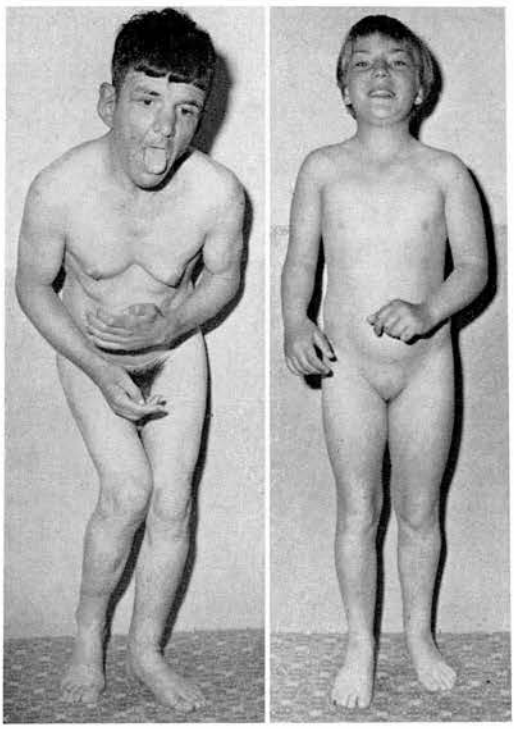
*Case No. 77/74* (Fig. 6)

Karyotype 47,XY,+mar (Fig. 3d)

Age 45 years, weight 60.2 kg, height 165 cm, head circumference 58 cm.

Parental ages: mother 32 years, father 34 years. Birth weight 4.10 kg.

Severely retarded man with fixed flexion deformities of the elbows and knees, and marked gynaecomastia with deeply pigmented nipples. Unusual facial appearance with very prominent antihelices (there is a small pit on the right tragus) and a bulky tongue which constantly protrudes. Suffers from grand mal epilepsy. Maternal karyotype normal, father not available for study.



6 7

Fig. 6. Case 77/74. Karyotype 47,XY,+mar

Fig. 7. Case 53/71. Karyotype 45,X/46,X,+mar/46,XX

*Case No. 53/71* (Fig. 7)

Karyotype 45,X/46,X,+mar/46,XX (Fig. 3e)

Age 12 years, weight 32 kg, height 125 cm, head circumference 49.5 cm.
Parental ages: mother 23 years, father 27 years. Birth weight 1.98 kg.

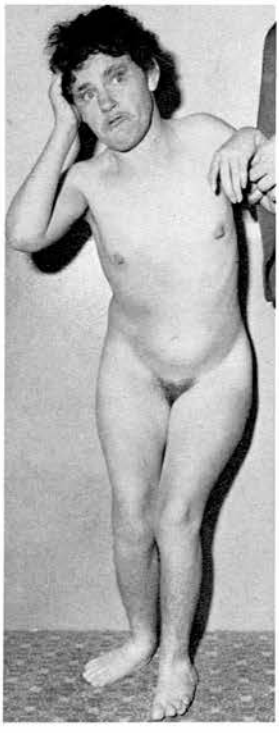
Moderately retarded short stocky girl with unusual facial appearance, low set ears with small tragi, shield chest with widely spaced nipples, increased carrying angles of the elbows and dimples over the greater trochanters. Epileptic since the age of 2 years. Severely abnormal E.E.G. with continuous subclinical epileptic discharges from the left middle and posterior temporal areas. Both parents have normal karyotypes.

*Case No. 485/73* (Fig. 8)

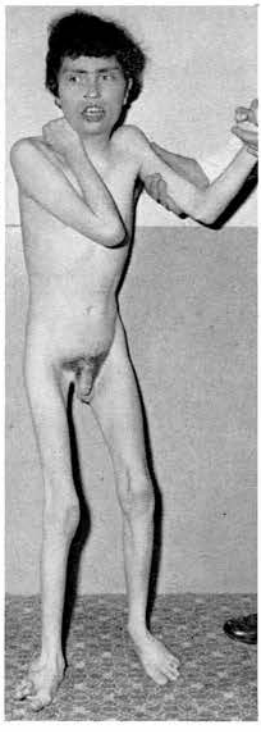
Karyotype 46,XX,r(13)

Age 23 years, weight 49.5 kg, height 142 cm, head circumference 50 cm.
Birth weight 1.57 kg.

Profoundly retarded, small brachycephalic microcephalic with eccentric constricted pupils and protuberant tongue. Long thin hands with tapering terminal phalanges. Secondary sexual characteristics normal and menstruation regular. Very hypertonic with sustained ankle clonus and maintains a bizzare posture with thighs flexed and legs crossed on chest. Both parents dead.



8



9

Fig. 8. Case 485/73. Karyotype 46,XX,r(13)

Fig. 9. Case 393/73. Karyotype 46,XY,r(18)

*Case No. 393/73* (Fig. 9)

Karyotype 46,XY,r(18)

Age 21 years, weight 28.9 kg, height 143 cm, head circumference 50 cm.
Parental ages: mother 29 years, father 28 years. Birth weight 2.50 kg.

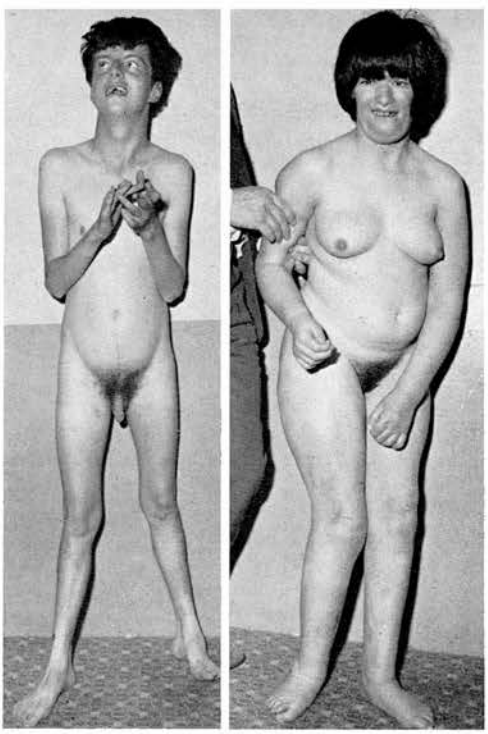
Numerous malformations: microcephaly, brachycephaly, mid-facial hypoplasia, enophthalmos, divergent strabismus, atretic ear canals, carp mouth. Left testis impalpable, large redundant scrotum. Leg musculature wasted, bilateral equinovalgus deformity with overriding of the third toes. Wrists hyperextensible, thumbs proximal and palmar creases normal. Repaired hiatus hernia. Vineland Social Maturity Scale score at 13 years was 1.0 years. Both parents have normal karyotypes.

*Case No. 143/70*

Karyotype 46,XX,5p-

Age 30 years, weight 61.8 kg, height 156 cm, head circumference 51.5 cm.
Parental ages: mother 31 years, father 33 years. Birth weight 3.29 kg.

Severely retarded microcephalic woman with multiple malformations: mild hypertelorism, divergent strabismus, mongolian slant to the palpebral fissures, and lumbar lordosis. Menstruates, normal secondary sexual characteristics. Violent and has to be kept away from children; had injured nursing staff. Did not have the "cri" characteristic of this syndrome. Both parents had normal karyotypes.



10 12

Fig. 10. Case 360/73. Karyotype 46,XY,ins(2)(2pter→2q21:: ?::2q21→2qter)

Fig. 12. Case 52/67. Karyotype 46,XX,der(9),t(9; ?)(p2?; ?)

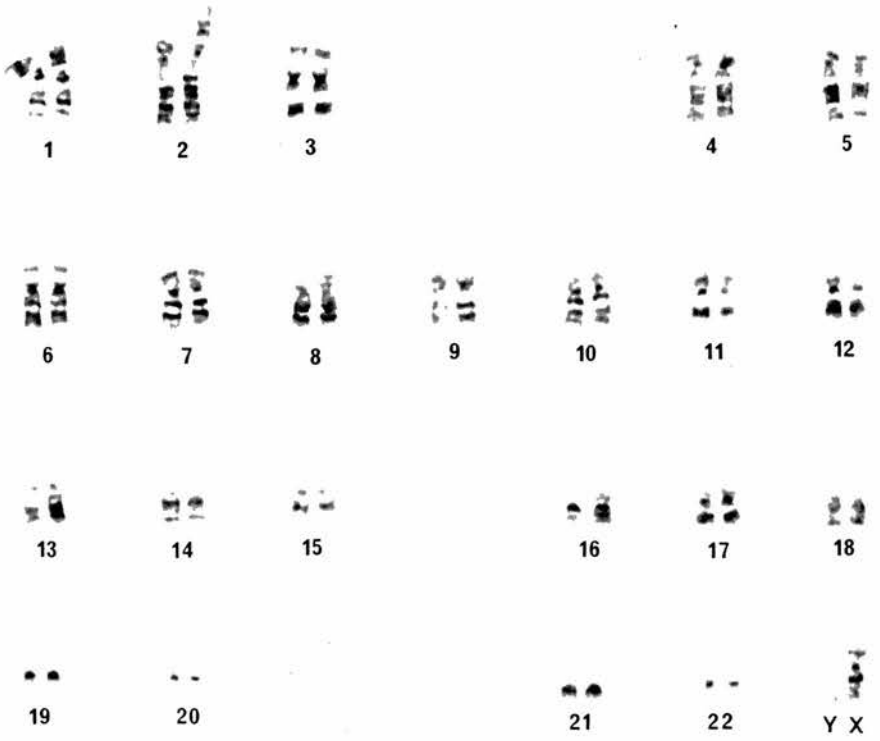



Fig. 11. Case 360/73. Karyotype 46,XY,ins(2)(2pter→2q21:: ?::2q21→2qter)

*Case No. 344/73*

Karyotype 46,XY,inv(19)(p13q13)mat.
(Full details of this patient have been published — Sutherland et al., 1976).

*Case No. 360/73* (Fig. 10)

Karyotype 46,XY,ins(2)(2pter→2q21:: ?::2q21→2qter) (Fig. 11)

Age 21 years, weight 45 kg, height 155 cm, head circumference 58 cm.
Parental ages: mother 29 years, father 35 years. Birth weight 2.41 kg.
Moderately retarded (IQ at 3 years 40—50) until the age of 17 years when he suffered ECHO virus encephalitis. After this became very violent but this settled and is now severely retarded (Vineland Social Maturity Scale score 3 years immediately post-encephalitis). Malformations include hypertelorism, broad base of nose, bilateral epicanthic folds, hypoplastic mandible, abnormal hands and feet. Marked camptodactyly of the left second and fifth digits with enlargement of the proximal interphalangeal joints and fixed flexion deformity, all toes except the first have varus deformity. Both parents have normal karyotypes.

*Case No. 52/67* (Fig. 12)

Karyotype 46,XX,der(9),t(9; ?)(p2 ?; ?) (Fig. 13)

Age 29 years, weight 50 kg, height 152 cm, head circumference 51.5 cm.
Parental ages: both 24 years. Birth weight 2.84 kg.

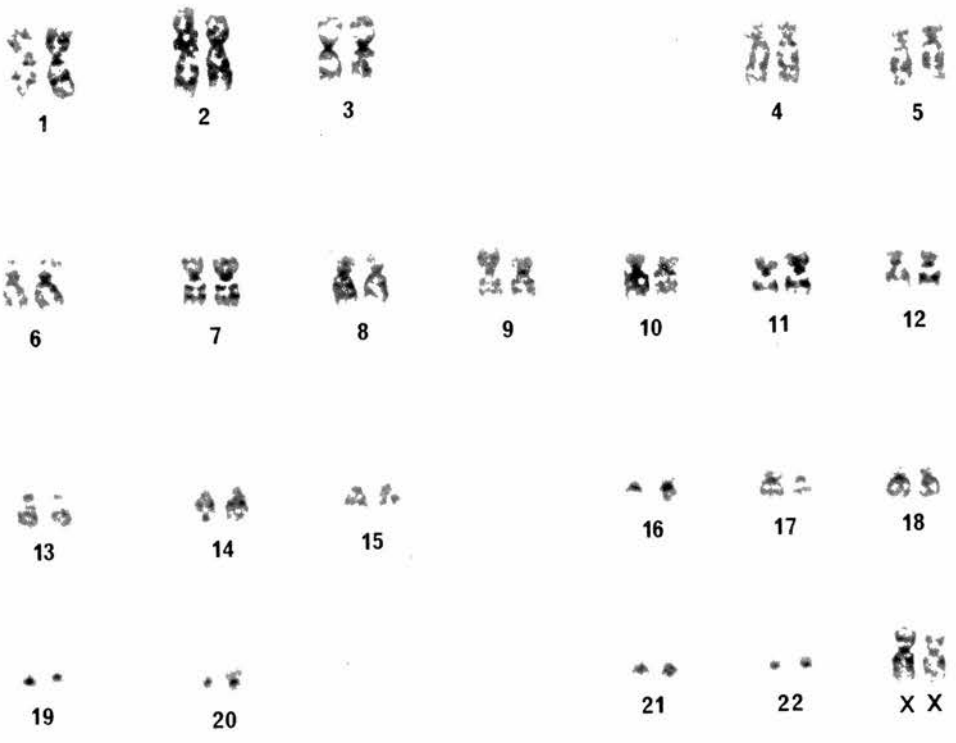



Fig. 13. Case 52/67. Karyotype 46,XX,der(9),t(9; ?)(p2 ?; ?)

Profoundly retarded, mildly microcephalic, with minimal phenotypic abnormality. Valgoid ankles, atrophic changes in the lower limbs. Grand mal epilepsy. Both parents have normal karyotypes.

*Case No. 330/74*

Karyotype 45,XX,t(13;14)(13qter→cen→14qter)
Age 25 years, weight 41.5 kg, height 152 cm, head circumference 54.5 cm.
Parental ages: mother 22 years, father 21 years. Birth weight 3.41 kg.

Severely retarded, hirsuite with excessive face and body hair, odd face with synophrys, short philtrum, upturned tip of nose and carp mouth (which may have been due to dental anomalies), palate high and arched. Epileptic since the age of 2 years; E.E.G. shows cortical damage in the right mid-temporal lobe which was epileptogenic. Mother has normal karyotype, father dead.

*Case No. 103/70*

Karyotype 46,XX,t(2;10)(q22;p13) (Fig. 14)
Age 8 years, weight 18.9 kg, height 122.5 cm, head circumference 49.5 cm.
Parental ages: mother 18 years, father 24 years. Birth weight 2.92 kg.

Small, profoundly retarded, low set ears (right ear also rotated anti-clockwise), divergent strabismus, broad shield-shaped chest with increased anterior-posterior diameter, mild pectus carinatum and widely spaced nipples. Camptodactyly of the fourth and fifth fingers bilaterally, mild talipes equinovarus and stooped gait with increased lumbar lordosis. E.E.G. at 8 months of age showed an excess delta rhythm but not hypsarrhythmia. Both parents have normal karyotypes.

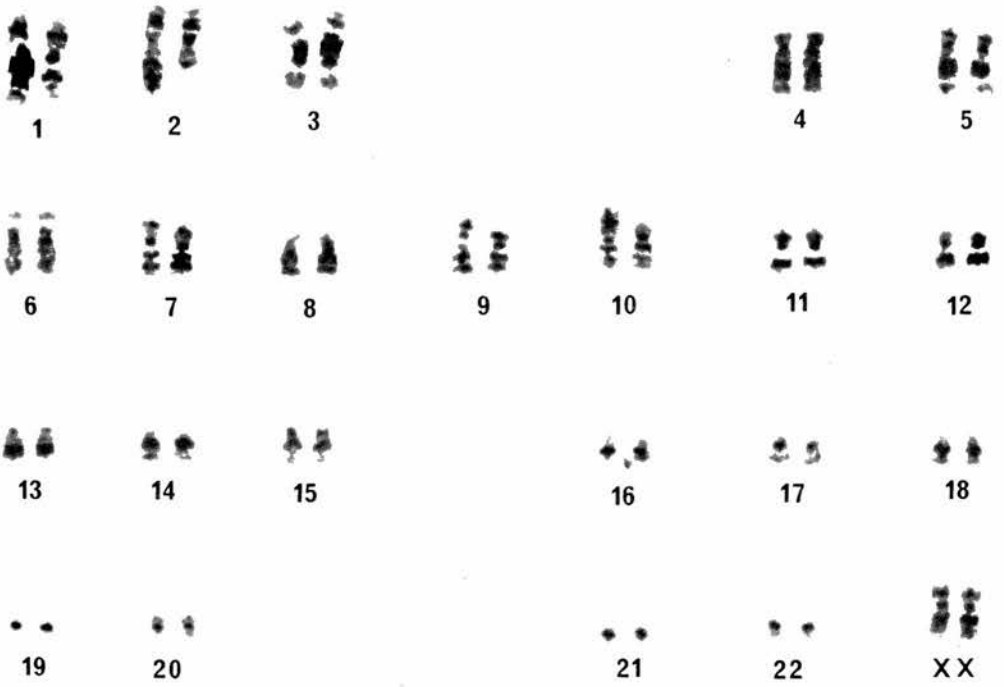



Fig. 14. Case 103/70. Karyotype 46,XX,t(2;10)(q22;p13)

*Acknowledgements.* We thank Lynene Day, Helen Eyre, Trudy Hocking and Erica Woollatt for technical assistance and the Director of Mental Health Services in South Australia, Dr. W. Dibden, for allowing us to carry out this study.

## References

Cassiman, J. J., Fryns, J. P., De Roover, J., Van den Berghe, H.: Sex chromatin and cytogenetic survey of 10,417 adult males and 857 children institutionalised in Belgian institutions for mentally retarded patients. Humangenetik **28**, 43—48 (1975)

Court Brown, W. M.: Sex chromosome aneuploidy in man and its frequency with special reference to mental subnormality and criminal behaviour. Int. Rev. Exp. Path. **7**, 31—97 (1969)

Dey, J.: Survey of 500 cases of Down's syndrome. Aust. J. ment. Ret. **1**, 154—159 (1971)

Fujita, H. M., Fujita, K.: A cytogenetic survey a mentally retarded children. Jap. J. hum. Genet. **19**, 175—176 (1974)

International Classification of Diseases (1967). Manual of the international statistical classification of diseases, injuries and causes of death. Eighth revision. World Health Organisation, Geneva

Jacobs, P. A.: Correlation between euploid structural chromosome rearrangements and mental subnormality in humans. Nature (Lond.) **249**, 164—165 (1974)

Newton, M. S., Jacobs, P. A., Price, W. H., Woodcock, G., Fraser, I. A.: A chromosome survey of a hospital for the mentally subnormal. Part 1: Sex chromosome abnormalities. Clin. Genet. **3**, 215—225 (1972a)

Newton, M. S., Cunningham, C., Jacobs, P. A., Price, W. H., Fraser, I. A.: A chromosome survey of a hospital for the mentally subnormal. Part. 2: Autosome abnormalities. Clin. Genet. **3**, 226—248 (1972b)

Nielsen, J., Sillesen, I.: Incidence of chromosome aberrations among 11,148 newborn children. Humangenetik **30**, 1—12 (1975)

Pitt, D., Roboz, P., Seidurs, E.: The spectrum of severe mental deficiency. Experiences with 1,400 cases. Aust. J. ment. Ret **2**, 40—46 (1972)

Sutherland, G. R., Gardiner, A. J., Carter, R. F.: Familial pericentric inversion of chromosome 19,inv(19) (p13q13) with a note on genetic counselling of pericentric inversion carriers. Clin. Genet. (**10**, 54—59, 1976)

Sutherland, G. R., Wiener, S.: Chromosome studies in a mental deficiency hospital: Total ascertainment. Aust. J. ment. Ret. **1**, 246—247 (1971)

Sutherland, G. R., Wiener, S.: Cytogenetics of 271 mongols. Aust. paediat. J. **8**, 90—91 (1972)

Dr. Grant R. Sutherland
Cytogenetics Unit
Department of Histopathology
The Adelaide Children's Hospital (Inc.)
North Adelaide, S.A. 5006
Australia

*Ann. Hum. Genet., Lond.* (1978), **42**, 173
*Printed in Great Britain*



# Chromosome studies at the paediatric necropsy

BY GRANT R. SUTHERLAND, RODNEY F. CARTER

*Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia*

RHONA BAULD, IAN I. SMITH AND A. DOUGLAS BAIN

*Department of Pathology, Royal Hospital for Sick Children, Edinburgh EH9 1LF, Scotland*

Studies by Machin (1974), Bauld, Sutherland & Bain (1974) and Kuleshov (1976) have shown that the incidence of major chromosome abnormalities in perinatal deaths is ten times greater than that in liveborn infants. This report extends the findings from Edinburgh (Bauld *et al.* 1974) to cover a full 4-year period ending in September 1976 and documents the results of a similar study carried out over a full 2-year period ending in May 1977 at the Adelaide Children's Hospital.

## MATERIALS AND METHODS

*Edinburgh.* All routine necropsies on late spontaneous abortions, therapeutic abortions following prenatal diagnostic studies, stillbirths and neonatal deaths in the Lothian area were carried out by the Department of Pathology at the Royal Hospital for Sick Children. Necropsies on older children dying in hospital were also included in the study as were sudden infant deaths examined under instruction from the Procurator Fiscal.

*Adelaide.* All routine necropsies following deaths within the Adelaide Children's Hospital were studied. In addition some stillbirths and neonatal deaths were referred by private practitioners for necropsy and all sudden infant deaths in Adelaide were examined under the authority of the Coroner.

Definition of the terms 'perinatal death' and 'older neonate' are given by Bauld *et al.* (1974). In addition, in this report the term infant is used to denote a baby aged more than 28 days but less than 366 days, and child refers to deaths occurring on or after the 366th day of life. In Edinburgh chromosome studies were carried out wherever possible and without selection in all abortus, perinatal, neonatal and infant necropsies but some selection was applied to childhood deaths.

In Adelaide chromosome studies were carried out in all necropsies and this series can be considered to be on unselected consecutive necropsies.

The methods of chromosome study for the Edinburgh series have been published (Bauld *et al.* 1974). Chromosome banding was carried out in most cases during the last two years of the period of study. In Adelaide virtually all results were obtained from skin fibroblast culture; 30 metaphases per case were examined microscopically and a karyotype prepared. Skin was collected for this purpose from the edge of the ventral incision at the beginning of the necropsy. Where chromosome studies had been carried out during life these results were used. Banding was employed in all cases of significant malformation except for isolated central nervous system and cardiac lesions, to delineate any chromosome abnormalities detected. G-, C- or Q-banding was used as required.

Table 1. *Chromosome results according to category of necropsy, Edinburgh series*

| Category | Total | Chromosome studies attempted | Successful | Normal karyotype | | Abnormal karyotype | |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | No. | % |
| Therapeutic abortion | 24 | 18 | 18 | 3 | 8 | 7 | 39 |
| Spontaneous abortion (less than 20 weeks) | 19 | 15 | 8 | 3 | 3 | 2 | 25 |
| Spontaneous abortion (more than 20 weeks) | 52 | 35 | 27 | 10 | 15 | 2 | 7·4 |
| Macerated stillbirth | 206 | 144 | 49 | 24 | 19 | 6 | 12·2 |
| Non-macerated stillbirth | 196 | 178 | 146 | 59 | 82 | 5 | 3·4 |
| Early neonatal death (less than 28 weeks gestation) | 32 | 30 | 29 | 17 | 11 | 1 | 3·4 |
| Early neonatal death (more than 28 weeks gestation) | 351 | 341 | 311 | 172 | 125 | 14 | 4·5 |
| Later neonatal death | 82 | 72 | 64 | 34 | 28 | 2 | 3·1 |
| Older than 28 days | 139 | 119 | 96 | 51 | 35 | 10 | 10·4 |
| Older than 1 year | 92 | 36 | 32 | 15 | 15 | 2 | 6·3 |
| Total | 1193 | 988 | 780 | 388 | 341 | 51 | 6·5 |

Table 2. *Chromosome results according to category of necropsy, Adelaide series*

| Category | Total | Chromosome studies attempted | Successful | Normal karyotype | | Abnormal karyotype | |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | No. | % |
| Therapeutic abortion | 6 | 6 | 5 | 2 | 2 | 1 | 20 |
| Spontaneous abortion (less than 20 weeks) | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| Spontaneous abortion (more than 20 weeks) | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| Macerated stillbirth | 10 | 10 | 7 | 3 | 3 | 1 | 14 |
| Non-macerated stillbirth | 13 | 13 | 11 | 6 | 5 | 0 | 0 |
| Early neonatal death | 52 | 52 | 48 | 36 | 12 | 0 | 0 |
| Later neonatal death | 29 | 29 | 22 | 7 | 12 | 3 | 13·6 |
| Older than 28 days | 116 | 116 | 107 | 56 | 47 | 4 | 3·7 |
| Older than 1 year | 102 | 99 | 94 | 58 | 29 | 7 | 6·4 |
| Total | 331 | 328 | 295 | 169 | 110 | 16 | 5·4 |

## RESULTS

All necropsies carried out in both series are shown according to category in Table 1 (Edinburgh) and Table 2 (Adelaide). In the Edinburgh series chromosome studies were attempted on 83% of necropsies and a chromosome result was obtained for 65%; the corresponding figures for the Adelaide series are 99% and 89%. Reasons for failure to attempt chromosome study included maceration (mainly in the earlier part of the Edinburgh series as the technique of amnion culture in these cases was not in use at that time), severe pyogenic infection, arrival of specimens in fixative, loss of samples over holiday periods, etc. From these tables it can be seen that the populations studied are very different; only 19% of the Edinburgh series was older than 28 days compared with 65% of the Adelaide series.

Table 3. *Chromosome results according to primary cause of death (therapeutic abortions and abortions of less than 20 weeks gestation excluded), Edinburgh series*

| Cause of death | Total | Chromosome studies attempted | Successful | Normal karyotype | | Abnormal karyotype | |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | No. | % |
| Macerated without malformation | 188 | 127 | 47 | 25 | 19 | 3 | 6·4 |
| Macerated with malformations | 29 | 20 | 3 | 0 | 0 | 3 | (100) |
| Prematurity associated disease | 180 | 172 | 157 | 102 | 53 | 2 | 1·3 |
| Primary C.N.S. malformation | 138 | 120 | 101 | 32 | 69 | 0 | 0 |
| Severe congenital malformations | 116 | 107 | 98 | 39 | 33 | 26 | 27 |
| Congenital heart malformation | 80 | 67 | 62 | 37 | 23 | 2 | 3·3 |
| Primary anoxia | 185 | 168 | 143 | 74 | 67 | 2 | 1·4 |
| Infection | 64 | 41 | 34 | 17 | 16 | 1 | 2·9 |
| S.I.D.S. | 58 | 56 | 40 | 17 | 21 | 2 | 5·0 |
| Leukaemia | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other malignancy | 12 | 2 | 1 | 0 | 0 | 1 | (100) |
| Trauma | 33 | 29 | 26 | 15 | 11 | 0 | 0 |
| Mendelian disorders | 22 | 16 | 15 | 7 | 8 | 0 | 0 |
| Miscellaneous | 40 | 30 | 27 | 17 | 10 | 0 | 0 |
| Total | 1150 | 955 | 754 | 382 | 330 | 42 | 5·6 |

The chromosome results according to primary cause of death for all the cases in each series, with the exception of abortions of less than 20 weeks and therapeutic abortions, are shown in Tables 3 and 4. A similar breakdown of findings pertaining only to perinatal deaths (to allow comparison of these series with other published series) are shown in Tables 5 and 6.

There are only two major groups in which chromosome abnormalities are not prominent; these are deaths due to primary central nervous system malformations (mainly anencephaly and spina bifida) and the sudden infant death syndrome (S.I.D.S.). Of the two infants in the latter group in the Edinburgh series the $48,XXYY/49,XXYY,+8$ had failed to thrive from birth but the $46,XY/47,XY,+8$ was a true case of S.I.D.S. Although traumatic death may have an association with chromosome abnormality via its association with mental subnormality and consequent increased accident mortality, the finding of a balanced translocation in this group in the Adelaide series is fortuitous. The chromosome abnormalities detected are shown in Tables 7 and 8. These abnormalities are of the type usually seen in live-born infants rather than abortuses with exception of the triploids and the two macerated foetuses who were mosaics for trisomy 2. These mosaics showed no malformations, although the one with karyotype $46,XY/47,XY,+ace/48,XY,+2,+ace$* has an unusual facial appearance with protuberant eyes, antimongoloid slanting, palpebral fissures and low-set ears with a maldeveloped helix; its placenta showed hydatidiform change and the possibility of triploidy had been considered.

Chromosome counts in the mosaics from the Edinburgh series are shown in Table 9. The mosaic in the Adelaide series showed karyotype $46,XX$ in 50 cells and $46,XX,t(9;14)(q34;q12)$ in 31 cells

* *ace* – small acentric fragment.

Table 4. *Chromosome results according to primary cause of death (therapeutic abortions and abortions of less than 20 weeks gestation excluded), Adelaide series*

| Cause of death | Total | Chromosome studies attempted | Successful | Normal karyotype | | Abnormal karyotype | |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | No. | % |
| Macerated without malformation | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| Prematurity associated disease | 13 | 13 | 12 | 8 | 4 | 0 | 0 |
| Primary C.N.S. malformation | 17 | 17 | 14 | 5 | 9 | 0 | 0 |
| Severe congenital malformations | 28 | 28 | 28 | 10 | 11 | 7 | 22·2 |
| Congenital heart malformation | 44 | 44 | 44 | 28 | 13 | 3 | 6·8 |
| Primary anoxia | 27 | 27 | 21 | 16 | 4 | 1 | 4·8 |
| Infection | 27 | 27 | 26 | 11 | 13 | 2 | 7·7 |
| S.I.D.S. | 78 | 78 | 66 | 39 | 27 | 0 | 0 |
| Leukaemia | 10 | 10 | 7 | 4 | 3 | 0 | 0 |
| Other malignancy | 13 | 12 | 10 | 7 | 2 | 1 | 10 |
| Trauma | 32 | 30 | 30 | 22 | 7 | 1 | 3·3 |
| Mendelian disorders | 10 | 10 | 10 | 6 | 4 | 0 | 0 |
| Miscellaneous | 23 | 23 | 21 | 11 | 10 | 0 | 0 |
| Total | 323 | 320 | 290 | 167 | 108 | 15 | 5·2 |

Table 5. *Perinatal deaths in the Edinburgh series*

| Cause of death | Total | Chromosome studies attempted | Successful | Normal karyotype | | Abnormal karyotype | |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | No. | % |
| Macerated without malformation | 179 | 125 | 46 | 24 | 19 | 3 | 6·5 |
| Macerated with malformations | 26 | 18 | 3 | 0 | 0 | 3 | (100) |
| Prematurity associated disease | 158 | 151 | 139 | 93 | 45 | 1 | 0·7 |
| Primary C.N.S. malformation | 116 | 106 | 88 | 27 | 61 | 0 | 0 |
| Severe congenital malformations | 74 | 72 | 67 | 28 | 24 | 15 | 22 |
| Congenital heart malformation | 30 | 30 | 26 | 15 | 11 | 0 | 0 |
| Primary anoxia | 152 | 143 | 123 | 67 | 54 | 2 | 1·6 |
| Infection | 11 | 9 | 7 | 1 | 5 | 1 | (14·3) |
| Malignancy | 2 | 2 | 1 | 0 | 0 | 1 | (100) |
| Trauma | 14 | 14 | 13 | 7 | 6 | 0 | 0 |
| Mendelian disorders | 7 | 7 | 7 | 2 | 5 | 0 | 0 |
| Miscellaneous | 16 | 16 | 15 | 8 | 7 | 0 | 0 |
| Total | 785 | 693 | 535 | 272 | 237 | 26 | 4·9 |

Table 6. *Perinatal deaths in the Adelaide series*

| Cause of death | Total | Chromosome studies attempted | Successful | Normal karyotype | | Abnormal karyotype | |
|---|---|---|---|---|---|---|---|
| | | | | Male | Female | No. | % |
| Macerated without malformation | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| Macerated with malformations | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Prematurity associated disease | 12 | 12 | 11 | 8 | 3 | 0 | 0 |
| Primary C.N.S. malformation | 2 | 2 | 2 | 1 | 1 | 0 | 0 |
| Severe congenital malformations | 11 | 11 | 11 | 5 | 6 | 0 | 0 |
| Congenital heart malformation | 12 | 12 | 12 | 10 | 2 | 0 | 0 |
| Primary anoxia | 26 | 26 | 20 | 15 | 4 | 1 | 5 |
| Infection | 3 | 3 | 3 | 2 | 1 | 0 | 0 |
| Malignancy | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Trauma | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| Mendelian disorders | 2 | 2 | 2 | 2 | 0 | 0 | 0 |
| Miscellaneous | 4 | 4 | 3 | 1 | 2 | 0 | 0 |
| Total | 75 | 75 | 66 | 45 | 20 | 1 | 1·5 |

examined. This mosaicism was found in fibroblasts from replicate primary cultures of skin. In addition to chromosome abnormalities a number of diseases due to single gene defects were recognized in both series of necropsies. These are listed in Table 10.

In five macerated stillbirths from the Edinburgh series the chromosomal sex and the phenotypic sex did not correspond. These results have not been included and are listed in the tables as though they had not been attempted. One of these was a male, almost certainly affected by Down's syndrome, yet amnion culture revealed a normal female karyotype. Enquiries suggested that it was highly probable that the placenta which accompanied the foetus at necropsy was the wrong one. Of the other cases three were male foetuses and one a female foetus. Whilst the possibility that some of these represent genuine discordance between phenotypic and genetic sex more plausible explanations are that the wrong placentae were sent for necropsy or possibly that amnion culture in some cases may yield maternal cell growth.

## DISCUSSION

Chromosome studies have now been carried out on unselected paediatric necropsies in four centres – Adelaide, Edinburgh, London and Moscow (Table 11). The results obtained from each centre are similar for deaths occurring in the perinatal period, the frequency of major chromosome abnormality being 1·5 % in Adelaide, 4·9 % in Edinburgh, 5·6 % in London and 6·9 % in Moscow. The incidence in Adelaide is lower than in the other centres, but this may be due to differences in the types of neonates admitted to the Adelaide Children's Hospital and the relatively small numbers in this part of the Adelaide series. Frequently the more severely malformed infants with chromosome abnormalities are not admitted to the Adelaide Children's Hospital but die during the perinatal period in maternity hospitals.

Table 7. *Chromosome abnormalities detected in the Edinburgh series*

| Karyotype | | Comments |
|---|---|---|
| 45, *X*/46*X*, *dic*(*X*)(*p*11) | | 2 months old, congenital heart disease |
| 47, *XXX* | (2) | 1 therapeutic abortion; 1 31-week stillbirth |
| 47, *XYY* | | 3 hours old, bilateral renal dysplasia |
| 48, *XXYY*/49, *XXYY*, +8 | | 8 months old, S.I.D.S. |
| 46, *XY*/47, *XY*, +*ace*/48, *XY*, +2, +*ace* | | 34-week stillbirth |
| 46, *XX*/47, *XX*, +2 | | 37 week macerated stillbirth |
| 47, *XY*, +C | | 35-week stillbirth |
| 47, *XX*, +9 | | 35-week stillbirth, trisomy 9 syndrome |
| 46, *XY*/47, *XY*, +C | (3) | 26-week abortion; 5-month multiple malformations; 36-week hydropic stillbirth |
| 46, *XX*/47, *XX*, +C | (2) | 2-month congenital heart disease; 2-days infectious death |
| 46, *XY*/47, *XY*, +8 | | 8 months old, S.I.D.S. |
| 47, *XX*, +13 | | 20 days old |
| 47, *XY*, +13 | | 12 hours old |
| 46, *XX*, −14, +*t*(13*q*14*q*) | | 6 days old, only malformations bicornuate uterus and ventricular septal defect |
| 47, *XY*, +18 | (4) | 2 stillbirths; 2 early neonatal deaths |
| 47, *XX*, +18 | (9) | 3 therapeutic abortions; 5 early neonatal deaths; 1 infant |
| 47, *XY*, +21 | (8) | 3 therapeutic abortions; 1 macerated stillbirth; 1 early neonatal death; 1 later neonatal death; 2 infants |
| 47, *XX*, +21 | (7) | 1 therapeutic abortion; 1 macerated stillbirth; 1 early neonatal death; 2 infants; 2 children |
| 45, *XX*, *t*(13*q*14*q*) | | 1 hour old, congenital hepatic haemangioma |
| 46, *XX*, *inv*(8)(*p*23*q*11) | | Stillbirth at term, anoxic death |
| 46, *XY*, *der*(13), *t*(7; 13)(*q*36; *q*22)*pat* | | 6 days old, multiple malformations |
| 69, *XXY* | (2) | 13-week abortion; 20-week abortion |

Table 8. *Chromosome abnormalities detected in the Adelaide series*

| Karyotype | | Comments |
|---|---|---|
| 45, *X* | (3) | 1 therapeutic abortion; 2 older neonates with congenital heart disease |
| 47, *XXY* | | 1 year old with congenital heart disease |
| 47, *XY*, +21 | (4) | 9 years old; 1 year old; 4 months old; 5 weeks old |
| 47, *XX*, +21 | | 8 years old |
| 47, *XX*, +18 | | 7 months old |
| 46, *XY*, 7*p*+ | | Unbalanced translocation, not maternal |
| 46, *XY*, *inv*(2)(*p*11*q*13) | | 8 days old, infectious death |
| 46, *XY*, *t*(12; 16)(*q*24; *p*11) | | Normal child, traumatic death |
| 46, *XX*, *t*(1; 5)(*q*25; *p*13) | | Normal child, rhabdomyosarcoma |
| 46, *XX*/46, *XX*, *t*(9; 14)(*q*34; *q*12) | | Normal child, part aboriginal, infectious death |
| 69, *XXY* | | Macerated 33-week foetus |

Only the series reported from Adelaide and Edinburgh have included studies on unselected paediatric autopsies beyond the perinatal period. These are summarized in Table 11. In macerated stillbirths it can be seen that chromosome abnormality has the highest incidence. In non-macerated stillbirths the frequency of chromosome abnormality drops to 3·8% but continues to increase with age until it reaches 7·4% in children. The increased incidence of chromosome abnormalities associated with death after the perinatal period has not been noted previously and is accounted for mainly by infants with Down's syndrome.

Table 9. *Chromosome counts in cases of mosaicism detected in the Edinburgh series*

| Karyotype | Tissue | Chromosome number | | | | |
|---|---|---|---|---|---|---|
| | | 45 | 46 | 47 | 48 | 49 |
| 45, $X$/46, $X$, *dic*($X$)($p11$) | Urine | 7 | 8 | — | — | — |
| | Pleural fluid | 32 | 27 | — | — | — |
| 48, $XXYY$/49, $XXYY$, +8 | Testis | — | — | — | 42 | 4 |
| | Pericardium | — | — | — | 5 | |
| 46, $XX$/47, $XX$, +$C$* | Pericardium | — | 28 | 4 | — | |
| 46, $XX$/47, $XX$, +$C$ | Pericardium | — | 20 | 5 | — | |
| 46, $XY$/47, $XY$, +$C$* | Testis | — | 49 | 6 | — | |
| 46, $XY$/47, $XY$, +$C$ | Testis | — | 70 | 5 | — | |
| 46, $XY$/47, $XY$, +$C$ | Amnion | 3 | 27 | 4 | — | |
| 46, $XY$/47, $XY$, +8 | Testis | 1 | 25 | 3 | — | |
| 46, $XX$/47, $XX$, +2 | Amnion | — | 43 | 20 | — | |
| 46, $XY$/47, $XY$, +*ace*/48, $XY$, +2, +*ace* | Amnion | — | 12 | 15 | 3 | |

* Previously published in Bauld *et al.* (1974).

Table 10. *Mendelian disorders detected in both series of necropsies*

| Edinburgh | | Adelaide | |
|---|---|---|---|
| Cystic fibrosis | (9) | Cystic fibrosis | (3)* |
| Gaucher disease | (3) | Hurler syndrome | (2) |
| Renal cystic dysplasia | (2) | $Gm_1$-gangliosidosis | (2)† |
| Hunter syndrome | | San Filipo A syndrome | |
| Hurler syndrome‡ | | Adrenoleukodystrophy | |
| Zellweger syndrome | | Cystinosis | |
| Apert syndrome | | Thanatophoric dwarf | |
| Lactic acidosis (familial) | | Metatrophic dwarf | |
| Neurolipidosis | | | |
| Arthrogryposis multiplex congenita | | | |
| Endocardial fibroelastosis (familial) | | | |
| Asphyxiating thoracic dystrophy | | | |

* One of these was in an infant with 47, $XY$, +21.
† One therapeutic abortion.
‡ Therapeutic abortion following prenatal diagnosis.

Table 11. *Results from four series of chromosome studies in unselected paediatric autopsies*

| Status | Machin (1974) London | Kuleshov (1976) Moscow | This report Edinburgh | This report Adelaide | Total | Abnormal (%) |
|---|---|---|---|---|---|---|
| Macerated stillbirth | 34 (3)* | 22 (3) | 49 (6) | 7 (1) | 112 (13) | 11·6 |
| Non-macerated stillbirth | 122 (5) | 61 (3) | 146 (5) | 11 (0) | 340 (13) | 3·8 |
| Early neonatal death | 344 (20) | 92 (6) | 340 (15) | 48 (0) | 824 (41) | 5·0 |
| Late neonatal death | — | — | 64 (2) | 22 (3) | 86 (5) | 5·8 |
| Infant | — | — | 96 (10) | 107 (4) | 203 (14) | 6·9 |
| Child | — | — | — | 94 (7) | 94 (7) | 7·4 |

* Number studied (number abnormal).

Chromosome abnormalities are of course not the only genetic disorders associated with paediatric death. Machin & Crolla (1974) recognized 9 infants with disorders due to genes of major effect amongst 728 perinatal necropsies (1·24 %). In the whole Edinburgh series (excluding early and therapeutic abortions) there were 22 such disorders recognized in 1,150 necropsies (1·91 %) and in Adelaide 10 in 323 (3·10 %). All these figures must be regarded as minimal since some of these disorders may not be recognized, especially in perinatal deaths.

A number of chromosome mosaics were diagnosed in this study. In view of reports (Littlefield & Mailhes, 1975; Harnden *et al.* 1976) of chromosomally abnormal clones arising in skin fibroblast cultures it is possible that some of the mosaics diagnosed could be due to artifact and not reflect constitutional chromosome status. The most frequent type of mosaicism involved a minor cell line with an additional C-group chromosome. The cell strains involved were often poorly growing and sufficient material could not be obtained to allow precise identification of the additional chromosomes. Harnden *et al.* (1976) state that abnormal clones have only been observed to arise in adult skin cultures and have not been seen in foetal skin cultures. Most of the tissues used in the present studies were not skin, and it is known that clones can arise in amniotic fluid cultures which are foetal in origin (Sutherland, Bowser-Riley & Bain, 1975). There can be little doubt about the validity of the results of the $45,X/46,X,dic(X)$ mosaic as this was found from two tissues. Similarly, the translocation mosaic in the Adelaide series was diagnosed from replicate primary cultures and is most unlikely to be artifact.

Amongst the 535 perinatal deaths which were successfully karyotyped in the Edinburgh series there were four cases of Down's syndrome. This is more than four times the accepted incidence of this condition at birth. Similarly Down's syndrome was disproportionately represented in the London and Moscow series. As has already been pointed out by Bain *et al.* (1976), these findings may help to explain the discrepancy between the epidemiologically derived risk figure of 1·3 % for older women having a child with Down's syndrome (Hook, 1976) and the figure of 4·6 % derived by Ferguson-Smith (1976) from prenatal diagnostic results. It is unlikely that the epidemiological data would have included macerated stillbirths and some of the infants dying in the first week of life as these would probably have remained undiagnosed.

There can be little doubt that chromosomal studies are an important part of the investigation of paediatric deaths. Information has been gained concerning selection against the chromosomally abnormal, which begins at conception and continues well into childhood. The chromosome results obtained are of great value in genetic counselling, even in those instances where the chromosomes are found to be normal since there is little point in offering prenatal chromosome studies to parents who have had an abnormal baby which was found to have a normal karyotype. In conclusion, it is suggested that chromosome studies should be an integral part of the paediatric necropsy, except for deaths due to primary central nervous system lesions, S.I.D.S. in apparently normal healthy infants and trauma. However, in dealing with S.I.D.S. it can be argued that it is essential to obtain as much information as possible; routine chromosome studies are perhaps justifiable on this basis.

## SUMMARY

The results of chromosome studies from 1193 consecutive paediatric necropsies in Edinburgh and 331 in Adelaide are given. In the Edinburgh series 51 major chromosome abnormalities were detected in 780 (6·5 %) necropsies where chromosome studies were successful and in Adelaide

16 in 295 (5·4 %) were found. It is suggested that chromosome studies should become an integral part of the paediatric necropsy except for deaths due to primary central nervous system lesions and trauma.

We thank the many people who have assisted with this study, including pathologists who conducted necropsies and collected samples for us, cytogeneticists from other institutions who allowed us to use chromosome results from patients studied during life and the following people for assistance with tissue culture and cytogenetics: Susan Bowser-Riley, Elizabeth Grace and Janet Watson in Edinburgh, and Lynene Day, Helen Eyre, Trudy Hocking and Erica Woollatt in Adelaide.

REFERENCES

Bain, A. D., Smith, I. I., Bauld, R., Bowser-Riley, S. & Watson, J. (1976). Prenatal diagnosis, stillbirths, and the macerated fetus. *Lancet* ii, 375.

Bauld, R., Sutherland, G. R. & Bain, A. D. (1974). Chromosome studies in the investigation of stillbirths and neonatal deaths. *Archs Dis. Child.* **49**, 782–788.

Ferguson-Smith, M. A. (1976). Prospective data on risk of Down syndrome in relation to maternal age. *Lancet* ii, 252.

Harnden, D. G., Benn, P. A., Oxford, J. M., Taylor, A. M. R. & Webb, T. P. (1976). Cytogenetically marked clones in human fibroblasts cultured from normal subjects. *Somat. Cell Genet.* **2**, 55–62.

Hook, E. B. (1976). Estimates of maternal age-specific risks of a Down-syndrome birth in women aged 34–41. *Lancet* ii, 33–34.

Kuleshov, N. P. (1976). Chromosome anomalies of infants dying during the perinatal period and premature newborn. *Humangenetik* **31**, 151–160.

Littlefield, L. G. & Mailhes, J. B. (1975). Observations of de novo clones of cytogenetically aberrant cells in primary fibroblast cell strains from phenotypically normal women. *Am. J. Hum. Genet.* **27**, 190–197.

Machin, G. A. (1974). Chromosome abnormality and perinatal death. *Lancet* i, 549–551.

Machin, G. A. & Crolla, J. A. (1974). Chromosome constitution of 500 infants dying during the perinatal period. *Humangenetik* **23**, 183–198.

Sutherland, G. R., Bowser-Riley, S. M. & Bain, A. D. (1975). Chromosomal mosaicism in amniotic fluid cell cultures. *Clin. Genet.* **7**, 400–404.

# DOWN'S SYNDROME IN SOUTH AUSTRALIA

GRANT R. SUTHERLAND, M.SC., PH.D.,*
SUSAN R. CLISBY, M.B., B.S.,†
GEOFFREY BLOOR, B.A., B.SOC.ADMIN.,‡
AND RODNEY F. CARTER, M.D., F.R.C.P.A., M.R.C.PATH.§

*Cytogenetics Unit, Adelaide Children's Hospital, and Intellectually Retarded Services, Adelaide*

*Med. J. Aust.*, 1979, 2: 58–61.

**In a survey of Down's syndrome in South Australia, 921 persons, both living and deceased, were identified; 717 individuals with the disorder were living in South Australia. Cytogenetic confirmation of the diagnosis had been made in 774 cases. From 1955 to 1977, the over-all incidence of Down's syndrome at birth was found to be 1.175/1000 live births. The incidence of Down's syndrome was significantly lower over the last five years of this period than for the first 18 years; thus it appears that the incidence of Down's syndrome in South Australia is falling. Analysis of maternal age changes with time has not revealed any changes to the maternal age-specific rates for Down's syndrome, although the rate for mothers aged 25 years or younger appears to be falling. The proportion of Down's syndrome babies born to women aged 35 years or more has decreased from 65.7% for those born before 1950 to 30.4% for those born from 1975 to 1977; similarly, the median maternal age has fallen from 37.12 years to 28.25 years. Regression analyses of maternal age rates for Down's syndrome by single years have produced figures suitable for genetic counselling. A plea is made that Down's syndrome should become a notifiable condition.**

No comprehensive study of the epidemiology of Down's syndrome has been carried out in Australia since that of Collmann and Stoller in Victoria, which covered the period from 1942 to 1957.[1] The pattern of maternal age at birth has been changing since that period, and this could be expected to change the incidence of Down's syndrome at birth. The effects of this parameter have been studied for the period from 1939 to 1964 in Victoria.[2] Furthermore, there had been a clinical impression that the mothers of children with Down's syndrome who were seen at Intellectually Retarded Services were younger than those seen in the past. With the advent of prenatal diagnostic programmes aimed at reducing the frequency of Down's syndrome at birth, up-to-date information on the epidemiology of Down's syndrome is essential for such programmes to be properly planned, and their impact to be assessed. A survey of Down's syndrome over the period from 1950 until the end of 1977 was carried out in South Australia to identify all births in this State which had resulted in children with Down's syndrome, and to identify all living persons with Down's syndrome in the State.

## METHODS

The records of all public institutions which may have had dealings with persons suffering from Down's syndrome were searched to identify cases. These included public hospitals, mental health institutions, special schools, sheltered workshops, and so on. Notices requesting information about persons with Down's syndrome were placed in the *AMA Gazette*, country medical practices were contacted by letter, and newspaper publicity was obtained asking parents or next of kin to contact the survey.

When an individual with Down's syndrome had been identified, the next of kin was approached by telephone, letter or personal visit, and asked to supply information regarding the time and place of conception and birth of the subject. Data concerning ages and occupations of the parents, and family structure, were also collected. If the subject was still alive, every effort was made to carry out cytogenetic studies, if these had not already been done. No diagnosis of Down's syndrome was accepted in the absence of chromosome studies, unless it had been made unequivocally by a medical practitioner. This report covers all cases born before January 1, 1978. Data on total live-birth occurrences and parental ages were supplied by the Australian Bureau of Statistics.

Hook and Chambers have used regression analysis to estimate rates of Down's syndrome by single-year maternal-age intervals.[3] Their regression was of the form $\ln y = C_1 x + C_0$, where $y$ is the rate of Down's syndrome births per 1000 live births, $x$ is maternal age, and $C_1$, $C_0$ are constants. They divided the maternal age into two ranges, from 20 years to 31 years and from 33 years to 49 years. Similar regression analysis was carried out by means of Statistical Package for the Social Sciences[4] for the data from 1960 to 1977, for the age ranges 20–31 and 33–47.5 years. The latter range has been determined as in Hook and Lindsjo.[5]

## RESULTS

A total of 921 individuals with Down's syndrome were identified, comprising 498 males and 423 females (sex ratio 1.18). The distribution of the Down's syndrome cases is shown in Table 1. Of the 717 living cases only 256 (36%) were institutionalized. The sex ratio suggests that there might have been an underascertainment of females. Hence, the sex ratio according to period of birth for those born in South Australia was calculated (Table 2), and this shows an increase in male:female sex ratio up to 1.56 for the period from 1975 to 1977. It is inconceivable that sex could be a factor in ascertainment in infancy. However, it should be noted that none of these sex ratios is significantly different from 1.06.

TABLE 1
*Distribution and Sex Ratio of 921 Cases of Down's Syndrome*

| Location | Number of Persons | | Sex Ratio |
|---|---|---|---|
| | Males | Females | |
| Unknown | 4 | 4 | 1.00 (1%) |
| Living at home | 238 | 196 | 1.21 (47%) |
| Strathmont Training Centre | 50 | 24 | 2.08 (8%) |
| Minda Home Inc. | 84 | 67 | 1.25 (17%) |
| Other institutions | 14 | 17 | 0.82 (3%) |
| Adopted/fostered | 13 | 6 | 2.17 (2%) |
| Dead | 95 | 109 | 0.87 (22%) |
| Total | 498 | 423 | 1.18 100 |

The diagnosis of Down's syndrome had been confirmed by chromosome analysis in 774 cases and the distribution of the various chromosomal forms are shown as follows.

| | |
|---|---|
| Regular trisomy 21 | 728 (94.0%) |
| Mosaic trisomy 21 | 16 ( 2.1%) |
| Translocation trisomy 21 | 24 (3.1%) |
| Miscellaneous | 6 (0.8%) |

Only 23 of the living persons with Down's syndrome have not had a chromosomal analysis.

There were 818 individuals with Down's syndrome, who had been born in South Australia; their birth dates ranged from 1888 to 1977. Of those born in South Australia all were conceived in South Australia, except for six who had been conceived in other States, and eight who had been conceived outside Australia. The places of conception (taken as places of residence of the mothers at the time of conception) of 28 of those born in South Australia were unknown.

Fluctuations in the incidence of Down's syndrome are shown in Figure 1. The incidence appears to be lower up to the early 1960s than from this time until 1971. The most likely explanation of this is under-ascertainment in the early part of this period. The drop in incidence since 1971 is unlikely to be due to this factor, and may represent a real decrease in the incidence of Down's syndrome. If the incidence of Down's syndrome over the last five years is compared with that of the period from 1955 to 1972 as in Table 3, then there has been a significant decrease.

* Chief Cytogeneticist.
† Medical Officer.
‡ Assistant Senior Social Worker.
§ Director of Histopathology.

Address for reprints: Dr G. R. Sutherland, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia.

TABLE 2
*Sex Ratio of Persons With Down's Syndrome According to Period of Birth*

| Period of Birth | Number of Affected Persons | | | Sex Ratio |
|---|---|---|---|---|
| | Total | Males | Females | |
| Before 1950 . . | 190 | 100 | 90 | 1.11 |
| 1950 to 1954 | 71 | 39 | 32 | 1.22 |
| 1955 to 1959 | 107 | 49 | 58 | 0.84 |
| 1960 to 1964 | 124 | 68 | 56 | 1.21 |
| 1965 to 1969 | 154 | 83 | 71 | 1.17 |
| 1970 to 1974 | 126 | 71 | 55 | 1.29 |
| 1975 to 1977 | 46 | 28 | 18 | 1.56 |
| Total . . | 818 | 438 | 380 | 1.15 |

The age of the mother at the birth of the Down's syndrome individual was known for 806 of the 818 such persons born in South Australia. This age ranged from 16 to 49 years (mean 33.67 ± standard deviation 7.72 years). There has been a fall in the mean and median maternal ages for mothers of children with Down's syndrome which has been more marked than the fall in the maternal age of the general population (Table 4, Figure 2). Because of the fall in the proportion of older mothers in the population, the proportion of Down's syndrome children born to

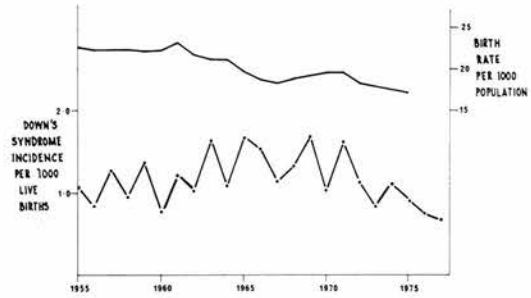



FIGURE 1: Fluctuations in the incidence of Down's syndrome in South Australia since 1955. Also shown is the birth rate in South Australia. The data from which the incidence figures were calculated can be obtained from the authors.

these women had fallen (Table 5). The mothers of the Down's syndrome persons in the survey who were born before 1950 were aged 40 years or older in 33.4% of cases, and 35 years or older in 65.7% of cases. In the last three years of the survey these proportions had fallen to 13.3% and 30.4% respectively.

To determine whether the maternal age-specific rates of Down's syndrome had changed with time these were calculated for quinquennial maternal age groups over time (Table 6). It appears that there has been no change in the age-specific rates over the period studied. The lower rates for the period from 1955 to 1959 are probably due to under-ascertainment, since it is to be expected that ascertainment would be more complete for the more recent

TABLE 3
*Comparison of Down's Syndrome Births Over the Last Five Years With Those in the Period from 1955 to 1972*

| Period | Number of Births | |
|---|---|---|
| | With Down's Syndrome | Without Down's Syndrome |
| 1955 to 1972 . . | 470 | 376 607 |
| 1973 to 1977 . . | 87 | 97 078 |

$\chi^2 = 7.82$; $P < 0.01$.

TABLE 4
*Maternal Age at Birth of Individuals With Down's Syndrome Born in South Australia: Changes With Time*

| Year of Birth | Number of Down's Syndrome Persons | Maternal Age (Years) | |
|---|---|---|---|
| | | Mean | Median |
| Before 1950 . . | 181 | 35.93 | 37.12 |
| 1950 to 1954 | 71 | 35.03 | 36.67 |
| 1955 to 1959 | 107 | 34.86 | 36.80 |
| 1960 to 1964 | 123 | 33.94 | 34.75 |
| 1965 to 1969 | 154 | 32.64 | 32.50 |
| 1970 to 1974 | 124 | 30.60 | 29.17 |
| After 1975 . . | 46 | 30.51 | 28.25 |
| Total . . | 806 | 33.67 | 34.87 |

years. The impact of prenatal diagnosis on these figures is minimal. There were only three pregnancies terminated for fetal Down's syndrome in the period from 1975 to 1977, one in the 35 to 39 years age group, and two in the 40 to 44 years age group. These have been excluded from all other considerations.

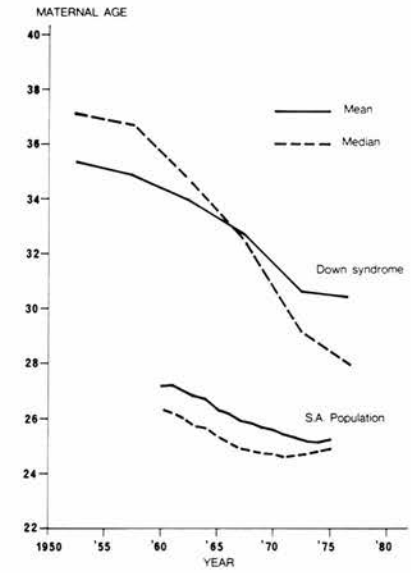



FIGURE 2: Changes in the mean and median maternal ages for mothers of Down's syndrome infants and for mothers of live-born infants in South Australia.

The regression equation obtained for the age group of 20 to 31 years was $1ny = 0.06325x - 1.98775$ (SE slope=0.02379; SE intercept=0.61229); and for the age group 33 to 47.45 years it was $1ny = 0.24823x - 7.89579$ (SE slope=0.01847; SE inter-

TABLE 5
*Proportions of Infants With Down's Syndrome and Total Live Infants Born to Women of Advanced Age in South Australia*

| Interval | Maternal Age | | | |
|---|---|---|---|---|
| | Greater than 34 Years | | Greater than 39 Years | |
| | Total Live Births | Down's Syndrome Births | Total Live Births | Down's Syndrome Births |
| Before 1950 . . | | 65.7% | | 35.4% |
| 1951 to 1954 . . | 13.5% | 66.2% | 3.1% | 30.9% |
| 1955 to 1959 . . | 13.4% | 59.3% | 3.1% | 32.4% |
| 1960 to 1964 . . | 13.0% | 51.2% | 3.0% | 30.9% |
| 1965 to 1969 . . | 9.8% | 44.2% | 2.5% | 22.1% |
| 1970 to 1974 . . | 5.9% | 35.5% | 1.3% | 21.0% |
| 1975 to 1977 . . | 4.1% | 30.4% | 0.73% | 13.3% |

TABLE 6
*Age-Specific Incidence Rates* for Down's Syndrome 1955 to 1977 in South Australia†*

| Maternal Age (Years) | 1955 to 1959 | 1960 to 1964 | 1965 to 1969 | 1970 to 1974 | 1975 to 1977 |
|---|---|---|---|---|---|
| 19 and younger | 0.324 | 0 | 0.541 | 0.679 | 0.181 |
| 20 to 24 | 0.429 | 0.588 | 0.587 | 0.651 | 0.351 |
| 25 to 29 | 0.459 | 0.560 | 1.000 | 0.807 | 0.831 |
| 30 to 34 | 0.767 | 1.189 | 1.785 | 1.263 | 0.792 |
| 35 to 39 | 2.850 | 2.353 | 4.439 | 3.650 | 3.603 |
| 40 to 44 | 9.997 | 11.07 | 10.87 | 18.72 | 18.37 |
| 45 and older | 31.41 | 27.62 | 44.20 | 22.22 | 0 |
| Total | 1.094 | 1.158 | 1.468 | 1.155 | 0.807 |

* Infants with Down's syndrome per 1000 live births.
† Copies of the data used to calculate these rates are available from the authors.

cept= 0.74371). The observed incidences and the regression lines are shown in Figure 3. Table 7 shows the observed and regression-derived rates for Down's syndrome by single years, and similar regression-derived rates from other studies.

## DISCUSSION

A major problem in a study such as this, where retrospective ascertainment was used, is to know how complete it has been. It is believed that ascertainment is virtually complete for living individuals, but becomes progressively incomplete for the deceased as one goes back in time. Although there was a constant excess of males, the sex ratio of the Down's syndrome cases does not differ significantly from that of the population. Hence, it can be assumed that there has been no ascertainment bias related to sex. Evans *et alii* found a non-significant excess of males with Down's syndrome, born in Manitoba between 1965 and 1974.[6]

Recently published surveys of Down's syndrome give incidences ranging from 1.501/1000 live births from Massachusetts for the period from 1958 to 1965,[7] to 1.148/1000 live births from Copenhagen for the period from 1960 to 1971.[8] Chromosome studies on 54 749 consecutive neonates gave an incidence of 1.15/1000.[9] The incidence of 1.175/1000 found in the present study for the period from 1955 to 1977 is consistent with that found in other recently published series, and not far below that of 1.32/1000 for the most recent period studied, from 1953 to 1957, by Collmann and Stoller.[2]

Fluctuations in the annual incidence of Down's syndrome were demonstrated in Victoria.[1] Similar fluctuations have been shown in South Australia. These fluctuations make it difficult to be certain that Down's syndrome is really decreasing in incidence in South Australia. While it appears that the incidence of Down's syndrome is falling, it may be a few years yet before it is possible to be certain of this. There have been 24 known cases of Down's syndrome in South Australia in 1978, and two more aborted after amniocentesis. Stoller has suggested that long cycles in incidence may operate in some instances.[10]

TABLE 7
*Total Number of Down's Syndrome and Live Births in South Australia 1960 to 1977 by Age of Mother**

| Age of Mother | Down's Syndrome Births | Live Births | Incidence per 1000 | Regression Incidence | New York[3] | Mass.[7] | Sweden[5] |
|---|---|---|---|---|---|---|---|
| <15 | 0 | 177 | 0 | | | | |
| 15 | 0 | 877 | 0 | | | | |
| 16 | 2 | 2833 | 0.706 | | | | |
| 17 | 4 | 6574 | 0.608 | | | | |
| 18 | 4 | 11158 | 0.358 | | | | |
| 19 | 5 | 15470 | 0.323 | | | | |
| 20 | 11 | 19514 | 0.564 | 0.49 | 0.52 | 0.57 | 0.64 |
| 21 | 12 | 24011 | 0.500 | 0.52 | 0.59 | 0.60 | 0.67 |
| 22 | 10 | 27315 | 0.366 | 0.55 | 0.65 | 0.64 | 0.71 |
| 23 | 14 | 29904 | 0.468 | 0.59 | 0.71 | 0.67 | 0.75 |
| 24 | 28 | 30568 | 0.916 | 0.63 | 0.77 | 0.70 | 0.79 |
| 25 | 29 | 29578 | 0.980 | 0.67 | 0.83 | 0.74 | 0.83 |
| 26 | 16 | 27109 | 0.590 | 0.71 | 0.89 | 0.77 | 0.87 |
| 27 | 21 | 23765 | 0.884 | 0.76 | 0.95 | 0.80 | 0.92 |
| 28 | 19 | 20723 | 0.917 | 0.81 | 1.01 | 0.84 | 0.97 |
| 29 | 10 | 17730 | 0.564 | 0.86 | 1.07 | 0.87 | 1.02 |
| 30 | 13 | 15171 | 0.857 | 0.91 | 1.13 | 0.90 | 1.08 |
| 31 | 14 | 12636 | 1.11 | 0.97 | 1.21 | 0.93 | 1.14 |
| 32 | 19 | 10787 | 1.76 | 1.09 (int) | 1.38 | 1.15 | 1.25 |
| 33 | 13 | 9093. | 1.43 | 1.34 | 1.69 | 1.55 | 1.47 |
| 34 | 14 | 7823 | 1.79 | 1.72 | 2.15 | 1.98 | 1.92 |
| 35 | 18 | 6897 | 2.61 | 2.21 | 2.74 | 2.53 | 2.51 |
| 36 | 11 | 5744 | 1.92 | 2.83 | 3.49 | 3.22 | 3.28 |
| 37 | 18 | 4931 | 3.65 | 3.63 | 4.45 | 4.11 | 4.28 |
| 38 | 17 | 4172 | 4.07 | 4.65 | 5.67 | 5.24 | 5.60 |
| 39 | 20 | 3416 | 5.85 | 5.96 | 7.21 | 6.68 | 7.32 |
| 40 | 16 | 2525 | 6.34 | 7.64 | 9.19 | 8.52 | 9.57 |
| 41 | 19 | 1876 | 10.13 | 9.79 | 11.71 | 10.86 | 12.51 |
| 42 | 17 | 1293 | 13.15 | 12.55 | 14.91 | 13.85 | 16.36 |
| 43 | 22 | 842 | 26.13 | 16.09 | 19.00 | 17.66 | 21.39 |
| 44 | 16 | 500 | 32.00 | 20.62 | 24.20 | 22.51 | 27.96 |
| 45 | 5 | 267 | 18.73 | 26.43 | 30.84 | 28.71 | 36.55 |
| 46 | 8 | 135 | 59.25 | 33.88 | 39.28 | 36.61 | 47.79 |
| 47 | 0 | 48 | 0 | 43.42† | 50.04 | 46.68 | 62.47 |
| 48 | 2 | 19 | 105.26 | 55.66† | 63.75 | 59.52 | 81.62 |
| 49 | 0 | 7 | 0 | 71.34† | 81.21 | 75.89 | 106.76 |

* Regression-derived estimates of incidence are compared with other published regression-derived incidence figures.
† By extrapolation of regression line.
int=interpolated.
Mass=Massachusetts.

Collmann and Stoller predicted that, with the fall in mean maternal age of the population, the incidence of Down's syndrome would fall.[2] They showed this to be so for three consecutive quinquennial periods in Victoria from 1943 to 1957. Evans *et alii*[6] reported an "apparent overall decrease" in the incidence of Down's syndrome, and suggest that this may have been due to falling maternal age. They also found that the age-specific incidence of Down's syndrome children born to women aged from 35 to 39 years increased over their period of study. Mikkelsen *et alii* made a similar observation.[8] In the present study, the age-specific incidence for women aged from 35 to 39 years has been fairly constant over the last 15 years. The low incidence of Down's syndrome in this study for the period from 1975 to 1977 may be due not only to a changing maternal age distribution but also to a marked drop in age-specific incidence of Down's syndrome children born to mothers aged less than 25 years, compared with the preceding 10 years.

The proportion of women over 35 years of age giving birth in South Australia has fallen since 1964. Similar findings of decreasing maternal age in the general population have been recorded in other communities.[6 9] This change in maternal age distribution appears to be associated with, and may indeed be responsible for, the apparent decreasing incidence of Down's syndrome in South Australia. Future trends will be difficult to interpret because of the increasing application of prenatal diagnosis to the older age groups.

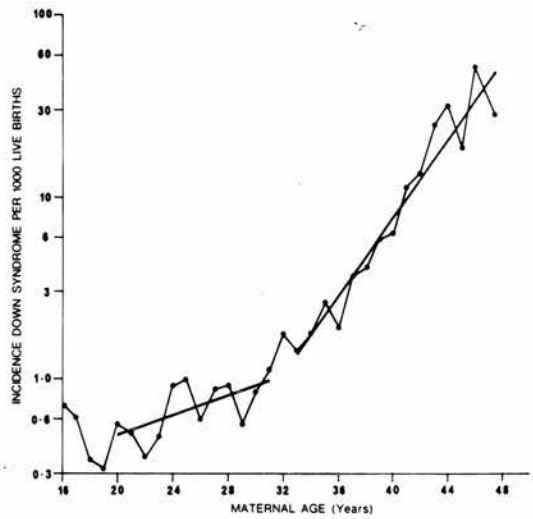



FIGURE 3: Rates of Down's syndrome per 1000 live births by maternal age. Actual values are plotted and regression lines are shown.

Over the last three years in South Australia, 30.4% of mothers of children with Down's syndrome have been aged 35 years or more, yet only 4.1% of the women giving birth were in this category. This fact gives support to the recent recommendation of the NHMRC[11] that all women over the age of 35 years should be offered amniocentesis. Adequate laboratory facilities for this already exist in South Australia, but are under-used.

The proportions of chromosomal types of Down's syndrome seen in South Australia are similar to those seen in New South Wales,[12] and Victoria.[13]

The risk figures for Down's syndrome to be used in genetic counselling have been the subject of considerable controversy.[14] Some authors have advocated the use of risk figures from epidemiological studies such as the present one,[15 16] whereas others have regarded the figures obtained from prospective prenatal diagnostic studies as being more appropriate.[17 18 19] Since the two sets of figures are rather different, the controversy is a real one. The reasons for the discrepancy may be the number of Down's syndrome fetuses lost during the second and third trimesters,[20] and in the perinatal period.[21]

For genetic counselling in South Australia, the best epidemiological figures are those derived from the regression equation (Table 7). These figures are similar to those obtained by means of a similar statistical treatment from New York,[3] Massachusetts,[7] and Sweden.[5] As yet, there are no figures for risks of Down's syndrome by single-year maternal-age intervals from prenatal diagnostic series based on large numbers for Australia. It is to be hoped that such figures will become available in the near future, as figures derived from such data are useful for genetic counselling.

The present survey will be maintained as an ongoing project to monitor Down's syndrome in South Australia. South Australia has only about 10% of the population of Australia, hence any changes in the occurrence of Down's syndrome in this State cannot be assumed to represent those in Australia as a whole, although they may do so. The task of monitoring Down's syndrome is made more difficult, because this is not a notifiable condition. A plea is made for this disorder to be made notifiable, and for the data to be kept in a central registry. Down's syndrome is the most common single identifiable cause of mental retardation in the community, yet its distribution and occurrence in the community are poorly known.

## ACKNOWLEDGEMENTS

We wish to thank the many people who assisted us in ascertaining individuals with Down's syndrome, and the relatives of these individuals for providing us with information about them. Mrs Margaret Carter diligently helped in the collection of many of the data. Mr Jim Golding provided computer assistance, Dr T. Heins gave statistical advice. The Australian Bureau of Statistics kindly provided data on live-birth occurrences and parental ages in South Australia. Dr W. A. Dibden, Director-General of Medical Services, Dr J. Clayer, Deputy Director of Mental Health Services, and Dr Norma Kent, Assistant Director of Mental Health Services (IRS) provided encouragement and support. This project was supported by the Adelaide Children's Hospital Research Trust, and Mental Health Services (South Australia).

## REFERENCES

1 COLLMANN, R. D., and STOLLER, A., A survey of mongoloid births in Victoria, Australia, 1942–1957, *Amer. J. publ. Hlth*, 1962, 52: 813.
2 COLLMANN, R. D., and STOLLER, A., Shift of childbirth to younger mothers, and its effect on the incidence of mongolism in Victoria, Australia, 1939–1964, *J. ment. Defic. Res.*, 1969, 13: 13.
3 HOOK, E. B., and CHAMBERS, G. M., Estimated rates of Down syndrome in live births by one year maternal age intervals for mothers aged 20–49 in a New York State study: Implications of the risk figures for genetic counselling and cost-benefit analysis of prenatal diagnosis programs, *Birth Defects, Orig. Art. Ser.*, 1977, 13: 123.
4 NIE, N. H., HULL, C. H., JENKINS, J. G., *et alii, Statistical Package for the Social Sciences*, 2nd ed., McGraw-Hill, New York, 1975.
5 HOOK, E. B., and LINDSJO, A., Down syndrome by single year maternal age interval in a Swedish study: Comparison with results from a New York study, *Amer. J. hum. Genet.*, 1978, 30: 19.
6 EVANS, J. A., HUNTER, A. G. W., and HAMERTON, J. L., Down syndrome and recent demographic trends in Manitoba, *J. med. Genet.*, 1978, 15: 43.
7 HOOK, E. B., and FABIA, J. J., Frequency of Down syndrome by single-year maternal age interval: Results of a Massachusetts study, *Teratology*, 1978, 17: 223.
8 MIKKELSEN, M., FISCHER, G., STENE, J., *et alii*, Incidence study of Down's syndrome in Copenhagen, 1960–1971: With chromosome investigation, *Ann. hum. Genet.*, 1976, 40: 177.
9 NIELSEN, J., and SILLESEN, I., Incidence of chromosome aberrations among 11,148 newborn children, *Hum. Genet.*, 1975, 30: 1.
10 STOLLER, A., The epidemiology of Down syndrome, *Rec. Adel. child. Hosp.*, 1977, 1: 269.
11 NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL, Prevention of birth defects, MED. J. AUST., 1978, 2: 378.
12 DEY, J., Survey of 500 cases of Down's syndrome, *Aust. J. ment. Ret.*, 1971, 1: 154.
13 SUTHERLAND, G. R., and WIENER, S., Cytogenetics of 271 mongols, *Aust. paediat. J.*, 1972, 8: 90.
14 EDITORIAL, Maternal age and Down's syndrome, *Lancet*, 1978, 2: 24.
15 HOOK, E. B., Risk of Down syndrome in relation to maternal age, *Lancet*, 1976, 2: 465.
16 WYATT, P. R., Aneuploidy and the older gravida: Which risk to quote, *Lancet*, 1972, 1: 1305.
17 FERGUSON-SMITH, M. A., Prospective data on risk of Down syndrome in relation to maternal age, *Lancet*, 1976, 2: 252.
18 FERGUSON-SMITH, M. A., Maternal age and Down syndrome, *Lancet*, 1978, 2: 213.
19 KARDON, N., KRAUSS, M., SELVERBERG, G., and DAVIS, J., Aneuploidy and the older gravida: Which risk to quote, *Lancet*, 1978, 1: 1305.
20 CREASY, M. R., and CROLLA, J. A., Prenatal mortality of trisomy 21 (Down's syndrome), *Lancet*, 1974, 1: 473.
21 SUTHERLAND, G. R., CARTER, R. F., BAIN, A. D., *et alii*, Chromosomé studies at the paediatric necropsy, *Ann. hum. Genet.*, 1978, 42: 173.

Hum Genet (1981) 57:176–179



Human Genetics



# Distribution of $\alpha_1$-Antitrypsin (PI) Phenotypes in Chromosome Abnormalities

John C. Mulley and Grant R. Sutherland

Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia

**Summary.** *PI* phenotypes (including subtypes) were determined for 168 individuals with chromosomal abnormalities ascertained in Adelaide. These included patients with mosaicism, trisomy 21, trisomy 13, trisomy 18, and various sex chromosome aberrations (45,X, 47,XXX, 47,XXY, 47,XYY, and 48,XXXY). Data did not support an existing proposition that mildly deficient *PI* phenotypes predispose to abnormal chromosome segregation during mitosis or meiosis. Phenotypic distributions of each group were statistically similar to control populations of cord bloods and bloods donors.

## Introduction

$\alpha_1$-Antitrypsin ($\alpha_1$AT) is the major regulator of proteolytic activity in humans. A glycoprotein, it occurs in many body fluids and inhibits a variety of proteases (Morse 1978a). Genetic heterogeneity is determined at the *PI* locus by a null allele and 32 confirmed codominant alleles (Cox et al. 1980). Most variants are infrequent, although the alleles *PI*S, PI*Z, PI*F,* and *PI*I* are relatively common and associated with reduced $\alpha_1$AT serum levels.

The clinical importance of $\alpha_1$AT relates primarily to severe deficiency associated with the PI Z phenotype. Severe $\alpha_1$AT deficiency predisposes to adult pulmonary emphysema and childhood cirrhosis of the liver (Fagerhol 1976; Morse 1978b). Although PI *S-Z* is also considered deficient, the association with these diseases is weaker. Clear disease associations have not been demonstrated with the common mildly deficient phenotypes PI S, PI M-S, and PI M-Z, or the less common PI F, PI I, PI F-I, PI I-Z, and PI F-Z phenotypes.

Apart from the established disease associations, PI phenotypes with reduced $\alpha_1$AT concentration have been tentatively implicated in the genesis of recurrent miscarriages (Aarskog et al. 1978), abnormal chromosome segregation during mitosis (Aarskog and Fagerhol 1970; Kueppers et al. 1975; Fineman et al. 1976), and meiotic nondisjunction with advanced maternal age (Fineman et al. 1976). This investigation extends the sample size of the chromosomal abnormalities previously studied, and examines PI phenotypes from additional groups with chromosome abnormalities from a population where phenotypic frequencies are known. All common $\alpha_1$AT variants, including alleles determining $\alpha_1$AT deficiency, are reliably classified isoelectric focusing (Hoffmann and van den Broek 1977; Ward et al. 1977) and PI M can be subdivided by high resolution isoelectric focusing (Frants and Eriksson 1978; Kueppers and Christopherson 1978).

*Offprint requests to:* J. C. Mulley

## Materials and Methods

All chromosomal aberrations were diagnosed amongst neonates, children, and adults referred to the Cytogenetics Unit of the Adelaide Children's Hospital up to September 30, 1980. Samples from trisomy 21 individuals were collected in conjunction with a survey of Down syndrome in South Australia (Sutherland et al. 1979). The control data were derived from a local survey of blood donors (Mulley 1980) and of cord bloods from a survey now in progres. $\alpha_1$AT was phenotyped as previously described (Mulley 1980). Genetic nomenclature follows the recommendations of Shows et al. (1979).

Contingency $\chi^2$ tests were applied with a correction for continuity. Phenotypes were classified for statistical purposes as either PI M, or not PI M which represented all mildly deficient phenotypes. All PI M subtypes were grouped as PI M since the $\alpha_1$AT concentration of subtypes is equivalent (Kueppers and Christopherson 1978), although the complete subtype classification is presented.

## Results

$\alpha_1$AT phenotypes from patients with a variety of deletions are given in Table 1. The exclusion of familial translocations and inversions by family study and paternity testing indicated that the deletions were probably sporadic in origin from at least eight of the propositi. Although two low frequency alleles (*PI*F* and *PI*Z*) were detected from only 11 families, there is no evidence of an association between non PI M phenotypes and the occurrence of spontaneous deletions.

**Table 1.** PI Phenotypes in patients with presumed sporadic deletions

| Case | Deletion | PI Phenotype | | | Probability of paternity |
|---|---|---|---|---|---|
| | | Proband | Mother | Father | |
| 1 | 4q(27→31) | M1 | M1–M3 | M1–M3 | 88% |
| 2 | 4q(27→31) | M1–M2 | [a] | [a] | — |
| 3 | 5p(14→pter) | M1–M2 | M1–M3 | M1–M2 | 79% |
| 4 | 7q(22) | M1 | M1 | M1 | 80% |
| 5 | 12p(12) | M1 | M1 | M1 | 95% |
| 6 | 12p(13) and 15q(15) | M1 | M1–M2 | M1 | 85% |
| 7 | 12p(ter) and 19p(ter) or 19q(ter) | M1–F | [a] | [a] | — |
| 8 | 18p(11) | M1 | M1 | M1–M2 | 61% |
| 9 | 18q(21→23) | M1 | M1–M2 | M1–Z | 94% |
| 10 | Xq(13→24) | M1–M3 | [a] | [a] | — |
| 11 | Xp(11→22) | M1 | M1 | M1 | 97% |

[a] Unavailable for study

0340-6717/81/0057/0176/$ 01.00

**Table 2.** Distribution of PI phenotypes among chromosome abnormalities arising from abnormal chromosome segregation

| Chromosome abnormality | PI Phenotype | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M1 | M1–M2 | M1–M3 | M2–M3 | M2 | M3 | M1–S | M2–S | M1–Z | M2–Z | M1–F | M3–F | M2–I |
| 1. Mosaics | 5 | 2 | 3 | — | — | — | 1 | 1 | — | — | — | — | — |
| a) Sex chromosome mosaics | 3 | 2 | 2 | — | — | — | — | 1 | — | — | — | — | — |
| b) Autosomal mosaics | 2 | — | 1 | — | — | — | 1 | — | — | — | — | — | — |
| 2. Trisomy 21[a] | 55 | 18 | 14 | 1 | 1 | 2 | 1 | — | 6 | — | 2 | — | 1 |
| a) Maternal age < 30 years | 8 | 7 | 1 | 1 | 1 | 1 | — | — | 1 | — | — | — | 1 |
| b) Maternal age > 35 years | 30 | 7 | 9 | — | — | 1 | 1 | — | 3 | — | 2 | — | — |
| 3. Trisomy 13 | 1 | 1 | — | — | 1 | — | — | — | — | — | — | — | — |
| 4. Trisomy 18 | — | — | 1 | — | — | — | — | — | — | — | — | — | — |
| 5. Sex chromosome | | | | | | | | | | | | | |
| Abnormalities (total) | 23 | 9 | 3 | — | 1 | — | 1 | 1 | — | 1 | — | 1 | — |
| a) 45,X | 6 | 1 | — | — | — | — | 1 | — | — | — | — | — | — |
| b) 47,XXX | 5 | — | — | — | 1 | — | — | — | — | — | — | — | — |
| c) 47,XXY | 11 | 7 | 2 | — | — | — | — | 1 | — | — | — | — | — |
| d) 47,XYY | — | 1 | 1 | — | — | — | — | — | — | 1 | — | 1 | — |
| e) 48,XXXY | 1 | — | — | — | — | — | — | — | — | — | — | — | — |

[a] Total trisomy 21 includes the subclasses where maternal age was unknown or between 30 and 35 years, in addition to the two subclasses presented (see text)

**Table 3.** *PI* allele frequencies from control populations and major test groups examined

| Population | No. | Allele frequency | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *PI*M1* | *PI*M2* | *PI*M3* | *PI*S* | *PI*Z* | *PI*F* | *PI*G* | *PI*I* |
| Controls | | | | | | | | | |
| Blood donors | 220 | 0.65 | 0.14 | 0.13 | 0.061 | 0.016 | — | — | 0.002 |
| Cord bloods | 220 | 0.70 | 0.16 | 0.08 | 0.027 | 0.014 | 0.005 | 0.002 | 0.002 |
| Trisomy 21 | 101 | 0.75 | 0.11 | 0.09 | 0.005 | 0.030 | 0.010 | — | 0.005 |
| Maternal age < 30 years | 21 | 0.60 | 0.26 | 0.10 | — | 0.024 | — | — | 0.024 |
| Maternal age > 35 years | 53 | 0.77 | 0.07 | 0.10 | 0.009 | 0.028 | 0.019 | — | — |
| Sex chromosome abnormalities[a] | 40 | 0.74 | 0.16 | 0.05 | 0.025 | 0.013 | 0.013 | — | — |
| 47,XXY | 21 | 0.74 | 0.19 | 0.05 | 0.024 | — | — | — | — |
| Mosaics[a] | 12 | 0.67 | 0.13 | 0.13 | 0.083 | — | — | — | — |
| Sex chromosome mosaics | 8 | 0.63 | 0.19 | 0.13 | 0.063 | — | — | — | — |
| Deletions | 11 | 0.82 | 0.09 | 0.05 | — | — | 0.05 | — | — |

[a] Subclasses with small sample sizes are omitted

**Table 4.** Mosaics studied

| Karyotype | PI Phenotype |
|---|---|
| Sex chromosome mosaics | |
| 46,XX/47,XXY | M1 |
| 46,XY/47,XXY | M1 |
| 46,XY/47,XYY | M1–M2 |
| 45,X/46,XX/47,XXX | M1–M2 |
| 45,X/46,X,dic(X)(p11) | M1 |
| 45,X/46,X,dic(X)(p11) | M1–M3 |
| 45,X/46,X,r(X) | M1–M3 |
| 45,X/46,X,i(Xq) | M2–S |
| Autosomal mosaics | |
| 46,XY/46,XY,del(18p) | M1–M3 |
| 46,XY/47,XY,+8 | M1 |
| 46,XY/46,XY,t(15;18)(p13;q12) | M1 |
| 46,XY/47,XY,+r(?) | M1–S |

The phenotypic distributions from the other classes of chromosome abnormality are presented in Table 2. Allele frequencies from all groups, and from subgroups with reasonable numbers, are given in Table 3. Standard errors of these estimates are considerable, given the small sample sizes involved. Phenotypic distributions were not significantly different between any test group and the control populations. The 12 mosaics (Table 4) show no significant increase in non PI M phenotypes compared with controls. It should be noted that only six of the mosaics studied involved malsegregation of normal chromosomes, rather than structurally altered ones. Similarly, neither autosomal trisomies nor sex chromosome abnormalities arising from meiotic nondisjunction show any increase of non PI M phenotypes compared with controls.

The trisomy 21 results were subdivided according to maternal age, where known. Of the 101 trisomy 21 individuals stud-

**Table 5.** Basis for proposed associations between mildly deficient phenotypes and chromosome aberrations

| Study | No. | Allele frequency | | | | |
|---|---|---|---|---|---|---|
| | | *PI*F* | *PI*M* | *PI*S* | *PI*Z* | *PI*MLAMB* |
| Mosaics | | | | | | |
| Aarskog and Fagerhol (1970) | 7 | 0.14 | 0.71 | 0.14 | — | — |
| Kueppers et al. (1975) | 21 | 0.02 | 0.88 | 0.07 | 0.02 | — |
| Fineman et al. (1976) | 19 | — | 0.92 | — | 0.03 | 0.05 |
| Trisomy 21 | | | | | | |
| Fineman et al. (1976) Maternal age > 35 years | 31 | — | 0.74 | 0.13 | 0.05 | 0.08 |

ied, maternal age axceeded 35 years in 53 individuals and was less than 30 years in 21 individuals. For the remaining 27, maternal age was either unknown or between 30 and 35 years. The phenotypic distribution of each subgroup was not significantly different from that of the control populations.

## Discussion

Samples of both adult blood donors and cord bloods were chosen as control populations because of age heterogeneity within test groups. Allele frequencies from the controls are given in Table 3, and are similar to recent estimates obtained by isoelectric focusing from North America (Kueppers and Christopherson 1978) and southern England (Arnaud et al. 1979). However, the *PI*S* frequency of 0.061 from adult blood donors and 0.027 from cord bloods causes significant heterogeneity ($0.01 < P < 0.05$) between the two control populations, with and without subtyping. This divergence is presumably a chance effect since pooling the control populations would in fact result in a *PI*S* frequency of 0.044, comparable to another Australian estimate of 0.045 from a Melbourne control group of cord bloods (McPhee et al. 1980).

Results of previous investigations suggestive of an association between non PI M phenotypes and chromosomal aberrations are summarised in Table 5. Significantly increased heterozygosity was due to higher frequencies of non PI*M alleles among sex chromosome mosaics, and among trisomy 21 where maternal age exceeded 35 years. The *PI*MLAMB* subtype reported by Fineman et al. (1976) was not recorded in the present study, indeed its existence as a distinct variant is unconfirmed (Cox et al. 1980) and the relationship with PI M subtypes is unclear.

No evidence supporting an association between PI phenotypes and cytogenetic aberrations was found in the present study. The preliminary report by Aarskog and Fagerhol (1970) and subsequent studies by Arnaud et al. (1976) and McPhee et al. (1980) showed no relationship between PI and trisomy 21, but information on maternal age was unavailable. Although trisomy 21 is strongly correlated with maternal age, random selections of cases will always include a significant proportion where maternal age is less than 35 years (see Table 3). Despite subdivision on the basis of maternal age, Guanti and di Loreto (1980) and the present study found no evidence of a possible causal relationship between PI phenotypes and trisomy 21 where maternal age exceeded 35 years. The initial hypothesis proposing such an association was probably based on a chance observation resulting from a small sample size.

A genetic predisposition leading to multiple births and/or chromosomal abnormalities has been discussed (Lieberfarb et al. 1978). However, $\alpha_1$AT would appear to be an unlikely component of such a genetic predisposition to meiotic nondisjunction. Further data is necessary to reject conclusively the suggested association between $\alpha_1$AT phenotypes and abnormal chromosome segregation during mitosis. While a degree of racial heterogeneity involving non *PI*M* alleles at the *PI* locus has been established, no evidence for a corresponding heterogeneity in the frequency of cytogenetic abnormalities has been documented. The aspect of a genetic predisposition to multiple births involving $\alpha_1$AT remains open to investigation.

*Acknowledgements.* We wish to thank Helen Eyre, Lynene Hinton, Trudy Hocking, and Erica Woollatt for technical assistance with the cytogenetics. Dr. Peggy Clarke confirmed the initial identification of *PI*F, PI*G,* and *PI*I.*

## References

Aarskog D, Aarseth P, Fagerhol MK (1978) Alpha-1-antitrypsin (Pi)-types in recurrent miscarriages. Clin Genet 13:81–84

Aarskog D, Fagerhol MK (1970) Protease inhibitor (Pi) phenotypes in chromosome aberrations. J Med Genet 7:367–370

Arnaúd P, Galbraith RM, Pagé Faulk W, Black C (1979) Pi phenotypes of alpha$_1$-antitrypsin in southern England: Identification of M subtypes and implications for genetic studies. Clin Genet 15:406–410

Arnaúd P, Burdash NM, Wilson GB, Fudenberg HH (1976) Alpha-1-antitrypsin (Pi) types in Down's syndrome. Clin Genet 10:239–243

Cox DW, Johnson AM, Fagerhol MK (1980) Report of nomenclature meeting for $\alpha_1$-antitrypsin. INSERM, Rouen/Bois-Guillaume-1978. Hum Genet 53:429–433

Fagerhol MK (1976) The genetics of alpha-1-antitrypsin and its implications. Postgard Med J (Supp 2) 52:73–83

Fineman RM, Kidd KK, Johnson AM, Breg WR (1976) Increased frequency of heterozygotes for $\alpha_1$ antitrypsin variants in individuals with either sex chromosome mosaicism or trisomy 21. Nature 260: 320–321

Frants RR, Eriksson AW (1978) Reliable classification of six Pi M subtypes by separator isoelectric focusing. Hum Hered 28:201–209

Guanti G, Di Loreto M (1980) $\alpha_1$-Antitrypsin quantitative and qualitative (Pi phenotyping) characterisation in Down syndrome subjects and in their parents. Am J Hum Genet 32:174–178

Hoffmann JJML, van den Broek WGM (1977) Application of isoelectric focusing in $\alpha_1$-antitrypsin phenotyping. Clin Chim Acta 75:233–242

Kueppers F, Christopherson MJ (1978) Alpha$_1$-antitrypsin: Further genetic heterogeneity revealed by isoelectric focusing. Am J Hum Genet 30:359–365

Kueppers F, O'Brien P, Passarge E, Rüdiger HW (1975) Alpha$_1$-antitrypsin phenotypes in sex chromosome mosaicism. J Med Genet 12:263–264

Liberfarb RM, Atkins L, Holmes LB (1978) Down syndrome in two of three triplets. Clin Genet 14:261–264

McPhee H, Ananthakrishnan R, Taft LI (1980) Antiproteases and Down's syndrome in an Australian population. J Med Genet 17: 170–173

Morse JO (1978a) Alpha$_1$-antitrypsin deficiency. (First of two parts). N Engl J Med 299:1045–1048

Morse JO (1978b) Alpha$_1$-antitrypsin deficiency. (Second of two parts). N Engl J Med 299:1099–1105

Mulley JC (1980) Distribution of the six $\alpha_1$-antitrypsin (PI) subtypes from a sample of blood donors. Aust J Exp Biol Med Sci 58:409–414

Shows TB, Alper CA, Bootsma D, Dorf M, Douglas R, Huisman T, Kit S, Klinger HP, Kozack C, Lalley PA, Lindsley D, McAlpine PJ, McDougal JK, Meera Khan P, Meisler M, Morton NE, Opitz JM, Partridge CW, Payne R, Roderick TH, Rubinstein P, Ruddle FH, Shaw M, Spranger JW, Weiss K (1979) Guidelines for human gene nomenclature. Cytogenet Cell Genet 25:96–116

Sutherland GR, Clisby SR, Bloor G, Carter RF (1979) Down's syndrome in South Australia. Med J Aust 2:58–61

Ward AM, Pickering JD, Fagerhol MK, Martin J-P (1977) Pi (antitrypsin) typing methods. Hum Hered 27:292–297

Received October 14, 1980

*Reprinted from J Clin Pathol* 1983;**36**:140–142




# Cytogenetic studies: an essential part of the paediatric necropsy

GRANT R SUTHERLAND, RODNEY F CARTER

*British Medical Association, Tavistock Square, London WC1H 9JR*

*J Clin Pathol* 1983;**36**:140–142

# Cytogenetic studies: an essential part of the paediatric necropsy

GRANT R SUTHERLAND, RODNEY F CARTER

*From the Department of Histopathology, Adelaide Children's Hospital, North Adelaide, SA 5006 Australia*

SUMMARY Chromosome studies were attempted on 97% of necropsies carried out in the Department of Histopathology of the Adelaide Children's Hospital over the four-year period ending May 1981. Results were obtained from 89% of necropsies of which 7·5% had major chromosome abnormalities. The chromosome results are analysed according to the category of the necropsy and to primary cause of death. It is recommended that cytogenetic studies be performed on all stillbirths and infants dying at less than 28 days of age except in cases of isolated CNS malformation, sudden infant death syndrome (SIDS), trauma, or known single gene defects.

Chromosome studies carried out on several series of paediatric necropsies up to 1977 were summarised by Sutherland *et al*[1] and the recommendation made that such studies should form part of most paediatric necropsies. The present report documents the cytogenetic findings on all necropsies at the Adelaide Children's Hospital over an additional period of four years and makes further, more detailed, recommendations concerning the application of chromosome studies at the paediatric necropsy.

## Material and methods

All routine paediatric necropsies at the Adelaide Children's Hospital were studied. During the period of study the annual necropsy rate fluctuated between 75% and 80%, the majority of those not coming to necropsy having died from long standing illnesses such as cystic fibrosis, thalassaemia and malignant disease. In addition some stillbirths and neonatal deaths occurring outside the Hospital were referred for necropsy and all sudden infant deaths in South Australia were examined under the authority of the Coroner. Such studies were carried out for a six-year period ending May 1981; the results of the first two years have been published,[1] hence this report covers the last four years of the period.

Methods of chromosome study have been previously recorded.[1] Perinatal deaths are defined as stillbirths of more than 28 weeks gestation and liveborn infants who lived for less than one week, regardless of gestation. The perinatal deaths were further subdivided into macerated stillbirths, non-macerated stillbirths and early neonatal deaths. Older neonate refers to a baby dying between 8 and 28 days after birth, infant to a death occurring between 29 and 365 days after birth and child to a death occurring on or after the 366th day of life.

Accepted for publication 25 August 1982

## Results

The chromosome results are shown by category of necropsy in Table 1. Chromosome results were obtained for 89% of necropsies, the major area of failure being macerated stillbirths where results were obtained from only 54% of those studied. Despite their high failure rate, macerated stillbirths are considered worthy of study since they yielded the second highest proportion of chromosome anomalies. Therapeutic abortions of course had the highest yield since in many cases these were performed as the result of a prenatal diagnosis of a chromosome abnormality.

The chromosome findings according to cause of death are shown in Table 2 for the whole series excluding the therapeutic abortions and spontaneous abortions of less than 20 wk gestation. The chromosome abnormalities found in the SIDS were a 46,XY/47,XYY mosaic and a t(X;5) in a male known to have this abnormality prior to death, having been investigated for severe hypospadias.

The chromosome abnormalities detected are shown in Table 3. The majority of these are typical of those found at prenatal diagnosis (mainly autosomal trisomies) or of those dying in childhood—for example, Down's syndrome. There was one case of the very rare 45,X male found in a babe dying on the

Table 1 *Chromosome results according to category of necropsy*

| *Category* | *Total* | *Chromosome studies attempted* | *Successful* | *Normal karyotype* | | *Abnormal karyotype* | |
|---|---|---|---|---|---|---|---|
| | | | | *Male* | *Female* | *No* | *%* |
| Therapeutic abortion | 31 | 29 | 28 | 6 | 9 | 13 | 46 |
| Spontaneous abortion (less than 20 wk) | 7 | 7 | 3 | 3 | 0 | 0 | 0 |
| Spontaneous abortion (more than 20 wk) | 4 | 4 | 3 | 3 | 0 | 0 | 0 |
| Macerated stillbirth | 51 | 48 | 26 | 12 | 9 | 5 | 19 |
| Non-macerated stillbirth | 21 | 20 | 19 | 7 | 10 | 2 | 11 |
| Early neonatal death | 38 | 38 | 38 | 20 | 13 | 5 | 13 |
| Later neonatal death | 25 | 25 | 24 | 15 | 9 | 0 | 0 |
| Older than 28 days | 210 | 208 | 199 | 107 | 87 | 5 | 2·5 |
| Older than 1 yr | 151 | 143 | 141 | 84 | 52 | 5 | 3·5 |
| Total | 538 | 522 | 481 | 257 | 189 | 35 | 7·5 |

Table 2 *Chromosome results according to primary cause of death (therapeutic abortions and abortions of less than 20 wk gestation excluded)*

| *Cause of death* | *Total* | *Chromosome studies attempted* | *Successful* | *Normal karyotype* | | *Abnormal karyotype* | |
|---|---|---|---|---|---|---|---|
| | | | | *Male* | *Female* | *No* | *%* |
| Macerated without malformation | 41 | 37 | 18 | 7 | 9 | 2 | 11 |
| Macerated with malformation | 3 | 3 | 2 | 0 | 0 | 2 | (100) |
| Prematurity associated disease | 3 | 3 | 3 | 2 | 1 | 0 | 0 |
| Primary CNS malformations | 41 | 41 | 38 | 17 | 21 | 0 | 0 |
| Severe congenital malformations | 49 | 49 | 47 | 14 | 9 | 23 | 49 |
| Congenital heart malformation | 56 | 56 | 56 | 29 | 25 | 2 | 3·6 |
| Primary anoxia | 23 | 22 | 19 | 11 | 7 | 1 | 5·3 |
| Infection | 27 | 27 | 25 | 14 | 11 | 0 | 0 |
| SIDS | 142 | 141 | 135 | 81 | 52 | 2 | 1·5 |
| Leukaemia | 15 | 15 | 15 | 9 | 6 | 0 | 0 |
| Other malignancy | 18 | 18 | 18 | 11 | 7 | 0 | 0 |
| Trauma | 54 | 45 | 44 | 28 | 16 | 0 | 0 |
| Mendelian disorders | 21 | 21 | 20 | 14 | 6 | 0 | 0 |
| Miscellaneous | 37 | 37 | 37 | 18 | 18 | 0 | 0 |
| Total | 530 | 575 | 477 | 255 | 188 | 32 | 6·7 |

Table 3 *Chromosome abnormalities detected*

| | | *Chromosome abnormality* | *No* |
|---|---|---|---|
| (a) | Amongst the therapeutic abortions | 47,XX or XY,+21 | 6 |
| | | 46,XX,rob(13;14)mat,+21 | 1 |
| | | triploid | 2 |
| | | 47,XX,+mar | 1 |
| | | 47,XY,+13 | 1 |
| | | 47,XX,+del(22q) | 1 |
| | | 46,XX,del(5p) | 1 |
| (b) | Amongst the others | (i) *Sex chromosome anomalies* | |
| | | 46,XY (female) | 1 |
| | | 45,X (male) | 1 |
| | | 45,X | 1 |
| | | 46,XY/47,XYY | 1 |
| | | (ii) *Autosomal anomalies* | |
| | | 47,XX or XY,+21 | 5 |
| | | 46,XY/47,XY,+21 | 1 |
| | | 46,XX or XY,+18 | 4 |
| | | 47,XY,+13 | 1 |
| | | 46,XX/47,XX,+2 | 1 |
| | | 45,XY,rob(14q21q) | 1 |
| | | 46,Y,t(X;5)(q13;p15) | 1 |
| | | 46,XX,14q+ | 1 |
| | | 46,XX,del(5p) | 1 |
| | | 46,XX,del(6p) | 1 |
| | | 46,XY,del(4q) | 1 |

second day of life from cardiac failure due to a hypoplastic left heart. His external genitals were normal as was testicular histology. The mosaic trisomy 2 had 17/27 cells examined showing trisomy 2. This was one of macerated twins stillborn at 37 wk gestation; there was a single placenta and chromosome studies were from amnion. The main necropsy finding was of intrauterine hypoxia and there was evidence of growth disturbance at the costochondral junction. The findings in the second twin were similar except that the karyotype was normal female. The infant with del(5p) was an intrapartum death and apart from borderline microcephaly no unusual findings were made at necropsy.

## Discussion

The incidence and type of chromosome abnormalities detected in this series is similar to other published series.[1–3] The repeated finding of mosaic trisomy 2 is of interest since there is some doubt as to whether this is a true chromosome abnormality or an in vitro chromosome change which is particularly likely to occur when amnion is used for chromosome study. In the present series the finding of mosaic trisomy 2 in one of probably monozygous twins, with the other having a normal karyotype, suggests

Table 4 *Results of chromosome studies on unselected paediatric necropsies**

| *Status* | *No studied* | *No abnormal* | *% abnormal* |
|---|---|---|---|
| Macerated stillbirth | 138 | 18 | 13 |
| Non-macerated stillbirth | 359 | 15 | 4·2 |
| Early neonatal death | 862 | 46 | 5·3 |
| Late neonatal death | 110 | 5 | 4·5 |
| Infant | 402 | 19 | 4·7 |
| Child | 235 | 12 | 5·1 |

*Taken from references 1–3 and the present series.

that this finding is most likely to be due to in vitro change.

In Table 4 the results of this series of paediatric necropsies have been added to those summarised previously.[1] In the macerated stillbirth group the incidence of chromosome abnormality is around 13% and in all other groups it is in the region of 4–5%. Apart from occasional sex chromosome abnormalities and balanced translocations, virtually all the chromosome abnormalities in infants and children had been recognised prior to necropsy.

The value of chromosome results, even the normal results, cannot be over-emphasised. Genetic counselling should be offered to all couples who lose a child in the perinatal period and since the question of prenatal diagnosis arises in most genetic counselling situations knowledge of the chromosome results of the dead child are particularly valuable. In the present series the finding of del(5p) in a stillbirth, potentially a case of the cri du chat syndrome, was of immense help to the parents. It provided them with a reason for the death, alleviated some of their grief with the knowledge that had the child survived it would have been severely handicapped, and after they themselves had been shown to have normal karyotypes, provided positive reassurance that the recurrence risk was very small.

There are several causes of death in which chromosome abnormalities are no more common than amongst unselected liveborn children. These include deaths due to primary CNS malformation (mainly anencephaly and spina bifida), SIDS, trauma and known single gene defects. Since these diagnostic categories will usually be known by the time of the necropsy, and since there are very few unknown chromosome abnormalities detected in babies who have lived for more than one month, the following guidelines are suggested for the use of chromosome studies at the paediatric necropsy. Studies should be done on all infants dying at less than 28 days of age and on all stillbirths, except in cases of isolated CNS malformation, SIDS, trauma or known single gene defects. It is important that these exceptions are properly identified and not interpreted too loosely. For example, it is important to ensure that a CNS malformation is an isolated defect and not just one of many malformations present as occurred in one case of trisomy 18 in the present series where the most obvious feature prior to detailed examination of the neonate was spina bifida. Obviously infants older than 28 days in whom there are indications for chromosome studies should be karyotyped if this has not been done prior to necropsy.

Assistance with the tissue culture and cytogenetics was provided by Elizabeth Baker, Helen Eyre, Trudy Hocking and Erica Woollatt. Some of the necropsies were carried out by Drs GF Binns, AJ Bourne and G Phillips. Mr J Stappers' care and diligence in collecting material for chromosome analysis at necropsy greatly contributed to the success of this study.

**References**

1 Sutherland GR, Carter RF, Bauld R, Smith II, Bain AD. Chromosome studies at the paediatric necropsy. *Ann Hum Genet Lond* 1978;**42**:173–81.

2 Machin GA. Chromosome abnormality and perinatal death. *Lancet* 1974;**i**:549–51.

3 Kuleshov NP. Chromosome anomalies of infants dying during the perinatal period and premature newborn. *Hum Genet* 1976;**31**:151–60.

Requests for reprints to: Dr GR Sutherland, Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, SA 5006, Australia.

*Aust. J. Ment. Ret.*
(1971) 1: 239-245

# SIX CASES OF THE CRI-DU-CHAT SYNDROME

**HELEN FLYNN , ALLAN CARMICHAEL and GRANT R. SUTHERLAND**

*Aust. J. Ment. Ret.*
(1971) 1: 239-245

# SIX CASES OF THE CRI-DU-CHAT SYNDROME*

**HELEN FLYNN[1], ALLAN CARMICHAEL[2] and GRANT R. SUTHERLAND[3]**

**SUMMARY**

Six cases of the cri-du-chat syndrome are presented. Brief case histories are given along with clinical description, cytogenetics and dermatoglyphics. The problem of diagnosing this syndrome in older children is discussed and it is pointed out that the 'cat cry' can persist into early adulthood. A case of Wolf's syndrome is also described so that comparison of these two cytogenetically similar syndromes can be made.

During the past six years six cases of cri-du-chat syndrome have been recognized at Children's Cottages, Kew. It is the purpose of this paper to describe them, and to compare the clinical features with those of another disorder, Wolf's syndrome, a case of which has recently been seen at St. Nicholas Hospital.

It is interesting to consider the reasons which prompted chromosome analysis for these children; three cases were discovered when a chromosomal survey was done of patients with epicanthic folds, and a history of 'cat cry' in infancy was subsequently obtained by questioning the parents. In two other cases a history of abnormal cry in the neo-natal period was given by the parents on admission, thus suggesting chromosome analysis. Another, a 17-year-old girl, was noticed by the Charge Nurse to have a wail like a cat: she mentioned this on several occasions to the doctors concerned in her care, but as her appearance was so unlike that described for cri-du-chat no action was taken. The Sister's persistence finally resulted in chromosome analysis being performed and her diagnosis was confirmed.

## SUMMARY OF THE CASE HISTORIES

**Case 1:** C. H. Female, born 2nd August, 1957. (Fig. 1)

She was the second child, with four normal siblings. Both parents were twenty five years at her birth. Pregnancy was normal, gestation 40 weeks, labor normal. Birth weight 2,750 gm. She would not suck and had a high weak cry. The doctor said to the mother, 'you have a pussycat for a baby'.

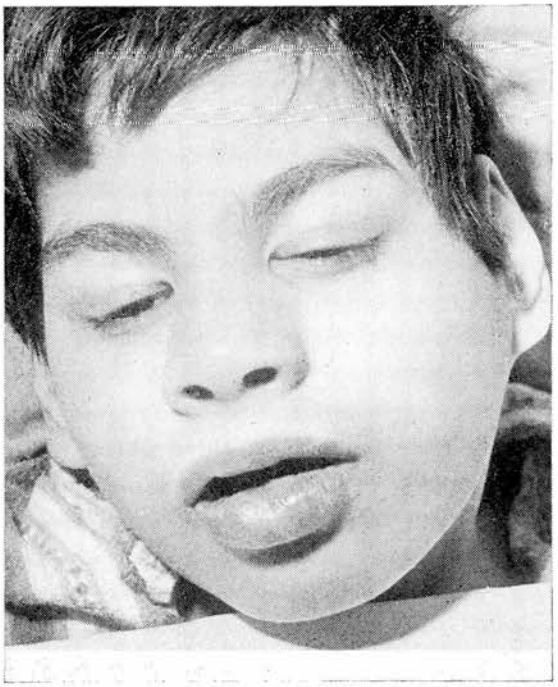


**Figure 1.** C.H. Note prominent lower lip, low set ears, epicanthic folds.

**Case 2:** E. H. Female born 21st August, 1949. (Fig. 2)

Mother was 26 years and father 34 years at her birth. She was the second child and had three normal siblings. The mother contracted mumps in the second trimester, and noticed that foetal movements were thenceforward sluggish. Delivery was normal. Birth weight

*Presented at AGSOMD Conference Adelaide SA Oct. 1970.

1. Medical Officer, Childrens Cottages, Kew Vic. Aust. 3101
2. Medical Officer, Alfred Hospital, Prahran Vic. Aust. 3181 Participation in this study was during an effective period whilst a fifth year Medical Student at Monash University.
3. Cytogenticist, St. Nicholas Hospital, Carlton Vic. Aust. 3053

**Figure 2.** R.H., E.H., W.W. Note dissimilar facial appearances.

3,430 gm. She did not suck well and failed to thrive. A friend hearing the baby's voice said, "Where's the cat?".

**Case 3:** R. H. Male, born 6th March, 1962. (Fig. 2)

A paternal first cousin had Down's Syndrome, and a paternal first cousin once removed was severely retarded. At the time of his birth, his mother was 36 years, his father 35 years. There were two normal siblings, two miscarriages, and one stillbirth. The patient was the youngest child. Gestation was 40 weeks, labor normal. Birth weight 3,070 gm. The cry was immediately noted to be abnormal like a kitten wailing. The child sucked poorly, had episodes of twitching and cyanosis, and failed to thrive.

**Case 4:** W. W. Male, born 31st January, 1960. (Fig. 2)

The mother was 24, the father 25 years, at his birth. He was the second child in a family of five. Three of his siblings were normal, and one mildly retarded. Labor was induced at thirty-six weeks, followed by a normal delivery with birth weight 2,590 gm. In the neo-natal period he cried like a kitten, and presented feeding difficulties.

**Case 5:** M. F. Female, born 5th December, 1952. (Fig. 3)

She was third born and has three normal siblings. Her mother was 23 years, her father 24 years at her birth. Labor was induced following an ante partum haemorrhage at thirty-five weeks, and birth weight was 2,650 gm.; there is no history available about the child's cry in infancy, but she would not suck and failed to thrive. Her cry is still recognizably feline, at the age of 18.

**Case 6:** A. W. Female, born 22nd August, 1962, and died 1969.

Her mother was 25, her father 33 years at birth. Her mother had one normal child, and then six miscarriages before birth of the patient. Labor occurred at thirty-six weeks, producing an infant whose birth weight was 1,680 gm. The baby remained in hospital for five weeks, and was always difficult to feed.

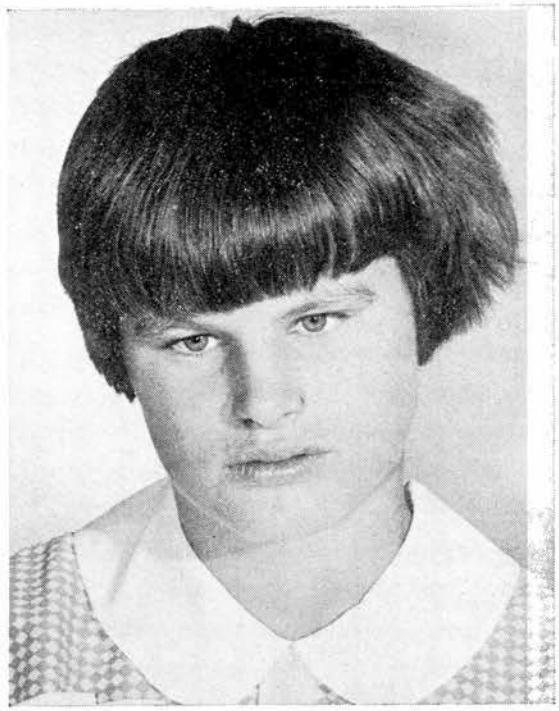


**Figure 3.** M.F. Note virtually normal appearance.

Later in hospital it was noted that she was noisy, "making her own peculiar sound", and this was recognized to be the cri-du-chat on chromosome analysis at 4 years. She had a bilateral congenital dislocation of the hips, diagnosed at birth, and was treated for 'coeliac disease' at eighteen months. She died in 1969 as a result of a febrile illness.

Since Lejeune et al. first described cri-du-chat in 1963 many other cases have been recorded. Apart from the cry the most constant features have been severe mental and physical retardation. Some fairly constant facial characteristics have been described, such as hypertelorism, epicanthus, low set ears, micrognathia, and oblique palpebral fissures.

The most striking feature is of course the cry. Three of our patients, aged respectively 8 years, 18 years, and 21 years, still have a recognizable 'cat cry'. The cry from the 21 year old girl recently started a search around the ward for "the fighting cats." In two other children there is no definite cat sound, but when distressed they produce a high pitched wail. Not all parents volunteered a history of cri-du-chat, but most remembered it when the direct question was asked. If this question were included in routine history taking of retarded children, an earlier diagnosis may result.

Growth retardation was common in our patients but not invariable. The height of one boy (R.H.) was at the seventy-fifth percentile, while one girl (M.F.) had a height at the fiftieth percentile. The others varied from below the third to the tenth percentile in height.

All these patients had extremely small heads. In each case the head circumference measured less than three standard deviations below the mean for age, and in one child (A.W.) it was 9 standard deviations below the mean.

Their facial appearance is not sufficiently characteristic to enable a diagnosis to be made on these grounds alone. Four of our cases are at present in-patients at Children's Cottages, Kew, and when seen together their resemblence to each other is not striking. The facial appearance of one girl (M.F.) is relatively normal.

The moon-shaped or round face frequently described in cri-du-chat cases was not seen in any of our patients. Their faces were variously long and thin, triangular, broad—in short they varied as much as do normal individuals. Perhaps if seen in infancy they would have presented round faces.

In five of these six patients epicanthic folds were present. The question of hypertelorism is more difficult to decide. A flat wide nasal bridge was present in every case, giving the impression of widely spaced eyes. Without exact measurements in ratio to the small and narrow head, hypertelorism cannot definitely be diagnosed.

A similar problem attaches to the description of oblique palpebral fissures. Assigning direction to the slant of the eyes is difficult in the presence of epicanthi. However, three patients did appear to have an anti-mongoloid slant, two a mongoloid slant, and one was normal. Four children had a convergent strabismus. We found no characteristic shape or position of the ears. Several patients had ears which were mildly abnormal in shape or size but no common pattern was seen. The position varied, but the ears were mainly low set. Micrognathia was an infrequent finding, appearing in only two children. The relative size of the mandible probably increases with age. Three of the six patients had a high arched palate. A constant feature was a wide generous mouth, with full lips. The mouth usually turned down at the corners.

The external genitalia were infantile in one male (R.H.) and one female patient (A.W.). An interesting finding was that two of the females (M.F. and E.H.) had a profuse growth of axillary hair which extended beyond the usual limits. These two females aged 18 and 21 years were both menstruating.

Abnormalities in the hands and feet were common. Four of the six patients have short incurved fifth fingers. One patient (A.W.) had an absent fifth toe on the left foot, and syndactyly of the fourth and fifth toes on the right foot. Three of the patients have abnormally short fourth toes. Two have hyperextensible elbows.

Neurological examination revealed hypertonicity of varying degree in all cases except one. Two children had ankle clonus, and three had a Babinski response. One boy (R.H.) with pale skin and fair hair, had fundi resembling an albino's. Although the retina was poorly pigmented, the optic discs were normal. One girl (C.H.) also has pale fundi, although her skin is dark.

On psychological assessment, five of the patients were found to be functioning in the profoundly retarded range. One girl (C.H.)

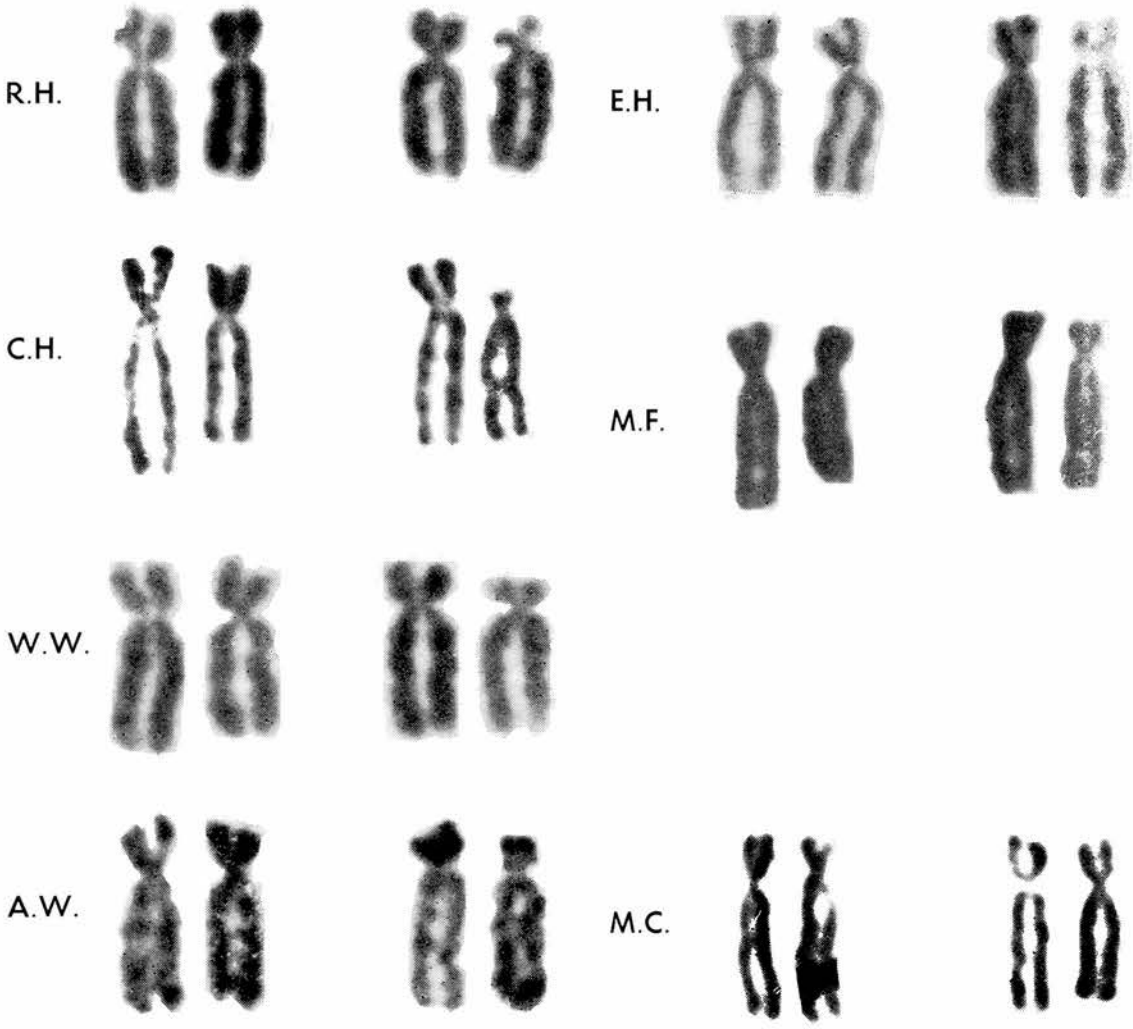



**Figure 4.** B-group chromosomes from all of the patients presented. In the six cases of 'cri-du-chat' syndrome the deleted chromosome is placed fourth, in the case of Wolf's syndrome it is placed second. Note variation in the amount of deleted material.

aged 13 years cannot yet walk without support. M.F. aged 18 years functions in the severely retarded range and has several words of speech. They are all amiable, friendly individuals, responsive to affection. The two males were notably hyperactive and distractible.

All patients showed marked susceptibility to infection, especially skin infection of the staphylococcal type. Self mutilation in the form of scratching and head banging was a common feature and the numerous skin infections may have been secondary to this. Respiratory infections were much more common than in other institutionalised patients. Five of these six patients have had infectious hepatitis at different times. This is a higher incidence than one would expect, and in conjunction with the other infections mentioned, may represent increased susceptibility to infection. Australia Antigen studies were done on our four in-patients, but were all negative.

## CYTOGENETICS

Leucocyte cultures on all the cases were prepared using standard methods. Where possible chromosome studies were carried out on the parents. In one case (C.H.) skin

cultures were performed (Pitt et al., 1966). In all cases the karyotype was 46 XX (or XY), Bp-, with the deletion being present in all cells. Two cases (E.H. and R.H.) were originally thought to have karyotypes 46 XX (or XY), Cinv. (Pitt et al. 1967), this error was corrected in a later paper (Roboz and Pitt, 1969). The amount of genetic material deleted varied from case to case. It ranged from most of the short arm (e.g. R.H.) to only about 1/3 of it (e.g. M.F.) (see Fig. 4.). In the five cases in which the parents could be studied all had normal karyotypes.

## DERMATOGLYPHICS

Some of the dermatoglyphic features of the patients are summarised in Table 1.

Fingerprint patterns showed a slight excess of whorls and a corresponding decrease in frequency of ulnar loops. The frequencies of radial loops and arches were normal. Total ridge counts were within the normal range. The axial triradii were slightly more distal than normal, being located mainly in the $t^1$ position. Every case had a pattern in the fourth interdigital area on at least one hand. Palmar creases were abnormal on one hand in five cases. There were 3 simian creases, one Sydney line and one transitional crease. Two cases had a single interphalangeal crease on the right fifth finger. There were no true thenar patterns and only one true hypothenar pattern and one parathenar pattern in the series. One case (A.W.) showed dermatoglyphic, but not clinical evidence of zygodactyly of the fingers. This is the child who had syndactyly of the toes. The hallucal patterns were mainly large distal loops.

The dermatoglyphic findings in the cri-du-chat cases are similar to those of Warburton and Miller (1967) with the exception that in our series there was not an excess of finger tip arch patterns.

**TABLE 1**

Some Dermatoglyphic Data on the Cases Presented

| Case | | Finger Print Patterns | | | | | Total Ridge Count | Areas with Interdigital Patterns | Maximal atd Angles in Degrees | Palmar Creases | Number of Fifth Digit Interphalangeal Creases | Hallucal Patterns |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | | | | | | |
| R.H. | R | W | R | U | U | U | 161 | IV | 46 | Normal | 2 | $L^d$ (large) |
| | L | W | W | U | U | U | | IV | 53 | Simian | 2 | $L^t$ ( |
| C.H. | R | U | A | U | U | U | 64 | IV | 47 | Normal | 2 | $L^d$ (large) |
| | L | U | U | U | U | U | | IV | 45 | Normal | 2 | $L^d$ (large) |
| W.W. | R | W | R | U | U | U | 98 | | 36 | Sydney | 1 | $L^d$ (small) |
| | L | W | R | W | W | U | | IV | 40 | Normal | 2 | $L^d$ (large) |
| A.W. | R | U | W | U | W | W | 137 | II | 74 | Simian | 1 | |
| | L | U | W | W | W | W | | IV | 63 | Normal | 2 | |
| E.H. | R | U | U | U | W | U | 169 | III | 48 | Normal | 2 | $L^d$ (large) |
| | L | U | W | W | W | W | | III IV | 50 | Simian | 2 | $L^d$ (large) |
| M.F. | R | W | A | U | W | U | 177 | IV | 34 | Transitional | 2 | $L^d$ (large) |
| | L | W | R | A | U | U | | | * | Normal | 2 | $L^d$ (large) |
| M.C. | R | W | U | U | W | U | 164 | | 45 | Normal | 2 | $L^d$ (large) |
| | L | W | U | U | U | U | | IV | 49 | Transitional | 2 | $L^d$ (large) |

* d-triradius absent, axial triradius in t position. There were no thenar or hypothenar patterns in this series except for W.W. who had a loop radial on the left hypothenar area and C.H. who had a parathenar loop distal on the right palm.

W = Whorl U = Ulnar loop L = Loop R = Radial loop A = Arch
superscripts — d = distal, t = Tibial

## OTHER INVESTIGATIONS

Four of our patients had skull X-rays and skeletal surveys. No abnormalities were found. The chest X-rays of the same patients are currently normal.

Haematological investigations were done on each patient but no unexpected abnormalities were found.

Electro-encephalograms were obtained from two patients only. The tracing was within normal limits in one (R.H.). Another (E.H.) had a non-specifically diffusely abnormal record.

Recently another child from St. Nicholas Hospital was found to have a karyotype of 46,XX,Bp-, however this child was shown to have Wolf's syndrome rather than the cri-du-chat syndrome.

In 1965 Wolf and others described a chromosome disorder in which there is deletion of the short arm of a chromosome number four, rather than a number five. A cytogenetic distinction between these two pairs of chromosomes can be made only by autoradiography. The physical appearance of the subjects with Wolf's syndrome and those with the cri-du-chat syndrome varies in certain important details, and in the absence of autoradiography the diagnosis can be made by considering the phenotype. The main difference is the absence of 'cat cry' in Wolf's syndrome. So far about 20 cases have been described in the literature, and the features reported include seizures, abnormal dermatoglyphics, cranial asymmetry, prominent eyes, ptosis, hypertelorism, flat nose, cleft or very high palate, pre-auricular tags and dimples.

The patient, a female, (M.C.) was born on 30-6-67. Her mother was 27 and her father 31 years at her birth. One normal child was born, then two miscarriages and one stillbirth preceded the birth of our patient. (see Fig. 5)

Maternal urinary oestriol levels were low from thirty weeks, and a Caesarean section was performed at thirty-eight weeks. Birth weight was 1,970 gm. Resuscitation was necessary and the baby did not breathe for two minutes. Congenital dislocation of the left hip was present. Nasolacrimal duct obstruction was a persistent problem in infancy. There have been frequent febrile convulsions. Rates of growth and development have been slow.

In view of her multiple anomalies, chromosome analysis was performed. The karyotype was 46,XX,Bp-, with the deletion being present in all cells. The parents both had normal karyotypes.

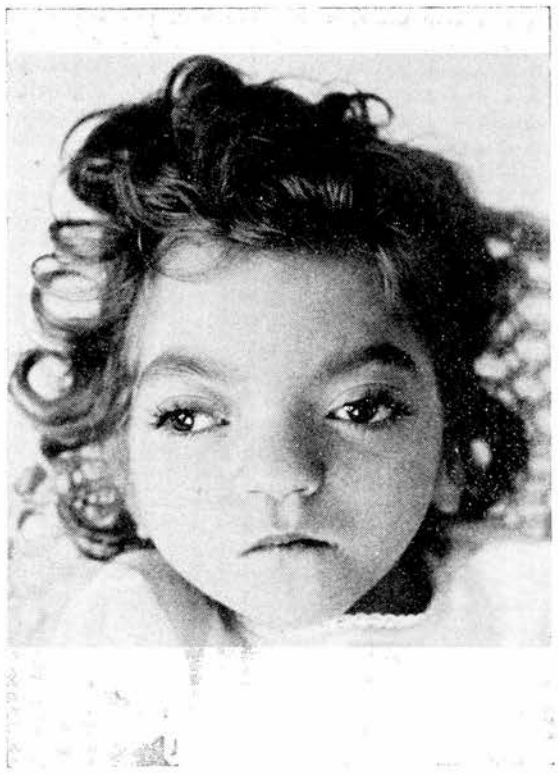


**Figure 5.** M.C. This is the child with Wolf's Syndrome.

When examined at the age of three years her height was 83.5 cms., below the third percentile, weight 9.25 Kg., below the third percentile, and her head circumference 41.5 cms., which is six standard deviations below the mean for age. She had marked cranial and facial asymmetry, and hypertelorism with a divergent strabismus. Her eyes were very prominent, and there was ptosis of the lids. Her ears were low set and asymmetrical, there was a pre-auricular pit on one side and a pre-auricular skin tag had been removed from the other. The nose was flat and the palate extremely high. There was a grade II systolic murmur present, maximal at the apex.

The left arm was one inch shorter than the right, the left leg one inch shorter than the right leg. She was generally hypotonic and had an extensor plantar response. Hair was present on the vulva. The hands had low set thumbs and flexion deformities of the fifth fingers. The

feet were equinus. The dermatoglyphics showed no striking features except for a bilateral mid-palmar ridge dysplasia which has been described by Van Kempen and Jongbloet (1967) as a distinguishing feature of Wolf's Syndrome.

She was unable to sit without support, had a clumsy grasp, and could eat only pureed food. She appeared to function at about a level of four months. Her cry, although described as 'peculiar and breathless' had never been thought feline or high pitched. X-rays of skull, chest and skeletal structures were normal. Urine chromatography gave a normal pattern. Considering the absence of a 'cat cry' the dermatoglyphics, and the clinical findings, the diagnosis of Wolf's syndrome was made.

## DISCUSSION

Six cases of cri-du-chat have been described, and one case of Wolf's syndrome. It may be useful to consider a composite picture of the cri-du-chat syndrome as it appears in relatively mature individuals. If the diagnosis is not made in infancy, the cry may still be the basis of diagnosis in adult life, for it can persist.

Even without a feline sound, cri-du-chat may be suspected in a patient with the following characteristics. He is small, profoundly retarded, and microcephalic. The eyes have epicanthic folds and a squint, the mouth is wide, the lips are full and the nasal bridge is flat and broad. There are minor anomalies of the hands and feet, and abnormal dermatoglyphics. Self-mutilation is common, and there has been a succession of skin infections and much respiratory disease.

Finally, the case history should be read. The psychologists' report on one case provided a clue. The report reads, in part: "Only one word sound was noted; this she produced in response to all questions or requests to imitate the examiner. She did, however produce the correct sound was noted ABLENR duce the correct sound when asked, 'What does a pussy say?' ".

## ACKNOWLEDGEMENTS

We thank Drs. David Pitt and Saul Wiener for their help and encouragement during this study, Dr. Dorothy Oliver for referring the patient with Wolf's Syndrome to us and the Mental Health Authority for permission to publish this report.

## REFERENCES

Lejeune, J., Lafourcade, J., Berger, R., Vialatte, J., Boeswillwald, M., Seringe, P., Turpin, R. (1963) Trois cas de délétion du bras court d'un chromosome 5. *C. R. Acad. Sci., Paris,* 257: 3098-3102.

Pitt, D. B., Wiener, S., Sutherland, G., Pearce, P. (1967) The Pericentric Syndrome. *Lancet (ii)* 568.

Pitt, D. B., Brasch, J., Wong, J. (1966). Another "cri-du-chat". *Med. J. Aust.* (2) 606-7.

Roboz, P., Pitt, D. B. (1969). Studies on 782 Cases of Mental Deficiency. Part IV. *Aust. Paed. J.* 5: 137-148.

Van Kempen, C., Jongbloet, P. H., (1967). Partial Deletion of the Short Arm of a Chromosome No. 4 *Maand. Kinder.* 35: 252-269.

Warburton, D., Miller, O, J., (1967). Dermatoglyphic features of patients with a partial short arm deletion of a B-group chromosome. **Ann.** *Hum. Genet., Lond.,* 31: 189-207.

Wolf, U., Porsch, R., Baitsch, H.. Reinwein, H., (1965). Deletion on short arms of a B-chromosome without 'cri-du-chat' syndrome. *Lancet (ii),* 769.

# Notes brèves

## A MALE WITH KARYOTYPE 46,XX

G.R. SUTHERLAND (*), S. WIENER, and A.A. BARTHOLOMEW

*Chromosome Laboratory, St. Nicholas Hospital, Melbourne, Australia.*
and
*H.M. Prison Pentridge, Coburg 3058 Australia.*

Whilst the usual phenotype associated with the 46,XX karyotype is that of a normal female, a number of near normal males have been described with this chromosome constitution. In a recent discussion of the nature and origin of these males de la Chapelle [1] knew of about 50 cases from the literature, personal communications and his own observations, the majority of whom had clinical findings consistent with Klinefelter's syndrome. When presenting the results of Xg blood group studies on persons with sex chromosome abnormalities, Sanger *et al.* [2] reported on 34 XX males. We report here another case of a male with the stigmata of Klinefelter's syndrome and a 46,XX karyotype.

### CASE HISTORY

The propositus, LAE 290451, was identified as having a normal female karyotype during a chromosomal survey of the patients in a security ward for the mentally defective [3]. He had been in the hospital only a short time prior to ascertainment and had been transferred to the security ward from the general section of the hospital for allegedly pestering female patients ; an offence which he denied.

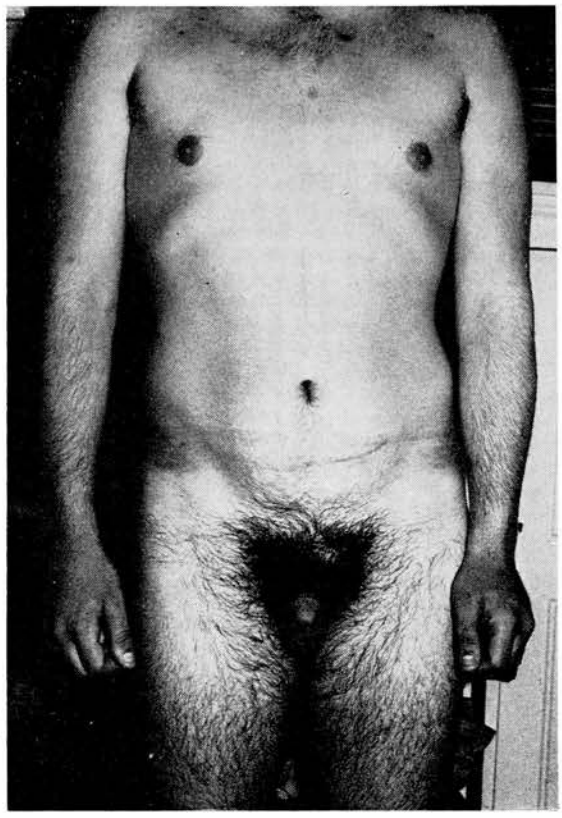
He was born in Queensland and what little is known of his early history was supplied to us from that State. He was an illegitimate child who spent most of his early years in orphanages. He was an object of ridicule in the orphanage due to his low intelligence level (IQ was assessed as 62) and he reacted with violence and aggression, eventually attempting to set fire to the orphanage. He was subsequently transferred to a Youth Hospital, classed as unmanageable, certified and admitted to a mental hospital at the age of 11 years. Electro-encephalographic investigation at this time demonstrated «marked dysrhythmia with much diffuse slow activity». His behaviour improved as he became older and he worked on unskilled tasks in the hospital grounds. He was discharged into his mother's care in 1968; at this stage being regarded as having a behaviour problem with mild mental retardation. Nothing more is known of him until he was admitted to Aradale Hospital (Victoria) in April, 1970, following his apprehension for travelling on a train without a ticket. He absconded before full clinical studies could be completed and he still remains at large.

#### Clinical Examination

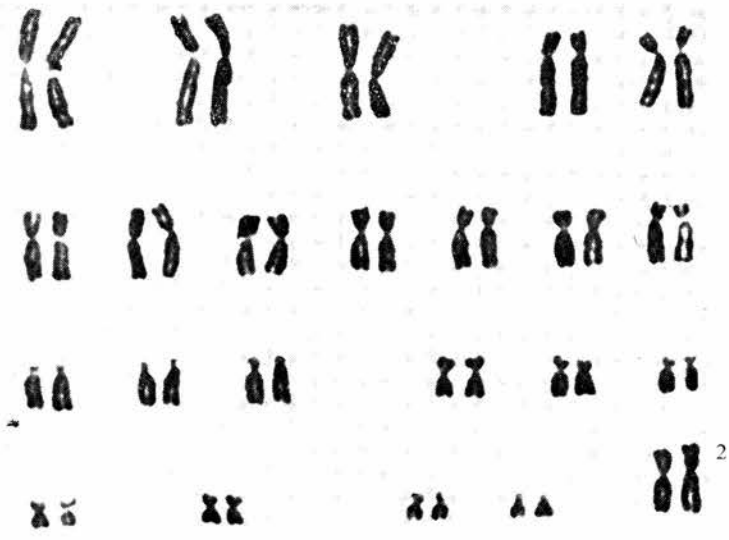
The patient appeared as a slightly dull, well built 19-year old man, 68 inches tall. He had severe acne on the face extending on to the chest, back and upper arms which presented as active pustules and much scarring. The facial scarring was so severe that we were uncertain whether his lack of facial hair was due to widespread destruction of the hair follicles or was primarily associated with his chromosome abnormality. Body hair distribution was that of a normal male and was extremely profuse, especially on the limbs (fig. 1). Clinically there was no abnormality detected in the cardio-respiratory system. The penis was normally developed but the scrotum contained small testes of normal consistency. The anus and perineum were clinically normal. He was unable to provide a sample of semen and according to the hospital staff he was not sexually active. His colour vision was normal and he was Xga+.

(*) Present Address: Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Sciennes Road, Edinburgh EH9 1LF, Scotland.

SUTHERLAND G.R., WIENER S., BARTHOLOMEW A.A. — A male with karyotype 46,XX. *Ann. Génét.*, *15*, n° 3, 187-189.



1



2

FIG. 1. — The propositus. Note acne scarring on chest, profuse body and apparently normal genitalia.

FIG. 2. — Karyotype of the propositus.

**Cytogenetic Studies**

Two blood leucocyte cultures were done at different times. Fifty cells were counted and examined for the presence of a Y chromosome or any evidence of a translocation, and a further fifty cells were scored for the number of G group chromosomes. There was no evidence to suggest that cells with other than a 46,XX chromosome complement were present (fig. 2). Skin fibroblast culture gave a similar result from the analysis of 42 cells. Buccal nucosal nuclei showed a normal female sex chromatin pattern. Fluorescent studies using quinacrine mustard on interphase nuclei showed no evidence of a Y chromosome in 200 buccal epithelial cell nuclei and 100 leucocyte nuclei. Control studies on XX, XY and XYY material yielded results appropriate to the number of Y chromosomes for both types of interphase nuclei. Fluorescent studies were not done on metaphase chromosomes.

## DISCUSSION

The reasons for the development of the Klinefelter phenotype in persons with an apparently normal female karyotype remain unclear. Possible explanations which have been tendered include undemonstrated mosaicism, Y to X or Y to autosome translocations, gene mutation, and the loss of the Y chromosome from an XXY zygote after sex differentiation has been initiated. This last explanation is supported by the Xg studies since the distribution of Xg types found in XX males most closely resembles that found in Klinefelter's syndrome rather than that in normal males or females [2].

The severity of the symptoms shown by XX males appears to vary greatly, ranging from normal members of the community whose only complaint is sterility, to mental hospital patients such as the one presented here. When more cases have been documented the degree of clinical variation may be as great as that seen in 47,XXY Klinefelter's syndrome, the only essential features being a 46,XX karyotype and testicular hypoplasia with subsequent sterility.

## ACKNOWLEDGMENTS

We thank Dr. P. WOOD for allowing us to study the patient who was under his care, Dr. Roslyn ANGELL for assistance with the fluorescent studies, the colour vision testing and for arranging the Xg blood group investigation to be done by Dr. R.T. SIMMONS, Miss Jean FERGUSON for performing the skin fibroblast cultures, Dr. V.L. MATCHETT for providing the report on the early history of the patient, and the Mental Health Authority for permission to publish this paper.

# A MALE WITH KARYOTYPE 46,XX

by G.R. SUTHERLAND, S. WIENER and A.A. BARTHOLOMEW

*Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Sciennes Road, Edinburgh EH9 1LF, Scotland*

(*Ann. Génét.*, 1972, *15*, n° 3, 187-189)

## SUMMARY

A 19-year old man with a normal female karyotype (46,XX) and the stigmata of Klinefelter's syndrome is described. He had a long history of antisocial behaviour and was ascertained when a security ward for the mentally defective was surveyed for chromosome abnormalities.

KEY-WORDS :

Klinefelter's syndrome* familial and genetic. — Mental retardation. — Phenotype. — Karyotyping.

# UNE OBSERVATION D'HOMME 46,XX

par G.R. SUTHERLAND, S. WIENER and A.A. BARTHOLOMEW

*Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Sciennes Road, Edinburgh EH9 1LF, Scotland*

(*Ann. Génét.*, 1972, *15*, n° 3, 187-189)

## RÉSUMÉ

Les auteurs rapportent l'observation d'un jeune homme de 19 ans ayant un caryotype féminin normal (46,XX) et atteint d'un syndrome de Klinefelter. Il avait un lourd parsé de conduite antisociale, et a été dépisté au cours d'une enquête sur les anomalies chromosomiques chez des débiles mentaux dangereux hospitalisés en milieu psychiatrique.

MOTS-CLÉS :

Syndrome de Klinefelter* familial et génétique. — Arriération mentale. — Phénotype. — Caryotype.

## REFERENCES

1. de la CHAPELLE A., 1972. — Nature and Origin of Males with XX Sex Chromosomes. *Amer. J. hum. Genet., 24,* 71.
2. SANGER R., TIPETT P., GAVIN J., 1971. — Xg Groups and Sex Abnormalities in People of Northern European Ancestry. *J. med. Genet., 8,* 427.
3. SUTHERLAND G., BARTHOLOMEW A.A., 1971. — Chromosome Survey in a mental Deficiency Security Ward: Total Ascertainment. *Aust. N.Z. J. Criminol., 4,* 82.

L'EXPANSION
— ÉDITEUR —
15, rue Saint-Benoît
— PARIS - 6e —

MONOGRAPHIES DES ANNALES DE GÉNÉTIQUE

# LES ANOMALIES CHROMOSOMIQUES AU COURS DES HÉMOPATHIES MALIGNES ET NON MALIGNES

## UNE ÉTUDE DE 171 CAS

*par Maria Cristina CORTINAS DE NAVA*

Un volume broché 21 × 27
90 pages, 22 figures
45 F, franco : 47,50 F

Extrait de la Table des Matières



*EN VENTE DANS TOUTES LES LIBRAIRIES SPÉCIALISÉES*
*ET A LA LIBRAIRIE DES FACULTÉS DE MÉDECINE ET DE PHARMACIE*
*174, BD SAINT-GERMAIN - PARIS-VIe*
*C.C.P. EXPANSION, LIBRAIRIE DES FACULTÉS, PARIS 5601-33*

# DIFFICULTY IN SHOWING MOSAICISM IN THE MOTHER OF THREE MONGOLS

BY
G. R. SUTHERLAND, MARGARET G. FITZGERALD,
and
D. M. DANKS

*Reprinted from Archives of Disease in Childhood, Vol. 47, No. 256 December 1972*



LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, LONDON WC1H 9JP

*Archives of Disease in Childhood*, 1972, **47**, 970.

# Difficulty in Showing Mosaicism in the Mother of Three Mongols

Parental mosaicism for trisomy-21 is a recognized reason for the birth of mongol children, and the possibility of parental mosaicism may be suspected especially in couples who produce more than one 'regular' mongol. However, in a recent review, Richards (1970) found only 11 documented instances of this occurrence. We wish to describe another woman in whom mosaicism was suspected clinically but was difficult to show by chromosome studies.

## Findings

This family presented for investigation at the Royal Children's Hospital in 1963 because the parents had two mongol children. The family pedigree is shown in the Fig. Chromosome studies were carried out at this time on the children, parents, and relatives as indicated. The two mongol children and the maternal uncle were shown to have regular trisomy-G and all the other relatives studied were normal. The results obtained in the abnormal individuals are shown in the Table.

The mother was short in stature and borderline in intelligence, and her facial appearance and head shape strongly suggested mongolism. A second blood sample and a bone marrow sample were therefore examined in an endeavour to find some trisomic cells. Unfortunately, a skin biopsy failed to grow. Even though mosaicism was not shown, it was considered so likely that the parents were told that the chance of having another mongol child could be as high as 50%.

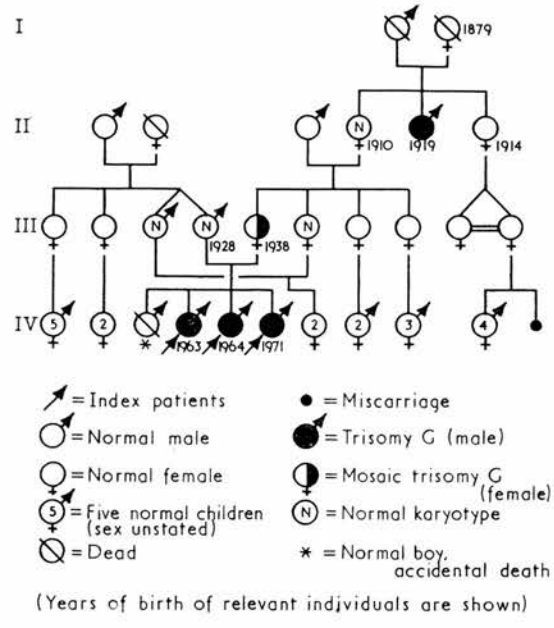



FIG.—*Family pedigree.*

In 1970 the family was referred to St. Nicholas Hospital for chromosome study by a clinician who was not aware that studies had already been performed. Leucocyte cultures showed a normal karyotype in the

TABLE

*Cytogenetic Data*

| Patient | Tissue | Chromosome Counts | | | | |
|---|---|---|---|---|---|---|
| | | 45 | 46 | 47 | Total | Karyotype |
| Mother (III.5) | Blood (1964) | | 22 | | 22 | |
| | Blood (1964) | | 30 | | 30 | 46,XX |
| | Bone marrow (1964) | 1 | 6 | | 7 | |
| | Blood (1970) | | 65 | 5 | 70 | 46,XX/47,XX,G+ |
| Son (IV.4) | Blood (1964) | | | 15 | 15 | 47,XY,G+ |
| | Blood (1970) | | | 22 | 22 | |
| Son (IV.5) | Blood (1964) | | | 25 | 25 | 47,XY,G+ |
| | Blood (1970) | | | 22 | 22 | |
| Mother's maternal uncle (II.4) | Blood (1964) | | | 18 | 18 | 47,XY,G+ |

father and G trisomy in the two mongol children, but revealed 5 cells with 47 chromosomes among 70 cells counted from the mother. In 4 of these cells the extra chromosome was from the G group, and in the remaining cell it was a C group chromosome. On these results maternal mosaicism with karyotype 46,XX/47, XX,G+ was diagnosed.

In 1971 the couple elected to have a further child despite the risks involved. A male mongol was born in December 1971. His karyotype has been confirmed as 47,XY,G+.

## Discussion

The difficulty of excluding chromosomal mosaicism has long been recognized, and this family re-emphasizes the need to study large numbers of cells from several tissues of both parents when two or more regular mongols are born to a couple. In this family, studies concentrated on the mother because she showed some clinical features of mongolism, but the initial investigations failed to reveal the mosaicism despite counting 59 cells from two tissues. Fortunately, appropriate genetic advice was still given.

The occurrence of another regular mongol in the family is also of interest, but his mother was 40 years old at his birth, so this may represent a chance occurrence.

## Summary

A case of maternal mosaicism leading to the production of three mongol children is reported. The difficulty of detecting the trisomic cell line and the need to examine large numbers of cells is emphasized.

We thank Drs. Ann Morgan, David Pitt, and Saul Wiener for their assistance with this study; Dr. O. Margaret Garson for the bone marrow studies and confirming the trisomic state of the third child; and the Mental Health Authority for permission to publish this paper.

### Reference

Richards, B. W. (1970). Observations on mosaic parents of mongol propositi. *Journal of Mental Deficiency Research*, **14**, 342.

G. R. Sutherland,* Margaret G. Fitzgerald, and D. M. Danks
*Chromosome Laboratory, St. Nicholas Hospital; Cytogenetics Laboratory, Royal Children's Hospital; Genetics Section, Royal Children's Hospital Research Foundation and Department of Genetics, University of Melbourne, Melbourne, Australia.*

*Correspondence to Dr. G. R. Sutherland, Department of Pathology, Royal Hospital for Sick Children, Edinburgh EH9 1LF.

*Letter to the Editor*
*reprinted from* THE LANCET, *July* 29, 1972, *p.* 231

## FAMILIAL INSERTIONAL TRANSLOCATION

SIR,—We were interested to read the report by Dr. Gray and colleagues (July 8, p. 92) in which they described an insertional translocation in a child with multiple congenital abnormalities. We have been studying a familial translocation in which an interstitial segment of the long arms of chromosome no. 7 has been inserted into the long arms of chromosome no. 3. The translocation was detected when chromosome studies were carried out on a 3-year-old girl referred on account of multiple congenital abnormalities; full clinical details will be described elsewhere.

Chromosome preparations were made from short-term leucocyte cultures of peripheral blood. The banded karyotypes were prepared using a modified Leishman technique.[1] The affected child had karyotype 46,XX,3q+mat and the mother and sister had karyotype 46,XX,t(3q+;7q−). The

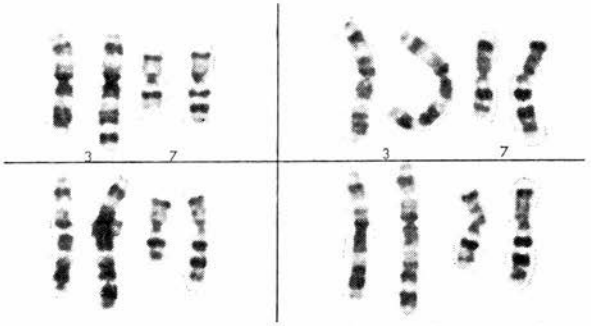



**Fig. 1—Chromosome pairs nos. 3 and 7 from four metaphases from the balanced karyotype showing banding patterns.**

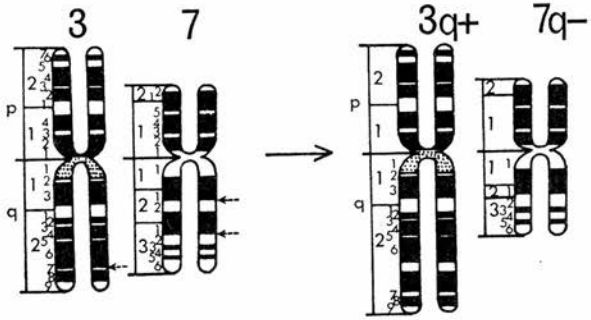



**Fig. 2—Idiogram of the banding patterns from the balanced karyotype.**
The break points are indicated thus ←– – –

maternal grandparents and a sister of the mother had normal karyotypes. An analysis of the banding patterns from the balanced karyotype was made to identify the break points. Metaphase spreads in which the chromosomes were least contracted were selected and photographed. Partial karyotypes of chromosome pairs nos. 3 and 7 from four of these cells is presented in fig 1. An idiogram (fig. 2) of the banding patterns of the translocated chromosomes was constructed according to the convention decided upon at the Fourth Standardisation Conference on Human Cytogenetics.[2]

The figures show that both a band and an interband have been removed, interstitially, from 7q. Examination of a number of metaphase spreads indicated that it was the distal broad band in 7q that had been translocated rather than the proximal. The break points were interpreted as being in the regions 7q22 and 7q32. The break in chromosome no. 3 was probably in the region 3q27, and this was the position in which the segment of 7q was inserted. Since there is no evidence of any reciprocal exchange of material from 3q to 7q, the simplest explanation of this translocation is that it arose by a three-break event in the gametes of one of the grandparents. The affected child is trisomic for the segment of 7q that has been inserted into 3q, and it is reasonable to assume that the clinical anomalies are directly associated with this trisomy.

Insertional translocations have been induced in *Drosophila*[3] and the mouse.[4] These translocations have been observed directly on the polytene chromosomes from *Drosophila* and in meiotic chromosome preparations from the mouse. Since the study of meiotic chromosome preparations in man is difficult because of the problems in obtaining suitable material, it is only since banding techniques have been developed that elucidation of chromosomal rearrangements has become possible. In *Drosophila*, insertional translocations are relatively rare when compared with terminal translocations, and the same will probably be true in man.

Department of Pathology,
Royal Hospital for Sick Children,
and University of Edinburgh,
Edinburgh EH9 1LF.

E. GRACE
G. R. SUTHERLAND
A. D. BAIN.

1. Grace, E., Bain, A. D. *J. clin. Path.* (in the press).
2. Fourth Standardisation Conference on Human Cytogenetics, Paris, September, 1971.
3. Lindsley, D. L., Grell, E. H. Genetic Variations of *Drosophila melanogaster*. Carnegie Institute, 1968.
4. Ohno, S., Cattanach, B. M. *Cytogenetics*, 1962, **1**, 129.

Printed in Great Britain

The Lancet Office,
7, Adam Street, Adelphi, London, W.C.2.

# PARTIAL TRISOMY OF 7q RESULTING FROM A FAMILIAL TRANSLOCATION

Elizabeth GRACE, G.R. SUTHERLAND, G.D. STARK, and A.D. BAIN

*Department of Pathology, Royal Hospital for Sick Children and the University of Edinburgh (Scotland).*

## INTRODUCTION

The development of banding techniques has enabled not only the accurate identification of individual chromosome pairs but also the localisation of the break points in chromosomes where rearrangements have occurred. Before the advent of these techniques the interpretation of chromosomal anomalies involving the C group had been difficult because of inadequate methods of identification. This report describes a 2 year old girl who presented with severe psychomotor retardation and who was found to be trisomic for part of the long arm of chromosome number 7. This trisomy was associated with a balanced insertional translocation in the mother. A detailed description of the morphology and origin of the translocation has been published previously (Grace, Sutherland and Bain, 1972).

## CASE REPORT

The proposita is the second child of healthy unrelated parents who were both aged 30 at the time of her birth. Her older sister is well and there is no family history of congenital anomaly or developmental retardation (fig. 1).

She was born by breech delivery at full term. Birth weight 2 800 g. She required endotracheal intubation and positive pressure ventilation for one minute before respiration was established. She was slow to feed initially but was fit for discharge at the age of two weeks.

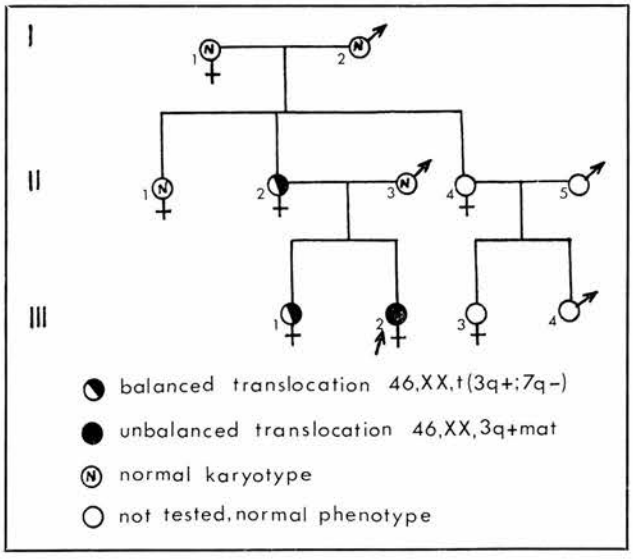



FIG. 1. — Pedigree of the family.

GRACE Elizabeth, SUTHERLAND G.R., STARK G.D., BAIN A.D. (1973). — Partial trisomy of 7q resulting from a familial translocation. *Ann. Génét., 16,* nº 1, 51-54.

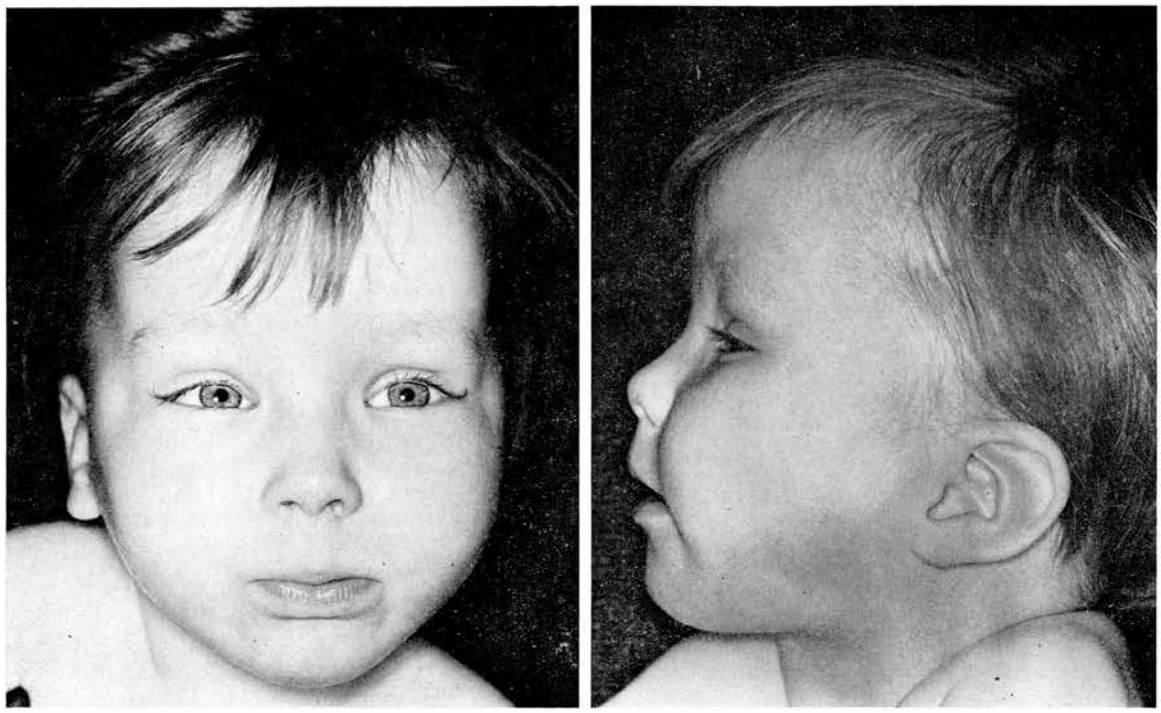


FIG. 2. — Full face and profile of the proposita at the age of 1 ¾ years.

She was first admitted to hospital at the age of 10 months suffering from severe bronchiolitis. Following recovery, she was noted to be severely retarded in all aspects of development. She was small for her age, her height (68 cm) and weight (7,6 kg) being below the 25th percentile. Occipito-frontal circumference was normal at 46 cm. The facial appearance was odd with bilateral epicanthic folds, almond-shaped eyes, flat nose and everted lower lip; the ears were large and low set (fig. 2). She had broad, proximally-placed thumbs and broad first toes.

She was extremely apathetic and did not smile nor could she follow objects with her eyes or localise sounds. The optic fundi were normal but there was a rotatory nystagmus and a left concomitant, convergent strabismus. There was some head lag when pulled up from supine and she was unable to sit without support. Her hands were open but she did not grasp or reach out for objects. The posture was one of generalised flexion; there was a slight increase in tone and in reflexes in the lower limbs with bilateral ankle clonus. Primitive reflexes were absent but parachute and Landau reactions were poorly developed. The clinical diagnosis was one of severe mental handicap and mild diplegia.

The patient was examined again at the age of 3 years when the visual and auditory abnormalities, which were noted at the age of 10 months, were attributed to severe mental retardation rather than to a physical defect.

**Cytogenetic studies.**

Chromosome preparations were made from short-term cultures of heparinised peripheral blood. The banded karyotypes were prepared according to the method of Grace and Bain (1972).

Orcein stained chromosome preparations from the proposita were first made at the age of 2 years and the abnormally long number 3 chromosome was noted. Cultures were set up from the family in order to obtain preparations for banding and the extra material on the number 3 chromosome was identified as being derived from part of the long arms of the number 7 chromosome. The proposita had the karyotype 46,XX,3q+mat and the mother and sister 46,XX,t(3q+;7q−) (figs 3a and 3b). Detailed examination of the banding patterns revealed the translocation to be insertional rather than terminal (Grace, Sutherland and Bain, 1972). The proposita is trisomic for an interstitial segment of the long arms of chromosome number 7. The maternal grandparents and one of the aunts had normal karyotypes; the other aunt is domiciled abroad and could not be examined. Table I lists the number of cells analysed and the karyotypes of the members of the family who were tested.

There was no evidence of mosaicism for the translocation in either the mother II.2 or the maternal grandparents I.1 and I.2.

**Dermatoglyphics.**

Some of the dermatoglyphic characteristics of the proposita, her mother and sister are shown in table II. The palmar and plantar configurations of the proposita are represented in figure 4. The unusual features in the dermatoglyphics include the presence of three radial loops on the fingertips and abnormal palmar creases. Finger prints were not of sufficient quality to enable an accurate ridge count to be made, probably due to the perpetual sucking of the fingers, but was estimated to be about 90. The only significant feature of the proposita which is apparent when

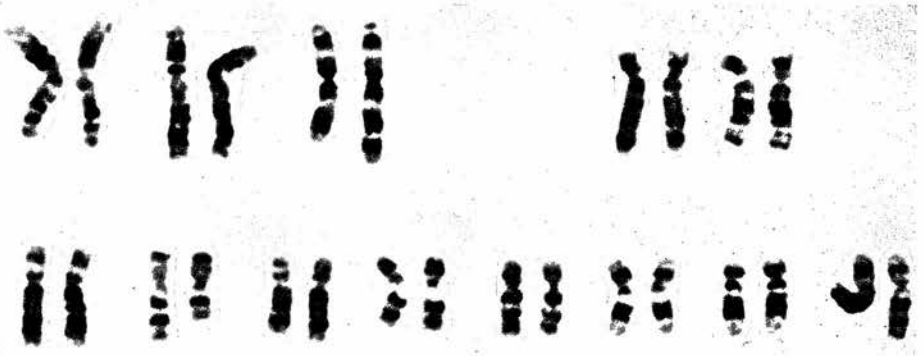


FIG. 3a. — Partial karyotype of the sister III,1.

FIG. 3b. — Partial karyotype of the proposita.

TABLE I

NUMBER OF METAPHASES EXAMINED FOR EACH FAMILY MEMBER AND THEIR KARYOTYPES

| Family Member | Number of Metaphases | Karyotype |
|---|---|---|
| I.1 | 30 | 46,XX |
| I.2 | 30 | 46,XY |
| II.1 | 30 | 46,XX |
| II.2 | 30 | 46,XX,t(3q+;7q−) |
| II.3 | 10 | 46,XY |
| III.1 | 30 | 46,XX,t(3q+;7q−) |
| III.2 | 30 | 46,XX,3q+mat. |

compared to her mother and sister is a slight decrease in pattern intensity evidenced by the lower total ridge count(TRC) and absence of hypothenar patterns.

**Other investigations.**

Prior to recognition of the chromosomal abnormality, investigations were directed towards excluding metabolic and degenerative diseases of the central nervous system.

On electro-encephalography, there was asymmetry of background activity but no epileptic discharges. Skeletal X.ray survey was negative but lumbar air encephalography revealed slight ventricular dilatation and an increase in the width of sulci suggesting cerebral atrophy. All biochemical investigations were within normal limits.

TABLE II

DERMATOGLYPHIC CHARACTERISTICS OF THE PROPOSITA, HER MOTHER AND HER SISTER

| | | Finger-print patterns | | | | | Maximal atd angle | TRC | Palmar formula (*) |
|---|---|---|---|---|---|---|---|---|---|
| | | i | ii | iii | iv | v | | | |
| Proposita | R ..... | U | RL | RL | U | U | 48° | approx. 90 | IV t 4 |
| | L ..... | U | RL | A | U | U | 45° | | IV t 4 |
| Mother | R ..... | U | U | A | U | U | 76° | 103 | $III^T$ H $\hat{H}$ t $t''t^b$ 4 |
| | L ..... | U | RL | U | U | U | 78° | | IV H $\hat{H}$ t $t''t^b$ 4 |
| Sister | R ..... | U | U | RL | U | U | 47° | 133 | IV $\hat{H}$ t $t^b$ 4 |
| | L ..... | U | U | U | U | U | 44° | | IV $\hat{H}$ t $t^b$ 4 |

(*) after Penrose and Loesch (1970). U = ulnar loop. RL = radial loop. A = arch.

## DISCUSSION

This is the first reported instance of a confirmed trisomy for part of the long arms of chromosome number 7. An examination of the banding patterns indicated that the broad band of 7q furthest from the centromere, together with a light interband, had been inserted into the long arms of chromosome number 3.

The clinical abnormalities which had been described in the proposita are assumed to be the direct consequence of the excess chromosomal material, however, a comparison of the present case with previous reports of partial trisomy for Cq is non-contributory to the discussion because of the uncertainty of identification in those cases. Differential staining techniques have proved valuable in this case and will help to bring some order to a large number of C group chromosome abnormalities which have previously been unclassifiable. A syndrome associated with trisomy of chromosome number 8 has already been described (Caspersson et al., 1972 ; de Grouchy et al., 1971), and two cases of partial trisomy of 10q have been identified (de Grouchy et al., 1972; Laurent, pers. comm.).

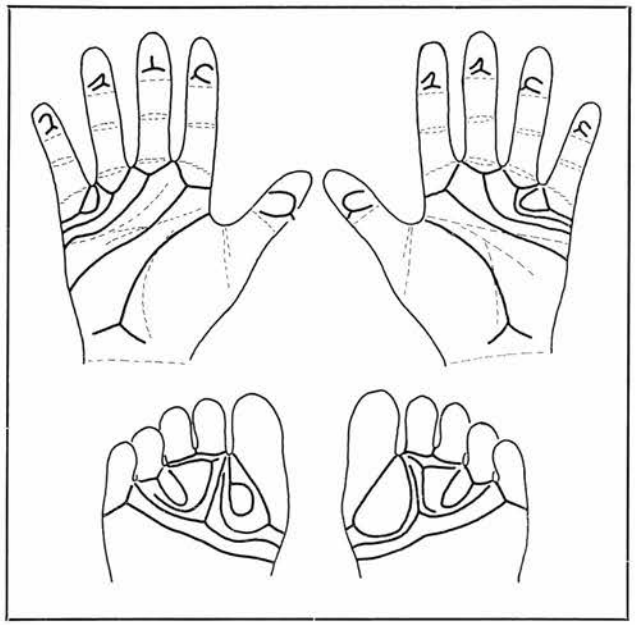


FIG. — 4. — Diagram of the plantar and palmar dermatoglyphic patterns of the proposita.

## PARTIAL TRISOMY OF 7q RESULTING FROM A FAMILIAL TRANSLOCATION

by Elizabeth GRACE, G.R. SUTHERLAND, G.D. STARK and A.D. BAIN

*Department of Pathology, Royal Hospital for Sick Children and the University of Edinburgh (Scotland)*

(*Ann. Génét.*, 1973, *16*, nº 1, 51-54).

### SUMMARY

This report describes a 2 year old girl who presented with severe psychomotor retardation associated with an odd facies involving bilateral epicanthic folds, almond-shaped eyes and low set ears. These features are attributed to trisomy for an interstitial segment of 7q arising from a familial translocation.

KEY-WORDS : Trisomy. — Chromosomes, human, 6-12. — Chromosome aberrations. — Chromosome abnormalities. — Psychomotor disorders.

## TRISOMIE PARTIELLE DE 7q DUE A UNE TRANSLOCATION FAMILIALE

par Elizabeth GRACE, G.R. SUTHERLAND, G.D. STARK et A.D. BAIN

*Department of Pathology, Royal Hospital for Sick Children and the University of Edinburgh (Scotland)*

(*Ann. Génét.*, 1973, *16*, nº 1, 51-54).

### RÉSUMÉ

Les auteurs décrivent le cas d'une fille de 2 ans atteinte de retard psychomoteur grave associé à une dysmorphie faciale avec epicanthus bilatéral, yeux en amande, et oreilles bas situées. Ces anomalies sont attribuées à une trisomie pour un segment interstitiel de 7q due à une translocation familiale.

MOTS-CLÉS : Trisomie. — Chromosomes humains 6-12. — Aberration chromosomique. — Anomalies par aberration chromosomiques. — Troubles psychomoteurs.

### REFERENCES

1. CASPERSSON T., LINDSTEN J., ZECH L., BUCKTON K.E., PRICE W.H. (1972). — Four patients with trisomy 8 identified by the fluorescence and Giemsa banding techniques. *J. med. Genet., 9,* 1.

2. GRACE E., BAIN A.D. (1972). — A simple method for chromosome banding. *J. Clin. Path., 25,* 910.

3. GRACE E., SUTHERLAND G.R., BAIN A.D. (1972). — Familial insertional translocation. *Lancet, ii,* 231.

4. GROUCHY J. DE, TURLEAU C., LEONARD C. (1971). — Etude en fluorescence d'une trisomie C mosaïque probablement 8 : 46,XY/47,XY,?8+. *Ann. Génét., 14,* 69.

5. GROUCHY J. DE, FINAZ Catherine, ROUBIN Michelle, ROY J. (1972). — Deux translocations familiales survenues ensemble chez chacune de deux sœurs, l'une équilibrée, l'autre trisomique partielle 10q. *Ann. Génét., 15,* 85.

6. PENROSE L.S., LOESCH D. (1970). — Topological classification of palmar dermatoglyphics. *J. ment. Defic. Res., 14,* 111.

*J. ment. Defic. Res.* (1974) **18**, 79



# A GIRL WITH WOLF-HIRSCHORN SYNDROME AND MOSAICISM 46,XX/46,XX,4p−

C. G. JUDGE, O. M. GARSON, D. B. PITT and G. R. SUTHERLAND

*Children's Cottages, Kew Victoria, Melbourne University Department of Medicine, St. Vincent's Hospital, Melbourne, and St. Nicholas Hospital, Melbourne, Australia*

SUMMARY

Chromosomal analysis of an eleven-year-old mentally retarded girl showed 46,XX/46,XX,4p− karyotype. Subsequent clinical examination demonstrated that she has features of Wolf-Hirschorn syndrome. (Sidbury *et al.*, 1964; Wolf *et al.*, 1965; Hirschorn *et al.*, 1965.)

## INTRODUCTION

The Wolf-Hirschorn syndrome has features, some of which are shared by the "cri-du-chat" syndrome, but others which are specific. Several authors (Miller, 1970; Wilson *et al.*, 1970; Passarge *et al.*, 1970) regard the phenotype as a distinct clinical entity. Others, however (Carter *et al.*, 1969), believe diagnosis to be difficult on clinical grounds alone. Possibly there is variability in the extent of the deletion and this could be a source of phenotypic variability (Taylor *et al.*, 1970).

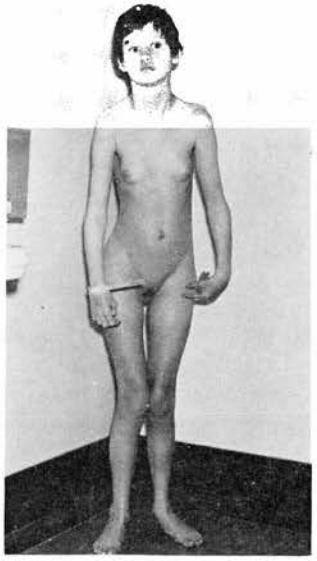


Fig. 1. Build of proposita

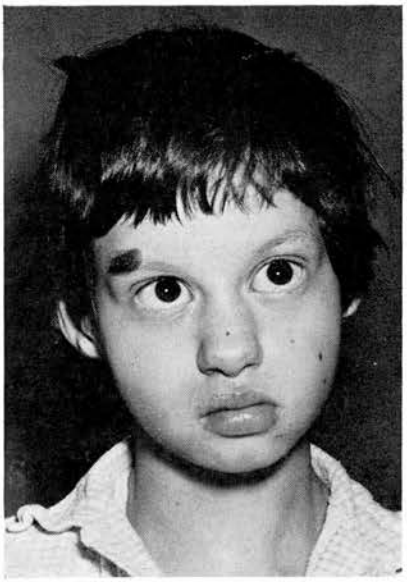


Fig. 2. Facies.

*Received 29th August, 1973*

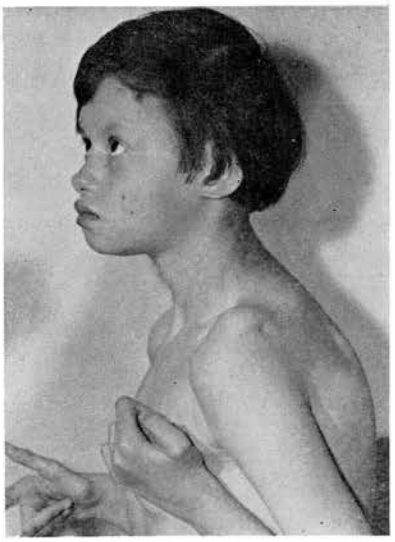


Fig. 3. Illustrates shoulder dimpling of the proposita.

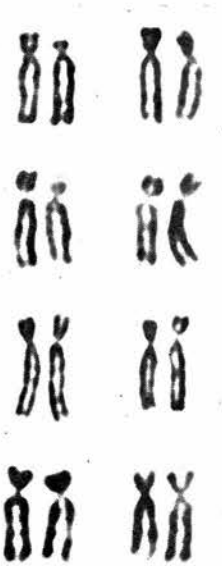


Fig. 4(right). B group chromosomes from four cells. The deletion is present in the upper two groups, not in the lower.

FAMILY HISTORY

The patient is the eldest child in a family of six children. The mother has had no miscarriages. She was aged twenty-seven years at the time of the patient's birth. Father is the same age. Both parents are of normal intelligence. Mother is mildly dependent on alcohol. The youngest child in the family suffers from strabismus and dyslalia.

CASE HISTORY

Mother states that there was poor foetal activity during the pregnancy. She took "nerve" tablets. The birth was normal and the birth weight 2282 gr. A small umbilical hernia was present.

The proband did not walk until five years of age. Speech commenced at seven years and is still delayed. At the age of two years she suffered her first epileptic seizure. She has attended a Day Centre for retarded children. Clitoral enlargement has always been present and breast development commenced at the age of eight years.

PRESENT STATUS

The patient's build is slight (see Fig. 1), her height being 138 cm (at the 25th percentile), her weight 25.4 kg (3rd-10th percentile) and her head circumference 46.5 cm (5 S.D. > mean). She has marked kyphosis.

The face is moon-shaped with proptosis of the eyes (see Fig. 2). The eye slant is upwards, and there are epicanthi. The eyebrows are sparse, particularly medially. The nose is flattened and has two tips rather than one. The ears are low set. There is poor development of the lobule and lower portions of the ears. She has gingivitis and the palate is high. There is micrognathia. The complexion is sallow and there is a hairy pigmented mole over the right eyebrow.

There is a systolic bruit at the apex, possibly due to a small septal defect. There are well-developed breasts and the clitoris is enlarged. The fingers are long and spidery with hyper-convex nails which are striated in an axial direction. The feet are valgoid. There is acrocyanosis of the hands and feet. The dimples over the posterior superior iliac spines are more prominent than normal. There are pronounced dimples over the spines of the scapulae (see Fig. 3).

Table 1

*Chromosome counts*

| *Patient* | *Tissue* | *44* | *45* | *46* | *46, 4p-* | *Total* | *Karyotype* |
|---|---|---|---|---|---|---|---|
| Proposita | Blood 1 | | 6 | 23 | 5 | 54 | 46,XX/46,XX,4p- |
| | Blood 2 | | 4 | 23 | 6 | 33 | |
| | Bone Marrow | 3 | 3 | 25 | 19 | 50 | |
| Mother | Blood | | | 30 | | 30 | 46,XX |
| Father | Blood | | 1 | 29 | | 30 | 46,XY |

Psychologically she tests in the severely retarded range (IQ 20-35) (Merrill-Palmer Scale). Radiography of the pubic and carpal bones showed the bone age to be normal. Electroencephalography showed a non-specifically diffusely abnormal record without frank paroxysmal discharges.

CYTOGENETIC STUDIES

Stimulated lymphocyte cultures were performed on the proposita and her parents by a modification of the method of Moorhead *et al.* (1960). The majority of the cells in the proposita

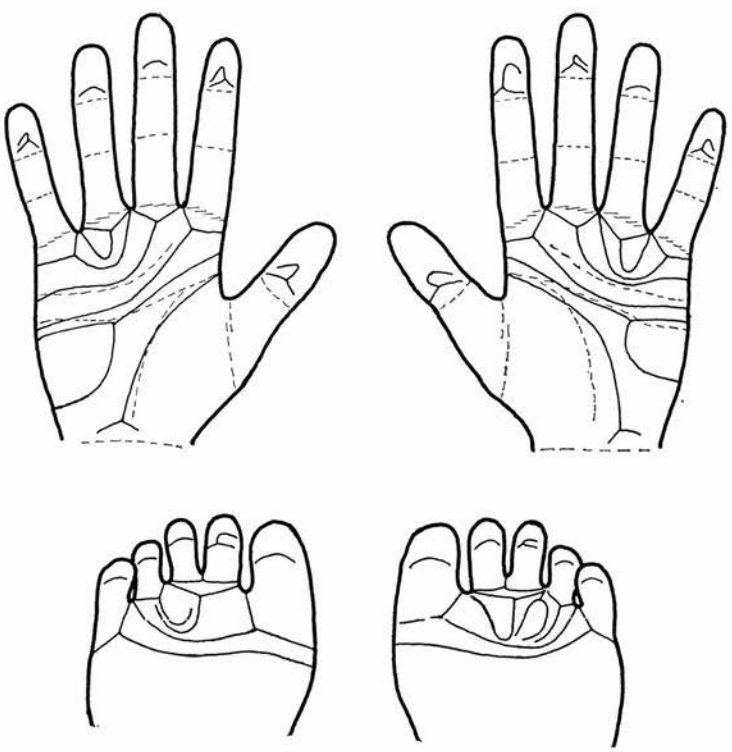


Fig. 5. Dermatoglyphic configurations of proposita.

Table 2
*Specific features in Wolf-Hirschorn syndrome*

| | Sidbury *et al.* | Wolf *et al.* | Hirschorn *et al.* | Miller *et al.* | Miller *et al.* | Miller *et al.* | Miller *et al.* | Miller *et al.* | Van Kempen *et al.* | Taylor *et al.* | Leas *et al.* | Arias *et al.* | Arias *et al.* | Flynn *et al.* | Passarge *et al.* | Georgi | Pfeiffer | Pfeiffer | Judge *et al.* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | F | F | M | M | M | M | F | F | F | M | M | M | M | F | M | F | F | M | F |
| Facial and cranial distortion | | + | | | | + | | | + | + | + | | | + | | | | | + |
| Midline cranial defects | | + | + | | | + | + | | + | + | | + | | | + | + | | | + |
| Iris deformities | + | + | + | | | + | | | + | + | | | + | + | | | | | + |
| Skin dimples or sinuses | | + | + | + | + | + | + | | + | + | + | + | + | | | + | + | | + |
| Absence of cat-cry | + | | | + | + | + | + | | + | | + | + | | | | + | | | + |
| Eyelid ptosis | | + | | | + | + | | | | | | | | + | | | | | |
| Hypospodias | + | | + | | + | + | | | | + | + | + | + | | + | + | + | | + |
| Enlarged clitoris | | + | | | | | + | | + | | | | | | | + | + | | + |
| Premature pubarche | | | | | | | | | + | | | | | + | | | | | + |
| Preauricular defects | | + | + | + | | | | + | + | | + | | | + | | + | | | + |
| Epilepsy | + | | + | | | + | | | + | | + | + | + | + | + | + | + | + | + |
| Ocular proptosis | | + | | | | | + | | + | | | | | + | | | | | + |
| Dislocated hip | + | | | | | | | | + | | | + | | + | | | | | + |
| Underdeveloped dermal ridge patterns | | + | | + | | | | | + | | | | | + | | | | | + |
| Mental retardation | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | + | + |
| Microcephaly | + | + | + | + | + | + | + | + | + | + | | + | + | + | | + | | + | + |
| Cardiac malformation | + | + | | | | | | | + | | | | | + | | + | | | + |

were of normal female chromosome constitution (46,XX), but on each of the two occasions there was a small percentage of cells (15-18 per cent) which showed a deletion of approximately two-thirds of the short arms of a B group chromosome (Fig. 4 and Table 1). Direct bone marrow preparations using a modification of the method of Kiossoglou *et al.* (1964) showed a similar mosaic picture, but the percentage of cells containing the deleted B group chromosome was 36 per cent. The hypodiploid cells present in all three cultures were due to a random loss of chromosomes and therefore the cells were probably broken in preparation.

The giemsa banding technique of Schnedl (1971) was applied to the bone marrow chromosomes, and allowed identification of the deleted B group chromosome as a No. 4. In the spreads with normal chromosomes, the B group chromosomes 4 and 5, could be readily paired, according to their banding patterns.

Chromosome studies in the lymphocytes of the parents showed no abnormality.

## DERMATOGLYPHICS

The dermatoglyphic features are shown in Fig. 5. The total finger ridge count is thirty-nine. The axial triradii are in the mid-palmar area (66°, 52, 6 per cent; 74°, 56, 6 per cent right and left respectively). The palmar creases are abnormal with the proximal transverse crease reaching the ulnar border of the palm, the so-called Sydney line (Purvis-Smith and Menser, 1968). There is ridge dysplasia on the peripheral areas of the fingerprints and on some parts of the soles; it is not evident on the palms.

## DISCUSSION

The clinical features of the Wolf-Hirschorn syndrome show an aggregation which is specific and which in only a few instances resembles the cri-du-chat syndrome. As with the majority of autosomal conditions associated with mental retardation there is the usual high incidence of short stature, microcephaly, micrognathia, low-set ears, epicanthi, strabismus, etc. A review of published cases is shown in Table 2 and examination of this table indicates the features specific to 4p- syndrome. Facial and cranial distortion, midline cranial defects, iris deformities, hypospadias in the male, skin dimples, and the absence of the cat-cry, were considered specific by some authors (Wilson *et al.*, 1970). Other authors have included eyelid ptosis, broad nasal base, a full upper lip, sparse eyebrows on the medial half (giving a startled expression), enlarged clitoris, premature pubarche, pre-auricular sinuses, epilepsy, proptosis, dislocated hip, dysplasis of the skin ridges of the hands, bone defects, "volar pads" (Van Kempen and Jongbloet, 1968; Miller *et al.*, 1970). In particular there is a high incidence of epilepsy in 4p- syndrome, as opposed to a low incidence in other autosomal conditions. The frequency of epilepsy in the 4p- syndrome appears to be a distinct point of differentiation from the 5p- syndrome. This may be related to the very low birth weight in 4p-, as it has been pointed out (Taylor *et al.*, 1970) that the birth weight in 4p- syndrome is the lowest mean birth weight of any chromosomal anomaly. The incidence of premature puberty and enlarged clitoris in the female and hypospadias in the male is also increased. Such genital anomalies do not occur with the cri-du-chat syndrome.

Clinical features in our case suggest a diagnosis of Wolf-Hirschorn syndrome, and this is confirmed by cytogenetic studies. Some modification of clinical features may be the result of mosaicism. We believe this to be the first case in which mosaicism for a deletion of the short arm of chromosome number 4 has been demonstrated. Mosascism

for deleted number 5 chromosome has been reported (Antich *et al.*, 1968) and phenotypically is consistent with the cri-du-chat syndrome. Chromosome studies on the mother of a child with 4p- syndrome (Taylor et al 1970) showed one cell out of one hundred with the deleted B chromosome, but the authors considered that the most likely explanation of this was temporary chimerism.

Features which are present, but which are common to both the 4p- and 5p- syndrome, include mental retardation, microcephaly, growth disorder, epicanthi, low birth-weight, hypotonia, strabismus, low-set ears, and a high arched palate. The features in our case which are specific to the 4p- syndrome are the absence of cat-cry, clitoral enlargement, early breast development, proptosis, epilepsy, and under-developed dermal ridge patterns. Minor features present, and again specific for the 4p- syndrome, include absence of the medial half of the eyebrows, the presence of sacral and shoulder dimples (Wilson *et al.*, 1970) and a possible heart defect.

Features which are absent in our case but which would have added further evidence in favour of the 4p- syndrome include a midline defect such as cleft lip and/or palate, iris deformity and a "fishlike" mouth. The dermatoglyphics are consistent with the diagnosis of Wolf's syndrome, especially in view of the ridge dysplasia and the low pattern intensity of the digits. The arch proximal patterns on the halluces are unusual. Similar patterns have been reported in three cases with deleted B-group chromosome (Warburton and Miller, 1967).

On clinical grounds the 4p- phenotype can be distinguished from that of the cri-du-chat syndrome but neither can be readily diagnosed in isolation from numerous other cases of mental retardation (with multiple congenital anomalies) without the aid of chromosomal analysis.

ACKNOWLEDGEMENTS

We wish to thank the Mental Health Authority for permission to publish this paper. We also thank Dr. Saul Wiener for chromosomal analysis and Miss Wendy Milligan for performing the banding techniques.

## REFERENCES

Antich, J., Ribas-Mundo, M., Prats, J. and Roca, M. (1968) Cri-du-chat with chromosomal mosaicism. *Lancet* **1**, 58.

Arias, D. (1970) Human chromosome deletion. Two patients with 4p- syndrome. *J. Ped.* **76**, 82.

Carter, R., Baker, E. and Hayman, D. (1969) Congenital malformations associated with a ring 4 chromosome. *J. med. Genet.* **6**, 224.

Flynn, H., Carmichael, A. and Sutherland, G. R. (1971) Six cases of the cri-du-chat syndrome. *Aust. J. Ment. Retard.* **1**, 239.

Georgi, P. L., Ceccarelli, M. and Paci, A. (1965) Su un case del sindrome del cri-du-chat con peculiari anomalie genotipiche. *Minerva pediat.* **17**, 1972.

Hirschorn, K., Cooper, H. L. and Firschein, I. L. (1965) Deletion of short arms of chromosome 4-5 in a child with defects of midline fusion. *Humangenet.* **1**, 479.

Kiossoglou, K. A., Mitus, W. J. and Dameshek, W. (1964) A direct method for chromosome studies of human bone marrow. *Amer. J. Clin. Path.* **41**, 183.

LEAO, J. C., NEU, R. and GARDNER, L. I. (1966) Hypospadias and other anomalies associated with partial deletion of short arms of chromosomes number 4. *Lancet* **1**, 493.

MILLER, O., BREG, W. R., WARBURTON, D., MILLER, D. A., DE CAPOA, A. and CHUTORIAN, A. M. (1966) Deleted late-replicating chromosome 4-5. *Lancet* **2**, 105.

MILLER, O. J., BREG, W. R. and WARBURTON, D. (1970) Partial deletion of the short arm chromosome No. 4 (4p-). Clinical studies in five unrelated patients. *J. Paediat.* **77**, 792.

MOORHEAD, P. S., NOWELL, P. C., MELLMAN, W. J., BATTIPS, D. M. and HUNGERFORD, D. A. (1960) Chromosome preparations of leukocytes cultures from human peripheral blood. *Exp. Cell Res.* **20**, 613.

PASSARGE, E., ALTROOGE, H. C. and RUDIGER, R. A. (1970) Human chromosomal deficiency: The 4p- syndrome. *Humangenet.* **10**, 51.

PFEIFFER, R. A. (1968) Neue Dokumentation zur abrenzung eines syndroms der deletion des Kurzen Arms eines Chromosoms Nr. 4. *Zschr. f. Kinderheilk.* **102**, 49.

PURVIS-SMITH, S. G. and MENSER, M. A. (1968) Dermatoglyphics in adults with congenital rubella. *Lancet* **11**, 141.

SCHNEDL, W. (1971) Banding patterns of human chromosomes. *Nature. New Biol.* **233**, 93.

SIDBURY, J. B. Jnr., SCHMICKEL, R. D. and GRAY, M. 1964 Findings of a patient with apparent deletion of the short arms of one of the B group chromosomes. *J. Ped.* **65**, 1098.

TAYLOR, A. I., CHALLACOMBE, D. N. and HOWLETT, R. M. (1970) Short arm deletion chromosome 4: A syndrome? *Ann. Hum. Genet.* **34**, 137.

VAN KEMPEN, C. and JONGBLOET, P. H. (1968) Partial deletion of short arm of chromosome 4. Wolf's syndrome. *Maandschrift voor Kindergeneskunde* **35**, 252.

WARBURTON, D. and MILLER, O. J. (1967) Dermatoglyphic features of patients with a partial short arm deletion of a B-group chromosome. *Ann. Hum. Genet., Lond.* **31**, 189.

WILSON, M. G., TOWNER, J. W. and NEGUS, L. D. (1970) Wolf-Hirschorn syndrome associated with an unusual abnormality of chromosome No. 4. *J. med Gen.* **7**, 164.

WOLF, U., PORSCH, R., BAITSCH, H. and REINWEIN, H. (1965) Deletion of short arms of a B-chromosome without "cri-du-chat" syndrome. *J. med. Gen.* **1**, 769.

Hum. Genet. 32, 133—140 (1976)


# Partial and Complete Trisomy 9: Delineation of a Trisomy 9 Syndrome

Grant R. Sutherland[1], Rodney F. Carter[1], and Lloyd L. Morris[2]

[1] Cytogenetics Unit, Department of Histopathology, and [2] Department of Radiology, The Adelaide Children's Hospital (Inc.), North Adelaide, Australia

Received January 15, 1976

*Summary.* Two infants with trisomy involving chromosome 9 are described. One had complete trisomy 9 and the other karyotype 47,XX,+der(9),t(7;9)(p22;q32)mat. A trisomy 9 syndrome is delineated, consisting of features of the trisomy 9p syndrome and various other malformations. These include abnormalities of the cardiovascular and urogenital systems, cranial suture anomalies, dislocation of the hips and knees and early death. A possible relationship of some of these findings to regions of 9q involved in cases of partial trisomy 9 is suggested.

## Introduction

Trisomy of the short arm of chromosome 9 results in a defined clinical syndrome (Rethoré et al., 1970, 1973; Rethoré and Lafourcade, 1974). However, the clinical syndrome associated with trisomy of the whole of chromosome 9 has not been fully characterised, indeed only 5 cases of complete (Juberg et al., 1970; Feingold and Atkins, 1973) or mosaic (Haslam et al., 1973; Bowen et al., 1974; Schinzel et al., 1975) trisomy 9 have been reported. The present paper records 2 similar cases, one with complete trisomy 9 and the other with trisomy of much of this chromosome resulting from 3:1 meiotic disjunction of a maternal translocation t(7;9)(p22;q32). As in the previously reported cases these 2 infants had features of the trisomy 9p syndrome and a variety of other malformations. An analysis of recorded cases of complete and partial trisomy 9 shows a pattern of malformations occurring in addition to those of the trisomy 9p syndrome as the long arm of the chromosome is involved in increasing amount, until finally a trisomy 9 syndrome can be delineated.

## Case Histories

### *Case 1*

The proposita was the first child born to a 28-year-old woman and her 33-year-old husband. The parents were of Greek origin. Delivery was normal with birth weight 1880 g and Apgar scores of 7 at 1 min and 8 at 4 min. Gestation immediately prior to delivery was estimated radiologically to be 35 weeks but was 40 weeks by dates. The infant was small (length 38 cm) and microcephalic (occipito-frontal circumference 30 cm) with many congenital malformations (Fig. 1). These included low hair-line, low-set ears, marked micrognathia and hypertelorism with microphthalmos and pinpoint pupils. The mouth was large and down-turned at the corners. There was a widely open sagittal suture with a large posterior fontanelle and the metopic suture was palpable. The sternum was short and prominent, there was a sacral dimple with

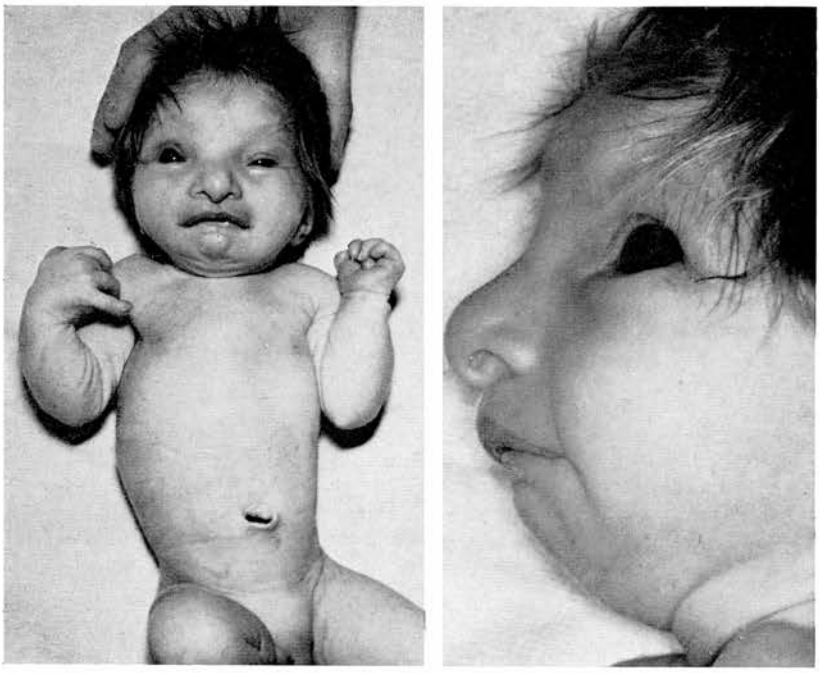


Fig. 1. The proposita, Case 1

associated tuft of hair, the anus was small. There were multiple limb abnormalities which included flexion deformities of the arms and hands, bilateral simian creases, absence of the terminal phalanges of the thumb and index fingers and hyperconvex nails. There was fixed dislocation of the hips, unstable dislocated knees and talipes calcaneovalgus. The grasp reflex could not be demonstrated but the Moro reflex was present.

Death from bronchopneumonia occurred at the age of 17 days. Autopsy findings included a persistent left superior vena cava with absence of the left innominate vein, and a widely patent ductus arteriosus. The right lung was bilobed. The ovaries were long (1.5 cm) and narrow but histologically normal. The kidneys were half normal size and the ureters were dilated and thin-walled. The caecum and ascending colon were abnormally suspended, being bound by a mesocolon to the underside of the liver. The cerebellar vermis was very hypoplastic and the fourth ventricle was correspondingly increased to about 4 times normal size.

*Radiological Findings.* Apart from confirming a number of the clinical findings several unusual radiographical features were present. These included bilateral hypoplasia of the pubic bones and an angulated appearance of the ischia. There was left dislocation of the head of the radius and hypoplasia of the distal humerus and the fibulae. There was hypoplasia of the ala of the sacrum with poorly developed sacro-iliac joints associated with lack of development of the iliac wings posteriorly.

*Cytogenetics.* The infant was found to have an extra chromosome which best fitted the C group, it showed a secondary constriction similar to that usually encountered in chromosome 9 and could be distinguished from the other C-group chromosomes. This chromosome was not fully identified before the child died. There was a single X-chromatin body in 25% of cells from a buccal mucosal smear, doubly chromatin positive nuclei not being seen. The father had a normal male karyotype but the mother carried an apparently balanced reciprocal translocation (Fig. 2), having a karyotype 46,XX,t(7;9)(p22;q32). The karyotype of the proposita was considered to be 47,XX,+der(9),t(7;9)(p22;q32)mat.

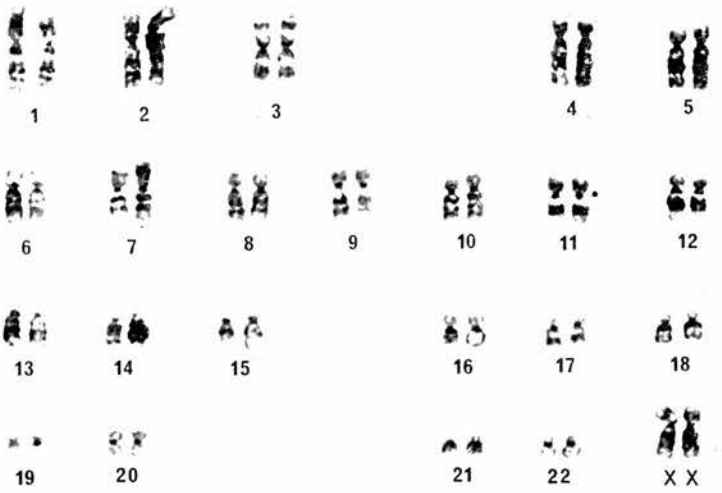



Fig. 2. Karyotype 46,XX,t(7;9)(p22;q32) from the mother of Case 1

*Case 2*

The propositus was the second child born to a 28-year-old woman and her 32-year-old husband. The parents were of Greek origin. The pregnancy was uneventful and ended in a breech delivery after 38 weeks' gestation. Birth weight was 1433 g, length 40 cm, head circumference 27.5 cm and the Apgar score was 7 at 1 min. There were multiple congenital malformations including hypertelorism, microcephaly with a large anterior fontanelle and widely open cranial sutures. The ears were low-set, there were epicanthic folds and micrognathia. The genitalia were ambiguous; the penis was small with hypospadias, there was a rudimentary divided scrotum and the testes were in the inguinal canal. A post-anal dimple was present. There were multiple limb abnormalities. The forearms were pronated and the hands showed ulnar deviation and flexion deformities with the index fingers tending to over-ride the middle fingers; the fifth fingers were clinodactylous. Simian creases were bilateral. There was talipes calcaneovalgus and bilateral dislocation of the hips, knees and elbows. Some of the toes on the left foot were hypoplastic. Moro and grasp reflexes were normal. The child failed to thrive and weighed only 2.3 kg when he died at the age of $3\frac{1}{2}$ months. The principle finding at autopsy was of a congenitally malformed heart. There was a persistent left superior vena cava, a widely patent ductus arteriosus, a large ventricular septal defect and a bicuspid pulmonary valve.

*Radiological Findings.* There were a number of unusual features detected radiographically. The pubic bones were absent on both sides and the iliac wings hypoplastic posteriorly. The fibulae were small and poorly developed and the distal humeri hypoplastic. The hands showed bilateral hypoplasia of the first metacarpals and absence of the distal phalanges of the thumbs and the mid-phalanges of the fifth fingers. The fifth segment of the sacrum was absent and there was hypoplasia of the inferior portion and ala resulting in poorly developed sacro-iliac joints.

*Cytogenetics.* An extra C-group chromosome was present in 58 out of 60 cells from leucocyte culture, the remaining 2 had apparently normal karyotypes by non-banded criteria so that the possibility of mosaicism cannot be excluded. The extra chromosome was identified as a number 9 using G-banding (Fig. 3). X-chromatin was not seen. The mother had a normal karyotype, the father was not available for study.

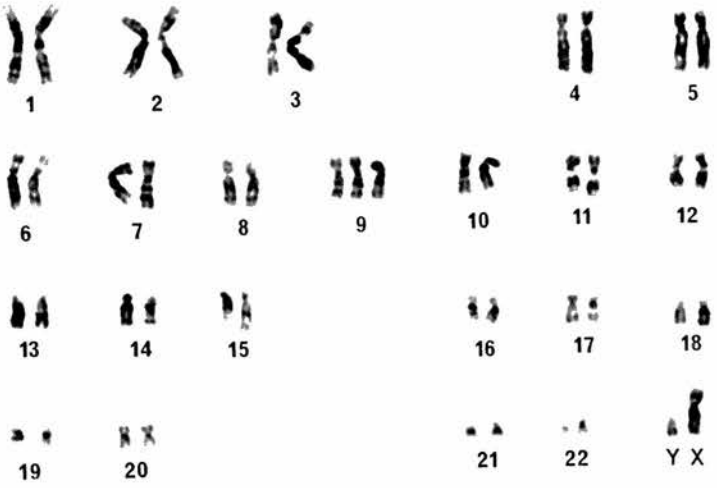



Fig. 3. Karyotype 47,XY,+9 from the propositus, Case 2

## Discussion

A number of cases involving trisomy for varying amounts of chromosome 9 have been recorded and a trisomy 9p syndrome has been delineated (Rethoré et al., 1970, 1973; Rethoré and Lafourcade, 1974). In most cases this results from a parental translocation involving chromosome 9. The trisomy arises either from 3:1 meiotic disjunction (Lindenbaum and Bobrow, 1975) in cases with 47 chromosomes (tertiary trisomy) or inheritance of an unbalanced form of the translocation in cases with 46 chromosomes. In tertiary trisomic cases there is also trisomy for the telomeric region of the other chromosome involved in the translocation. Whilst this additional imbalance cannot be ignored it probably has minimal phenotypic effect since such telomeric regions are most probably heterochromatic (Ford, 1973).

The clinical features of the trisomy 9p syndrome are shown in Table 1. Table 2 lists recorded cases of trisomy involving more of chromosome 9 than just the

Table 1. Principle clinical findings which constitute the trisomy 9p syndrome (modified from Rethoré et al., 1973)

| |
|---|
| Mental retardation |
| Craniofacial malformations<br>Microcephaly, prominent forehead, antimongoloid slant of the palpebral fissures, hypertelorism, enophthalmos, prominent fleshy nose, low-set abnormal ears |
| Skeletal anomalies<br>Hypoplastic phalanges, clinodactyly of the fifth finger, hypoplastic nails |
| Dermatoglyphic characteristics<br>Simian creases, absence or fusion of palmar triradii b and c |

Table 2. Clinical findings apart from those seen in trisomy 9p in cases with additional trisomy of 9q

| Case report | Trisomy | Urogenital anomalies | Micrognathia | Cranial suture anomalies | Age at death | Cardiac malformations | Dislocated knee(s) | Dislocated hips |
|---|---|---|---|---|---|---|---|---|
| Rethoré et al. (1973) Case 1 | 9pter → 9q11 | ± | 0 | 0 | alive 22 months | 0 | 0 | 0 |
| Rethoré et al. (1973) Case 2 | 9pter → 9q12 | 0 | 0 | 0 | alive 2 years | 0 | 0 | 0 |
| Fujita et al. (1974) | 9pter → 9q12 | ± | 0 | 0 | alive 11 years | 0 | 0 | 0 |
| Turleau et al. (1974) Case 1 | 9pter → 9q13 | 0 | 0 | 0 | alive 15 months | 0 | 0 | 0 |
| Penchaszadeh and Coco (1975) | 9pter → 9q13 | + | + | + | alive 8 months | 0 | 0 | 0 |
| Rethoré et al. (1974) | 9pter → 9q21 | + | + | + | alive 3 months | 0 | 0 | 0 |
| Mason et al. (1975) | 9pter → 9q21 | + | 0 | 0 | alive 23 years | 0 | 0 | 0 |
| Rott et al. (1971) | 9pter → 9q21 | + | + | + | 15 months | 0 | 0 | 0 |
| Schwanitz et al. (1974) | 9pter → 9q22 | + | 0 | + | 17 months | + | 0 | 0 |
| Centerwall et al. (1975) | 9pter → 9q22 | + | + | 0 | alive 6 months | ± | 0 | + |
| This Report, Case 1 | 9pter → 9q32 | + | + | + | 17 days | + | + | + |
| Schinzel et al. (1974) | mosaic inv(9) | ± | + | + | alive 2 months | 0 | 0 | 0 |
| Haslam et al. (1973) | mosaic 9 | + | + | 0 | 9 years | + | + | + |
| Bowen et al. (1974) | mosaic 9 | + | + | 0 | 42 days | + | + | + |
| Feingold and Atkins (1973) | 9 | + | + | 0 | 26 days | + | ± | 0 |
| Juberg et al. (1970) | C | + | + | 0 | 1 hour | 0 | + | + |
| This Report, Case 2 | 9 | + | + | + | 3½ months | + | + | + |

+ present, ± mild form, 0 absent or not stated to be present.

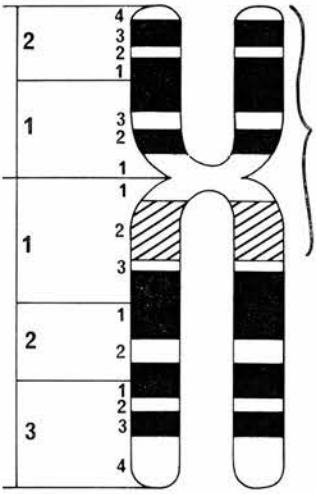



Fig. 4. Provisional relationship of clinical findings to chromosomal region as determined from cases of partial trisomy 9

short arm. Most of these cases have features of the trisomy 9p syndrome indeed 8 of the cases listed were considered to have this syndrome when they were reported. As the amount of chromosome 9 involved in the trisomy increases so additional clinical features become more constant. Addition of region 9q1 to the trisomy appears to have little phenotypic effect over and above the trisomy 9p syndrome. This is not surprising as most of this region is heterochromatic. Once region 9q2 is also added the following anomalies appear: micrognathia, abnormalities of the cranial sutures involving widely patent fontanelles, malformations of the urogenital and cardiovascular systems, and early death.

Once region 9q3 begins to be included then abnormalities of articulation of the hips and knees are found. Other abnormalities of articulation include dislocation of the head of the radius, seen in both cases in this report and one other (Feingold and Atkins, 1973). There were no apparent additional findings when the whole of chromosome 9 was involved. Mosaicism appeared to have little effect with the possible exception that life may be prolonged. Both cases described in this report had multiple bony abnormalities which were only detected radiologically. There was considerable similarity between the two cases and a more detailed radiological assessment will be published elsewhere.

Trisomy of part of the long arm of 9 without the associated trisomy 9p has been reported in 2 cases (Turleau et al., 1975). Clinically these did not closely resemble the cases summarised in Table 2. Prenatal diagnosis of trisomy 9 has been reported (Francke et al., 1975); examination of the foetus showed a number of malformations but detailed pathology was obscured by autolysis.

Delineation of a trisomy 9 syndrome can now be attempted. Over and above the findings in trisomy 9p (Table 1) there will be abnormalities of the urogenital and cardiovascular systems, micrognathia, abnormalities of the cranial sutures

with widely patent fontanelles, abnormalities of articulation, especially of the hips and knees, and death at an early age. In cases of partial trisomy 9 involving more than 9p then the clinical findings will depend on the amount of 9q present. A tentative outline of the possible relationship between clinical findings and the amount of extra chromosome is given in Figure 4. In view of the very wide clinical expression associated with autosomal trisomy, both the constitution of the trisomy 9 syndrome and the relationship of the various clinical findings to regions of the chromosome will almost certainly require modification as further cases are described.

*Acknowledgements.* We thank Drs. T. H. Beare and H. G. Rischbieth for allowing us to use the clinical records of their patients, Mrs. Trudy Hocking and Mrs. Erica Woollatt for technical assistance, and the Hospital's Department of Photography for photographic help.

## References

Bowen, P., Ying, K. L., Chung, G. S. H.: Trisomy 9 mosaicism in a newborn infant with multiple malformations. J. Pediat. **85**, 95—97 (1974)

Centerwall, W. R., Mayeski, C. A., Cha, C. C.: Trisomy 9q—. A variant of the 9p trisomy syndrome. Humangenetik **29**, 91—98 (1975)

Feingold, M., Atkins, L.: A case of trisomy 9. J. med. Genet. **10**, 184—187 (1973)

Ford, E. H. R.: Human chromosomes, p. 34. London-New York: Academic Press 1973

Francke, U., Benirschke, K., Jones, O. W.: Prenatal diagnosis of trisomy 9. Humangenetik **29**, 243—250 (1975)

Fujita, H., Abe, T., Yamamoto, K., Furuyama, J.: Possible complex translocation t(9;14;13)(q12;1?;q31) in a mother of a child with 9p trisomy syndrome. Humangenetik **25**, 83—92 (1974)

Haslam, R. H. A., Broske, S. P., Moore, C. M., Thomas, G. H., Neill, C. A.: Trisomy 9 mosaicism with multiple congenital anomalies. J. med. Genet. **10**, 180—183 (1973)

Juberg, R. C., Gilbert, E. F., Salisbury, R. S.: Trisomy C in an infant with polycystic kidneys and other malformations. J. Pediat. **76**, 598—603 (1970)

Lindenbaum, R. H., Bobrow, M.: Reciprocal translocations in man. 3:1 meiotic disjunction resulting in 47— or 45— chromosome offspring. J. med. Genet. **12**, 29—43 (1975)

Mason, M. K., Spencer, D. A., Rutter, A.: A case of partial (9p) trisomy in a family with a balanced translocation 46,XX,t(1p+9q—). J. med. Genet. **12**, 310—314 (1975)

Penchaszadeh, V. B., Coco, R.: Partial 9 trisomy by 3:1 segregation of balanced maternal translocation (7q+;9q—). J. med. Genet. **12**, 301—305 (1975)

Rethoré, M. O., Ferrand, J., Dutrillaux, B., Lejeune, J.: Trisomie 9p par t(4;9)(q34;q21)mat. Ann. Génét. **17**, 157—161 (1974)

Rethoré, M. O., Hoehn, H., Rott, H. D., Couturier, J., Dutrillaux, B., Lejeune, J.: Analyse de la trisomie 9p par dénaturation ménagée. A propos d'un nouveau cas. Humangenetik **18**, 129—138 (1973)

Rethoré, M. O., Lafourcade, J.: Trisomie du bras court du chromosome 9: Syndrome +9p. Journées parisiennes de pédiatrie, pp. 379—390. Paris: Flammarion 1974

Rethoré, M. O., Larget-Piet, L., Abonyi, D., Boeswillwald, M., Berger, R., Carpentier, S., Cruveiller, J., Dutrillaux, B., Lafourcade, J., Penneau, M., Lejeune, J.: Sur quatre cas de trisomie pour le bras court du chromosome 9. Individualisation d'une nouvelle entite morbide. Ann. Génét. **13**, 217—232 (1970)

Rott, H. D., Schwanitz, G., Grosse, K. P.: Partielle Trisomie C9 bei balancierter B4/C9-Translokation bei der Mutter. Z. Kinderheilk. **109**, 292—299 (1971)

Schinzel, A., Hayashi, K., Schmid, W.: Mosaic-trisomy and pericentric inversion of chromosome 9 in a malformed boy. Humangenetik **25**, 171—177 (1974)

Schwanitz, G., Schamberger, U., Rott, H. D., Wieczorek, V.: Partial trisomy 9 in the case of familial translocation 8/9mat. Ann. Génét. **17**, 163—166 (1974)

Turleau, C., de Grouchy, J., Chavin-Colin, F., Roubin, M., Brissand, P. E., Repessé, G., Safar, A., Borniche, P.: Partial trisomy 9q: A new syndrome. Humangenetik **29**, 233—241 (1975)
Turleau, C., de Grouchy, J., Chavin-Colin, F., Roubin, M., Langmaid, H.: Trisomie 9p: Deux nouvelles observations. Ann. Génét. **17**, 167—174 (1974)

Dr. G. R. Sutherland
Cytogenetics Unit
Department of Histopathology
The Adelaide Children's Hospital (Inc.)
72 King William Road
North Adelaide 5006
Australia

*Clinical Genetics* 1976: **10**: 54–59

# Familial pericentric inversion of chromosome 19,inv(19) (p13q13) with a note on genetic counselling of pericentric inversion carriers

GRANT R. SUTHERLAND, ANDREW J. GARDINER, AND RODNEY F. CARTER
The Adelaide Children's Hospital (Inc.), North Adelaide, South Australia

An inmate of a hospital for the mentally retarded was found during a cytogenetic screening programme to have karyotype 46,XY,inv(19)(p13q13)mat. Clinical, cytogenetic and family findings are presented and it is concluded that the chromosome abnormality was probably not the cause of the patient's retardation. The problem of genetic counselling of inversion carriers is examined in some detail and estimates of risk are given.

*Received 19 December 1975, revised 5 February, accepted for publication 11 February 1976*

Abnormalities of the F-group chromosomes are encountered infrequently in clinical cytogenetics. There are reports of translocations involving chromosomes of this group, but no studies on a pericentric inversion have been published. During the cytogenetic screening of the inmates of a hospital for the mentally retarded, a patient was found to have a pericentric inversion of chromosome 19, which was shown to be familial. Carriers of pericentric inversions are detected from time to time; the problems of counselling these individuals are discussed.

## Case History

The propositus was the first child born to a primiparous 32-year-old woman and her 42-year-old husband, both of Italian origin. After a normal vaginal delivery at term the infant was cyanosed with an Apgar score of 5. Birth weight was 3.05 kg. Several cyanotic episodes occurred in the neonatal period and head circumference increased from 33.5 to 35.5 cm in the first 7 days of life, but subdural haematoma was excluded. Development progressed slowly with many seizures, up to 8–10 per day, which were very difficult to control, and with episodes of aggressive behaviour which caused problems in management. At 5.3 years of age his score on the Vineland Social Maturity Scale was 2.9 years. At 7 years his air encephalogram showed dilation of the left cerebral ventricle consistent with diffuse atrophic changes in the left hemisphere.

On examination at age 16 the propositus was found to be a severely retarded microcephalic (head circumference 52 cm) youth who showed no other external malformations. He had a prominent mandible and large hands and feet. He had a left divergent strabismus, held his mouth open with constant drooling and showed gingival hypertrophy. He was still having frequent grand mal epileptic seizures and outbursts of ag-

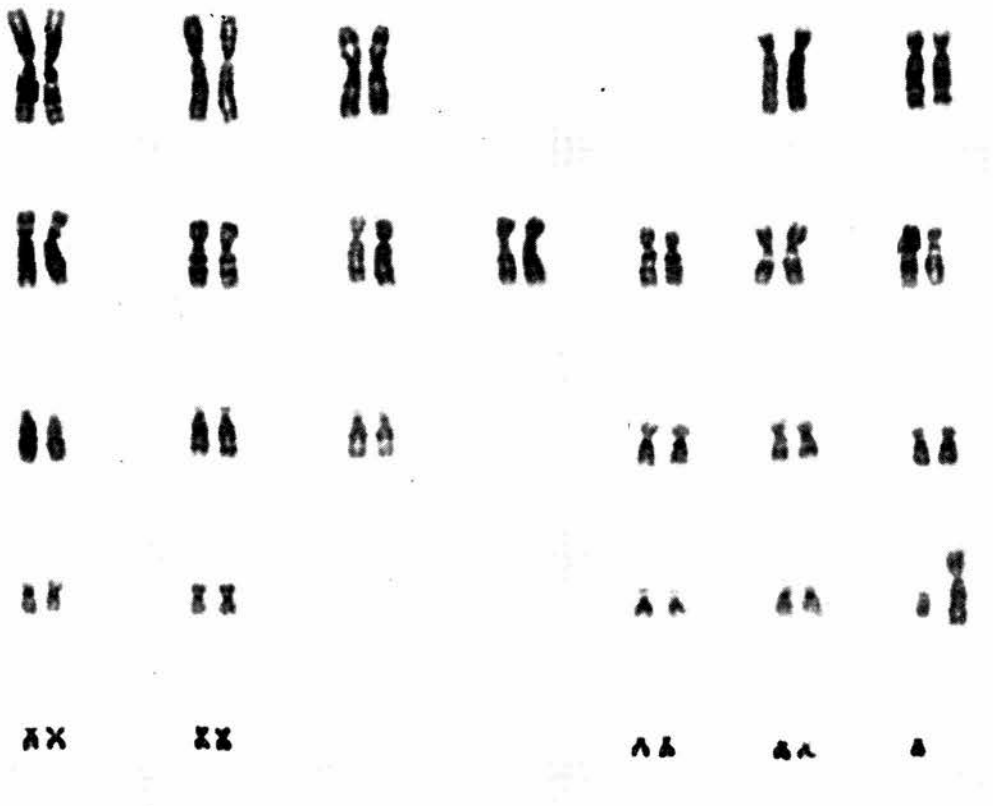


**Fig. 1.** G-banded karyotype 46,XY,inv(19)(p13q13) of the propositus. Unbanded F- and G-group chromosomes from another cell are also shown.

gressive behaviour. Chest, heart, abdomen and external genitalia were all clinically normal. He was 155 cm tall and weighed 60 kg.

### Cytogenetic Studies

Analysis of G-banded chromosomes from lymphocyte culture showed that the propositus had a pericentric inversion of chromosome 19 (Fig. 1). The banding pattern of chromosome 19 is such that the location of break points is difficult to determine, but since the 12 bands in the propositus were not separated by the inversion, his karyotype was interpreted as 46,XY,inv(19)(p13q13). Family studies showed the presence of an identical inversion chromosome in the mother and both normal siblings of the propositus (Fig. 2).

### Discussion

Pericentric inversions of chromosome 19 have been recorded by Jacobs et al. (1974) in a male with delayed onset of puberty whose father was an inversion heterozygote, and by Hamerton et al. (1975) in a child

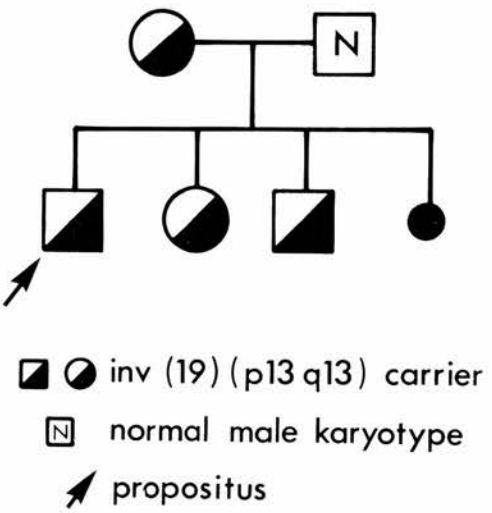



**Fig. 2.** The family pedigree.

with Down's syndrome whose mother carried the inversion. There is no evidence that these inversions are other than chance findings. In the present case the birth history, lack of obvious congenital malformation, and similarity of the inversion chromosome in unaffected individuals, suggest that the chromosome abnormality is not associated with the mental retardation of the propositus.

When phenotypically normal individuals are identified as inversion heterozygotes a problem arises as far as genetic counselling is concerned. Translocations ascertained via balanced carriers have not been reported to produce unbalanced forms (Jacobs et al. 1970). Similarly, pericentric inversions ascertained via inversion heterozygotes have not produced recombinant forms; hence some reassurance can be given to inversion carriers so ascertained. There is also evidence that small inversions where both break points are close to the centromere and involve mainly centric heterochromatin, as in chromosomes No. 9 (Madan & Bobrow 1974), No. 3 (Soudek et al. 1974) and No. 10 (de la Chapelle et al. 1974) can be regarded as variants without pathological significance.

When a pericentric inversion is known to have produced a recombinant form the situation becomes more difficult. It is not possible to define accurately the risk for the production and survival of recombinant gametes; it will depend on the size of the inverted segment and the frequency of chiasmata. It should be noted than an uneven number of chiasmata between two chromatids in an inversion loop will always produce two unbalanced gametes with complementary recombinant chromosomes which should be cytologically recognisable. One recombinant chromosome will be smaller than the non-recombinant chromosomes and the other larger, provided that the original inversion produced an alteration in arm ratio. Warter et al. (1973) recorded a family in which a male with an inv(5) had offspring with both recombinant forms.

Families in which pericentric inversions segregate and which have been ascertained via a recombinant form are shown in Table 1. The inversions involved are different, and grouping them together is probably invalid. However, these data are all that are available on which to base estimates of recurrence risks. In assembling this table the progeny of inferred carriers of unknown sex were not included as this would lead to an excess of inversion heterozygotes (Hamerton 1970). Unkaryotyped individuals with a normal phenotype or those in whom there was no suggestion of chromosomal pathology were listed separately ("normals"), as were others who could be inferred to be recombinants (this did not include abortions). If the data in Table 1 are corrected for single ascertainment (by elimination of all probands), then the recurrence risk for recombinants is 2/59 (3.4 %); and if all the inferred recombinants are included, the figures become 8/77 (10.4 %). In either in-

**Table 1**

Segregation patterns of pericentric inversions ascertained via a recombinant form

| Authors | Inverted chromosome | Total karyotyped segregants | Normal karyotyped segregants | Inversion heterozygote segregants | Karyotyped recombinants | "Normal" segregants not karyotyped | Inferred recombinants not karyotyped | Sex of parent of karyotyped recombinants† |
|---|---|---|---|---|---|---|---|---|
| Ferguson-Smith (1967) | C (10) | 3 | 1 | 1 | 1 | 2 | 3 | F |
| Wilson et al. (1970) | 4 | 8 | 2 | 5 | 1 | 2 | – | F |
| Parrington & Edwards (1971) | 13* | 3 | 2 | – | 1 | – | 1 | F |
| Hauksdóttir et al. (1972) | 13 | 26 | 13 | 11 | 2 | 6 | 1 | F, F |
| Surana & Conen (1972) | D | 3 | 1 | 1 | 1 | – | – | M |
| Dutrillaux et al. (1973) | 10 | 2 | – | 1 | 1 | – | – | F |
| Taysi et al. (1973) | 13 | 5 | 1 | 3 | 1 | – | – | F |
| Warter et al. (1973) | 5 | 3 | 1 | – | 2 | – | – | M |
| Escobar et al. (1974) | 13 | 2 | – | 1 | 1 | – | – | F |
| Jacobs et al (1974)** | 18 | 4 | 3 | – | 1 | – | – | F |
| Fujimoto et al. (1975) | 8 | 11 | 5 | 5 | 1 | 2 | 1 | F |
| Total | | 70 | 29 | 28 | 13 | 12 | 6 | 2 M, 10 F |

** Also Frackiewicz (1975) pers. comm.
* McDermott & Parrington (1975).
† F – female; M – male.

stance these figures will be minimal, as ascertainment may not be complete.

If the sex of the inversion heterozygote who produced the recombinant is considered (Table 1), then of 12 heterozygotes, 10 were females and 2 were males. Such ascertainment bias is also noted when t(DqGq) heterozygotes are identified by their offspring with Down's syndrome; however, this reflects the increased risk that such female heterozgotes will produce unbalanced forms (Hamerton 1970). By analogy, female inversion heterozygotes may be more likely to have recombinant offspring. In addition, if the higher genetic recombination fractions in females (e.g. Weitkamp et al. 1973) are a reflection of increased chiasma frequency, and if this holds for inverted chromosomes, then the probability of recombinants from female inversion heterozygotes would be greater than from males.

Apart from the production of recombinants, there is the possibility that an inverted chromosome can interfere with normal meiotic disjunction of other chromosomes (Leonard et al. 1975). These authors reviewed the occurrence of aneuploidy associated with pericentric inversions and found three cases of Turner's syndrome associated with an inv(2), and five autosomal trisomies associated with other inverted chromosomes. In view of the frequency of about 1 in 7,500 (Nielsen & Sillesen 1975) of autosomal inversions, the few associations with aneu-

ploidy may just be chance. Nondisjunction of recombinant chromosomes leading to partial trisomies is another source of abnormal offspring. Gray et al. (1962) recorded a woman with a pericentric inversion of chromosome 21 who produced a child with Down's syndrome in whom both recombinant chromosomes from an inversion loop cross-over were present; Jacobs et al. (1974) detected an infant with karyotype 47,XX, inv(18),+rec(18) whose mother was an inversion heterozygote.

## Conclusion

The risks for the production of abnormal offspring by inversion heterozygotes were derived by pooling heterogeneous data and must be regarded as unreliable. Nevertheless at the present time they provide the only available guide in a difficult area. For the practical purposes of genetic counselling, risks of 5 % for male and 10 % for female inversion heterozygotes to produce recombinants, when a previous recombinant is known within the family, should be accurate enough for patients to arrive at informed decisions. In a family in which an inversion has segregated without producing recombinants a nominal risk of about 1 % could be offered as guidance. These risk figures would include any risk for the production of aneuploids as well as recombinants. Either circumstance would be adequate indication for prenatal chromosome analysis.

## Acknowledgements

We thank Mrs. Trudy Hocking and Mrs. Erica Woollatt for technical assistance; the Director of Mental Health Services in South Australia, Dr. W. Dibden, for allowing us access to the patient; and Dr. D. L. Hayman for helpful discussions during the preparation of this report.

## References

de la Chapelle, A., J. Schröder, K. Stenstrand, J. Fellman, R. Herva, M. Saarni, I. Anttolainen, I. Tallila, L. Tervilä, L. Husa, G. Tallqvist, E. B. Robson, P. J. L. Cook & R. Sanger (1974). Pericentric inversions of human chromosomes 9 and 10. *Amer. J. hum. Genet.* **26**, 746–766.

Dutrillaux, B., C. Laurent, J. M. Robert & J. Lejeune (1973). Inversion péricentrique, inv (10), chez la mère et aneusomie de recombinaison, inv(10), rec(10) chez son fils. *Cytogenet. Cell Genet.* **12**, 245–253.

Escobar, J. I., O. Sanchez & J. J. Yunis (1974). Trisomy for the distal segment of chromosome 13. *Amer. J. Dis. Child.* **128**, 217–220.

Ferguson-Smith, M. A. (1967). Clinical Cytogenetics. *Proceedings of the 3rd International Congress of Human Genetics, Chicago 1966*, ed. Crow, J. F. & J. V. Neel. Baltimore, Johns Hopkins Press, pp. 69–75.

Fujimoto, A., M. G. Wilson & J. W. Towner (1975). Familial inversion of chromosome No. 8. An affected child and a carrier fetus. *Hum. Genet.* **27**, 67–73.

Gray, J. E., D. E. Mutton & D. W. Ashby (1962). Pericentric inversion of chromosome 21. A possible further cytogenetic mechanism in mongolism. *Lancet* **i**, 21–23.

Hamerton, J. L. (1970). Robertsonian translocations: Evidence on segregation from family studies. *Human Population Cytogenetics*, ed. Jacobs, P. A., W. H. Price & P. Law. Edinburgh, Edinburgh University Press, pp. 64–80.

Hamerton, J. L., N. Canning, M. Ray & S. Smith (1975). A cytogenetic survey of 14,069 newborn infants. I. Incidence of chromosome abnormalities. *Clin. Genet.* **8**, 223–243.

Hauksdóttir, H., S. Halldórsson, O. Jensson, M. Mikkelsen & A. McDermott (1972). Pericentric inversion of chromosome No. 13 in a large family leading to duplication deficiency causing congenital malformations in three individuals. *J. med. Genet.* **9**, 413–421.

Jacobs, P. A., J. Aitken, A. Frackiewicz, P. Law, M. S. Newton & P. G. Smith (1970). The inheritance of translocations in man: Data from families ascertained through a balanced heterozygote. *Ann. hum. Genet.* **34**, 119–131.

Jacobs, P. A., K. E. Buckton, C. Cunningham & M. Newton (1974). An analysis of the break points of structural rearrangements in man. *J. med. Genet.* **11**, 50–64.

Leonard, C., P. Hazael-Massieux, L. Bocquet, L. Larget-Piet & J. Boué (1975). Inversion péricentrique inv(2)(p11q13) dans des familles non apparentées. *Hum. Genet.* **28**, 121–128.

Madan, K. & M. Bobrow (1974). Structural variation in chromosome No. 9. *Ann. Génét.* **17**, 81–86.

McDermott, A. & J. M. Parrington (1975). Elucidation of a pericentric inversion of a D-group chromosome in the mother of a child with Patau's Syndrome. *Ann. hum. Genet.* **38**, 305–307.

Nielsen, J. & I. Sillesen (1975). Incidence of chromosome aberrations among 11,148 newborn children. *Hum. Genet.* **30**, 1–12.

Parrington, J. M. & J. H. Edwards (1971). Patau's Syndrome with $D_1$ duplication-deficiency derived from a maternal D-group pericentric inversion. *Ann. hum. Genet.* **35**, 35–45.

Soudek, D., S. O'Shaughnessy, P. Laraya & B. D. McCreary (1974). Pericentric inversion of "fluorescent" segment in chromosome No. 3. *Hum. Genet.* **22**, 343–346.

Surana, R. B. & P. E. Conen (1972). Inherited pericentric inversion of a group D (13–15) chromosome. *J. med. Genet.* **9**, 105–110.

Taysi, K., M. Bobrow, S. Balci, K. Madan, M. Atasu & B. Say (1973). Duplication/deficiency product of a pericentric inversion in man: A cause of $D_1$ trisomy syndrome. *J. Pediat.* **82**, 263–268.

Warter, S., J.-V. Ruch & M. Lehmann (1973). Karyotype with chromosomal abnormality with various inherited defects in the offspring (recombination aneusomy). *Hum. Genet.* **20**, 355–359.

Weitkamp, L. R., J. J. Van Rood, E. Thorsby, W. Bias, M. Fotino, S. D. Lawler, J. Dausset, W. R. Mayr, J. Bodmer, F. E. Ward, J. Seignalet, R. Payne, F. Kissmeyer-Nielsen, R. A. Gatti, J. A. Sachs & L. U. Lamm (1973). The relation of parental sex and age to recombination in the HL-A system. *Hum. Hered.* **23**, 197–201.

Wilson, M. G., J. W. Towner, G. S. Coffin & I. Forsman (1970). Inherited pericentric inversion of chromosome No. 4. *Amer. J. hum. Genet.* **22**, 679–690.

Address:
*Grant R. Sutherland, Ph.D.*
*Cytogenetics Unit*
*Department of Histopathology*
*The Adelaide Children's Hospital (Inc.)*
*72 King William Road*
*North Adelaide 5006*
*Australia*

# 46,XX/46,XX,r(2) (p25 q37) MOSAICISM : CLINICAL AND CYTOGENETIC STUDIES

G.R. SUTHERLAND, R.F. CARTER

# 46,XX/46,XX,r (2) (p25 q37) MOSAICISM : CLINICAL AND CYTOGENETIC STUDIES

G.R. SUTHERLAND, R.F. CARTER.

*Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital Inc., North Adelaide, South Australia 5006 (Australia).*

Whilst ring chromosomes have been reported for the sex chromosomes and most of the autosomes, a ring chromosome 2 has not been described. This paper reports the first case of 46,XX/46,XX,r(2) mosaicism which was found in a severely mentally retarded and physically handicapped girl. Observations on the behaviour of the ring chromosome are recorded.

## CASE REPORT

The proposita (fig. 1) was the second child born to 42-year-old parents. The first child born three years previously was well. The mother had Addison disease (adrenal insufficiency) for which she had been under treatment for some years prior to and during her pregnancy. Delivery was by elective lower segment caesarean section at 39 weeks gestation because of cephalopelvic disproportion and maternal Addison disease. Apgar score was 8 and there were no resuscitation problems. Microcephaly (head circumference 29cm) and multiple anomalies were noted at birth. Birth weight was 2.2 kg. There were feeding difficulties and the child was kept in hospital until five weeks of age. Developmental milestones were grossly delayed. She could sit at 18 months but never learned to walk and can only say one or two words. At seven years she was functioning at the level of 6 — 8 months. Examination at the age of seven years showed a small (height 106 cm, span 97cm) obese (26.7 kg) profoundly retarded girl. Her head was small (circumference 43 cm) and unusually shaped having a very flattened occiput. She had an old facial appearance with bilateral epicanthi, flattened nose and absent ear lobes. Skull X-ray confirmed the gross microcephaly and showed fusion of the coronal and sagittal sutures; there was no intracranial calcification or other specific feature noted. She could see and hear, ear canals and eyes appeared normal. There was a high arched palate. The neck was short and webbed. There was no breast tissue and the chest appeared shield-like with widely spaced nipples. There was a blind sacro-coccygeal sinus; anus and external genitalia were normal. The fat distribution was unusual with buffalo hump and marked truncal obesity but small forearms, hands and feet. Multiple abnormalities of the limbs and their articulation were present. The forearms could not be fully pronated, there were flexion deformities of the elbows and shoulders. The wrists were hypermobile and appeared to readily subluxate, the thumbs were incurving and there was a right simian crease. Radiography showed that the fourth metacarpals were abnormally short. A congenital dislocation of the left hip had been treated satisfactorily at six months of age but the left leg was held in external rotation and there was decreased abduction of the right hip.

SUTHERLAND G.R., CARTER R.F. — 46,XX/46,XX,r(2) (p25q37) mosaicism : clinical and cytogenetic studies. *Ann. Génét.*, 1978, *21*, n° 3, 164-167.

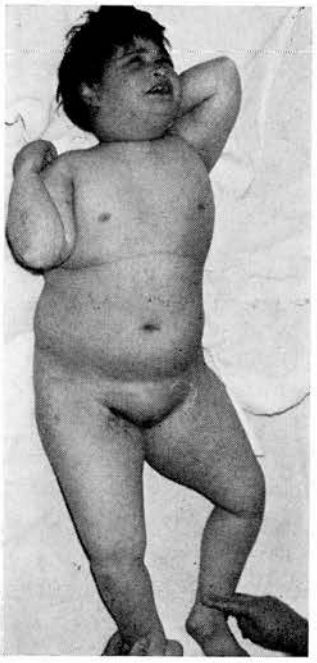


Fig. 1. — The proposita aged seven years.

### Cytogenetics

Cytogenetic studies on blood lymphocytes had been carried out in this Department on two occasions shortly after birth of the proposita. The first when she was six days of age and again at seven weeks of age. Unfortunately the preparations had deteriorated and were unsuitable for re-examination. However results obtained from 100 metaphases scored on each occasion at that time indicated that 5 and 11 percent respectively of the cells were tetraploids containing rings and that of the diploid cells about half had a normal female karyotype and half contained a monocentric ring in place of a normal chromosome number two.

Chromosome studies on blood lymphocytes were repeated in 1977 when the child was seven years of age and these results are show in table I and figs. 2 and 3. 193 consecutive metaphases were examined of which 46 were tetraploid (tetraploidy = 22 per cent) and a further 16 tetraploid cells were scored. These results strongly suggest that the frequency of tetraploid cells has increased and the frequency of normal diploid cells has decreased since chromosome studies were carried out in the neonatal period. Of the diploid cells only 14 percent appeared to have a normal female karyotype.

The great majority of cells showed a monocentric ring chromosome identified by banding to be a number 2 with break points at p25 and q37 (fig. 2). This chromosome seemed to be remarkably stable in diploid cells ; the only evidence of instability was in three of these cells in which the ring had apparently formed a rod-shaped chromosome equivalent in size and morphology to chromosome 1, in two cells in which the ring chromosome had been lost, in one cell in which there were two rings and in one cell in which the size of the ring had altered significantly and was replaced by a small ring. In the diploid cells there was no evidence of rings with more than one centromere and no open rings or pulverised rings were seen. There was much more evidence of ring instability in the tetraploid cells (fig. 3). Of the 62 cells examined 32 showed a dicentric ring and three cells showed two monocentric rings equivalent in size and morphology to the monocentric rings seen in the diploid cells. Instability was evidenced by open rings and the formation of large dicentrics presumably due to breaking of the ring, however these dicentrics appeared as rod-shaped chromosomes. A number of the tetraploid cells showed partial pulverisation of the chromosomes, however in no case was a tetraploid cell seen in which only the ring was pulverised. One octoploid cell was seen in which there were apparently interlocking dicentric rings (fig. 3 f).

TABLE I

CYTOGENETIC STUDIES CARRIED OUT AT AGE 7 YEARS

Diploid Cells

| Normal | −2 | $R_1$ | $R_1R_1$ | Rod * * | Ro | Total |
|---|---|---|---|---|---|---|
| 21 | 2 | 119 | 1 | 3 | 1 | 147 |

Tetraploid Cells

| $R_2$ | $R_2R_2$ | $R_1R_1$ | Dicentric | Pulverised | $R_2$ open | $R_2$ open +Ro | $R_2R_2$ both open | $R_1R_1$ open | No Rings | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| 32 | 3 * | 3 | 7 | 5 | 5 | 2 | 1 | 3 | 1 * * | 62 |

Ro = small monocentric ring. * = interlocking in one cell. * * = abnormal rod chromosomes.
$R_1$ = monocentric ring. $R_2$ = dicentric ring.

## DISCUSSION

Formation of the r(2) chromosome has resulted in very little loss of chromosomal material and is present in mosaic form with normal cells, and yet the proposita is severely physically and mentally handicapped. It is possible that this degree of maldevelopment is less related to deletion of genetic material than to some disturbance in the regulation of cell division since the instability of the ring appears to be manifesting itself by the induction of tetraploidy. Hoo *et al.* (1974) suggested that the clinical stigmata in a child with a r(13) chromosome could have been due to the subsequent behaviour of the ring chromosome rather than the deletion *per se* which was minimal. Furthermore it has been

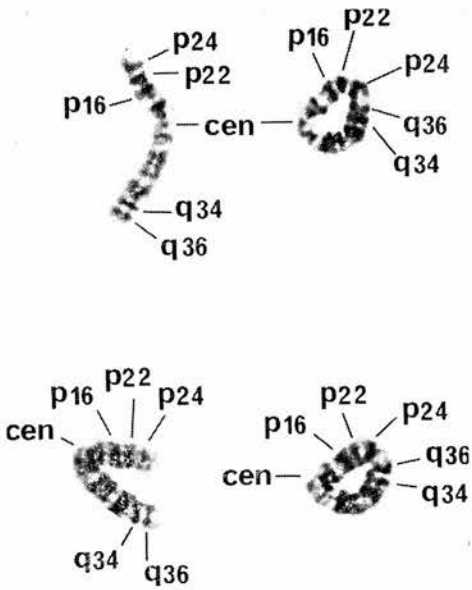



Fig. 2. — G. banded chromosomes 2 from two diploid metaphases showing break points at p25 and q37.

pointed out (Zdansky *et al.*, 1975) that the phenotypic consequences of ring chromosomes are variable and that there are no well defined clinical syndromes associated with ring autosomes.

Few reports of ring chromosomes mention tetraploid cells and when referred to these are usually present only at the expected frequency of 1-2 per cent for lymphocyte cultures. Bobrow *et al.* (1973) found about 10 per cent tetraploid cells in lymphocyte metaphases from a patient with a r(1) chromosome. The only dicentric rings observed in that patient were in the tetraploid cells, the ring being fairly stable in the diploid cells in that less than 5 per cent of diploid cells showed other than a normal sized monocentric ring. The behaviour of this r(1) chromosome was similar to that of the r(2) in the present case. In a patient with a r(4) previously described from this Department (Carter *et al.*, 1969) the frequency of tetraploidy was 5 per cent Hecht (1969) recorded 4-5 per cent tetraploidy in another child with a r(4). The model of ring chromosome behaviour put forward by Pathak and Sinha (1972) does not account for the production of tetraploidy which would appear to be a consequence of dicentric ring formation in some cases, particularly when the ring chromosome is a large chromosome. However, Kirstemacher and Punnett (1970) concluded from an extensive literature survey that the stability of ring chromosomes in Man was unrelated to their size. The mechanism by which tetraploid formation occurs is unclear but Hoo *et al.* (1974) suggested that the formation of tetracentric rings led to the formation of tetraploid cells by disturbing anaphase in a case with a r(13).

Most reports of mosaicism involving ring chromosomes have not recorded changes in the frequency of the various cell types. Packer *et al.* (1974) reported a child with 46,XX/46,XX,r(4) mosaicism in which the proportion of cells with the ring rose from 9 p. cent to 72 p. cent over a period of seven years. Hecht (1969) reported an increase in frequency of cells with a ring chromosome from 54 p. cent to 95 p. cent in a child with 45,XY,-4/46, XY,r(4) mosaicism over a four year period. In this latter case the changes in proportion of the cell types is unexpected since at conception this child was probably 46,XY,r(4) with subsequent loss of the ring from some cells. However, it would appear that cells containing a ring are at a selective advantage *in vivo* in some cases.

ACKNOWLEDGEMENTS

Dr. J.F. Smallhorn conducted the detailed physical examination of the proposita, Dr. D.L. Hayman provided constructive criticism of the manuscript.

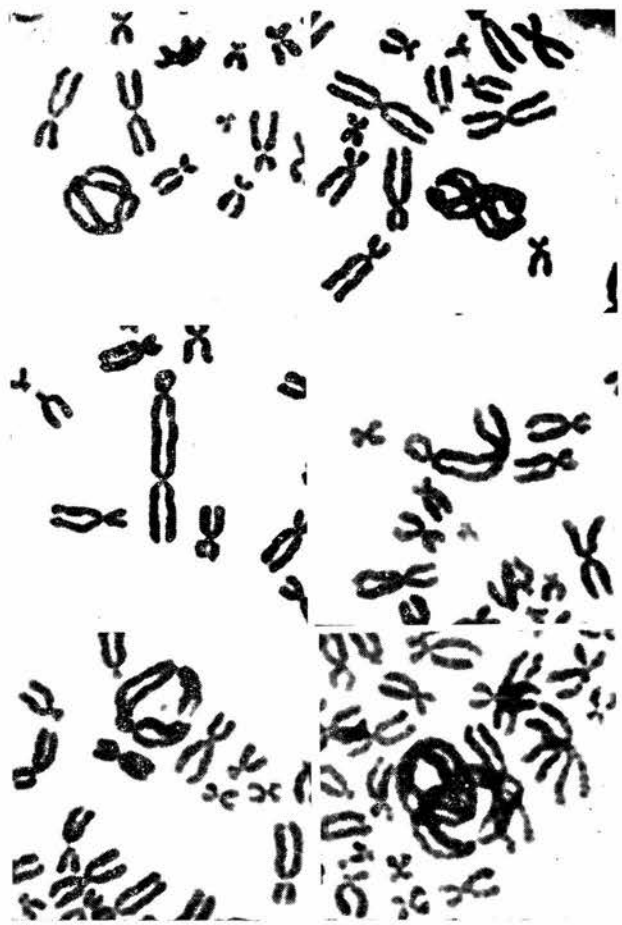


Fig. 3. — Appearance of the ring chromosome and its derivatives in polyploid cells. (a) and (b) dicentric rings in tetraploid cells, (c) and (d) large dicentrics showing sister chromatird reunion in tetraploid cells, (e) open dicentric ring in a tetraploid cell, (f) interlocking dicentric rings in an octoploid cell.

SUMMARY. — A severely mentally retarded and physically handicapped girl is described who has 46,XX/46,XX.r(2)(p25q37) mosaicism. This is the first ring 2 chromosome to be described in Man. Studies of the behaviour of the ring showed that it was stable in diploid cells which had increased in frequency over a period of seven years, but unstable in tetraploid cells which were at a much higher frequency than in normal individuals. It is concluded that in some cases the phenotypic consequences of ring chromosome formation may be due more to their disturbing the regulation of cell division than to the loss of genetic material. Current models of ring chromosome behaviour do not account for the induction of tetraploidy.

KEY-WORD : Ring chromosome 2.

MOSAÏQUE 46,XX/46,XX,r(2)(p25q37) : ÉTUDES CLINIQUE ET CYTOGÉNÉTIQUE

RÉSUMÉ. — Une malade sévèrement encéphalopathe et porteuse d'une mosaïque : 46,XX/46,XX,r(2)(p25q37) est décrite. Cette observation est la première d'un chromosome 2 en anneau chez l'homme. Celui-ci était stable dans les cellules diploïdes et la fréquence s'était accrue pendant une période de 7 ans, mais instable dans les cellules tétraploïdes dont la fréquence était plus élevée que chez des individus normaux. Les auteurs concluent que dans certains cas les conséquences phénotypiques des chromosomes en anneau peuvent être dues davantage à leur action sur la régulation de la division cellulaire qu'à la perte de matériel génétique. Les modèles proposés jusqu'à présent dans la mécanique des chromosomes en anneau ne rendent pas compte de la production des cellules tétraploïdes.

MOT-CLÉ : Chromosome 2 en anneau.

## REFERENCES

1. BOBROW M., EMERSON P.M., SPRIGGS A.I., ELLIS H.L. — Ring -1 chromosome, microcephalic dwarfism and acute myeloid leukemia. *Am. J. Dis. Child.*, 1973, *126*, 257-260.
2. CARTER R., BAKER E., HAYMAN D. — Congenital malformations associated with a ring 4 chromosome. *J. Med. Genet.*, 1969, *6*, 244-227.
3. KIRSTENMACHER M.L., PUNNETT H.H. — Comparative behaviour of ring chromosomes. *Am. J. Hum. Genet.*, 1970, *22*, 304-318.
4. HECHT F. — Ring -4 chromosome: ring autosomes, lorelei of clinical-karyotype corelation and deletion mapping. *Birth Defects: Orig. Art. Ser.*, 1969, *V*, *5*, 106-113.
5. HOO J.J., OBERMANN U., CRAMER H. — The behaviour of ring chromosome 13. *Humangenetik*, 1974, *24*, 161-171.
6. PARKER C.E., ALFI O.S., DERENCSENYI A., MAVALWALA J., DONNELL G. — A child with a ring -4 chromosome (46.XX/46,XX,r4). *Am. J. Dis. Child.*, 1974, *128*, 371-374.
7. PATHAK S., SINHA A.K. — An alternative model for ring chromosome perpetuation in a human subject. *Ann. Hum. Genet., Lond.*, 1972, *35*, 471-476.
8. ZDANSKY R., ANDRLE M., BUHLER E., TSUCHIMOTO T., MAYR W.R., RETT A. — Irregular phenotypic expression of ring chromosomes. *Humangenetik*, 1975, *26*, 193-198.

*Records of the Adelaide Children's Hospital, Vol. 2, 1978*

# 46,XY Females

**by G. R. Sutherland, G. F. Binns, R. F. Carter, and Ruth Dow**
**from the Department of Histopathology, Adelaide Children's Hospital, and the Department of Gynaecology, Royal Adelaide Hospital**

In man the Y chromosome is strongly male-determining and individuals with a normal Y chromosome are almost always males. There are exceptions to this, however, and a few conditions are known in which phenotypic females have apparently normal male chromosome complements. The two best defined disorders of this type are testicular feminization and XY pure gonadal dysgenesis (Swyer syndrome). Whilst relatively rare these conditions are of importance in that they can be familial and XY females are at high risk of tumour development in their dysgenetic gonads. This paper reports three XY females with pure gonadal dysgenesis, two of whom are sisters.

## CASE 1

The proposita was the result of the first pregnancy of a 22-year-old woman and her non-consanguineous 24-year-old husband. Hydrocephaly had been diagnosed antenatally and birth at term was by instrumental delivery. Investigations during the second week of life showed marked asymmetry of the skull with prominence of the right parietal region, widening of sutures, agenesis of the corpus callosum, moderate ventricular dilatation and a very large Dandy-Walker cyst. A cisterno-peritoneal shunt was installed, but intermittent shunt blockages occurred from the age of seven months so it was converted to a cisterno-atrial shunt. From the age of 16 months she began having convulsions and final admission to hospital was at the age of 22 months. She was then shown to have hypertension and chronic renal failure; an intravenous pyelogram showed small shrunken, malrotated kidneys. She was treated with diuretics and hypotensive agents but gradually deteriorated, with increasing oedema and persistently high plasma urea and creatinine. She died at the age of 23 months.

The main relevant autopsy findings were confined to the kidneys and brain. The right kidney was about half normal size and of an abnormal shape with the hilum in the centre of the anterior surface. The arterial supply was highly abnormal, two small arteries of about 1.5 mm external diameter arising from the posterior aspect of the lower abdominal aorta just above the bifurcation and entering the kidney on the posterior aspect of its lower pole. A few small unilocular cysts about 1.5 mm diameter and filled with clear fluid were present in the superficial cortex and visible on the external surface. Microscopically these cysts were similar to those seen in multicystic dysplasia although interpretation of the renal pathology was somewhat hindered by the advanced pyelonephritic changes present. Two larger cystic areas about 15 mm diameter protruded from the upper pole. On incision these were seen to be filled partly by honeycombed grey tissue which microscopically proved to consist of primitive to well-differentiated renal tubules and occasional glomeruli lying between numerous cysts lined by either flattened or obvious renal-type epithelium.

The left kidney was about one-third normal size but of normal shape. The surface was marked by slightly elevated fine cysts up to 2 mm in diameter. Vascular anomalies were again present, two veins arising from the posterior aspect of the lower pole, running behind the aorta, and then fusing before entering the inferior vena cava just above the junction of the common iliac veins. Cysts of

the smaller type were again present with similar distribution and microscopical appearances, and this kidney also showed pyelonephritic changes.

The abnormalities in the kidneys clearly indicate renal dysgenesis but of a complex type that cannot be readily classified within any of the currently accepted systems of nomenclature. The pyelonephritic changes were considered to be secondary and undoubtedly the cause of the child's death from renal failure.

The brain showed hydrocephalus due to a Dandy-Walker malformation and there was associated agenesis of the corpus callosum.

The internal and external genitalia were thought to be normal macroscopically so only the right ovary was taken for routine histological study. Microscopically there was an outer shell of tissue resembling ovarian stroma which covered an internal mass of tumour. This was considered to be a gonadoblastoma arising in a streak ovary. Section through the outer end of the fallopian tube showed fimbria externally but internally tubules resembling epididymis and, in one area, rete testis.

Routine skin fibroblast culture showed a normal male karyotype, 46,XY. Chromosome studies on the parents, siblings, and mother's sisters were all normal. An extensive family history did not suggest that the XY pure gonadal dysgenesis was familial.

## CASE 2

The proposita approached her family doctor at age 16 years for advice regarding lack of breast development. On initial examination she was noted to have no breast development and infantile external genitalia; she had never menstruated. Investigation included chromosome studies which revealed a normal male karyotype, 46,XY. Detailed gynaecological examination at the age of 17 years revealed a prepubertal appearance in a girl 168 cm tall. There was no axillary hair, there was no evidence of any breast tissue, and the nipples were of male type. She was, however, not muscular but of definitely feminine habitus. There were a few long dark streaky hairs over the mons and the external genitalia were infantile; there was no clitoral enlargement. Vaginal examination was not possible as there was a tight tender hymen; rectal examination

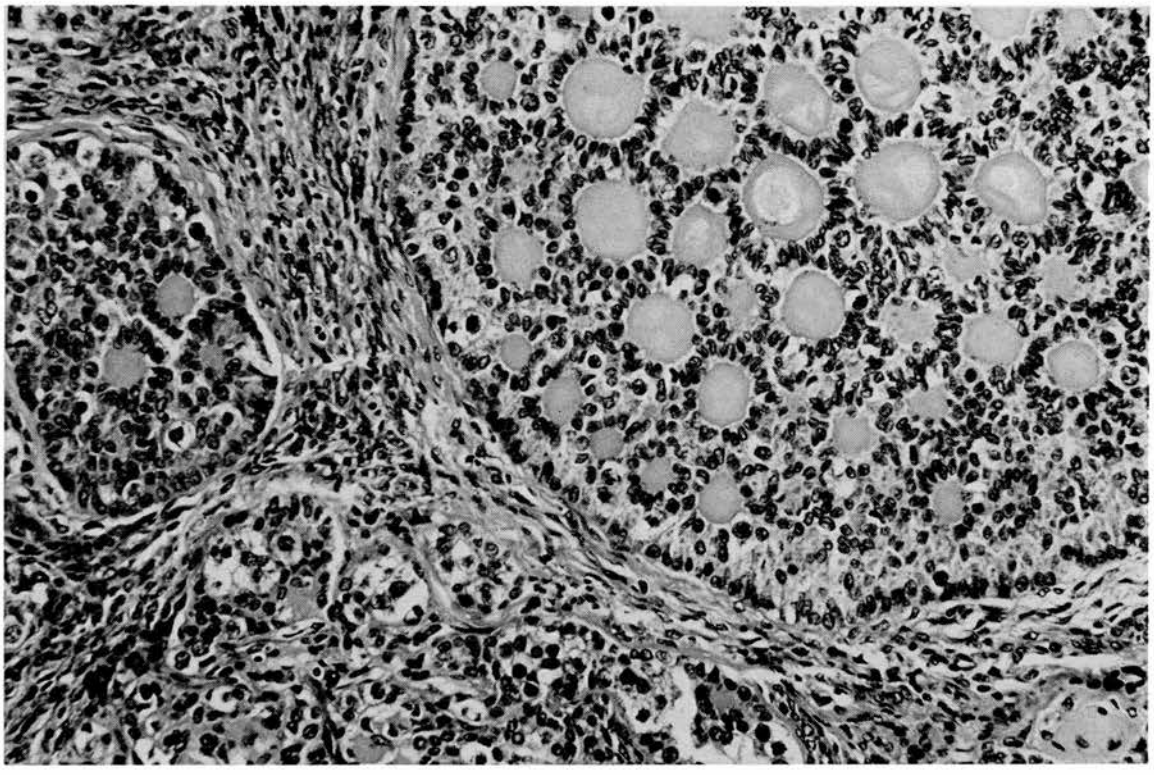


**FIG. 1: Section of right gonad of Case 2 showing typical appearance of gonadoblastoma. (Haematoxylin & Eosin X360.)**

was normal. The girl enjoyed good health, was of normal intelligence and socially female.

Gonadectomy was performed and both gonads measured 1.5 x 0.5 x 0.5 cm. Microscopically the right ovary showed the typical appearance of a streak gonad without evidence of neoplasia. The left ovary showed the typical appearance of a gonadoblastoma but without any evidence of transformation to other tumour types (Figure 1). There was a small infantile uterus and fallopian tubes were present. Hormone replacement therapy was instituted and one year later there has been little in the way of breast development but periodic withdrawal bleeding occurs.

Family studies indicated that the proposita had three sisters (Figure 2), and one of these was found to have a normal male karyotype, 46,XY. Extensive investigation of more distant relatives did not reveal any individuals in whom this condition might have been suspected.

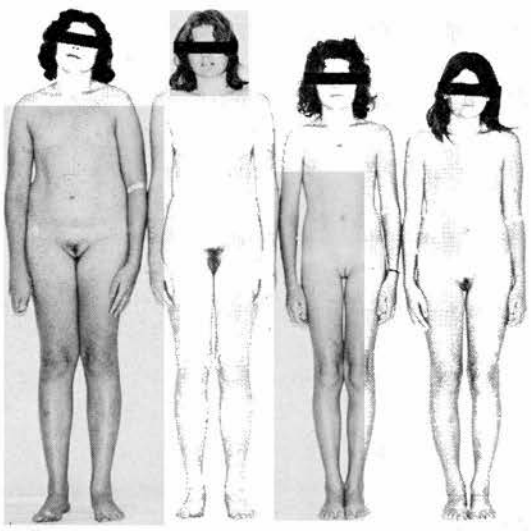


**FIG. 2: Case 2 (left) and her three siblings, two of whom are normal females and one (second from right) is Case 3.**

## CASE 3

This girl is a sister of Case 2 and was ascertained by family studies. Detailed gynaecological examination at the age of 14 years showed an underdeveloped girl 158 cm tall. There was no breast development and no axillary or pubic hair. She had never menstruated. External genitalia were infantile in appearance with no clitoral enlargement. Vaginal examination was not possible because of the presence of a tight hymen. Rectal examination was normal except that there was a fullness and tenderness on the right of the pelvis suggestive of a small mass.

Gonadectomy was performed and the left ovary measured 2 x 0.5 x 0.5 cm and microscopically had the appearance of a streak ovary. The right gonad measured 2.5 x 1 x 1 cm and microscopically was shown to be a gonadoblastoma without any evidence of transformation to other tumours. The internal genitalia were similar to those of Case 2. Her response to hormone replacement therapy has been similar to her older sister's. The two other sisters with normal female karyotypes showed completely normal development for their ages.

## DISCUSSION

Associations of hydrocephalus with renal anomalies are well-known (D'Agostino *et al.*, 1963, Cases 9 and 10; Goldston *et al.*, 1963; Miranda *et al.*, 1972) as are associations of pseudohermaphroditism with renal anomalies (Franks and Northcutt, 1963; Drash *et al.*, 1970; Barakat *et al.*, 1974). The association of renal and cerebral anomalies with XY pure gonadal dysgenesis shown in our Case 1 may be fortuitous as such an association has not been previously recorded.

The familial occurrence of XY pure gonadal dysgenesis is best shown in the families reported by Sternberg *et al.* (1968) and Espiner *et al.* (1970). In these families there were affected individuals in three sibships. Siblings with XY gonadal dysgenesis have been reported on numerous occasions. The inheritance pattern seen is strongly suggestive of X-linkage but the possibility of sex-limited dominant transmission cannot be excluded. XY gonadal dysgenesis probably represents a heterogeneous group of disorders. The majority of patients are of normal to slightly increased height, lack secondary sexual characteristics, have streak gonads, and are not masculinized. However, others have been reported to show some features of Turner syndrome and some are masculinized to a greater or lesser extent. Differences in expression of the gene responsible may account for some of this variation. Barr *et al.* (1967) described a family in which one sibling had XY pure gonadal dysgenesis, another had male pseudohermaphroditism, and a son of their sister had marked hypospadias. Some of the phenotypic variations, particularly

masculinization, may well be due to tumour formation. Not all patients with gonadal tumours are virilized but when there is clinical evidence of tumour hormonal secretion this is usually androgenic (Scully, 1970).

Dysgenetic gonads in individuals who carry a Y chromosome are prone to tumour development. Manuel *et al.* (1976) estimated that 27.5 per cent of patients with XY gonadal dysgenesis will develop tumours by the age of 30 years. The tumours are most commonly gonadoblastomas but frequently are dysgerminomas and more rarely are choriocarcinomas and embryonal carcinomas. Case 1 in this report appears to be the youngest patient recorded to have developed a gonadoblastoma. Manuel *et al.* (1976) did not find gonadal tumours in individuals below the age of five years and suggested that in XY gonadal dysgenesis only about 2 per cent will develop tumours before the age of 10 years.

In view of the risk of tumour development in individuals with XY gonadal dysgenesis, and considering that the gonads will be functionless in any case, we believe they should be removed at the time the diagnosis is made. There can be no good reason for delaying gonadectomy for, as the first case in this report shows, tumour can be present before the age of two years. Moreover, in addition to the risk of malignant transformation and even metastasis occurring before the primary tumour has been detected, there is also the possibility of inappropriate virilization occurring in some cases.

## ADDENDUM

Swanson and Chapler (1978) have reported two XY females with renal anomalies (horseshoe kidneys) and suggest that all XY females should have their urologic systems evaluated.

## ACKNOWLEDGEMENTS

We thank Dr. J. Croall for referring Case 2 and for his assistance with the family studies. Dr. R. E. Scully reviewed the gonadal pathology in Case 1.

## REFERENCES

Barakat, A. Y., Papadopoulou, Z. L., Chandra, R. S., Hollerman, C. E., and Calcagno, P. L. (1974): Pseudohermaphroditism, nephron disorder and Wilms' tumour: a unifying concept. *Pediatrics*, **54**, 366.

Barr, M. L., Carr, D. H., Plunket, E. R., Soltan, H. C., and Wiens, R. G. (1967): Male pseudohermaphroditism and pure gonadal dysgenesis in sisters. *American Journal of Obstetrics and Gynecology*, **99**, 1047.

D'Agostino, A. N., Kernohan, J. W., and Brown, J. R. (1963): The Dandy-Walker syndrome. *Journal of Neuropathology and Experimental Neurology*, **22**, 450.

Drash, A., Sherman, F., Hartmann, W. H., and Blizzard, R. M. (1970): A syndrome of pseudohermaphroditism, Wilms' tumour, hypertension, and degenerative renal disease. *The Journal of Pediatrics*, **76**, 585.

Espiner, E. A., Veale, A. M. O., Sands, V. E., and Fitzgerald, P. H. (1970): Familial syndrome of streak gonads and normal male karyotype in five phenotypic females. *New England Journal of Medicine*, **283**, 6.

Franks, R. C., and Northcutt, R. (1963): Female pseudohermaphroditism and renal anomalies. *American Journal of Diseases of Children*, **105**, 490.

Goldston, A. S., Burke, E. C., D'Agostino, A., and McCaughey, W. T. E. (1963): Neonatal polycystic kidney with brain defect. *American Journal of Diseases of Children*, **106**, 484.

Manuel, M., Katayama, K. P., and Jones, H. W. (1976): The age of occurrence of gonadal tumours in intersex patients with a Y chromosome. *American Journal of Obstetrics and Gynecology*, **124**, 293.

Miranda, D., Schinella, R. A., and Finegold, M. J. (1972): Familial renal dysplasia. Microdissection studies in siblings with associated central nervous system and hepatic malformations. *Archives of Pathology*, **93**, 483.

Scully, R. E. (1970): Gonadoblastoma. A review of 74 cases. *Cancer*, **25**, 1340.

Sternberg, W. H., Barclay, D. L., and Kloepfer, H. W. (1968): Familial XY gonadal dysgenesis. *New England Journal of Medicine*, **278**, 695.

Swanson, J. A., and Chapler, F. K. (1978): Renal anomalies in the 'XY female'. *Obstetrics and Gynecology*, **51**, 237.

B 29

# Picture of the Month

Sydney S. Gellis, MD, Murray Feingold, MD; Contributed by Grant R. Southerland, PhD, John G. Rogers, MD

Fig 1.

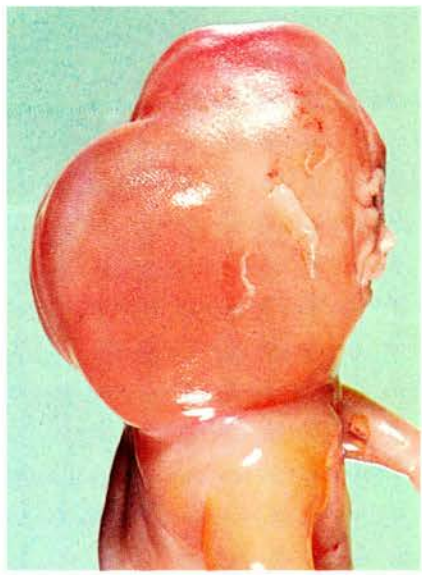


Fig 2.

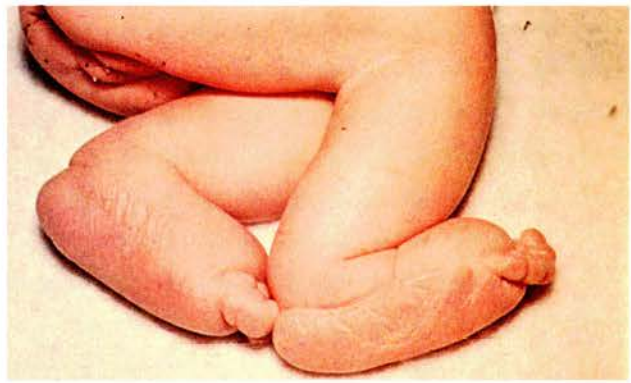


Fig 3.

From the Cytogenetics Unit, Adelaide Children's Hospital, North Adelaide (Dr Southerland) and the Genetics Research Unit, Royal Children's Hospital, Melbourne, Australia (Dr Rogers).

Reprint requests to Boston Floating Hospital, 20 Ash St, Boston, MA 02111 (Dr Gellis).

## Denouement and Discussion

# Fetal Turner's Syndrome

Fig 1.—Fetus with Turner's syndrome depicting marked cervical sac and generalized lymphedema.

Fig 2.—Posterior view of the cervical sac.

Fig 3.—Lymphedema on the dorsum of the feet.

### Manifestations

In the fetus the main features of Turner's syndrome are gross lymphedema with large cervical fluid-filled sacs. The cervical sacs may be extremely large and are most likely associated with the webbing of the neck found in patients with Turner's syndrome. There have been reports of elevated α-fetoprotein levels in the amniotic fluid of fetuses with Turner's syndrome. Some believe that this is due to obtaining fluid from the cervical sac rather than the amniotic fluid. Although the majority of patients both with Turner's syndrome survive, a substantial number of fetuses with Turner's syndrome do not come to term. The reason for this is not known.

### Genetics

The karyotype in the majority of patients with Turner's syndrome is 45,X. However, 20% to 30% have various types of mosaicism including X/XX, X/XY, and X/XX/XY or a structurally altered chromosome such as an isochromosome X, ring X, or partial deletion of the X chromosome. There is generally no parental carrier state predisposing to Turner's syndrome and therefore the recurrence risk is not much greater than that of the general population. This condition can be detected by performing chromosomal analysis on amniotic fluid.

### Treatment

There is no treatment for the fetus with Turner's syndrome. There is a considerable risk of malignancy in individuals with mosaic-type Turner's syndrome that have a cell line containing a Y chromosome (eg, XO/XY).

### References


1. Sing RP: Hygroma of the neck in XO abortuses. *Am J Clin Pathol* 53:104-107, 1970.
2. Ferguson-Smith MA: Karyotype-phenotype correlation in gonadal dysgenesis and their bearing on the pathogenesis of malformations. *J Med Genet* 2:142-155, 1965.
3. Sutherland GR, Holt D, Rogers JG: Amniotic-fluid α-fetoprotein in Turner syndrome. *Lancet* 1:649-650, 1977.


*The Editors welcome contributions to "Picture of the Month" and "Radiological Case of the Month." Those who wish to contribute should send their manuscripts to Dr Gellis (Picture of the Month), Boston Floating Hospital, 20 Ash St, Boston, MA 02111, or Dr Young (Radiological Case of the Month), Department of Radiology, Children's Hospital of Pittsburgh, 125 DeSoto St, Pittsburgh, PA 15213. Articles and photographs accepted for publication will bear the contributor's name. There is no charge for reproduction and printing of color illustrations.*

NEUROLOGY NOW

# Genetic counselling for neurological disorders

Grant R. Sutherland

Whilst it is clear that many neurological disorders are the result of environmental factors, such as anoxia, trauma or infection, there are also many which have a genetic component, such as epilepsy and the neural tube defects, or are wholly genetic such as Down syndrome or Huntington disease. Those disorders which are genetic or partly genetic have recurrence risks which vary from 50 per cent for the dominant disorders, such as Huntington disease, down to less than one per cent for the recurrence of regular Down syndrome to a young mother. Some disorders are readily amenable to prenatal diagnosis and others are not.

## What can genetic counselling offer?

(i) An explanation of what went wrong, with consequent alleviation of guilt.

Grant R. Sutherland

Grant R. Sutherland, MSc (Melb), PhD (Edin) is Chief Cytogeneticist at the Adelaide Children's Hospital and Honorary Consulting Geneticist to The Queen Victoria Hospital, South Australia. He was previously senior Cytogeneticist at the Royal Hospital for Sick Children in Edinburgh and an Honorary Fellow in the Faculty of Medicine of the University of Edinburgh.

This article continues the series being provided by the Australian Neurological Foundation.

(ii) A statistical figure for recurrence given in comprehensible terms.

(iii) A discussion of alternative courses of action which may include:
- continue reproduction;
- cease reproduction with possible mention of abortion, contraception, sterilization or adoption;
- prenatal diagnosis for future pregnancies;
- donor insemination, either if the husband is the cause of the problem or if both parents contribute to the risk.

The 'counselling' rather than the 'genetic' aspects of genetic counselling cannot be overemphasized. When parents produce an abnormal child they usually experience the emotional reactions of loss, grief and guilt. It is essential to cope with these reactions as well as giving information, otherwise the information will be poorly understood and might be rejected. There can be considerable strains put on marital and other family relationships (for example, with the in-laws). In spite of these problems, full diagnostic evaluation of any child with a potential genetic disorder should be carried out as soon as possible after the problem has been recognized. Family studies which may be necessary should also be carried out at this time, as there might be others at risk of having abnormal offspring.

The involvement of a social worker in helping families cope with the problems of adjustment can be helpful. The stress on genetics in this article merely reflects the bias of its author.

From a geneticist's viewpoint, it is convenient to classify neurological disorders with a genetic component according to the type of inheritance pattern they follow. This may have little relevance to any clinical classification, but is useful since familial aggregation may be a clue to diagnosis.

## Autosomal recessive (AR) disorders

Both parents of an individual with an AR disease must be carriers of the gene involved. Carriers of such genes are normal individuals; indeed, virtually everyone is a carrier of two or three different recessive genes. There is usually no relevant family history, although in the rarer disorders the parents may be related to each other; this is more common in some of the immigrant communities where cousin marriage is often encountered. Examples of AR disorders which can present as neurological problems are shown in *Table 1*.

**Table 1**
**Some autosomal recessive disorders**
Friedreich ataxia
Hurler syndrome (mucopolysaccharidosis)
Muscular atrophy (various forms)
Pain insensitivity (analgia)
Phenylketonuria (PKU)
Tay-Sachs disease

McKusick[3] lists 521 proven AR disorders: many of these are very rare and not associated with neurological disease, but many are. Once parents have had a child with an AR disorder, the recurrence risk for each and every subsequent pregnancy is 1 in 4, or 25 per cent. This risk is high enough to deter most couples from further reproduction, especially when the disorder is severe and untreatable. A number of AR disorders, such as phenylketonuria (PKU) can be successfully treated.

● *Accurate diagnosis, at the chromosomal or molecular level, is often an essential basis for the provision of reliable genetic counselling.*

Possible courses of action include prenatal diagnosis, as many of the AR disorders are amenable to this procedure. For this reason, it is important that every effort be made to reach a diagnosis as soon as a child is recognized as having a problem, so that recurrence can be prevented.

Diseases which can be prenatally diagnosed include the neuronal storage diseases, such as Tay-Sachs and Gaucher diseases, which are degenerative conditions associated with mental deficit. The accuracy of diagnosis needed in this area is demonstrated by the mucopolysaccharidoses, which were previously called gargoyles, where all are AR diseases except Hunter's syndrome, which is X-linked.

Another possible course of action is donor insemination, which is applicable to AR disorders. Similarly, in any AR case, if the parents separate and remarry there should be no recurrence provided their new partners are not related to their previous ones, or to themselves. Again, correct diagnosis is essential as donor insemination would be useless in X-linked diseases.

## Autosomal dominant (AD) disorders

These are conditions which are passed directly from parent to offspring. When a person is affected with an AD disorder, half his/her children would be expected to have it also. *Table 2* lists a number of dominant disorders.

**Table 2**
**Some autosomal dominant disorders**
Charcot-Marie-Tooth disease (most common form)
Huntington disease (chorea)
Motor neurone disease (some families)
Neurofibromatosis (Von Recklinghausen disease)
Spastic paraplegia (some families)
Tuberous sclerosis
Waardenburg syndrome

● *A detailed family history can often give a clue to the type of inheritance a disease is following, but care must be taken as a common environment can also produce multiple affected family members.*

Counselling is often difficult in dominant disorders. In almost all cases there is no objective diagnosis possible, that is, there is no consistent biochemical or chromosomal abnormality demonstrable. This means that clinical conditions which appear identical may have different aetiologies. For example, motor neurone disease is usually not familial, but some families have this following an AD inheritance pattern. Charcot-Marie-Tooth disease is most commonly dominant, but AR and X-linked forms also exist. A detailed family history is particularly helpful in differentiating patterns of inheritance.

Further problems are encountered with AD disorders due to differences in penetrance and expression. Penetrance simply refers to the occurrence or otherwise of the disease in an individual who carries the gene for it; it is an all or none phenomenon. Expression is the degree of severity of the disease. These phenomena are important in a condition like tuberous sclerosis. The majority of individuals with this disorder are the result of a new mutation. This means that the parents are running only a very small risk that the condition may recur in subsequent offspring, but half the offspring of the new mutant would be expected to have the disease.

The severity of tuberous sclerosis varies greatly, such that in some carriers of the gene it is not penetrant. Before deciding that a child is a new mutant, the parents should be examined under Wood's light for white macules, have skull radiographs for intracranial calcification and undergo fundoscopic examination for phakomata.

In Huntington disease, the age of onset of symptoms ranges from early teenage until the seventh decade. Consequently, members of a family in which this condition

is present can never be certain that their unaffected ancestors did not carry the gene, particularly if they died in middle age from some intervening cause.

Genetic technology cannot offer a great deal to members of a family with an AD disorder. Only a handful of these conditions are amenable to prenatal diagnosis, and the risk of recurrence is high (50 per cent). When a male is affected then donor insemination can be discussed, but for females there is no practical alternative to abstaining from reproduction. Counselling can, however, put the problem into perspective and pedigree analysis can often show that the risks being run are small.

## X-linked (XL) disorders

In this type of inheritance the gene responsible is located on the X chromosome. The majority are recessive in that they do not cause disease in females who have two X chromosomes, but do in males who have only one X chromosome. Some XL recessive disorders are shown in *Table 3*.

**Table 3**
**Some X-linked recessive disorders**

| |
|---|
| Colour blindness |
| Fabry disease |
| Hunter type mucopolysaccharidosis |
| Hydrocephalus (aqueduct stenosis type) |
| Menke syndrome |
| Mental retardation (i) with marker X chromsome |
| (ii) without marker X chromosome |
| Muscular dystrophy, Duchenne and Becker types |

In families with XL recessive disorders, any males who reproduce will have all normal children but all their daughters will be carriers. Carrier females who reproduce will have normal daughters (although half of them will be carriers) but half their sons will be affected.

It is often possible to detect the carrier status in females with a fair degree of accuracy. For example, by measuring creatinine phosphokinase levels in females from families with Duchenne type muscular dystrophy, it is found that about 70 per cent of carriers have increased levels of this enzyme. For females in such families, pedigree analysis in combination with biochemical studies can usually establish carrier status with considerable reliability.

Most XL recessive disorders are not amenable to specific prenatal diagnosis; however, since only males are affected, prenatal sexing and termination of male foetuses can allow couples where the wife is a carrier to have normal girls without the risk of having an affected male. Foetal blood sampling may be available in the near future, and should allow prenatal diagnosis of Duchenne muscular dystrophy in male foetuses at risk for this disorder.

XL mental retardation (Renpenning syndrome) has been proposed to account for 20 per cent of retarded males with IQs between 30 and 55 by Turner and Turner[7] in New South Wales, and is probably the commonest form of familial mental retardation. It is a heterogeneous group of conditions. In one form there is macro-orchidism which is now associated with a visible chromosome lesion known as a fragile site *(Figure 1)*. In other forms without the chromosome lesion, the diagnosis can only be made by study of the family tree and exclusion of other aetiologies.

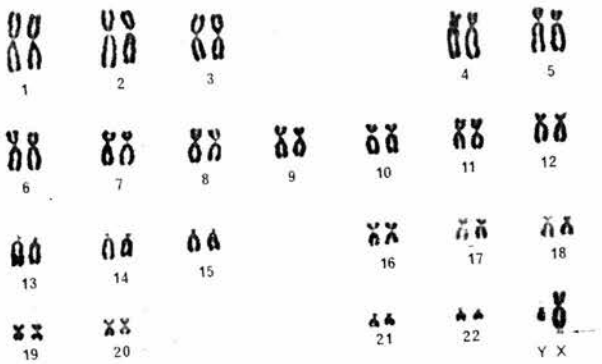



**Figure 1.** Unbanded karyotype of a male with the form of X-linked mental retardation showing a fragile site (arrow) on the end of the X chromosome.

XL dominant disorders are rare. An example is the oro-facial-digital syndrome in which males are so severely affected that death *in utero* occurs, but females survive with varying expression of the condition.

● *All couples at risk for having children with neural tube defects should be informed that these conditions are amenable to prenatal diagnosis.*

## Multifactorial disorders

There are many conditions which follow a multifactorial pattern of inheritance *(Table 4)*. There are a number of properties of multifactorial inheritance which can be noted:

(i) The incidence in first degree relatives (parents, siblings, offspring) is usually five to 20 times that of the general population. In more distant relatives the incidence falls off rapidly.

(ii) The more affected members there are in the family, the greater the chance that subsequent members will be affected.

(iii) The incidence of the disorder will vary from one population or ethnic group to another.

**Table 4**
**Some disorders which follow a multifactorial inheritance pattern**
Epilepsy
Febrile convulsions
Neural tube defects
Schizophrenia

(iv) One sex may be more commonly affected than the other.

Two groups of disorders which follow multifactorial inheritance are worthy of more detailed discussion because they are not rare, and frequently give rise to enquiries.

**Neural tube defects**

The most frequently encountered neural tube defects are anencephaly and spina bifida and, more rarely, encephaloceles. Whilst these forms of neural tube defect are diverse clinically they are similar genetically, since they are all manifestations of failure of neural tube closure during embryogenesis.

In Australia, the incidence of neural tube defects at birth is 1.94/1,000 births[4], of which about half have spina bifida. The risk of recurrence of neural tube defects for parents after the birth of an affected child is three to five per cent, after two affected children 10 per cent, and for the children of an individual with a neural tube defect (usually spina bifida) two to three per cent. The risks are increased for persons with a family history of neural tube defect, and for those with minor spinal dysraphisms or spina bifida occulta, but the magnitude of the increase in these cases is not well established. It should be noted that precise diagnosis is again important, since on casual inspection the AR Meckel syndrome can mimic a simple encephalocele.

What can be done for couples at risk for children with neural tube defects? Those neural tube defects which are open lesions, (that is, not covered with skin) can be prenatally diagnosed by measurement of amniotic fluid alphafoetoprotein, which is raised in concentration in such instances.[1] Generally speaking, the closed lesions are much less frequent and not nearly so handicapping as open ones, hences couples at risk can be assured they can have a child who will almost certainly not have an open neural tube defect if at prenatal diagnosis their amniotic fluid alphafoetoprotein is normal. Prenatal diagnosis will thus identify a second affected child in a family. Identification of the first affected child would be of much greater benefit.

Maternal serum alphafoetoprotein is high in nearly all pregnancies where the foetus is anencephalic, and in about 60 per cent of those when the foetus has a different open neural tube defect. Since the normal range of serum alphafoetoprotein varies markedly with the gestational age, the time at which the serum sample is collected and an accurate gestational age are important. Sampling should be at 16 weeks' gestation; sampling earlier than this gives less reliable results.

In Glasgow, Ferguson-Smith *et al*[2] showed that raised serum alphafoetoprotein was associated with a foetal neural tube defect in one in two cases after amniotic fluid alphafoetoprotein has been measured. Pilot programmes of maternal serum alphafoetoprotein screening are currently well advanced in Australia.

● *Before attempting to give genetic counselling in epilepsy, complete neurological assessment is essential.*

**Epilepsy**

Epilepsy can be difficult to define accurately, and is a heterogeneous group of disorders ranging from isolated infantile convulsions through to congenital malformation complexes, of which fitting is a component to the temporal focal epilepsy with its characteristic EEG changes. In many instances the occurrence of seizures can be positively related to trauma, neoplasm or infection; in such cases genetic contribution will be minimal and the risk to sibs or offspring probably not increased. Where the epilepsy is related to congenital malformations the aetiology of the epilepsy is bound up with that of the malformation which may be genetic.

Studies on epilepsy where the above factors are eliminated, are complicated by the discordance between clinical and EEG findings. In both centrencephalic epilepsy (this group includes many cases of petit mal) and temporal focal epilepsy, almost half first degree relatives have EEG changes which would suggest that these conditions follow an AD pattern of inheritance with incomplete penetrance. The risks to sibs and offspring in such cases are about 1 in 8 to 1 in 10.

In some families, analysis of a pedigree suggests both AR and AD patterns of inheritance. One form of myoclonic epilepsy is certainly autosomal recessive.

● *One in 150 live born children, and half of all early spontaneous abortuses, have a major chromosome abnormality.*

## The chromosome disorders

**Down syndrome**

The most commonly encountered chromosome abnormality is Down syndrome. This is the commonest single cause of moderate to severe mental retardation in the community, there being about 350 new cases born in

Australia each year. Down syndrome is due to the presence of an extra chromosome number 21, affected individuals usually having 47 instead of the normal 46 chromosomes. Down syndrome is usually sporadic in occurrence but a number of familial cases which result from a translocation in a parent occur. The sporadic cases are strongly related to maternal age.

● *Every child with Down syndrome should have its chromosomes examined.*

Before giving genetic counselling to parents of a Down syndrome child, the child's chromosomes must be examined. If the child has a 'regular' karyotype, that is 47 chromosomes, then it is a waste of resources to study the parents' chromosomes; about 95 per cent of Down syndrome cases have regular karyotypes *(Figure 2)*. Counselling can proceed on the basis that Down syndrome is not familial.

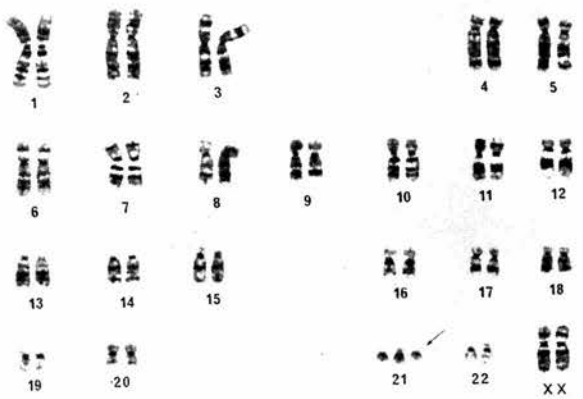



**Figure 2.** Banded karyotype of a 'regular' Down syndrome female. Note the presence of the three number 21 chromosomes.

When the karyotype is regular, there is little evidence that the risk of recurrence is greater than if the parents had not had a child with Down syndrome. There are, however, rare instances where one parent is mosaic: that is, has both normal and 47 chromosome cells within its body: here, the recurrence risk can be up to 50 per cent. (When Down syndrome females reproduce, half their children are normal and the other half have Down syndrome). Because of this the recurrence risk is sometimes quoted[5] at twice that for parents who have not had a child with Down syndrome *(Table 5)*.

Parents who have had a child with Down syndrome are often referred for amniocentesis in subsequent pregnancies. Experience has shown[6] that chromosome abnormalities are not encountered any more frequently in this group than would be expected by chance. Amniocentesis may well be indicated for reasons of anxiety, but in young parents there is certainly no genetic indication. When amniocentesis is carried out on this group, about one per cent of pregnancies will have a major chromosome abnormality indicating foetal abnormality.

**Table 5**
**Risks and recurrence risk of Down syndrome according to maternal age**

| Maternal age | Risk of Down syndrome child | Risk of Down syndrome child after having one regular Down syndrome child |
|---|---|---|
| 20-24 | 1/1600 | 1/800 |
| 25-29 | 1/1100 | 1/550 |
| 30-34 | 1/ 750 | 1/370 |
| 35 | 1/ 400 | 1/200 |
| 37 | 1/ 270 | 1/135 |
| 39 | 1/ 130 | 1/ 65 |
| 40-44 | 1/ 80 | 1/ 40 |

Although there is some evidence that the age of the father is relevant to the risk of producing a child with Down syndrome, and it can be demonstrated cytologically that the extra chromosome is of paternal origin in a proportion of cases, the only available risk figures for Down syndrome are based on the maternal age. *(Table 5)*

● *Couples with a family history of Down syndrome should be referred for genetic counselling, not amniocentesis.*

If the child has an 'irregular' karyotype, then parental chromosomes must be studied. If these are normal the recurrence risk is virtually nil; there have been no reports of more than one irregular Down syndrome individual in a sibship where parental chromosomes are normal. The commonest irregular karyotype is a 14/21 translocation; in about two thirds of these cases parents will also carry the translocation, thus having a total of only 45 chromosomes. Recurrence risk is then significantly increased. If the mother carries the translocation it is about 20 per cent, if the father carries the translocation it is about two to three per cent.

Women with a family history of Down syndrome are often referred for amniocentesis without reference to a genetic counsellor. In many cases the amniocentesis is quite unnecessary because investigation of the family, possibly with chromosome analysis of the Down syndrome individual involved, will in most cases show that the Down syndrome is of the regular variety and most unlikely to recur. Even in cases where the Down syndrome is the result of a familial translocation, the particular woman involved may not be a carrier of the translocation and be at no increased risk of having a child with Down syndrome.

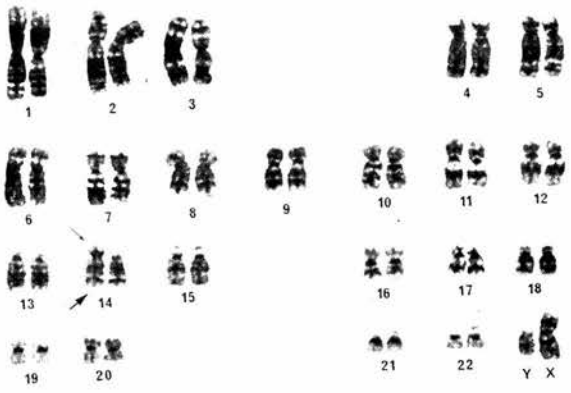



**Figure 3.** Banded karyotype of an 'irregular' Down syndrome male. Note the chromosome 21 (small arrow) translocated onto chromosome 14 (large arrow); there are two other number 21 chromosomes present.

### Other chromosome disorders

There are many other chromosome disorders which cause mental retardation, often in association with congenital malformations. Those which involve an extra normal chromosome being present all (except for males with two Y chromosomes) increase in frequency with maternal age. *Table 6* lists some of the more common chromosome abnormalities. The presence of an extra autosome, apart from chromosome 21, always leads to severe malformation syndromes incompatible with prolonged survival. Extra X chromosomes can cause mild intellectual deficit in a proportion of those affected.

**Table 6**
**Some chromosome abnormalities seen in live-born children**

Edwards syndrome (trisomy 18)
Patau syndrome (trisomy 13)
Cri-du-chat syndrome (absence of short arm of chromosome 5)
Klinefelter syndrome (male with extra X chromosome)
Triple-X female (female with extra X chromosome)
Trisomy 9p (trisomy for only the short arm of chromosome 9)

Since chromosome banding has come into regular use, many clinical syndromes associated with extra or missing parts of chromosomes have been delineated. Almost all of these involve mental deficit to a greater or lesser extent. Many of these new syndromes can be familial if they arise from malsegregation of parental translocations.

A high proportion of individuals with chromosome abnormalities die around the time of birth. Chromosome studies should be part of every perinatal autopsy; chromosome abnormality is now a more common finding (about five per cent of cases) at perinatal autopsy than Rhesus disease.

● *Every pregnant woman over the age of 35 should be informed that she is running an increased risk of chromosome abnormalities which are prenatally diagnosable.*

### Prenatal diagnosis and parental age

It can be seen from *Table 5* that the risk of having a child with Down syndrome increases with maternal age. Many of the other chromosome abnormalities also increase in incidence with maternal age, although the rate of increase is not as marked as for Down syndrome. Consequently, if women of advanced age were offered prenatal diagnosis of chromosome abnormalities, the incidence of these disorders in the community could be reduced and the individual couples saved much unhappiness. Indeed, it is a recommendation of the Australian National Health and Medical Research Council that where facilities are available, women older than 35 years should be offered prenatal diagnosis.

The incidence of chromosome abnormality detected by prenatal diagnosis is higher than that obtained from epidemiological studies of live born infants. *Table 7* shows the incidence of total chromosome abnormalities and of Down syndrome detected at prenatal diagnosis. The reasons for these risks being so high are uncertain: however, there can be no doubt that some of these pregnancies would have been lost as late abortions or stillbirths.

**Table 7**
**Incidence of chromosome abnormalities found at amniocentesis at 16 weeks according to mother's age**

| Maternal age | Total chromosome abnormalties | Down syndrome |
|---|---|---|
| 35-39 | 1/70 | 1/120 |
| 40-44 | 1/25 | 1/ 45 |
| >45 | 1/15 | 1/ 20 |

When counselling women in these age groups, it is important to inform them of the risk of an abnormality being detected which will indicate termination of their pregnancy. It should be noted that if a woman is not prepared to have a pregnancy terminated on the grounds of foetal abnormality, there is little point in her having amniocentesis. The reasons for this are that the procedure is a costly one to the community and that there are definite risks, albeit very small ones, associated with amniocentesis. To pointlessly expose a foetus which is most likely to be normal to even a small risk without any possiblity of benefit, must be regarded as unwarranted.

Whilst there are many theoretical risks involved in amniocentesis, the only one which is realized significantly is induction of abortion, which probably occurs after less than 0.5 per cent of amniocenteses.

● *Amniocentesis is a safe procedure for mother and foetus; the only real risk is that of inducing abortion, and this occurs in less than 0.5 per cent of cases.*

**Summary:** When parents have a child with any problem, they usually become concerned about the chances of this problem recurring if they have further children, or of appearing in their grandchildren. Similarly, persons with any congenital disorder themselves or family history of such disorder, often worry about their future children. Genetic counselling can in most cases provide reassurance that there is little need for concern.

In those instances where the risks are real, they can be put into perspective and often effectively circumvented by procedures such as prenatal diagnosis. Pregnant women of advanced age run increased risks of having children with chromosome disorders; it is of benefit to individual couples and to the community to refer such women for amniocentesis.

## References

1. Brock, D. J. H. (1977). The prenatal diagnosis of neural tube defects. *Records of the Adelaide Children's Hospital,* **1:** 226.
2. Ferguson-Smith, M. A. *et al.* (1978). Avoidance of anencephalic and spina bifida births by maternal serum-alphafoetoprotein screening. *Lancet,* **1:** 1330.
3. McKusick, V. A. (1978). Mendelian Inheritance in Man. Fifth Edition, The Johns Hopkins University Press, Baltimore.
4. Simpson, D. A. (1977). The epidemiology of neural tube defects. *Records of the Adelaide Children's Hospital,* **1:** 218.
5. Stevenson, A. C., Davidson, B. C. C. (1976). Genetic Counselling. Second Edition, William Heinemann Medical Books Ltd., London.
6. Sutherland, G. R. (1977). Prenatal diagnosis of chromosome abnormalities. *Records of the Adelaide Children's Hospital,* **1:** 260.
7. Turner, G., Turner, B. (1974). X-linked mental retardation. *J. med. Genet.,* **11:** 109.

Reprint rrequests —
Dr. G. R. Sutherland,
Cytogenetics Unit,
Adelaide Children's Hospital,
North Adelaide, 5006.

course of pregnancy. Washington, D.C.: National Academy of Sciences, 1970:5-40.
5. Duncan JM. On sterility in woman. Philadelphia: Blakiston, 1884:111-5.
6. Siiteri PK. Postmenopausal estrogen production. In: van Keep PA, Lauritzen C, eds. Estrogens in the postmenopause. Front Horm Res. 1975; 3:40-4.
7. McMahon B. Risk factors in endometrial cancer. Gynecol Oncol. 1974; 2:122-9.

## INCREASED SISTER-CHROMATID EXCHANGE IN MULTIPLE SCLEROSIS

*To the Editor:* The analysis of rates of sister-chromatid exchange (SCE) has been shown to be a sensitive method of measuring DNA damage.[1] SCE is increased after exposure to many mutagens and carcinogens[2] and in some viral illnesses.[3] During a study of persons exposed to a variety of environmental agents during pregnancy, we encountered a woman with multiple sclerosis (MS) who had an unexplained elevation of SCE. This incidental finding was followed up, and it was found that SCE is increased in MS.

Volunteers were sought from the South Australian MS-rehabilitation unit, and blood samples were collected from inpatients with MS; data on current and long-term drug usage and smoking habits were collected. The control groups consisted of healthy persons between the ages of eight and 80 years. None were taking drugs except for oral contraceptives in some women, and only six smoked cigarettes. Lymphocyte cultures were established from venous blood samples in Ham's F10 culture medium; harvesting and differential staining were performed as previously described.[4] SCE rates were determined by examination of 20 metaphases; SCE's at the centromere were included in the count. All SCE rates were determined from coded slides by one observer (E.B.).

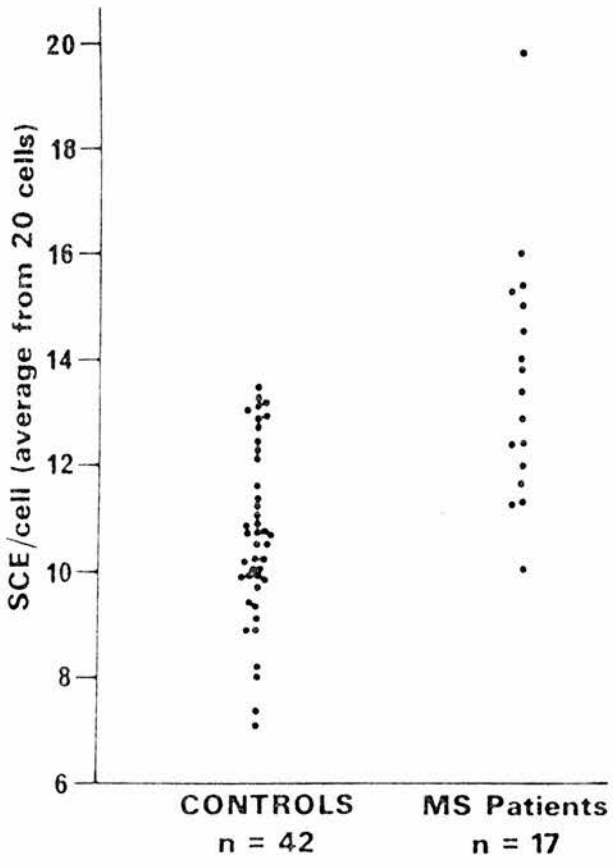



Figure 1. Rates of Sister-Chromatid Exchange (SCE) in Patients with Multiple Sclerosis (MS) and Controls.

The results are shown in Figure 1. The SCE rate in MS patients was higher than that of the control group ($P<0.01$ by Wilcoxon rank-sum test), and this significance remained at the same level even if the highest SCE rate of 19.8 exchanges per cell was omitted from consideration. Half the patients with MS smoked cigarettes, but there was no difference in SCE rates between the smokers and nonsmokers in this group. All but five of the MS patients were taking one or more of the following drugs: methenamine mandelate, diazepam, laxatives, steroids, cimetidine (one patient), and imipramine in various combinations. The possibility that these drugs could be responsible for the SCE findings must be considered, but we are not aware of evidence that these agents cause any increase in SCE. The finding of a high SCE rate among MS patients may reflect the origin of the condition or may be due to some metabolite produced by the disease. The possibility that MS may be caused by a slow virus has been suggested,[5] and viral infections have been shown to cause an increase in SCE.[3] However, we are aware of no data on SCE in slow virus infections.

Our findings may indicate a basic DNA abnormality in MS that could be responsible for the clinical manifestations of the disorder. Inherited disorders of DNA repair can be associated with neurologic impairment, as seen in such diseases as ataxia telangiectasia and some forms of xeroderma pigmentosum.[6] Field et al.[7] claim that there is a strong genetic component in MS. The possibility that MS may result from an inherited abnormality of DNA structure or repair when chromosomes are subjected to some chemical or biologic agent merits consideration.

GRANT R. SUTHERLAND, PH.D.
ELIZABETH BAKER
North Adelaide, South Australia 5006
Adelaide Children's Hospital

R. S. SESHADRI, M.D., F.R.A.C.P.
Bedford Park, South Australia 5062
Flinders Medical Centre

ANDREW BLACK, M.B., F.R.A.C.P.
Woodville, South Australia 5011
The Queen Elizabeth Hospital

1. Nakanishi Y, Schneider EL. *In vivo* sister-chromatid exchange. A sensitive measure of DNA damage. Mutat Res. 1979; 60:329-37.
2. Perry P, Evans HJ. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature. 1975; 258:121-5.
3. Kurvink K, Bloomfield CD, Cervenka J. Sister chromatid exchange in patients with viral disease. Exp Cell Res. 1978; 113:450-4.
4. Sutherland GR, Baker E, Seshadri RS. Heritable fragile sites on human chromosomes. V. A new class of fragile site requiring BrdU for expression. Am J Hum Genet. (in press).
5. Fraser KB. Multiple sclerosis: a virus disease. Br Med Bull. 1977; 33:28-32.
6. Hecht F, McCaw BK. Chromosome instability syndromes. In: Mulvihill JJ, Miller RW, Fraumeni JF, eds. Genetics of human cancer. New York: Raven Press, 1977:105-23.
7. Field EJ, Meyer-Rienecker HJ, Shenton BK, Jenssen HL, Köhler H. Anomalous lymphocyte-antigen reaction in relatives of multiple sclerosis patients: a study of a possible genetic factor in the disease. J Neurol. 1977; 216:135-46.

## PASTEURELLA MULTOCIDA SEPTICEMIA AND PERITONITIS IN A CIRRHOTIC COCK TRAINER WITH A PET PIG

*To the Editor: Pasteurella multocida* has been reported rarely as a cause of spontaneous bacterial peritonitis and septicemia in patients with hepatic cirrhosis.[1-3] Among four previous case reports in cirrhotic patients with clinically discernible peritonitis, the organism was isolated only from the peritoneal fluid in two patients,[1-3] from the blood alone in one,[2] and from both the blood and the peritoneal fluid in one.[1] We wish to report on a patient in whom we isolated *P. multocida* from both the blood and the peritoneal fluid.[3]

The patient, a 48-year-old man, presented to the Veterans Administration Hospital, University of Alabama School of Medicine, and reported hematemesis. Although he had a history of poor general health from alcohol abuse and previous upper gastrointestinal-tract bleeding from esophageal varices associated with portal hypertension and alcoholic cirrhosis, he had felt well enough before

# ADDITIONS TO THE EXCLUSION MAP OF MAN

J.C. MULLEY, G.D. BRYANT, G.R. SUTHERLAND

ERRATUM :

Additions to the Exclusion Map of Man

Ann. Génét. 1980, 23, 198-200.

J.C. Mulley, G.D. Bryant, G.R. Sutherland

Cytogenetics Unit, Department of Histopathology,
Adelaide Children's Hospital Inc., Adelaide, S.A. 5006 (Australia).

This paper reported the exclusion of the MNS blood group from (4)(q27→31). Whilst the paper was in press a blood grouping error was detected, and as a consequence this exclusion is now retracted.

# ADDITIONS TO THE EXCLUSION MAP OF MAN

J.C. MULLEY [1], G.D. BRYANT [2],
G.R. SUTHERLAND [1]

**SUMMARY**

**Exclusion mapping was applied to individuals with monosomic segments defined by chromosomal banding. A range of genetic markers and blood groups was determined resulting in new exclusions for unassigned markers at the following segments : (3)(p25→pter) -** JK, GPT, PI ; **(4)(q27→31) -** MNS, JK, PI, C3, F13A, F13B ; **(7)(q22) -** LU, F13A ; **(12)(p12) -** MNS ; **(12)(p13) and (15)(q15) -** GPT, C3 ; **(12)(pter) and (19)(p or qter) -** MNS, GPT, PI ; **and (18)(q21→23) -** MNS, JK, F13A. **These exclusions may be useful for narrowing the regional localisations of any genetic markers subsequently assigned to the chromosome involved in the exclusion. No new gene assignments were made from cases where family data was available.**

**KEY-WORDS : Deletion mapping. — Exclusion mapping. — Chromosomal deletions.**

**RÉSUMÉ**

**Suppléments à la carte génique humaine par exclusions** La technique d'exclusion a été appliquée pour l'obtention de la carte génique chez des individus monosomiques partiels. Une série de marqueurs génétiques et de groupes sanguins ont été déterminés et permettent de nouvelles exclusions pour des marqueurs non encore localisés aux segments suivants : (3)(p25→pter) - **JK, GPT, PI ;** (4)(q27→31) - **MNS, JK, PI, C3, F13A, F13B** ; (7)(q22) - **LU, F13A** ; (12)(p12) - **MNS** ; (12)(p13) et (15)(q15) - **GPT, C3** ; (12)(pter) et (19)(p or qter) - **MNS, GPT, PI** ; et (18)(q21→23) - **MNS, JK, F13A.** Ces conclusions peuvent être utiles pour préciser les localisations des marqueurs ultérieurement localisés sur des chromosomes intéressés par l'exclusion. Aucune localisation nouvelle n'a pu être obtenue à partir des cas pour lesquels des données familiales étaient disponibles.

**MOTS-CLÉS : Technique de délétion. — Technique d'exclusion. — Délétions chromosomiques.**

Gene mapping by exclusion is applicable to cases having unbalanced chromosomal rearrangements with defined monosomic segments. The exclusion of codominant loci provides supplementary mapping data to that derived from somatic cell hybridisation and segregation analysis. Test loci are excluded when an individual with a deletion is heterozygous. Gene assignments are indicated when obligate heterozygotes (on the basis of parental phenotypes) are shown to be hemizygous. Possible misclassification, the presence of null or silent alleles and incorrect paternity require careful investigation for the confirmation of assignments by deletion mapping. While deletions can be useful for narrowing regional localisations within chromosomes, the technique is relatively inefficient for gene assignment. Exclusion data is readily interpretable and considerable information has been obtained since the advent of chromosomal banding (Aitken et al., 1975 [1] ; Aitken and Ferguson-Smith, 1978 [2]).

## MATERIALS AND METHODS

Genetic markers were examined from patients with deleted autosomal segments (3)(p25→pter), (4)(q27→31), (7)(q22), (12)(p12), (12)(pter), (12)(p13), (15)(q15), (18)(p11), (18) (q21→23) and (19)(p or qter) and a fetus with deleted (5)(p14→pter).

The (3)(p25→pter) deletion resulted from a familial pericentric inversion inv(3)(p25q23). Apart from the ten cases of autosomal deletion, another

1. *Cytogenetics Unit, Department of Histopathology ;*
2. *Department of Haematology,*
*The Adelaide Children's Hospital Inc., ADELAIDE, S.A. 5006 (Australia).*

MULLEY J.C., BRYANT G.D., SUTHERLAND G.R. — Additions to the exclusion map of man. **Ann. Génét.,** 1980, **23,** n° 4, 198-200.

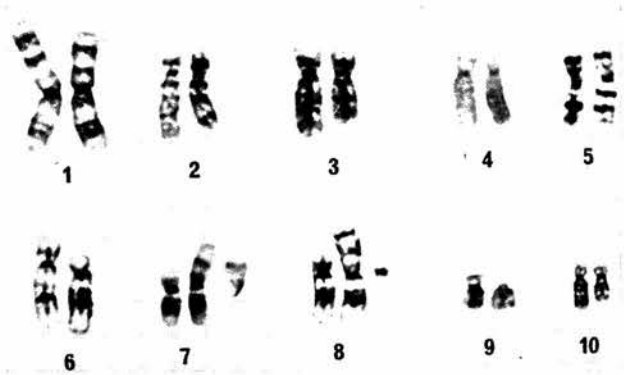



**Fig. 1. — Partial karyotypes from 10 cases with autosomal deletions as shown in table I. Full karyotypes are as follows : 1. 46,XY,rec(3)dup(q)del(p)inv(3)(p25q23)pat., 2. and 3. 46,XY,del(4)(q27q31), 4. 46.XY,del(5)(p14), 5. 46,XY,inv(7)(q11q22)del(7)(q22q22), 6. 46,XY,del(12)(p11p13), 7. 45,XY,t(12;15)(p13;q15), 8. 45,XX, ter rea (12;19) (p13;p or q13), 9. 46,XX,del(18)(p11), 10. 46,XX,del(18) (q21q23).**

family included a female proposita with del(X)(p11p22). Deletions were delineated by trypsin-Leishman G-banding and partial karyotypes for the autosomal deletions are given in figure 1.

Samples of serum, plasma, red cells or fibroblasts where available were stored and subsequently typed by standard blood grouping and electrophoretic procedures. *PI* was completely subtyped by isoelectric focusing (Mulley, 1980 [11]) and *F13A* and *F13B* typed by agarose gel electrophoresis (Board, 1979, 1980 [3]). Locus designations follow the recommendations of Shows et al. (1979) [13].

## RESULTS

The results of autosomal genetic marker studies are presented in table I. No gene assignments were made. New exclusions or extensions to existing exclusions were determined for the unassigned loci *JK, MNS, LU, GPT, PI, FI3A, FI3B* and *C3*. These may prove useful for regional localisation if subsequent gene assignment is made to the chromosome involved in the exclusion. Although new exclusions were determined for the assigned markers *JK, RH, FY, PGM1, PGM3, AK1, ACP1, GLO1, PGP, ESD* and *HPA*, these were irrelevant for regional localisation as all are assigned to other autosomes.

The *XG* result from the family of the propositus with del(X)(p11→p22) indicated anomalous inheritance involving the proposita (fig. 2). Paternity testing (apart from *XG*) indicated that the probability of correct paternity was 97 %. This family either suggests the localisation of *XG* to (X)(p11→p22), or demonstrates inactivation of the paternal *XG* allele present on the deleted chromosome.

TABLE I. — **List of exclusions for both assigned and unassigned loci from ten cases with autosomal deletions.**

| Patient n° | Segment deleted | Assigned Loci | Unassigned Loci |
|---|---|---|---|
| 1 | (3) (p25→pter) | RH, PGM1, PGP, ACP1, PGM3, HPA | MNS*, JK, GPT, PI |
| 2 | (4) (q27→31) | JK, PGM3, HPA | F13B |
| 3 | (4) (q27→31) | RH, ESD, PGM1 | MNS, JK, PI, C3, F13A |
| 4 | (5) (p14→pter) | AK1, PGM3 | MNS *, LU, |
| 5 | (7) (q22) | ACP1, PGM1 | GPT *, F13A |
| 6 | (12) (p12) | RH, FY, GLO1, PGP | MNS |
| 7 | (12) (p13) and (15) (q15) | PGM1, PGM3, ESD, HPA | GPT, C3 |
| 8 | (12) (pter) and (19) (p or qter) | ACP1, GLO1, ESD, HPA | MNS, GPT, PI |
| 9 | (18) (p11) | HPA* | |
| 10 | (18) (q21→23) | HPA* | MNS, JK, F13A |

* Previously excluded from the segment investigated (Aitken et al., 1975 ; Aitken and Ferguson-Smith, 1978).

## DISCUSSION

The localisation of *ACP1* to the short arm of chromosome 2 (Ferguson-Smith et al., 1973 [7]) remains the only locus to be assigned by deletion mapping. Meanwhile, other unassigned loci have been eliminated from significant regions of the genome : *MNS* being the most notable (Aitken and Ferguson-Smith, 1978 [2]). Additional portions of the genome in the vicinity of assigned markers may be excluded by segregation analysis when the absence of linkage is demonstrated between the unassigned markers and markers previously localised. Recent examples of regional localisations arising from exclusion mapping are *HPA* to (16)(cent→q22) (Ferguson-Smith and Aitken, 1978 [8], *ACP1* to the distal

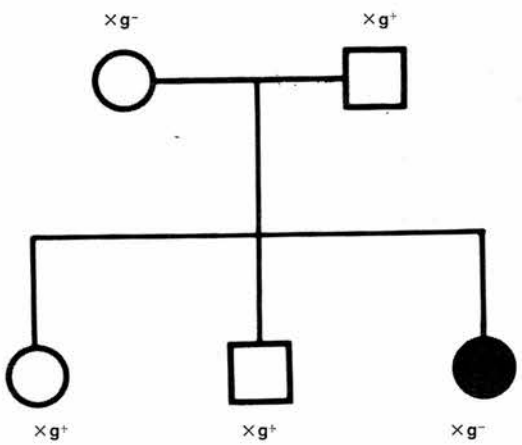



**Fig. 2. — Pedigree showing anomalous XG inheritance in the proposita with caryotype 46,X,del(X)(p11p22).**

portion of band (2)(p23) (Emanuel et al., 1979 [6]) and *ESD* to (13)(q31) or (13)(q32) pending confirmation of a previous assignment to (13)(q3) (Turleau et al., 1978 [14]).

*XG* is not normally inactivated (Fialkow, 1970 [9] ; Fialkow et al., 1970 [10] ; Ducos et al., 1971 [5] unlike other studied X-linked loci which are subject to random inactivation. On this basis, *XG* is apparently localised to (X)(p11→p22) given the anomalous inheritance ; but this conclusion is invalidated by the occurrence of inactivation of *XG* alleles carried on structurally abnormal X chromosomes (Race, 1971 [12]). The alternative explanation for the observed result is the inactivation of the abnormal X of paternal origin.

ACKNOWLEDGEMENTS

We wish to thank Elizabeth Baker, Helen Eyre, Lynene Hinton, Trudy Hocking and Erica Woollatt for tissue culture and technical assistance with cytogenetics, and Janine Van Leeuwen for assistance with blood grouping.

## REFERENCES

1. AITKEN D.A., FERGUSON-SMITH M.A., DICK H.M. Gene mapping by exclusion : the current status, pp. 256-265, Baltimore Conference, 1975.
2. AITKEN D.A., FERGUSON-SMITH M.A. — The exclusion map updated. *Cytogenet. Cell Genet.*, 1978, *22*, 613-617.
3. BOARD P.G. — Genetic polymorphism of the A subunit of human coagulation factor XIII. *Amer. J. Hum. Genet.*, 1979, *31*, 116-124.
4. BOARD P.G. — Genetic polymorphism of the B subunit of human coagulation factor XIII. *Amer. J. Hum. Genet.* 1980, *32*, 348-353.
5. DUCOS J., MARTY Y., SANGER R., RACE R.R. — Xg and X chromosome inactivation. *Lancet*, 1971, *11*, 219-220.
6. EMANUEL B.S., ZACKAI E.H., VAN DYKE D.C., SWALLOW D.M., ALLEN F.H., MELLMAN W.J. — Deletion mapping : further evidence for the localisation of acid phosphatase ($ACP_1$) within 2p23. *Amer. J. Med. Genet.*, 1979, *4*, 167-172.
7. FERGUSON-SMITH M.A., NEWMAN B.F., ELLIS P.M., THOMPSON D.M.G., RILEY I.D. — Assignment by deletion of human red cell acid phosphatase gene locus to the short arm of chromosome 2. *Nat. New Biol.*, 1973, *243*, 271-274.
8. FERGUSON-SMITH M.A., AITKEN D.A. — Heterozygosity at the α-haptoglobin locus associated with a deletion, 16q22→16qter, *Cytogenet. Cell Genet.*, 1978, *22*, 513.
9. FIALKOW P.J. — X-chromosome inactivation and the Xg locus. *Amer. J. Hum. Genet.*, 1970, *22*, 460-463.
10. FIALKOW P.J., LISKER R., GIBLETT E.R., ZAVALA C. — Xg locus : failure to detect inactivation in females with chronic myelocytic leukemia. *Nature*, 1970, *226*, 367-368.
11. MULLEY J.C. — Distribution of six $\alpha_1$-antitrypsin (PI) subtypes from a sample of blood donors. *Aust. J. Exp. Biol. Med. Sci.*, 1980, *58*, 409-414.
12. RACE R.R. — Is the Xg blood group locus subject to inactivation ? pp. 312-314, Proc. 4th Int. Congr. Hum. Genet, Paris, Excerpta Medica, Amsterdam, 1971.
13. SHOWS T.B., ALPER C.A., BOOTSMA D. et al. — Guidelines for human gene nomenclature. *Cytogenet. Cell Genet.*, 1979, *25*, 96-116.
14. TURLEAU C., SÉGER J., GROUCHY J. de, DORÉ F., JOB J.C. — Del(13)(q33). Exclusion de estérase D (ESD) de 13q33 et q34. *Ann. Génét.*, 1978, *21*, 189-192.

# Sister Chromatid Exchange in Aplastic Anemia

**Ram Seshadri, Elizabeth Baker, Peter Leonard, Grant Sutherland, and Alexander A. Morley**

**ABSTRACT:** *The incidence of sister chromatid exchanges (SCE) in the lymphocytes of patients with aplastic anemia (AA) was determined before and after exposure to mitomycin C (MMC). The "baseline" SCE rate was significantly higher in AA, but MMC-induced SCE rate was not different compared to controls. It is suggested that some patients with AA may have an underlying DNA damage.*

Idiopathic aplastic anemia (AA) is a syndrome of diverse etiology. Epidemiological evidence suggests many cases are due to direct chemical toxicity to marrow cells, but the infrequency of development of the disease in the total population at risk suggests the possible existence of some individual predisposition, either genetic or acquired. Our studies of an animal model of the disease and of the observations that lymphocytes of individuals with AA have heightened susceptibility to the action of bleomycin, a cytotoxic agent known to interact with DNA, suggests that injury to marrow cells might be the result of damage to DNA [1].

Although the relationship between sister chromatid exchange (SCE) and DNA damage is still unclear, analysis of SCE has been shown to be a sensitive index of damage to and repair of chromosomal DNA. Recent in vivo studies indicate that the incidence of SCE may be used to detect exposure to potential mutagens and carcinogens [2–4]. In this report we present our observations of the baseline and mitomycin C (MMC)-induced SCE rate in the peripheral blood lymphocytes of individuals with AA.

## METHODS

Thirteen patients with AA were studied. Clinical and hematological data are presented in Table 1. Three patients were studied during the acute phase of the disease, but the rest had recovered to variable extents after supportive care and androgen treatment. The suspected etiological agents in these patients are also shown in Table 1. None of the patients were smokers. The control group consisted of 43 healthy volunteers who had not been on any drugs except for contraceptives in some women. Their ages ranged from 8–80 years.

Whole blood lymphocyte cultures were established in Hams $F_{10}$ tissue culture medium (Commonwealth Serum Laboratories, Melbourne, Australia) supplemented

From the Department of Haematology, Flinders Medical Centre, Bedford Park, South Australia.

*Address requests for reprints to Dr. R. Seshadri, Department of Haematology, Flinders Medical Centre, Bedford Park, South Australia 5042.*

*Received March 27, 1980; accepted June 18, 1980.*





Cancer Genetics and Cytogenetics **3**, 81–84 (1981)
0165-4608/81/01008104$02.50

**Table 1** Clinical data

| Patient No. | Age | Sex | Time since diagnosis | Suspected etiology | Treatment | Hematological findings at the time of study |
|---|---|---|---|---|---|---|
| 1 | 31 | M | 5 years | idiopathic | androgens | normal |
| 2 | 12 | F | 6 months | idiopathic | androgens/steroids | severe pancytopenia |
| 3 | 67 | F | 2 years | idiopathic | androgens | pancytopenia |
| 4 | 31 | F | 1 year | idiopathic | androgens | pancytopenia |
| 5 | 51 | M | 7 years | idiopathic | androgens | thrombocytopenia |
| 6 | 13 | M | 2 years | idiopathic | nil | pancytopenia |
| 7 | 55 | F | 7 years | gold | nil | normal |
| 8 | 48 | F | 1 year | idiopathic | androgens | normal |
| 9 | 77 | M | 8 years | idiopathic | androgens | normal |
| 10 | 46 | F | 4 months | penicillamine | androgens/prednisolone | severe pancytopenia |
| 11 | 8 | F | 2 years | idiopathic | androgens/prednisolone | moderate pancytopenia |
| 12 | 25 | M | 1 year | carbimizole | androgens/prednisolone | moderate pancytopenia |
| 13 | 26 | F | 4 years | idiopathic | nil | thrombocytopenia |

with 20% fetal bovine serum to which 10 $\mu$g/ml of bromodeoxyuridine (Sigma) had been added. Cultures were maintained for 70 hr in the dark and harvested after 2 hr exposure to colchicine. In four patients and controls, SCE rate was estimated after exposing the cells during the last 24 hr of culture to varying concentrations of MMC (Sigma). Differential chromatid staining was done by a modification of the method of Korenberg and Freedlender [5]. Twenty metaphases from each subject were scored for SCE. The chromosome preparations were also analyzed for excessive number of breaks and unusual abnormalities.

## RESULTS AND DISCUSSION

None of the patients showed excessive chromosome breaks or any other specific chromosome abnormality. Table 2 shows the frequencies of SCE in the controls and in AA. The mean baseline frequency of SCE/cell was 10.68 in controls and 12.66 in the AA group. The difference was statistically significant ($p < 0.01$ Wilcoxon's rank sum test). Both control and aplastic patients showed an increase in SCE rate after exposure to MMC. The degree of increase in SCE was not significantly different in the two groups (Table 3).

Our observation supports the previous study that some AA patients may have an underlying DNA abnormality leading to bone marrow failure. Our recent biochemical studies have also shown that lymphocyte DNA in AA is abnormal as indicated by the increased number of strand breaks when compared with age-matched controls [6]. These observations strongly point to an abnormality of DNA, either a propensity for damage or an abnormality of repair. A recent preliminary report by Tsuboyama et al. [7] suggested an abnormality in repair of ultraviolet damage to DNA in AA lymphocytes.

Although the relation between SCE and DNA repair is unclear, analysis of SCE formation has been used to differentiate between inherited diseases with defects in DNA repair. Thus, in Bloom's syndrome an elevated SCE frequency has been observed. In contrast, normal SCE frequencies were detected in Fanconi's anemia, ataxia telangiectasia, and xeroderma pigmentosum (reviewed in [8]). An SCE "stress test" with MMC, developed to probe potential defects in SCE formation, showed a failure of lymphocytes of Fanconi's anemia to respond to MMC with a normal increase in SCE formation [9]. In contrast, lymphocytes from patients with xeroderma pigmento-

**Table 2** Baseline SCE in AA lymphocytes

| Subject | Age (years) | Mean SCE/metaphase |
|---|---|---|
| Aplastic anemia | | |
| 1 | 31 | 12.20 |
| 2 | 12 | 12.35 |
| 3 | 67 | 19.80 |
| 4 | 31 | 12.45 |
| 5 | 51 | 11.65 |
| 6 | 13 | 14.20 |
| 7 | 55 | 13.00 |
| 8 | 48 | 14.25 |
| 9 | 77 | 12.30 |
| 10 | 46 | 10.73 |
| 11 | 8 | 7.55 |
| 12 | 25 | 11.75 |
| 13 | 26 | 12.35 |
| Group mean | 37.7 | 12.66[a] |
| ±2 SD | 43.2 | 5.62 |
| SEM | – | 0.75 |
| Controls ($N = 43$) | | |
| Group mean | 40.1 | 10.68 |
| ±2 SD | 52.9 | 2.36 |
| SEM ± | – | 0.26 |

[a] $p < 0.01$ (Wilcoxon rank sum test); $p < 0.02$ if patient 3 were to be excluded from the analysis.

sum develop a much greater increase in SCE than do identically treated normal cells [10]. We did not find any significant difference between control and AA patients in the "MMC stress test."

Since there is a linear relationship between carcinogen-induced mutation rate and SCE frequency in cultured cells [11, 12], SCE formation may be a useful indicator of those specific and probably rare DNA lesions that are responsible for mutagenesis

**Table 3** MMC-induced SCE in AA lymphocytes

| | | Mean SCE/metaphase | | | |
|---|---|---|---|---|---|
| | | | Concentrations of MMC (μg/ml) | | |
| Subjects | | Baseline[b] | $1 \times 10^{-2}$ | $3 \times 10^{-2}$ | $1 \times 10^{-1}$ |
| AA[a] | 1 | 8.15 | 12.87 | 21.00 | 29.83 |
| | 2 | 13.95 | 15.20 | 23.00 | 32.35 |
| | 3 | 8.87 | – | 23.10 | – |
| | 4 | 7.60 | – | 21.00 | – |
| Controls | 1 | 9.65 | 17.20 | 26.00 | 35.65 |
| | 2 | 10.80 | 15.05 | 27.00 | 39.30 |
| | 3 | 10.05 | – | 27.10 | – |
| | 4 | 9.95 | – | 25.05 | – |

[a] Patient numbers do not correspond to Table 2.

[b] Concentration of bromodeoxyuridine was 5 μg/ml.

and possibly carcinogenesis. Our observation of heightened SCE in AA is of interest, as AA patients have an increased incidence of leukemia compared to the general population [13].

This study was supported by a grant from The Channel 10 Children's Medical Research Foundation of South Australia.

## REFERENCES

1. Morley AA, Seshadri R, Trainor KJ, Sorrell J (1978): Is aplastic anemia due to alteration of DNA? Lancet 2, 9–11.
2. Stetka DG, Wolff S (1976): Sister chromatid exchange as an assay for genetic damage induced by mutagen-carcinogens. I. In vivo test for compound requiring metabolic activation. Mutat Res 41, 333–342.
3. Marquardt H, Bayer U (1977): The induction in vivo of sister chromatid exchanges in the bone marrow of the Chinese hamster. I. The sensitivity of the system (Methyl methane sulphonate). Mutat Res 56, 169–176.
4. Boszinsky-Köcher G, Basler A, Röhrborn G (1979): Mutagenicity of polycyclic hydrocarbons. V. Induction of sister-chromatid exchanges in vivo. Mutat Res 66, 65–67. 65–67.
5. Korenberg JR, Freedlender EF (1974): Giemsa technique for the detection of sister chromatid exchanges. Chromosoma (Berl) 48, 355–360.
6. Turner D, Morley AA, Sorrell J, Seshadri R (1980): Lymphocyte DNA in aplastic anemia. manuscript in preparation.
7. Tsuboyama A, Takaku F, Sakamoto S, Kano Y (1979): DNA repair synthesis in lymphocytes from patients with aplastic anemia, personal communication.
8. Wolff S (1977): Sister chromatid exchange. Annu Rev Genet 11, 183–201.
9. Latt SA, Stetten G, Juergens LA, Buchanan GR, Gerald PS (1975): Induction by alkylating agents of sister chromatid exchange and chromatid breaks in Fanconi's anemai. Proc Natl Acad Sci USA 72, 4066–4070.
10. Wolff S, Rodin A, Cleaver JE (1977): Sister-chromatid exchanges induced by carcinogens and mutagens in xeroderma pigmentosum cells. Nature 265, 347–348.
11. Carrano AV, Thompson LH, Lindl PA, Minkler JL (1978): Sister chromatid exchange as an indicator of mutagenesis. Nature 271, 551–553.
12. Bradley MO, Hsu CI, Harris CC (1979): Relationship between sister chromatid exchange and mutagenicity, toxicity and DNA damage. Nature 282, 318–320.
13. Pierre RV (1974): Preleukaemic states. Semin Hematol 11, 73–92.

*Clinical Genetics* 1981: **19**: 331–334

# Two unusual G-band variants of the short arm of chromosome 9

GRANT R. SUTHERLAND AND HELEN EYRE
Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, Australia

Two familial G-band variants of the short arm of chromosome 9 are described. One is associated with C-band material, but the other is not and appears to involve euchromatin.

*Received 23 October 1980, accepted for publication 10 February 1981*

*Key words:* Chromosome 9; G-banding; normal variant.

Structural variation of chromosome no. 9 has been recognized for many years, and with the advent of chromosome banding techniques this variation has been shown to be due mainly to the amount and position of the C-band material (Madan & Bobrow 1974). Buckton et al. (1980) reported a variant of the short arm of chromosome 9 in which there was an additional dark band proximal to the centromere in the short arm and which did not involve C-band material.

This report describes two families, ascertained when a phenotypically abnormal child referred for chromosome studies was found to have an additional dark G-band in the short arm of chromosome 9. The variant chromosomes were initially suspected of being pathologically significant, but were shown by family studies to be harmless variants.

## Materials and Methods

Cytogenetic studies were carried out using standard lymphocyte culture methods. C-banding was performed on aged slides using saturated barium hydroxide, followed by incubation in 2 × SSC and staining with Leishmann. The G-banding on freshly prepared slides was carried out using a hydrogen peroxide treatment prior to trypsinization, followed by staining with Leishmann. Replication banding was carried out after BrdU incorporation for 6 h prior to harvest, followed by staining with Hoechst 33258, exposure to UV light and staining with Leishmann.

*Case 1.* A 13-month-old female infant was referred for chromosome studies because of spastic quadriplegia and microcephaly. There was a well-documented history of intrauterine and perinatal hypoxia. Her no. 9 chromosomes are shown in Figure 1a. Chromosome studies were carried out on other members of the family (Fig. 2a) and several were found to have apparently the same variant (Fig. 1b). All other chromosomes were normal by C- and G-banding.

*Case 2.* A 15-month-old female infant with developmental delay, hypotonic cerebral palsy and abnormal cervical vertebrae was referred for chromosome studies. Her no. 9 chromosomes are shown in Figure 1c.

0009–9163/81/050331–04$02.50/0 

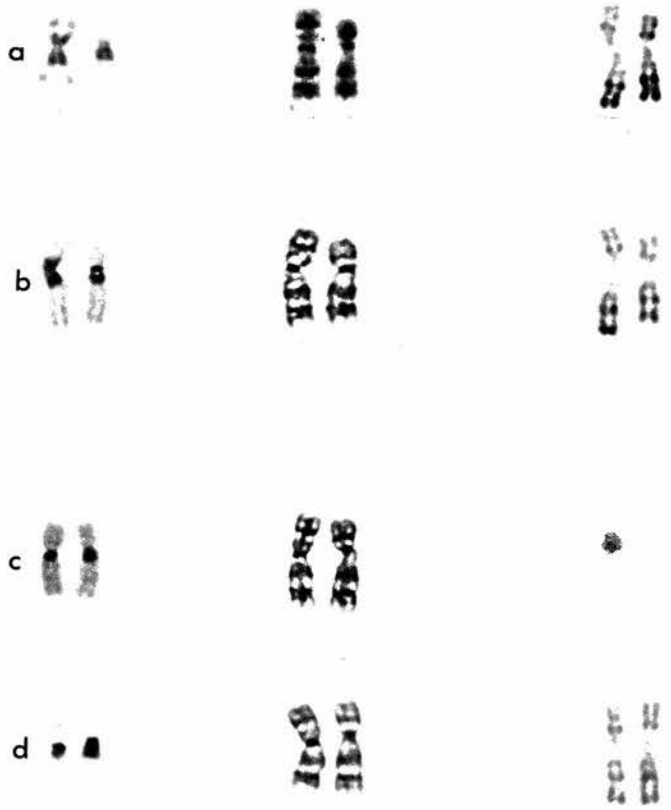



**Fig. 1.** C-banded, G-banded and BrdU labelled chromosome no. 9 pair from (a) the proposita, and (b) a phenotypically normal member of Family 1; (c) the proposita, and (d) a phenotypically normal member of Family 2. The variant is on the left of each pair.

Family studies (Fig. 2b) showed that other normal individuals had karyotypes identical to that of the proposita (Figs. 1d and 3).

*Cytogenetics*

On G-banding, the variant chromosomes from both families had an additional dark band inserted into the middle of band 9p13. This appeared as an additional light band on replication banding. This extra material gave the variant chromosome a more metacentric appearance than the usual no. 9 chromosome, and the initial impression was that it was possibly associated with an inversion of C-band material.

C-band staining revealed an average amount of heterochromatin, which was confined to the long arm of the variant chromosome in Family 2. In this family the additional material was undoubtedly C-band negative. In Family 1 there was a large C-band with the centromere in the middle of it. The C-band extended over the position of the dark G-band; hence, in this family the variant dark G-band was also C-band positive.

## Discussion

The variation in the size of chromosome 9 has been long recognized, originally as a secondary constriction of variable size (Fer-

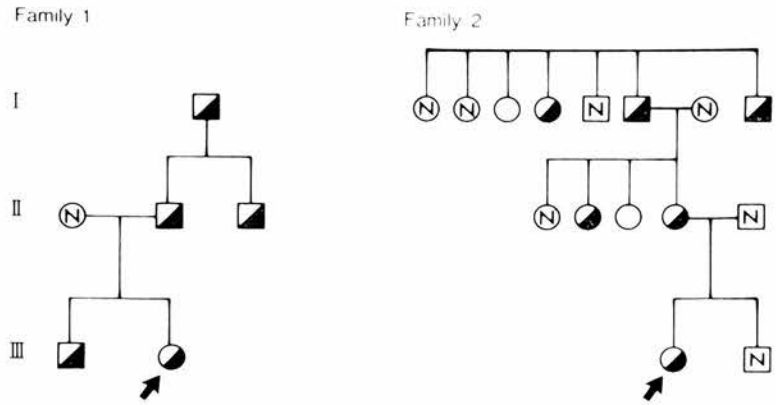



**Fig. 2.** Family pedigrees. Shaded symbols indicate presence of variant no. 9; N indicates its absence.

guson-Smith et al. 1962). The presence of the C-band heterochromatin in the short arm of 9, as opposed to the long arm, the so-called pericentric inversion of C-band heterochromatin, was demonstrated by Wahrman et al. (1972) and shown to be a not uncommon variant (Buckton et al. 1976, 1980). Madan & Bobrow (1974) demonstrated that the size of the C-band heterochromatin was highly variable and could range from very small to greater than the entire length of the short arm of

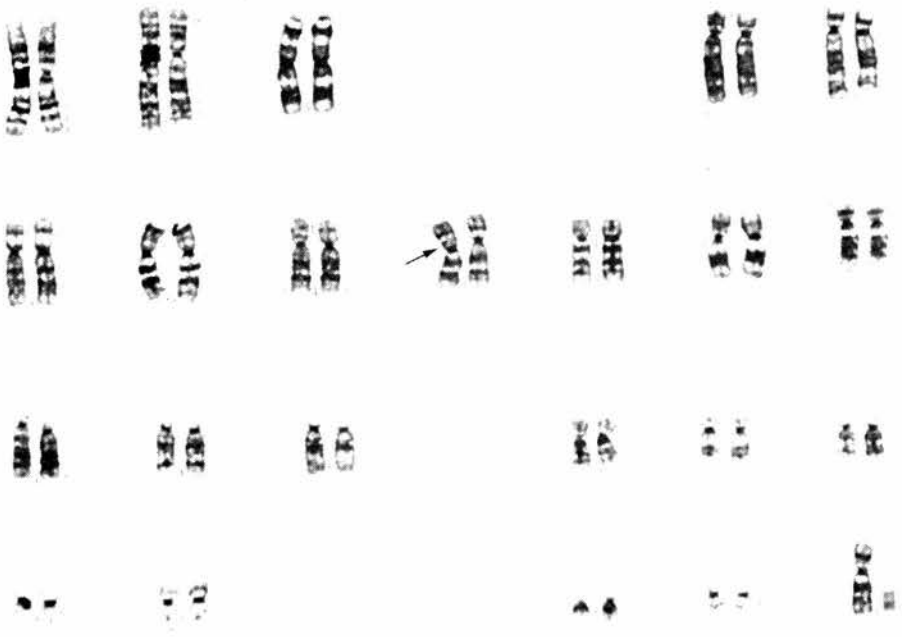


**Fig. 3.** G-banded karyotype from the father of the proposita of Family 2; the variant no. 9 is arrowed.

this chromosome in apparently normal individuals. Hence, variation in chromosome 9 has been well established, but it is due almost entirely to variations in the amount and position of C-band heterochromatin. Madan (1978) recorded a G-band variant of the long arm of chromosome 9 which was within the region which is normally dark-staining on C-banding; this was seen only in individuals with large C-bands. In the first family in the present report, it would appear that a similar type of variant is segregating; that is, a segment of a large C-band is also expressed as a dark G-band. Another G-band variant of chromosome 9 has been described by Berg et al. (1980). On G-banding it was similar to the present variants, but on C-banding it was different, although the dark G-band was C-band positive, as in Family 1.

The G-band variant of the short arm of chromosome 9 in the second family in this report appears to be very similar to that recorded by Buckton et al. (1980). This variant chromosome would appear to be the only one involving chromosome 9 which is not associated with C-band material. It is important in clinical cytogenetics that this variant be recognized as such, since the presence of additional autosomal material, which is apparently euchromatic, is generally associated with phenotypic abnormality.

#### Acknowledgment

We thank Elizabeth Baker for assistance with the cytogenetics.

#### References

Berg, J. M., H. A. Gardner, R. J. M. Gardner, E. G. Goh, V. D. Markovic, N. E. Simpson & R. G. Worton (1980). Dic(21;21) in a Down's syndrome child with an unusual chromosome 9 variant in the mother. *J. med. Genet.* **17**, 144–148.

Buckton, K. E., M. L. O'Riordan, P. A. Jacobs, J. A. Robinson, R. Hill & H. J. Evans (1976). C- and Q-band polymorphisms in the chromosomes of three human populations. *Ann. hum. Genet.* **40**, 99–112.

Buckton, K. E., M. L. O'Riordan, S. Ratcliffe, J. Slight, M. Mitchell, S. McBeath, A. J. Keay, D. Barr & M. Short (1980). A G-band study of chromosomes in liveborn infants. *Ann. hum. Genet.* **43**, 227–239.

Ferguson-Smith, M. A., M. E. Ferguson-Smith, P. M. Ellis & M. Dickson (1962). The sites and relative frequencies of secondary constrictions in human somatic chromosomes. *Cytogenetics* **1**, 325–343.

Madan, K. (1978). An extra band in human 9qh+ chromosomes. *Hum. Genet.* **43**, 259–264.

Madan, K. & M. Bobrow (1974). Structural variation in chromosome No. 9. *Ann. Genet.* **17**, 81–86.

Wahrman, J., J. Atidia, R. Goitein & T. Cohen (1972). Pericentric inversions of chromosome 9 in two families. *Cytogenetics* **11**, 132–144.

Address:

*Grant R. Sutherland, Ph.D.*
*Cytogenetics Unit*
*Department of Histopathology*
*The Adelaide Children's Hospital Inc.*
*North Adelaide, S. A. 5006*
*Australia*

# A LARGE KINDRED WITH AN INV(3)(p25q23) : CLINICAL, CYTOGENETIC AND GENETIC MARKER STUDIES

G.R. SUTHERLAND, J.C. MULLEY, E. GOLDBLATT

# A LARGE KINDRED WITH AN INV(3)(p25q23) : CLINICAL, CYTOGENETIC AND GENETIC MARKER STUDIES

G.R. SUTHERLAND[1], J.C. MULLEY[1], E. GOLDBLATT[2]

SUTHERLAND G.R., MULLEY J.C., GOLDBLATT E. — A large kindred with an inv(3)(p25q23) : clinical, cytogenetic and genetic marker studies.
**Ann. Génét.**, 1981, **24**, n° 4, 202-205.

SUMMARY : A large kindred in which an inv(3)(p25q23) is segregating is described. At least two malformed children with the recombinant chromosome rec(3)dup(q23→qter)del(p25→pter) have been produced, both of whom have the characteristic trisomy 3q syndrome. Genetic marker studies showed that **PI** and **GM** are not linked to the inversion break points. The **PI-GM** linkage group has been excluded from much of chromosome 3 by deletion mapping and linkage analysis of this kindred.

KEY-WORDS : Chromosome 3. — Pericentric inversion. — Recombinant chromosome. — Genetic markers.

SUTHERLAND G.R., MULLEY J.C., GOLDBLATT E. — Une grande famille avec une inv(3)(p25q23) : études clinique, cytogénétique, et des marqueurs génétiques. *(In English).*
**Ann. Génét.**, 1981, **24**, n° 4, 202-205.

RÉSUMÉ : Les auteurs décrivent une grande famille dans laquelle ségrègue une inv(3)(q25q23). Au moins deux enfants malformés avec le chromosome recombiné rec(3)dup(q23→qter)del(p25→ter) sont nés l'un et l'autre avec le phénotype caractéristique de la trisomie 3q. Une étude des marqueurs génétiques a montré que **PI** et **GM** ne sont pas liés au point de cassure de l'inversion. Le **PI-GM** groupe de linkage a été exclu d'une grande partie du chromosome 3 par une analyse de linkage de l'utilisation des délétions dans cette famille.

MOTS-CLÉS : Chromosome 3. — Inversion péricentrique. — Chromosome recombiné. — Marqueurs génétiques.

## INTRODUCTION

A number of large pericentric inversions of chromosome 3 have been ascertained via malformed children with recombinant chromosomes (Hirschhorn et al., 1973 ; Fineman et al., 1978 ; Rivera et al., 1979 ; Kawashima and Maruyana, 1979). One large kindred has been described in which such an inversion has resulted in more than 20 recombinants or presumed recombinants (Allderdice et al., 1975). The present report documents another large kindred which was ascertained via a recombinant child in South Australia in 1978. This kindred was independently ascertained in Western Australia and the phenotype of the recombinant described (Mulcahy et al., 1979). The clinical features of the South Australian propositus are recorded, and cytogenetic and genetic marker studies of the family presented.

## CASE REPORT

The propositus was the second child born to healthy unrelated parents. Delivery was by caesarean section at 38 week's gestation and birth weight was 3.01 kg. The appearance was unusual (fig. 1) and the following features were noted on examination : slanting, curved palpebral fissures, an unusual nose, high arched palate, micrognathia, odd ears with pits on the helices, redundant skin folds on the back of the neck, inverted nipples, bilateral simian creases, right talipes calcaneo-valgus, left ectopic and right undescended testes. Investigations at the age of three months showed a hypertonic severely retarded infant, with a clinically diagnosed ventricular septal defect and probably atrial septal defect. Intravenous pyelography showed a dysplastic right kidney with dilated ureter. Radiography revealed retarded skeletal maturity, closed or closing sagittal suture, scapho-

1. *Cytogenetics Unit, Department of Histopathology ;*
2. *Department of Cardiology,*
*The Adelaide Children's Hospital Inc., NORTH ADELAIDE, S.A. 5006 (Australia).*

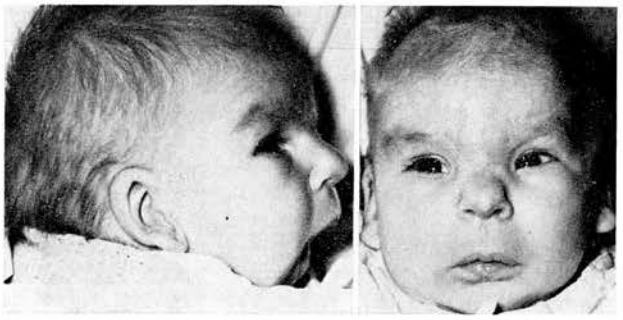


**Fig. 1. — The propositus.**

cephaly, mild cervical and sacrococcygeal kyphosis, hypoplastic third right rib and a mildly enlarged heart. The child died in another hospital at 22 months of age; an autopsy was not performed.

### Cytogenetics

G-banded chromosome studies (fig. 2) showed that the propositus had a recombinant chromosome rec(3)dup(q23→qter)del(p25→pter) resulting from a paternal inv(3)(p25q23).

### Family studies

Family studies (fig. 3) showed that the inv(3) chromosome was present in many other members of the family. A number of stillbirths and neonatal deaths had occurred, but records were inadequate to indicate whether or not these were due to recombinant chromosomes.

### Genetic marker studies

The blood groups *LU*, *MNS*, *JK*, *ABO*, *RH*, *FY*, *K* (two loci) and *P*, and enzyme and protein polymorphisms *GALT*, *GPT*, *PGP*, *ACP1*, *AK1*, *ADA*, *GLO1*, *PGD*, *ESD*, *PGM1*, *HPA*, *E2*, *AMY2* and *GM* were determined using standard serological and electrophoretic techniques. Genetic marker results from the propositus and the *GM* results from infants aged less than six months were not used for linkage analysis. *PI* was completely subtyped by isoelectric focusing (Mulley, 1980) and *F13A* and *F13B* typed by agarose electrophoresis (Board, 1979 and 1980). In no instance were any of the genetic marker results inconsistent with Mendelian inheritance or paternity. The results of the genetic marker studies which were informative are shown in table I and the corresponding lod scores in table II.

TABLE I. — **Informative phenotypes for segregation analysis with the marker inv(3)(p25q23).**

| Subject | | Locus | | |
|---|---|---|---|---|
| | | PI | GALT | GM |
| II | 10 | M1 | — | 1,2,3,5 |
| III | 34 | M1-M3 | N—D | 1,2 (*) |
| Husband of | 34 | M1-M2 | N | 3,5 |
| IV | 23 | M2-M3 | N—D | 1,3,5 |
| | 24 | M1 | — | 1,3,5 |
| | 25 | M1-M3 | — | 1,2,3,5 |
| | 26 | M1 | — | 1,3,5 |
| V | 13 | M1-M2 | N | 3,5 |

(*) Phenotypes of offspring indicate genotype to be GM 1/1,2.

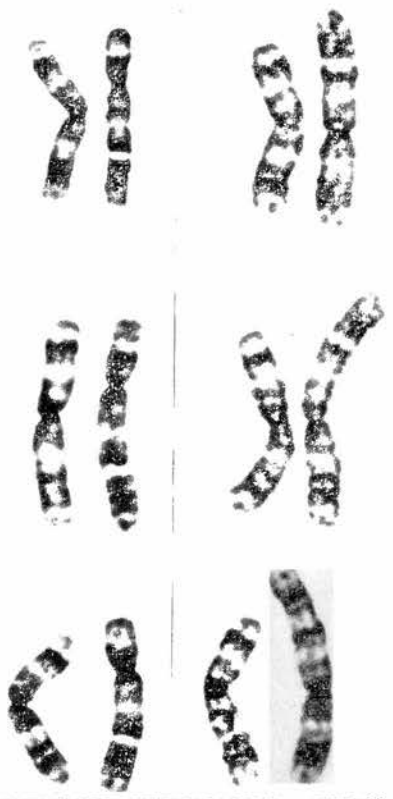


**Fig. 2. — G-banded chromosomes from three metaphases of the propositus (right) showing the recombinant chromosome, and his father (left) showing the inverted chromosome.**

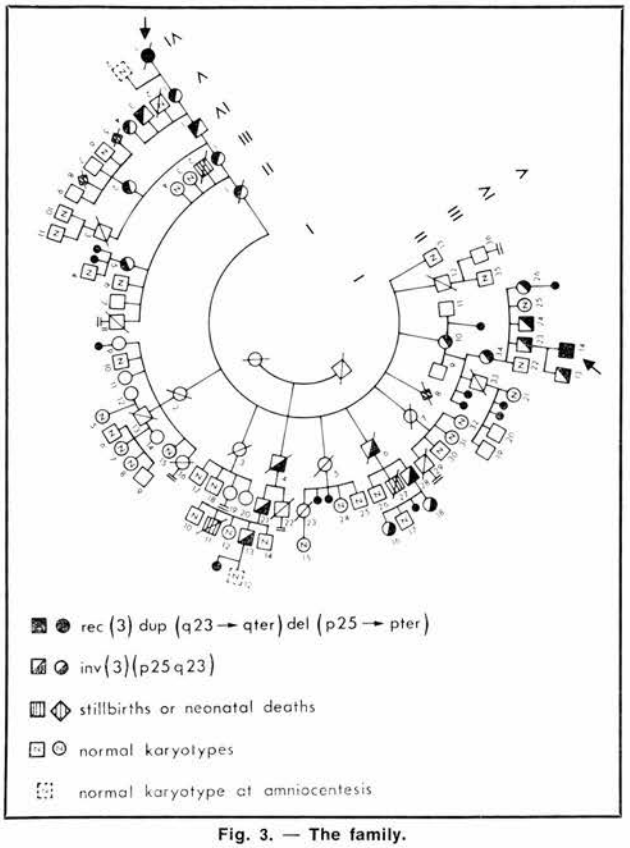



**Fig. 3. — The family.**

TABLE II. — **Lod scores between the inversion and PI, GM and GALT.**

| | θ | | | | | | |
|---|---|---|---|---|---|---|---|
| | .01 | .05 | .10 | .15 | .20 | .25 | .30 |
| **PI** | —2.508 | —1.164 | —0.632 | —0.355 | —0.184 | —0.074 | —0.005 |
| **GM** | —8.495 | —5.000 | —3.495 | —2.615 | —1.990 | —1.505 | —1.109 |
| **GALT** | —3.398 | —2.000 | —1.398 | —1.046 | —0.796 | —0.602 | —0.444 |

## DISCUSSION

The phenotype of the propositus is typical of the syndrome produced by partial trisomy 3q, either as a result of recombination within an inversion or malsegregation of a balanced translocation involving this chromosome segment (Mulcahy et al., 1979). The risk of inversion carriers producing recombinant offspring has been previously discussed (Sutherland et al., 1976). In a more recent review Winsor et al. (1978) concluded that the risk of an abnormal child resulting from recombination within a pericentric inversion was greater for « large » inversions than for « small » ones. In the present kindred it is not possible to determine such a risk because of uncertainty about the number of recombinants in the family. On the basis of medical records III.27 was almost certainly a recombinant, and VI.1 was documented as such by Mulcahy et al. (1979). Since the family was investigated one pregnancy has been monitored by amniocentesis (V.12) and shown to have a 46,XY karyotype and confirmed after birth as a normal male infant. This is in addition to the pregnancy monitored antenatally by Mulcahy et al. (1979) in Western Australia (VI.2). Allderdice and Frecker (1979) found two recombinants among eight pregnancies monitored antenatally, where one parent was a member of their inv (3) kindred.

Large inversions are potentially valuable as markers for gene mapping by exclusion when offspring with duplication/deficiency chromosomes are available. Mulley et al. (1980) excluded the unassigned loci *JK*, *GPT* and *PI* from (3) (p25→pter) by deletion mapping of the propositus. Furthermore, the lod scores between *PI* and the inversion indicate that close linkage of this locus to 3p25 or 3q23 is unlikely. Similarly, lod scores between *GM* and the inversion suggest that all but loose linkage with 3p25 or 3q23 is unlikely. In view of the loose linkage between *PI* and *GM* (Noades and Cook, 1976) this linkage groupe is excluded from most of chromosome 3 on the basis of deletion mapping and linkage analysis within this family. The only other significant lod score was that for *GALT* and the inversion if II.10 was assumed to have the phenotype GALT N, a fair assumption given the absence of other *GALT* alleles in 16 of her nieces and nephews tested. This is, however, of minimal significance now that *GALT* has been assigned to chromosome 9 (Sparkes et al., 1980).

In natural populations where pericentric inversions are present as adaptive polymorphisms there is no recombination within the inverted segments in structural heterozygotes (White and Morley, 1955). However, newly arisen inversions in insects (White and Morley, 1955 ; White, 1961) and in man do not behave in this fashion. Renwick (1971) has considered the theoretical use of inversions for gene mapping. Recombination within the inversion is reduced, especially between inversion breakpoints and markers within small inversions. For large inversions in man recombination can occur within the inverted segment as a result of two-strand double crossovers at meiosis. Van der Linden et al. (1975) observed three out of 40 inversion loops resulting from an inv (4) which they interpreted at diakinesis as having two crossovers within the inverted sequence. However, according to Haldane (1931), « ...if crossing over occurs in any section, the probability of crossing over in adjacent sections is reduced... », hence any inversion would need to be of a minimum size before more than one chiasma would occur within it. If it were possible to determine the minimum size of an inversion necessary for two-strand double crossovers then inversions below this size would be very useful for gene mapping since the observation of a single event of recombination between such an inversion and a gene locus would exclude that locus from within the inversion.

ACKNOWLEDGEMENTS

We thank Helen Eyre, Lynene Hinton, Trudy Hocking and Erica Woollatt for technical assistance, the parents of the propositus for their help with the family studies, Dr M.T. Mulcahy for details of the branch of the family in Western Australia, Dr D.N. Propert for the *GM* typing, Dr P.J.L. Cook for particularly helpful comments on the use of inversions for gene mapping and Pr M.J.D. White for his constructive criticism of a draft of this paper.

### REFERENCES

1. ALLDERDICE P.W., FRECKER M. — Karyotypic results for eight inv(3)(p25q21) carriers requesting prenatal diagnosis. (Abstract). *Amer. J. Hum. Genet.*, 1979, *31*, 86 A.
2. ALLDERDICE P.W., BROWNE N., MURPHY D.P. — Chromosome 3 duplication q21→qter deletion p25→pter syndrome in children of carriers of a pericentric inversion inv(3)(p25q21). *Amer. J. Hum. Genet.*, 1975, *27*, 699-718.
3. BOARD P.G. — Genetic polymorphism of the A subunit of human coagulation factor XIII. *Amer. J. Hum. Genet.*, 1979, *31*, 116-124.
4. BOARD P.G. — Genetic polymorphism of the B subunit of human coagulation factor XIII. *Amer. J. Hum. Genet.*, 1980, *32*, 348-353.
5. FINEMAN R.M., HECHT F., ABLOW R.C., HOWARD R. O., BREG W.R. — Chromosome 3 duplication q/deletion p syndrome. *Pediatrics*, 1978, *61*, 611-618.

6. HALDANE J.B.S. — The cytological basis of genetical interference. *Cytologia,* 1931, *3,* 54-65.
7. HIRSCHHORN K., LUCAS M., WALLACE I. — Precise identification of various chromosomal abnormalities. *Ann. Hum. Genet. (Lond.),* 1973, *36,* 375-379.
8. KAWASHIMA H., MARUYANA S. — A case of chromosome 3 duplication q deletion p syndrome born to the mother with a pericentric inversion, inv(3)(p25q21). *Jap. J. Hum. Genet.,* 1979, *24,* 9-12.
9. MULCAHY M.T., PEMBERTON P.J., SPRAGUE P. — Trisomy 3q : two clinically similar but cytogenetically different cases. *Ann. Génét.,* 1979, *22,* 217-220.
10. MULLEY J.C. — Distribution of the six $\alpha^1$-antitrypsin (PI) subtypes from a sample of blood donors. *Aust. J. Exp. Biol. Med. Sci.,* 1980, *58,* 409-414.
11. MULLEY J.C., BRYANT G.D., SUTHERLAND G.R. — Additions to the exclusion map of man. *Ann. Génét.,* 1980, *23,* 4, 198-200.
12. NOADES J.E., COOK P.J.L. — Family studies with the *Gm-Pi* linkage group. *Cytogenet. Cell Genet.,* 1976, *16.* 341-344.
13. RENWICK J.H. — Assignment and map position of human loci using chromosomal variation. *Ann. Hum. Genet. (Lond.),* 1971, *35,* 79-97.
14. RIVERA H., HERNANDEZ A., ROLON A., IBARRA B., ALCARAZ A., CANTU J.M. — Familial pericentric inversion (3)(p14q24). *Ann. Génét.,* 1979, *22,* 1, 43-46.
15. SPARKES R.S., EPSTEIN P.A., KIDD K.K., KLISAK I., SPARKES M.C., CRIST M., MORTON L.A. — Probable linkage between the human galactose-1-P uridyl transferase locus and 9qh. *Amer. J. Hum. Genet.,* 1980, *32,* 188-193.
16. SUTHERLAND G.R., GARDINER A.J., CARTER R.F. — Familial pericentric inversion of chromosome 19, inv(19)(p13q13) with a note on genetic counselling of pericentric inversion carriers. *Clin. Genet.,* 1976, *10,* 54-69.
17. VAN DER LINDEN A.G., PEARSON P.L., VAN DE KAMP J.J. — Cytological assessment of meiotic exchange in a human male with a pericentric inversion of chromosome n° 4. *Cytogenet. Cell Genet.,* 1975, *14,* 126-139.
18. WHITE M.J.D. — Cytogenetics of the grasshopper *Moraba scurra.* VI. A spontaneous pericentric inversion. *Aust. J. Zool.,* 1961, *9,* 784-790.
19. WHITE M.J.D., MORLEY F.H.W. — Effects of pericentric rearrangements on recombination in grasshopper chromosomes. *Genetics,* 1955, *40,* 604-619.
20. WINSOR E.J.T., PALMER C.G., ELLIS P.M., HUNTER J.L.P., FERGUSON-SMITH M.A. — Meiotic analysis of a pericentric inversion, inv(7)(p22q32), in the father of a child with a duplication-deletion of chromosome 7. *Cytogenet. Cell Genet.,* 1978, *20,* 169-184.

*Mutation Research*, 97 (1982) 139–146
Elsevier Biomedical Press



# Sister-chromatid exchange (SCE) analysis in mothers exposed to DNA-damaging agents and their newborn infants

Ram Seshadri [1], Elizabeth Baker [2] and Grant R. Sutherland [2]*

[1] *Department of Haematology, Flinders Medical Centre, Bedford Park, S.A. 5042 and* [2] *Cytogenetics Unit, Adelaide Children's Hospital, North Adelaide, S.A. 5006 (Australia)*

(Received 2 June 1981)
(Revision received 11 August 1981)
(Accepted 14 August 1981)

**Summary**

The incidence of SCE in the lymphocytes of mothers and their newborn infants was determined. A detailed antenatal history of parental habits such as smoking, alcohol consumption and possible exposure to DNA-damaging agents was documented. The results showed that the SCE rate in the newborn is significantly less than that of their mothers. Mothers who consumed alcohol, but not cigarette smokers, had a significantly increased SCE rate compared to control mothers. However, these maternal habits did not affect the SCE rate of their infants. Neonates with neural tube defects showed a significantly increased SCE rate compared to normal babies.

---

Sister-chromatid exchange (SCE) represents the reciprocal interchange of DNA between chromatids at apparently homologous loci. Although the precise nature and mechanism involved in the formation of SCE are unknown, SCE analysis has come into use as a sensitive means of detecting DNA damage. SCE analysis has been shown to be useful for assessing the affect upon DNA of cytotoxic drugs (Perry and Evans, 1975; Nakanishi and Schneider, 1979), viruses (Kurvink et al., 1978) and environmental pollutants in man (Crossen et al., 1978).

The exposure of the fetus to agents which damage DNA might result in birth defects or cancer. Kram et al. (1979) showed SCE analysis to be a sensitive method for the detection of the mutagenic affects of cyclophosphamide, mitomycin C and adriamycin in female mice and their fetuses. Funes-Cravioto et al. (1977) observed

* Reprint requests.

0165-1161/82/0000–0000/$02.75 

that children of mothers occupationally exposed to organic solvents had an increased SCE rate implying thay they had been exposed in utero to DNA-damaging agents. The present study was undertaken to determine whether SCE analysis of mothers and their neonates would detect in utero exposure of the fetus to DNA-damaging agents and to determine whether neonates with birth defects of unknown aetiology may have been exposed to DNA-damaging agents.

## Materials and methods

*Study subjects*

Mothers for this study were chosen from the antenatal clinics of a large maternity hospital and a detailed interview was conducted prior to delivery. The following parental data were recorded: smoking habits, use of drugs, alcohol, coffee, hair dyes, details of the environment in which the mother lived during the antenatal period and details of acute or chronic illness. At the time of delivery details of the procedures involved and neonatal observations such as the nature of anaesthetic agents used, obstetric complications, Apgar scores, birth weight and presence of birth defects were recorded. The data-collection interviews were conducted by a trained interviewer and the information was not available to the investigators until after SCE scoring had been completed. Cord-blood samples were collected from neonates, and venous samples from their mothers within a few hours of delivery.

Samples were also obtained from babies with multiple birth defects, neural tube defects and Down syndrome; in some of these instances the maternal history and blood samples were not available. A control group of 20 healthy adults (age range 21–40 years, mean 29.9 years) and a group of 19 healthy children aged 10 years or less (age range 1–10 years, mean 7.7 years) were also studied. No members of these groups were on drugs (except for oral contraceptives in some women) or smoked cigarettes.

*Cytogenetics*

Preparations suitable for SCE determination were made as previously described (Seshadri et al., 1981). All SCE counts were made by one observer from coded slides. 20 metaphases were scored from each subject; exchanges at the centromere were included in the count (Tice et al., 1975).

*Control mother and baby groups*

The control population of 30 mothers (age range 17–38 years, mean 29.4 years) were those who had a normal antenatal period, normal delivery and normal healthy infants. None of these mothers were smokers or used hair dyes. 11 of the mothers had a history of alcohol consumption during the antenatal period but the amount consumed was small and the frequency less than once a week. 15 of the mothers had general anaesthetics during delivery. Mothers included in this control group lived within the Adelaide metropolitan area and to the best of their knowledge had no other exposure to environmental pollutants. All babies included in this control group had normal birth weights, gestations, physical findings and karyotypes.

*Definition of smokers*

This group of 23 mothers (age range 17–43 years, mean 23.3 years) were similar to the control group except that they smoked cigarettes during the antenatal period. The degree of smoking ranged from 5 to more than 15 cigarettes per day. The alcohol consumption of these mothers was similar to the control group. The babies in this group were clinically normal.

*The "alcoholic" mother group*

This group consisted of 9 mothers (age range 17–35 years, mean 23.2 years) who had alcohol consumption ranging from moderate amounts daily to abuse. All these mothers smoked more than 15 cigarettes a day and many had used marijuana, LSD and other illicit narcotics. Only 2 of these mothers were regarded clinically as alcoholics. All infants born to this group of mothers were clinically normal.

*Miscellaneous groups*

13 mothers had various chronic problems such as mental retardation, diabetes, malignancy and cardiac problems. 7 mothers were epileptics who had been on anticonvulsants during pregnancy. All the infants born to the mothers in these groups were clinically normal.

## Results

Figs. 1 and 2, and Table 1, summarise the data on the number of sister-chromatid exchanges observed per mitotic figure in the groups studied. The SCE rates in smoker mothers and normal age matched controls were not different from each other. The "alcoholic" mothers had an SCE rate which was significantly higher than

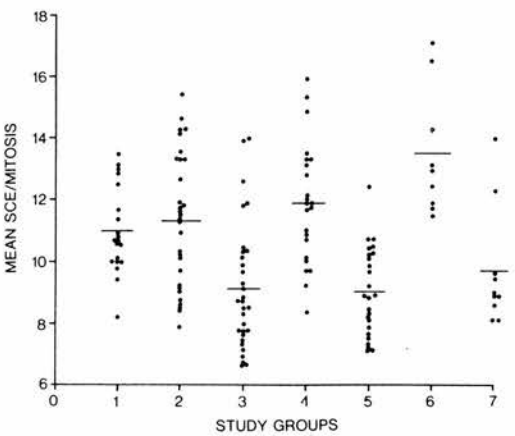



Fig. 1. Mean SCE rate per mitosis in the following study groups. (1) Normal adults. (2) Normal mothers. (3) Normal babies (cord blood). (4) Smoker mothers. (5) Babies born to smoker mothers. (6) "Alcoholic" mothers. (7) Babies born to the "alcoholic" mothers.

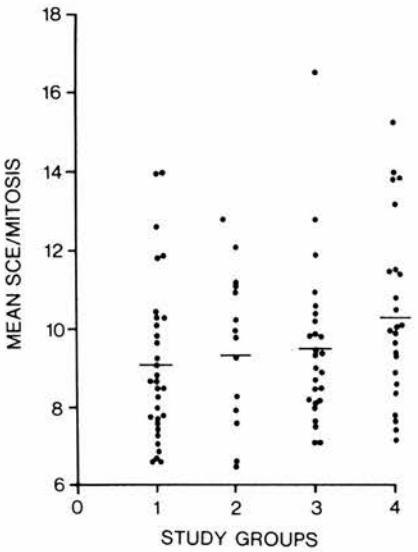



Fig. 2. Mean SCE rate per mitosis in babies. (1) Normal babies. (2) Babies with Down syndrome. (3) Babies with multiple congenital malformations. (4) Babies with neural tube defects.

the above groups (Wilcoxon rank sum test, $p < 0.01$). The SCE rates of babies born to the control mothers, the smoking mothers and the "alcoholic" mothers were not significantly different from one another.

Table 2 shows the SCE rates in mothers with chronic illnesses and their babies.

TABLE 1

DATA ON SCE IN THE GROUPS STUDIED

| Group | No. | Mean SCE per cell | S.E.M. |
|---|---|---|---|
| *Adults* | | | |
| Controls | 20 | 10.95 | 0.32 |
| Control mothers | 30 | 11.35 | 0.40 |
| Smoker mothers | 23 | 11.88 | 0.41 |
| Alcoholic mothers | 9 | 13.5 | 0.23 |
| *Babies* | | | |
| Control mothers' babies | 30 | 8.95 | 0.37 |
| Smoker mothers' babies | 25 | 9.01 | 0.28 |
| "Alcoholic" mothers' babies | 10 | 9.71 | 0.61 |
| Multiple congenital malformations | 26 | 9.50 | 0.39 |
| Neural tube defects | 24 | 10.34 | 0.46 |
| Down syndrome | 13 | 9.37 | 0.50 |
| *Children* | | | |
| 1–20 years, normal | 19 | 9.23 | 0.33 |

TABLE 2

SCE DATA ON MOTHERS WITH CHRONIC ILLNESS AND THEIR INFANTS

| Age | Disorder | Treatment [a] | Number of cigarettes smoked | Alcohol intake | Mean SCE/mitosis | |
|---|---|---|---|---|---|---|
| | | | | | in mother | in baby |
| 24 | Epilepsy | Dilantin and Tegretol | Nil | Nil | 9.45 | 15.05 [b] |
| 19 | Epilepsy | Tegretol | Nil | Nil | 11.50 | 9.05 |
| 23 | Epilepsy | Dilantin | Nil | Nil | 11.20 | 10.00 |
| 23 | Epilepsy | Dilantin | Nil | Nil | 11.35 | 8.80 |
| 19 | Epilepsy | Dilantin | <5/day | Small | 9.13 | 7.20 |
| 22 | Epilepsy | Dilantin | <15/day | Small | 10.85 | 12.35 |
| 30 | Epilepsy | Mysolin | Nil | Moderate | 9.05 | 6.65 |
| 17 | Mental retardation | Nil | Nil | Nil | – | 10.45 |
| 23 | Mental retardation | Nil | Nil | Nil | 8.13 | 8.60 |
| 32 | Psychiatric | Phenothiazine | Nil | Small | 7.53 | 7.35 |
| 21 | Psychiatric | Phenothiazine | >15/day | Small | 10.65 | 7.40 |
| 40 | Psychiatric | Phenothiazine | Nil | Nil | 14.15 | 8.50 |
| 26 | Diabetes | Insulin | >15/day | Small | 14.05 | 10.75 |
| 31 | Diabetes | Insulin | Nil | Nil | 10.25 | 8.90 |
| 31 | Pituitary malignancy | Dilantin and radiotherapy [a] | Nil | Nil | 14.25 | 9.20 |
| 27 | Hodgkin's disease | Chemotherapy and radiotherapy [a] | Nil | Nil | 14.05 | 14.25 [b] |
| 28 | Malignant melanoma | Surgical excision [a] | Nil | Small | 12.95 | 8.20 |
| 29 | Cardiac | Nil | Nil | Nil | 10.55 | 8.20 |
| 30 | Multiple scletosis | Nil | >15/day | Nil | 19.86 [b] | – |
| 34 | Benign intra-cranial tension | Lumbar puncture | Nil | Small | 9.75 | 7.55 |

[a] All treatment occurred during pregnancy, except for treatment of neoplasms.
[b] SCE level >2 S.D. above the control mean for babies.

Only 1 mother had an SCE rate which was more than 2 S.D. above the mean of the control group, this was a mother with multiple sclerosis, the SCE rate in her baby was not determined. 2 babies had SCE rates that were significantly elevated, 1 born to an epileptic and 1 born to a woman who had Hodgkin's disease.

The SCE rate of babies with neural tube defects (Table 1) was significantly elevated compared to the control babies ($p < 0.01$). The mean SCE rate of babies with multiple congenital malformations and with Down syndrome were no different from normal infants (Table 1). However, 1 baby with multiple congenital malformations had an SCE rate of 16.6. In this baby and in those with neural tube defects the maternal history did not point to any difference in the maternal environment during

pregnancy compared to mothers in the normal group. The SCE rates of the parents of some of the children with neural-tube defects were studied and found to be within the normal range. The SCE rates in normal newborns and normal children under the age of 10 were not significantly different from one another. However, the normal adult controls over the age of 20 had SCE rates which were significantly higher than those of the normal newborns ( $p < 0.01$).

## Discussion

Before attempting to examine the affects of environmental agents upon SCE rates in mothers and infants it was important to establish control ranges for SCE in mothers immediately post-partum and for cord bloods. A group of mothers and their neonates were studied by Ardito et al. (1980) but they did not establish that there was no difference in SCE between mothers and controls who had not recently been pregnant. Our data shows that there is no difference in SCE rate between our control mother, smoker mother and healthy adult control groups.

The SCE rate measured from cord blood, capillary or venous blood in infants was found to be highly significantly less than that of adults. Whilst we did not specifically examine the affect of age on SCE we are unable to support the contention of Morgan and Crossen (1977) that SCE is not affected by age. Our data is in agreement with that of Ardito et al. (1980) who found a considerably lower SCE rate in cord blood than in mothers blood, with Husgafvel-Pursiainen et al. (1980) who found that children with a mean age of 1.5 years had significantly lower SCE than adults and is supported by the animal studies of Kram et al. (1980) who found female mice to have higher SCE than their fetuses.

There is controversy concerning the affect of cigarette smoking on SCE. Cigarette smoke condensate has been shown to be a potent inducer of SCE in vitro (Hopkin and Evans, 1979) but studies of SCE on smokers themselves have yielded inconsistent findings. Higher SCE levels in smokers than non-smokers have been found in some studies (Husgafvel-Pursiainen et al., 1980; Lambert et al., 1978; Murthy, 1979), but not in others (Ardito et al., 1980; Hollander et al., 1978). Our data, like that of Ardito et al. (1980) was not able to establish any difference between smokers and non-smokers (Sutherland et al., 1980). Ethanol, via its first metabolite acetaldehyde, has been shown to increase SCE in vitro (Ristow and Obe, 1978; Obe and Ristow, 1979; Obe et al., 1979) and in vivo in alcohol fed mice (Obe et al., 1979; Alvarez et al., 1980). Obe et al. (1980) have shown that clinically diagnosed alcoholics have an increase in chromosome aberrations in lymphocytes. The finding in the present study of an increase in SCE in the alcoholic mother group is complicated because all this group smoked cigarettes and others used a variety of illicit drugs. Interaction between cigarette smoke and alcohol in the aetiology of malignancy has been described (Tuyns, 1979).

Of the mothers suffering from chronic illness, one with multiple sclerosis had an elevated SCE rate; it appears that patients with MS have increased SCE rates (Sutherland et al., 1980). Only one group of abnormal infants had elevated SCE

rates and this was those with neural tube defects. It should be noted that most of these infants with neural-tube defects were studied in the first few days or weeks of life and many had either been recently operated upon or were taking medications such as panadol or chloral hydrate which could have influenced the results. When studied the parents of this group had normal SCE and this elevation of SCE in neural-tube defects is worthy of further investigation.

All the infants born to the epileptic and "alcoholic" mothers in this study were normal and had normal SCE rates. Explanations for this could include a diminished ability of fetal cells to form SCE, although this is unlikely in view of the animal studies of Kram et al. (1979, 1980); the inability of relevant metabolic products to cross the placenta; or possible metabolic differences in mothers which may influence the conversion of putative mutagens to active components which could cross the placenta. It would be of interest to study SCE in the abnormal offspring of epileptics and alcoholics to determine whether there was any evidence of DNA damage.

The finding that the SCE rate of infants was not influenced by the exposure of their mothers to a variety of environmental pollutants and drugs indicates that neonatal SCE rate is probably not a useful indicator of intrauterine exposure of normal infants to DNA-damaging agents. Kram et al. (1980) found in their mouse study that fetal SCE response to DNA-damaging agents decreased with gestation, but also concluded that the mutagen concentrations required to produce a marked increase in SCE were well below those required for teratogenesis. In our study only one infant with non-specific multiple malformations had an elevated SCE level. Although there was no specific maternal history of exposure to potential DNA-damaging agents in this instance the possibility that some infants with multiple malformations result from intrauterine exposure to DNA-damaging agents, which is reflected in an elevated SCE at birth, requires further study.

## Acknowledgements

We thank Dr. G. Gibson, Mr. D. Colley and Mrs. J. Jonas of the Queen Victoria Research Foundation for collecting data on the mothers and infants studied, and the Queen Victoria Hospital Labour Ward staff for collecting maternal and cord bloods. This project was supported by a grant from the Channel 10 Children's Medical Research Foundation of South Australia Inc.

## References

Alvarez, M.R., L.E. Cimino and T.J. Pusateri (1980) Induction of sister chromatid exchanges in mouse fetuses resulting from maternal alcohol consumption during pregnancy, Cytogenet. Cell Genet., 28, 173–180.

Ardito, G., L. Lamberti, E. Ansaldi and P. Ponzetto (1980) Sister chromatid exchanges in cigarette-smoking human females and their newborns, Mutation Res., 78, 209–212.

Crossen, P.E., W.F. Morgan, J.J. Horan and J. Stewart (1978) Cytogenetic studies of presticide and herbicide sprayers, New Zeal. Med. J., 88, 192–195.

Funes-Cravioto, F., C. Zapata-Gayon, B. Kolmodin-Hedman, B. Lambert, J. Lindsten, E. Norberg, M. Nordenskjöld, R. Olin and A. Swensson (1977) Chromosome aberrations and sister-chromatid exchange in workers in chemical laboratories and a rotoprinting factory and in children of women laboratory workers, Lancet, 2, 322–325.

Hollander, D.H., M.S. Tockman, Y.W. Liang, D.S. Borgaonkar and J.K. Frost (1978) Sister chromatid exchanges in the peripheral blood of cigarette smokers and in lung cancer patients; and the effect of chemotherapy, Hum. Genet., 44, 165–171.

Hopkin, J.M., and H.J. Evans (1979) Cigarette smoke condensates damage DNA in human lymphocytes, Nature (London), 279, 241–242.

Husgafvel-Pursiainen, K., J. Mäki-Paakkanen, H. Norppa and M. Sorsa (1980) Smoking and sister chromatid exchange, Hereditas, 92, 247–250.

Kram, D., G.D. Bynum, G.C. Senula and E.L. Schneider (1979) In utero sister chromatid exchange analysis for detection of transplacental mutagens, Nature (London), 279, 531.

Kram, D., G.D. Bynum, G.C. Senula, C.K. Bickings and E.L. Schneider (1980) In utero analysis of sister chromatid exchange: Alterations in susceptibility to mutagenic damage as a function of fetal cell type and gestational age, Proc. Natl. Acad. Sci. (U.S.A.), 77, 4784–4787.

Kurvink, K., C.D. Bloomfield and J. Cervenka (1978) Sister chromatid exchange in patients with viral disease, Exp. Cell Res., 113, 450–453.

Lambert, B., A. Linblad, M. Nordenskjöld and B. Werelius (1978) Increased frequency of sister chromatid exchanges in cigarette smokers, Hereditas, 88, 147–149.

Mitelman, F., and J. Wadstein (1978) Chromosome aberrations in chronic alcoholics, Lancet, 1, 216.

Morgan, W.F., and P.E. Crossen (1977) The incidence of sister chromatid exchanges in cultured human lymphocytes, Mutation Res., 42, 305–312.

Murthy, B.P.K. (1979) Frequency of sister chromatid exchanges in cigarette smokers, Hum. Genet., 52, 343–345.

Nakanishi, Y., and E.L. Schneider (1979) In vivo sister-chromatid exchange: A sensitive measure of DNA damage, Mutation Res., 60, 329–337.

Obe, G., and H. Ristow (1979) Mutagenic, cancerogenic and teratogenic effects of alcohol, Mutation Res., 65, 229–259.

Obe, G., A.J. Natarajan, M. Meyers and A. den Hertog (1979) Induction of chromosomal aberrations in peripheral lymphocytes of human blood in vitro, and of SCEs in bone marrow cells of mice in vivo by ethanol and its metabolite acetaldehyde, Mutation Res., 68, 291–294.

Obe, G., D. Göbel, H. Engeln, J. Herha and A.T. Natarajan (1980) Chromosomal aberrations in peripheral lymphocytes of alcoholics, Mutation Res., 73, 377–386.

Perry, P., and H.J. Evans (1975) Cytological detection of mutagen–carcinogen exposure by sister chromatid exchange, Nature (London), 258, 121–125.

Ristow, H., and G. Obe (1978) Acetaldehyde induces cross-links in DNA and causes sister chromatid exchanges in human cells, Mutation Res., 58, 115–119.

Seshadri, R.S., E. Baker, P. Leonard, G.R. Sutherland and A.A. Morley (1981) Sister chromatid exchange in aplastic anaemia, Cancer Genet. Cytogenet., 3, 81–84.

Sutherland, G.R., E. Baker, R.S. Seshadri and A. Black (1980) Increased sister-chromatid exchange in multiple sclerosis, N. Engl. J. Med., 303, 1126.

Tice, R., J. Chaillet and E.L. Schneider (1975) Evidence derived from sister chromatid exchanges of restricted rejoining of chromatid subunits, Nature (London), 256, 642–644.

Tuyns, A.J. (1979) Epidemiology of alcohol and cancer, Cancer Res., 39, 2840–2843.

# FURTHER EXCLUSIONS BY DELETION MAPPING

J.C. MULLEY, G.R. SUTHERLAND

# FURTHER EXCLUSIONS BY DELETION MAPPING

J.C. MULLEY, G.R. SUTHERLAND

MULLEY J.C., SUTHERLAND G.R. — Further exclusions by deletion mapping.
**Ann. Génét.**, 1982, **25**, n° 3, 152-153.

SUMMARY : Exclusions for unassigned markers were determined by deletion mapping at the following segments : (4)(p15.1→16.1) - *F13A,, F13B, TF* ; (4)(q27→31) - *TF* ; (9)(p22→pter) - *F13B* ; (12)(p12) - *TF* ; (12)(pter) - *TF* ; (18)(q22) - *F13A, K* and *LU* ; (19)(p or qter) - *TF* ; (22)(pter→q11) - *F13B. GC* was excluded from (4)(p15.1→16.1) and (4)(q27→31). *GALT* was excluded from (9)(p22→pter).

KEY-WORDS : Deletion mapping. — Exclusion mapping. — Chromosomal deletions.

MULLEY J.C., SUTHERLAND G.R. — Nouvelles exclusions déterminées par la méthode des délétions. *(En anglais).*
**Ann. Génét.**, 1982, **25**, n° 3, 152-153.

RÉSUMÉ : Les exclusions suivantes pour les localisations des marqueurs génétiques non encore localisés ont été déterminées par la méthode des délétions : (4)(p15.1→16.1) - *F13A, F13B, TF* ; (4)(q27→31) - *TF* ; (9)(p22→pter) - *F13B* ; (12)(p12) - *TF* ; (12)(pter) - *TF* ; (18)(q22) - *F13A, K* et *LU* ; (19)(p ou qter) - *TF* ; (22)(pter→q11) - *F13B, GC* a été exclu de (4)(p.15.1→16.1) et de (4)(q27→31). *GALT* a été exclu de (9)(p22→pter).

MOTS-CLÉS : Méthode des délétions. — Méthode d'exclusion. — Délétions chromosomiques.

Further exclusions arising from deletion mapping are presented. These were derived from patients with monosomic segments defined by trypsin-Leishman G-banding or BrdU replication banding. The detection of two alleles implies heterozygosity and clearly excludes that locus from the defined monosomic segment in the corresponding individual.

## MATERIALS AND METHODS

A large range of genetic markers were examined from individuals with the deleted autosomal segments listed in table I. All deletions examined were spontaneous in origin except the deletion in patient 1 resulting from a familial pericentric inversion inv(3)(p25q23) (Sutherland, Mulley and Goldblatt, 1981) and the deletions in patients 7 and 8 resulting from familial translocations t(9;22)(p22;q11) and t(10;18)(q26;q21) respectively. Partial karyotypes for patients 1, 3, 4, 6, 9, 10 and 12 in table I were given previously (Mulley et al., 1980) and for patients 2, 5, 7, 8, 11 and 13 are shown in figure 1. Isoelectric focusing was used to subtype GC 1 into 1S and 1F, PI M into M1, M2 and M3 and TF C into C1, C2 and C3. No GM results were presented for infants younger than six months of age.

## RESULTS AND DISCUSSION

The results of genetic marker studies are given in table I. *F13A, F13B, TF, K* and *LU* are unassigned, although *TF* may be located on chromosome 3 (Oslo Conference, 1981). The exclusions shown for these unassigned markers, and markers known to be syntenic with monosomic segments, are potentially valuable for regional localisation. New exclusions are : *F13A* from (4)(p15.1↠16.1), (18)(q22) ; *F13B* from (4)(p15.1↠16.1), (9)(p22↠pter), (22)(pter↠q11) ; *TF* from (4)(p15.1↠16.1), (4)(q27↠31), (12)(p12), (12)(pter), (19)(p or qter) ; *K* and *LU* from (18)(q22) (table I). Recent gene assignments have been *JK* to chromosome 2, *MNS* to chromosome 4, *GM* and *PI* to chromosome 14, *GPT* to chromosome 16 and *C3* to chromosome 19 (Oslo Conference, 1981). The numerous exclusions given for these loci (table I) are trivial if assignments for these loci are correct.

*Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, 72, King William road, NORTH ADELAIDE, S.A. 5006 (Australia).*

TABLE I. — **Exclusions determined by deletion mapping.**

| Patient No. | Segment deleted | Locus excluded |
|---|---|---|
| 1 | (3)(p25→pter) | *GC* |
| 2 | (4)(p15.1→16.1) | *F13A, F13B, GC, PI, TF, GALT, PGM1, PGP* |
| 3 | (4)(q27→31) | *GC, GPT* |
| 4 | (4)(q27→31) | *TF* |
| 5 | (5)(p13) | *PI, ADA, ESD, PGM1* |
| 6 | (7)(q22) | *GC* |
| 7 | (9)(p22→pter) and (22)(pter→q11) | *C3, F13B, GC, PI, ACP1, ADA, GALT, GLO, PGM1, PGP* |
| 8 | (10)(q26) | *HPA*, PI, AK1, ESD, GLO1, PGP* |
| 9 | (12)(p12) | *GM, TF* |
| 10 | (12)(pter) and (19)(p or qter) | *TF* |
| 11 | (18)(p11) | *C3, GC*, AK1, GLO1, PGM1** |
| 12 | (18)(q21→23) | *GM* |
| 13 | (18)(q22) | *F13A, FY*, JK*, K, LU, MNS*, RH, GC*, ACP1*, GALT, PGM1** |

* Previously excluded from the segment investigated (Aitken et al., 1975 [1]).

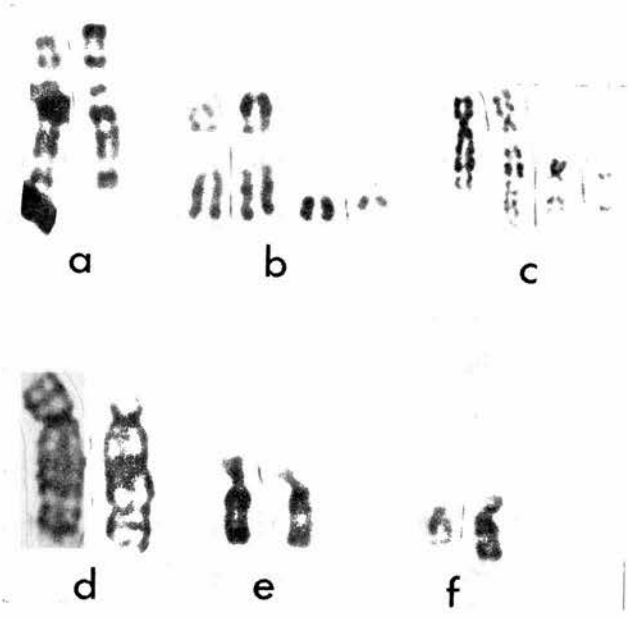



**Fig. 1. — Partial karyotypes from patients 2, 5, 7, 8, 11 and 13. BrdU banded karyotypes : (a) patient 2, del(4)(p15.1→p16.1) ; (b) balanced form of t(9;22)(p22;q11), patient 7 had karyotype 45,XY, —9,—22,+der(9)t(9;22)(p22;q11) ; (c) balanced form of t(10;18)(q26;q21), patient 8 had karyotype 46,XY,der(10)t(10;18)(q26;q21) ; and G-banded karyotypes ; (d) patient 5, del(5)(p13) ; (e) patient 11, del(18)(p11) and (f) patient 13, del(18)(q22).**

*GC* and *GALT* are each excluded from portions of the chromosome to which they are assigned. *GC* is excluded from (4)(p15.1→16.1) and (4)(q27→31) (table I) which is compatible with the regional assignment of *GC* to (4)(q11→13). *GALT* is excluded from (9)(p22→pter) (table I) which is compatible with the regional localisation of *GALT* to (9)(p13) and tight linkage between *GALT* and 9qh variants with a lod score of 3.67 at $\theta = 0$ (Sparkes et al., 1980). Close linkage of *GALT* to the centromere is further supported in « family 2 » of Sutherland and Eyre (1981) which is segregating for an unusual G-band variant near be centromere of the short arm of chromosome 9. Portion of this pedigree is informative giving a positive lod score of 0.90 at $\theta = 0$, ($z_1$ 4:0), resulting in a total positive lod score of 4.57 at $\theta = 0$.

ACKNOWLEDGEMENTS

We wish to thank Elizabeth Baker, Helen Eyre, Trudy Hocking, Barbara Menzies and Erica Woollatt for tissue culture and technical assistance with the cytogenetics, Catherine Nicholls for blood-grouping and Dr. D. Propert for GM typing.

## REFERENCES

1. AITKEN D.A., FERGUSON-SMITH M.A., DICK H.M. — Gene mapping by exclusion : the current status *Cytogenet. Cell Genet.*, 1975, *14*, 256-265.
2. MULLEY J.C., BRYANT G.D., SUTHERLAND G.R. — Additions to the exclusion map of man. *Ann. Génét.*, 1980, *23*, 198-200.
3. Oslo Conference. — Proceeding of the 6th International Workshop on Human Gene Mapping, 1981, *Cytogenet. Cell Genet.* (In press).
4. SPARKES R.S., EPSTEIN P.A., KIDD K.K., KLISAK I., SPARKES M.C., CRIST M., MORTON L.A. — Probable linkage between the human galactose-1-phosphate uridyl transferase locus and 9qh. *Amer. J. Hum. Genet.*, 1980, *32*, 188-193.
5. SUTHERLAND G.R., EYRE H. — Two unusual G-band variants of the short arm of chromosome 9. *Clin. Genet.*, 1981, *19*, 331-334.
6. SUTHERLAND G.R., MULLEY J.C., GOLDBLATT E. — A large kindred with an inv(3)(p25q23) : clinical, cytogenetic and genetic marker studies. *Ann. Génét.*, 1981, *24*, 202-205.

In Press : Mutation Res



# SCE, X-radiation sensitivity and mutation rate in multiple sclerosis

Ram Seshadri, Grant R. Sutherland*, Elizabeth Baker*,
Robert Kutlaca, Dianne Wigmore and Alexander A. Morley.

Haematology Unit
Flinders University and Medical Centre
Bedford Park
South Australia 5042
and
* Genetics Department
Adelaide Children's Hospital
North Adelaide
South Australia 5006

Summary

In order to detect any underlying DNA abnormality that may be present in multiple sclerosis (MS), the incidence of SCE, X-radiation sensitivity, and the frequency of 6-thioguanine resistant cells were determined in the lymphocytes of 34 MS patients. As a group, MS patients showed an increase in the SCE rate compared to control. However, there was no increase in X-radiation sensitivity. The frequency of 6-thioguanine resistant mutant cells was also normal.

In recent years several methods have been described to detect DNA damage and repair processes. Of these, biological methods such as the analysis of rates of sister chromatid exchange (SCE)(Nakarishi and Schneider, 1970), and assessment of cell survival following X-irradiation (Agarwal et al., 1977; Taylor et al., 1975; Weichselbaum et al., 1980) are being used because of their simplicity. A new method for measuring DNA damage in man may be the enumeration of 6-thioguanine-resistant presumptively mutant lymphocytes; this approach seems capable of detecting an increased number of mutants although there is uncertainty as to its specificity when the number of mutants is low (Strauss and Albertini, 1979; Morley et al., 1982). The evidence that 6TG resistant cells are mutants is indirect, and is based on the observation that increased frequency of resistant cells occur after cancer therapy with mutagens (Strauss and Albertini, 1979) and that X-irradiation of lymphocytes in vitro increases the frequency of 6TG resistant cells (Evans and Vijayalaxmi, 1981; Dempsey and Morley, 1982).

Although each of these tests appears to be a sensitive method of detecting DNA damage, a detailed analysis of the correlation between these tests has not been performed. SCE rate is increased after exposure to many mutagens and carcinogens (Perry and Evans, 1975), and there appears to be a correlation between SCE and mutation (Carrano et al., 1978). The assessment of cell survival following X-irradiation has been valuable in detecting inherited disorders with DNA repair abnormalities (Taylor et al., 1975; Weichselbaum et al., 1980). However, the relationship between SCE rate, X-radiation sensitivity and somatic mutation as measured by the frequency of 6TG resistant cells is unclear.

In an earlier communication, we reported that multiple sclerosis (MS) patients have an increased rate of SCE (Sutherland et al., 1980). Since Gipps and Kidson (1981) observed that lymphoblastoid cells of MS patients have a greater sensitivity to ionizing radiation than those of normal individuals, we investigated the correlation between SCE rate, X- radiation sensitivity and spontaneous mutation rate as measured by the 6TG resistant cells in MS patients and appropriate controls.

## Materials and Methods

Volunteers were obtained from the South Australian MS Rehabilitation Unit, and blood samples were collected from inpatients with MS; data on current and long-term drug usage, alcohol consumption and smoking habits were collected. The control group consisted of healthy persons between the ages of eight and 80 years. None were taking drugs except for oral contraceptives in some women, and only six smoked cigarettes.

## SCE determination

Lymphocyte cultures were established from venous blood samples in Ham's F10 culture medium; harvesting and differential staining were performed as previously described (Seshadri et al., 1981). SCE rates were determined by examination of 20 metaphases; SCE's at the centromere were included in the count. All SCE rates were determined from coded slides by one observer (E.B.). Where possible those patients with elevated SCE rates were re-studied at a later date.

T-lymphocyte colony survival following X-irradiation

Hypaque-Ficoll separated peripheral blood lymphocytes were cloned in soft agar in the presence of phytohaemagglutinin and irradiated feeder cells, and colony survival following X-irradiation was determined as described (Kutlaca et al., 1982) for each patient and an age-matched control. All MS patients and controls were aged less than 60 years.

Mutation assay

The assay is based on the principle that lymphocytes with a mutation at the HGPRT locus are resistant to 6-thioguanine (6TG) and, when grown in the presence of 6TG, are able to enter DNA synthesis, incorporate $^{3}$H-thymidine and become identifiable using autoradiography. Full details of the method have been described elsewhere (Morley et al., 1982).

Results

SCE rate

Thirty-four patients with MS of varying severity were studied. The SCE rate in MS patients was higher than that of the control group ($p<0.01$ by Wilcoxon rank sum test) (Figure 1). Half of the patients with MS smoked cigarettes but none had excessive consumption of alcohol. All but 10 of the MS patients were taking one or more of the following drugs: methenamine mandelate, diazepam, laxatives, steroids, cimetidine and imipramine.

Repeat studies were done in three patients showing initial SCE rates (Table) above the normal adult range of 11.2 ± 3.3 exchanges/cell determined in this laboratory. Although in one case the number of cells suitable for SCE analysis was small it is apparent that even after a considerable time the SCE rates were still at the top of or above the control range.

Radiation sensitivity

The results of lymphocyte colony survival following X-irradiation are shown in Figure 2 and it can be seen that there is no evidence of increased radiation sensitivity of lymphocyte colonies of MS patients.

Mutation assay

Figure 3 shows the frequency of 6TG resistant cells in MS patients and control. There was no evidence of increased frequency of 6TG resistant cells in MS patients compared with controls. Radiation sensitivity and 6TG-resistant cell frequency in four patients who had a high SCE rate showed no abnormalities (data not shown).

Discussion

The present study confirms the previous preliminary observation (Sutherland et al., 1980) that the SCE rate in MS patients is higher than that of normal individuals. Although half the patients with MS smoked cigarettes there was no difference in SCE rates between the smokers and non-smokers in this group (data not shown). In a previous study there was also no difference in the SCE rate of smokers and non-smokers

(Seshadri et al., 1982) and it is therefore unlikely that the increase in the SCE rate of the MS patient group is due to the inclusion of smokers. Since many of the MS patients were taking various drugs, the possibility that the drugs could be responsible for the increased SCE rate should be considered, but there is no evidence that the drugs used by the MS patients caused any increase in SCE. The possibility that MS may be caused by a slow virus has been suggested (Fraser, 1977), and viral infections have been shown to cause an increase in SCE (Kurvink et al., 1978).

The observation that SCE rate is elevated in MS patients in conjunction with the observation of Gipps and Kidson (1981) that some patients with MS have an increased sensitivity to ionizing radiation may indicate a basic DNA abnormality in MS that could be responsible for the clinical manifestations of the disorder. However, the present study does not confirm that patients with MS are sensitive to X-rays. Furthermore, there was no increase in the somatic mutation frequency.

Thus, it appears that MS patients do have a higher SCE frequency than appropriate controls but that a search for other abnormalities of DNA has been unrewarding. The reasons for and the significance of the increased SCE rate in MS remain unknown.

Acknowledgement

This study was partly supported by a grant from the National Multiple Sclerosis Society of Australia.

Table: Results of repeat SCE studies on MS patients with initial high SCE levels

| Patient | Date of Study | Mean SCE/Cell + SD | No. of Cells |
|---|---|---|---|
| W | August 1981 | 16.4 ± 7.4 | 20 |
| | February 1982 | 15.3 ± 6.0 | 20 |
| E | June 1979 | 15.1 ± 3.4 | 20 |
| | February 1982 | 14.2 ± 4.6 | 18 |
| O | September 1979 | 15.5 ± 6.2 | 20 |
| | February 1982 | 13.3 ± 2.3 | 6 |

References

Agarwal, S.S., D.Q. Brown, E.J. Katz and L.A. Loeb (1977) Screening for deficits in DNA repair by the response of irradiated human lymphocytes to phytohaemagglutinin, Cancer Res., 37, 3594-3598.

Carrano, A.V., L.H. Thompson, P.A. Lindl and J.L. Minkler (1978) Sister-chromatid exchange as an indicator of mutagenesis, Nature, 271, 551-553.

Dempsey, J.L. and A. A. Morley (1982) Evidence that thioguanine-resistant lymphocytes detected by autoradiography are mutant cells, accepted for publication (Mutation Res.).

Evans, H.J. and Vijayalaxmi (1981) Induction of 8-azaguanine resistance and sister-chromatid exchange in human lymphocytes exposed to mitomycin C and X-rays in vitro, Nature, 292, 601-605.

Fraser, K.B. (1977) Multiple sclerosis: a virus disease, Brit. Med. Bull., 33, 28-32.

Gipps, E. and C. Kidson (1981) Ionizing radiation sensitivity in multiple sclerosis, Lancet, 1, 8226; 947.

Kurvink, K., C.D. Bloomfield and J. Cervenka (1978) Sister-chromatid exchange in patients with viral disease, Exp. Cell Res., 113, 450-454.

Kutlaca, R., S.J. Alder, R.S. Seshadri and A.A. Morley (1982) Radiation sensitivity of human lymphocytes, Mutation Res., 94, 125-131.

Morley, A.A., S. Cox, D. Wigmore, R. Seshadri and J.L. Dempsey (1982) Enumeration of thioguanine resistant lymphocytes using autoradiography, Mutation Res., 95, 363-375.

Nakariski, Y. and E.L. Schneider (1970) In vivo sister-chromatid exchange. A sensitive measure of DNA damage, Mutation Res., 60, 329-337.

Perry, P. and H.J. Evans (1975) Cytological detection of mutagen-carcinogen exposure by sister-chromatid exchange, Nature, 259, 121-125.

Seshadri, R., E. Baker, P. Leonard, G. Sutherland and A. A. Morley (1981) Sister-chromatid exchange in aplastic anaemia, Cancer Genetics and Cytogenetics, 3, 81-84.

Seshadri, R., E. Baker and G. Sutherland (1982) Sister-chromatid analysis in mothers exposed to DNA-damaging agents and their newborn infants, Mutation Res., 97, 139-146.

Strauss, G.H. and R.J. Albertini (1979) Enumeration of 6-thioguanine resistant peripheral blood lymphocytes in man as a potential test for somatic cell mutations arising in vivo, Mutation Res., 61, 353-379.

Sutherland, G.R., E. Baker, R. S. Seshadri and A. Black (1980) Increased sister-chromatid exchange in multiple sclerosis, N. Engl. J. Med., 303, 1126.

Taylor, A.M.R., D.G. Harnden, C.F. Arlett, S.A. Harcourt, R.R. Lehmann, S. Stevens and B.A. Bridges (1975) Ataxia telangiectasia: a human mutation with abnormal radiation sensitivity, Nature, 258, 427-429.

Weichselbaum, R.R., J. Nove and J.B. Little (1980) X-ray sensitivity of fifty-three human diploid fibroblast cell strains from patients with characterized genetic disorders, Cancer Res., 40, 920-925.

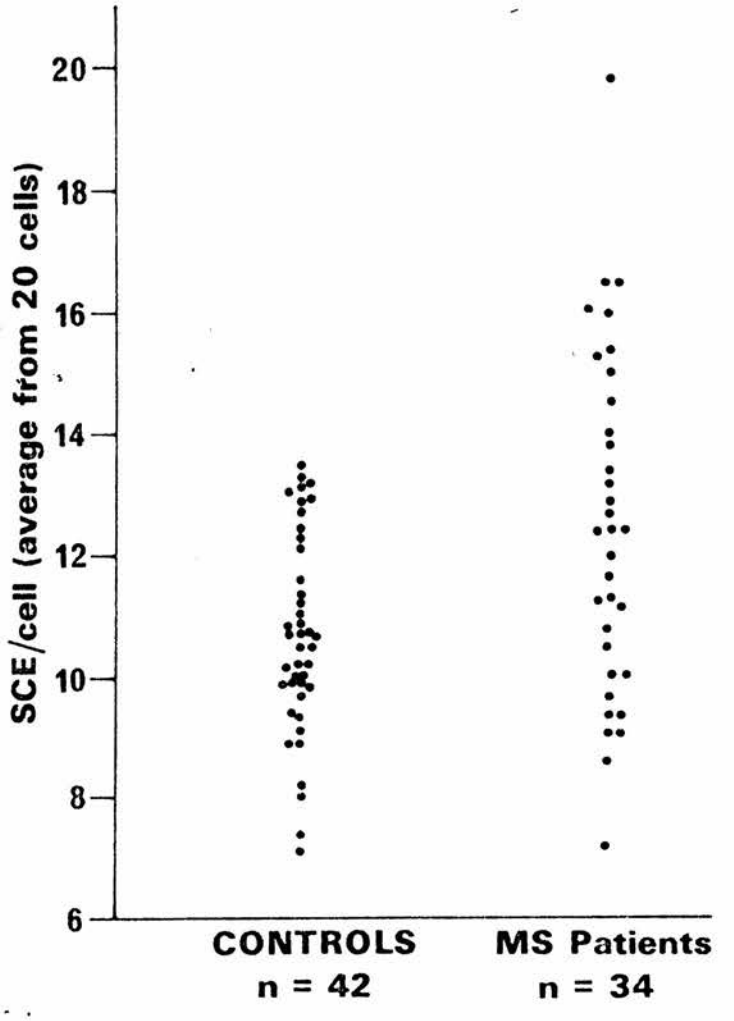



Figure 1: SCE rates in controls and MS patients ($p<0.01$ by Wilcoxon rank sum test)

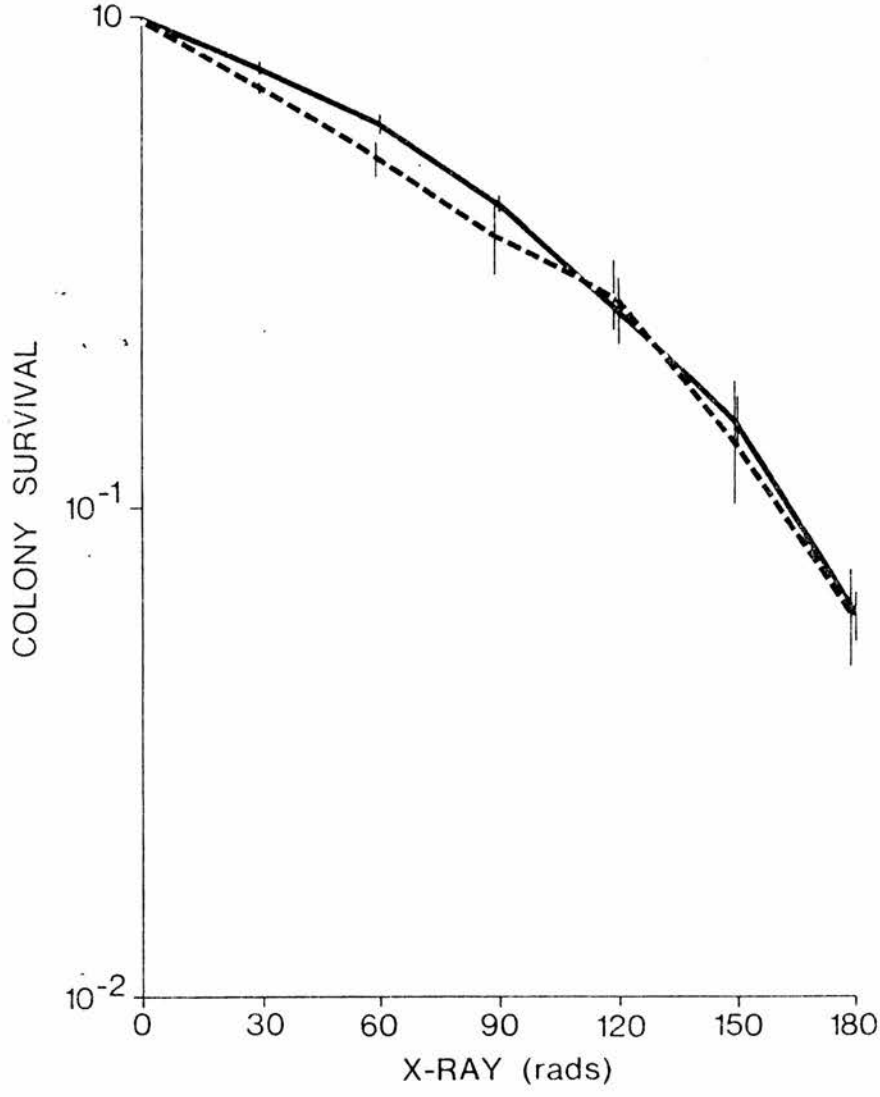



Figure 2: Lymphocyte colony survival following X-irradiation
——— normal controls, ----- MS patients (mean ± standard error).

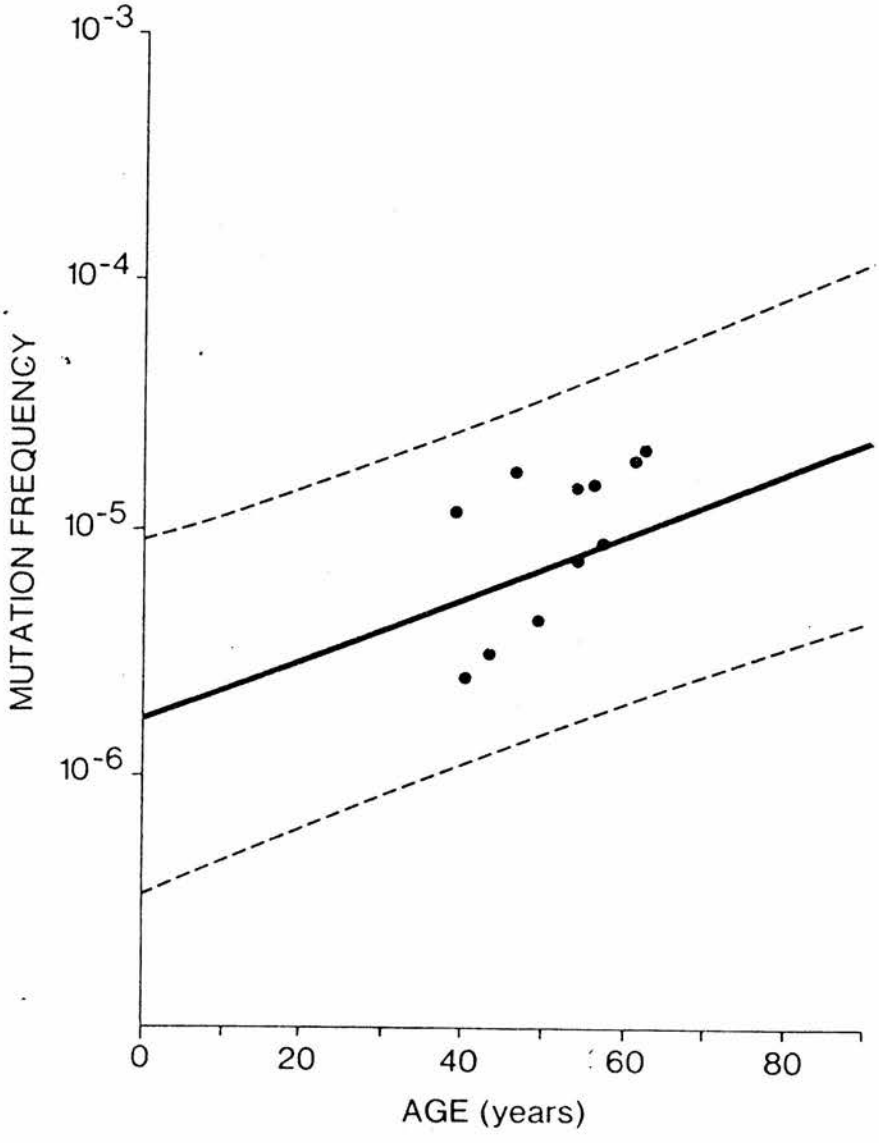



Figure 3: Mutation frequency: the thick and interrupted line shows the mean and the 95% confidence limits, respectively, of the frequency of 6TG-resistant cells in normal controls. Dots represent the values in MS patients (see text for details).

Hum Genet (1983) 63:73–74

Human Genetics



*Short Communications*



# A New Allele of $\alpha_1$-Antitrypsin: *PI*NADELAIDE*

J. C. Mulley[1], D. W. Cox[2], and G. R. Sutherland[1]

[1] Cytogenetics Unit, Department of Histopathology, The Adelaide Children's Hospital Inc., North Adelaide, S.A. 5006, Australia
[2] Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Canada M5G IX8

**Summary.** The allele *PI*NADELAIDE (PI*NADE)* was named in accord with nomenclature guidelines and specifies a new co-dominant variant of $\alpha_1$AT. Discovery was achieved by IEF and the isoelectric point of NADE is between N and NHAM. Familial inheritance of *PI*NADE* was demonstrated and both PI M2NADE and PI M3NADE phenotypes were observed. The mobility of PI*NADE is identical to PI M by both starch and agarose electrophoresis. *PI*NADE* apparently confers normal $\alpha_1$AT serum concentrations and is probably unrelated to disease.

## Introduction

$\alpha_1$-Antitrypsin ($\alpha_1$AT) is the major regulator of protease activity in serum. Phenotypes are determined by multiple alleles at a locus designated *PI,* for protease inhibitor system. The frequency of the *PI*S* and *PI*Z* variants exceed 0.01 in most populations; and *PI* is highly polymorphic when the common phenotype (PI M) is subtyped. The remainder of the 32 alleles recognised at the nomenclature meeting in 1978 (Cox et al. 1980) occur with frequencies of less than 0.01, and many are extremely rare. All alleles recognised to date have recently been summarised (Cox 1981). Another allele is described in this report.

## Materials and Methods

The family studied was of Anglo-Saxon origin in which there was a fragile site at 10q23 segregating (Family Ay of Sutherland 1979). The propositus and his brother were mentally retarded, but otherwise healthy. Sera and plasma were collected and stored at −20° C until studied.

Separator isoelectric focusing (IEF) as described by Frants and Eriksson (1978) detected an unknown PI variant. Details of IEF differed from Mulley (1980) only by the use of LKB ampholine in the pH 3.5–5 range rather than the mixture of pH 3.5–5 and pH 4–6 ranges. Subsequently, acid starch gel electrophoresis and agarose electrophoresis were carried out as previously described (Cox 1981). $\alpha_1$AT was quantitated by radial immunodiffusion (Mancini et al. 1965). Allelic and phenotypic designations follow standard recommendations (Shows et al. 1979). Procedures for naming alleles and the confirmation of new variants follow recommendations of the International PI Committee (Cox et al. 1980).

*Offprint requests to:* J. C. Mulley

## Results and Discussion

The isoelectric point of the unknown variant was shown to lie cathodal to that of PI N and anodal to PI NHAMPTON (NHAM) (Fig. 1). The allele responsible for this new variant was named *PI**-

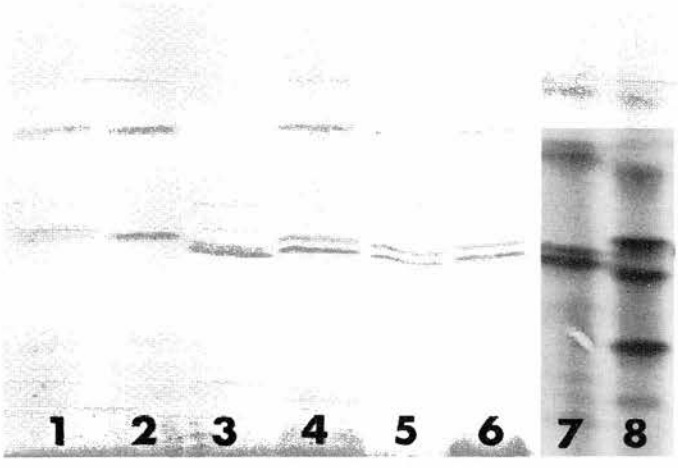



**Fig. 1.** Variants as seen by IEF. Anode at top. *1* — M1M2, *2* — M2M3, *3* — M2NADE, *4* — M1N, *5* — M3NADE, *6* — M1N, *7* — M3NADE, *8* — NHAMS

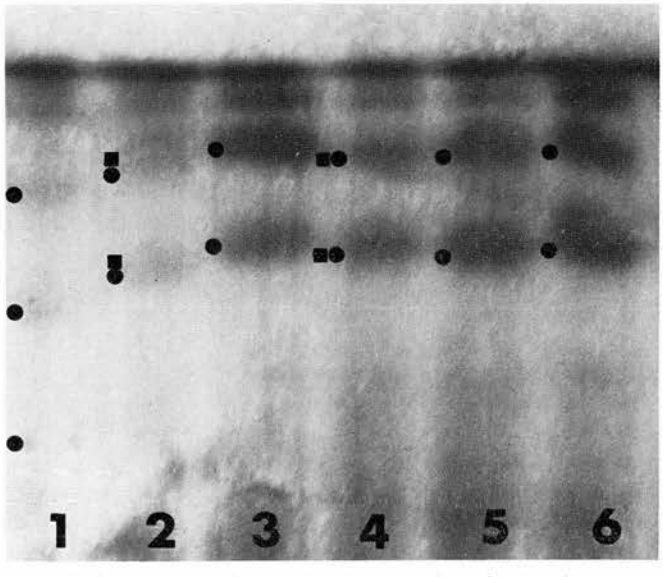



**Fig. 2.** Variants as seen by starch gel electrophoresis. Anode at top. *1* — NHAMZ, *2* — M2NADE, *3* — M1, *4* — M3NADE, *5* — M3, *6* — M1. ■ indicates position of NADE band. • indicates position of other variant

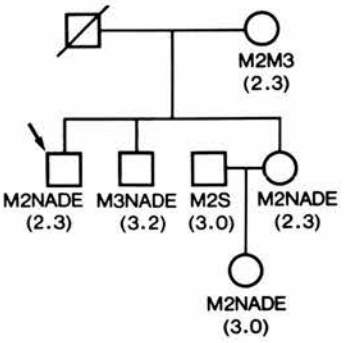



**Fig. 3.** Pedigree showing inheritance of *PI*NADE*. $\alpha_1$AT concentrations (g/l) are shown in parentheses

*NADELAIDE (PI*NADE).* Bands were confirmed as $\alpha_1$AT by immunofixation after IEF. By acid starch gel electrophoresis and agarose electrophoresis, M1 = M3 = NADE. However, on the runs with especially good resolution, M1 > M3 = NADE > M2, with both M3 and NADE only slightly cathodal to M1 (Fig. 2).

The pedigree is presented together with observed phenotypes and $\alpha_1$AT concentrations (Fig. 3). There is no evidence that the *PI*NADE* allele reduces $\alpha_1$AT concentration as all carriers were within the normal range (2–4 g/l). The mean $\alpha_1$AT concentration for the four carriers was 2.7 g/l which is similar to the normal mean value of 3.1 g/l.

The family study described has confirmed the inheritance of *PI*NADE* and demonstrated expression consistent with co-dominance. The same PI pattern was obtained at repeat collection from the propositus. Three generations were investigated and the variant was present in four family members from two generations. The deceased father of the propositus must also have carried *PI*NADE.*

*Acknowledgements.* We thank the National Health and Medical Research Council of Australia for support, Shirley Smyth for technical assistance in the laboratory of D. W. Cox whose work is supported by a grant from the Medical Research Council of Canada (MA-5426), Dr. Phillippe Arnaud for supplying the NHAMPTON standard for the electrophoretic comparison and the Department of Chemical Pathology of the Adelaide Children's Hospital for $\alpha_1$AT quantitations.

## References

Cox DW (1981) New variants of $\alpha_1$-antitrypsin: comparison of PI typing techniques. Am J Hum Genet 33:354–365

Cox DW, Johnson AM, Fagerhol MK (1980) Report of nomenclature meeting for $\alpha_1$-antitrypsin. (INSERM, Rouen/Bois — Guillaume — 1978). Hum Genet 53:429–433

Frants RR, Eriksson AW (1978) Reliable classification of six PiM subtypes by separator isoelectric focusing. Hum Hered 28:201–209

Mancini G, Carbonara O, Heremans JF (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235

Mulley JC (1980) Distribution of the six $\alpha_1$-antitrypsin (*PI*) subtypes from a sample of blood donors. Aust J Exp Biol Med Sci 58:409–414

Shows TB, Alper CA, Bootsma D, Dorf M, Douglas R, Huisman T, Kit S, Klinger HP, Kozack C, Lalley PA, Lindsley D, McAlpine PJ, McDougal JK, Meera Khan P, Meisler M, Morton NE, Opitz JM, Partridge CW, Payne R, Roderick TH, Rubinstein P, Ruddle FH, Shaw M, Spranger JW, Weiss K (1979) Guidelines for human gene nomenclature. Cytogenet Cell Genet 25:96–116

Sutherland GR (1979) Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects, and cytogenetics. Am J Hum Genet 31:136–148

Received November 11, 1982

*Aust. J. Ment. Ret.*
(1972) 2: 85-92

# THE ROLE OF AMNIOCENTESIS IN GENETIC COUNSELLING

**GRANT R. SUTHERLAND**

*Aust. J. Ment. Ret.*
(1972) 2: 85-92

# THE ROLE OF AMNIOCENTESIS IN GENETIC COUNSELLING

GRANT R. SUTHERLAND[1]

SUMMARY

The development of methods of diagnosing genetic disorders in the early fetus via amniocentesis are discussed. The role that this technique can play in genetic counselling is emphasised and it is stressed that this service should be offered to all couples who have a high risk of producing children with a disease that can be diagnosed *in utero*. It is also suggested that the screening of fetuses at moderate to low risk would be an economically viable public health measure which could significantly reduce the incidence of mongolism in the community.

One of the difficulties facing the genetic counsellor is that he can usually only give prospective parents an indication of the risk involved in having an affected child. If this risk is high then the parents may choose to have no children and request termination of any pregnancies which may arise. This can create ethical problems which were summarised by Pitt (1969) when he said "that to perform an abortion for a one in four risk of abnormality is to run a three in four chance of losing a normal infant". In cases where the risk is as high as one in four, many parents would not be prepared to take the chance of having, what would be to the majority in this situation, another affected child. When the risks are lower, say of the order of 5%, then decisions regarding further children and those of terminating pregnancy become more difficult. Such problems could be largely overcome if any fetal abnormality could be accurately determined early enough for therapeutic abortion of affected fetuses. This would allow parents with a high risk of having an affected child to have a normal family and should go much of the way to solving the problem outlined by Pitt.

The problem of attempting to find out whether a pregnancy will result in an affected child has received increasing attention in the last few years. The main method of this fetal diagnosis is by a study of the amniotic fluid. In a procedure known as amniocentesis a sample of about 10 ml. of this fluid can be fairly readily obtained by puncture of the uterus from early in the second trimester onwards. The fluid so obtained is a clear amber colour and it contains a number of cells which are almost certainly of fetal origin (Votta et al., 1968) although their exact source is uncertain.

A full discussion of the techniques used to obtain amniotic fluid is beyond the scope of this paper. Early workers tried the transvaginal approach and had a high incidence of fetal complications. Riis and Fuchs (1966) had three spontaneous abortions and one stillbirth out of eleven cases using this method. The transabdominal approach (see Thiede, 1968 for details of this technique) seems to carry little risk. Gerbie et al. (1971) reported no complications, either fetal or maternal, after 256 such procedures and knew of 170 others which had been performed without complication. Ferguson-Smith et al. (1971) have had no complications in 30 cases and now use ultrasonography to locate the placental site and to exclude multiple pregnancy which is a contra-indication to amniocentesis.

A sample of amniotic fluid provides three avenues for study, the composition of the fluid itself, the cells present in the fluid and the culture of these cells.

## Amniotic Fluid

There is controversy regarding the origin of amniotic fluid in early pregnancy (Emery,

1. (Mr.) Cytogeneticist, Department of Pathology, Royal Hospital for Sick Children, Edinburgh EH9 1LF, Scotland (Address for Reprints).

1970). However, there is little doubt that fetal urine contributes to the fluid and that this contribution becomes more significant as gestation proceeds. The total volume of amniotic fluid increases throughout pregnancy from 50 ml. at 12 weeks to 150 ml. at 15 weeks and 450 ml. at 20 weeks (Emery, 1970). As amniotic fluid is composed partly of fetal urine it would be reasonable to assume that it could be put through the series of tests which are normally applied to urine when screening for metabolic disorders. However, it will not be until normal values for all relevant components (as a function of gestational age) have been determined that the validity of this approach will be known. At present it is not known how the ability of the fetal kidney to secrete various metabolites varies with gestation and, possibly, with the diet of the mother. Nadler et al. (1970) reported a case of Pompe's disease in which the enzyme normally absent, $\alpha$-1,4-glucosidase, was present in the amniotic fluid but not in the cells cultured from it; some enzymes in amniotic fluid may be maternal in origin. Matalon et al. (1970) reported that Hurler's syndrome could be diagnosed by the presence of excessive amounts of heparatin sulphate in the amniotic fluid at 14 weeks, Brock et al. (1971) failed to confirm this and missed diagnosing a case using similar criteria; genetic heterogeneity of this disease may explain this apparent contradiction. Jeffcoate et al. (1965) have diagnosed the adrenogenital syndrome by demonstrating grossly elevated levels of 17-ketosteroids and pregnanetriol in the amniotic fluid shortly before birth. As the source of these steroids is presumably the fetal urine and as the fetal adrenal cortex functions by the fourteenth week, the possibility of diagnosing this syndrome early enough for termination of the pregnancy arises. However, Merkatz et al. (1969) were unable to diagnose this syndrome in early pregnancy using these criteria, in fact, the steroid levels were only suggestive of the diagnosis at term.

To date, amniotic fluid studies have been generally equivocal and much more data on the composition of this fluid in normal and diseased states will be necessary before confident diagnosis of genetic disorders can be made early in pregnancy. When the production of an abnormal metabolite is not present at birth but depends on diet (e.g. phenylketonuria) it is difficult to see how the condition could be diagnosed from the amniotic fluid.

**Amniotic Fluid Cell Studies**

At present the information which can be obtained from the cells without tissue culture is limited to sex chromatin determination, the ABO blood groups and, recently, electron microscopy studies.

**Sex Chromatin**

In 1956, several workers independently found that fetal sex could accurately be determined by X-chromatin studies on the cells present in amniotic fluid. This technique has been extended, and probably increased in reliability, since the discovery of the quinacrine fluorescence pattern of the Y chromosome in interphase nuclei (Pearson et al., 1970) was extended to amniotic fluid cells (Rook et al., 1971). By combining these methods it should be possible to accurately determine the sex chromosome complement in all cases.

The determination of X-chromatin on amniotic fluid cells was first used by Riis and Fuchs (1960) in the management of the pregnancies of two women who were carriers of the gene for haemophilia. The technique is, however, applicable to all cases where an X-linked gene is responsible for a disease which cannot be specifically diagnosed. When a woman carries such a gene, half her sons would be affected but all her daughters would be normal. By terminating the pregnancies where the fetus is a male the parents concerned can have a family of normal girls. This does not completely solve the problem of Pitt (1969) although it reduces the chance of losing a normal infant from three in four to one in two and has been used by numerous genetic counsellors to manage pregnancies involving X-linked conditions (e.g., Gerbie et al. 1971) where male fetuses have been aborted. Edwards (1970) has suggested that fetal sex determination could be used as the basis for aborting the female fetuses fathered by men who carry X-linked diseases (e.g. Haemophilia) as this "... could allow the reduction of such diseases to the level maintained by mutation, the virtual elimination of familial cases, and progress in therapy without the prospect of increasing the incidence of disease."

**Blood Groups**

The determination of the fetal ABO blood groups is possible using cells from the amniotic fluid as early as 11 weeks gestation (Sachs et al., 1956). However, attempts to demonstrate the Rh groups have been unsuccessful (Scott et al., 1969). Such information is of little use

in genetic counselling at present but may become useful when genetic maps of the autosomes have been constructed, especially if it becomes possible to determine other blood groups from these cells. In cases of close linkage where the probability of recombination is small, it may be easier to determine the blood group than to assay for its closely linked disease-producing gene. So far, the only such close linkage known is that of the ABO locus to the gene for nail-patella syndrome, and possibly the MNS blood group locus to that for sclerotylosis (Renwick, 1969).

**Electron Microscopy Studies**

The ultrastructure of normal amniotic fluid cells has been studied by Hoyes (1968) who found two recognisable cell types. Bartman (1971) studied cultured amniotic fluid cells and found only one cell type similar to fibroblasts cultured from other sources. Hug et al. (1970) found that amniotic fluid cells obtained during the 36th week of gestation from a woman carrying a child subsequently shown to have Pompe's disease, had abnormal lysosomes and suggested that it may be possible to diagnose this disease early in pregnancy by electron microscopy of uncultured amniotic fluid cells.

**Amniotic Fluid Cell Culture Studies**

This aspect of fetal diagnosis will probably prove to be by far the most important. Two broad fields of study are opened up by culturing the cells present in the amniotic fluid, viz., cytogenetics and biochemistry. Although much work has been done on the methodology of culturing these cells, the optimum conditions have probably not yet been found. Hence, the time required to produce results is longer than ideal especially when a result may indicate termination of a pregnancy; to produce a karyotype requires two to three weeks and a biochemical result may take significantly longer.

**Biochemical Studies**

Davidson (1970) lists about 30 different cell phenotypes which can be distinguished in cultures of fibroblasts grown from skin biopsy. Harris (1970) estimates that these represent about one-third of the metabolic disorders in which the biochemical defect is known. This knowledge cannot be directly applied to fetal diagnosis until it has been established that the cells cultured from amniotic fluid cells similarly and consistently express the same biochemical phenotypes as those grown from skin. It would be necessary to establish this for each enzyme or other property separately and any variations during gestation would need to be known. For these reasons work on fetal diagnosis of biochemical disorders is proceeding slowly; each disorder requires a separate technique for diagnosis and each technique will require highly skilled staff, separate reagents and often expensive laboratory equipment. At present, biochemical techniques can only be applied to individual families who have already produced an affected child, the detection of heterozygotes in the population is not feasible unless the detection is reasonably simple and the gene frequency in the population is high, e.g., screening for carriers of Tay-Sachs disease in some Jewish populations where their frequency is as high as 1 in 30.

Biochemical studies on cultured cells involve three main types of approach, autoradiography, histochemistry and the assaying of enzyme levels on cell homogenates. These are all sophisticated methods which are prone to technical difficulties. The procedures are also lengthy, autoradiographs usually requiring at least a week to prepare after sufficient cells have been grown. However Fujimoto et al. (1968) reported the autoradiographic diagnosis of a female fetus heterozygous for the Lesch-Nyhan syndrome 22 days after amniocentesis. The main histochemical technique is the staining for metachromatic granules in the cytoplasm using toluidine blue. These granules have been demonstrated in a number of conditions including the polysaccharidoses and cystic fibrosis of the pancreas. It is, however, difficult to distinguish homozygotes from heterozygotes and the metachromasia may not develop until after two to three months in cell culture (Fratantoni et al., 1969), in fact this technique should not be used for diagnostic purposes at its present stage of development. The assaying of enzyme levels in cultured cell homogenates generally requires a large number of cells which may take several weeks to produce. However, specific enzyme deficiencies in cultured cells have been demonstrated for galactosemia, metachromatic leukodystrophy, lysosomal acid phosphatase deficiency and Pompe's disease (Nadler and Gerbie, 1971), in each case making intrauterine diagnosis possible. Regan et al., (1971) have diagnosed xeroderma pigmentosum, an autosomal recessive disease in which there is a lack of functional ultraviolet endonuclease, from cultured amniotic fluid cells. Their technique which was able to clearly distinguish normals from homozygotes and from heterozygotes was

based on shifts in the DNA sedimentation constants after the cells had repaired ultraviolet induced lesions in the presence of 5-bromodeoxyuridine and then been subjected to further irradiation.

**Cytogenetic Studies**

Shortly after Klinger first grew amniotic fluid cells in tissue culture, two small series of cultures reported by Steele and Breg (1966) and by Thiede et al. (1966) confirmed that the cells would grow in culture and that they could be used for chromosome analysis, thus making available a technique for the antenatal diagnosis of chromosome abnormalities. The bringing of this technique to perfection and its widespread application has been slow and is far from complete at present. It is, however, better developed than the majority of biochemical techniques, largely because the methods used are few, well established and largely within the competence of established cytogenetic units. Nevertheless, only one large series of cases of chromosome analysis as a result of amniocentesis has been reported, that of Gerbie et al. (1971) who successfully karyotyped 238 out of 250 samples of amniotic fluid. Such remarkable success has not been widespread, Nelson and Emery (1970) were successful in culturing only 49 out of 90 samples; although Ferguson-Smith et al. (1971) were successful in 29 out of 30 cases referred for genetic counselling (repeat amniocentesis was, however, required in five cases) they managed to obtain karyotypes in only 17 of 24 samples obtained at hysterotomy.

The results of successful amniocentesis performed for genetic counselling purposes which required chromosome analysis are summarised in Table 1. The results reveal some surprises. The finding of three pregnancies with Down's syndrome amongst 85 where the mother had had a previous regular mongol is, as Ferguson-Smith et al. (1971) point out, much higher than the generally accepted recurrence risk for this syndrome. Also the 5 abnormal fetuses (three cases of Down's syndrome, one of Klinefelter's syndrome and one XYY male) found in 130 women with advanced age at the time of pregnancy (older than 40 years in most cases) is more than expected. Advanced maternal age is a factor in the production of both Down's and Klinefelter's syndromes but the risk figures are generally believed to be less than found here.

Whilst the theoretical expected risk of producing unbalanced offspring from Robertsonian translocations involving non-homologous chromosomes is 33%, it has been found in practice to be in the region of 4-9% depending

**TABLE 1**

**Cases in Which Fetal Chromosome Analysis via Amniocentesis has been Successfully used for Genetic Counselling**

| Reason for amniocentesis | Valenti and Kehaty (1969) | Butler and Reiss (1970) | Gertner et al. (1970) | Nadler and Gerbie (1971) | Kersey et al. (1971) | Ferguson-Smith et al. (1971) | Total |
|---|---|---|---|---|---|---|---|
| Previous child with regular mongolism | | | 5(1) | 67(2) | | 13 | 85(3) |
| Previous child with other chromosome abnormality | | | | | | 1 | 1 |
| Parent with balanced translocation | 1(1) | 2(1) | | 41(10) | 1(1) | 4 | 49(13) |
| Maternal mosaic for mongolism | | | | | | 1 | 1 |
| Increased maternal age | 1 | | 6(1) | 119(4) | | 4 | 130(5)+ |
| Family history of mongolism | | | 1 | | | 3 | 4 |
| Exposure to mutagens | 2 | | 6 | | | 1 | 9 |
| Total | 4(1) | 2(1) | 18(2) | 227(16) | 1(1) | 27 | 279(21) |

+ Three mongols, one XXY and one XYY male

Figures in parentheses indicate number of abnormal fetuses diagnosed and, except for two cases of Nadler and Gerbie, also indicate that these fetuses were therapeutically aborted.

on the sex of the carrier parent and the exact chromosomes involved (Hamerton, 1970). The reasons for the finding of 13 out of 49 affected fetuses from balanced translocation carriers, apparently in aggreement with the theoretical figure, is not known. Apart from the small number of cases, a possible reason for the finding of an apparent excess of abnormal fetuses in all the groups studied could be that these findings in mid-pregnancy should not be directly compared with expected frequencies of chromosome abnormalities in liveborn neonates. With two exceptions, all the chromosomally abnormal fetuses were therapeutically aborted. It is possible that some of these would have spontaneously aborted had they continued, this could have reduced the incidence of neonates with chromosome abnormalities amongst these groups closer to the expected levels. This is, however, unlikely because most chromosomally abnormal abortuses are lost during the first trimester, and amongst mid-trimester spontaneous abortuses there are only about 3% with chromosome abnormalities (Carr, 1970).

Hence it can be seen that antenatal chromosome analysis is an effective means of predicting the chromosomal status of the fetus and, if followed by abortion of affected fetuses, can reduce the incidence of chromosomal disorders at birth. Unfortunately, misdiagnosis has occurred and a normal fetus has been aborted on at least one occasion (Kohn and Robinson, 1970), however, no child with an unknown chromosome abnormality has been born after amniocentesis although in the series of Gerbie et al. (1971) two fetuses diagnosed as female by amniocentesis were in fact males with normal karyotypes. The reason for this error was probably an overgrowth of contaminant maternal cells.

**Future Prospects**

When any relatively safe technique which can prevent or improve the prognosis of a disease is discovered the question of screening populations at risk arises. Amniocentesis is such a technique, and it is worth considering whether it could be usefully employed as a screening test for disorders which can be diagnosed *in utero,* either with a view to terminating the affected pregnancies or initiating treatment of the fetus or the subsequent neonate. The decision regarding termination or treatment or neither will depend on the particular disease, the attitudes of parents, doctors and the law. In a liberal atmosphere fetuses with treatable disorders (e.g. phenylketonuria if it becomes detectable at this stage) would probably be terminated as would those with any sex chromosome abnormality and perhaps even those with balanced autosomal rearrangements, the termination of this last group is however difficult to justify on genetic grounds (Hamerton, 1970). In a more restrictive climate, probably only those with severely debilitating and untreatable disorders would be terminated.

Littlefield (1970) suggested that amniocentesis was appropriate for four distinct groups of women ranging from those with high risks of producing abnormal fetuses to those with a low to moderate risk. I would suggest that in the high risk cases amniocentesis is not only appropriate but its offer should be an essential part of genetic advice (where facilities are available) in the following instances:

1. Either parent carries a chromosomal rearrangement or has a chromosome abnormality, e.g. where the mother is XXX or the father XYY.
2. The parents are known to be carriers of autosomal recessive genes for any disease diagnosable *in utero.* These would normally have been ascertained via a previously affected child.
3. When one parent carries an autosomal dominant condition which can be diagnosed *in utero.*
4. When the mother is known to be the carrier of an X-linked condition, even when the specific condition cannot be diagnosed.

Where facilities are available amniocentesis is being offered in most of these cases at present and it can be expected that in the near future most major centres will have at least one unit capable of providing antenatal diagnostic services for such cases.

Apart from cytogenetic abnormalities, screening for other disorders is not feasible at present. This is because each requires a separate technique and even with considerable selection the incidence of fetuses with any single disorder is very low. The most common would probably be Tay Sachs disease with an incidence of about one per thousand in some Jewish populations but even here it is preferable to screen prospective parents for heterozygosity.

Apart from the high risk groups outlined above, there are two other groups which are probably worth screening for chromosome abnormalities. These are:

(a) Women who have produced a child with a chromosome abnormality.

(b) Women who become pregnant towards the end of their reproductive life when the chances of producing children with chromosome abnormalities is increased.

In general women in these categories are not being offered amniocentesis for several reasons. These include the inability of present cytogenetic units to handle the numbers which would be involved and reluctance on the part of obstetricians to perform a new procedure when its risks and benefits may not be fully understood and who are worried about the problem of technical failure to produce a result, necessitating repeat amniocentesis. The first problem can be overcome fairly easily because when the costs involved are compared to the resulting benefits, the saving to the community is great, hence government support for the establishment of adequate cytogenetic facilities should be forthcoming. The complications of amniocentesis, both fetal and maternal have been discussed and although present data suggests that this is a procedure which involves very low risks, more information is required from large series before this can be fully confirmed. The problem of failure will probably diminish as more experience is gained by laboratories in amniotic fluid cell culture.

If such a screening programme were applied to Australia, what could we expect? The mothers who had produced a previous child with a known chromosome abnormality would constitute only a small group and could probably be handled without expansion of existing facilities; this group will not be considered further. If 40 years was taken as an age above (and including) which women would be screened then in Australia in 1967 there would have been about 5,400 out of 230,000 mothers included (Sutherland, 1970). The chromosome abnormalities which this group would have been responsible for are estimated in table 2.

**TABLE 2**

**Chromosome Abnormalities which would have been produced by 5,400 women 40 years or older in Australia in 1967**

| Abnormality | | No. of Cases |
|---|---|---|
| Down's syndrome | (47,G+) | 65 |
| Edward's syndrome | (47,E+) | 3 |
| Patau's syndrome | (47,D+) | 3 |
| Klinefelter's syndrome | (47,XXY) | 15 |
| Triple-X female | (47,XXX) | 5 |
| "YY-males" | (47,XYY) | 5 |
| Other abnormalities | | 10 |

The cost to the community of these abnormalities in human terms cannot be calculated; it is sufficient to say that they will result in considerable anguish to the families concerned. It is, however, easier to calculate the cost in financial terms, especially for the cases of Down's syndrome which constitute the most significant group. If each case required 20 years institutionalisation at a cost of $2000 per year, this amounts to $2,600,000. It is more difficult to estimate the costs to the community of the other abnormalities which would be found. The Edward's and Patau's syndrome cases could not be expected to live for more than six months but would require hospital care for most of the time till death. It is difficult to estimate the costs to the community of the cases with sex chromosome abnormalities. The XXY and XXX individuals do have a tendency towards mental deficiency, behavioural disturbance and subsequent institutionalisation (Bartholomew and Sutherland, 1971). They can develop normally except for sterility in the case of XXY, however the proportion of each which develops normally is not accurately known. The XYY males present even more of a problem, Australian experience (Sutherland et al., 1972) suggests that they have a tendency towards institutionalisation but this is denied in Britain (Jacobs et al., 1971). This is a problem which still requires much more study.

I would estimate that to screen these women over 40 years would cost about $500,000 per annum (this allows for a centre in each state). Hence the spending of about half a million dollars would save about three million. Although these cost estimates are extremely approximate they are of the correct order of magnitude, hence there would be about a tenfold/fivefold return on money spent on this type of preventative medicine. The problem of "discounting" is beyond the scope of this review but it will tend to reduce the magnitude of the return on money spent on such population screening. However if the age above which pregnant women were screened was lowered to 35 years then it has been estimated (W.H.O., 1972) that "the cost of such a programme would be substantially less than half that of institutional care for the trisomic population." If further experience with the technique of amniocentesis confirms that the risks involved are almost negligible, or at least significantly less than the chance of having a child with a chromosome abnormality, then screening could

be extended to younger women, e.g. those in the 35-39 age group. Carter (1970) stated that in Britain, women of 35 and over produce about half the mongols, yet comprise only about 10% of the pregnant populuation.

I can only conclude by wholeheartedly agreeing with Ferguson-Smith et al. (1971) when they say " . . . it is important that amniocentesis should not be undertaken . . . for trivial purposes such as choosing the sex of offspring".

**ACKNOWLEDGEMENTS**

I thank Dr. A. D. Bain for helpful discussions during the preparation of this paper. This work was supported in part by a grant to the University of Edinburgh from the Distillers Company Ltd.

**REFERENCES**

Bartholomew, A. A., and Sutherland, G. R. (1971). Psychopathy and sex chromosome abnormalities. Paper presented at World Psychiatric Association Conference, Mexico, November, 1971.

Bartman, J. (1971). Ultrastructure of cultivated amniotic fluid cells. *Obstet. Gynec.*, **38:** 838.

Brock, D. J. H., Gordon, H. Seligman, H., and Lobo, E. de H. (1971). Antenatal detection of Hurler's syndrome. *Lancet,* (ii): 1324.

Butler, L. J., and Reiss, H. E. (1970). Antenatal detection of chromosome abnormalities. *J. Obstet. Gynec. Br. Cwlth.*, **77:** 902.

Carr, D. H. (1970). Chromosome abnormalities and spontaneous abortions. *Pfizer Medical Monographs,* **5:** 103. Edinburgh University Press.

Carter, C. O. (1970). Prospects in genetic counselling. In Emery, A. E. H., Editor, Modern Trends in Human Genetics — **1**, London, Butterworths.

Davidson, R. G. (1970). Application of cell culture techniques to human genetics. In Emery, A. E. H., Editor, Modern Trends in Human Genetics — **1**, London, Butterworths.

Edwards, J. H. (1970). Uses of amniocentesis. *Lancet,* (i): 608.

Emery, A. E. H. (1970). Antenatal diagnosis of genetic disease. In Emery, A. E. H, Editor, Modern Trends in Human Genetics — **1**, London, Butterworths.

Ferguson-Smith, M. E., Ferguson-Smith, M. A., Nevin, N. C. and Stone, M. (1971). Chromosome analysis before birth and its value in genetic counselling. *Br. med. J.*, **4:** 69.

Fratantoni, J. C., Neufeld, E. F., Uhlendorf, B. W., and Jacobson, C. B. (1969). Intrauterine diagnosis of the Hurler and Hunter syndromes. *New Engl. J. Med.*, **280:** 686.

Fujimoto, W. Y., Seegmiller, J. E., Uhlendorf, B. W., and Jacobson, C. B. (1968). Biochemical diagnosis of an X-linked disease *in utero. Lancet,* (ii): 511.

Gerbie, A. B., Nadler, H. L., and Gerbie, M. V. (1971). Amniocentesis in genetic counselling. Safety and reliability in early pregnancy. *Amer. J. Obstet. Gynec.*, **109:** 765.

Gertner, M., Hou, L. Y. F., Martin, J., and Hirschhorn, K. (1970). The use of amniocentesis for prenatal genetic counselling. *Bull. N.Y. Acad. Med.*, **46:** 916.

Hamerton, J. L. (1970). Fetal sex. *Lancet,* (i): 516.

Harris, H (1970). in Amniocentesis: *ad hoc* meeting of experts. Report of M.R.C. Conference, London, 1970.

Hoyes, A. D. (1968). Ultrastructure of the cells in the amniotic fluid. *J. Obstet. Gynec. Br. Cwlth.* **75:** 164.

Hug, G., Schubert, W., and Soukup, S. (1970). Prenatal diagnosis of type-II glycogenosis. *Lancet,* (i): 1002.

Jacobs, P. A., Price, W. H., Richmond, S., and Ratcliff, R. A. W. (1971). Chromosome surveys in penal institutions and approved schools. *J. med. Genet.*, **8:** 49.

Jeffcoate, T. N. A., Fliengner, J. R. H., Russell, S. H., Davis, J. C. and Wade, A. P. (1965). Diagnosis of the adrenogenital syndrome before birth. *Lancet,* (ii): 553

Kersey, J. H., Yunis, J. J., Lee, J. C., Bendel, R. P. (1971). Antenatal detection of partial C(6-12) trisomy. *Lancet,* (i): 702.

Klinger, H. (1965). Personal communication to Steele and Breg (1966).

Kohn, G., and Robinson, A. (1970). Tetraploidy in cells cultured from amniotic fluid. *Lancet,* (ii): 778.

Littlefield, J. W. (1970). The pregnancy at risk for a genetic disorder. *New Engl. J. Med.*, **282:** 627.

Matalon, R., Dorfman, A. Nadler, H. L., and Jacobson, C. (1970). A chemical method for the antenatal diagnosis of mucopolysaccharidoses. *Lancet,* (i): 83.

Merkatz, I. R., New, M. I., Peterson, R. E., and Seaman, M. P. (1969). Prenatal diagnosis of adrenogenital syndrome by amniocentesis. *J. Pediat.* **75:** 977.

Nadler, H. L., Bigley, R. H., and Hug, G. (1970). Prenatal detection of Pompe's disease. *Lancet,* (ii): 369.

Nadler, H. L., and Gerbie, A. (1971). Present status of amniocentesis in intrauterine diagnosis of genetic defects. *Obstet. Gynec.*, **38:** 789.

Nelson, M. M., and Emery, A. E. H. (1970). Amniotic fluid cells; Prenatal sex prediction and culture. *Br. Med. J.*, **1:** 523.

Pearson, P. L., Bobrow, M., and Vosa, C. G. (1970). Technique for indentifying Y chromosomes in human interphase nuclei. *Nature,* (Lond.), **226:** 78.

Pitt, D. (1969). The likelihood of congential disease. *Med. J. Aust.*, **2:** 712.

Regan, J. D, Setlow, R. B., Kabak, M. M., Howell, R. R., Klein, E., and Burgess, G. (1971). Xeroderma pigmentosum: A rapid sensitive method for prenatal diagnosis. *Science,* **174:** 147.

Renwick, J. H. (1969). Progress in mapping human autosomes. *Brit. Med. Bull.*, **25:** (i), 65.

Riis, P., and Fuchs, F. (1960). Antenatal determination of fetal sex in prevention of hereditary diseases. *Lancet,* (i): 383.

Riis, P., and Fuchs, F. (1966). Sex chromatin and antenatal sex diagnosis. In Moore, K. L., Editor, The Sex Chromatin. London and Philadelphia, Saunders.

Rook, A., Hsu, L. Y., Gertner, M., and Hirschhorn, K. (1971). Identification of Y and X chromosomes in amniotic fluid cells. *Nature* (Lond.), **230:** 53.

Sachs, L. Feldman, N., and Danon, M. (1956). Prenatal identification of blood group antigens. *Lancet,* (ii): 356.

Scott, J. S., Coulson, A., and Goulden, R. (1969). Antenatal determination of fetal blood groups. *J. Obstet. Gynec. Br. Cwlth.,* **76:** 330.

Steele, M. W., and Breg, W. R. (1966). Chromosome analysis of human amniotic fluid cells. *Lancet,* (i): 383.

Sutherland, G. R. (1970). Chromosome abnormalitie in newborn babies. *Aust. J. Ment. Ret.,* **1:** 77.

Sutherland, G. R., Weiner, S., Bartholomew, A. A., and Fitzgerald. M. G. (1972). XYY males in Victoria. *Med. J. Aust.,* **1:** 1249.

Thiede, H. A. (1968). Obstetricians should learn the technic of amniocentesis. *Obstet. Gynec.,* **31:** 146.

Thiede, H. A., Creasman, W. T., and Metcalf, S. (1966). Antenatal analysis of the human chromosomes. *Amer. J. Obstet. Gynec.,* **94:** 589.

Valenti, C., and Kehaty, T. (1969). Culture of cells obtained by amniocentesis. *J. Lab. clin. Med.,* **73:** 355.

Votta, R. A., de Gagneten, C. B., Parad, O., and Giulietti, M. (1968). Cytologic study of amniotic fluid in pregnancy. *Amer. J. Obstet. Gynec.,* **102:** 571.

World Health Organisation Technical Report Series (1972), No. 497. Genetic Disorders: Prevention, Treatment and Rehabilitation. World Health Organisation, Geneva.

(*Reprinted from Nature, Vol. 239, No. 5369, p. 231, September 22, 1972*)

# Culture of Cells from the Urine of Newborn Children

THE increasing importance of amniocentesis for the diagnosis of disease *in utero* has focused attention on the cells obtained by this procedure. Culture of amniotic fluid cells is becoming an established procedure for the identification of foetuses with chromosomal and metabolic abnormalities. Cytogenetic and biochemical studies of these cells in culture and morphological studies of the uncultured cells have shown that they are of foetal origin[1–3]. However, the exact origin of the cells which grow remains speculative and several possibilities have been suggested including exfoliated cells from the amnion or from foetal skin[2,3] and also from the foetal respiratory[4] and urogenital[3,4] tracts. Since foetal urine undoubtedly contributes to the amniotic fluid as gestation progresses[5] it seemed reasonable to assume that some of the cells cultured from amniotic fluid could be derived from the foetal urinary tract.

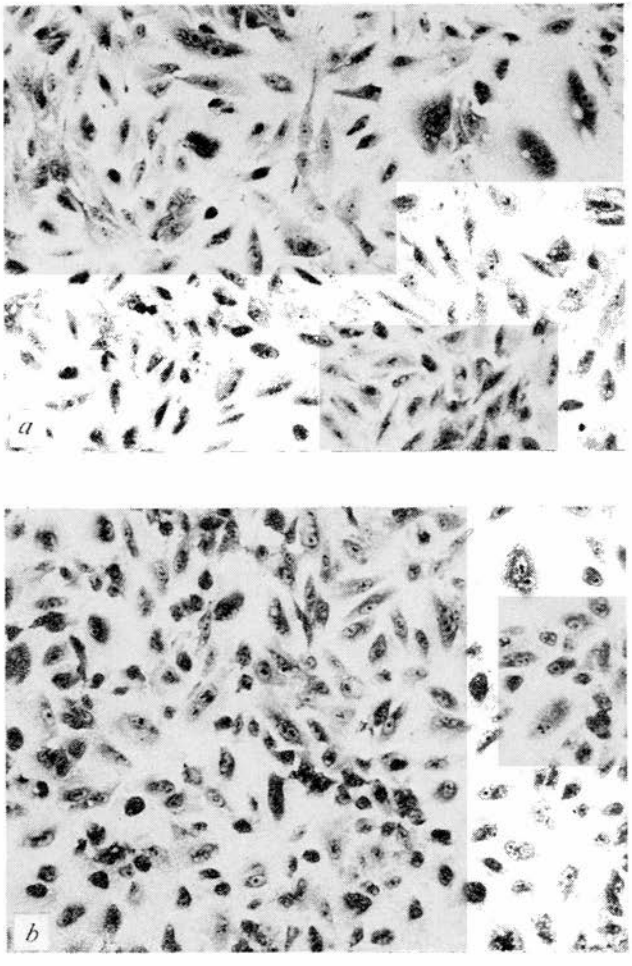



**Fig. 1** Cells cultured from (*a*) urine and (*b*) amniotic fluid as seen 24 h after the third subculture (stain toluidine blue, × 100).

Samples of urine were collected in sterile bags from four infants less than two days old. For tissue culture, the urine samples were then treated as amniotic fluids; they were centrifuged at 1,000 r.p.m. for 10 min, the supernatant decanted, the cells resuspended in tissue culture medium and then transferred to Petri dishes containing cover slips. Ham's F10 TCM containing streptomycin and penicillin with the addition of

30% foetal calf serum was used. Cultures were incubated at 37° C in an atmosphere of 5% $CO_2$ in air. Although one culture showed contamination within 24 h, the remaining three were successfully maintained and proliferating cells were first observed at 6, 9 and 10 days respectively. Sufficient cells were present to allow harvesting for chromosome studies at 12 days in the first case and at 17 days for the other two. These culture times are similar to those being obtained at the present time in this laboratory for amniotic fluid cell cultures.

Using light microscopy the cultured urine cells appear morphologically similar to those cultured from amniotic fluid (Fig. 1); but further characterization of these two types of cell strain is required. Cell cultures have now been set up using urines from a total of eight infants aged less than 2 days and of gestational ages ranging from 31 to 40 weeks. Seven of these cultures have produced primary cell strains, one of which has now reached the seventh pass. A small number of cultures from older children have failed to grow. The only previous attempt to culture cells from urine is that of Jacobson and Barter[4] who set up cultures from sheep urines and bladder aspirates from four human foetuses; however, only one of the former was successful.

The culture of cells from neonatal urine, embodying simplicity in collection and in culture technique, could provide a non-traumatizing procedure for cytogenetic investigations when studies on tissue other than blood are required. Moreover, the most potentially useful application of this technique may be the production of primary cell strains for use in the investigation of inborn errors of metabolism at a biochemical and molecular level.

We thank Dr F. Cockburn for collecting the urine samples.

GRANT R. SUTHERLAND
A. DOUGLAS BAIN

*Department of Pathology,*
*Royal Hospital for Sick Children,*
*and the University of Edinburgh*

[1] Van Leeuwen, L., Jacoby, H., and Charles, D., *Acta Cytol.*, **9**, 442 (1965).
[2] Votta, R. A., de Gagneten, C. B., Parada, O., and Giulietti, M.. *Amer. J. Obstet. Gynec.*, **102**, 571 (1968).
[3] Wachtel, E., Gordon, H., and Olsen, E., *J. Obstet. Gynaec. Br. Commonw.*, **76**, 596 (1969).
[4] Jacobson, C. B., and Barter, R. H., *Amer. J. Obstet. Gynec.*, **99**, 796 (1967).
[5] Emery, A. E. H., in *Modern Trends in Human Genetics*—1 (edit. by Emery, A. E. H.), 268 (Butterworths, London, 1970).

Printed in Great Britain by Flarepath Printers Ltd., St. Albans, Herts.

## CCA Report

CCA 5108

# Lysosomal enzymes in amniotic fluid

J. BUTTERWORTH, GRANT R. SUTHERLAND, A.D. BAIN and W.M. McCRAE

*Department of Pathology, Royal Hospital for Sick Children, and the University of Edinburgh, Edinburgh (U.K.)*

(Received March 10, 1972)

The possible use of amniotic fluid for the prenatal detection of inborn errors of metabolism due to lysosomal enzyme defects is of increasing interest. However, Emery[1] and Nadler and Gerbie[2] have stated that the lack of data on the normal levels of enzymes in amniotic fluid is a limiting factor. We have measured the levels of certain lysosomal enzymes in amniotic fluid in an attempt to provide some of this information.

Amniotic fluid was obtained from hysterotomy specimens and from amniocentesis on rhesus isoimmunized women. The cells were removed by centrifugation. Lysosomal enzymes were assayed after van Hoof and Hers[3] using 4-methylumbelliferone phosphate or glycoside conjugates as substrates. For α-fucosidase, however, the nitrophenol glycoside was used. Protein was determined by the method of Miller[4]. The results obtained to date are given in Table I.

Although at the moment this is only a small series, the results seem to indicate, particularly when displayed graphically, that the enzyme levels vary with gestational age. Apparently β-glucuronidase, α-mannosidase and protein show peak levels at around 25 weeks' gestation. Acid phosphatase and *N*-acetyl-β-D-glucosaminidase appear to increase and β-glucosidase and α-arabinosidase to decrease as gestation proceeds. Another feature of the results is that at any particular gestation age each enzyme showed considerable variation in activity.

The use of amniotic fluid for the detection of lysosomal enzyme defects may be of limited value in view of the variation in enzyme level at any gestational age. Owing to this variation, enzyme values on amniotic fluid alone are unlikely to be reliable and assays will generally be required on cultured cells. It is hoped that further work in progress along these lines may clarify the position.

We thank Drs. F. Cockburn, J.G. Robertson and J. Scrimgeour for the amniotic fluids.

TABLE I

LYSOSOMAL ENZYMES IN AMNIOTIC FLUID

| *Enzyme* * | *Gestation (weeks)* | | | |
|---|---|---|---|---|
| | *12–17* | *18–24* | *25–30* | *31+* |
| β-Glucuronidase | 0.06–0.25 [a]<br>0.2 [b] | 0.30–1.55<br>0.7 | 0.64–1.97<br>1.1 | 0.26–0.52<br>0.4 |
| α-Mannosidase | 0.02–0.32<br>1.8 | 0.23–0.60<br>3.8 | 0.37–1.10<br>6.9 | 0.17–0.32<br>2.6 |
| Acid phosphatase | 0.47–2.00<br>1.4 | 1.41–7.40<br>3.4 | 2.53–9.93<br>5.3 | 1.93–3.87<br>2.7 |
| *N*-acetyl-β-D-glucosaminidase | 1.82–13.44<br>6.5 | 7.25–21.30<br>12.0 | 10.45–23.73<br>16.0 | 6.80–22.60<br>12.2 |
| β-Glucosidase | 0.97–7.25<br>3.0 | 0.67–9.59<br>4.5 | 0.26–5.50<br>2.0 | 0.03–0.26<br>0.2 |
| α-Arabinosidase | 0.51–2.83<br>1.4 | 0.10–3.33<br>1.6 | 0.67–1.13<br>0.9 | 0.0–1.26<br>0.4 |
| α-Fucosidase | 0.10–1.36<br>0.7 | 0.63–2.43<br>1.4 | 1.47–3.31<br>2.1 | 0.44–1.56<br>1.1 |
| Protein | 0.55–4.80<br>2.5 | 3.25–9.02<br>5.1 | 3.62–6.83<br>5.2 | 2.52–5.87<br>4.0 |
| Number of samples | 9 | 4 | 3 | 9 |

* nmoles 4-methylumbelliferone/min/ml of amniotic fluid
[a] Range; [b] Mean

REFERENCES

1 A.E.M. Emery, in *Modern Trends in Human Genetics*, Butterworths, London, 1 (1970) 267.
2 H.L. Nadler and A. Gerbie, *Amer. J. Obstet. Gynec.*, 38 (1971) 789.
3 F. van Hoof and H.G. Hers, *Eur. J. Biochem.*, 7 (1968) 34.
4 G.L. Miller, *Anal. Chem.*, 31 (1959) 964.

Humangenetik 17, 273—275 (1973)


# Metaphase Chromosomes from Neonatal Urine

Grant R. Sutherland, Elizabeth Grace, and A. Douglas Bain

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF

Received August 28, 1972

*Summary.* Tissue culture of the cells present in the urine of neonates has been demonstrated to be a suitable means of obtaining metaphase chromosomes whenever cytogenetic studies are required on cells other than blood lymphocytes. The advantages of this type of cell culture compared with the use of fibroblast culture from skin biopsy are discussed.

*Zusammenfassung.* Es konnte gezeigt werden, daß Gewebekulturen von Zellen aus dem Urin Neugeborener sich zur Gewinnung von Metaphasenchromosomen eignen, wenn cytogenetische Untersuchungen nicht allein an Blutlymphocyten durchgeführt werden sollen. Es werden die Vorteile diskutiert, die diese Art der Zellkultur gegenüber der Fibroblastenkultur aus Hautbiopsien bietet.

Mitotic metaphase preparations for chromosome analyses in the neonate are usually obtained from blood lymphocyte cultures but if further studies are indicated then fibroblasts cultured from a skin biopsy are most commonly used. Skin fibroblast culture has the disadvantage that results are not obtained for many weeks, skin biopsy is a traumatising procedure and is consequently not readily repeatable. It is now possible to culture cells from the urine of the neonate (Sutherland and Bain, 1972) and these can be used for cytogenetic studies.

## Materials and Methods

Urine is collected into standard sterile urine collecting bags and transported to the laboratory. The urine is centrifuged at 1000 r.p.m. for 10 min and the supernatant removed. The pellet of cells is resuspended in tissue culture medium and pipetted into a 50 mm plastic (Nunclon) petri dish containing 4 or 5 glass coverslips (6×22 mm). Duplicate cultures are set up, the cells from approximately 5 ml of urine being used for each. The tissue culture medium is Ham's F10 with 30% fetal calf serum added. Antibiotics incorporated in the medium are kanamycin, or penicillin and streptomycin in combination. The cultures are incubated at 37°C in an atmosphere of 5% $CO_2$ in air and left relatively undisturbed for 1 week after which time half the medium is replaced thrice weekly. The cultures are examined regularly for signs of cell proliferation (almost always first seen on one of the coverslips) which are usually observed after a period of 7 to 14 days.

When an area of proliferating cells appear large enough for harvesting, the medium is fully changed and incubation continued for a further 16 hrs. The coverslip, on which the cells to be harvested are attached, is then removed to a separate petri dish or suitable tube and covered in fresh medium to which Colcemid at a final concentration of 1 μg/ml has been added. Incubation is continued for a further 5—6 hrs after which time the medium is aspirated from the tube and replaced by warm (37°C) 0.075 M KCl. Hypotonic treatment is terminated after 5 min by the addition of an equal volume of fixative (1 part acetic acid to 3 parts methanol)

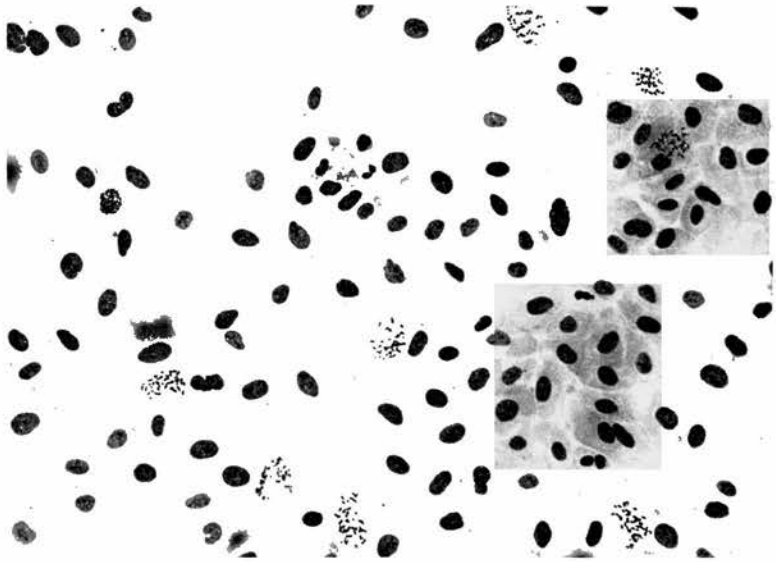


Fig. 1. Primary culture of urine cells harvested for cytogenetic studies after 12 days in culture and 5 hrs exposure to colcemid. (Stain Giemsa, ×125)

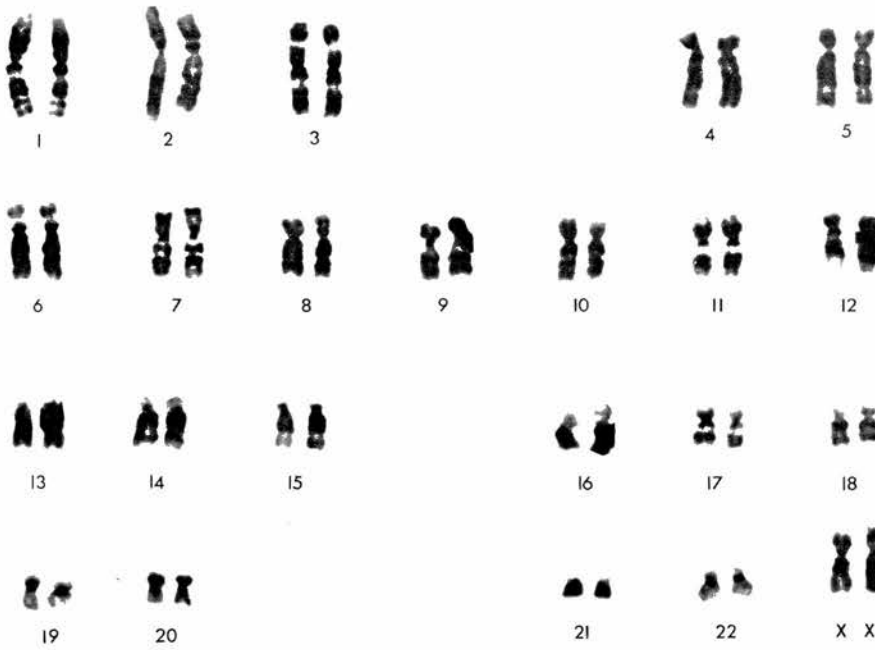



Fig. 2. Banded karyotype prepared using the method of Grace and Bain (1972) from a primary urine cell strain in the 13th passage

to the KCl. Half the KCl/fixative mixture is removed after 1 min and replaced by fixative. This step is repeated twice following which the coverslip is put through two changes of fixative, each of about 5 min. The coverslip is allowed to dry in air at room temperature, stained with Giemsa and mounted on a slide.

By allowing the cells remaining in the original petri dish to proliferate they can be subcultured to produce a primary cell strain suitable for further cytogenetic studies.

## Results

A number of neonates have now been karyotyped successfully by tissue culture of urine. High mitotic activity is present in primary cultures harvested by this method (Fig. 1) and preparations suitable for fluorescent and banding studies have been obtained (Fig. 2).

## Discussion

The technique of culturing urine cells for the purpose of cytogenetic study should prove a valuable addition to the techniques already in widespread use. It has several advantages over skin biopsy in that no trauma is involved in collecting the urine and repeat specimens can be readily obtained if contamination or other problems arise. The method is simple and should be within the capability of any laboratory involved in tissue culture. Results are obtained in 2—3 weeks, sooner than is usually possible in the case of skin biopsy and much less technical work is involved.

The main difficulty encountered is in the collection of the urine samples. Care should be taken as the urine may be contaminated by organisms which will multiply in the culture medium in spite of the antibiotics used. This problem could be overcome by collecting the urine via suprapubic bladder aspiration, a procedure certainly less traumatic than skin biopsy.

In the absence of microbial contamination the proportion of successful cultures has been high and would appear to be independent of the gestational age of the baby but the success rate almost certainly decreases as post-natal age increases. Possible factors which may influence the ability of the cells present in urine to proliferate in tissue culture are being investigated.

We are grateful to the staff of the Paediatric Special Care Unit of the Simpson Memorial Maternity Pavilion, especially Dr. F. Cockburn and Sisters B. Elliott and J. Shaw for collecting the urine specimens.

## References

Grace, E., Bain, A. D.: A simple method for banded chromosomes. J. clin. Path. **25**, 910—911 (1972).

Sutherland, G. R., Bain, A. D.: Culture of cells from the urine of newborn children. Nature (Lond.) **239**, 231 (1972).

Grant R. Sutherland, M.Sc.
Department of Pathology
Royal Hospital of Sick Children
and University of Edinburgh
Edinburgh EH9 1LF
Scotland

Reprinted from

*Clinica Chimica Acta*, 44 (1973) 295–299



CCA 5547

# LYSOSOMAL ENZYMES OF CULTURED AMNIOTIC FLUID CELLS

J. BUTTERWORTH, G. R. SUTHERLAND, D. M. BROADHEAD AND A. D. BAIN
*Department of Pathology, Royal Hospital For Sick Children, Edinburgh (U.K.)*
(Received October 25, 1972)

## SUMMARY

1. The levels of some lysosomal enzymes in a minimum of 38 amniotic fluid cell strains cultured to the third passage were as follows (mean ± standard deviation):

*N*-acetyl-β-D-glucosaminidase 52.1 ± 16.70, acid phosphatase 7.73 ± 2.82, β-galactosidase 4.59 ± 1.97, β-glucuronidase 1.30 ± 0.52, β-glucosidase 0.072 ± 0.030, α-galactosidase 0.42 ± 0.15, α-arabinosidase 0.53 ± 0.25, α-glucosidase 0.38 ± 0.18 and α-mannosidase 0.65 ± 0.30 nmole 4-methylumbelliferone/min/mg protein.

2. No correlation was observed between the levels of these enzymes and gestational age.

3. The levels of these enzymes in uncultured cells tended to be lower than in the corresponding cultured cells. Occasionally, very low levels of certain of the enzymes were found in uncultured cells, although normal levels were found in the corresponding cultured cells.

## INTRODUCTION

Amniocentesis combined with biochemical analysis of the amniotic fluid or cells is increasingly being utilized for the antenatal diagnosis of genetic disease. The uses and possibilities of this procedure have been reviewed[1–3]. Great caution must be exercised if amniotic fluid or amniotic fluid cells are used directly for enzyme analysis[2]. A more reliable indication of the presence of enzyme activity in the foetus is obtained from cultured amniotic fluid cells[4]. However, the variation in the activity of lysosomal enzymes of cultured amniotic fluid cells has not previously been investigated in detail and is presented here.

## MATERIALS AND METHODS

Amniotic fluid samples were obtained from hysterotomy specimens and by amniocentesis on rhesus isoimmunised women with no history of inborn errors of metabolism involving lysosomal enzymes. Primary cultures of cells from these fluids were established[5] and subsequently subcultured, using 0.25% trypsin in phosphate-buffered saline to remove the cells from the Petri dish, into a glass culture bottle with

a growth surface area of approximately 20 $cm^2$. The cells were then similarly subcultured into and maintained in bottles twice this size. Ham's F10 tissue culture medium (Flow Lab. Ltd.) supplemented with 30% foetal calf serum (Bio-Cult Lab. Ltd.) was used; antibiotics incorporated were kanamycin (100 $\mu$g/ml) or penicillin (60 $\mu$g/ml) and streptomycin (100 $\mu$g/ml) in combination. Culture bottles were flushed with 5% $CO_2$ in air prior to stoppering.

The cells were harvested on reaching confluency after the third subculture. Trypsin (0.25%) in phosphate-buffered saline was used to remove the cells from the glass. After washing in phosphate-buffered saline and centrifuging at 600 $\times g$ for 5 min at 5° the cells were taken up in 4 ml of ice-cold 100 mM sodium chloride and sonicated for 15 sec at maximum power using a "Soniprobe" (Dawes Instruments). The homogenates were centrifuged at 25000 $\times g$ for 10 min at 5° and stored at −65°.

The methods of enzyme estimation were those of Butterworth *et al.*[6] with two modifications:

$\beta$-glucuronidase—1 mM conjugate, 0.1 M acetate buffer pH 4.0, 30 min

$\alpha$-glucosidase—2 mM conjugate, 0.2 M phosphate/citrate buffer pH 4.5, 60 min.

## RESULTS

The results obtained for the enzymes assayed in a series of cultured amniotic fluid cells are presented in Figs. 1 and 2. Table I gives the mean, standard deviation and range for each enzyme. The levels of the enzymes differed greatly, varying from 52.1 ± 16.7 nmole/min/mg protein for *N*-acetyl-$\beta$-D-glucosaminidase to 0.072 ± 0.030 nmole/min/mg protein for $\beta$-glucosidase. Each of the enzymes showed a fairly narrow range of values except for a few cell strains showing unusually high

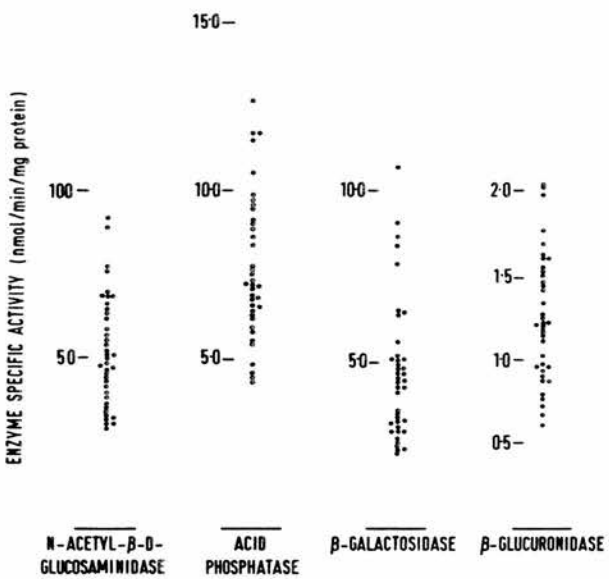



Fig. 1. Specific activities (nmole 4-methylumbelliferone/min/mg protein) of *N*-acetyl-$\beta$-D-glucosaminidase, acid phosphatase, $\beta$-galactosidase, $\beta$-glucuronidase in amniotic fluid cells after the third passage of culture.

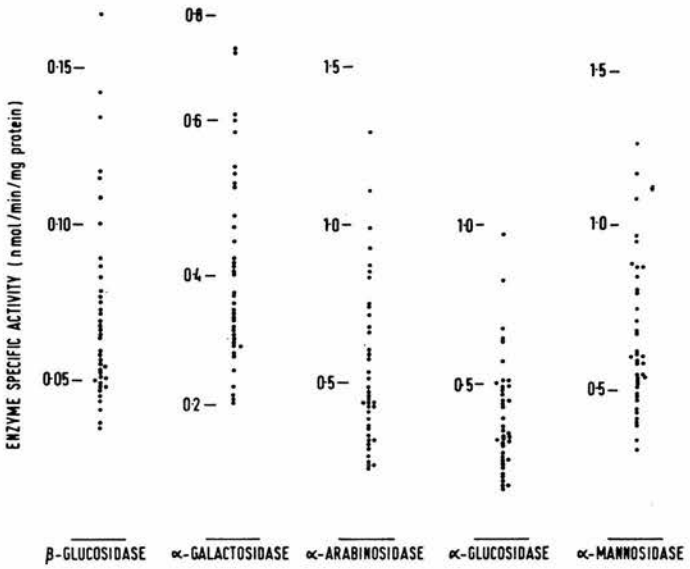



Fig. 2. Specific activities (nmole 4-methylumbelliferone/min/mg protein) of β-glucosidase, α-galactosidase, α-arabinosidase, α-glucosidase and α-mannosidase in amniotic fluid cells after the third passage of culture.

levels of enzyme activity. Consequently 95% confidence limits based on a normal distribution produce lower limits for the enzyme specific activities which are well below those actually measured. Hence Table I gives the upper and lower values (range) actually measured. The correlation coefficients ($r$) for the enzyme specific activities with gestational age are given in Table I from which it can be seen that none of the enzymes exhibits any relationship with this factor.

In Table II a comparison is made between the levels of the enzymes assayed for uncultured and cultured amniotic fluid cells derived from three amniotic fluid samples. One feature, with five exceptions, is the lower level of these enzymes in the

TABLE I

LYSOSOMAL ENZYMES OF CULTURED AMNIOTIC FLUID CELLS

| *Enzyme* | *Number of cell strains* | *Enzyme specific activity*[a] | | | *Correlation coefficient (r) of enzyme activity with gestational age*[b] |
|---|---|---|---|---|---|
| | | *Mean* | *Standard Deviation* | *Range* | |
| *N*-Acetyl-β-D-glucosaminidase | 39 | 52.10 | 16.70 | 28.70–91.85 | −0.196 |
| Acid phosphatase | 38 | 7.73 | 2.82 | 4.34–12.69 | 0.198 |
| β-Galactosidase | 41 | 4.59 | 1.97 | 2.37–10.69 | −0.135 |
| β-Glucuronidase | 38 | 1.30 | 0.52 | 0.62–2.02 | 0.284 |
| β-Glucosidase | 39 | 0.072 | 0.030 | 0.03–0.17 | −0.083 |
| α-Galactosidase | 38 | 0.42 | 0.15 | 0.22–0.78 | −0.158 |
| α-Arabinosidase | 40 | 0.53 | 0.25 | 0.22–1.30 | −0.081 |
| α-Glucosidase | 40 | 0.38 | 0.18 | 0.17–0.97 | 0.218 |
| α-Mannosidase | 39 | 0.65 | 0.30 | 0.28–1.27 | −0.093 |

[a] nmole 4-Methylumbelliferone/min/mg protein.
[b] $(r) \geq 0.325$ $P(r = 0) \leq 0.05$ $df$ 35.

TABLE II

SPECIFIC ACTIVITY[a] OF LYSOSOMAL ENZYMES OF UNCULTURED AND CULTURED AMNIOTIC FLUID CELLS

| *Enzyme* | *Sample*[b] | | | | | |
|---|---|---|---|---|---|---|
| | *Uncultured*[1] | *Cultured* | *Uncultured*[2] | *Cultured* | *Uncultured*[3] | *Cultured* |
| *N*-Acetyl-β-D-glucosaminidase | 8.28 | 52.37 | 23.18 | 47.26 | 4.46 | 31.40 |
| Acid phosphatase | 7.20 | 7.55 | 17.59 | 7.63 | 3.26 | 4.84 |
| β-Galactosidase | 0.10 | 4.36 | 0.30 | 3.91 | 0.25 | 4.19 |
| β-Glucuronidase | 2.20 | 0.94 | 2.94 | 1.34 | 0.73 | 1.02 |
| β-Glucosidase | 0.012 | 0.089 | 0.028 | 0.122 | 0.012 | 0.035 |
| α-Galactosidase | 0.023 | 0.37 | 0.075 | 0.35 | 0.018 | 0.32 |
| α-Arabinosidase | 0.008 | 0.43 | 0.040 | 0.43 | 0.040 | 0.60 |
| α-Glucosidase | 0.05 | 0.42 | 0.57 | 0.30 | 0.45 | 0.25 |
| α-Mannosidase | 0.25 | 0.55 | 0.28 | 0.62 | 0.20 | 0.50 |

[a] nmole 4-Methylumbelliferone/min/mg protein.
[b] Gestational age: Sample 1: 23 weeks; 2: 21 weeks; 3: 16 weeks.

uncultured compared with the cultured cells. The low level in uncultured cells of some of these enzymes, such as β-galactosidase, α-galactosidase and α-arabinosidase, would make it very difficult to assert that an absence of enzyme in this material was due to a possible inborn error of metabolism. The need for great caution in the use of uncultured amniotic fluid cells for antenatal diagnosis is clearly demonstrated by sample 1. In this sample, the levels of α-glucosidase and α-arabinosidase were very low in the uncultured cells, but were normal in the corresponding cultured cells.

## DISCUSSION

Nadler[7] suggested that the activity of glucose-6-phosphate dehydrogenase in cultured amniotic fluid cells may show a correlation with gestational age and remarked that there was no such correlation for acid phosphatase, β-glucuronidase and α-glucosidase. Indeed, in the present study, this latter group of enzymes, together with the other lysosomal enzymes assayed, did not show a correlation with gestational age. A similar lack of correlation for some lysosomal enzymes has been reported in uncultured amniotic fluid cells[8].

The results presented here form part of an investigation of factors affecting the levels of lysosomal enzyme activity in cultured amniotic fluid cells. The third passage was the earliest at which it was possible to obtain sufficient cells for the enzyme assays and still maintain all of the cell strains. The enzyme levels in the third passage can, however, be taken as representative of at least the first five passages[9]. The use of cells from the third passage results in some delay after amniocentesis before the enzymes can be estimated. This delay varied from 4–10 weeks, although 75% of the cell strains could be assayed before 6 weeks. This time-lapse could be a problem in relation to antenatal diagnosis. However, even by using the first passage a maximum of only 2 weeks could be saved.

The relative merits of uncultured and cultured amniotic fluid cells for the biochemical studies involved in antenatal diagnosis of inborn errors of metabolism are not yet resolved. The assay of uncultured cells, if feasible, would avoid the uncertainty of culture and permit early diagnosis[10]. Indeed Tay–Sachs' disease[11] and

Pompe's disease[12] have been diagnosed *in utero*. However, the reliability of using uncultured cells for diagnosing these diseases has been seriously questioned[13–15]. Nadler and Gerbie[2] have emphasised the need for great caution in the direct assay of uncultured amniotic fluid cells due to the possibility of maternal blood cell contamination, enzyme instability and inadequate numbers of viable cells. They suggested that the enzyme of interest should be related to another control enzyme in order to minimise any such problems. However, our results indicate that this could be hazardous as it is possible for uncultured cells to have some enzymes virtually absent and others normal, whilst the corresponding cultured cells have all their enzyme activities in the control range. In contrast, no enzyme level in cultured cells from the whole series of normal amniotic fluid samples was low enough to suggest that a particular enzyme was absent. However, the suggestion of Nadler and Gerbie[2] should be applied when using cultured amniotic fluid cells for antenatal diagnosis of inborn errors of metabolism involving lysosomal enzymes in order to check that the culture and assay conditions have not produced uniformly low enzyme levels.

ACKNOWLEDGEMENTS

This work was supported by the Scottish Advisory Committee on Medical Research and the Secretary of State for Scotland.

REFERENCES

1 A. MILUNSKY, J. W. LITTLEFIELD, J. N. KANFER, E. H. KOLODNY, V. E. SHIH AND L. ATKINS, *New Engl. J. Med.*, 283 (1970) 1370, 1441, 1498.
2 H. L. NADLER AND A. B. GERBIE, *Amer. J. Obstet. Gynecol.*, 103 (1969) 710.
3 B. HULTBERG, P. A. ÖCKERMAN AND S. SJOBLAD, *Europ. Neurol.*, 7 (1972) 101.
4 P. F. BENSON, *Proc. Roy. Soc. Med. (London)*, 64 (1971) 1137.
5 G. R. SUTHERLAND AND A. D. BAIN, *Nature*, 239 (1972) 231.
6 J. BUTTERWORTH, F. SCOTT, W. M. MCCRAE AND A. D. BAIN, *Clin. Chim. Acta*, 40 (1972) 139.
7 H. L. NADLER, *Biochem. Genet.*, 2 (1968) 119.
8 W. R. DEN TANDT, *Clin. Chim. Acta*, 40 (1972) 199.
9 J. BUTTERWORTH, G. R. SUTHERLAND, D. M. BROADHEAD AND A. D. BAIN, (1972) unpublished observations.
10 R. G. SUTCLIFFE AND D. J. BROCK, *Clin. Chim. Acta*, 31 (1971) 363.
11 L. SCHNECK, C. VALENTI, D. AMSTERDAM, J. FRIEDLAND, M. ADACHI AND B. W. VOLK, *Lancet*, i (1970) 582.
12 H. L. NADLER AND A. M. MESSINA, *Lancet*, ii (1969) 1277.
13 R. G. DAVIDSON AND M. C. RATTAZZI, *Clin. Chem.*, 18 (1972) 179.
14 H. L. NADLER, *Pediatrics*, 49 (1972) 329.
15 R. NAVON AND B. PADEH, *Brit. Med. J.*, 4 (1971) 17.

Life Sciences Vol. 13, pp. 713-722, 1973. Pergamon Press
Printed in Great Britain

# LYSOSOMAL ENZYME LEVELS IN HUMAN AMNIOTIC FLUID CELLS IN TISSUE CULTURE. I. α-GLUCOSIDASE AND ß-GLUCOSIDASE

John Butterworth, Grant R. Sutherland, David M. Broadhead and A. Douglas Bain

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Scotland

(Received 29 May, 1973; in final form 30 July, 1973)

## SUMMARY

A number of factors which may correlate with the levels of α-glucosidase and ß-glucosidase in cultured amniotic fluid cells have been investigated. Fluctuations in enzyme activity occurred as passage numbers increased. Whereas α-glucosidase showed a consistently lower activity in the earlier passages compared to the later ones, the results for ß-glucosidase were equivocal. Both enzymes showed an increase in activity correlated with the time taken by the cells to reach confluency in the third passage. When replicate cultures were assayed daily after subculture, neither enzyme showed any change correlated with time. When cultures were grown in parallel in Ham's F10 and Eagle's M.E.M. tissue culture media, the activity of both enzymes was unaffected. Cell strains cultured from serial samples of amniotic fluid from the same woman had differing enzyme levels unrelated to gestational age.

A number of diseases have been shown to be due to a deficiency in the activity of a lysosomal enzyme (1,2,3,4). These enzymes are normally present in cultured amniotic fluid cells (5,6,7) and it has been found that an enzyme deficiency in the foetus is reflected in these cells (8,9,10). Although studies have been undertaken on the fluctuations in the activity of lysosomal enzymes in cultured fibroblasts (11-18), very little attention (11,19) has been paid to the variations in lysosomal enzymes in cultured amniotic fluid cells. With the rapidly expanding demand for antenatal diagnosis of inborn errors of lysosomal enzyme metabolism, a greater knowledge of the variations

in the levels of these enzymes in cultured amniotic fluid cells is necessary. Hence an investigation of some of the conditions of cell culture that may affect the levels of α-glucosidase and ß-glucosidase was undertaken.

## METHODS

Amniotic fluid samples were obtained from hysterotomy specimens and by amniocentesis on Rhesus iso-immunised women who had no history of inborn errors of metabolism involving lysosomal enzymes. Primary cell cultures were set up (20) then subcultured, harvested, homogenized (7), and the lysosomal enzymes assayed using 4-methylumbelliferone conjugates (7,21).

The reproducibility of the combined culture and enzyme assay techniques was tested by establishing twelve replicate cultures of one cell strain at two different passages. The twelve replicates were harvested when they had simultaneously reached confluency and then assayed for enzyme activity. To study enzyme activity with respect to passage, six cell strains were subcultured for up to fifteen passages and the enzyme levels assayed at intervals. All the cell strains were assayed at the third passage and the time interval between subculture and harvest (confluency) noted. The variation in enzyme level within a passage was followed by establishing a series of replicate cultures which were harvested for enzyme assay at daily intervals for up to seven days. The effect of culture medium on enzyme activity was followed by performing enzyme assays on six cell strains cultured in parallel for three passages in Ham's F10 and Eagle's M.E.M. tissue culture media (supplemented with 30% foetal calf serum).

## RESULTS

The reproducibility of the combined culture and assay system for each enzyme is presented in Table I. The variation of the total experimental procedure (coefficient of variance) was less than 10% for both enzymes. The variation in the levels of the enzymes with passage is given for six cell strains in Figs. 1 and 2. The coefficient of variance of the mean enzyme levels

TABLE I

Reproducibility of Culture and Assay System

| Enzyme | Mean* | Standard Deviation | Coefficient of Variance (%) |
|---|---|---|---|
| α-GLUCOSIDASE | 0.41[a] | 0.035 | 8.5 |
| | 0.81[b] | 0.027 | 3.3 |
| ß-GLUCOSIDASE | 0.154[a] | 0.012 | 7.8 |
| | 0.053[b] | 0.005 | 9.3 |

* Enzyme activity in nmoles 4-methylumbelliferone/ min./mg. protein for twelve replicates.
a - Assay tenth passage; b - Assay twentieth passage.

for the different cell strains was 8.9 - 42.6% for α-glucosidase and 32.9 - 65.0% for ß-glucosidase. These values are much greater than for the experimental procedure and indicate that there are changes occurring in the enzyme levels in any one cell strain as culture progresses.

Marked variations in the levels of both enzymes occurred with passage. In the case of α-glucosidase there was a rise in activity as culture progressed. This was shown by the lower activity ($P < 0.005$) of this enzyme in the first five passages

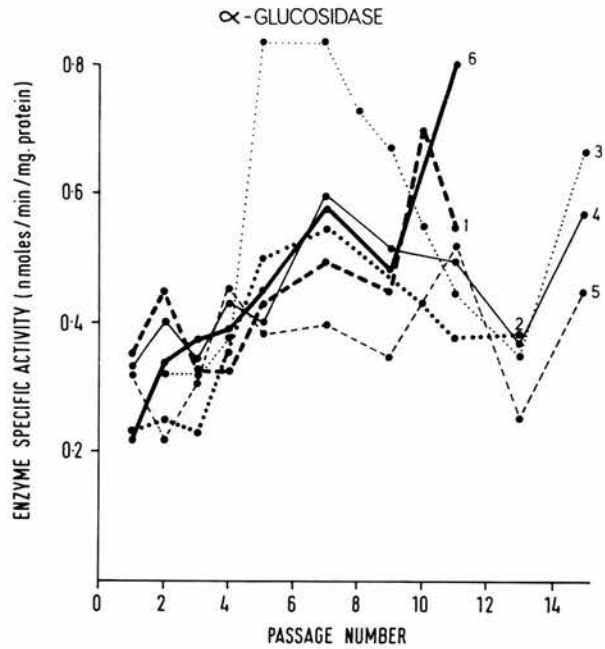



FIG. 1

The activity of α-glucosidase in serial passages of six strains of cultured amniotic fluid cells.

compared with the next ten. In addition the mean activity in the third passage was lower ($P < 0.01$) than that in the tenth passage of ten strains studied. For ß-glucosidase cell strains 1 and 6 showed a positive correlation ($P < 0.05$) and strains 3,4 and 5 a similar negative one with passage number. Although the levels of this enzyme were also lower in the first five passages ($P < 0.05$) than in the next ten, the comparison of activities at the third and tenth passages showed no difference.

The relationship of the enzyme activity in cell strains at the third passage to the time at which confluency (harvest) was reached is given in Fig. 3. Both α-glucosidase ($r = 0.48$) and ß-glucosidase ($r = 0.63$) showed a significant positive correlation with this factor. The correlation was still significant even if

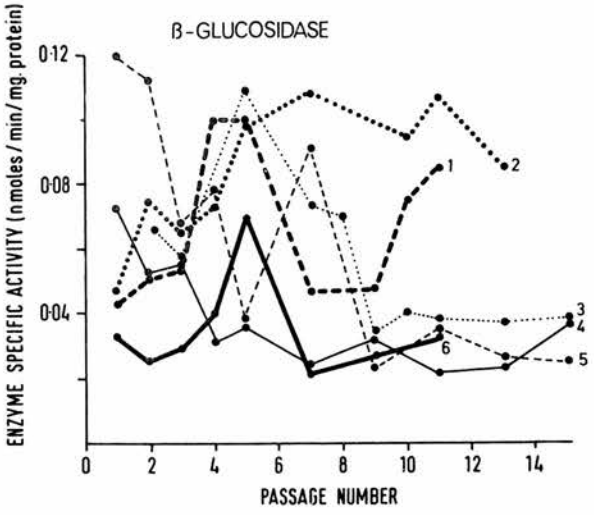



FIG. 2

The activity of ß-glucosidase in serial passages of six strains of cultured amniotic fluid cells.

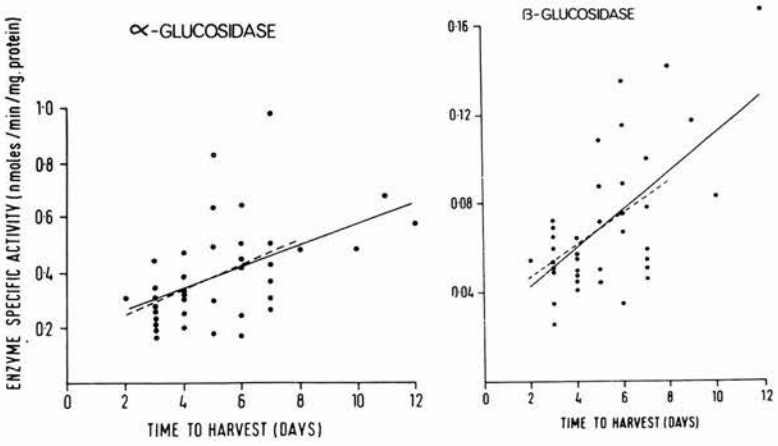



FIG. 3

Relationship of the activity of α-glucosidase and ß-glucosidase in cultured amniotic fluid cells with time to reach confluency (harvest).
——— All days ------ Without 9-12 days.

the few cell strains requiring 10-12 days to reach confluency were not considered.

The fluctuations in the levels of the two enzymes were not related to the time after subculture and no consistent change was associated with the point at which confluency was reached (Fig. 4).

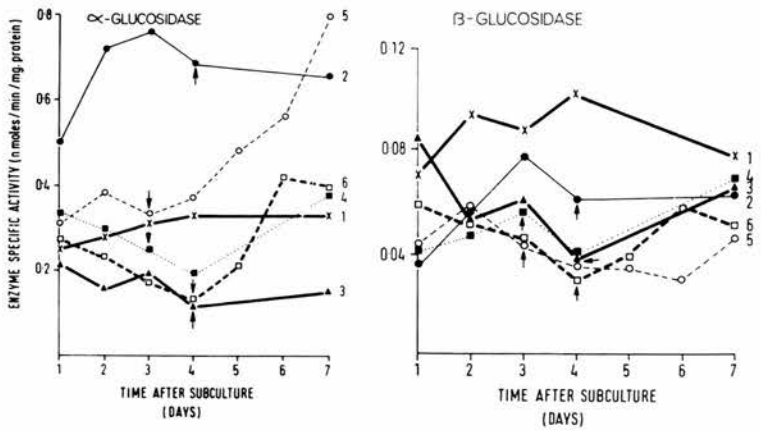



FIG. 4

The activity of α-glucosidase and ß-glucosidase in six strains of amniotic fluid cells in relation to time since subculture.

↑ Confluency; Strain 1 not confluent by 7 days.

Analysis of variance of the mean enzyme levels over three passages for each cell strain in the two culture media was performed. No significant difference was found for either α-glucosidase ($F = 2.1$, $F_{0.05}(1,5) = 6.61$) or ß-glucosidase ($F = 1.0$).

Cell strains were grown from serial samples of amniotic fluid obtained from several Rhesus iso-immunised women and the levels of the enzymes, at the third passage, are given in Table II. The activity of the two enzymes showed considerable variability within each series, but this did not seem to be related to the gestational age.

TABLE II

Enzyme Levels* in Cell Strains at the Third Passage Cultured from Serial Samples of Amniotic Fluid Obtained from Four Women

| SERIES | GESTATIONAL AGE (WEEKS) | α-GLUCOSIDASE | ß-GLUCOSIDASE |
|---|---|---|---|
| 1 | 18 | 0.30 | 0.059 |
| | 22 | 0.47 | 0.057 |
| | 24 | 0.33 | 0.048 |
| | 33 | 0.58 | 0.081 |
| | 35 | 0.24 | 0.025 |
| 2 | 23 | 0.38 | 0.064 |
| | 26 | 0.44 | 0.072 |
| | 28 | 0.20 | 0.069 |
| 3 | 22 | 0.57 | 0.167 |
| | 26 | 0.67 | 0.117 |
| 4 | 27 | 0.63 | 0.050 |
| | 34 | 0.31 | 0.049 |

* Enzyme activity - nmoles 4-Methylumbelliferone/ min./mg. protein.

## DISCUSSION

Several studies have been undertaken of the influence of culture conditions on the levels of lysosomal enzymes in fibroblast-like cells (11-18). However, very little work of this nature has been reported using cultured amniotic fluid cells (11). This may well be due to the supposed difficulty in obtaining cell strains from amniotic fluid (22,23). However, in our laboratory it has been possible to culture the majority of the amniotic fluid cell strains to the tenth passage with some still growing well at the twentieth (24).

The fluctuations observed in the levels of the two enzymes

studied with passage has not been previously reported for amniotic fluid cells, although it has been noted for fibroblasts (25). The increase in the level of α-glucosidase with passage observed in this study probably reflects the decrease in the ratio of the epithelioid to fibroblast-like cells which occurs with time in culture (22). The situation with regard to ß-glucosidase is equivocal. Since the activity of ß-glucosidase, unlike α-glucosidase, can show opposite trends in different cell strains, it seems very unlikely that these trends can be related to changes in cell type. This interpretation is supported by the data of Gerbie et al. (19) on the activity of these two enzymes in epithelioid and fibroblast-like cultured amniotic fluid cells.

The positive correlation between the levels of both enzymes and time to harvest (confluency) is an unexpected result and may reflect the proportion of dividing to resting cells in cultures growing at different rates. The lack of any relationship between the enzyme activities and time after subculture is perhaps not surprising as such a relationship has only been demonstrated using homogeneous cell populations (13,16,17,18).

A significantly lower level of ß-glucosidase has been found in fibroblasts grown in Ham's F10 compared with Eagle's M.E.M. (11,17). Beutler et al. (11) claimed that this enzyme was also lower in activity in amniotic fluid cells grown in an enriched medium. In the present study, however, the high concentration of serum supplement used may have tended to obscure any real difference in enzyme activity attributable to the chemically defined components of the medium.

There has been no previous report on the levels of lysosomal enzymes in cells cultured from amniotic fluid obtained from the

same women at different stages of pregnancy. The apparent lack of a correlation of enzyme activity with the gestational age of the amniotic fluid agrees with results obtained using samples from different women (7).

The marked variations due to conditions of culture which occur in the levels of α-glucosidase and β-glucosidase do not preclude the antenatal diagnosis of Pompe's or Gaucher's disease, but they would render the detection of heterozygotes very difficult. A complete understanding of the causes of these variations will require further investigation.

## ACKNOWLEDGEMENTS

This work was supported by the Advisory Committee on Medical Research and the Secretary of State for Scotland.

## REFERENCES

1. A. Milunsky, J.W. Littlefield, J.N. Kanfer, E.H. Kolodny, V.E. Shih and L. Atkins, New Engl. J. Med. 283 1370-1381, 1441-1447, 1498-1503 (1970).

2. H.L. Nadler, Birth Defects 6 26-33 (1970).

3. H.L. Nadler and A. Gerbie, Obstet. Gynec. 38 789-799 (1971).

4. B. Hultberg, P.A. Ockerman and S. Sjoblad, Euro. Neurol. 7 101-118 (1972).

5. M.M. Kabach and R.E. Cooke, Paediat. jap. 18 79-87 (1970).

6. P.F. Benson, Proc. R. Soc. Med. 64 1137-1139 (1971).

7. J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Chim. Acta 44, 295-299 (1973).

8. H.L. Nadler and A.M. Messina, Lancet 2 1277-1278 (1969).

9. B. Padeh and R. Navon, Israel J. Med. Sci. 1 259-263 (1971)

10. I.S. Salafsky and H.L. Nadler, J. Pediat. 79 794-798 (1971)

11. E. Beutler, W. Kuhl, F. Trinidad, R. Teplitz and H.L. Nadler, Am. J. hum. Genet. 23 62-66 (1971).

12. V.J. Cristofalo and D. Kritchevsky, Prog. Immunobiol. Stand. 3 99-105 (1969).

13. R. DeMars, Natn. Cancer Inst. Monogr. 13 181-195 (1964).

14. A. Maciera-Coelho, E. Garcia-Giralt, M. Adrian, Proc. Soc. exp. Biol. Med. 138 712-718 (1971).

15. S. Okada, M.L. Veath, J. Leroy and J.S. O'Brien, Am. J. hum. Genet. 23 55-61 (1971).

16. S.B. Russell, J.D. Russell and J.W. Littlefield, J. med. Genet. 8 441-443 (1971).

17. C.A. Ryan, S.Y. Lee and H.L. Nadler, Expl. Cell Res. 71 388-392 (1972).

18. J. Leroy and R. DeMars, Science 157 804-806 (1967).

19. A. Gerbie, S.B. Melancon, C. Ryan and H.L. Nadler, Am. J. Obstet. Gynec. 114 314-320 (1972).

20. G.R. Sutherland and A.D. Bain, Nature 239 231 (1972).

21. J. Butterworth, F. Scott, W.M. McCrae and A.D. Bain, Clin. Chim. Acta 40 139-142 (1972).

22. M.M. Nelson. In Antenatal Diagnosis of Genetic Disease (ed. A.E.H. Emery) Churchill Livingstone, London, 69-81 (1973).

23. J.W. Littlefield, Birth Defects 7 15-17 (1971).

24. G.R. Sutherland, R. Bauld and A.D. Bain. In preparation.

25. C.W. Castor, Arthritis Rheum. 14 41-54 (1971).

26. S.B. Melancon, S.Y. Lee and H.L. Nadler, Science 173 627-628 (1971).

Humangenetik 20, 251—255 (1973)


# Antenatal Diagnosis of Inborn Errors of Metabolism: Tissue Culture Aspects

Grant R. Sutherland and A. Douglas Bain

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh

Received August 12, 1973

*Summary.* The cells from 62 amniotic fluids have been cultured to the stage at which biochemical studies could have been undertaken. Although all cultures showed initial signs of cellular proliferation in only 90% of these were sufficient cells obtained for biochemical assay. If a time limit of 6 weeks was to be imposed, only 58% of the cultures could have been regarded as successful. The problems involved in culturing amniotic fluid cells for the antenatal diagnosis of inborn errors of metabolism are discussed.

*Zusammenfassung.* Von 62 Amnionflüssigkeits-Proben wurden die Zellen bis zu einem Stadium kultiviert, in dem biochemische Untersuchungen möglich wurden. Obwohl alle Kulturen anfänglich Zeichen einer Zellproliferation zeigten, wurden nur in 90% genügend Zellen für biochemische Untersuchungen gewonnen. Unter Annahme einer Zeitbegrenzung von 6 Wochen konnten sogar nur 58% aller Kulturen als erfolgreich betrachtet werden. Die Probleme bei der Kultivierung von Amnionzellen für die pränatale Diagnose angeborener Stoffwechselstörungen werden diskutiert.

Cultured amniotic fluid cells are almost always required when the antenatal diagnosis of an inborn error of metabolism is to be made. Except where histochemical techniques are available, this usually means many more cells are required than for cytogenetic studies. Gerbie *et al.* (1971) successfully karyotyped 95% of 250 amniotic fluids and other groups have reported similar high success rates. Little comparable data exists for success in culturing amniotic fluid cells to the stage where biochemical assays are possible. The time is usually limited to between 6 and 8 weeks for the culture of sufficient cells if a pregnancy may be terminated on the result. Hence it is valuable to report the experience in culturing cells from all the amniotic fluids received in this laboratory over a 1-year period.

## Materials and Methods

Amniotic fluids were obtained from hysterotomy specimens and by amniocentesis of Rhesus iso-immunised women. The 62 amniotic fluids were taken from 47 women. When more than one sample was collected from any woman it was always from the same pregnancy. This study includes all such amniotic fluids received in this laboratory over the year ending November 1972.

The methods used for primary culture have been previously described (Sutherland *et al.*, 1973). The primary culture vessel was a 50 mm Nunclon plastic petri dish which contained four or five 6 × 22 mm glass coverslips. In the majority of cases one or two of these coverslips were removed from the culture for cytogenetic studies (Sutherland *et al.*, 1973). The primary

cultures were subcultured according to Butterworth *et al.* (1973) into a glass culture bottle with a growth surface area of 20 $cm^2$. On reaching confluency the cell strains were further subcultured into a glass culture bottle with a growth surface area of 40 $cm^2$. When the cell strains reached confluency after this second subculture the time since the amniotic fluid had been collected was noted and this was regarded as the time required to produce enough cells for a biochemical assay. Cell homogenates prepared from this quantity of cells contain ½—1 mg of protein and are adequate for a number of biochemical assays (Butterworth *et al.*, 1973).

## Results

The results are summarized in Table 1 and Fig. 1. There were 62 amniotic fluids, 21 from hysterotomy specimens and 41 from Rhesus iso-immunised women. All cultures showed cell proliferation as observed under the inverted microscope.

Table 1. Data on the origin and cultural behaviour of the amniotic fluids in this series

| Origin | No. | Gestational ages (weeks) | | No. of biochemical failures | Time to biochemical result (days) | |
|---|---|---|---|---|---|---|
| | | mean | range | | mean | range |
| Rhesus | 41 | 28.0 | 18—36 | 3 | 42.4 | 25—74 |
| Hysterotomy | 21 | 16.8 | 12—23 | 3 | 39.5 | 29—63 |
| Total | 62 | 24.2 | 12—36 | 6 | 41.4 | 25—74 |

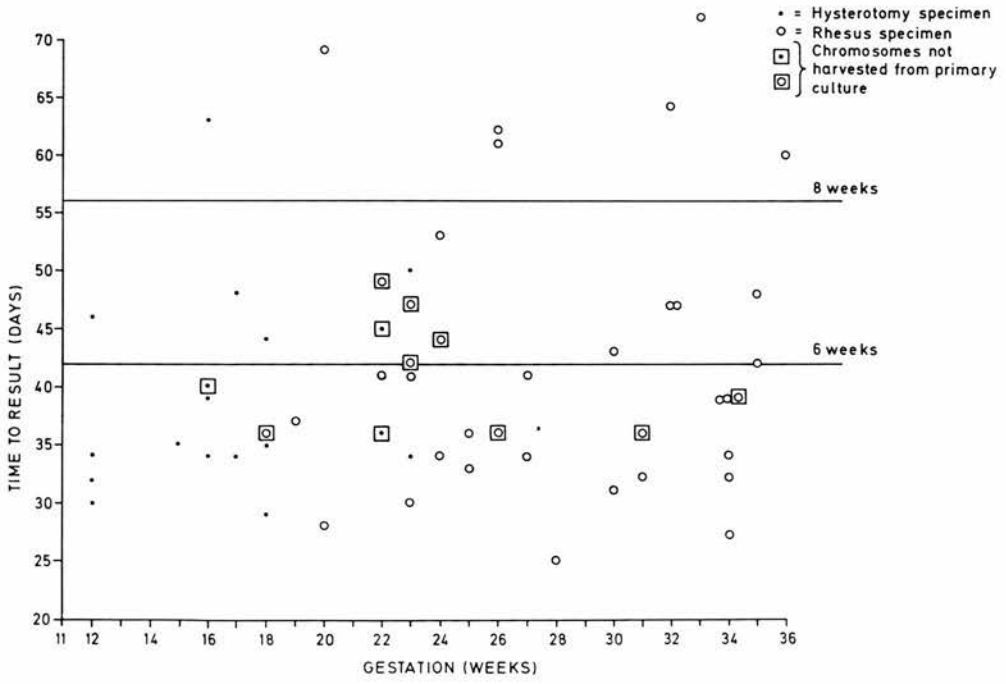



Fig. 1. Distribution of times taken to achieve sufficient cells for biochemical study

Table 2. Times required for biochemical and cytogenetic results from serial samples of amniotic fluid collected from three pregnancies

| Pregnancy | Gestation | Time to biochemical result (days) | Time to cytogenetic result (days) |
|---|---|---|---|
| 1 | 23 | 42 | N |
| | 26 | 36 | N |
| | 28 | 25 | 12 |
| 2 | 24 | 44 | N |
| | 30 | 43 | 18 |
| | 32 | 64 | 14 |
| 3 | 18 | 42 | N |
| | 22 | 41 | 26 |
| | 24 | 34 | 12 |
| | 33 | 74[a] | 21 |
| | 35 | 42 | 19 |

[a] This cell strain degenerated after the third subculture.

N = Cytogenetic preparations not made in the minimum time.

There were two cytogenetic failures due to degeneration of the primary culture before a chromosome result could be obtained. Four other cultures were successful cytogenetically but failed from the biochemical point of view, one degenerated in primary culture after a chromosome result had been obtained, two were lost to microbial contamination after the first subculture and one degenerated after the first subculture. Hence, if there was no limit on the time taken to produce enough cells for biochemical assay, the success rate was 56 out of 62. If a time limit of 8 weeks was to be imposed then the success rate would fall to 49 out of 62 and if the time limit was to be reduced to 6 weeks then the success rate would fall to 36 out of 62 or 58%.

There is no correlation between gestational age and time to a biochemical result, either for the whole series or within the Rhesus or hysterotomy groups. Similarly, the mean time to such a result for specimens of less than 20 weeks gestation is not different from the time required by those of 20 weeks or more.

All except 11 primary cultures had at least one coverslip harvested for cytogenetic studies. It might be expected that removal of some of the earliest proliferating cells would increase the time required for biochemical success. This was not so as the time to result was not significantly different for the two groups.

The collection of serial samples of fluid from women with Rhesus iso-immunisation problems affords the opportunity of studying the behaviour of these serial samples in tissue culture. Three women contributed three or more samples and data on these are shown in Table 2. The times taken to reach a biochemical result within each series are no more related to each other than to the series as a whole. Although there appears to be some relationship between the times taken to achieve cytogenetic and biochemical results, a quick cytogenetic result does not necessarily mean that a quick biochemical result will follow.

## Discussion

Commenting on tissue culture aspects of antenatal diagnosis, Brock (1973) has said "... that success to the biochemist is more elusive than success to the cytogeneticist". The main problem is one of time. Only 4 out of 62 amniotic fluids failed to reach a biochemical result because of their inability to proliferate sufficiently. Two were lost to microbial contamination; this should not happen and highlights the need for meticulous technique. In practice, if more than 6 to 8 weeks were required to produce enough cells any antenatal diagnosis for the purpose of terminating affected pregnancies would have to be regarded as a failure.

There is little data in the literature on this problem. Most authors discussing amniotic fluid cell culture have claimed the successful repeated subculture of primary cultures but the proportion in which this is achieved is usually not stated. The ability of these cells to proliferate sufficiently has been questioned by Littlefield (1971). Nadler and Gerbie (1970) were only able to subculture 75% of 155 successfully cultivated amniotic fluid cell strains more than three times. The most useful study in this regard is that reported by Uhlendorf (1970) who managed to achieve "massive cultures" from 77% of 114 amniotic fluid samples. 4—11 weeks were required to produce two 32 oz. prescription bottle monolayers, several subcultures being necessary. The time range was very similar to that in the present study although a higher number of cells were taken as the end point. In many cases "fairly large amounts" of amniotic fluid were used to initiate cultures whereas in the present series the volumes of fluid used were always less than 20 ml and often less than 10 ml.

The results from the serial samples taken during individual pregnancies indicate that each sample of fluid can behave differently. If as Uhlendorf (1970) has stated, there are less than 10 colony forming cells per millilitre of amniotic fluid, fluctuations in the number in any given sample of, say, 5 ml could be considerable. It is perhaps significant that although the cellular content of amniotic fluid increases throughout pregnancy (Nelson and Emery, 1970) the time required for a biochemical result is not related to gestation.

One problem not apparent in antenatal cytogenetic diagnosis is the degeneration of primary cultures. If an amniotic fluid culture for cytogenetic purposes is not proliferating after 7 to 10 days, amniocentesis can usually be repeated and another sample of fluid cultured. Most reported series of cytogenetic antenatal assessments contain a proportion of such instances. If, however, the primary culture grows well initially and then degenerates, it is usually too late to repeat amniocentesis and start again. A similar situation arises if degeneration takes place after the first or second subculture. A means of overcoming this problem, and of producing a quicker result in all cases, may be in the application of histochemical techniques. Uhlendorf (1970) described the use of histochemistry for the antenatal diagnosis of $G_{M1}$-gangliosidosis by staining cells for $\beta$-galactosidase activity. Hill and Puck (1973) have devised an autoradiographic method for the detection of galactosaemia which utilizes less than 1000 cultured cells. Such techniques could be carried out on primary cultures and perhaps produce a biochemical result in the same time as required for a cytogenetic result.

Nadler and Gerbie (1970) have stated ". . . the major limiting factor appears to be the difficulty in obtaining an adequate number of cells for biochemical analysis". Unless this problem can be overcome the chances of success in this type of antenatal diagnosis will remain low enough to give doubts as to its clinical application. Unless the techniques for the culture of amniotic fluid cells can be improved then efforts should be made towards developing histochemical techniques and micro assay methods for the enzymes involved in inborn errors of metabolism.

*Acknowledgement.* This work was supported in part by the Scottish Advisory Committee on Medical Research and the Secretary of State for Scotland.

## References

Brock, D. J. H.: Biochemical studies on amniotic fluid cells. In: Emery, A. E. H., Ed., Antenatal diagnosis of genetic disease, pp. 82—112. Edinburgh-London: Churchill Livingstone 1973

Butterworth, J., Sutherland, G. R., Broadhead, D. M., Bain, A. D.: Lysosomal enzymes of cultured amniotic fluid cells. Clin. chim. Acta **44**, 295—299 (1973)

Gerbie, A. B., Nadler, H. L., Gerbie, M. V.: Amniocentesis in genetic counselling. Amer. J. Obstet. Gynec. **109**, 765—770 (1971)

Hill, H. Z., Puck, T. T.: Detection of inborn errors of metabolism: Galactosemia. Science **179**, 1136—1139 (1973)

Littlefield, J. W.: Problems in the use of cultured amniotic fluid cells for biochemical diagnoses. Birth Defects **VII**, 15—17 (1971)

Nadler, H. L., Gerbie, A. B.: Role of amniocentesis in the intrauterine detection of genetic disorders. New Engl. J. Med. **282**, 596—599 (1970)

Nelson, M. M., Emery, A. E. H.: Amniotic fluid cells; prenatal sex prediction and culture. Brit. med. J. **1970 I**, 523—526

Sutherland, G. R., Grace, E., Bain, A. D.: Metaphase chromosomes from neonatal urine. Humangenetik **17**, 273—275 (1973)

Uhlendorf, B. W.: Use of amniotic fluid and reliability of diagnostic procedures. In: Harris, M., Ed., Early diagnosis of human genetic defects. Fogarty International Proceedings No. 6, pp. 149—168 (1970)

Grant R. Sutherland
Department of Pathology
Royal Hospital for Sick Children
and University of Edinburgh
Edinburgh EH9 1LF
Scotland

*Clinical Genetics* 1974: **5**: 110–112

# The antenatal diagnosis of trisomy 18

GEORGE GORDON, GRANT R. SUTHERLAND, RHONA BAULD, AND A. DOUGLAS BAIN

Cresswell Maternity Hospital, Dumfries, Department of Pathology,
Royal Hospital for Sick Children, and University of Edinburgh, Edinburgh, Scotland

The antenatal diagnosis of a fetus with trisomy 18 in a 41-year-old woman is reported. The pregnancy was terminated and the diagnosis confirmed cytogenetically and morphologically. The pathological findings in the fetus are discussed.

*Received 23 July, accepted for publication 18 August 1973*

The antenatal screening of mothers of advanced age for fetal chromosome abnormalities has been advocated by a number of authors. Although the main reason for this screening is the prevention of Down's syndrome (e.g., Stein et al. 1973), other chromosome abnormalities will inevitably be detected. We wish to report the antenatal diagnosis of a fetus with trisomy 18 in a 41-year-old mother and the subsequent pathological findings in the abortus.

## Case Report

*Pregnancy History*

The pregnancy was the fifth pregnancy of a 41–year-old woman but the first with her second husband. In her first marriage two uncomplicated pregnancies were followed by two spontaneous abortions at 9 and 13 weeks.

She was first seen at 12 weeks of pregnancy when she expressed anxiety about the possibility of having a mongol child because of her age. In view of this anxiety amniocentesis was performed at 16 weeks' gestation when 8 ml of amniotic fluid was withdrawn. This fluid was unsatisfactory in culture (see below); consequently the amniocentesis was repeated at 19 weeks' gestation when 15 ml of fluid was withdrawn. At this time the uterus was found to be smaller than expected for the gestation. Negative Kleihauer counts were obtained after each amniocentesis and the fetal heart, monitored by Sonicaid, was unaffected. From this second sample of amniotic fluid the fetus was shown to have trisomy 18. Lower segment hysterotomy through a small Pfannenstiel incision was performed at 23 completed weeks of gestation and the conceptus removed intact. At this time the uterus was again noted to be small, as was the placenta. Post-operative recovery was uneventful. The mother is contemplating another pregnancy on the understanding that amniocentesis will again be performed.

*Cytogenetic Findings*

The first amniotic fluid sample contained very few cells and showed poor growth in culture. After 2 weeks there were only a few

This work was supported in part by the Scottish Advisory Committee on Medical Research and the Secretary of State for Scotland.

scattered proliferating cells, growing too slowly for chromosome studies. The cells obtained from the second sample did grow and after 20 days in culture a fetal karyotype of 47,XX,+18 was established.

The pregnancy was terminated by hysterotomy and cultures from fetal heart blood, skin and fascia all confirmed the antenatal diagnosis of trisomy 18 (see Table 1).

*Pathological Findings*

The fetus (Fig. 1) had a crown-rump length of 18.5 cm and a crown-heel length of 27 cm. There were multiple external abnormalities. The ears were low set with underdeveloped helices which were adherent to the scalp. There was a small preauricular skin tag on the right, and two subcutaneous nodules in the left preauricular area. There was micrognathia but no cleft lip and the palate appeared normal. Both hands showed flexion deformities of the fingers and the palmar creases were atypical. Both feet had a rocker-bottom appearance and the first toes were shorter than the second. There was only a single umbilical artery.

Internally there also were multiple malformations. There was complete esophageal atresia with a tracheo-esophageal fistula. The heart showed a high interventricular septal defect. There was a complete horseshoe kidney. A Meckel's diverticulum was present with a small nodule of whitish tissue at its tip; histologically this proved to be pancreatic tissue without islet cells. The only abnormality of the brain was an anterior defect in the falx cerebri.

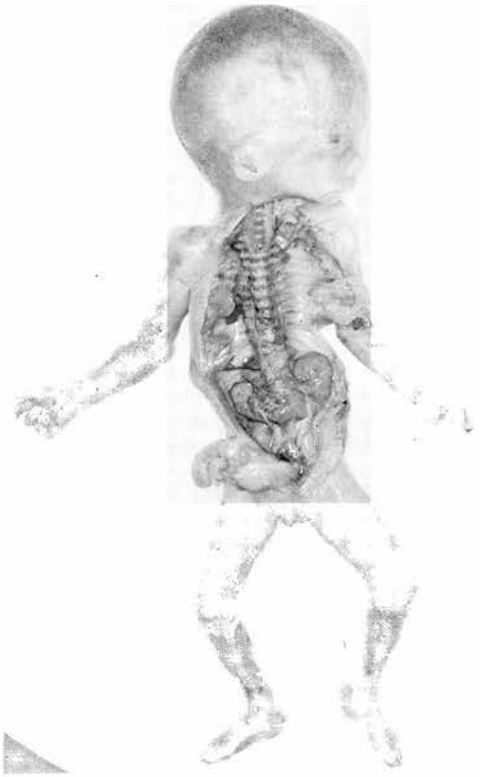


**Fig. 1.** The fetus at necropsy. Note low set ears, micrognathia, flexion deformities of the fingers and the horse-shoe kidney.

**Table 1**

Cytogenetic data

| Tissue | Cells examined | Karyotype |
|---|---|---|
| Fetus amniotic fluid | 9 | 47,XX,+18 |
| heart blood | 30 | 47,XX,+18 |
| fibroblasts (a) skin | 15 | 47,XX,+18 |
| (b) fascia | 15 | 47,XX,+18 |
| Mother blood | 30 | 46,XX |
| Father blood | 30 | 46,XY |

## Discussion

The case illustrates two main points. The first is the value of antenatal diagnosis of fetal abnormality when this can be followed by abortion. The second is that chromosome abnormalities other than mongolism will be encountered when fetal karyotyping is performed on women of advanced age (Sutherland 1972). The prevention of children with these other chromosome abnormalities is an

added benefit in any attempt to reduce the incidence of Down's syndrome (Stein et al. 1973).

The amniocenteses were apparently without any ill effect. There were no maternal complications, the sac was intact at hysterotomy and the fetus showed no evidence of injury.

There are only two previously reported instances of the antenatal diagnosis of trisomy 18. The first, diagnosed at 31 weeks' gestation, proceeded to term and resulted in a stillbirth with the features of Edwards' syndrome (Milunsky et al. 1972). The second (Hsu et al. 1973) was a case similar to the present one, diagnosed in a 40-year-old woman and aborted at 20 weeks' gestation.

The pathological findings in the fetus are typical of those seen in neonates and older children with this syndrome (Taylor 1968). It is of interest that this fetus and the one described by Hsu et al. (1973) both had a Meckel's diverticulum with a small nodule at its tip. In the present case this proved to be heterotopic pancreatic tissue. One of the cases described by Taylor (1968) also had heterotopic pancreatic tissue but apparently not in association with a diverticulum.

The fetus had a crown-rump length which corresponded to only 20 weeks' gestation and the placenta was small. The finding of a small placenta in this syndrome has been recorded by Hecht (1963) and by Hsu et al. (1973). The study of fetuses such as this may help to determine the time of onset of the retardation in growth and development usually associated with chromosome abnormality.

## References

Hecht, F. (1963). The placenta in trisomy 18 syndrome: Report of 2 cases. *Obstet. Gynec.* **22**, 147–148.

Hsu, L. Y., L. Strauss, E. Dubin & K. Hirschhorn (1973). Prenatal diagnosis of trisomy 18. *Amer. J. Dis. Child.* **125**, 290–292.

Milunsky, A., L. Atkins & J. Littlefield (1972). Amniocentesis for prenatal genetic studies. *Obstet. Gynec.* **40**, 104–108.

Stein, Z., M. Susser & A. Guterman (1973). Public screening programme for prevention of Down's syndrome. *Lancet* **i**, 305–310.

Sutherland, G. R. (1972). The role of amniocentesis in genetic counselling. *Aust. J. ment. Retardation* **2**, 85–92.

Taylor, A. (1968). Autosomal trisomy syndromes: A detailed study of 27 cases of Edward's syndrome and 27 cases of Patau's syndrome. *J. med. Genet.* **5**, 227–252.

Address:
*Grant R. Sutherland*
*Department of Pathology*
*Royal Hospital for Sick Children*
*Edinburgh*
*Scotland*

Reprinted from

*Clinica Chimica Acta*, 52(1974) 211—217


CCA 6322

# LYSOSOMAL ENZYME VARIATIONS IN THIRTEEN CELL STRAINS CULTURED FROM ONE AMNIOTIC FLUID

G.R. SUTHERLAND, J. BUTTERWORTH, D.M. BROADHEAD and A.D. BAIN
*Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF (U.K.)*

(Received December 12, 1973)

## Summary

Fifteen primary amniotic fluid cultures were established from a single sample of amniotic fluid. Three different methods were used to set up these cultures which yielded 13 cell strains. Nine lysosomal enzymes (acid phosphatase, β-glucuronidase, β-galactosidase, α-galactosidase, α-glucosidase, α-mannosidase, α-arabinosidase, *N*-acetyl-β-D-glucosaminidase and arylsulphatase A) were assayed in these 13 cell strains. The coefficients of variation of these enzyme levels were less than the coefficients for enzyme levels in cell strains grown from different samples of amniotic fluid but greater than those for the combined culture and assay system used. No assay values were found which could have suggested a possible enzyme deficiency disease.

## Introduction

Lysosomal enzymes are involved in a number of inborn errors of metabolism, many of which can now be diagnosed antenatally [1]. The activities of these enzymes fluctuate in amniotic fluid cell strains [2—4] and fibroblast-like cell strains [5,6] assayed at different stages of culture. Multiple fibroblast-like cell strains grown from a single skin biopsy have been shown to have considerable variation in the activity of five lysosomal enzymes [7]. This variation was so great as to suggest that a lysosomal enzyme deficiency disease might have been wrongly diagnosed. The possibility that a similar situation may exist for cell strains cultured from a single sample of amniotic fluid has been investigated.

## Materials and Methods

Amniotic fluid was collected from a pregnancy of 18 weeks gestation, terminated because of severe maternal pre-eclamptic toxaemia. The fluid was divided into 15 aliquots of 5 ml and one primary culture was established from

each aliquot. Three different methods were used to establish the primary cultures:

(I) Five cultures were set up using the method of Gray et al. [8]. The amniotic fluid was added to the culture vessel and left for one week. At this time half the amniotic fluid was replaced by culture medium and thereafter half the medium changed three times per week.

(II) Five cultures were set up [9] by centrifuging the amniotic fluid at 200 × *g* for 10 min, the cell pellet was resuspended in culture medium and added to the culture vessel. After one week half the culture medium was replaced three times per week.

(III) Five cultures were set up as in method II except that half the medium was changed after 24 h and thereafter three times per week.

In all cases the culture vessel was a 50 mm plastic Nunclon Petri dish and the culture medium was Ham's F10 with 30% fetal calf serum supplement and antibiotics [9]. The cultures were subcultured and maintained as previously described [10]. After subculture of the primary culture the fetal calf serum supplement in the culture medium was reduced to 15%. Enzyme assays were carried out on each cell strain at the third and sixth passage. Cells were harvested for enzyme assay on the day they reached confluency.

The cells were washed twice with phosphate-buffered saline, scraped off the glass into 4 ml of water, sonicated for 30 s at maximum power using a "Soniprobe" (Dawes Instruments), centrifuged at 800 × *g* for 10 min at 5° and stored at −65°. Protein levels were estimated [11] using bovine serum albumin as standard. Arylsulphatase A was assayed [12] using *p*-nitrocatechol sulphate as the substrate. For all the other enzymes 4-methylumbelliferone conjugates were utilized [10] under the following conditions:

| | | |
|---|---|---|
| Acid phosphatase | — | 1 mM conjugate, 0.1 M acetate buffer pH 5.0, 30 min incubation. |
| β-Glucuronidase | — | 1 mM, 0.1 M acetate buffer pH 4.0, 60 min. |
| β-Galactosidase | — | 1 mM, 0.2 M phospho-citrate buffer pH 4.0 + 100 mM NaCl, 60 min. |
| α-Galactosidase | — | 2 mM, 0.1 M acetate buffer pH 4.8, 60 min. |
| α-Glucosidase | — | 2 mM, 0.2 M phospho-citrate buffer pH 4.5, 60 min. |
| α-Mannosidase | — | 2 mM, 0.1 M acetate buffer pH 4.0, 60 min. |
| α-Arabinosidase | — | 1 mM, 0.2 M phospho-citrate buffer pH 4.5, 60 min. |
| *N*-Acetyl-β-D glucosaminidase | — | 2 mM, 0.2 M phospho-citrate buffer pH 4.5, 30 min. |

## Results

Cultures established by Method I were very slow growing, one degenerated in primary culture and could not be subcultured and another did not survive after the first subculture. Hence there were 13 cell strains available for enzyme assay. Details of times in culture for the three methods of establishing cultures are given in Table I. Comparison of these times shows that Method I was significantly worse than Methods II and III which did not differ. On the basis of behaviour in culture the cell strains were divided into one group of 10 and another group of 3 derived from the cultures set up using Method I.

TABLE I

TIMES IN CULTURE FOR THE 3 METHODS OF ESTABLISHING CULTURES

Behaviour of the 15 amniotic fluid cultures (a—o) with time in primary culture and time from the first subculture until the cells in the third and sixth passages were harvested for enzyme assay.

| Method of primary culture and cell strain designation | | Days in primary culture | Days until 3rd passage harvest | Days until 6th passage harvest |
|---|---|---|---|---|
| I | a | 43 | —* | — |
| | b | —** | — | — |
| | c | 38 | 46 | 60 |
| | d | 36 | 53 | 78 |
| | e | 36 | 51 | 82 |
| II | f | 25 | 37 | 51 |
| | g | 28 | 39 | 58 |
| | h | 25 | 39 | 58 |
| | i | 22 | 37 | 53 |
| | j | 22 | 37 | 51 |
| III | k | 22 | 39 | 56 |
| | l | 23 | 37 | 58 |
| | m | 22 | 39 | 60 |
| | n | 22 | 39 | 58 |
| | o | 22 | 37 | 58 |

* Cell strain died in the first passage.
** Primary culture degenerated and could not be subcultured.

The assay values of the nine lysosomal enzymes measured at the third and sixth passages are shown in Table II. To compare the variations in enzyme level in the two passages and to establish their relationship with the variations found in a series of amniotic fluid cell strains cultured from different samples of amniotic fluid [10], the coefficients of variation were calculated and are shown in Table III. For this calculation the values for the three cell strains derived from primary cultures established using Method I were excluded because of their different behaviour in tissue culture. The coefficients of variation of the combined assay and culture system, determined by assays on replicate cultures as previously described [2], are also included in Table III.

The coefficients of variation for each enzyme in the third and sixth passages are similar in magnitude for all enzymes except α-mannosidase and α-arabinosidase. The coefficients for all the enzyme levels in the third and sixth passages are smaller than those found for the control series of cell strains cultured from different samples of amniotic fluid and greater than those for the combined culture and assay system.

## Discussion

Cultured amniotic fluid cells are the most reliable material for enzyme assay on which antenatal diagnoses of inborn errors of lysosomal enzyme metabolism are to be based [1,13,14]. It is thus essential that the two principle techniques involved in such diagnoses, tissue culture and enzyme assay, must combine to produce reliable and reproducible results. The establishment of a

TABLE III

COEFFICIENT OF VARIATION VALUES OBTAINED

Mean, standard deviation and coefficient of variation for the lysosomal enzymes in the third and sixth passages of culture and the coefficient of variation of a control series of cell strains cultured from different samples of amniotic fluid and for the combined assay and culture system. N.D. = not done.

| Cell strains | Acid phosphatase | β--Glucuronidase | β--Galactosidase | α--Galactosidase | α--Glucosidase | α--Mannosidase | α--Arabinosidase | *N*-Acetyl-β-D--glucosaminidase | Arylsulphatase A |
|---|---|---|---|---|---|---|---|---|---|
| Third passage | | | | | | | | | |
| Mean | 5.19 | 1.12 | 7.25 | 0.587 | 0.444 | 1.062 | 0.204 | 44.5 | 1.93 |
| S.D. | 0.63 | 0.28 | 1.27 | 0.058 | 0.125 | 0.129 | 0.49 | 5.58 | 0.416 |
| Coefficient of variation | 12.1 | 25.2 | 17.6 | 9.93 | 28.1 | 12.2 | 23.8 | 12.5 | 21.5 |
| Sixth passage | | | | | | | | | |
| Mean | 5.73 | 0.88 | 6.50 | 0.566 | 0.416 | 0.767 | 0.165 | 39.8 | 2.16 |
| S.D. | 0.82 | 0.21 | 0.70 | 0.054 | 0.091 | 0.203 | 0.024 | 4.95 | 0.413 |
| Coefficient of variation | 14.4 | 23.7 | 10.8 | 9.51 | 21.9 | 26.5 | 14.6 | 12.4 | 19.1 |
| Control (third passage) | | | | | | | | | |
| Coefficient of variation | 36.5 | 40.0 | 42.8 | 35.7 | 47.4 | 46.1 | 47.2 | 32.1 | N.D. |
| Combined system | | | | | | | | | |
| Coefficient of variation | 7.4 | 4.5 | 5.2 | 3.3 | 8.5 | 6.5 | 5.2 | 7.1 | N.D. |

TABLE II

SPECIFIC ACTIVITIES OF THE LYSOSOMAL ENZYMES IN THE THIRTEEN CELL STRAINS ASSAYED AT THE THIRD AND SIXTH PASSAGES OF CULTURE

Results are expressed in nmoles 4-methylumbelliferone/min per mg protein, except for arylsulphatase A which is expressed in nmoles nitrocatechol/min per mg protein.

| Method of primary culture | Cell strain | Acid phosphatase | β--Glucuronidase | β--Galactosidase | α--Galactosidase | α--Glucosidase | α--Mannosidase | α--Arabinosidase | *N*-Acetyl-β-D--glucosaminidase | Arylsulphatase A |
|---|---|---|---|---|---|---|---|---|---|---|
| I | c | 4.00* | 0.94 | 5.93 | 0.51 | 0.60 | 1.27 | 0.16 | 35.8 | 1.14 |
| | | 5.90** | 0.87 | 5.66 | 0.59 | 0.45 | 0.83 | 0.19 | 39.1 | 2.37 |
| | d | 3.62 | 1.30 | 11.46 | 0.49 | 0.64 | 1.83 | 0.28 | 47.3 | 4.08 |
| | | 4.98 | 0.64 | 11.27 | 0.58 | 0.64 | 1.09 | 0.25 | 62.3 | 5.40 |
| | e | 4.31 | 0.51 | 4.87 | 0.50 | 0.32 | 1.39 | 0.11 | 37.0 | 1.98 |
| | | 5.97 | 0.24 | 3.72 | 0.36 | 0.21 | 0.65 | 0.10 | 30.7 | 2.78 |
| II | f | 5.01 | 1.40 | 7.89 | 0.57 | 0.47 | 1.00 | 0.21 | 43.2 | 1.82 |
| | | 4.92 | 0.91 | 5.59 | 0.60 | 0.29 | 1.02 | 0.13 | 37.5 | 2.37 |
| | g | 6.18 | 0.86 | 6.88 | 0.60 | 0.43 | 1.11 | 0.21 | 44.7 | 1.67 |
| | | 5.92 | 0.60 | 5.77 | 0.55 | 0.32 | 0.77 | 0.14 | 33.6 | 1.59 |
| | h | 4.88 | 0.64 | 6.48 | 0.52 | 0.39 | 1.11 | 0.20 | 38.0 | 1.72 |
| | | 5.09 | 0.75 | 6.40 | 0.53 | 0.35 | 0.68 | 0.15 | 36.6 | 1.89 |
| | i | 4.18 | 1.41 | 7.28 | 0.53 | 0.53 | 1.00 | 0.22 | 41.2 | 1.90 |
| | | 5.56 | 0.95 | 7.13 | 0.47 | 0.51 | 0.99 | 0.18 | 35.9 | 2.53 |
| | j | 4.61 | 0.96 | 5.92 | 0.69 | 0.29 | 1.09 | 0.15 | 42.6 | 1.80 |
| | | 4.47 | 1.24 | 7.59 | 0.62 | 0.49 | 1.12 | 0.20 | 47.7 | 2.46 |
| III | k | 5.49 | 1.04 | 7.52 | 0.56 | 0.39 | 1.09 | 0.21 | 43.3 | 2.64 |
| | | 6.21 | 1.13 | 6.95 | 0.61 | 0.42 | 0.66 | 0.18 | 43.5 | 2.74 |
| | l | 5.05 | 1.32 | 6.42 | 0.68 | 0.33 | 1.02 | 0.16 | 41.9 | 1.46 |
| | | 6.24 | 0.97 | 6.44 | 0.57 | 0.36 | 0.61 | 0.16 | 39.7 | 2.49 |
| | m | 6.10 | 0.91 | 9.74 | 0.60 | 0.58 | 1.33 | 0.29 | 49.7 | 2.61 |
| | | 7.42 | 0.60 | 6.87 | 0.60 | 0.49 | 0.56 | 0.19 | 44.7 | 2.07 |
| | n | 4.90 | 1.49 | 8.71 | 0.54 | 0.69 | 1.06 | 0.26 | 58.1 | 2.18 |
| | | 5.82 | 0.95 | 6.79 | 0.62 | 0.56 | 0.70 | 0.18 | 44.5 | 1.80 |
| | o | 5.49 | 1.13 | 5.63 | 0.58 | 0.34 | 0.81 | 0.13 | 42.7 | 1.52 |
| | | 5.69 | 0.76 | 5.47 | 0.49 | 0.37 | 0.56 | 0.14 | 34.5 | 1.63 |

* Assayed in the third passage of culture.
** Assayed in the sixth passage of culture.

number of primary cultures from a single large sample of amniotic fluid and enzyme assay on the cell strains subsequently produced allows both laboratory procedures to be tested simultaneously.

Much of the delay in achieving sufficient cells for a biochemical assay is taken up by the time in primary culture. Hence three methods of primary culture were tested. The first method used has been claimed to produce such rapid growth of primary cultures that sufficient cells for cytogenetic studies could be obtained in an average of seven days [8]. No other published method of primary culture has reported such rapid cell growth. The second method used has been employed to study the delay due to tissue culture in the antenatal diagnosis of inborn errors of metabolism [15]. The third method involved a modification of the culture maintenance schedule of the second, similar to that of other published methods [16,17]. The results show that the first method was inferior to the other two for this sample of amniotic fluid. In addition, the variation in the enzyme levels in the three cell strains eventually derived from the primary cultures set up using the first method was greater than that seen in the 10 strains derived from the other primary cultures.

There are two main features of this variation in enzyme levels in a series of cell strains derived from one sample of amniotic fluid. The first is that this variation is considerably less than that found in a series of cell strains derived from different amniotic fluid samples. There are several reasons for this other than the common origin of the cell strains; they were all in tissue culture at the same time, receiving the same batch of culture medium and fetal calf serum and all the enzymes were assayed at the same time. In spite of these constant conditions of tissue culture and enzyme assay the highest value of some enzymes was more than twice the lowest. Such variation would cast doubt upon heterozygote identification based on enzyme assay of a single sample of cultured cells. Some cell strains even retained widely different enzyme levels in both passages as, for example, the $\alpha$-glucosidase level in strain *n* which was approximately twice that in strain *o* in both passages assayed. If the cell strains derived from cultures established by method I are considered then this variation is even more marked. Strain *d* had $\beta$-galactosidase and arylsulphatase A levels which were much greater than in the other cell strains although the rest of the enzymes in this culture were within the range for the other cell strains.

The second main feature of the results is their relationship to the only comparable study [7], in which cultured fibroblast-like cells were used. Great variations were found in the levels of several lysosomal enzymes in multiple fibroblast-like cell strains grown from a single human foreskin. For $\beta$-galactosidase, there was more than a 3-fold difference between the highest and lowest values whereas in the present study this difference was relatively small. The two series agree on the finding for *N*-acetyl-$\beta$-D-glucosaminidase which showed little variation and for $\beta$-glucuronidase in which there was a 2.5-fold difference between the highest and lowest assay values. The results of the present study for arylsulphatase A are not comparable with those quoted by Milunsky et al. [7] as their substrate and experimental conditions were not specific for arylsulphatase A [18,19].

The results of this study on cell strains derived from one sample of amniotic fluid are in agreement with previous findings [10] on cell strains derived

from different amniotic fluids. Enzyme assay of normal amniotic fluid cell strains should not produce values suggestive of an enzyme deficiency disease.

## Acknowledgement

This work was supported in part by the Medical Research Fund of the Secretary of State for Scotland.

## References

1 A. Milunsky. The Prenatal Diagnosis of Hereditary Disorders, Charles C. Thomas, Springfield, 1973.
2 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Life Sci., 13 (1973) 713.
3 G.R. Sutherland, J. Butterworth, D.M. Broadhead and A.D. Bain, Clin. Genet., (1974) in the press .
4 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Genet., (1974) in the press.
5 W.R. Den Tandt and A. Schaberg, Pathol. Eur., 8 (1973) 3.
6 B. Hultberg, S. Sjöblad and P.A. Ockerman, Acta Paediat. Scand., 62 (1973) 474.
7 A. Milunsky, C. Spielvogel and J.N. Kanfer, Life Sci., 11 (2) (1972) 1101.
8 C. Gray, R.G. Davidson and M.M. Cohen, J. Pediat., 79 (1971) 119.
9 G.R. Sutherland, E. Grace and A.D. Bain, Humangenetik, 17 (1973) 273.
10 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Chim. Acta, 44 (1973) 295.
11 G.L. Miller, Anal. Chem., 31 (1959) 964.
12 H. Baum, K.S. Dodgson and B. Spencer, Clin. Chim. Acta, 4 (1959) 453.
13 R.G. Davidson and M.C. Rattazzi, Clin. Chem., 18 (1972) 179.
14 H.L. Nadler, R.H. Bigley and G. Hug, Lancet, ii (1970) 369.
15 G.R. Sutherland and A.D. Bain, Humangenetik, 20 (1973) 251.
16 M.E. Ferguson-Smith, M.A. Ferguson-Smith, N.C. Nevin and M. Stone, Brit. Med. J., 4 (1971) 69.
17 H.L. Nadler, Pediatrics, 42 (1968) 912.
18 A.S. Perumal and E. Robins, J. Neurochem., 21 (1973) 459.
19 M.T. Porter, A.L. Fluharty, S.D. de la Flor and H. Kihara, Biochim. Biophys. Acta, 258 (1972) 769.

Reprinted from AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY, St. Louis
Vol. 119, No. 6, Pages 821-828, July 15, 1974 (Printed in the U. S. A.)




# Lysosomal enzymes of amniotic fluid in relation to gestational age

J. BUTTERWORTH, M.SC., PH.D.
D. M. BROADHEAD, B.SC.
G. R. SUTHERLAND, M.SC.
A. D. BAIN, M.D., M.R.C.P.E., F.R.C.PATH.
*Edinburgh, Scotland*

*The activities of ten lysosomal enzymes were assayed in more than 100 amniotic fluids. Wide variations in enzyme activity were found at any particular gestational age. However, various patterns of enzyme activity were apparent in relation to gestational age. Three enzymes, β-glucosidase, α-glucosidase, and α-arabinosidase, showed high initial values, falling rapidly to a very low level after 20 weeks' gestation. The enzymes β-glucuronidase and α-mannosidase showed a peak of activity at about 26 weeks, and N-acetyl-β-D-glucosaminidase and α-fucosidase had higher values at midgestation, declining toward term. Acid phosphatase exhibited no obvious pattern. Higher values of α-galactosidase and β-galactosidase were noted near term, but at all times the levels of these two enzymes were minimal. The findings are discussed in relation to possible sources of the enzymes and antenatal diagnosis of inborn errors of metabolism.*

INCREASING attention has been focused on the analysis of the constituents of amniotic fluid as an indicator of the health of the fetus.[1-7] In order that any particular parameter of amniotic fluid may be utilized in this connection it is necessary to obtain the normal range of values throughout gestation.[5] An earlier report[8] indicated that lysosomal enzymes may show changes related to gestational age and the results on a larger series of amniotic fluids are presented confirming these trends.

*From the Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh.*

*This work was supported by the Advisory Committee on Medical Research and the Secretary of State for Scotland, and the Cystic Fibrosis Research Trust.*

*Received for publication August 8, 1973.*

*Revised December 10, 1973.*

*Accepted December 20, 1973.*

*Reprint requests: Dr. J. Butterworth, Department of Pathology, Royal Hospital for Sick Children, Edinburgh, Scotland.*

## Materials and methods

Samples of amniotic fluid were obtained at different stages of pregnancy. For the gestational range of 11 to 23 weeks amniotic fluid samples were obtained by aspiration of the intact amniotic sac following hysterotomy (n = 19) performed for social reasons and by transabdominal amniocentesis (n = 10) when performed for antenatal chromosome analysis. Between 21 and 37 weeks the samples (n = 76) were obtained by transabdominal amniocentesis of rhesus isoimmunized women. For 38 to 41 weeks, samples were obtained at the induction of labor by Drew-Smythe catheter. Cells and debris were removed by centrifugation at 1,500 g for five minutes at 5° C. and the fluids stored at −70° C. The enzymes were assayed[9] with 4-methylumbelliferone conjugates except for α-fucosidase, where the p-nitrophenol glyco-

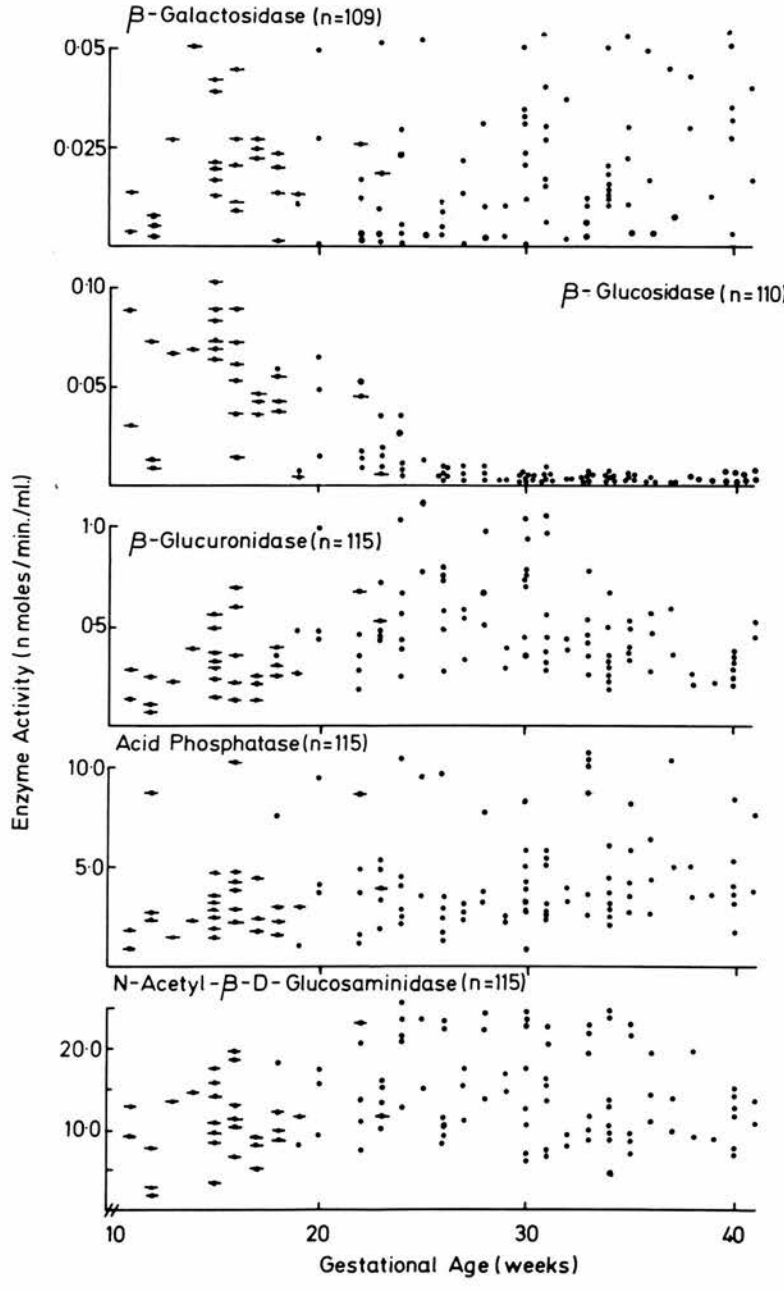



Fig. 1. Activities of β-galactosidase, β-glucosidase, β-glucuronidase, acid phosphatase, and N-acetyl-β-D-glucosaminidase in aminotic fluid. -●-, Hysterotomy/antenatal diagnostic samples; ●, rhesus up to 27 weeks; 38+ weeks term samples.

side was utilized. Protein was determined[10] with bovine serum albumin as the standard.

## Results

Although all 10 enzymes assayed could be detected, the activities of β-galactosidase, α-galactosidase, and α-arabinosidase were very low. The variations in the activity of the lysosomal enzymes in amniotic fluid with gestational age are presented in Figs. 1 and 2. The enzyme activities were expressed on a volume basis as the protein concentration

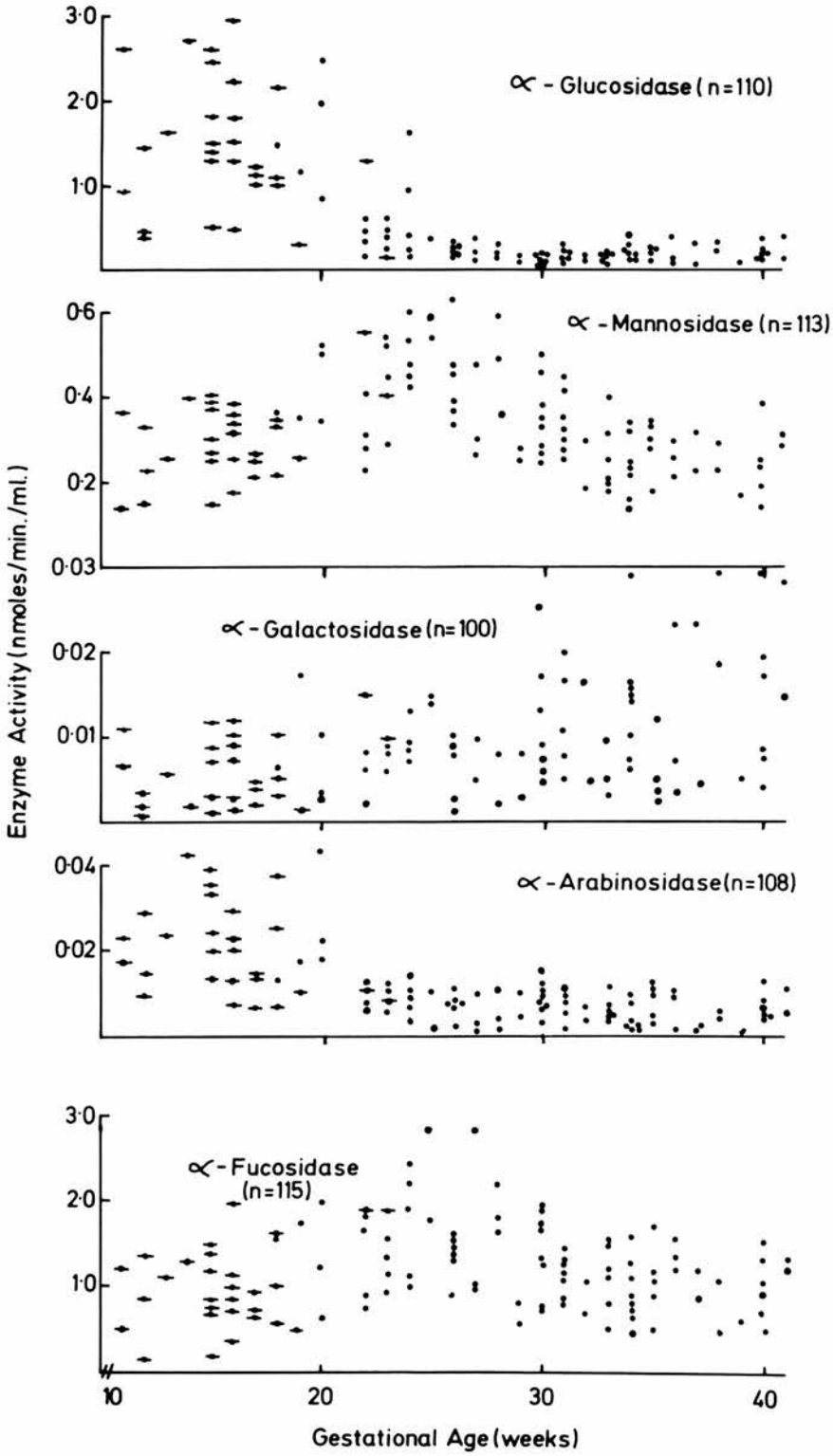



Fig. 2. Activities of α-glucosidase, α-mannosidase, α-galactosidase, α-arabinosidase, and α-fucosidase in amniotic fluid. -●-; Hysterotomy/antenatal diagnostic samples; ●, rhesus up to 27 weeks; 38+ weeks term samples.

**Table I.** Lysosomal enzyme activity* in serial samples of amniotic fluid from six rhesus isoimmunize

| *Gestational age (wk.)* | *Δ O.D.†* | *Protein (mg./ml.)* | *N-acetyl-β-D-gluco-saminidase* | *Acid phosphatase* | *β-Galactosidase* | *β-Glucuroni-dase* |
|---|---|---|---|---|---|---|
| 18 | 0.16 | 5.39 | 18.2 | 7.6 | 0.020 | 0.33 |
| 22 | 0.20 | 7.48 | 23.5 | 3.7 | 0.013 | 0.29 |
| 24 | 0.18 | 6.13 | 26.0 | 2.0 | 0.003 | 0.36 |
| 33 | 0.08 | 3.05 | 21.8 | 3.6 | 0.011 | 0.34 |
| 35 | 0.08 | 2.71 | 21.1 | 4.1 | 0.011 | 0.33 |
| 20 | 0.14 | 4.09 | 15.5 | 3.9 | 0.027 | 0.44 |
| 23 | 0.14 | 4.56 | 13.1 | 2.0 | 0.003 | 0.46 |
| 26 | 0.08 | 5.49 | 21.4 | 2.5 | 0.005 | 0.73 |
| 29 | 0.06 | 4.31 | 14.5 | 2.1 | 0.010 | 0.39 |
| 18 | 0.08 | 4.00 | 10.3 | 3.5 | 0.023 | 0.45 |
| 25 | 0.17 | 7.53 | 14.9 | 3.5 | 0.003 | 0.77 |
| 31 | 0.08 | 7.67 | 22.2 | 5.4 | 0.027 | 0.97 |
| 19 | 0.12 | 4.67 | 8.00 | 4.4 | 0.013 | 0.47 |
| 30 | 0.07 | 4.31 | 12.3 | 3.2 | 0.023 | 0.78 |
| 33 | 0.05 | 3.83 | 9.7 | 2.5 | 0.012 | 0.77 |
| 23 | 0.16 | 5.18 | 15.6 | 4.9 | 0.009 | 0.47 |
| 26 | 0.28 | 7.81 | 20.7 | 2.8 | 0.009 | 0.46 |
| 28 | 0.50 | 8.51 | 24.7 | 3.7 | 0.009 | 0.50 |
| 24 | 0.20 | 6.29 | 12.5 | 4.2 | 0.023 | 0.42 |
| 30 | 0.11 | 2.99 | 6.9 | 3.4 | 0 | 0.44 |
| 32 | 0.11 | 3.18 | 7.7 | 3.2 | 0 | 0.43 |

*Nmole 4-methylumbelliferone/min./ml.; α-fucosidase, nmole p-nitrophenol/min./ml.

†Optical density difference at 450 nm.[11]

(Fig. 3) of amniotic fluid varied with gestational age, showing a peak at about 26 weeks' gestation. The results obtained for the enzymes in the serial samples of amniotic fluid from a number of women are given in Table I and show the same trends observed in the large series from different women. A comparison of the optical density differences at 450 nm. for the rhesus isoimmunized amniotic fluids and enzyme activity indicated that they were apparently unrelated.

Although a wide variation in enzyme activity was found at any particular gestational age, various patterns of enzyme activity were apparent (Table II). Three enzymes, β-glucosidase, α-glucosidase, and α-arabinosidase, showed a declining pattern of activity, reaching a very low level by 20 weeks. When enzyme activity was expressed on a protein basis, this pattern was still clearly apparent (Fig. 3). Two enzymes, β-glucuronidase and α-mannosidase, showed a peak of activity at about 26 weeks' gestation. Acid phosphatase showed no pattern, although occasional high values were found throughout gestation. N-acetyl-β-D-glucosaminidase and α-fucosidase had higher values at midterm and declined toward term, although neither of these patterns was clear-cut. For β-galactosidase and α-galactosidase higher values were found near term, but at no time was the activity of these two enzymes more than minimal.

## Comment

Amniotic fluid provides a potential means of investigating fetal development and predicting fetal disease. It is clearly necessary to obtain accurate information on the normal level of any constituent in relation to gestational age prior to its use in assessing fetal health.[12] A knowledge of variations in lysosomal enzyme levels in amniotic fluid could be of use for following both fetal development and the detection of inborn errors of metabolism.

ɔmen

| β-Glucosidase | α-Galactosidase | α-Glucosidase | α-Arabinosidase | α-Mannosidase | α-Fucosidase |
|---|---|---|---|---|---|
| 0.058 | 0.010 | 1.41 | 0.013 | 0.37 | 1.56 |
| 0.015 | 0.008 | 0.45 | 0 | 0.40 | 1.81 |
| 0.004 | 0.007 | 0.13 | 0 | 0.38 | 1.75 |
| 0.003 | 0 | 0.20 | 0 | 0.39 | 1.22 |
| 0.004 | 0 | 0.26 | 0 | 0.35 | 1.11 |
| 0.049 | 0.003 | 0.83 | 0.018 | 0.51 | 1.97 |
| 0.019 | 0 | 0.25 | 0.011 | 0.28 | 1.35 |
| 0.009 | 0 | 0.21 | 0.011 | 0.46 | 1.39 |
| 0.003 | 0 | 0.12 | 0.010 | 0.25 | 0.81 |
| 0.059 | 0.003 | 1.81 | 0.025 | 0.57 | 0.90 |
| 0.012 | 0 | 0.35 | 0.010 | 0.54 | 1.77 |
| 0.003 | 0 | 0.26 | 0.010 | 0.42 | 1.28 |
| 0.046 | 0.017 | 1.16 | 0.017 | 0.35 | 1.74 |
| 0.029 | 0.017 | 0.12 | 0.009 | 0.37 | 1.84 |
| 0.020 | 0.003 | 0.14 | 0.007 | 0.20 | 1.45 |
| 0.035 | 0.008 | 0.41 | 0.006 | 0.52 | 0.90 |
| 0.011 | 0.006 | 0.19 | 0 | 0.47 | 1.64 |
| 0.007 | 0.006 | 0.14 | 0 | 0.47 | 1.77 |
| 0.020 | 0.013 | 0.38 | 0.010 | 0.41 | 0.99 |
| 0.013 | 0 | 0.07 | 0.006 | 0.28 | 0.46 |
| 0.010 | 0 | 0.10 | 0.006 | 0.18 | 0.68 |

**Table II.** Enzyme-specific activity* in relation to gestational age

| Enzyme | Gestational age (wk.) | | | | | |
|---|---|---|---|---|---|---|
| | 11-15 | 16-20 | 21-25 | 26-30 | 31-35 | 36-41 |
| β-Glucosidase | 0.066† | 0.043 | 0.020 | 0.004 | 0.003 | 0.002 |
| | 0.033‡ | 0.024 | 0.015 | 0.003 | 0.002 | 0.001 |
| α-Glucosidase | 1.53 | 1.40 | 0.51 | 0.18 | 0.16 | 0.21 |
| | 0.80 | 0.68 | 0.42 | 0.07 | 0.07 | 0.12 |
| α-Arabinosidase | 0.024 | 0.018 | 0.008 | 0.008 | 0.006 | 0.005 |
| | 0.012 | 0.011 | 0.003 | 0.003 | 0.004 | 0.004 |
| α-Mannosidase | 0.31 | 0.34 | 0.44 | 0.39 | 0.28 | 0.26 |
| | 0.10 | 0.11 | 0.12 | 0.11 | 0.09 | 0.06 |
| β-Glucuronidase | 0.25 | 0.38 | 0.57 | 0.63 | 0.44 | 0.35 |
| | 0.15 | 0.23 | 0.27 | 0.24 | 0.21 | 0.13 |
| N-acetyl-β-D-glucosaminidase | 9.94 | 11.85 | 16.54 | 16.01 | 13.70 | 12.25 |
| | 5.17 | 4.43 | 5.66 | 6.16 | 6.42 | 3.69 |
| α-Fucosidase | 0.87 | 1.05 | 1.59 | 1.45 | 1.04 | 1.06 |
| | 0.48 | 0.50 | 0.60 | 0.54 | 0.33 | 0.35 |
| α-Galactosidase | 0.005 | 0.006 | 0.009 | 0.008 | 0.011 | 0.016 |
| | 0.004 | 0.004 | 0.004 | 0.006 | 0.006 | 0.011 |
| β-Galactosidase | 0.020 | 0.022 | 0.018 | 0.016 | 0.020 | 0.029 |
| | 0.017 | 0.013 | 0.018 | 0.014 | 0.016 | 0.018 |
| Acid phosphatase | 2.84 | 3.94 | 4.62 | 3.75 | 4.63 | 4.84 |
| | 1.92 | 2.61 | 2.97 | 2.29 | 2.75 | 2.38 |
| Protein (mg./ml.) | 2.85 | 3.99 | 6.75 | 5.38 | 3.53 | 2.81 |
| | 1.26 | 1.42 | 1.96 | 2.07 | 1.31 | 0.82 |

*Nmole 4-methylumbelliferone/min./ml.; α-fucosidase, nmole p-nitrophenol/min./ml.

†Mean.

‡Standard deviation.

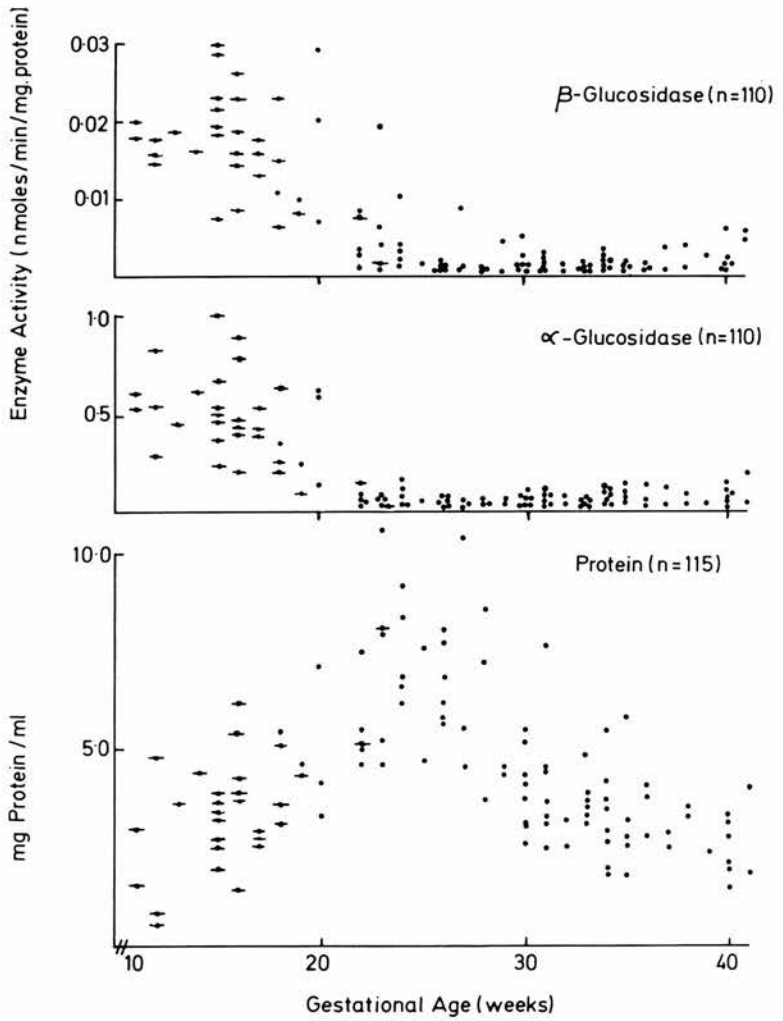



**Fig. 3.** Protein levels and activities of $\beta$-glucosidase and $\alpha$-glucosidase on a protein basis in amniotic fluid. -●-, Hysterotomy/antenatal diagnostic samples; ●, rhesus up to 27 weeks; 38+ weeks term samples.

The present results together with previous work[8, 13, 14] demonstrate that lysosomal enzymes show patterns of activity related to gestational age. The fall in activity of $\alpha$-glucosidase to a very low level by 20 weeks' gestation has previously been reported.[8, 13, 14] No clear pattern was found[14] for N-acetyl-$\beta$-D-glucosaminidase. A similar lack of a relationship was noted for acid phosphatase,[15] although a rise near term has been demonstrated.[14] Jonasson[15] demonstrated that the acid phosphatase level was similar in samples of amniotic fluid from normal and rhesus isoimmunized pregnancies, indicating that the latter source of amniotic fluid could be included in obtaining normal patterns of activity. This would also seem to be the case for the enzymes in the present study.

The average levels of $\beta$-galactosidase and N-acetyl-$\beta$-D-glucosaminidase found in the present studies differ from those of Lowden and associates.[2] The average level of N-acetyl-$\beta$-D-glucosaminidase was about twice as high as that found by Lowden and associates.[2] The average level of $\beta$-galactosidase was about ten times lower in the present study and was effectively absent, a finding in agreement with other reports.[16, 17] It is also

noticeable that the values for these enzymes in cultured amniotic fluid cells given by Lowden and associates[2] are far in excess of those found by other workers.[3, 18, 19, 20]

The dynamic state of amniotic fluid is clearly shown by the differing patterns obtained for many constituents.[1, 5, 6, 21, 22, 23] It is of interest that the total amniotic fluid volume follows a pattern,[24] reaching a peak at about 34 weeks' gestation, to which changes in constituents do not relate. The causes of the observed fluctuations in the levels of the lysosomal enzymes in amniotic fluid are unknown. It is noteworthy, however, that many enzymes show a change in their level of activity at about 20 weeks' gestation, a time when the fetal skin becomes impermeable to water[25] and fetal urine becomes an important source of amniotic fluid.[26] The finding of a decrease in the activity of the lysosomal enzymes of the placenta[16] at about 20 weeks may also be relevant. The disappearance of $\alpha$-glucosidase at midpregnancy has been suggested[13] to reflect the complete assumption of glucose homeostasis by the fetal liver. However, this would not explain the changes observed for the other enzymes and their relationship to fetal development must await further investigation.

The levels of certain lysosomal enzymes have been utilized in the detection of inborn errors of metabolism.[2, 27, 28] However, prior to using amniotic fluid for antenatal diagnosis it is necessary to demonstrate that the enzyme being measured is the one involved in the disease. In the case of $\alpha$-glucosidase, its use was seriously questioned[29] and it was later shown[30] that the enzyme present in amniotic fluid is not the one missing in Pompe's disease. The N-acetyl-$\beta$-D-glucosaminidase present in amniotic fluid has been shown[31] to be similar to that involved in the Tay-Sachs complex. However, while an absence of this enzyme could be used for an unequivocal diagnosis of Sandhoff's disease,[19] the much lower percentage of N-acetyl-$\beta$-D-glucosaminidase A present as compared to that in cultured amniotic fluid cells[3] leaves much less room for error in the diagnosis of Tay-Sachs disease.[27] The low level of certain enzymes, the wide range in enzyme levels at a particular gestational age, and the variations with gestational age make difficult the use of amniotic fluid lysosomal enzyme levels for antenatal diagnosis. Any such diagnosis made on the basis of amniotic fluid should be confirmed by enzyme assay of cultured amniotic fluid cells.[18]

We thank Drs. J. G. Robertson and J. B. Scrimgeour for the amniotic fluids and the former for the optical density difference values of the rhesus isoimmunized amniotic fluid samples.

## REFERENCES

1. Emery, A. E. H., Burt, D., Nelson, M. M., and Scrimgeour, J. B.: Lancet **1:** 1307, 1970.
2. Lowden, J. A., Cutz, E., Conen, P. E., Rudd, N., and Doran, T. A.: N. Engl. J. Med. **288:** 225, 1973.
3. O'Brien, J. S., Okada, S., Fillerup, D. L., Veath, M. L., Adornato, B., Brenner, P. H., and Leroy, J. G.: Science **172:** 61, 1971.
4. Nakamura, J., Roux, J. F., Brown, E. G., and Sweet, A. Y.: Am. J. Obstet. Gynecol. **113:** 363, 1972.
5. Scott, C. R., Teng, C. C., Sagerson, R. N., and Nelson, T.: Pediatr. Res. **6:** 659, 1972.
6. Shah, S. I., Alderman, M., Queenan, J. T., Brasel, J. A., and Winick, M.: Am. J. Obstet. Gynecol. **114:** 250, 1972.
7. Spellacy, W. N., and Buhi, W. C.: Obstet. Gynecol. **39:** 852, 1972.
8. Butterworth, J., Sutherland, G. R., Bain, A. D., and McCrae, W. M.: Clin. Chim. Acta **40:** 275, 1972.
9. Butterworth, J., Scott, F., McCrae, W. M., and Bain, A. D.: Clin. Chim. Acta **40:** 139, 1972.
10. Miller, G. L.: Anal. Chem. **31:** 964, 1959.
11. Robertson, J. G.: Am. J. Obstet. Gynecol. **103:** 713, 1969.
12. Nelson, G. H., and Freedman, D. S.: Am. J. Obstet. Gynecol. **111:** 930, 1971.
13. Fluharty, A. L., Scott, M. L., Porter, M. T., and Kihara, H.: Biochem. Med. **7:** 39, 1973.
14. Sutcliffe, R. G., Brock, D. J. H., Robertson, J. G., Scrimgeour, J. B., and Monaghan, J. M.: J. Obstet. Gynaecol. Br. Commonw. **79:** 895, 1972.
15. Jonasson, L-E.: Acta Obstet. Gynaecol. Scand. **52:** 113, 1973.
16. Wiederschain, G. Y., Rosenfeld, E. L., Bru-

silovsky, A. I., and Kolibaba, L. G.: Clin. Chim. Acta **35:** 99, 1971.
17. Huijing, F., Warren, R. J., and McLeod, A. G. W.: Clin. Chim. Acta **44:** 453, 1973.
18. Butterworth, J., Sutherland, G. R., Broadhead, D. M., and Bain, A. D.: Clin. Chim. Acta **44:** 295, 1973.
19. Desnick, R. J., Krivit, W., and Sharp, H. L.: Biochem. Biophys. Res. Commun. **51:** 20, 1973.
20. Okada, S., Veath, M. L., Leroy, J., and O'Brien, J. S.: Am. J. Hum. Genet. **23:** 55, 1971.
21. Anteby, S. C., Zukerman, H., Gedella, I., and Weistreich, V.: J. Obstet. Gynaecol. Br. Commonw. **80:** 27, 1973.
22. Jonasson, L-E.: Acta Obstet. Gynaecol. Scand. **51:** 187, 1972.
23. Sutcliffe, R. G., and Brock, D. J. H.: J. Obstet. Gynaecol. Br. Commonw. **79:** 902, 1972.
24. Queenan, J. T., Thompson, W., Whitfield, C. R., and Shah, S. I.: Am. J. Obstet. Gynecol. **114:** 34, 1972.
25. Parmley, T. H., and Seeds, A. E.: Am. J. Obstet. Gynecol. **108:** 123, 1970.
26. Lind, T., Kendall, A., and Hytten, F. E.: J. Obstet. Gynaecol. Br. Commonw. **79:** 289, 1972.
27. Friedland, J., Perle, G., Saifer, A., Schneck, L., and Volk, B. W.: Proc. Soc. Exp. Biol. Med. **136:** 1297, 1971.
28. Nadler, H. L., and Messina, A. M.: Lancet **2:** 1277, 1969.
29. Nadler, H. L., Bigley, R. H., and Hug, G.: Lancet **2:** 369, 1970.
30. Salafsky, I. S., and Nadler, H. L.: J. Pediatr. **79:** 794, 1971.
31. Murphy, J. V.: Life Sci. **11:** 309, 1972.

*Clinical Genetics* 1974: **5**: 351–355

# Lysosomal enzyme levels in human amniotic fluid cells in tissue culture

## II. α-galactosidase, β-galactosidase and α-arabinosidase

G. R. Sutherland, J. Butterworth, D. M. Broadhead, and A. D. Bain

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Scotland

Changes in the activities of α-galactosidase, β-galactosidase and α-arabinosidase in amniotic fluid cells with time in culture were studied. Marked fluctuations in all three enzymes occurred with passage. In certain cell strains, β-galactosidase showed a marked rise in activity correlated with passage. The activity of all three enzymes, in amniotic fluid cells at the third passage, was correlated with the total time taken to reach confluency. There was no consistent pattern of enzyme activity associated with the time after subculture. Enzyme levels in cell strains derived from serial samples of amniotic fluid from several women showed large differences in activity unrelated to gestational age.

*Received 12 December 1973, accepted for publication 7 January 1974*

The number of diseases resulting from a known deficiency of a lysosomal enzyme has increased rapidly in recent years. With the demonstration that these deficiencies can be detected in cultured amniotic fluid cells, antenatal diagnosis for inborn errors of lysosomal metabolism has become practicable (Brady 1973, Nadler 1970, 1972, Prescott et al. 1972). However, little attention has been paid to possible fluctuations in the levels of lysosomal enzymes in cultured normal amniotic fluid cells (Beutler et al. 1971, Butterworth et al. 1973a, b, Gerbie et al. 1972). The effect of culture time on the activity of lysosomal enzymes in normal amniotic fluid cell strains has, therefore, been investigated. The results for α-galactosidase, β-galactosidase and α-arabinosidase are presented as part of this study.

### Methods

Amniotic fluid samples were obtained from hysterotomy specimens and by amniocentesis of Rhesus iso-immunised women who had no history of inborn errors of lysosomal metabolism. Primary cell cultures were set up (Sutherland et al. 1973), then subcultured, harvested and homogenized (Butterworth et al. 1973a), and the lysosomal enzymes α-galactosidase, β-galactosidase and α-arabinosidase assayed using 4-methylumbelliferone glycosides (Butterworth et al. 1972). Protein concentration was assayed (Miller 1959) using bovine serum albumin as standard.

The reproducibility of the combined culture and enzyme assay methods was tested by establishing 12 replicate cultures of one

This work was supported by the Advisory Committee on Medical Research for Scotland.

cell strain at two different passages. The 12 replicates were harvested when they had simultaneously reached confluency, and then assayed for enzyme activity. To study enzyme activity with respect to passage, six cell strains were subcultured for up to 15 passages and the enzyme levels assayed at intervals. All the cell strains were assayed at the third passage, and the time interval between subculture and harvest (confluency) noted. The variation in enzyme level within a single passage was followed by establishing a series of replicate cultures, which were harvested for enzyme assay at daily intervals for up to 7 days.

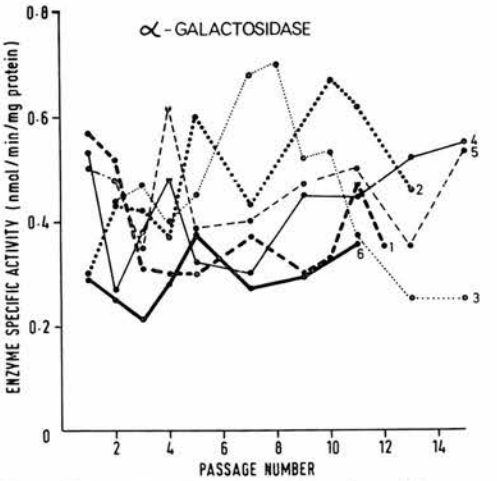



**Fig. 1.** The activity of α-galactosidase in serial passages of six strains of cultured amniotic fluid cells.

## Results

The reproducibility of the combined culture and assay system for each enzyme is presented in Table 1. The variation of the total experimental procedure (coefficient of variation) was below 6.0% for all the enzymes. The variation in the levels of the three enzymes with passage is given for six cell strains in Figs. 1–3. The coefficient of variation of the mean enzyme levels for the different cell strains was 20–33% for α-galactosidase, 27–68% for β-galactosidase and 18–39% for α-arabinosidase. Thus, all three enzymes showed much greater fluctuations with passage than could be attributed to experimental error alone.

A comparison of the enzyme levels in the first five passages with those in the next 10 for these six cell strains showed that β-galactosidase activity was lower ($P < 0.005$) in the earlier passages. However, the mean activity of β-galactosidase in the third and tenth passages of these six strains, plus an additional six strains, was not sig-

**Table 1**

Reproducibility of culture and assay system

| Enzyme | Mean* | s. d. | Coefficient of variation (%) |
|---|---|---|---|
| α-Galactosidase | 0.90[a] | 0.03 | 3.1 |
| | 0.40[b] | 0.01 | 3.3 |
| β-Galactosidase | 6.11[a] | 0.32 | 5.2 |
| | 8.58[b] | 0.32 | 3.7 |
| α-Arabinosidase | 0.86[a] | 0.05 | 5.2 |
| | 0.61[b] | 0.02 | 3.3 |

* Enzyme activity in nmol 4-methylumbelliferone $min^{-1}$ $mg^{-1}$ protein for 12 replicates

a – Assay tenth passage; b– Assay twentieth passage

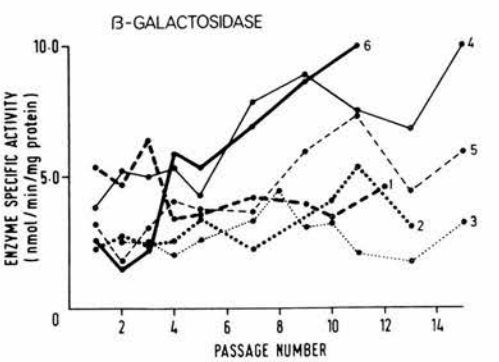



**Fig. 2.** The activity of β-galactosidase in serial passages of six strains of cultured amniotic fluid cells.

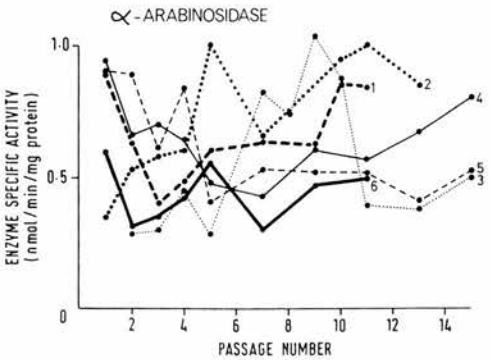



**Fig. 3.** The activity of $\alpha$-arabinosidase in serial passages of six strains of cultured amniotic fluid cells.

nificantly different. In these 12 strains the activities of $\alpha$-galactosidase and $\alpha$-arabinosidase at the third and tenth passages did not differ significantly. The contradictory results for $\beta$-galactosidase could be accounted for by three of the six strains examined in detail (strains 4–6, Fig. 2), which showed a marked rise in activity with passage not apparent in the other strains.

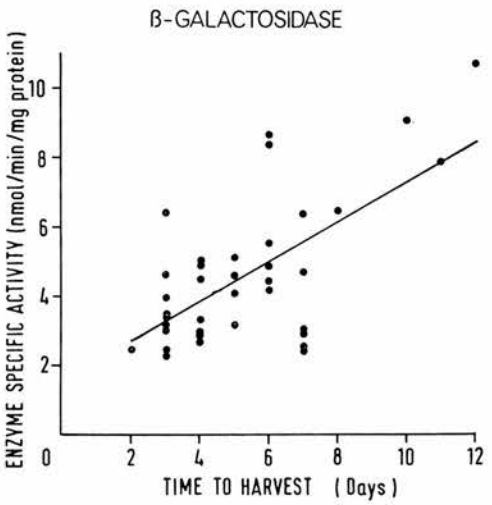



**Fig. 5.** Relationship of the activity of $\beta$-galactosidase in cultured amniotic fluid cells with time to reach confluency (harvest).

The relationship of the enzyme activity in cell strains at the third passage to the

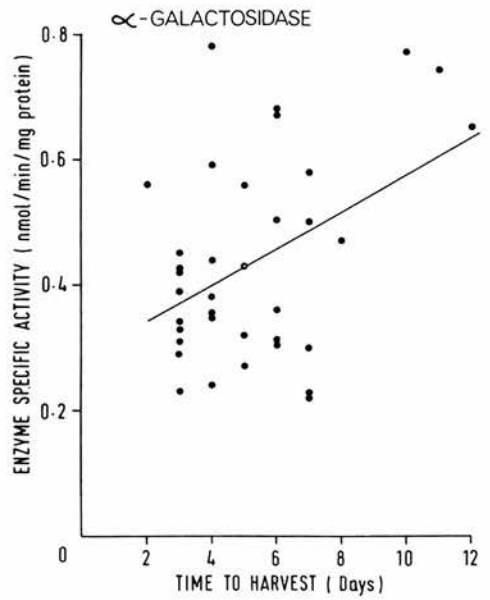



**Fig. 4.** Relationship of the activity of $\alpha$-galactosidase in cultured amniotic fluid cells with time to reach confluency (harvest).

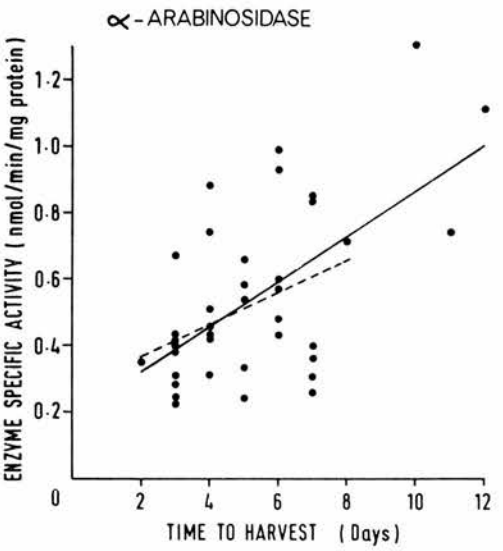



**Fig. 6.** Relationship of the activity of $\alpha$-arabinosidase in cultured amniotic fluid cells with time to reach confluency (harvest).
——— All days - - - - Without days 10-12

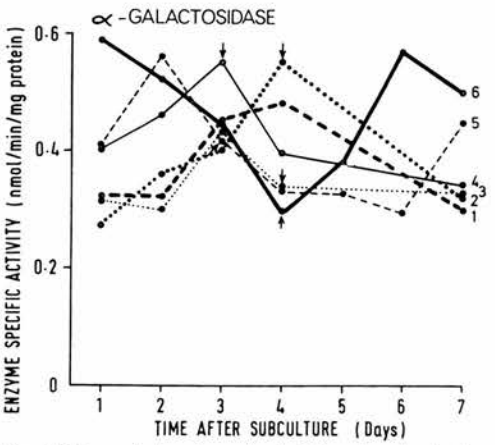



**Fig. 7.** The activity of $\alpha$-galactosidase in six strains of amniotic fluid cells in relation to time since subculture.
↑ Confluency; Strain 1 not confluent by 7 days

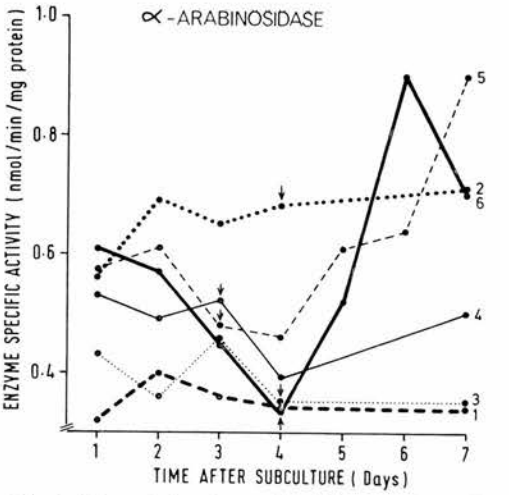



**Fig. 9.** The activity of $\alpha$-arabinosidase in six strains of amniotic fluid cells in relation to time since subculture.
↑ Confluency; Strain 1 not confluent by 7 days

time at which confluency (harvest) was reached is given in Figs. 4–6. A significant correlation between these two parameters was found for $\alpha$-galactosidase ($r = 0.42$), $\beta$-galactosidase ($r = 0.64$) and $\alpha$-arabinosidase ($r = 0.61$). However, if the few cell strains requiring 10–12 days to reach confluency were not considered, only the correlation for $\alpha$-arabinosidase ($r = 0.37$) was still significant.

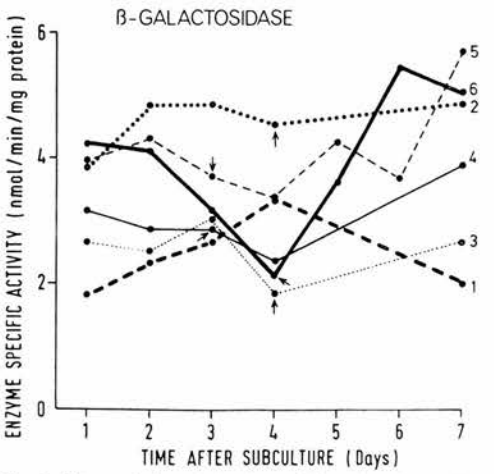



**Fig. 8.** The activity of $\beta$-galactosidase in six strains of amniotic fluid cells in relation to time since subculture.
↑ Confluency; Strain 1 not confluent by 7 days

**Table 2**

Enzyme levels* in cell strains at the third passage cultured from serial samples of amniotic fluid obtained from four women.

| Series | Gestational age (weeks) | $\alpha$-Galacto-sidase | $\beta$-Galacto-sidase | $\alpha$-Arabino-sidase |
|---|---|---|---|---|
| 1 | 18 | 0.30 | 2.87 | 0.47 |
| | 22 | 0.59 | 4.51 | 0.74 |
| | 24 | 0.78 | 5.01 | 0.80 |
| | 33 | 0.30 | 9.55 | 0.66 |
| | 35 | 0.44 | 5.48 | 0.38 |
| 2 | 23 | 0.35 | 2.87 | 0.48 |
| | 26 | 0.33 | 4.63 | 0.38 |
| | 28 | 0.29 | 4.96 | 0.22 |
| 3 | 22 | 0.65 | 10.76 | 1.11 |
| | 26 | 0.37 | 7.85 | 0.74 |
| 4 | 27 | 0.32 | 3.17 | 0.53 |
| | 34 | 0.35 | 2.67 | 0.31 |

* Enzyme activity – nmol 4-methylumbelliferone $min^{-1}$ $mg^{-1}$ protein

The fluctuations in the levels of the three enzymes in relation to time after subculture are given in Figs. 7–9. No consistent change was associated with the time after subculture. There was also no consistent change in enzyme activity after the time at which the cells became confluent.

Cell strains were grown from serial samples of amniotic fluid obtained from several Rhesus iso-immunised women and the levels of the enzymes, at the third passage, are given in Table 2. The activity of all three enzymes showed considerable variation within each series, that did not relate to gestational age.

## Discussion

The activities of α-galactosidase, β-galactosidase and α-arabinosidase in normal amniotic fluid cells have been shown to vary with the time in tissue culture. The significance of the results will be considered in relation to similar studies on other lysosomal enzymes in the following paper (Butterworth et al. 1974).

## References

Beutler, G. H., W. Kuhl, F. Trinidad, R. Teplitz & H. L. Nadler (1971). β-glucosidase activity in fibroblasts from homozygotes and heterozygotes for Gaucher's disease. *Amer. J. hum. Genet.* **23**, 62–66.

Brady, R. O. (1973). The abnormal biochemistry of inherited disorders of lipid metabolism. *Fed. Proc.* **32**, 1660–1667.

Butterworth, J., F. Scott, W. M. McCrae & A. D. Bain (1972). Lysosomal enzymes of cultured fibroblasts of Cystic Fibrosis patients. *Clin. chim. Acta* **40**, 139–142.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1973a). Lysosomal enzymes of cultured amniotic fluid cells. *Clin. chim. Acta* **44**, 295–299.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1973b). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. I. α-glucosidase and β-glucosidase. *Life Sci.* **13**, 713–722.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1974). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. III. β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase. *Clin Genet.* **5**, 356–362.

Gerbie, A., S. B. Melancon, C. Ryan & H. L. Nadler (1972). Cultivated epithelial-like cells and fibroblasts from amniotic fluid: Their relationship to enzymatic and cytologic analysis. *Amer. J. Obstet. Gynec.* **114**, 314–320.

Miller, G. L. (1959). Protein determination for large numbers of samples. *Analyt. Chem.* **31**, 964.

Nadler, H. L. (1970). Newer procedures in the preconceptional, prenatal and early postnatal diagnosis of birth defects. *Birth Defects: Original Article Series* VI, 26–33.

Nadler, H. L. (1972). Tissue culture and antenatal detection of molecular diseases. *Biochimie* **54**, 677–681.

Prescott, G. H., R. E. Magenis & N. R. M. Buist (1972). Amniocentesis for antenatal diagnosis. *Postgrad. Med.* **51**, 212–217.

Sutherland, G. R., E. Grace & A. D. Bain (1973). Metaphase chromosomes from neonatal urine. *Hum. Genet.* **17**, 273–275.

Address:
*J. Butterworth Ph. D.*
*Department of Pathology*
*Royal Hospital for Sick Children*
*Edinburgh EH9 1LF*
*Scotland*

*Clinical Genetics* 1974: **5**: 356–362

# Lysosomal enzyme levels in human amniotic fluid cells in tissue culture

## III, β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase

J. Butterworth, G. R. Sutherland, D. M. Broadhead, and A. D. Bain

Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Scotland

Fluctuations in the levels of β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase in amniotic fluid cells with time in culture were studied. The four enzymes fluctuated markedly with passage; no consistent trends were apparent. The activity of α-mannosidase in amniotic fluid cell strains at the third passage was correlated with the time taken to reach confluency. There was no consistent pattern of enzyme activity associated with the time after subculture. Enzyme levels in cell strains derived from serial samples of amniotic fluid from several women showed large differences in activity unrelated to gestational age. The significance of the findings are discussed in relation to antenatal diagnosis of inborn errors of lysosomal metabolism.

*Received 12 December 1973, accepted for publication 7 January 1974*

Previous reports (Butterworth et al. 1973a, Sutherland et al. 1974) have shown that the levels of five lysosomal enzymes (α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, α-arabinosidase) in amniotic fluid cells fluctuate with time in tissue culture. Similar studies have been carried out on four other lysosomal enzymes (β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase, acid phosphatase), all of which are involved in inborn errors of metabolism.

### Methods

The amniotic fluid cell strains were cultured under the experimental conditions described in previous papers (Butterworth et al. 1973a, Sutherland et al. 1974). The cells were harvested and the enzymes β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase assayed using 4-methylumbelliferone conjugates as substrates (Butterworth et al. 1972, 1973b).

### Results

The reproducibility of the combined culture and assay system for each enzyme is presented in Table 1. The variation of the total experimental procedure (coefficient of variation) was below 7.5% for all four enzymes. The variations in the levels of

This work was supported by the Advisory Committee on Medical Research for Scotland.

**Table 1**

Reproducibility of culture and assay system

| Enzyme | Mean* | s. d. | Coefficient of variation (%) |
|---|---|---|---|
| β-Glucuronidase | 2.02[a] | 0.09 | 4.3 |
| | 0.81[b] | 0.03 | 4.0 |
| N-acetyl-β-D-Glucosaminidase | 87.56[a] | 4.43 | 5.1 |
| | 41.60[b] | 2.97 | 7.1 |
| α-Mannosidase | 0.95[a] | 0.05 | 5.3 |
| | 1.11[b] | 0.07 | 6.5 |
| Acid Phosphatase | 7.11[a] | 0.37 | 5.2 |
| | 7.38[b] | 0.55 | 7.4 |

* Enzyme activity in nmol 4-methylumbelliferone $min^{-1}$ $mg^{-1}$ protein for 12 replicates
a – assay tenth passage; b – assay twentieth passage

the four enzymes with passage is given for six cell strains in Figs. 1–4. The coefficient of variation of the mean enzyme levels for the different cell strains was 27–38% for β-glucuronidase, 16–38% for N-acetyl-β-D-glucosaminidase, 23–52% for α-mannosidase and 18–45% for acid phosphatase. All four enzymes, therefore, showed fluctuations with passage greater than could be expected from experimental error. None

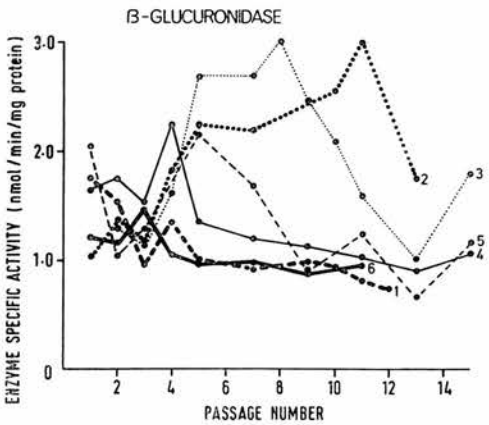



**Fig. 1.** The activity of β-glucuronidase in serial passages of six strains of cultured amniotic fluid cells.

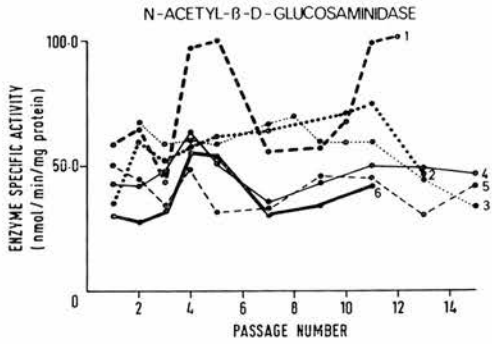



**Fig. 2.** The activity of N-acetyl-β-D-glucosaminidase in serial passages of six strains of cultured amniotic fluid cells.

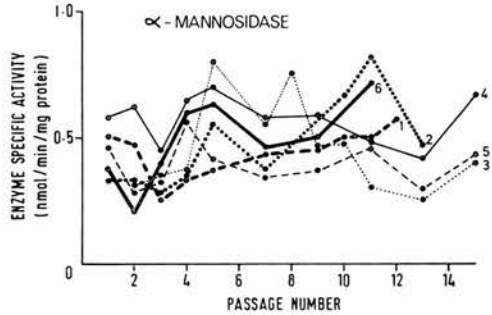



**Fig. 3.** The activity of α-mannosidase in serial passages of six strains of cultured amniotic fluid cells.

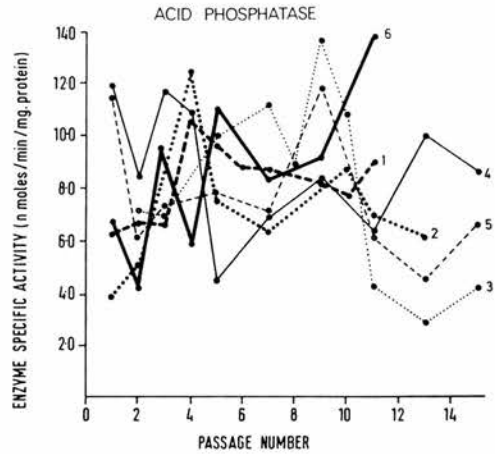



**Fig. 4.** The activity of acid phosphatase in serial passages of six strains of cultured amniotic fluid cells.

of these fluctuations in enzyme levels showed a consistent pattern of activity related to passage, as judged by previously described criteria (Butterworth et al. 1973a, Sutherland et al. 1974).

The results were examined to determine whether the enzyme levels of the cell strains at the third passage were related to the time at which confluency (harvest) was reached. The only significant correlation (r = 0.58) was for α-mannosidase (Fig. 5). This was still significant (r = 0.42) even if the few

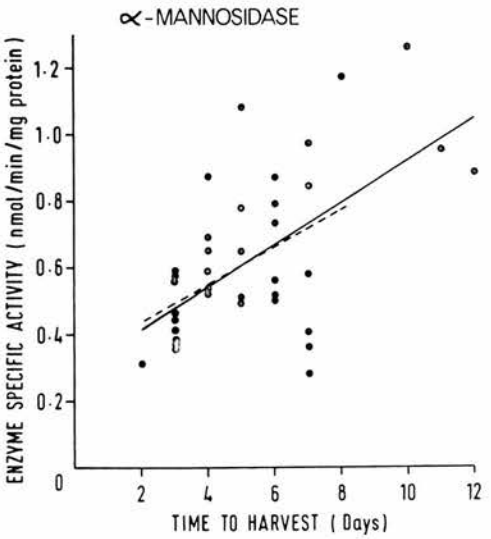



**Fig. 5.** Relationship of the activity of α-mannosidase in cultured amniotic fluid cells with time to reach confluency (harvest).
——— All days – – – – Without days 10-12

cell strains requiring 10–12 days to reach confluency were not included.

Figs. 6–9 show the fluctuations in the levels of the four enzymes related to the time after subculture for six amniotic fluid cell strains. No consistent pattern of enzyme activity related to this parameter was noted. Moreover, no consistent increase in enzyme activity was noted after the cell strains had become confluent.

Amniotic fluid cells were cultured from

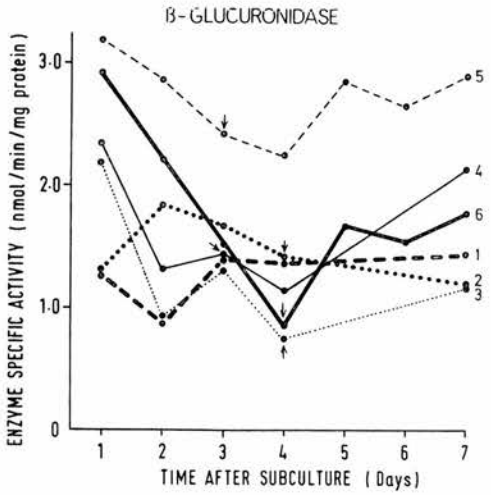



**Fig. 6.** The activity of β-glucuronidase in serial strains of amniotic fluid cells in relation to time since subculture.
↑ Confluency; Strain 1 not confluent by 7 days

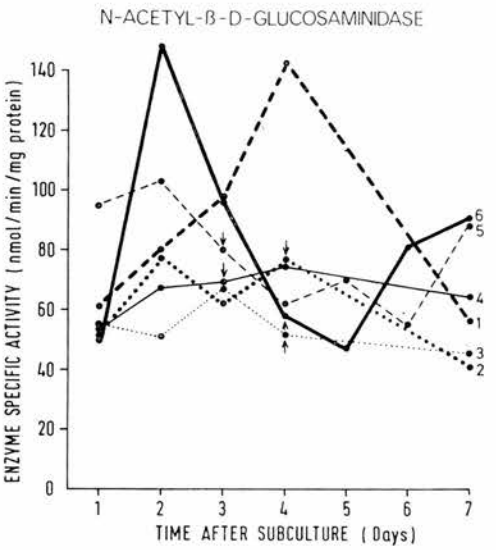



**Fig. 7.** The activity of N-acetyl-β-D-glucosaminidase in serial strains of amniotic fluid cells in relation to time since subculture.
↑ Confluency; Strain 1 not confluent by 7 days

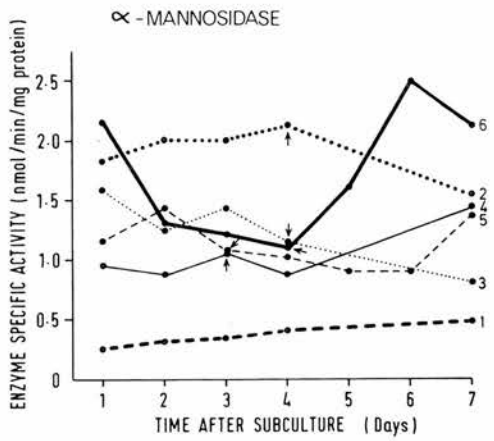



**Fig. 8.** The activity of $\alpha$-mannosidase in serial strains of amniotic fluid cells in relation to time since subculture.
↑ Confluency; Strain 1 not confluent by 7 days

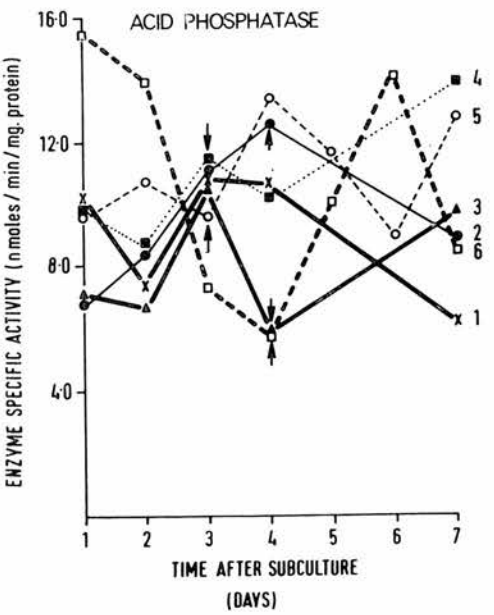



**Fig. 9.** The activity of acid phosphatase in serial strains of amniotic fluid cells in relation to time since subculture.
↑ Confluency; Strain 1 not confluent by 7 days

serial samples of amniotic fluid obtained from four Rhesus iso-immunised women. The levels of the four enzymes in the third passage of these cell strains are given in Table 2. Within each series the activity of the four enzymes showed considerable variability, unrelated to gestational age.

## Discussion

The known number of inborn errors of metabolism due to a lysosomal enzyme deficiency has increased rapidly in the last few years (Brady 1973, Milunsky 1973, Nadler 1972). Post-natal diagnosis of these diseases is made by enzyme assay using leukocytes and cultured fibroblasts. The properties and variations in activity of lysosomal enzymes in these cell types have been reported (Hultberg et al. 1973, Milunsky et al. 1972, Ryan et al. 1972).

With the demonstration that lysosomal enzymes are present in cultured amniotic fluid cells (Kaback & Cooke 1970), and that an enzyme deficiency in the fetus is reflected in these cells (Padeh & Navon 1971, Salafsky & Nadler 1971), antenatal diagnosis of inborn errors of lysosomal metabolism became possible. Indeed, there are now several reports (Brady et al. 1971, Desnick et al. 1973, Kaback et al 1973, O'Brien et al. 1971, van der Hagen et al. 1973) of the successful antenatal diagnosis of such enzyme deficiency diseases. This very success has had the result of increasing the demand for antenatal diagnosis. However, whilst there are many reports concerning lysosomal enzyme deficiencies in cultured amniotic fluid cells (Milunsky 1973), much less attention has been paid to fluctuations of these enzymes in cells cultured from normal amniotic fluids (Beutler et al. 1971, Butterworth et al. 1973a, 1973b, Gerbie et al. 1972). A study of the activities of nine lysosomal enzymes in cultured amniotic fluid cells has been

**Table 2**

Enzyme levels* in cell strains at the third passage cultured from serial samples of amniotic fluid obtained from four women

| Series | Gestational age (weeks) | β-Glucuronidase | N-acetyl-β-D-Glucosaminidase | α-Mannosidase | Acid Phosphatase |
|---|---|---|---|---|---|
| 1 | 18 | 0.60 | 44.4 | 0.28 | 6.35 |
| | 22 | 0.83 | 64.0 | 0.87 | 12.69 |
| | 24 | 0.76 | 57.3 | 0.95 | 11.69 |
| | 33 | 0.63 | 61.3 | 0.81 | 10.05 |
| | 35 | 0.75 | 32.2 | 0.42 | 4.41 |
| 2 | 23 | 0.43 | 68.5 | 0.52 | 7.15 |
| | 26 | 1.09 | 76.3 | 0.37 | 5.90 |
| | 28 | 0.42 | 34.4 | 0.41 | 11.52 |
| 3 | 22 | 0.98 | 91.9 | 0.88 | 9.15 |
| | 26 | 0.90 | 62.1 | 0.96 | 10.52 |
| 4 | 27 | 0.66 | 66.3 | 1.08 | 6.85 |
| | 34 | 0.64 | 39.4 | 0.59 | 9.67 |

* Enzyme activity – nmol 4-methylumbelliferone $min^{-1}$ $mg^{-1}$ protein

undertaken. The findings for five lysosomal enzymes have been reported (Butterworth et al. 1973a, Sutherland et al. 1974) and these will be considered together with the present results.

The levels of all the lysosomal enzymes investigated showed marked fluctuations with passage in amniotic fluid cell strains, a finding in agreement with the results of Milunsky et al. (1972) for cultured skin fibroblasts. Some amniotic fluid cell strains showed a significant pattern of increase in specific activity with passage for certain enzymes such as β-glucosidase and β-galactosidase, but only for α-glucosidase was this increase consistent. This contrasts with the general rise in lysosomal enzyme activity with passage noted in cultured fibroblasts by Hultberg et al. (1973). The ratio of epithelioid to fibroblast-like cells in amniotic fluid cell culture has been reported (Melancon et al. 1971) to change with passage. As the lysosomal enzyme levels differ between these cell types (Gerbie et al. 1972), it is possible that the patterns of change observed in the present studies reflect this change in cell type.

In cultured fibroblasts, acid phosphatase (Christofalo & Kritchevsky 1969), N-acetyl-β-D-glucosaminidase (Okada et al. 1971) and β-glucuronidase (DeMars 1964, Leroy & DeMars 1967, Russell et al. 1971) levels have been shown to be related to the time after subculture. No such relationship was consistently found for the lysosomal enzymes in cultured amniotic fluid cells. Similarly, the rise in the level of N-acetyl-β-D-glucosaminidase (Okada et al. 1971) and β-glucosidase (Ryan et al. 1972) activity in cultured fibroblasts after confluency was not apparent in cultured amniotic fluid cells. However, a positive correlation was noted between the activities of some of the enzymes in amniotic fluid cells and the time taken to reach confluency. This apparent relationship may reflect a difference between the cell types found in slowly and rapidly growing cell strains. The lack of a correlation between gestational age and the level of the lysosomal enzymes in cells

cultured from serial samples of amniotic fluid is consistent with previous findings using only one sample from any pregnancy (Butterworth et al. 1973b).

Hence, cells cultured from amniotic fluid of any gestational age will provide suitable control material for antenatal diagnosis of a disease involving any of these lysosomal enzymes. Similarly, cells at any stage of culture up to at least the fifteenth passage can be used as control material. One possible exception is $\alpha$-glucosidase, where cells in later passages may have higher enzyme levels. The possibility still remains, however, that an error in diagnosis could arise due to a change in the isoenzyme pattern with gestational age or time in culture. These possibilities clearly need to be taken into consideration when making an antenatal diagnosis of a disease where only one isoenzyme is deficient.

The marked variations in the activity of the nine lysosomal enzymes described in this series of studies clearly have implications with regard to antenatal diagnosis of inborn errors of lysosomal metabolism. The fluctuations of enzyme activity in amniotic fluid cell strains with culture time, and even in cells cultured from serial samples of amniotic fluid from the same woman, result in a wide range of normal values which would make identification of heterozygotes extremely difficult. When undertaking antenatal diagnosis of recessively inherited diseases, half the fetuses encountered will be heterozygotes. If the variation in enzyme activity is as large for these heterozygotes as for the normals, then in those diseases in which the enzyme deficiency is only partial, the heterozygote could well be mistaken for an affected homozygote.

### References


Beutler, E., W. Kuhl, F. Trinidad, R. Teplitz & H. L. Nadler (1971). $\beta$-glucosidase in fibroblasts from homozygotes and heterozygotes for Gaucher's disease. *Amer. J. hum. Genet.* **23**, 62–66.

Brady, R. O., B. W. Uhlendorf & C. B. Jacobson (1971). Fabry's disease: antenatal detection. *Science* **172**, 174–175.

Brady, R. O. (1973). The abnormal biochemistry of inherited disorders of lipid metabolism. *Fed. Proc.* **32**, 1660–1667.

Butterworth, J., F. Scott, W. M. McCrae & A. D. Bain (1972). Lysosomal enzymes of cultured fibroblasts of Cystic Fibrosis patients. *Clin. chim. Acta* **40**, 139–142.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1973a). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. I. $\alpha$-glucosidase and $\beta$-glucosidase. *Life Sci.* **13**, 713–722.

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1973b). Lysosomal enzymes of cultured amniotic fluid cells. *Clin. chim. Acta* **44**, 295–299.

Cristofalo, V. J. & D. Kritchevsky (1969). Enzyme activity during the growth and aging of human cells. *Progr. immunobiol. Standard.* **3**, 99–105.

DeMars, R. (1964). Some studies of enzymes in cultured human cells. *Nat. Cancer Inst. Monogr.* **13**, 181–195.

Desnick, R. J., W Krivit & H. L. Sharp (1973). In utero diagnosis of Sandhoff's disease. *Biochem. Biophys. Res. Commun.* **51**, 20–26.

Gerbie, A. B., S. B. Melancon, C. Ryan & H. L. Nadler (1972). Cultivated epithelial-like cells and fibroblasts from amniotic fluid: Their relationship to enzymatic and cytologic analysis. *Amer. J. Obstet. Gynec.* **114**, 314–320.

Hultberg, B., S. Sjobland & P. A. Ockerman (1973). Properties of five acid hydrolases in human skin fibroblast cultures. *Acta paediat. scand.* **62**, 474–480.

Kaback, M. M. & R. E. Cooke (1970). Intrauterine diagnosis of fetal disorders. *Paediat. jap.* **18**, 79–87.

Kaback, M. M., H. R. Sloan, M. Sonneborn, R. M. Herndon & A. K. Percy (1973). $G_{M1}$-gangliosidosis type 1: In utero detection and fetal manifestations. *J. Pediat.* **82**, 1037–1041.

Leroy, L. & R. DeMars (1967). Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. *Science* **157**, 804–806.

Melancon, S. B., S. Y. Lee & H. L. Nadler (1971). Histidase activity in cultivated human amniotic fluid cells. *Science* **173**, 627–628.

Milunsky, A. (1973). *The Prenatal Diagnosis*

*of Hereditary Disorders*. Springfield, Illinois, Charles C Thomas.

Milunsky, A., C. Spielvogel & J. N. Kanfer (1972). Lysosomal enzyme variations in cultured normal skin fibroblasts. *Life Sci.* **11**, 1101–1107.

Nadler, H. L. (1972). Tissue culture and antenatal detection of molecular diseases. *Biochimie* **54**, 677–681.

O'Brien, J. S., S. Okada, D. L. Fillerup, M. L. Veath, B. Adornato, P. H. Brenner & J. G. Leroy (1971). Tay-Sachs disease: Prenatal diagnosis. *Science* **172**, 61–64.

Okada, S., M. L. Veath, J. G. Leroy & J. S. O'Brien (1971). Ganglioside $G_{M2}$ storage disease: Hexosaminidase deficiencies in cultured fibroblasts. *Amer. J. hum. Genet.* **23**, 55–61.

Padeh, B. & B. Navon (1971). Diagnosis of Tay-Sachs disease by hexosaminidase activity of leukocytes and amniotic fluid cells. *Israel J. med. Sci.* **7**, 259–263.

Russell, S. B., J. D. Russell & J. W. Littlefield (1971). $\beta$-glucuronidase activity in fibroblasts cultured from persons with and without Cystic Fibrosis. *J. med. Genet.* **8**, 441–443.

Ryan, C. A., S. Y. Lee & H. L. Nadler (1972). Effect of culture conditions on enzyme activities in cultivated human fibroblasts. *Exp. Cell Res.* **71**, 388–392.

Salafsky, I. S. & H. L. Nadler (1971). Alpha-1,4 glucosidase activity in Pompe's disease. *J. Pediat.* **79**, 794 798.

Sutherland, G. R., J. Butterworth, D. M. Broadhead & A. D. Bain (1974). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. II. $\alpha$-galactosidase, $\beta$-galactosidase and $\alpha$-arabinosidase. *Clin. Genet.* **5**, 351–355.

van der Hagen, C. B., A-L Børresen, K. Molne, G. Oftedal, K. Bjøro & K. Berg (1973). Metachromatic leukodystrophy. 1. Prenatal detection of arylsulphatase deficiency. *Clin. Genet.* **4**, 256–259.

Address:
*J. Butterworth Ph. D.*
*Department of Pathology*
*Royal Hospital for Sick Children*
*Edingburgh EH9 1LF*
*Scotland*

Reprinted from

*Clinica Chimica Acta*, 53 (1974) 239—246


CCA 6442

# EFFECT OF SERUM CONCENTRATION, TYPE OF CULTURE MEDIUM AND pH ON THE LYSOSOMAL ENZYME ACTIVITY OF CULTURED HUMAN AMNIOTIC FLUID CELLS

J. BUTTERWORTH*, G.R. SUTHERLAND, D.M. BROADHEAD and A.D. BAIN
*Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF (U.K.)*

(Received January 31, 1974)

## Summary

1. Possible changes in lysosomal enzyme activity of cultured amniotic fluid cells with serum concentration, type of medium and the pH of the medium were studied.

2. Apart from a small, but significant, decrease in the activity of β-galactosidase, none of the enzymes changed with increasing serum concentration.

3. No significant changes in enzyme activity were found between cells cultured in Ham's F10 and Eagle's MEM medium.

4. Lysosomal enzyme levels were unaffected by culturing cells for up to 9 days at pH 7.0, 7.4 and 7.9.

## Introduction

As cultured amniotic fluid cells are now being used in the antenatal diagnosis of inborn errors of lysosomal enzyme metabolism, a better understanding of the reasons for the fluctuations observed in the levels of lysosomal enzymes in normal cultured amniotic fluid cells [1—4] is clearly required. It is also important to determine whether the different normal levels of enzyme activity obtained by different laboratories [5] could be attributed to variations in cell culture. Hence the effects of serum concentrations, type of tissue culture medium (Ham's F10, Eagles's MEM) and the pH of the medium on the levels of some lysosomal enzymes in cultured normal amniotic fluid cells were investigated.

* To whom correspondence should be addressed.

## Methods

Cell strains were initiated [6], subcultured and maintained [1] as previously described.

To study the effects of fetal calf serum concentration in the culture medium on lysosomal enzyme activity, 3 lots of culture medium (Ham's F10) were prepared with fetal calf serum concentrations of 15, 20 and 30%. The Ham's F10 (Flow Lab. Ltd) and fetal calf serum (Biocult Labs. Ltd) came from single batches: this condition was adhered to for all the culture experiments. A cell strain which had been maintained in medium with a 30% fetal calf serum concentration was used to seed 12 replicate culture bottles. 4 cultures were maintained at each serum concentration until harvested at confluency for enzyme assay. This procedure was carried out for 3 cell strains.

The effect of using two commercially available culture media, Ham's F10 (Flow Lab. Ltd) and Eagle's MEM (Wellcome Ltd) on the enzyme levels was compared. Both media were supplemented with 15% fetal calf serum. 6 cell strains which had been maintained in Ham's F10, were used for this experiment. Duplicate cultures which had been maintained in these two media for two passages were harvested at confluency for enzyme assay.

The effect of the pH of the medium on the enzyme levels was studied using Ham's F10 culture medium supplemented with 15% fetal calf serum and buffered with tricine [7]. Solutions of M tricine were prepared and the pH adjusted with 6 *N* NaOH so that when added to medium to give a final concentration of 50 mM, pH values of 7.0, 7.4 and 7.9 were obtained. In an initial experiment 12 replicate cultures were grown to confluency in Ham's F10 with 15% fetal calf serum and a bicarbonate buffer system. At this point 4 bottles were maintained in medium buffered at each of the three pH values and the cells harvested for enzyme assay 24 h later. Since this initial experiment indicated that pH did not affect the enzyme levels, another experiment was designed to determine whether growing the cells in media at different pH values from the time of seeding would affect the enzyme levels. 15 replicate cultures were prepared and medium at each pH was added to 5 bottles. To minimize pH changes the culture medium was changed every day. One culture at each pH was harvested for enzyme assay on days 1, 3, 5, 7 and 9 after the cultures had been set up. The experiment was carried out on two cell strains simultaneously, one an amniotic fluid derived fibroblast-like cell strain and the other, a similarly derived cell strain of mixed epithelioid type II and III cells [8].

The preparation of cell homogenates, protein estimation and the assay of the lysosomal enzymes were carried out as previously described [9].

## Results

The levels of the lysosomal enzymes in three strains of amniotic fluid cells cultured with 15, 20 and 30% fetal calf serum supplement in the medium are given in Table I. Analysis of variance of the results indicated that there was a small, but significant, decrease in the activity of β-galactosidase ($P < 0.05$) from 15 to 30% serum levels. None of the other comparisons of enzyme activity were significantly different. There was a significantly higher level of protein ($P < 0.05$) for cell strains cultured in 30% compared to 15% serum.

TABLE I

LYSOSOMAL ENZYME ACTIVITIES OF THREE AMNIOTIC FLUID CELL STRAINS CULTURED USING DIFFERENT CONCENTRATIONS OF FETAL CALF SERUM

Figures represent the means of 4 experiments; standard deviations in parentheses.

| | Enzyme activity (nmoles/min/mg protein); fetal calf serum concentration (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 15 | 20 | 30 | 15 | 20 | 30 | 15 | 20 | 30 |
| Acid phosphatase | 8.06 (0.43) | 7.37 (0.42) | 8.90 (0.92) | 5.45 (0.35) | 5.94 (0.29) | 5.18 (0.66) | 9.62 (1.40) | 9.71 (1.23) | 11.19 (1.12) |
| *N*-Acetyl-β-D-glucosaminidase | 36.2 (1.9) | 36.6 (1.5) | 33.4 (4.3) | 36.5 (0.7) | 33.2 (2.6) | 31.7 (2.6) | 88.9 (8.4) | 91.0 (11.0) | 101.7 (11.8) |
| β-Galactosidase | 6.42 (0.84) | 6.27 (0.74) | 5.94 (0.86) | 4.51 (0.16) | 4.19 (0.32) | 3.70 (0.34) | 10.96 (1.26) | 10.38 (0.88) | 10.36 (0.60) |
| β-Glucuronidase | 0.52 (0.01) | 0.50 (0.04) | 0.55 (0.05) | 0.76 (0.06) | 0.68 (0.04) | 0.62 (0.05) | 0.75 (0.17) | 1.00 (0.22) | 1.29 (0.12) |
| α-Galactosidase | 0.27 (0.01) | 0.27 (0.02) | 0.28 (0.04) | 0.28 (0.02) | 0.28 (0.01) | 0.27 (0.03) | 0.55 (0.06) | 0.55 (0.02) | 0.50 (0.06) |
| α-Arabinosidase | 0.24 (0.01) | 0.23 (0.01) | 0.20 (0.02) | 0.18 (0.01) | 0.16 (0.01) | 0.14 (0.01) | 0.34 (0.04) | 0.34 (0.04) | 0.34 (0.03) |
| α-Mannosidase | 0.64 (0.03) | 0.64 (0.03) | 0.60 (0.06) | 0.78 (0.03) | 0.74 (0.06) | 0.68 (0.04) | 1.41 (0.22) | 1.15 (0.10) | 1.39 (0.16) |
| α-Glucosidase | 0.44 (0.03) | 0.40 (0.04) | 0.35 (0.04) | 0.70 (0.04) | 0.63 (0.06) | 0.56 (0.06) | 1.71 (0.11) | 1.49 (0.15) | 1.46 (0.16) |
| β-Glucosidase | 0.07 (0.02) | 0.08 (0.02) | 0.06 (0.01) | 0.06 (0.01) | 0.07 (0.02) | 0.06 (0.02) | 0.27 (0.06) | 0.27 (0.07) | 0.34 (0.06) |
| Arylsulphatase A | 0.98 (0.07) | 1.11 (0.11) | 1.09 (0.16) | 1.07 (0.17) | 0.98 (0.05) | 0.84 (0.11) | | Not done | |
| Protein (mg/ml) | 0.30 (0.02) | 0.33 (0.02) | 0.38 (0.05) | 0.24 (0.02) | 0.24 (0.02) | 0.28 (0.03) | 0.07 (0.01) | 0.09 (0.02) | 0.14 (0.02) |

TABLE II

LYSOSOMAL ENZYME ACTIVITIES OF AMNIOTIC FLUID CELLS CULTURED USING HAM'S F10 AND EAGLE'S MEM MEDIUM

Figures represent the means of 12 experiments; standard deviations in parentheses.

| Enzyme | Enzyme activity (nmoles/min/mg protein) | |
|---|---|---|
| | Ham's F10 | Eagle's MEM |
| Acid phosphatase | 10.34 (3.92) | 12.11 (3.64) |
| *N*-Acetyl-β-D-glucosaminidase | 34.38 (7.69) | 42.56 (10.02) |
| β-Galactosidase | 6.02 (2.05) | 6.41 (2.22) |
| β-Glucuronidase | 0.89 (0.39) | 0.93 (0.40) |
| α-Galactosidase | 0.41 (0.12) | 0.45 (0.14) |
| α-Arabinosidase | 0.12 (0.06) | 0.10 (0.06) |
| α-Mannosidase | 1.21 (0.40) | 1.36 (0.60) |
| α-Glucosidase | 0.78 (0.34) | 0.92 (0.25) |
| Arylsulphatase A | 2.38 (0.73) | 2.70 (1.08) |
| Protein (mg/ml) | 0.083 (0.009) | 0.059 (0.013) |

The levels of the lysosomal enzymes in amniotic fluid cells cultured in Ham's F10 and Eagle's MEM medium are given in Table II. Whilst some of the enzymes in certain of the cell strains showed differences between the two media, a comparison of the results (*t*-test) indicated that there were no significant differences for any of the enzymes. However, the level of protein in cell strains cultured in Ham's F10 was significantly higher ($P < 0.05$) than when using Eagle's MEM.

The initial experiment on the effects of pH showed that there were no significant differences between the lysosomal enzyme activities of amniotic fluid cells when monolayer cultures were maintained at three different pH values (7.0; 7.4; 7.9) for 24 h. The results of the second experiment in which cell strains were cultured for up to 9 days at these three pH values are given in Figs 1—3. The pattern of activity with time in culture of some of the enzymes, such as α-mannosidase (Fig. 2) and arylsulphatase A (Fig. 3), was different for the two cell strains. Inspection of Figs 1—3 indicated that for only β-glucuronidase was there an apparent effect of pH, in that the enzyme activity at pH 7.9 was lower than that at 7.4 and 7.0 after 5 days of culture. However, a comparison (analysis of variance) of the mean enzyme activities throughout the period of culture indicated that there was no significant difference between the enzyme levels at the three pH values.

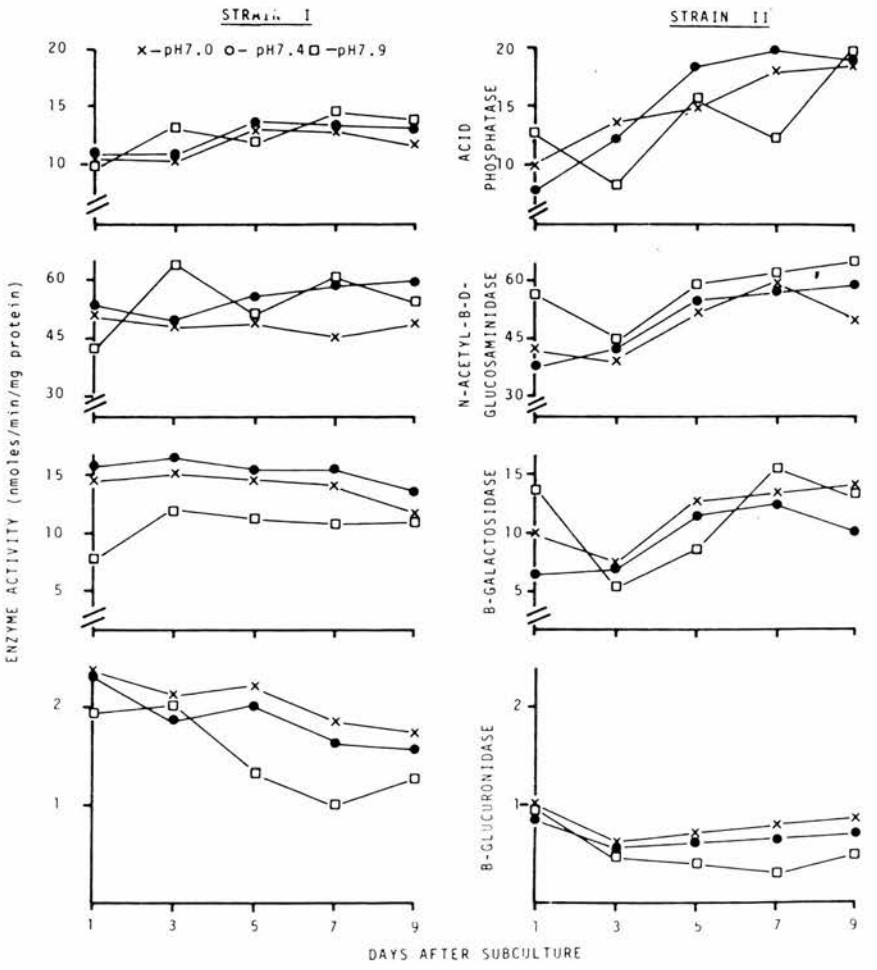



Fig. 1. The levels of acid phosphatase, *N*-acetyl-β-D-glucosaminidase, β-galactosidase and β-glucuronidase in amniotic fluid cells cultured for 9 days in medium at three pH values.

## Discussion

Apart from the effects of culture time [2—4], the study of variations in lysosomal enzyme activity of amniotic fluid cells with culture variables has been neglected. The effect of serum concentration on cell growth and enzyme activity has been largely confined to cell lines requiring very low levels of serum and few studies have been undertaken using high concentrations [10,11]. Growth of rat fibroblasts was shown [10] to increase as the serum was increased from 5 to 30% with a concomitant increase in mucopolysaccharide production. A change in serum from 10 to 20% had no effect on the growth rate of human fibroblasts, but an increase to 30% led to a reduction in cell growth [11]. In the present study cell growth, as judged by protein levels, increased with serum concentration from 15 to 30% although enzyme specific

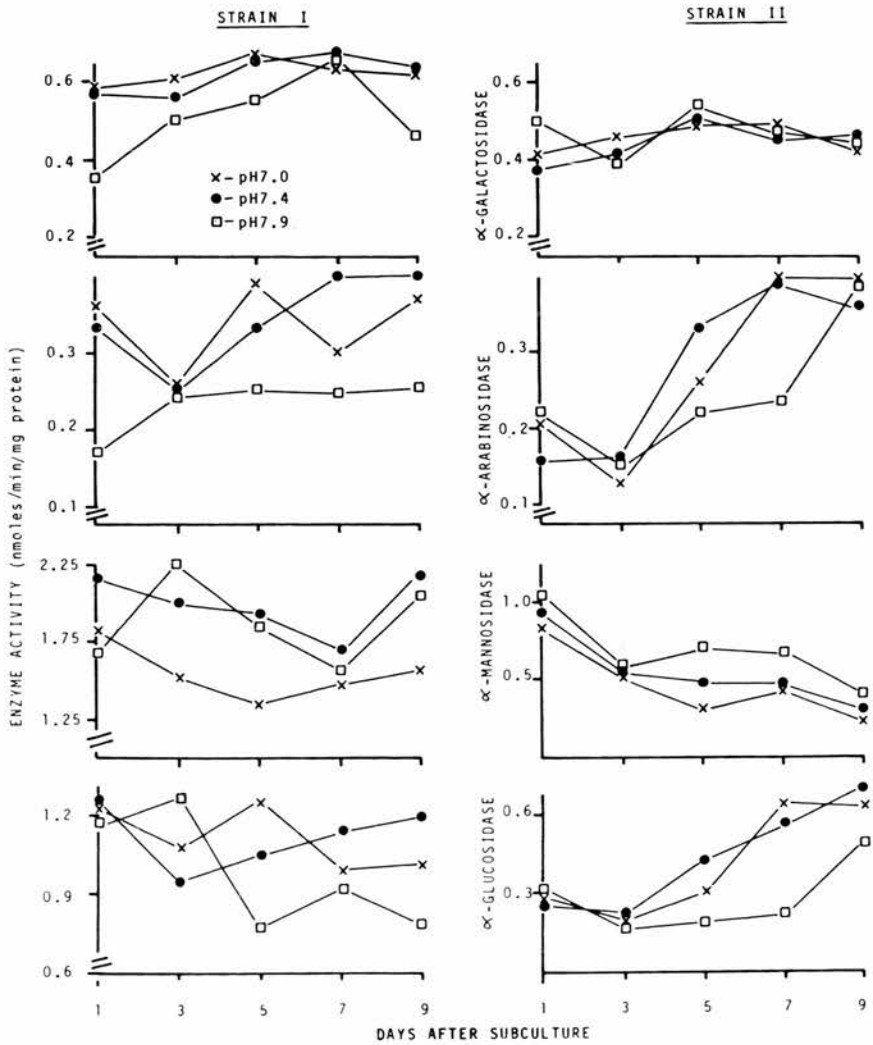



Fig. 2. The levels of α-galactosidase, α-arabinosidase, α-mannosidase and α-glucosidase in amniotic fluid cells cultured for 9 days in medium at three pH values.

activity remained constant. A possible explanation for this is that the cell strains used were adapted to growing in 30% serum. Attempts to increase the serum concentration to 30% once amniotic fluid cells were adapted to growing in 15% were unsuccessful, due to marked reduction in cell growth. In view of these effects of serum concentration on cell growth and metabolism, it is perhaps surprising that the lysosomal enzymes of amniotic fluid cells are unaffected; the statistically significant finding for β-galactosidase is probably fortuitous and of little biological significance. The finding [12] that the level of acid phosphatase in mouse fibroblasts remained unaltered when the serum concentration was changed from 0 to 10% is in accord with the present study.

The effect of using different types of media on enzyme activity has re-

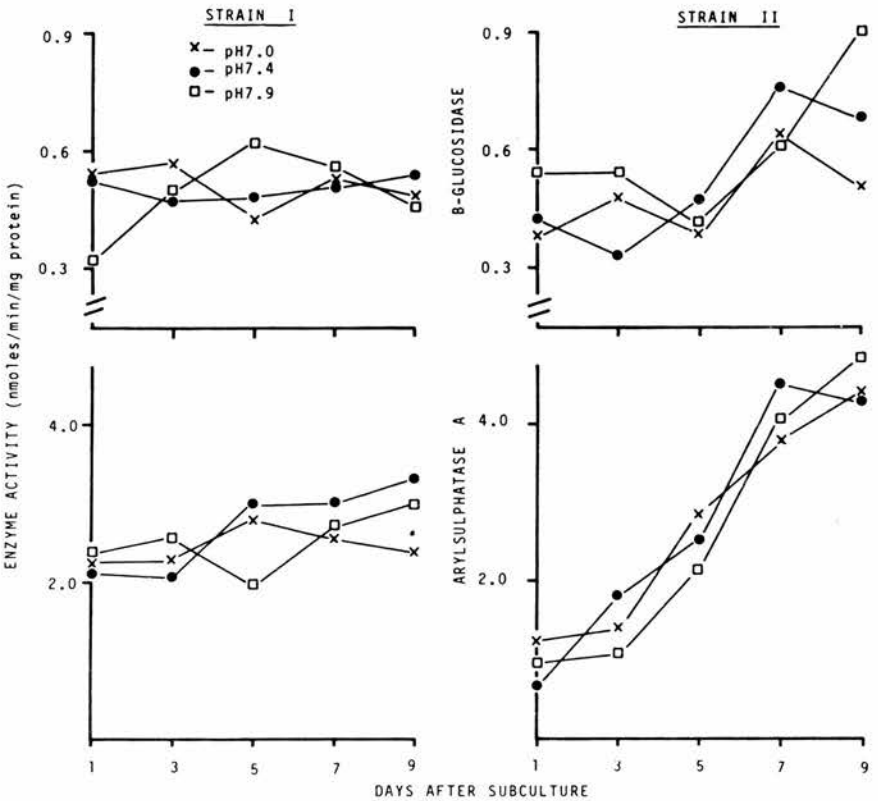



Fig. 3. The levels of β-glucosidase and arylsulphatase A in amniotic fluid cells cultured for 9 days in medium at three pH values.

ceived little attention [5,13,14]. The specific activity of lactate dehydrogenase and glucose-6-phosphate dehydrogenase has been shown [13] to be affected by the type of medium in which Earle's L cells were cultured. In cultured human fibroblasts the level of β-glucosidase was lower in cells grown in Ham's F10 than Eagle's MEM [5,14]. In the present study on amniotic fluid cells the level of acid phosphatase was found to be unaffected by these two media, in agreement with a finding for cultured human fibroblasts [14]. Although the level of arylsulphatase A has been found to be lower in fibroblasts [14] cultured in Ham's F10, no such affect was observed in amniotic fluid cells. The higher protein levels for amniotic fluid cells grown in Ham's F10 may reflect a genuine difference in the ability of these two media to support amniotic fluid cell culture, but it is more likely to be a consequence of having used cell strains adapted to growing in Ham's F10.

Although the effect of the pH of the medium on lysosomal enzyme activity has been little investigated, its affects on other aspects of metabolism have been studied. Collagen synthesis by human fibroblasts was found [15] to be greater at pH 7.4 and 8.0 than at 7.0. The effect of pH on mucopolysaccharide metabolism is perhaps of more relevance to studies involving lysosomal enzymes. The production of mucopolysaccharides by rat fibroblasts was found

[16] to be greater at pH 6.6 than pH 7.4. It was also shown [16,17] that [$^{35}$S]sulphate was incorporated differently into the various mucupolysaccharides at these pH values. In contrast, human fibroblast mucopolysaccharide content was found to increase with a change in the pH of the medium from 6.65 to 7.9 and this appeared to be due to a difference in degradation rather than synthesis [18]. This resulted in a great increase in intracellular membrane-bound bodies producing an abnormal ultrastructure in cells growing at their optimal pH. As acid phosphatase, a lysosomal marker, was shown [19] histochemically to be increased in activity at the higher pH there seems to be a paradox of increased lysosomal enzyme activity and at the same time decreased mucopolysaccharide degradation. In the present study biochemical assay of lysosomal enzyme levels did not reveal any difference between the activity in amniotic fluid cells cultured at about pH 7.8 as compared to lower pH values. However, the demonstration of enzyme activity in a cell homogenate or by histochemical techniques does not necessarily prove that the enzyme is functional in the living cell. It should be borne in mind that the changes in growth and metabolism of cells in a normal culture system which occur as the pH of the medium falls, may be the result of the accumulation of a metabolite, such as lactate [20], rather than to a pH change as such.

The lack of any changes in lysosomal enzyme activity of amniotic fluid cells due to the serum concentration, type of medium (Ham's F10, Eagle's MEM) and the pH of the medium indicates that these factors are unrelated to previously observed [2-4] fluctuations in these enzymes during amniotic fluid cell culture.

## References


1 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Chim. Acta, 44 (1973) 295
2 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Life Sci., 13 (1973) 713
3 G.R. Sutherland, J. Butterworth, D.M. Broadhead and A.D. Bain, Clin. Genet., in the press
4 J. Butterworth, G.R. Sutherland, D.M. Broadhead and A.D. Bain, Clin. Genet., in the press
5 E. Beutler, W. Kuhl, F. Trinidad, R. Teplitz and H. Nadler, Am. J. Hum. Genet., 23 (1971) 62
6 G.R. Sutherland, E. Grace and A.D. Bain, Humangenetik, 17 (1973) 273
7 R. Gardner, J. Cell. Biol., 42 (1969) 320
8 G.R. Sutherland, R. Bauld and A.D. Bain, J. Med. Genet., in the press
9 G.R. Sutherland, J. Butterworth, D.M. Broadhead and A.D. Bain, Clin. Chim. Acta, 52 (1974) 211
10 P.-D. Kittlick, G. Neupert and V. Lumkemann, Exp. Pathol., 8 (1973) 194
11 J.C. Houck and R.F. Cheng, J. Cell Physiol., 81 (1973) 257
12 D.C. Fish and J.P. Dobbs, In Vitro, 6 (1971) 441
13 C. de Luca, Expl. Cell Res., 43 (1966) 39
14 C.A. Ryan, S.Y. Lee and H.L. Nadler, Exp. Cell Res., 71 (1972) 388
15 T.P. Nigra, G.R. Martin and H. Eagle, Biochem. Biophys. Res. Commun., 53 (1973) 272
16 P.-D. Kittlick, G. Neupert and K.-J. Stiller, Exp. Pathol., 7 (1972) 109
17 P.-D. Kittlick and G. Neupert, Exp. Pathol., 7 (1972) 117
18 S.O. Lie, V.A. McKusick and E.F. Neufeld, Proc. Natl. Acad. Sci. U.S., 69 (1972) 2361
19 S.O. Lie, B.H. Schofield, H.A. Taylor and S.B. Doty, Pediat. Res., 7 (1973) 13
20 P.-D. Kittlick and G. Neupert, Exp. Pathol., 7 (1972) 125

# OBSERVATIONS ON HUMAN AMNIOTIC FLUID CELL STRAINS IN SERIAL CULTURE

BY

GRANT R. SUTHERLAND, RHONA BAULD, and A. DOUGLAS BAIN

*Reprinted from Journal of Medical Genetics*
*Volume* 11, *No.* 2, *pages* 190–195, June 1974



LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, WC1H 9JR

*Journal of Medical Genetics* (1974). **11,** 190.

# Observations on human amniotic fluid cell strains in serial culture

GRANT R. SUTHERLAND, RHONA BAULD, and A. DOUGLAS BAIN

*Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, Edinburgh EH9 1LF*

**Summary.** Observations made on 31 amniotic fluid cell strains serially cultured until senescent are recorded. The cell strains had an average life in culture of 13·9 passages (range 3–29). The source of the amniotic fluid from which the cultures were initiated did not influence the behaviour of the cell strains. The behaviour of the cell strains was unrelated to the growth characteristics of the primary cultures from which they were derived. Cell strains derived from serial samples of amniotic fluid from three women were compared and their characteristics were no more related to each other than to the group as a whole. The cell types found in amniotic fluid cultures are described. The karyology of 12 of the cell strains was monitored and no significant changes from normal diploidy were seen. Possible reasons for the highly variable and unpredictable behaviour of amniotic fluid cell strains are discussed.

Cultured amniotic fluid cells are now in widespread use for the antenatal diagnosis of chromosome disorders and inborn errors of metabolism (Emery, 1970; Sutherland, 1972; Emery, 1973; Milunsky, 1973). An accurate knowledge of the behaviour of normal cultured amniotic fluid cell strains is therefore essential for their use in genetic studies. However, little appears to be known about the characteristics of these cells in tissue culture. It has been stated (Melancon, Lee, and Nadler, 1971) that there are at least two morphologically distinguishable cell types in amniotic fluid cell cultures—an epithelial type which cannot be subcultured more than two to five times and a fibroblast-like cell which can be cultured for more than 30 passages. On the other hand, Littlefield (1971) has said that '. . . 6 to 8 weeks of rapid culture can approach the limit of the growth potential of amniotic fluid cells . . .'. This report records observations made during the long-term culture of 31 amniotic fluid cell strains.

## Materials and Methods

Amniotic fluids were obtained from hysterotomy specimens (HS), by amniocentesis on Rhesus isoimmunized women (RhS) and by artificial rupture of membranes (ARM) for the induction of labour. The primary cultures were set up as previously described (Sutherland and Bain, 1972) and subcultured according to the method of Butterworth *et al* (1973). The cell strains were maintained in 250-ml glass vessels with a growth surface area of 40 $cm^2$ and regularly subcultured on reaching confluency, using a 1:2 subculture ratio. Throughout this study, all cell strains were cultured in Ham's F10 tissue culture medium supplemented with 30% fetal calf serum and with the antibiotics kanamycin, or penicillin and streptomycin in combination. The methods used for cytogenetic studies on primary cultures have been described (Sutherland, Grace, and Bain, 1973b). Once cell strains became established, chromosome preparations were made using standard methods. Cell strains were stored in 10% dimethyl sulphoxide in culture medium in the vapour phase of a liquid nitrogen unit.

## Results

The 31 cell strains were derived from amniotic fluid samples taken from 25 women, ie, one woman contributed four samples, another three, and another two at different stages of pregnancy. The gestational ages, means of collection, and chromosomal sexes of the amniotic fluids are shown in Table I. All had normal karyotypes with the exception of two samples from one pregnancy which had a karyotype 46,XY,t(1;12)(p21;q21).

Received 5 November 1973.

TABLE I

GESTATIONAL AGES, ORIGINS AND CHROMOSOMAL SEX OF THE 31 AMNIOTIC FLUIDS WHICH YIELDED CELL STRAINS

| Origin of Amniotic Fluid | Gestation (weeks) | Chromosomal Sex | |
|---|---|---|---|
| | | Male | Female |
| *HS (n = 8)* | | 2 | 6 |
| Mean | 16·5 | | |
| Range | 11–23 | | |
| *ARM (n = 1)* | 39 | 1 | |
| *RhS (n = 22)* | | 9 | 13 |
| Mean | 28·3 | | |
| Range | 18–35 | | |
| *Total (n = 31)* | | 12 | 19 |
| Mean | 25·6 | | |
| Range | 11–39 | | |

TABLE II

DATA ON 31 AMNIOTIC FLUID CELL STRAINS

| Origin of Amniotic Fluid | No. of Passages | Days in 1° Culture | Days as Cell Strain | Mean Interval between Subculture |
|---|---|---|---|---|
| *HS (n = 8)* | | | | |
| Mean | 12·0 | 27·37 | 76·63 | 6·38 |
| Range | 4–20 | 18–29 | 19–135 | 4·8–9·6 |
| *ARM (n = 1)* | 11 | 27 | 111 | 10·1 |
| *RhS (n = 22)* | | | | |
| Mean | 14·77 | 25·91 | 96·63 | 7·36 |
| Range | 3–29 | 19–41 | 31–146 | 3·0–12·0 |
| *Total (n = 31)* | | | | |
| Mean | 13·90 | 26·32 | 91·94 | 7·19 |
| Range | 3–29 | 18–41 | 19–146 | 3·0–12·0 |

Some data on the cell strains are shown in Table II. The differences between the HS and RhS are not significant for any of these parameters. The number of passages is the number of times a cell strain could be subcultured, using a 1:2 subcultivation ratio, before the cell strain died. Death of a cell strain was defined as the point at which it would no longer grow to confluency after subculture. The time as a cell strain was the interval between subculturing the primary culture and the death of the cell strain. The mean interval between subcultures was the time as a cell strain divided by the number of passages till death, and was a crude estimator of the overall rate of growth of a cell strain. The distribution of the number of passages is shown in Table III.

The data were examined to see if any of the parameters of the amniotic fluid were related to the behaviour of the cell strain in culture. There was no correlation between gestational age and the following: time in primary culture, the number of passages

TABLE III

THE DISTRIBUTION OF THE NUMBER OF PASSAGES FOR WHICH THE 31 AMNIOTIC FLUID CELL STRAINS COULD BE CULTURED UNTIL DEATH

| No. of passages | 0–4 | 5–8 | 9–12 | 13–16 | 17–20 | 21–24 | > 25 |
|---|---|---|---|---|---|---|---|
| No. of cell strains | 3 | 3 | 8 | 7 | 5 | 3 | 2 |

to which a cell strain could be cultured or the mean interval between subcultures. Similarly, the number of passages was not correlated with the time in primary culture. There was, however, a significant ($p < 0{\cdot}01$) correlation ($r = -0{\cdot}550$) between mean subculture interval and the number of passages (Fig. 1).

All cell strains which survived more than five or six passages were stored in the liquid nitrogen unit. Thirteen strains have been retrieved and then cultured to senescence. The mean number of passages before death of the 13 strains was 14·5 (range 5–25) and after storage the mean was 13·4 (range 5–24). Most of the retrieved cell strains died within $\pm 3$ passages of the corresponding strain which had not been stored. The greatest difference was one strain which survived for 17 passages before storage and for only 12 passages after being stored at the fourth passage.

When more than one amniotic fluid sample was collected at different stages from a single pregnancy it was possible to compare the cell strains derived from these samples. Some data relating to three such groups of cell strains are shown in Table IV.

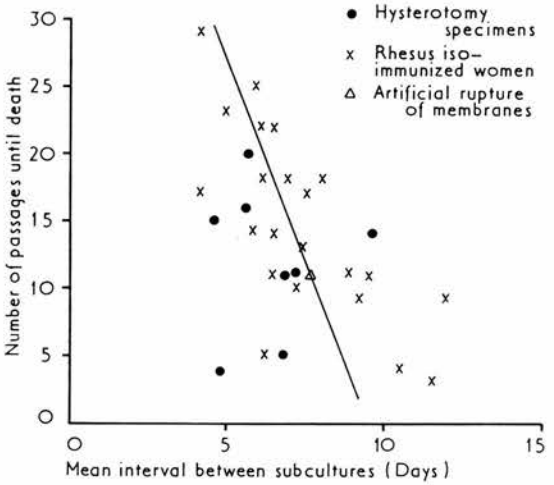



FIG. 1. Relationship between the number of passages for which cell strains could be cultured before death and mean interval between passages. ($r = -0{\cdot}550$.)

TABLE IV

DATA ON 9 AMNIOTIC FLUID CELL STRAINS COLLECTED FROM THREE PREGNANCIES

| Group | Chromosomal Sex | Gestation (weeks) | Days in 1° Culture | No. of Passages | Days as Cell Strain | Mean Interval between Subcultures |
|---|---|---|---|---|---|---|
| 1* | Male | 22 | 28 | 11 | 106 | 9·7 |
| | | 26 | 41 | 4 | 42 | 10·5 |
| 2 | Female | 18 | 28 | 18 | 144 | 7·8 |
| | | 22 | 24 | 8 | 108 | 12·0 |
| | | 24 | 20 | 15 | 110 | 7·3 |
| | | 35 | 36 | 17 | 69 | 4·1 |
| 3 | Female | 23 | 26 | 18 | 125 | 6·9 |
| | | 26 | 26 | 22 | 129 | 5·9 |
| | | 28 | 19 | 10 | 71 | 7·1 |

* This group has a constitutional chromosome abnormality (see text).

The cell strains in each group do not show uniform behaviour, they vary as greatly as cell strains derived from different pregnancies. This may indicate that the genetic make-up of the cells is not responsible for the variable behaviour of the cell strains.

The cell morphology was monitored throughout the period of culture by direct observation under the inverted microscope and by the examination of Giemsa-stained monolayers grown on flying coverslips. There were five readily recognizable cell types.

**1. Macrophages** (Fig. 2). These were visible the day after primary cultures had been set up. They appear to divide in culture but cannot be subcultured. They degenerate and are overgrown by other cell types. Very few macrophages are seen in amniotic fluid cell cultures except when the amniotic fluid comes from an anencephalic pregnancy, when they are present in considerable numbers (Sutherland, Brock, and Scrimgeour, 1973a).

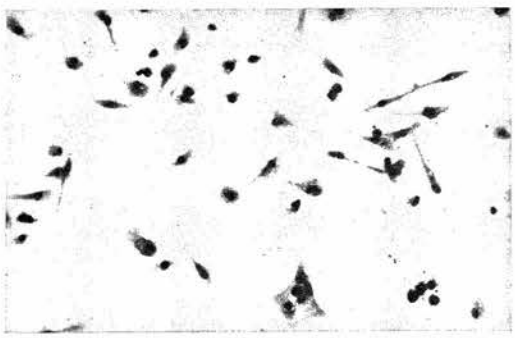


FIG. 2. Macrophages from anencephalic amniotic fluid after 20 hours in culture. (Giemsa stain, × 200.)

**2. Epithelioid Type I Cells** (Fig. 3) which grow in 'islands' in primary culture. These cells can apparently be subcultured but lose their tendency to grow in 'islands' after subculture.

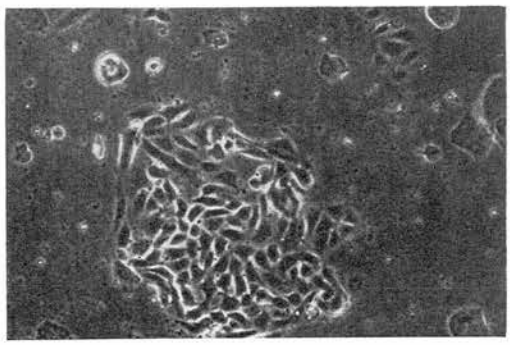


FIG. 3. Epithelioid type I cells after 18 days in primary culture. (Phase contrast, × 200.)

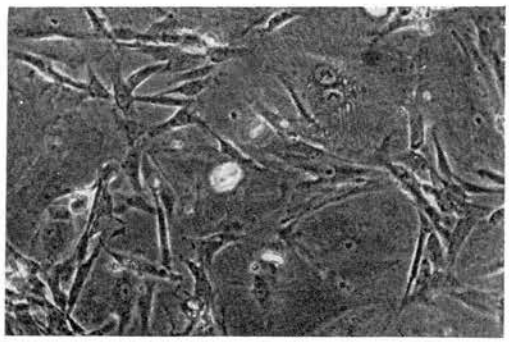


FIG. 4. Epithelioid type II cells after 23 days in primary culture. There are fibroblast-like cells growing between the epithelioid cells. Note one binucleate epithelioid cell and the fibrillar nature of the cytoplasm. (Phase contrast, × 200.)

**3. Epithelioid Type II Cells** (Fig. 4). These are very large cells with much cytoplasm and sometimes with more than one nucleus. Under phase contrast the cytoplasm of these cells has a fibrillar appearance. These cells can be subcultured at least 20 times. Almost all primary cultures of amniotic fluid contain these cells.

**4. Epithelioid Type III Cells** (Fig. 5) which morphologically resemble type I except that they do not grow in islands in primary cultures. In their growth patterns these cells show some of the features of the next cell type. These cells can be readily subcultured.

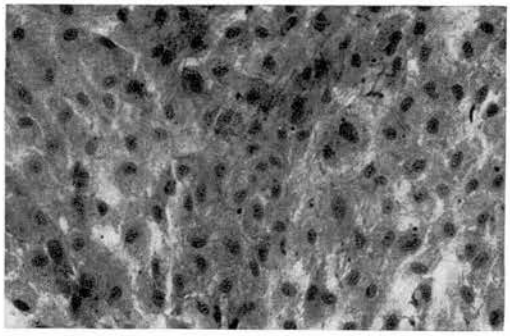


FIG. 5. Epithelioid type III cells in the twenty-second passage of culture. (Giemsa stain, × 200.)

**5. Fibroblast-like Cells** (Fig. 6) which can be readily subcultured. These are morphologically indistinguishable from fibroblast-like cells cultured from human embryonic lung or skin biopsy.

Most cell strains were a mixture of cell types throughout their life in culture. However, in some cases one type or the other predominated. In this series there was a tendency for the more fibroblastic cultures to live longer; however, some died after less than 10 passages whereas strains in which epithelioid cells predominated lived for more than 20 passages.

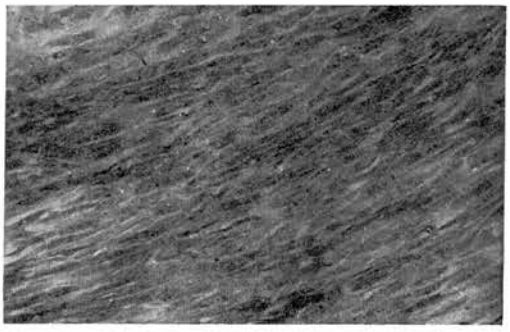


FIG. 6. Fibroblast-like cells in the fourth passage of culture. (Giemsa stain, × 200.)

The karyology of 12 of the cell strains was monitored to within at least three passages before cell death. At least 10 metaphases per cell strain were examined at this stage and no departure from normal diploid complements was seen, except for that present in two of the cell strains as a constitutional abnormality.

All except five cell strains were tested for mycoplasma contamination on at least one occasion near the end of their life in culture. All cell strains tested were reported free of mycoplasmas.

## Discussion

It is apparent that amniotic fluid cell strains have a highly variable yet finite life-span in culture. In the series reported here senescent strains and actively proliferating strains were in culture at the same time, receiving the same culture medium and being kept under apparently identical conditions. This would suggest that culture factors were not responsible for the variation. The longest surviving cell strain in this series survived 29 subcultures. This, however, is not the upper limit of life of an amniotic fluid cell strain; Melancon *et al* (1971) have reported that some can be cultured for more than 30 passages, and other cell strains since this series was completed have survived more than 50 passages.

The reasons for the variability in the life of the cell strains remain unclear. In all cases they are derived from fetal tissue and as the survival in culture was not correlated with gestational age, it is unlikely that the variable age of the cells which initiate the primary culture is the reason. Martin, Sprague, and Epstein (1970) have shown that the life span of fibroblast-like cells in tissue culture depends on the tissue used to establish the culture. Skin-derived fibroblasts survived longer than those derived from muscle, testis, or bone marrow spicules. Although the tissue of origin of the cells in amniotic fluid is uncertain they probably come from more than one source (Sutherland and Bain, 1972). Some of the variability seen could possibly be due to different samples of amniotic fluid having different proportions of cells from each possible source, each with a different survival potential in tissue culture.

As there are only a relatively small number of points of outgrowth in the primary culture the number of cell doublings would be much higher than the

number of passages would imply. This may well be the reason why amniotic fluid cell strains appear to have a shorter life in culture than cell strains grown from other fetal tissues. However, if this was the reason for the variation in the length of life in culture, a correlation between number of passages till death and time in primary culture would be expected: this was not found. The fact that the life in culture is finite is not surprising as Hayflick (1965) has shown that human diploid cells have a limited life span of $50 \pm 10$ passages when grown from fetal tissue.

The finding that the faster growing cell strains live longer is unexpected. This has not been previously reported for human diploid cell strains and the reasons for it remain unknown. Hay (1970) has in fact reported the exact opposite effect for chick embryo fibroblasts.

The variability in life-span of the amniotic fluid cell strains is very like that described by Swim and Parker (1957) for fibroblast-like cell strains. These workers found that the number of times they could subculture cell strains grown from neonatal foreskins ranged from 10 to 34 with a mean of 24. Cell strains grown from other tissues, including human embryonic lung, survived from two to 32 subcultures. They attributed this variability to inadequate tissue culture medium. Perhaps the same explanation may apply to the amniotic fluid cell strains. Using medium similar to that used in the present series but with only 15% fetal calf serum supplement, Nadler and Gerbie (1970) were able to subculture only 75% of 155 successful primary amniotic fluid cell cultures more than three times. Sutherland and Bain (1973) found that 90% of 62 primary cultures could be subcultured to yield cell strains which could be maintained for at least two subcultures.

The findings in the groups of cell strains grown from serial samples of amniotic fluid are not surprising. As none of the properties studied are related to gestation there is no reason why these cell strains should show either constant features or any trends within the groups. It would be of interest to culture a number of cell strains from a single amniotic fluid sample and to see if they showed the same degree of variation as seen in these groups.

Melancon *et al* (1971) suggested that cell type may influence length of life in culture. If cell type did affect life in culture then the number of passages till death would be expected to show a multimodal distribution. In this series the distribution of the number of passages is fairly evenly around the mean.

The variety of cell types present in amniotic fluid has received little attention. Gerbie *et al* (1972) have said that amniotic fluid cell strains are composed of two major cell types, epithelial-like cells and fibroblast-like cells. Melancon *et al* (1971) have commented that the epithelial-like cells are difficult to subculture and can be maintained for only two to five passages. It is recognized that medium composition can alter cell morphology (eg, Griffiths, 1973). In this regard it should be noted that for this series the medium was prepared in large batches and that the various cell types not only co-existed in the same culture vessel but cell strains of predominantly different morphologies were being cultured concurrently. The only comprehensive report of the various cell types is that of Uhlendorf (1970). He has described fibroblast-like cells and epithelial-like cells corresponding to types II and III of this report. The 'small fusiform cells' which he describes probably correspond to the type I epithelioid cells of this report. When the islands in which these cells grow become large, the cells in the centre of the island become crowded and take on an appearance very similar to that shown in Uhlendorf's Fig. 2. In addition, Uhlendorf described a cell type not recognized in the present series (Fig. 5 in Uhlendorf, 1970).

It is clear that amniotic fluid cell strains are composed of a number of different cell types and that some, if not all, of these can persist as long as the cell strain survives. If the various tissue culture media in general use and the variety of conditions under which these cells can be cultured had the effect of selecting against any particular cell type then this could explain the apparent differences reported in the behaviour of amniotic fluid cell strains. The very act of subculturing may in fact be such a selective mechanism as the fibroblast-like cells are more readily removed from the growth surface than epithelial-like ones. Melancon *et al* (1971) have indeed used this feature to select cell strains of different morphology.

The findings of normal karyology in the cell strains monitored until senescence is not surprising. The types of chromosome changes which have been described in senescent human diploid fibroblast-like cells (Saksela and Moorhead, 1963) would not have been demonstrated due to the small number of cells examined but any major departure from euploidy would have been detected. No cell lines or cell strains other than those derived from amniotic fluid or urine (Sutherland and Bain, 1972) were handled in the laboratory during the time this work was in progress.

The main question posed by this work is, why cannot all amniotic fluid cell strains be maintained

for the same length of time as cell strains derived from other fetal tissues? One reason is probably the low cell number used to initiate cultures but this is only part of the answer. The reasons for the variability in life expectancy of these cell strains remain speculative. The factors which prevent these cultures from having a prolonged existence should be identified. The loss of valuable amniotic fluid cell strains at an early passage is a hindrance to building up adequate controls for the antenatal diagnosis of genetic disease using cultured amniotic fluid cells.

We are grateful to Professor A. E. H. Emery for helpful criticisms during the preparation of this report, and to Dr S. Stewart for the Mycoplasma testing. This work was supported in part by the Scottish Advisory Committee on Medical Research and the Secretary of State for Scotland.

## References

Butterworth, J., Sutherland, G. R., Broadhead, D. M., and Bain, A. D. (1973). Lysosomal enzymes of cultured amniotic fluid cells. *Clinica Chimica Acta*, **44,** 295–299.

Emery, A. E. H. (1970). *Modern Trends in Human Genetics*, vol. 1. Butterworth, London.

Emery, A. E. H. (1973). *Antenatal Diagnosis of Genetic Disease*. Churchill Livingstone, Edinburgh and London.

Gerbie, A. B., Melancon, S. B., Ryan, C., and Nadler, H. L. (1972). Cultivated epithelial-like cells and fibroblasts from amniotic fluid: their relationship to enzymatic and cytologic analysis. *American Journal of Obstetrics and Gynecology*, **114,** 314–320.

Griffiths, J. B. (1973). The effects of adapting human diploid cells to grow in glutamic acid media on cell morphology, growth and metabolism. *Journal of Cell Science*, **12,** 617–629.

Hay, R. J. (1970). Cell strain senescence in vitro: cell culture anomaly or an expression of a fundamental inability of normal cells to survive and proliferate. In *Aging in Cell and Tissue Culture*, ed. by E. Holečková and V. J. Cristofalo, pp. 7–24. Plenum Press, New York.

Hayflick, L. (1965). The limited *in vitro* lifetime of human diploid cell strains. *Experimental Cell Research*, **37,** 614–636.

Littlefield, J. W. (1971). Problems in the use of cultured amniotic fluid cells for biochemical diagnoses. *Birth Defects: Original Article Series*, **7,** pt. 5, pp. 15–17. National Foundation—March of Dimes, New York.

Martin, G. M., Sprague, C. A., and Epstein, C. J. (1970). Replicative life-span of cultivated human cells. Effects of donor's age, tissue and genotype. *Laboratory Investigation*, **23,** 86–92.

Melancon, S. B., Lee, S. Y., and Nadler, H. L. (1971). Histidase activity in cultivated human amniotic fluid cells. *Science*, **173,** 627–628.

Milunsky, A. (1973). The prenatal diagnosis of hereditary disorders. Charles C. Thomas, Springfield.

Nadler, H. L. and Gerbie, A. B. (1970). Role of amniocentesis in the intrauterine detection of genetic disorders. *The New England Journal of Medicine*, **282,** 596–599.

Saksela, E., Moorhead, P. S. (1963). Aneuploidy in the degenerative phase of serial cultivation of human cell strains. *Proceedings of the National Academy of Sciences, U.S.A.*, **50,** 390–395.

Sutherland, G. R. (1972). The role of amniocentesis in genetic counselling. *Australian Journal of Mental Retardation*, **2,** 85–92.

Sutherland, G. R. and Bain, A. D. (1972). Culture of cells from the urine of newborn children. *Nature*, **239,** 231.

Sutherland, G. R. and Bain, A. D. (1973). Antenatal diagnosis of inborn errors of metabolism: Tissue culture aspects. *Humangenetik*, **20,** 251–255.

Sutherland, G. R., Brock, D. J. H., and Scrimgeour, J. B. (1973a). Amniotic-fluid macrophages and anencephaly. *Lancet*, **2.** 1098–1099.

Sutherland, G. R., Grace, E., and Bain, A. D. (1973b). Metaphase chromosomes from neonatal urine. *Humangenetik*, **17,** 273–275.

Swim, H. E. and Parker, R. F. (1957). Culture characteristics of human fibroblasts propagated serially. *American Journal of Hygiene*, **66,** 235–243.

Uhlendorf, B. W. (1970). Use of amniotic fluid and reliability of diagnostic procedures. In *Early Diagnosis of Human Genetic Defects*, ed. by M. Harris. Fogarty International Center Proceedings No. 6, pp. 149–168. Government Printing Office, Washington.

# AMNIOTIC FLUID MACROPHAGES AND THE ANTENATAL DIAGNOSIS OF ANENCEPHALY AND SPINA BIFIDA

BY

GRANT R. SUTHERLAND, D. J. H. BROCK, and J. B. SCRIMGEOUR

*Reprinted from Journal of Medical Genetics*
*Volume* 12, *No.* 2, *pages* 135–137, June, 1975



LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, WC1H 9JR

*Journal of Medical Genetics* (1975). **12,** 135.

# Amniotic fluid macrophages and the antenatal diagnosis of anencephaly and spina bifida

GRANT R. SUTHERLAND,* D. J. H. BROCK, and J. B. SCRIMGEOUR

*Department of Pathology, Royal Hospital for Sick Children and University of Edinburgh, and the Departments of Human Genetics and Obstetrics and Gynaecology, University of Edinburgh*

**Summary.** The macrophage content of amniotic fluid has been measured and the upper limit of normal on an arbitrary scale is 41. Amongst 65 amniotic fluids collected for antenatal diagnostic studies before 22 weeks' gestation there were eight which had macrophage counts ranging from 82 to 6226, three of these were shown to have anencephaly and two spina bifida. The reasons for three apparently false positives are as yet undetermined. Rhesus iso-immunized amniotic fluids were found to have macrophage counts of up to 276 and a possible explanation for this is considered. It is argued that an elevated amniotic fluid macrophage count may indicate a CNS defect or possibly other fetal abnormality.

The finding by Sutherland, Brock, and Scrimgeour (1973) of large numbers of macrophages in the amniotic fluid from two cases of anencephaly has been used by Nelson, Ruttiman, and Brock (1974) to make the antenatal diagnosis of a further case. The macrophage content of spina bifida and anencephalic amniotic fluids has been quantitated and compared with that from normal pregnancies.

## Materials and methods

Amniotic fluids were obtained by amniocentesis for antenatal chromosome studies, α-fetoprotein estimation, during the management of Rhesus iso-immunized pregnancies and at induction of labour in cases where the fetus was known to have anencephaly.

Amniotic fluid cultures were established as previously described (Sutherland, Grace, and Bain, 1973). The culture vessel was a 50 mm Petri dish which contained five or six 6 × 22 mm glass coverslips. The culture medium was Ham's F10 supplemented with 30% fetal calf serum. The day after cultures had been set up (usually about 20 hours later) one of the coverslips was aseptically removed from the Petri dish, washed well in phosphate buffered saline, fixed with methanol, stained with Giemsa, and mounted on a slide. The number of glass adherent cells in 10 low power fields (× 100) were counted and this number was adjusted to relate to 10 ml of amniotic fluid, ie, if the cells from 5 ml of amniotic fluid had been inoculated into the Petri dish then the number was multiplied by two. The resultant macrophage count (glass adherent cells/10 low power fields/10 ml of amniotic fluid) has been used to compare different samples of amniotic fluid. Amniotic fluid α-fetoprotein was measured according to Brock and Sutcliffe (1972).

Received 21 June 1974.

* Present address and reprint requests to: Cytogenetics Unit, Department of Pathology, The Adelaide Children's Hospital, North Adelaide 5006, Australia.

## Results

The macrophage counts for the 94 fluids studied are shown in Fig. 1. All the fluids classed as 'normal' had low macrophage counts (range 0–41), and there was no reason to suspect fetal CNS abnormality either because the infant had been delivered or because the amniotic fluid α-fetoprotein levels were not elevated. Four amniotic fluids from cases of hydramnios of unknown aetiology had macrophage counts within the normal range. A number of samples of amniotic fluid showing different degrees of blood staining had normal macrophage counts. Rhesus iso-immunized amniotic fluids had highly variable macrophage counts ranging up to 276. Where serial samples were obtained from these pregnancies the macrophage counts tended to decrease as gestation progressed.

Details of the cases with anencephaly or spina bifida are shown in Table I. In all except one of these fluids the macrophage count was elevated,

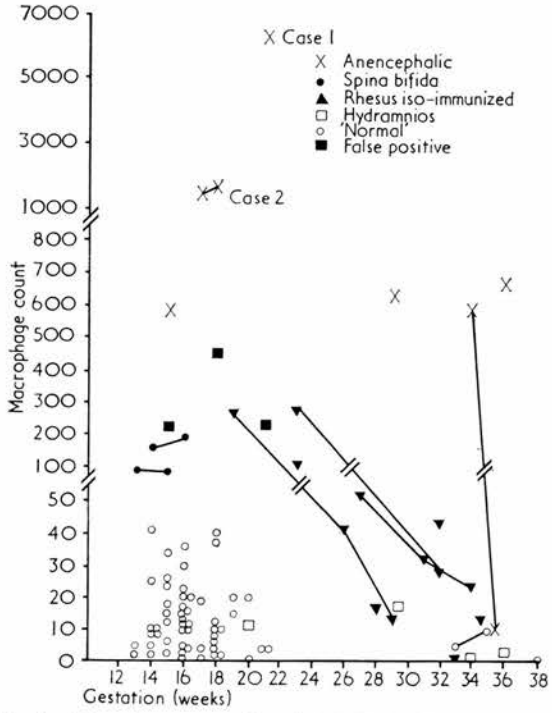



FIG. 1. Macrophage counts of amniotic fluids in this study in relation to gestation. Cases 1 and 2 have been previously reported (Sutherland *et al*, 1973a).

TABLE I

DATA PERTAINING TO EIGHT CASES WITH CNS ABNORMALITIES IN THIS STUDY

| Case No. | Gestation (weeks) | Macrophage Count | α-Fetoprotein (μg/ml) | CNS Lesion* |
|---|---|---|---|---|
| 1 | 21 | 6226 | 238 | ASB |
| 2 | 17 | 1540 | 254 | ASB |
| | 18 | 1640 | 250 | |
| 3 | 36 | 660 | 6.4 | ASB |
| 4 | 33 | 590 | 26 | ASB |
| | 35 | 10 | 21 | |
| 5 | 29 | 630 | 44 | ASB |
| 6 | 15 | 576 | 680 | ASB |
| 7 | 14 | 168 | 181 | SB |
| | 16 | 189 | 173 | |
| 8 | 13 | 82 | 143 | SB |
| | 15 | 84 | 118 | |

* ASB = anencephaly and spina bifida; SB = spina bifida.

ranging from 82 to 189 for the spina bifida fluids and from 576 to 6226 for the anencephalics. The fluid in which the macrophage count was not elevated was the second sample received from a twin pregnancy in which one of the twins was normal and the other anencephalic. Details of this case are shown in Table II.

There are three cases, listed as 'false positive' in Fig. 1 which are a problem in that they had elevated macrophage counts and normal α-fetoprotein

TABLE II

DATA ON AMNIOTIC FLUIDS FROM THE TWIN PREGNANCY IN WHICH ONE TWIN WAS ANENCEPHALIC

| Twin | Gestation (weeks) | α-feto-protein (μg/ml) | Macrophage Count | Chromo-somal Sex |
|---|---|---|---|---|
| I (anen-cephalic) | 33 | 26 | 590 | Female |
| | 35 | 21 | 10 | Female |
| II (normal) | 33 | 1 | 6 | Male |
| | 35 | 3.5 | 10 | Male |

levels. These pregnancies were allowed to continue and the one which had the highest macrophage count went into premature labour at 33 weeks' gestation and resulted in a normal male infant. The placenta showed multiple recurrent infarctions, there was a small succenturiate lobe and the cord had many false knots. The two other similar pregnancies are continuing.*

The possibility that interfering with the amniotic fluid cultures on the day after they have been set up may affect their success was tested by comparing the times required to achieve cytogenetic results from the first 25 normal cultures on which macrophage counts were performed (mean 13.0 days) with the 20 similar cultures studied before macrophage counts were being done routinely (14.5 days). In all cases where a cytogenetic result was required after macrophage counts had been performed this was achieved. It was not possible to differentiate between amniotic fluids with normal and raised macrophage counts (except in those cases where the macrophage count was grossly elevated) by direct inspection of the cultures with the inverted microscope. Quantitation of stained material was necessary for this purpose and its preparation did not prejudice the success of the cultures in any way.

## Discussion

The antenatal diagnosis of anencephaly and spina bifida can be achieved with a high degree of success using amniotic fluid α-fetoprotein measurements (*Lancet*, 1974). Ideally all samples of amniotic fluid, collected for any reason before mid-pregnancy, should have α-fetoprotein estimations carried out. The two cases of spina bifida in this report were diagnosed from amniotic fluids collected primarily for antenatal chromosome studies.

The nature and origin of the glass-adherent cells in the amniotic fluid must be considered before their clinical relevance is discussed. All the glass-adherent cells in amniotic fluid are probably not macrophages but erythrophagocytic properties

* These subsequently led to the birth of full-term normal infants.

(Sutherland *et al*, 1973a) and ultrastructural studies (G. R. Sutherland, unpublished observations) have shown that at least a proportion of them are. In the cases where there is an open CNS lesion there are two likely sources of the macrophages. The CSF is in free communication with the amniotic fluid and the CSF is known to contain macrophages (Greenfield and Carmichael, 1925; Chester, Penny, and Emery, 1971). In these cases with open lesions there is considerable exposure of highly vascularized tissue, with only a membrane between the fetal circulation and the amniotic fluid; this should present little hindrance to the passage of circulating macrophages into the amniotic fluid. In the absence of a fetal lesion the origin of the macrophages is unclear, however cytological (Casadei *et al*, 1973) and ultrastructural (Hoyes, 1968) studies of normal amniotic fluid have revealed the presence of macrophages. The finding of increased macrophage counts in Rhesus iso-immunized amniotic fluids is of interest since an increase in placental macrophages (Hofbauer cells) has been described in this condition (Fox, 1967). In the Rhesus iso-immunized fetus there is an increase in extramedullary haemopoiesis (Morison, 1961), hence it is possible that an increase in circulating macrophages could be responsible for the increase in both the Hofbauer cells and the amniotic fluid macrophages. The mechanism of escape of the macrophages into the amniotic fluid is unknown but migration via the lungs and kidney tubules are possibilities.

There are a number of conditions which might lead to elevated amniotic fluid macrophage counts. Such conditions could include any fetal lesion which is not covered with skin, apart from the CNS defects exomphalos is one possibility. Conditions other than Rhesus iso-immunization in which there is increased extramedullary haemopoiesis such as the chondrodystrophies (Potter, 1961) and intra-uterine infection (Bain *et al*, 1956) might be expected to show an increase in amniotic fluid macrophages.

Macrophage counts would appear to be of value in two circumstances. Firstly, as an independent confirmation of an antenatal diagnosis based on $\alpha$-fetoprotein levels; this may be particularly helpful if $\alpha$-fetoprotein levels are equivocal. Second, where $\alpha$-fetoprotein is not being routinely measured on amniotic fluids which are collected for other purposes a raised macrophage count would be a strong indication that $\alpha$-fetoprotein levels should be estimated. There have been no false negative results in this series except for the unaccountable finding from the second sample from the twin pregnancy. Hence it appears unlikely that the macrophage count would be normal in cases of open fetal CNS defects. The main problem appears to be false positive results, the proportion of which will depend on the local incidence of CNS lesions. In the present series there were eight out of 65 amniotic fluids of less than 22 weeks' gestation examined blindly which had elevated macrophage counts and five of these proved to have open CNS lesions. Perhaps macrophage counts greater than 500 will prove to be diagnostic of anencephaly but at present it would be unwise to diagnose fetal abnormality on the basis of a raised macrophage count alone.

Further studies of the macrophages in amniotic fluid are required to assess fully the usefulness of this parameter in antenatal diagnosis. The false positives require special attention to try and determine whether they are of any significance. It may well be that a raised amniotic fluid macrophage count is a non-specific indication that all is not well with the fetus.

We would like to thank the obstetricians who provided some of the samples of amniotic fluid included in this study, especially Drs M. J. Butterworth, G. Gordon, A. M. Khan, and Professor J. S. Scott; Dr J. G. Robertson provided the Rhesus iso-immunized samples; Miss Sandra Brown assisted with the $\alpha$-fetoprotein determinations. We are grateful to Dr A. D. Bain for his helpful criticism during the preparation of this report. D.J.H.B. acknowledges support from the Association for Spina Bifida and Hydrocephalus.

## References

Bain, A. D., Bowie, J. H., Flint, W. F., Beverley, J. K. A., Beattie, C. P. (1956). Congenital toxoplasmosis simulating haemolytic disease of the newborn. *Journal of Obstetrics and Gynaecology of the British Empire*, **63**, 826–832.

Brock, D. J. H. and Sutcliffe, R. G. (1972). Alpha-fetoprotein in the antenatal diagnosis of anencephaly and spina bifida. *Lancet*, **2**, 197–199.

Casadei, R., D'Ablaing, G., Kaplan, B. J., Schwinn, C. P. (1973). A cytologic study of amniotic fluid. *Acta Cytologica*, **17**, 289–298.

Chester, D. C., Penny, S. R., and Emery, J. L. (1971). Fat-containing macrophages in the cerebrospinal fluid of children with hydrocephalus. *Developmental Medicine and Child Neurology*, **13**, Suppl. No. 25, 33–39.

Fox, H. (1967). The incidence and significance of Hofbauer cells in the mature human placenta. *Journal of Pathology and Bacteriology*, **93**, 710–717.

Greenfield, J. G. and Carmichael, E. A. (1925). *The Cerebro-Spinal Fluid in Clinical Diagnosis*. MacMillan, London.

Hoyes, A. D. (1968). Ultrastructure of the cells of the amniotic fluid. *Journal of Obstetrics and Gynaecology of the British Commonwealth*, **75**, 164–171.

*Lancet* (1974). Towards the prevention of spina bifida. (Leader.) *Lancet*, **1**, 907.

Morison, J. E. (1961). *Foetal and Neonatal Pathology*. Butterworth, London.

Nelson, M. M., Ruttiman, M. T., and Brock, D. J. H. (1974). Predictive value of amniotic-fluid macrophages in gross C.N.S. defects. *Lancet*, **1**, 504.

Potter, E. L. (1961). *Pathology of the Fetus and Infant*, 2nd edition. Year book Medical, Chicago.

Sutherland, G. R., Brock, D. J. H., and Scrimgeour, J. B. (1973a) Amniotic fluid macrophages and anencephaly. *Lancet*, **2**, 1098–1099.

Sutherland, G. R., Grace, E., and Bain, A. D. (1973b). Metaphase chromosomes from neonatal urine. *Humangenetik*, **17**, 273–275.

*Clinical Genetics* 1975: **7**: 400–404

# Chromosomal mosaicism in amniotic fluid cell cultures

GRANT R. SUTHERLAND*, SUSAN M. BOWSER-RILEY AND A. DOUGLAS BAIN

Department of Pathology, Royal Hospital for Sick Children, and University of Edinburgh, Edinburgh, Scotland

Six cases of chromosomal mosaicism detected in amniotic fluid cultures are described. In five of these there was no evidence of fetal mosaicism. In one case fetal mosaicism was demonstrated but only by the study of fibroblasts since blood cultures showed only normal cells. The implications of amniotic fluid mosaicism are discussed and it is concluded that this usually does not indicate fetal mosaicism. The value of repeated amniocentesis in the diagnosis of fetal mosaicism was demonstrated by findings in three of the cases. It is recommended that amniotic fluid cultures be harvested *in situ* for chromosome studies and that cytogenetic results be expressed as number of colonies karyotyped rather than as number of cells analyzed.

*Received 27 November 1974, accepted for publication 24 January 1975*

The occurrence of chromosomal mosaicism in amniotic fluid cell cultures has been reported by several authors (Kajii 1971, Lee et al. 1972, Hsu et al. 1973, Cox et al. 1974) but only once has this been shown to reflect the fetal chromosome constitution (Bloom et al. 1974). Six further cases of mosaicism in amniotic fluid cell culture are described; however, in only one of these was fetal mosaicism demonstrated.

## Materials and Methods

Amniotic fluids collected by transabdominal amniocentesis, for a variety of reasons, are studied in this laboratory. The culture methods and chromosome harvesting techniques have been previously described (Sutherland et al. 1973). The culture vessel was a 50 mm plastic Petri dish containing a number of 6 × 22 mm glass coverslips. When coverslips were ready for harvesting they were removed aseptically from the Petri dish, exposed to colchicine in another vessel and processed with the cells *in situ*. Hence a harvested coverslip contained the cells from one or more colonies fixed *in situ*. When the cells remaining in the Petri dish became confluent they were subcultured and chromosome preparations made using standard trypsin suspension methods. Chromosome studies on fetuses and neonates were carried out using standard methods. Fetal C.S.F. was treated similarly to amniotic fluid.

* Present address: Department of Histopathology, The Adelaide Children's Hospital, Adelaide, Australia.

### Results

Six amniotic fluid cultures have been examined which showed chromosomal mosaicism. Since each case was different they will be presented individually.

*Case 1.* Amniocentesis was carried out at 14 weeks' gestation on a 26-year-old woman who had had a child with Down's syndrome. Routine amniotic fluid α-fetoprotein estimation indicated the presence of a fetal CNS abnormality and the pregnancy was terminated (Case 7, Sutherland et al. 1975) at which time a further sample of amniotic fluid was obtained and fetal tissues were collected for fibroblast culture. The cytogenetic findings are summarized in Table 1. The fetus was found to have an open myelomeningocele but no malformations indicative of autosomal trisomy. The colonies on the first coverslip harvested were very diffuse so that it was not possible to define where one ended and the next began. The best quality metaphases had karyotype 47,XX,+D, but a few of the poorer quality cells appeared to be normal. Subsequent coverslips contained only normal cells but subculture of the remaining cells in the Petri dish produced a cell strain which was about 30 % trisomic. The pregnancies relating to all the amniotic fluid cultures in the laboratory at that time have now concluded and there has been no unexpected D-trisomic infant.

**Table 1**

Cytogenetic data pertaining to Case 1

| Culture | Karyotype 46,XX | | 47,XX,+D | |
|---|---|---|---|---|
| | Colonies | Cells | Colonies | Cells |
| First amniotic fluid: | | | | |
| first coverslip | 1* | 3 | 2* | 17 |
| subsequent coverslips | 9 | | 0 | |
| after subculture | | 19 | | 9 |
| Second amniotic fluid: | | | | |
| after subculture | | 60 | | 0 |
| Fetal tissues: | | | | |
| C.S.F. | | 37 | | 0 |
| Pericardium | | 8 | | 0 |

* see text.

**Table 2**

Cytogenetic data pertaining to Case 2

| Culture | Karyotype 46,XX | | 45,XX,−G | |
|---|---|---|---|---|
| | Colonies | Cells | Colonies | Cells |
| First amniotic fluid: | | | | |
| coverslips | 8 | | 1 | |
| after subculture | | 73 | | 7 |
| Second amniotic fluid: | | | | |
| after subculture | | 65 | | 1 |
| Fetal skin | | 30 | | 0 |

*Case 2.* Amniocentesis at 13 weeks' gestation was carried out on a 39-year-old woman because of the risk of Down's syndrome. Amniotic fluid α-fetoprotein estimation indicated the presence of a fetal CNS defect and the pregnancy was terminated (Case 8, Sutherland et al. 1975) at which time a further sample of amniotic fluid and fetal tissues were obtained for culture. One cell colony from the first sample of amniotic fluid showed karyotype 45,XX,−G. The cytogenetic findings are summarized in Table 2. The finding of one cell with a G-group chromosome missing in 66 from the second sample of amniotic fluid was not regarded as significant.

*Case 3.* Amniocentesis was carried out at 21 weeks' gestation on a 31-year-old woman who had had a child with Down's syndrome. The amniotic fluid culture showed sex chromosome mosaicism; the cytogenetic findings are summarized in Table 3. A normal male infant was born at term but it has not been possible to carry out cytogenetic

studies. The mosaicism was presumably due to maternal cellular contamination of the amniotic fluid.

*Case 4.* Amniocentesis at 16 weeks' was carried out on a 35-year-old woman because of the risk of Down's syndrome. Two colonies of cells were found with a karyotype interpreted as 45,X, the remaining colonies being of normal male karyotype. The cytogenetic data are summarized in Table 3. Cultures were tested for mycoplasma and reported not to contain these organisms. The pregnancy was allowed to continue even though the possibility that the fetus was a mosaic could not be ruled out. A phenotypically normal male infant was delivered but sufficient studies could not be carried out to exclude the possibility of mosaicism as only cord blood cultures were examined.

**Table 3**

Cytogenetic data pertaining to Cases 3, 4 and 5

| Culture | Karyotype 46,XX | | 46,XY | |
|---|---|---|---|---|
| Case 3 | Colonies | Cells | Colonies | Cells |
| Amniotic fluid: coverslips | 3 | | 2 | |
| after subculture | | 5 | | 25 |

| | 45,X | | 46,XY | |
|---|---|---|---|---|
| Case 4 | Colonies | Cells | Colonies | Cells |
| Amniotic fluid: coverslips | 2 | | 13 | |
| after subculture | | 0 | | 50 |
| Cord blood | | 0 | | 100 |

| | 46,XX | | 46,XX, t(2p−;F?+) | |
|---|---|---|---|---|
| Case 5 | Colonies | Cells | Colonies | Cells |
| Amniotic fluid: coverslips | 4 | | 1 | |
| after subculture | | 40 | | 0 |
| Cord blood | | 100 | | 0 |

**Table 4**

Cytogenetic data pertaining to Case 6

| Culture | Karyotype 46,XY | | 47,XY,+mar | |
|---|---|---|---|---|
| | Colonies | Cells | Colonies | Cells |
| Amniotic fluid: | | | | |
| 27 weeks: coverslips | 1 | | 1 | |
| 29 weeks: coverslips | 1 | | 2 | |
| 34 weeks: coverslips | 9 | | 8 | |
| Tissues: | | | | |
| Blood | | 100 | | 0 |
| Skin | | 9 | | 21 |
| Cord | | 10 | | 20 |
| Amnion | | 26 | | 4 |

*Case 5.* Amniocentesis at 13 weeks' was carried out on a 29-year-old woman who had a previous child with spina bifida. One colony of cells was found with an apparently balanced translocation t(2p−;F?+). The cytogenetic findings are summarized in Table 3. The cultures were tested for mycoplasma and reported not to contain these organisms.

*Case 6.* Amniotic fluid was collected at 27 weeks' from a 41-year-old woman due to Rhesus isoimmunization to be used for other studies in the laboratory; the chromosomes, however, were routinely checked. Since an extra small metacentric chromosome was found in the cells of one colony, further samples of amniotic fluid, collected during the management of the Rhesus isoimmunization, were studied and the mosaicism confirmed. Because of the severe Rhesus isoimmunization the pregnancy was interrupted by Caesarean section at 34 weeks' and a male infant delivered. Skin, cord,

amnion and cord blood were cultured for chromosome study. The cytogenetic findings are summarized in Table 4. The infant survives as a small boy and at 6 months of age has no detectable phenotypic abnormality which could be attributed to his karyotype.

## Discussion

The finding of mosaicism in amniotic fluid cultures causes difficulties in interpretation of the results and raises a difficult clinical problem. When there are two (or more) cell populations present in an amniotic fluid culture there are a number of possibilities: 1) twin pregnancy; 2) if one of the cell populations is female there might be mixture of fetal and maternal cells; 3) the fetus is not a mosaic but for some reason the culture shows mosaicism; or 4) the fetus is a mosaic.

The possibility of twins leading to culture mosaicism does not seem to have been realized, as there are reports of twins following amniocentesis where culture mosaicism was not evident.

When one cell population is female the question of maternal cellular contamination of the amniotic fluid sample arises. This is presumably the explanation in the case of the XX/XY mosaic in this series and in other similar cases (e.g. Macintyre 1971).

True fetal mosaicism will remain difficult to detect antenatally for two reasons. The first is the difficulty in the interpretation of chromosome mosaicism detected in amniotic fluid cultures. Repeated amniocentesis should be most helpful in this regard. In the present series, mosaicism was not apparent in the second sample of the two cases (1 and 2) in which repeat samples were examined. In the case where fetal mosaicism was present the mosaicism was seen in cultures from three samples of amniotic fluid collected at different times during pregnancy.

The second reason for the difficulty is that even if large numbers of metaphases are available for analysis these may come from a small number of colonies. The monokaryology of colonies in mosaic cultures suggests that these are clones. This suggestion is supported by the work of Uhlendorf (1970) on amniotic fluid cultures heterozygous for the Lesch-Nyhan syndrome. Cox et al. (1974) found that on average three colonies per Petri dish were analyzable cytogenetically, hence an analysis performed on two Petri dishes would yield a result based on only about six fetal cells. In this laboratory there are usually one to three colonies per coverslip available for analysis, but up to six coverslips can be harvested from a single Petri dish.

Another problem arises in either confirming or excluding fetal mosaicism by extrauterine studies once it has been diagnosed *in utero*. The only case of fetal mosaicism detected in the present series would have gone unconfirmed if only blood cultures had been examined. Some of the other fetuses in this and other series where blood was the only tissue studied may have been mosaics.

The reasons for mosaicism in amniotic fluid cultures, apart from cellular contamination, remain obscure. The possibility of a colony arising from a single aberrant cell seems most likely but in at least two of the cases in the present series more than one aberrant colony appeared to be present. It is of some interest that the six Cases presented in this report occurred within a 2-month period at the beginning of 1974; over this time about 20 % of the amniotic fluid cultures studied were mosaics. During the 3 years in which this laboratory has been karyotyping amniotic fluid cultures, no other mosaics have been detected and no cytogenetic errors, including sex determination, have been made from the study of over 200 fluids. This clustering in a short

time may be due to chance, or perhaps during this period there was some agent in the culture medium causing aneuploidy, although the fetal mosaic and the XX/XY mosaic were probably not due to influences of the culture conditions. Schneider et al. (1974) have shown that Mycoplasma contamination of cultures could cause structural rearrangements likely to lead to diagnostic errors. In the present cases no cultures were found to contain mycoplasmas when tested.

The cytogenetic analysis of amniotic fluid cultures must be approached with greater caution than that of other tissues. As Cox et al. (1974) have pointed out, the harvesting of cultures *in situ* has advantages over other methods, especially in interpreting results, if more than one cell population is detected. The expression of results as number of colonies karyotyped is more meaningful than if expressed as number of cells analyzed. In the majority of cases when more than one cell population is detected in an amniotic fluid culture the fetus will not be a mosaic. Repeat amniocentesis would be, however, essential in order to confirm this.

#### Acknowledgments

We thank Prof. J. S. Scott and Drs. F. Cockburn, J. G. Robertson and J. B. Scrimgeour for allowing us to report details of patients under their care. Mrs. Rhona Bauld assisted with some of the cytogenetics and tissue culture. This work was supported in part by grants from the Distillers Co. Ltd. and the Medical Research Fund of the Secretary of State for Scotland.

#### References

Bloom, A. D., R. Schmickel, M. Barr & A. R. Burdi (1974). Prenatal detection of autosomal mosaicism. *J. Pediat.* **84**, 732–733.

Cox, D. M., V. Niewczas-Late, M. I. Riffell & J. L. Hamerton (1974). Chromosomal mosaicism in diagnostic amniotic fluid cell cultures. *Pediat. Res.* **8**, 679–683.

Hsu, L. Y. F., E. C. Dubin, T. Kerenyi & K. Hirschhorn (1973). Results and pitfalls in prenatal cytogenetic diagnosis. *J. med. Genet.* **10**, 112–119.

Kajii, T. (1971). Pseudomosaicism in cultured amniotic-fluid cells. *Lancet* **ii**, 1037.

Lee, J. C., R. P. Bendel & D. C. Brooker (1972). Aneuploidy in cultured amniotic cells. *J. Amer. med. Ass.* **219**, 1211.

Macintyre, M. N. (1971). Chromosomal problems of intrauterine diagnosis. *Birth Defects: Original Article Series VII* No. **5**, 10–13.

Schneider, E.L., E. J. Stanbridge, C. J. Epstein, M. Golbus, G. Abbo-Halbasch & G. Rodgers (1974). Mycoplasma contamination of cultured amniotic fluid cells: potential hazard to prenatal chromosomal diagnosis. *Science* **184**, 477–479.

Sutherland, G. R., E. Grace & A. D. Bain (1973). Metaphase chromosomes from neonatal urine. *Humangenetik* **17**, 273–275.

Sutherland, G. R., D. J. H. Brock & J. B. Scrimgeour (1975). Amniotic fluid macrophages and the antenatal diagnosis of anencephaly and spina bifida. *J. med. Genet.* In press.

Uhlendorf, B. W. (1970). Use of amniotic fluid and reliability of diagnostic procedures. *Early diagnosis of Human Genetic Defects,* ed. Harris, M. Bethesda, Fogarty International Center Proceedings No. 6.

Address:
*Grant R. Sutherland*
*Department of Histopathology*
*The Adelaide Children's Hospital*
*Adelaide, S.A. 5006*
*Australia*

*Clinical Genetics* 1976: **9**: 505–507

# Lysosomal enzyme levels in human amniotic fluid cells in tissue culture

## IV. %A N-acetyl-ß-D-glucosaminidase

J. Butterworth, G. R. Sutherland, G. J. Guy, S. Bowser-Riley, and A. D. Bain

Department of Pathology, Royal Hospital for Sick Children, and University of Edinburgh, Edinburgh, Scotland

Total and % A hexosaminidase were similar for primary cultures and later passages of amniotic fluid cells. The culture variables – through serial passage, within a passage and replicate primary cultures – resulted in some variation in total hexosaminidase, but an insignificant change in % A hexosaminidase. They are unlikely to give rise to any problems in the antenatal diagnosis of Sandhoff's and Tay-Sachs diseases.

*Received 7 October, accepted for publication 22 December 1975*

Although the activity of lysosomal enzymes, including hexosaminidase (Butterworth et al. 1974), is known to fluctuate in amniotic fluid cells during culture, the extent to which % A hexosaminidase varies during culture has not previously been reported.

### Methods

To investigate the effect of passage on hexosaminidase, primary amniotic fluid (second trimester) cell strains were divided between plastic T-flasks and petri-dishes containing three 5 × 22 mm glass coverslips. At confluency the T-flasks were similarly subdivided until the tenth passage, and two of the coverslips were removed for biochemical studies and the other for morphological study at each passage. To find the effect of subculture, amniotic fluid cells were added to a petri-dish containing eleven 6 × 22 mm glass coverslips; and one was removed for assay on each successive day. To study variation in enzyme activity between primary cultures derived from the same amniotic fluid, five 6 × 22 mm glass coverslips were removed from six separate primary cultures for assay.

For cell extraction, the coverslips were washed twice with phosphate-buffered saline and placed in 0.5 ml distilled water. The cells were scraped off, the extract spun at 800 × *g* for 10 min at 5°C and stored at −65°C until assay.

The activity of hexosaminidase was estimated using 2mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch-Light Lab. Ltd.) in 0.2M phosphate / 0.1M citrate buffer pH 4.5 and related to protein (Lowry et al. 1951). The % A hexosaminidase was estimated using a DEAE-cellulose batch technique (Butterworth 1975).

### Results

At least for the four strains used, total hexosaminidase activity and % A did not show

**Table 1**

Total activity* and % A of hexosaminidase in five replicates of six primary amniotic fluid cell cultures

| Strain | 1 | | | | | 2 | | | | | 3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Replicate | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| Total activity | 62 | 62 | 44 | 61 | 72 | 57 | 38 | 59 | 51 | 42 | 51 | 55 | 58 | 68 | 62 |
| % A | 78 | 77 | 76 | 79 | 78 | 81 | 75 | 75 | 77 | 81 | 74 | 67 | 73 | 66 | 71 |

* nmol/min/mg protein

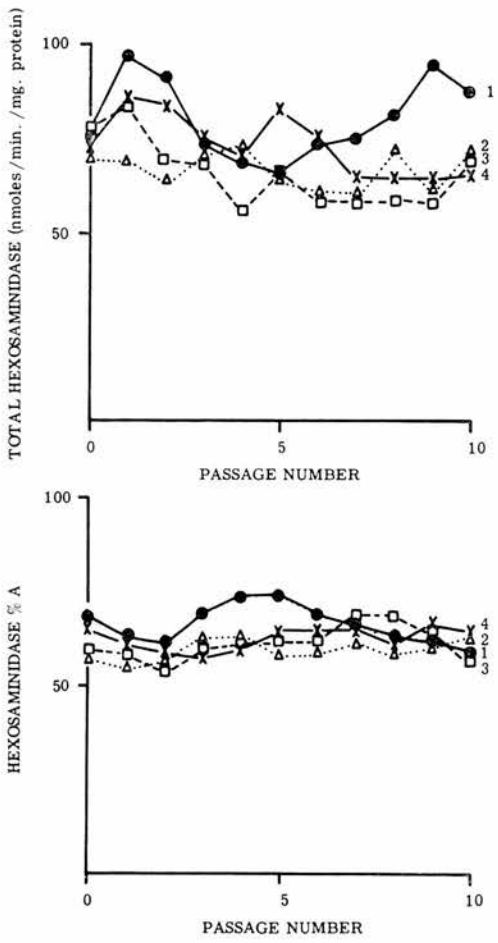



**Fig. 1.** The activity of total and % A hexosaminidase in serial passages of four strains of cultured amniotic fluid cells.

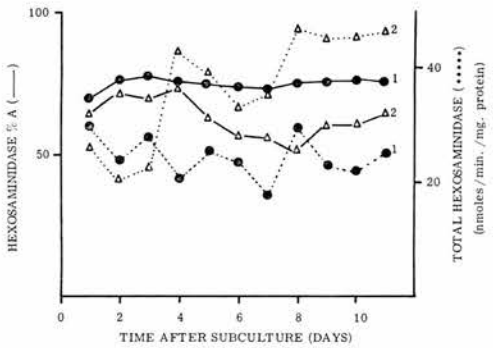



**Fig. 2.** The activity of total and % A hexosaminidase in two strains of amniotic fluid cells in relation to time since subculture.

marked variations or a consistent trend with passage (Fig. 1) and differences in morphological cell type – strains 1 and 2 were epithelioid and strains 3 and 4 fibroblastic – had little effect. The primary culture levels did not show any consistent difference from the later passages.

Although cell strain 1 (epithelioid) exhibited more fluctuations during a subculture than strain 2 (fibroblastic), the variations in total enzyme activity and % A were not marked (Fig. 2). Total hexosaminidase activity fluctuated more markedly in primary replicates than the % A which fell within a narrow range for each cell strain (Table 1).

**Table 1**
(cont.)

| Strain | 4 | | | | | 5 | | | | | 6 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Replicate | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| Total activity | 40 | 45 | 46 | 51 | 52 | 55 | 64 | 49 | 52 | 43 | 57 | 53 | 53 | 55 | 48 |
| % A | 75 | 77 | 73 | 79 | 78 | 73 | 71 | 75 | 72 | 71 | 69 | 75 | 68 | 71 | 73 |

In general, whilst fluctuations in the % A occurred in amniotic fluid cells through serial passage, within a single passage and in primary replicates, they were less than for total hexosaminidase activity.

## Discussion

The present study indicates that amniotic fluid cells cultured on glass coverslips can be used for the investigation of enzyme activity in relation to culture variables, and that replicate enzyme results can be obtained at the primary culture stage without the possibility of losing the culture at subculture. Hence an antenatal diagnosis can be undertaken in a shorter time than by conventional techniques, and the method has already been utilized to screen for Sandhoff's disease (Butterworth & Broadhead 1975).

Unlike results obtained with cultured skin fibroblasts (Okada et al. 1971, Milunsky et al. 1972, Hultberg et al. 1973), cultured amniotic fluid cells showed no consistent trend in % A within a subculture, less replicate variation and no increasing hexosaminidase activity with passage. The constancy of % A found in amniotic fluid cells with passage, within a passage and replicate culture, indicates that it is unlikely that an error due to these culture variables would occur in the antenatal diagnosis of Tay-Sachs disease.

## Acknowledgments

This work was supported by the Scottish Advisory Committee on Medical Research and the Secretary of State for Scotland.

## References

Butterworth, J., G. R. Sutherland, D. M. Broadhead & A. D. Bain (1974). Lysosomal enzyme levels in human amniotic fluid cells in tissue culture. III, β-glucuronidase, N-acetyl-β-D-glucosaminidase, α-mannosidase and acid phosphatase. *Clin. Genet.* **5**, 356–362.

Butterworth, J. (1975). Unpublished method.

Butterworth, J. & D. M. Broadhead (1975). Unpublished results.

Hultberg, B., S. Sjöblad & P. A. Öckerman (1973). Properties of five acid hydrolases in human skin fibroblast cultures. *Acta paediat. scand.* **62**, 474–480.

Lowry, O. H., N. J. Rosebrough, A. L. Farr & R. J. Randall (1951). Protein measurement with the folinphenol reagent. *J. Biol. Chem.* **193**, 265–275.

Milunsky, A., C. Spielvogel & J. N. Kanfer (1972). Lysosomal enzyme variation in cultered normal skin fibroblasts. *Life Sci.* **11**, 1101–1107.

Okada, S., M. L. Veath, J. G. Leroy & J. S. O'Brien (1971). Ganglioside $GM_2$ storage diseases: Hexosaminidase deficiencies in cultured fibroblasts. *Amer. J. hum. Genet.* **23**, 55–61.

Address:
*J. Butterworth, Ph. D.*
*Department of Pathology*
*Royal Hospital for Sick Children*
*Edinburgh, EH9 1LF*
*Scotland*

*Reprinted from* THE LANCET *March 19, 1977, p. 649-650*

## AMNIOTIC-FLUID ALPHA-FETOPROTEIN IN TURNER SYNDROME

SIR,—There have been conflicting reports about the level of amniotic-fluid α-fetoprotein (A.F.P.) when a fetus has Turner syndrome.[1,2] We have studied a pregnancy in which Turner syndrome was diagnosed antenatally. The pregnancy was terminated by hysterotomy at 20 weeks' gestation, and the intact gestation sac was removed (Dr R. Wurm) and transported to the laboratory within an hour. Cord-blood lymphocyte culture confirmed the fetal karyotype to be 45,X.

**Fetus with Turner syndrome after 30 ml of fluid had been aspirated from left cervical pouch.**

During collection of amniotic fluid from the sac one of the large fetal cervical pouches was inadvertently punctured, and 30 ml of clear amber liquid which looked like amniotic fluid was obtained without difficulty. Subsequent inspection of the cervical pouches showed a needle mark in the left pouch but little apparent decrease in size (see figure). Amniotic fluid was obtained from the sac.

Analyses of the amniotic and pouch fluids are shown in the table. The cord-blood A.F.P. was 1045 mg/l. Cells from both fluids grew in culture to show 45,X karyotypes, although only the pouch fluid had a raised macrophage-count.[3] Neither fluid was macroscopically bloodstained. The A.F.P. level in the fluid obtained at the diagnostic amniocentesis at 15 weeks' gestation was 16 mg/l (normal range 5–32 mg/l).

We suggest that the high levels of amniotic-fluid A.F.P. reported in some instances of Turner syndrome[1] may result

COMPOSITION OF AMNIOTIC AND CERVICAL-POUCH FLUID AT 20 WEEKS' GESTATION

| Component | | Amniotic fluid | Pouch fluid |
|---|---|---|---|
| Urea | (mmol/l) | 4·2 | 3·7 |
| Creatinine | (μmol/l) | 50 | 45 |
| Potassium | (mmol/l) | 4·4 | 3·6 |
| Protein | (g/l) | 9·6 | 7·8 |
| A.F.P. | (mg/l) | 10 | 572 |

from aspiration of the fetal cervical pouches at amniocentesis. The fluid obtained would macroscopically and chemically resemble amniotic fluid. Fluid from a cervical pouch may have an increased macrophage-count and A.F.P. level as in this case. There is no reason to suggest that the pouches leak[4] A.F.P. into the fluid, because they are covered with skin.

Cytogenetic Unit,
Department of Histopathology,
Adelaide Children's Hospital,
North Adelaide, South Australia 5006 — GRANT R. SUTHERLAND

Department of Chemical Pathology,
Adelaide Children's Hospital — DENISE HOLT

Genetics Research Unit,
Royal Children's Hospital Research Foundation,
Melbourne — JOHN G. ROGERS

1. Hunter, A., Hammerton, J. L., Baskett, T., Lyons, E. *Lancet*, 1976, i, 596.
2. Seller, M. J. *ibid.* p. 807.
3. Sutherland, G. R., Brock, D. J. H., Scrimgeour, J. B. *J. med. Genet.* 1975, **12**, 135.
4. Seller, M. J., Creasy, M. R., Alberman, E. D. *Br. med. J.* 1974, ii, 524.

Printed in Great Britain

# Prenatal Diagnosis of Chromosome Abnormalities

**by Grant R. Sutherland, Cytogenetics Unit, Department of Histopathology, The Adelaide Children's Hospital**

It is now 13 years since Fuchs and Philip (1963) claimed, in an abstract published in Norwegian, that they had successfully cultured the cells from two out of three samples of amniotic fluid but that they had not managed to complete chromosome studies. Three years later Steele and Breg (1966) were the first to fully document the culture of, and chromosome studies from, amniotic fluid cells. This ability to karyotype amniotic fluid was rapidly confirmed by several other groups (e.g. Thiede *et al.*, 1966; Jacobson and Barter, 1967). Prenatal diagnosis of chromosome abnormalities as a practical procedure was demonstrated when Gerbie *et al.* (1971) reported successfully karyotyping 238 out of 250 successive samples of amniotic fluid. Not all subsequent published series have recorded such high success rates; however most laboratories now have a success rate of 90-95 per cent for karyotyping samples of amniotic fluid and better than 95 per cent success in prenatal diagnosis.

## PREREQUISITES FOR SUCCESSFUL AMNIOTIC FLUID CELL CULTURE

1. The laboratory facilities must be adequate and the staff experienced in all aspects of tissue culture. Constant attention to the many details of this technique is essential as success or failure in tissue culture is not usually the result of any single factor. A reliable source of high quality tissue culture medium is also essential. The serum component of the medium can present considerable problems and each new batch of serum to be used requires extensive testing to ensure that its growth promoting properties are adequate. Different batches of serum can result in different average times required to produce results and poor serum can result in culture failure (Laxova *et al.*, 1975).

2. The sample of amniotic fluid should be of adequate volume, not contaminated with blood and must not be maternal urine. Whilst most laboratories can produce results from small volumes of grossly blood-stained fluid such material is obviously undesirable. The differentiation of maternal urine from amniotic fluid is relatively simple and should be routinely performed (Pirani *et al.*, 1976; Guibaud *et al.*, 1976) by quantitation of such components as urea, creatinine or potassium.

## SOURCES OF CULTURE FAILURE

Even the most successful laboratories are unable to obtain satisfactory growth in tissue culture from 1–2 per cent of amniotic fluids. It would appear that this small proportion of fluids contains inadequate numbers of colony-forming cells for culture to be successful. Other failures are due to inadequate laboratory technique; although some workers claim that heavy blood-staining of the fluid can be a source of culture failure and delayed results (Laurence and Gregory, 1976; Milunsky and Atkins, 1975), others find that this does not lead to problems (Robinson *et al.*, 1973). Sources of failure due to inadequate laboratory technique include microbial infection of cultures, laboratory accidents, use of inadequately tested culture medium and failure of cytogenetic processing techniques (Milunsky and Atkins, 1975; Laurence and Gregory, 1976).

## SOURCES OF ERROR

All laboratory techniques are prone to a number of errors, such as confusion, loss and mislabelling of samples, accidents, etc., but these will not be discussed further.

### Maternal cell growth

It has been estimated that in about 1 per cent of cases of amniotic fluid cell culture, maternal cells and not fetal cells are cultivated (Milunsky, 1973). Whilst this cannot be readily overcome there are a number of steps which may be

**TABLE I Chromosome analysis of colonies in amniotic fluid cell cultures in which mosaicism has been detected**

| *Authors* | *Colony Counts* Normal | Abnormal | *Amniotic Fluid Karyotype* | *Infant Karyotype* |
|---|---|---|---|---|
| Cox *et al.* (1974) | | | | |
| Case 1 | 32 | 1 | 46,XX/46,XX,t(2;E) | 46,XX |
| Case 2 | 33 | 2 | 46,XY/46,XY,t(B;C)/47,XY,+C | 46,XY |
| Case 3 | 42 | 1 | 46,XY/47,XY,+2 | 46,XY |
| Case 4 | 79 | 1 | 46,XX/47,XX,+C | 46,XX |
| Sutherland et al (1975b) | | | | |
| Case 1 | 10 | 2 | 46,XX/47,XX,+D | 46,XX |
| Case 2 | 8 | 1 | 45,XX,−G/46,XX | 46,XX |
| Case 3 | 3 | 2 | 46,XX/46,XY | 46,XY |
| Case 4 | 13 | 2 | 45,X/46,XY | 46,XY |
| Case 5 | 4 | 1 | 46,XX/46,XX,t(2;F) | 46,XX |
| Case 6 | 11 | 11 | 46,XY/47,XY,+mar | 46,XY/47,XY,+mar* |
| Nuzzo *et al.* (1975) | | | | |
| Case 1 | 1 | 1 | 46,XX/46,XX,t(3;12) | 46,XX |
| Case 2 | 9 | 1 | 46,XY/46,XY,del(5q) | 46,XY |

* Not seen in 100 cells from blood but present in cultures of skin, cord and amnion.

taken to recognize the problem. Sex determination of the amniotic fluid, by X and Y chromatin study, can usually identify those cases in which the fetus is male but the chromosome results predict a female. Furthermore, harvesting of chromosomes from more than one culture vessel and the harvest of primary cultures *in situ* can also help overcome this problem (Macintyre, 1971; Sutherland *et al.*, 1975b). Apparent XX/XY mosaicism will almost always be due to maternal cellular contamination and the fetus will have a normal male karyotype; XX/XY mosaicism in the fetus will be extremely rare. A greater difficulty is presented when the fetus is female and maternal cells are cultured. The only practical means of overcoming this problem is a study of chromosomal variants using C- and Q-banding techniques which should differentiate fetal from maternal karyotypes (Hauge *et al.*, 1975; Müller *et al.*, 1975; Alfi *et al.*, 1975). This, of course, requires that paternal chromosomes be studied, hence collection of blood from both parents at or near the time of amniocentesis for such studies has much to recommend it (Mulcahy and Jenkyn, 1973; Alfi *et al.*, 1975). This should not be left until a fetal karyotype presenting difficulties of interpretation is encountered as requesting parental blood samples two to three weeks after an amniocentesis (Macintyre *et al.*, 1974) which has not yet produced a result can only cause parental anxiety. It is, however, doubtful whether the routine study of chromosomal variants in both parents and all female amniotic fluid cultures is worthwhile. If one in 100 female amniotic fluids is in fact a culture of maternal cells, and if one amniotic fluid in 30 is abnormal, then this will lead to an erroneous diagnosis in about one case in 3,000. If this is the only error inherent in this procedure it must be one of the most precise laboratory examinations known.

It has been recently suggested (Hsu *et al.*, 1976b) that the presence of increased numbers of macrophages in amniotic fluid cell cultures is evidence of maternal cellular contamination. This is not so (Sutherland, 1976). Increased numbers of macrophages are more likely to be an indication of a fetal CNS lesion than of maternal cellular contamination (Sutherland *et al.*, 1975a). The report attributing this relationship to maternal cellular contamination (Hsu *et al.*, 1976b) suggested that these maternal macrophages divided in culture and were capable of being subcultured. It is highly improbable that mature maternal macrophages will divide in tissue culture and almost certain that they are not capable of being subcultured.

## Mosaicism

Mosaics have been diagnosed in amniotic fluid cultures on numerous occasions (Sutherland *et al.*, 1975b; Nuzzo *et al.*, 1975; Neirmeijer *et al.*, 1976; Laurence and Gregory, 1976) but confirmation of mosaicism in the

fetus or subsequently born infant has only rarely occurred (Sutherland *et al.*, 1975b). Hence it is apparent that mosaicism in amniotic fluid cultures is in most cases an artefact. A possible explanation for this lies in terms of the growth patterns of amniotic fluid cells in tissue culture. Each colony in an amniotic fluid cell culture appears to be a clone. The relative size of the numerous clones within any one culture varies considerably to the extent that one clone may tend to predominate within a culture vessel. If such a clone had arisen from a single cell carrying a chromosome aberration, and the culture was harvested for chromosome studies, most of the cells would have the particular chromosome abnormality concerned. Most workers now advocate the harvesting of amniotic fluid cultures *in situ* rather than subculturing them prior to harvest (Ferguson-Smith *et al.*, 1971; Cox *et al.*, 1974; Sutherland *et al.*, 1975b; Nuzzo *et al.*, 1975). Cultures harvested for chromosome studies *in situ* can be analysed in such a way that the chromosomal constitution of each colony is determined. The finding of one colony with a chromosome aberration can generally be regarded as of little significance (Table I). Antenatal diagnosis of true mosaics is obviously fraught with difficulty. However, the finding of more than one aberrant clone would strongly suggest this diagnosis, especially if it was found in more than one culture vessel. The confirmation of such a diagnosis should really rest on a repeat amniocentesis yielding the same findings (Sutherland *et al.*, 1975b). The possibility that twin pregnancy could give rise to apparent mosaicism has not been recorded as only one sac is usually entered at amniocentesis unless a deliberate attempt is made to sample each sac (Bang *et al.*, 1975).

Two prenatal diagnostic errors have been recorded in which some form of hidden mosaicism was advanced (Kardon *et al.*, 1972) as a plausible reason for the error. Kardon *et al* (1972) found a karyotype 45,X in an amniotic fluid culture but on termination the fetus was found to have a normal male karyotype. Katayama *et al.* (1974) reported a normal male karyotype in an amniotic fluid culture and a female Down syndrome infant with regular karyotype was born.

The contamination of amniotic fluid cell cultures with organisms such as mycoplasmas can cause chromosomal damage which may suggest mosaicism (Schneider *et al.*, 1974). Whilst in practice this should present little problem, amniotic fluid cultures should be regularly monitored for the presence of mycoplasma contamination.

## Misinterpretation

The analysis of chromosomes from amniotic fluid cell cultures is a situation in which a cytogenetic diagnosis is required with virtually no clinical information. Hence it becomes of use to know the sex of the fetus (as mentioned earlier) and to have access to chromosome preparations from the parents. In the absence of parental chromosome studies the interpretation of chromosomal variants can present problems (Wahlstrom, 1975). Stengel-Rutkowski *et al.* (1976) have recommended the routine use of G-banding when analysing amniotic fluid cell cultures. These authors present a number of cases of misinterpretation of karyotypes without G-banding, for example the confusion of a female Down syndrome with a Klinefelter karyotype and the confusion of a male Down syndrome with an XYY karyotype. However, the routine use of such techniques on karyotypes which are apparently normal by unbanded criteria is probably unnecessary as the number of errors which will result in the birth of a defective fetus as a result of not using banding techniques will be virtually nil. All necessary techniques should, however, be used to confirm the precise nature of any abnormal karyotype detected in amniotic fluid cell culture.

The finding of apparently balanced *de novo* translocations causes difficulties (Epstein *et al.*, 1972; Niermeijer *et al.*, 1976) as it is usually impossible to be certain that the translocation is balanced. There is some suggestion (Jacobs, 1976) that such *de novo* translocations may be associated with mental retardation.

The early errors of interpretation encountered with tetraploid cells (Kohn and Robinson, 1970) should no longer cause problems as it is well known that a high proportion of cells from amniotic fluid cultures can be tetraploid even though the fetus will virtually always be normal in these circumstances (Walker *et al.*, 1970; Tegenkamp and Hux, 1974). The antenatal diagnosis of fetal tetraploidy or tetraploid/diploid mosaicism would be virtually impossible, however such conditions are extremely rare in liveborn infants.

## APPLICATION OF PRENATAL CHROMOSOME STUDY

The technique of prenatal chromosome study is thus established as a reliable, accurate and safe (Hsu *et al.*, 1976a) procedure. The next question which arises is to which pregnancies should it be directed.

The obvious answer is any pregnancy in which there is a significant risk of aneuploidy in the fetus. The decision as to what risk is significant must vary according to the facilities available, the frequency of morbidity associated with amniocentesis and the attitudes of the patients. Niermeijer *et al.* (1976) regarded a risk of greater than 1 per cent as adequate indication for prenatal diagnostic studies. The most obvious high risk group is that in which one of the parents has a chromosome abnormality. Here the risk of aneuploidy can be as high as 50 per cent but is usually lower than this, ranging from 2–20 per cent.

Another high risk group to which prenatal chromosome study is applicable is that in which the mother carries an X-linked disorder which cannot be specifically diagnosed antenatally. Half the male fetuses in this group will be affected, hence fetal sexing with termination of male fetuses is often the procedure of choice in such cases. Fetal sexing should be done on chromosome preparations and should not rely solely on X and Y chromatin study of uncultured amniotic fluid cells (Laurence and Gregory, 1976).

All other groups to which prenatal chromosome study may be applied are at a lower risk of producing aneuploid offspring. These include women of advanced age and couples who have had a previous child with Down syndrome. It can be shown, however, that there is considerable financial benefit to the community in screening these groups, particularly the older mothers (Stein *et al.*, 1973; Hagard and Carter, 1976).

A previous child with regular Down syndrome is often regarded as an indication for prenatal chromosome study. Epidemiological studies have suggested that the risk of having a child with Down syndrome is not influenced by having had such a child previously (Kirman, 1972). However, maternal anxiety about such recurrence is often adequate justification for amniocentesis. A family history of Down syndrome always requires careful investigation before amniocentesis is offered as this is not in itself an indication for amniocentesis (Laxova *et al.*, 1975). Such families deserve a thorough genetic workup, indeed a geneticist should be part of the team evaluating any request for amniocentesis before it is performed. Most laboratories handling amniotic fluid receive samples which were collected for inappropriate reasons.

## PROGRESS

The results of a number of series of diagnostic prenatal chromosome studies are shown in Table II. This table incorporates the North American data assembled by Milunsky and Atkins (1975) and series published from elsewhere which contained more than 50 cases.

Examination of the data in Table II shows that the frequency of chromosome abnormalities detected is much higher than epidemiological data would suggest for all groups, with the possible exception of parental translocation carriers. A review of 6,000 prenatal diagnoses by Galjaard (1976), (cited by Niermeijer *et al.*, 1976) indicated that in mothers older than 38–40 years the risk of chromosome abnormality was 4.6 per cent. Using epidemiological

**TABLE II Cumulative prenatal diagnostic chromosome studies***

| *Indication for Study* | *Number of Cases* | *Major Cytogenetic Abnormalities* | |
|---|---|---|---|
| | | *Number* | *Frequency* |
| Translocation carrier | 135 | 23 | 1/5.9 |
| Maternal age $\geqslant$ 40 years | 718 | 27 | 1/26.6 |
| Maternal age $\geqslant$ 35 $<$ 40 years | 498 | 8 | 1/62.3 |
| Previous Down syndrome | 793 | 11 | 1/72.1 |
| Family history of D.S. | 88 | 1 | 1/88 |
| Miscellaneous | 407 | 7 | 1/58.1 |
| Total | 2,339 | 77 | 1/30.4 |

* From Milunsky and Atkins (1975) and other major ($>$ 50 cases) series published outside North America (Philip *et al.*, 1974; Wahlstrom *et al.*, 1974; Karjalainen and Aula, 1975; Laxova *et al.*, 1975; Laurence and Gregory, 1976; Niermeijer *et al.*, 1976).

data and correcting for incomplete ascertainment Hood (1976) estimated the risk of Down syndrome in mothers aged 35–39 to be 0.4 per cent and in mothers 40–44 to be 1.3 per cent. Analysis of prenatal diagnostic findings in such women by Ferguson-Smith (1976) gave risks of gross chromosome abnormality to mothers aged 35–39 to be 1.5 per cent and those aged 40 or more to be 5.2 per cent. There are a number of possible reasons for the apparent excess of chromosome abnormalities found by prenatal diagnosis. Most epidemiological data are probably based on fairly incomplete ascertainment of chromosome abnormalities. It has been shown that about 5–6 per cent of infants dying in the perinatal period have chromosome abnormalities (Machin, 1975; Bauld *et al.*, 1974). In addition, a number of chromosomally abnormal fetuses would probably be spontaneously aborted even if not prenatally diagnosed. It has been estimated that 65 per cent of trisomy 21 conceptuses are lost spontaneously between 10 and 26 weeks' gestation (Creasy and Crolla, 1974). Such chromosome abnormalities are most unlikely to be included in epidemiological data.

Two groups from Table II require close scrutiny. Firstly, those studied because of a family history of Down syndrome. Only one chromosome abnormality was detected in this group of 88 pregnancies; this was a translocation form of Down syndrome (Milunsky and Atkins, 1975). Presumably this was a familial translocation as the Down syndrome was familial although no information about this was provided. Genetic investigation of familial Down syndrome would ensure that translocation carriers at risk were offered amniocentesis and that in other instances where the risk of recurrence is small the procedure would not be unnecessarily carried out.

The group where the indications for chromosome analysis were miscellaneous is shown in more detail in Table III for those series which provided adequate information. Most of the miscellaneous indications were amongst the following: previous child with chromosome abnormality other than Down syndrome, parental anxiety, previous malformed child and exposure to radiation/viral hazards. Niermeijer *et al.* (1976) also included some showing intrauterine growth retardation in the second trimester. There were six alleged abnormalities detected in this group. However, four of them should not perhaps be included. The findings in the case of chromosome fragmentation from radiation/viral damage are of unknown significance, as acknowledged by the authors (Atkins *et al.*, 1974). The case of Down syndrome found by Laxova *et al.* (1975) in their miscellaneous group was in a 35-year-old woman; age alone would have been adequate justification for the amniocentesis and this case should not be regarded as miscellaneous. Niermeijer *et al.* (1976) found two abnormalities in their group. The first was detected at 35 weeks' gestation and cannot be strictly considered as part of a prenatal diagnostic series as it was too late for termination of the pregnancy. Their second case, an XYY male, was detected as the result of a miscellaneous indication, the mother having had two spontaneous abortions and retarded relatives. Laurence and Gregory (1976) detected two abnormalities in their miscellaneous group. The first, with karyotype

**TABLE III Details of prenatal diagnoses carried out for miscellaneous reasons**

| *Authors* | *No.* | *Abnormals* | *Comment* |
|---|---|---|---|
| Milunsky and Atkins (1975) | 116 | "fragmentation" | ? radiation/viral damage, normal child born |
| Hsu and Hirschhorn (1974) | 58 | — | — |
| Laurence and Gregory (1976) | 25 | +21 | maternal anxiety, age not given |
| | | Gp+ | pregnancy terminated, no other details |
| Niermeijer *et al.* (1976) | 19 | +18 | diagnosed at 35 weeks |
| | | XYY | 2 previous abortions and retarded relatives |
| Allen *et al.* (1974) | 16 | — | — |
| Laxova *et al.* (1975) | 16 | +21 | maternal age 35 years, previous "abnormal" child |
| Wahlstrom *et al.* (1974) | 14 | — | — |
| Rary *et al.* (1974) | 10 | — | — |
| Philip *et al.* (1974) | 7 | — | — |
| Doran *et al.* (1974) | 6 | — | — |
| Robinson *et al.* (1973) | 6 | — | — |
| Total | 293 | 6 | |

46,XY,Gp+, was found in a woman who had a previous abnormal child of unspecified type, no further information was provided about this except that the pregnancy was terminated. If this was a genuinely abnormal karyotype then it is probable that it was familial and again should not be included in the miscellaneous group. Their second abnormality was a case of Down syndrome in a woman of unspecified age manifesting anxiety as to the outcome of her pregnancy, again this case would seem to be one of few detected as a result of a miscellaneous indication.

Hence there are only two cases of chromosome abnormality detected where the indications for the test can be genuinely considered to be miscellaneous. This is no more than would be expected from unselected pregnancies as it is not different from the incidence of chromosome abnormality expected in unselected liveborn neonates (Nielsen and Sillesen, 1975). Whilst it can be seen that where the indications for prenatal chromosome study are miscellaneous there is apparently no increased risk that the fetus will be aneuploid, this does not necessarily mean such studies are contraindicated as marked parental anxiety can be a very good reason for this procedure. Some of the other miscellaneous indications should not indicate prenatal diagnostic studies. This is especially the case where the parents have had a previous malformed child which has not been specifically diagnosed. A previous child with a structural chromosome abnormality, when parental chromosomes are normal, should not indicate prenatal chromosome studies as the recurrence risk here is very small. Trisomy of other than chromosome 21 may well be a reasonable indication as a few cases have been reported of recurrent numerical chromosome abnormalities, for example Atkins *et al.* (1974) recorded a recurrence of trisomy 18. Boué and Boué (1973) have presented data which suggest that trisomy in one pregnancy may lead to an increased risk of trisomy in subsequent pregnancies.

In conclusion it should be stressed that prenatal chromosome analysis is a reliable and safe procedure which, if correctly applied, can be of immense benefit to individual couples and the community. If incorrectly applied or inadequately performed it can, like any other procedure, bring disrepute to itself and those involved with it. The procedure should be offered to all those who can benefit from it but they must be adequately investigated and counselled from a genetic viewpoint before amniocentesis is performed.

## DISCUSSION

*R. Hope, Adelaide:*
I have two questions. Firstly, you mentioned to us that there was a 1 per cent risk of culturing maternal cells rather than fetal cells. You said that the risk in making a misclassification is 1 in 3,000. It was not obvious to me that the risk was not higher than this. Perhaps you could elaborate. Secondly, would it be possible to make use of genetic markers to identify maternal cells? I am thinking specially of the HL-A locus which is highly polymorphic. In a number of situations it would be possible, using say a fluorescently labelled antiserum, to distinguish fetal cells from maternal cells quite rapidly.

*G. R. Sutherland:*
Firstly, you're right in saying that if we're growing maternal cells 1 per cent of the time, we will be making an error in 1 per cent of cases. However, as I think I showed, most of the group that we looked at had a frequency of chromosome abnormality that would indicate termination of pregnancy of about 1 in 30. I think we can multiply these figures together so that it will only be in about 1 in 3,000 cases that this error will be serious and I regard a serious error as the birth of a child with a chromosome abnormality, which, had it been detected, would have been aborted. The second question is something that, perhaps, you may know more about than I do. The problem is, I think, getting adequate numbers of cultured cells in time.

*R. F. Carter, Adelaide:*
You've mentioned the probability of finding a chromosomal abnormality in random samples of amniotic fluid as being around 1 per cent. You know that we and many other people karyotype fluids sent up for alphafetoprotein estimation or for the detection of inborn metabolic errors. In view of your attitude, which seems to me to be a little bit against looking at these random fluids, do you think we ought to go on karyotyping these as they are really in your miscellaneous group?

*G. R. Sutherland:*
Yes, I think we should. I think once an amniocentesis has been performed it's up to us to do as much as is reasonably practical. I

think every amniotic fluid collected for any reason should have chromosome studies and alphafetoprotein done on it as a bare minimum.

*M. A. Ferguson-Smith, Glasgow:*
Grant, you mentioned whether or not you should do banding of amniotic cell cultures. I think really one ought to but perhaps the solution to getting the diagnosis rapidly to the parents would be to give a preliminary report on the basis of conventional techniques. We do this. It takes longer to get banding results. We use the banding results for confirmation. We still have plenty of time to do something if a result has to be retracted.

In our case with the 9p+ syndrome, we would not have been able to diagnose this antenatally without using banding.

The second point is, have you any adequate explanation for the reason why the abnormality rate in mothers over the age of 40 is greater than 5 per cent rather than $1\frac{1}{2}$ per cent as predicted by epidemiological studies and similarly that the rate in women 35–39 is about $1\frac{1}{2}$ per cent, rather than 0.3 per cent? Thirdly, the techniques of prenatal diagnosis have been available to us for a number of years now. We're concerned that the numbers of cases being studied are really very small indeed. I think the opportunity of prenatal diagnosis has been offered to very few people at risk, because in the West of Scotland in the last seven years, we've only done 1,000 amniocenteses; we reckon that only one-tenth of the women who really need it are having it made available to them. And I wonder if you have any thoughts about how we can put this right?

*G. R. Sutherland:*
I will answer the questions in the order that they were presented. I wonder how often you've had to retract a preliminary report after you've done banding studies?

*M. A. Ferguson-Smith:*
Never.

*G. R. Sutherland:*
In cases of unbalanced translocations or potential unbalanced translocations, you may require banding. With regard to the discrepancy between the frequency of chromosome abnormalities at prenatal diagnosis and what epidemiology predicts: I'm not sure of the reasons but I think that a number of chromosome abnormalities would be lost spontaneously if those pregnancies weren't terminated immediately after the prenatal diagnosis. Creasy and Crolla recently suggested that 65 per cent of conceptions with trisomy 21 were lost between 10 and 26 weeks. We also know from work that Geoffrey Machin did in London, that I did in Edinburgh, and Kuleshov and colleagues in Russia have done, that about 5 per cent of all infants dying in the perinatal period have a chromosome abnormality. I think a lot of these haven't been included in the past in the epidemiological information on which the recurrence figures that we use in counselling are based. The problem of the small number of cases that come to prenatal diagnosis has been perplexing me for a long time too. I think it's unfortunate that we have such a reliable, safe procedure which isn't being taken advantage of and which has been shown to be of benefit to the individuals involved and to the community as a whole. The community can make money by promoting prenatal diagnosis and I am at a loss, like Professor Ferguson-Smith, to know exactly why this is so slow in getting off the ground.

## REFERENCES


Alfi, O., Derencsenyi, A., and Donnell, G. (1975): Chromosome polymorphism and prenatal diagnosis. *The Lancet*, **1**, 1253.

Allen, H. H., Sergovich, F., Stuart, E. M., Pozsonyi, J., and Murray, B. (1974): Infants undergoing antenatal genetic diagnosis: A preliminary report. *American Journal of Obstetrics and Gynecology*, **118**, 310.

Atkins, L., Milunsky, A., and Shahood, J. M. (1974): Prenatal diagnosis: detailed chromosomal analysis in 500 cases. *Clinical Genetics*, **6**, 317.

Bang, J., Nielsen, H., and Philip, J. (1975): Prenatal karyotyping of twins by ultrasonically guided amniocentesis. *American Journal of Obstetrics and Gynecology*, **123**, 695.

Bauld, R., Sutherland, G. R., and Bain, D. (1974): Chromosome studies in investigation of stillbirths and neonatal deaths. *Archives of Disease in Childhood*, **49**, 782.

Boué, J., and Boué, A. (1973): Chromosomal analysis of two consecutive abortuses in each of 43 women. *Humangenetik*, **19**, 275.

Cox, D. M., Niewczas-Late, V., Riffell, M. I., and Hamerton, J. L. (1974): Chromosomal mosaicism in diagnostic amniotic fluid cell cultures. *Pediatric Research*, **8**, 679.

Creasy, M. R., and Crolla, J. A. (1974): Prenatal mortality of trisomy 21 (Down's syndrome). *The Lancet*, **1**, 473.

Doran, T. A., Rudd, N. L., Gardner, H. A., Lowden, J. A., Benzie, R. J., and Liedgren, S. I. (1974): The antenatal diagnosis of genetic disease. *American Journal of Obstetrics and Gynecology*, **118**, 314.

Epstein, C. J., Schneider, E. L., Conte, F. A., and Friedman, S. (1972): Prenatal detection of genetic disorders. *American Journal of Human Genetics*, **24**, 214.

Ferguson-Smith, M. A. (1976): Prospective data on risk of Down syndrome in relation to maternal age. *The Lancet*, **2**, 252.

Ferguson-Smith, M. E., Ferguson-Smith, M. A., Nevin, N. C., and Stone, M. (1971): Chromosome analysis before birth and its value in genetic counselling. *British Medical Journal*, **4**, 69.

Fuchs, F., and Philip, J. (1963): Mulighed for antenatal undersøgelse af fosterets kromosomer. (Abstract). *Nordisk Medicin*, **69**, 572.

Gerbie, A. B., Nadler, H. L., and Gerbie, M. V. (1971): Amniocentesis in genetic counselling. *American Journal of Obstetrics and Gynecology*, **109**, 765.

Guibaud, S., Bonnet, M., Dury, A., Thoulon, J. M., and Dumont, M. (1976): Amniotic fluid or maternal urine? *The Lancet*, **1**, 746.

Hagard, S., and Carter, F. A. (1976): Preventing the birth of infants with Down's syndrome: a cost-benefit analysis. *British Medical Journal*, **1**, 753.

Hauge, M., Poulsen, H., Halberg, A., and Mikkelsen, M. (1975): The value of fluorescence markers in the distinction between maternal and fetal chromosomes. *Humangenetik*, **26**, 187.

Hook, E. B. (1976): Estimates of maternal age-specific risks of a Down syndrome birth in women aged 34–41. *The Lancet*, **2**, 33.

Hsu, L. Y. F., and Hirschhorn, K. (1974): Prenatal diagnosis of genetic disease. *Life Sciences*, **14**, 2311.

Hsu, L. Y. F., Yahr, F., Godmilow, L., and Hirschhorn, K. (1976a): Amniocentesis for prenatal diagnosis. *The Lancet*, **1**, 206.

Hsu, L. Y. F., Serotkin, A., and Hirschhorn, K. (1976b): Identification of macrophages in amniotic-fluid cell-cultures. *The Lancet*, **1**, 799.

Jacobs, P. A. (1974): Correlation between euploid structural chromosome rearrangements and mental subnormality in humans. *Nature*, **249**, 164.

Jacobson, C. B., and Barter, R. H. (1967): Intrauterine diagnosis and management of genetic defects. *American Journal of Obstetrics and Gynecology*, **99**, 796.

Kardon, N. B., Chernay, P., Hsu, L. Y. F., Martin, J. L., and Hirschhorn, K. (1972): Pitfalls in prenatal diagnosis resulting from chromosomal mosaicism. *Journal of Pediatrics*, **80**, 297.

Karjalainen, O., and Aula, P. (1975): Intrauterine diagnosis of chromosome anomalies. *Annales Chirurgiae et Gynaecologiae Fenniae*, **64**, 146.

Katayama, K. P., Park, I. J., Heller, R. H., Barakat, B. Y., Preston, E., and Jones, H. W. (1974): Errors of prenatal cytogenetic diagnosis. *Obstetrics and Gynecology*, **44**, 693.

Kirman, B. (1972): Genetic counselling. *British Medical Journal*, **2**, 46.

Kohn, G., and Robinson, A. (1970): Tetraploidy in cells cultured from amniotic fluid. *The Lancet*, **2**, 778.

Laurence, K. M., and Gregory, P. (1976): Prenatal diagnosis of chromosome disorders. *British Medical Bulletin*, **32**, 9.

Laxova, R., Lewis, B. V., and Suddaby, M. (1975): A clinical service for prenatal diagnosis. *The Lancet*, **2**, 964.

Machin, G. A. (1974): Chromosome abnormality and perinatal death. *The Lancet*, **1**, 549.

Macintyre, M. N. (1971): Chromosomal problems of intrauterine diagnosis. *Birth Defects: Original Article Series*, **7**, 10.

Macintyre, M. N., Rustad, R. C., and Turk, K. B. (1974): Prenatal evaluation in a case of familial Y chromosome long arm deletion (Yq–). *Journal of Medical Genetics*, **11**, 367.

Milunsky, A. (1973): *The Prenatal Diagnosis of Hereditary Disorders*. Charles C. Thomas, Springfield.

Milunsky, A., and Atkins, L. (1975): Prenatal diagnosis of genetic disorders. In *The Prevention of Genetic Disease and Mental Retardation*, Milunsky, A. (ed.). W. B. Saunders, Philadelphia.

Mulcahy, M. T., and Jenkyn, J. (1973): Prenatal diagnosis: Results of cytogenetic analysis of amniotic fluid cell cultures. *Medical Journal of Australia*, **1**, 979.

Müller, H., Klinger, H. P., and Glasser, M. (1975): Chromosome polymorphism in a human newborn population. II. Potentials of polymorphic chromosome variants for characterizing the idiogram of an individual. *Cytogenetics and Cell Genetics*, **15**, 239.

Nielsen, J., and Sillesen, I. (1975): Incidence of chromosome aberrations among 11,148 newborn children. *Humangenetik*, **30**, 1.

Niermeijer, M. F., Sachs, E. S., Jahodova, M., Tichelaar-Klepper, C., Kleijer, W. J., and Galjaard, H. (1976): Prenatal diagnosis of genetic disorders. *Journal of Medical Genetics*, **13**, 182.

Nuzzo, F., Simoni, G., Mottura, A., Larizza, L., Stefanini, M., Dambrosio, F., Zuffardi, O., and Maraschio, P. (1975): Deviant karyotypes in amniotic fluid cell cultures: a problem in prenatal diagnosis. *Journal de Génétique Humaine*, **23**, 225.

Philip, J., Bang, J., Hahnemann, N., Mikkelsen, M., Niebuhr, E., Rebbe, H., and Weber, J. (1974): Chromosome analysis of fetuses in risk pregnancies. *Acta Obstetrica et Gynecologica Scandinavica*, **53**, 9.

Pirani, B. B. K., Doran, T. A., and Benzie, R. J. (1976): Amniotic fluid or maternal urine? *The Lancet*, **1**, 303.

Rary, J. M., Park, I. J., Heller, R. H., Jones, H. W., and Baramki, T. A. (1974): Prenatal cytogenetic analysis of women with high risk for genetic disorders. *The Journal of Heredity*, **65**, 209.

Robinson, A., Bowes, W., Droegemueller, W., Puck, M., Goodman, S., Shikes, R., and Greenshur, A. (1973): Intrauterine diagnosis: potential complications. *American Journal of Obstetrics and Gynecology*, **116**, 937.

Schneider, E. L., Stanbridge, E. J., Epstein, C. J., Golbus, M., Abbo-Halbasch, G., and Rodgers, G. (1974): Mycoplasma contamination of cultured amniotic fluid cells: potential hazard to prenatal chromosomal diagnosis. *Science*, **184**, 477.

Steele, M. W., and Breg, W. R. (1966) : Chromosome analysis of human amniotic-fluid cells. *The Lancet*, **1**, 383.

Stein, Z., Susser, M., and Guterman, A. V. (1973) : Screening programme for prevention of Down syndrome. *The Lancet*, **1**, 305.

Stengel-Rutkowski, S., Wirtz, A., Hahn, B., Hofmeister, A., and Murken, J. D. (1976) : Routine G-banding in prenatal diagnosis of chromosomal disorders. *Human Genetics*, **31**, 231.

Sutherland, G. R. (1976) : Macrophages in amniotic-fluid cell-cultures. *The Lancet*, **1**, 1077.

Sutherland, G. R., Brock, D. J. H., and Scrimgeour, J. B. (1975a) : Amniotic fluid macrophages and the antenatal diagnosis of anencephaly and spina bifida. *Journal of Medical Genetics*, **12**, 135.

Sutherland, G. R., Bowser-Riley, S. M., and Bain, A. D. (1975b) : Chromosomal mosaicism in amniotic fluid cell cultures. *Clinical Genetics*, **7**, 400.

Tegenkamp, T. R., and Hux, C. H. (1974) : Incidence of tetraploidy as related to amniotic fluid cell types. *American Journal of Obstetrics and Gynecology*, **120**, 1066.

Thiede, H. A., Creasman, W. T., and Metcalfe, S. (1966) : Antenatal analysis of the human chromosomes. *American Journal of Obstetrics and Gynecology*, **94**, 589.

Wahlstrom, J. (1975) : Three cases of minor chromosomal aberrations discovered by prenatal chromosome determination. *Humangenetik*, **27**, 223.

Wahlstrom, J., Bartsch, F. K., and Lundberg, J. (1974) : Prenatal chromosome determination. A study of 219 cases. *Clinical Genetics*, **6**, 184.

Walker, S., Lee, C. L. Y., and Gregson, N. M. (1970) : Polyploidy in cells cultured from amniotic fluid. *The Lancet*, **2**, 1137.

Reprinted from
15 July 1977, Volume 197, pp. 265-266

SCIENCE

# Fragile Sites on Human Chromosomes: Demonstration of Their Dependence on the Type of Tissue Culture Medium

Grant R. Sutherland

# Fragile Sites on Human Chromosomes: Demonstration of Their Dependence on the Type of Tissue Culture Medium

Abstract. *The observation of heritable fragile sites on human chromosomes prepared from lymphocyte cultures has been shown to depend on the type of tissue culture medium in which the lymphocytes are grown. The sites are observed at a much greater frequency when medium 199 is used than when RPMI 1640, Ham's F10, Eagle's (basal), and CMRL 1969 are used. One site on the X chromosome is of clinical significance in that it is a marker for X-linked mental retardation.*

Heritable fragile sites have been described on a number of human chromosomes, including numbers 2, 12, 16, 17, X, and unidentified C-group chromosomes (*1*, *2*). When the autosomes are involved these sites are apparently of no clinical significance, but the site on the X is associated with some forms of X-linked mental retardation (*3*).

I recently attempted to reexamine a fragile site on chromosome 2 which had been identified and shown to be familial some years before. Initial studies were puzzling because the fragile site appeared to have vanished. Investigation of this led to the finding that the frequency of lesions at the fragile site depended on the type of tissue culture medium in which the lymphocytes used to produce the chromosomes were cultured. This observation was extended to and confirmed for fragile sites on the X, 10, and 20 (Fig. 1).

Chromosome preparations from lymphocyte cultures grown in parallel in several different commercially available tissue culture media were scored for the presence or absence of a lesion at the fragile site on a number of occasions. The results are shown in Table 1. The frequency of lesions at the fragile site was always much greater when medium 199 was used than with any of the other media. This difference was not so pronounced when the frequency of the lesions was low. When a medium other than 199 was used the frequency of these sites was so low that they would have escaped detection by routine clinical chromosome analysis.

The reasons for these differences in frequency of breakage at the fragile sites according to the type of culture medium are unknown. They may represent either a nonspecific phenomenon related to factors such as *p*H or osmolarity, or be due to a specific chromosome breaking agent present at higher concentrations in medium 199 than the other media used or only present in 199. Delineation of the mechanisms responsible for this effect would be of considerable use.

The findings reported here are of importance in the study of X-linked mental

Table 1. Frequency of observation of fragile sites according to the type of tissue culture medium used. Tissue culture media used were purchased at single strength (except for Eagle's basal medium which was purchased as a × 10 concentrate) from Commonwealth Serum Laboratories, Melbourne, Australia. Lymphocyte cultures consisting of 4 ml of medium, 1 ml of fetal bovine serum, 0.1 ml of phytohemagglutinin, and 0.2 ml of venous blood were harvested according to standard methods after 72 hours of incubation; colchicine was applied for 2 hours and the hypotonic solution used was 0.075*M* KCl. A cell was considered to display evidence of a fragile site if one or both chromatids were broken at the site, if there was chromosome material either in addition or missing which corresponded to the parts of the chromosome on either side of the fragile site, or if a triradial figure was present. Results are expressed as number of cells showing a lesion at the fragile site over the total number of cells examined.

| Subject No. | Fragile site | Date studied | Tissue culture medium | | | | |
|---|---|---|---|---|---|---|---|
| | | | 199 | RPMI 1640 | Ham's F10 | Eagle's (basal) | CMRL 1969 |
| 1 | 2q1 | September 1972 | 23/50 | | | | |
| | | May 1976 | 34/55 | 3/55 | | | |
| | | June 1976 | 25/50 | 0/50 | 3/50 | 3/50 | 2/50 |
| | | September 1976 | 34/50 | | 0/50 | | |
| 2 | 10q23 | October 1973 | 36/50 | | | | |
| | | May 1976 | 22/55 | 1/55 | | | |
| | | June 1976 | 7/50 | 0/50 | 1/50 | 1/50 | 0/50 |
| | | September 1976 | 23/50 | | 0/50 | | |
| 3 | 20p11 | November 1973 | 33/50 | | | | |
| | | May 1976 | 13/50 | 5/50 | 3/50 | 2/50 | |
| | | September 1976 | 24/50 | 1/50 | 0/50 | 2/50 | |
| 4* | 20p11 | September 1976 | 22/50 | | 0/50 | 2/50 | |
| 5† | Xq27 or 8 | May 1976 | 8/50 | 5/50 | | 0/50 | |
| 6‡ | Xq27 or 8 | May 1976 | 4/50 | 0/50 | 0/50 | 0/50 | |
| | | June 1976 | 5/50 | 0/50 | 0/50 | 0/50 | |

*Sibling of subject No. 3. †Male. ‡Mother of subject No. 5.

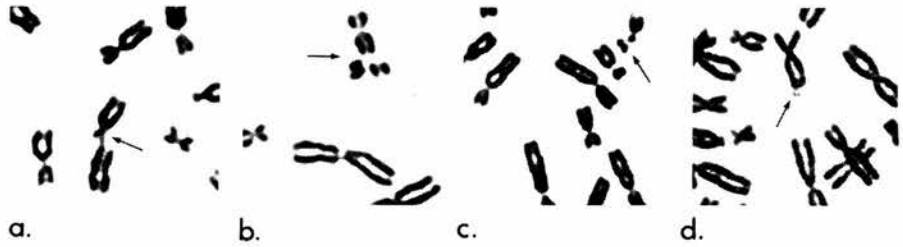



Fig. 1. Different appearances of lesions (arrowed) at fragile sites. (a) Breakage of a single chromatid at 2q1. (b) Triradial figure caused by duplication of chromatids distal to lesion at 10q23. (c) Unstained region of both chromatids at 20p11. (d) Unstained region of both chromatids at Xq27 or 8.

retardation, which has been estimated to account for one-fifth of males with an IQ in the 30 to 55 range (*4*). Most descriptions of X-linked mental retardation have included chromosome studies, but with few exceptions (*3*) these have shown nothing unusual. The fragile site on Xq, which is directly associated with at least one form of X-linked mental retardation, would probably have been missed if such studies were not carried out using medium 199; consequently, they should be repeated.

This association of the fragile site on Xq with one form of mental retardation could provide a means of prenatal diagnosis if the fragile site can be demonstrated in fibroblast cultures. Most reports of fragile sites do not include studies on fibroblast cultures, although Ferguson-Smith (*1*) found the frequency of lesions at a site at 2q to be much lower in these cultures than in lymphocyte cultures. I have been unable to demonstrate the fragile sites in fibroblast cultures from any of the cases in Table 1 regardless of the type of culture medium used.

Further studies of fragile sites are required to determine whether they all behave similarly, what exactly their phenotypic effects are, their frequency in the population, and their relationship to chromosome structure. Identification of the factors in medium 199 responsible for their induction would greatly facilitate this work.

GRANT R. SUTHERLAND

*Cytogenetics Unit,*
*Department of Histopathology,*
*Adelaide Children's Hospital Inc.,*
*North Adelaide, S.A. 5006*
*Australia*

**References and Notes**

1. M. A. Ferguson-Smith, *Ann. Genet.* **16**, 29 (1973).
2. F. Giraud, S. Ayme, J. F. Mattei, M. G. Mattei, *Hum. Genet.* **34**, 125 (1976).
3. H. A. Lubs, *Am. J. Hum. Genet.* **21**, 231 (1969); J. Harvey, C. Judge, S. Wiener, *J. Med. Genet.* **14**, 46 (1977); F. Giraud, S. Ayme, J. F. Mattei, M. G. Mattei; *Hum. Genet.* **34**, 125 (1976); G. R. Sutherland, unpublished data.
4. G. Turner and B. Turner, *J. Med. Genet.* **11**, 109 (1974).

3 December 1976; revised 16 February 1977

tant portion of the administered L-tryptophan (via L-5-HTP) to serotonin. From this hypothesis, it may be inferred that the raphe nuclei that contain most of the brain serotonin cell bodies — the latter being the site of synthesis of L-tryptophan hydroxylase — were either intact or only partially degenerated. Furthermore, an extensive post-mortem study of the brain of our Case 2 has revealed no detectable lesion in the midline structures of the brainstem.[5] If it is accepted that neuronal loss exceeding 50 per cent can be detected when cell counts are impracticable, our negative neuroanatomic findings suggest that at least 50 per cent of the neurons in the raphe nuclei had survived to anoxic damage.

In the light of these data, our findings suggest that this apparent reduction of serotoninergic mechanisms reflect not only moderate, if any, postanoxic neuronal degeneration in raphe nuclei but also functional alteration in serotoninergic activity of intact neurons in raphe nuclei.

Jacques De Léan, M.D.
Hôpital du St-Sacrement
Québec, PQ, Canada G1S 4L8 1050 Chemin Ste-Foy

1. Lhermitte F, Peterfalvi M, Marteau R, et al: Analyse pharmacologique d'un cas de myclonies et d'action post-anoxiques. Rev Neurol (Paris) 124:21-31, 1971
2. De Léan J, Richardson JC, Hornykiewicz O: Beneficial effects of serotonin precursors in postanoxic action myoclonus. Neurology (Minneap) 26:863-868, 1976
3. Moir ATB, Eccletion D: The effects of precursor loading in the cerebral metabolism of 5-hydroxyindoles. J Neurochem 15:1093-1108, 1968
4. Eccleston D, Ashcroft GW, Crawford TBB: Effects of tryptophan administration on 5-HIAA in cerebrospinal fluid in man. J Neurol Neurosurg Psychiatry 33:269-272, 1970
5. Richardson JC, Rewcastle B, De Léan J: Hypoxic myoclonus, Clinical and Pathological Observations in Proceedings Sir Gordon Holmes Centennial Meeting, London, March, 1976. Edinburgh, Blackwood (in press)

## CREUTZFELDT-JAKOB DISEASE AND SHEEP BRAIN (CONT.)

*To the Editor:* The postulated relation of Creutzfeldt-Jakob disease to the consumption of animal brain by Alter et al. (Alter M, Hoenig E, Pratzon G: Creutzfeldt-Jakob disease: possible association with eating brains. N Engl J Med 296:820-821, 1977) continues to be based on circumstantial evidence. A focus of relatively high incidence among Libyan Jews in Israel implies, according to that hypothesis, that the etiologic agent is prevalent in domestic animals, especially in sheep, in the Middle East. If ingestion of sheep brain can be responsible for transmission of the virus from animal to man parenteral inoculation of the brain tissue should also be likely to do so. That "experiment" is conducted daily on large numbers of persons who are exposed to rabies in Cairo, Egypt. The rabies vaccine in current (1975) use in Egypt is a suspension of rabies-virus-infected sheep brain treated with formalin. The dose is 3 ml. taken from a pool of refrigerated suspension in a beaker. There is no record of Creutzfeldt-Jakob disease as a result of the inoculations although the slow "viruses" are notoriously resistant to formalin inactivation (nor are there records of transmission of viral hepatitis by the common needle, nor of postvaccinal encephalitis, which might be expected from the injection of so much brain tissue).

Follow-up studies of vaccinated persons in Cairo might yield abundant data on the relation of sheep brain to Creutzfeldt-Jakob disease, or lack of it. If affirmative, the data could point to enzootically infected flocks.

J. Frederick Bell, M.D.
Hamilton, MT 59840 USPHS Rocky Mountain Laboratory

## MARKER X CHROMOSOMES AND MENTAL RETARDATION

*To the Editor:* It has recently been shown[1,2] that one form of X-linked mental retardation is associated with a fragile site on the long arm of the X chromosome in band Xq27 or 8. I have studied two families in which X chromosomes with such a fragile site are segregating and producing mental retardation in males who received the marker chromosome from their carrier mothers. I have found[3] that it is necessary to culture lymphocytes for chromosome preparations in culture medium 199 if the fragile sites are to be seen. In other commonly used culture mediums (Ham's F10, Eagle's BEM, CMRL 1969 and RPMI 1640 have been tried) the fragile sites are seen either not at all or at such a low frequency that they would not be ascertained by routine clinical cytogenetic analysis. The finding also applies to autosomal fragile sites at 2q1, 10q23, 11q13 and 20p11. As a result of these observations, my laboratory has reverted to using medium 199 for lymphocyte cultures for chromosome analysis.

Detection of the marker chromosome in carrier females is more difficult. Harvey et al [2] could not find the marker X chromosome in two women who were obligate carriers. In both families that I have studied I have not been able to demonstrate the marker chromosome in some of the females who are obligate carriers. I have an impression from the small numbers involved that the older the female, the less likely the demonstration of the marker chromosome.

Prenatal diagnosis of an affected fetus is also likely to be difficult. I have examined fibroblast cultures grown in medium 199 and in Ham's F10 from four retarded boys in two families and have not been able to detect any evidence of the fragile site at Xq27 or 8 although this evidence is present in lymphocyte cultures. Similarly, I have not been able to detect autosomal fragile sites, known to be present in lymphocyte chromosomes, in skin fibroblast chromosomes regardless of the type of culture medium in which they have been grown. Consequently, the suggestion[2] that mental retardation is amenable to prenatal diagnosis should be treated with caution at present, and the offer of anything more than fetal sexing would be unwise until the factors responsible for the induction of fragile sites present in medium 199 can be isolated and perhaps used to induce the sites in fibroblast chromosomes.

North Adelaide, S.A. 5006, Australia Grant R. Sutherland, Ph.D.
Adelaide Children's Hospital, Inc.

1. Giraud F, Ayme S, Mattei JF, et al: Constitutional chromosomal breakage. Hum Genet 34:125-136, 1976
2. Harvey J, Judge C, Wiener S: Familial X-linked mental retardation with an X chromosome abnormality. J Med Genet 14:46-50, 1977
3. Sutherland GR: Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. Science (in press)

## EVALUATION OF BURN CARE

*To the Editor:* The recent paper by Linn, Stephenson and Smith[1] provides some useful insights, particularly into the importance and difficulty of measurements of severity in burn injury, and the quantification of the economic penalties accruing to hospitals providing specialized burn care.

Given the exceptional costs of specialized burn facilities, the questions raised in the article concerning the benefits of these facilities are highly important. The authors note that "Patients in hospitals with special facilities for burn care never did significantly better statistically in mortality or morbidity than patients in hospitals without such special facilities." Two important additional bits of information are essential before any conclusions can be drawn from this finding. In the first place, there is great variability among facilities for treatment of burns. From the article it is not possible to determine what capabilities the three specialized facilities in Florida have. Secondly, whenever a negative conclusion is drawn in a statistical test it is important to know what size difference the test is likely to be able to find. This is a question concerning the statistical power of the test, which can be calculated for matched-pair designs by the method of Miettinin.[2] If the power at important levels of difference is low, these levels of difference are quite likely not to be discovered by the study. The data necessary to calculate this power are not in the article. Thus, we encourage the authors to provide this power calculation. Without such power estimates, negative findings may be misleading.

Adolescent Health, which took place at a recent meeting in Sydney on the subject of the health and medical care of young people. The Association aims to promote the health of young people in Australia, and to encourage the development of appropriate education, research and services in this field. Membership is open to medical practitioners and members of other professional disciplines; inquiries should be directed to me, as interim convener of the Association, at this address.

MURRAY WILLIAMS.
Canberra College of Advanced Education,
P.O. Box 1,
Belconnen, A.C.T. 2616.

## PANIC MEASLES VACCINATION

SIR: The publicity in your press and elsewhere of the current measles epidemic and vaccination seems unfortunate. Surely many children in the late incubation phase or in the early prodroma of measles will now be vaccinated with live attenuated virus. Surely there must be a risk of the vaccination aggravating an existing attack before antibodies become effective. Such an hypothesis could explain the reported high incidence of serious complications, for example, encephalitis (*Daily Telegraph*, October 17). If so, caution should be exercised in vaccination during measles epidemics.

37 Earlsfield Road, TERENCE LEE.
London, SW18 3DB, England.

---

SIR: Terence Lee raises several interesting points regarding the current measles epidemic. Immunization tends to be neglected until an epidemic brings home forcefully to parents the need for protecting their children against certain infectious diseases. Of the viral exanthemata endemic in our community, measles is the most serious. While it occurs every year, the incidence is usually higher in alternate years. As the attenuated measles virus vaccine is both safe and effective it is recommended for all children between the ages of 12 months and 15 months. The use of the vaccine markedly reduces the risks of measles complications, especially pneumonia, encephalitis and subacute sclerosing panencephalitis (SSPE). The attack rate of encephalitis after immunization by live attenuated measles vaccine is about one per million vaccinated persons, compared with one per thousand cases of natural measles.[1] The incidence of SSPE after natural measles was recently estimated to be five to 20 times greater than that after measles vaccination.[2]

If immunization is carried out at the time of an epidemic there is, as Lee points out, the possibility that the vaccine may be given to a susceptible child who has already been exposed to natural measles. No untoward effects have been observed.[1] During the long incubation period of viral exanthemata the numbers of viral particles in the host increase in logarithmic fashion in many tissues, reaching levels in the order of $10^8$ particles per gram of tissue by the late incubation or early prodromal stages of the disease.[3,4,5] The injection of vaccine virus at this time represents an infinitesimal amount of added virus compared with that already present in the host. Should vaccination be carried out shortly after the child has been infected with measles virus it is possible that active immunity may result from the milder vaccine induced attack (which may have a slightly shorter incubation period than wild measles), and so attenuate the attack of natural measles.[1] The best way of protecting a susceptible child contact in whom, because of current ill-health, unmodified measles is inadvisable, is by the administration of pooled human immune globulin.

The article in the *Daily Telegraph* to which Terence Lee refers (Tuesday, October 17, 1978) did not actually state that the incidence of serious complications in the present measles epidemic is any higher than expected. It stated that the epidemic was extensive and that Fairfield Hospital, Sydney, had received 26 serious cases including six patients in a critical condition because of complications, four with encephalitis and two with pneumonia; similarly, 23 patients had been admitted to Liverpool District Hospital with severe measles in the past two months. Patients referred to hospital usually represent the tip of the iceberg, that is, those patients with severe attacks or complications.

These figures confirm what has already been said that measles is a severe disease, it always has been, and all children should now be protected against it by means of active immunization.

C. R. BOUGHTON.
The Prince Henry Hospital,
Anzac Parade,
Little Bay, N.S.W. 2036.

[1] Krugman, S., and Ward, R., *Infectious Diseases of Children and Adults*, 5th ed., C. V. Mosby, St Louis, 1973: 439.

[2] *Morbidity and Mortality Weekly Report*, Subacute sclerosing panencephalitis and measles, United States Department of Health, Education and Welfare, 1977, September 23, No. 26: 309.

[3] Fenner, F., *Lancet*, 1948, 2: 915.

[4] Fenner, F., *J. Path. Bact.*, 1948, 65: 29.

[5] Mims, C. A., *The Pathogenesis of Infectious Diseases*, Academic Press, London, 1976.

## CARRIER DETECTION IN X-LINKED MENTAL RETARDATION

SIR: Dr Mulcahy (Journal, November 4) has suggested that the report by Harvey *et alii*[1] of an association between "terminal appendages" on the distal end of the long arm of the X chromosome and one form of X-linked mental retardation is unproven.

Data from this laboratory [2-6] have shown that the "terminal appendages" are due to the presence of a fragile site in bands Xq27 or 28. For this fragile site to be expressed in lymphocyte cultures it is necessary that the concentration of folic acid and thymidine in the culture medium be close to zero. The fragile site on the X chromosome has not been seen in bone marrow or skin fibroblast derived chromosomes. The fragile site at Xq27 or 28 behaves similarly with regard to folic acid and thymidine sensitivity as other heritable fragile sites at 2q1, 10q23, 11q13, 16p12 and 20p11, but differently from the one at 16q22.

The association of the fragile site with mental retardation[1] has been confirmed in four extended families and a number of other retarded males. The form of X-linked retardation is almost certainly the same[6] as that which has been described in association with macro-orchidism,[7-9] this clinical finding being present well before puberty (the youngest such child examined was $3\frac{1}{2}$ years old).[6] There has never been any suggestion that all X-linked mental retardation is associated with the fragile site on Xq: two further families suspected of having X linked mental retardation in them do not have the fragile site.

There are considerable difficulties in carrier detection. The proportion of metaphases from any individual showing the fragile site can be increased by manipulation of the culture medium. The "best recipe" which can be readily prepared is culture medium 199 buffered with 20mM hepes to pH 7.6 to pH 7.8. There is a strong correlation between pH and the proportion of metaphases showing the fragile site. However, even using this medium, carrier detection is difficult. I have not been able to demonstrate the fragile site in obligate carrier females over the age of 35 years, although at least six such females have been extensively studied. In females up to the age of about 20 years the fragile site can be readily demonstrated although the reliability with which this can be done is uncertain.

I would disagree with Dr Mulcahy that carrier detection is unwarranted and unwise. In some families with X-linked mental retardation the demonstration of the fragile site and macro-orchidism can establish the diagnosis unequivocally. In families without the fragile site the diagnosis of X-linked retardation can only be suggested from the pedigree: there is no definitive diagnosis in these families. Because of the difficulty of carrier detection in older females it can be extremely difficult to determine whether a single case in a family is the result of mutation, but if the mother is young and there are related young females in the family available for examination the quality of genetic counselling can be greatly enhanced by carrier detection studies.

I would agree with Dr Mulcahy that prenatal diagnosis presents considerable problems. Since the fragile site is not seen in skin fibroblast derived chromosomes it is unlikely that it would be present in amniotic fluid derived chromosomes. One can speculate that, if chromosome study can be successfully carried out on fetal blood samples obtained at fetoscopy, the fragile site would be expressed and definitive prenatal diagnosis would be possible.

Since this form of mental retardation is not rare (there are 18 males in South Australia known to suffer from it) any diagnostic clinical cytogenetic study of a retarded male in which the possibility of a fragile site at Xq27 or 28 is not excluded must be regarded as incomplete.

GRANT R. SUTHERLAND.
Cytogenetics Unit,
Adelaide Children's Hospital Inc.,
North Adelaide, S.A. 5006.

[1] Harvey, J., Judge, C., and Wiener, S., *J. med. Genet.*, 1977, 14: 46.

[2] Sutherland, G. R., *Science*, 1977, 197: 265.

[3] Sutherland, G. R., *New Engl. J. Med.*, 1977, 296: 1415.

[4] Sutherland, G. R., *Amer. J. hum. Genet.*, 1979 *(a)*, in the press.

[5] Sutherland, G. R., *Amer. J. hum. Genet.*, 1979 *(b)*, in the press.

[6] Sutherland, G. R., and Ashforth, P. L. C., 1978 submitted for publication.

[7] Turner, G., Eastman, C., Casey, J., *et alii*, *J. med. Genet.*, 1975, 12: 367.

[8] Cantú, J. M., Scaglia, H. E., Medina, M., *et alii*, *Hum. Genet.*, 1976, 33: 23.

[9] Cantú, J. M., Scaglia, H. E., González Diddi, M., *et alii*, *Hum. Genet.*, 1978, 41: 331.

---

SIR: We would like to support Grant Sutherland's letter advocating screening for Xq27h in families with X-linked mental retardation. Our findings are almost identical to those which he reports. We have found that, in one third of families with histories consistent with X-linked mental retardation, all of the affected males have had the Xq27h abnormality. All of these affected males have also had macro-orchidism.

In complete contrast to the uniform presence of Xq27h in the affected males, not all of the carriers demonstrate this abnormality. Eight such families have been studied. Four of the 11 obligatory carriers and five of the 16 possible carriers have had the X chromosome marker. Our evidence supports Dr Sutherland's contention that any study of X-linked mental retardation should include Xq27h studies. This research was supported by a grant from the Apex Foundation for Research into Mental Retardation Limited.

GILLIAN TURNER,
ROSALIND GILL,
ART DANIEL.
Department of Preventive Paediatrics
and Cytogenetics Unit,
Prince of Wales Children's Hospital,
Randwick, N.S.W. 2031.

*Am J Hum Genet* 31:125–135, 1979

# Heritable Fragile Sites on Human Chromosomes I. Factors Affecting Expression in Lymphocyte Culture

GRANT R. SUTHERLAND[1]

SUMMARY

The expression of heritable fragile sites on human chromosomes has been shown to be dependent upon composition of the tissue medium for sites at 2q11, 10q23, 11q13, 16p124, 20p11 and Xq27 or 28 but not for the site at 16q22. Expression of the fragile sites is inhibited by folic acid, thymidine, folinic acid, and probably bromodeoxyuridine, and induced by methotrexate. In addition, there is a correlation between frequency of expression of the sites and pH of the culture medium for the sites on 2q, 10q and Xq. Possible reasons for these findings are discussed, and a definition and classification of fragile sites is proposed.

Heritable fragile sites on human chromosomes have been the subject of numerous reports since that of Dekaban [1]. There are now eight well documented heritable fragile sites and others awaiting confirmation [2]. Expression of fragile sites in metaphase chromosomes obtained from lymphocyte cultures only occurs when culture medium 199 is used compared to several other media tested [3]. Medium 199 is relatively deficient in folic acid. Preliminary studies showed folic acid to be important in that it inhibited expression of some of the sites which could also be induced by the folic acid antagonist methotrexate. Some mutant cell lines grow in the presence of folic acid inhibitors if thymidine, glycine, and hypoxanthine are present [4]. These compounds were tested, but only thymidine was found to affect expression of fragile sites. It was found, incidentally, that the pH of the culture medium had an effect on some fragile sites. A systematic examination of these factors was carried out, and a definition and classification of fragile sites is proposed.

Received August 29, 1978; revised November 1, 1978.

[1] Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A., 5006 Australia.

## MATERIALS AND METHODS

Fragile sites studied were from individuals and families documented in the following paper [2]. Lymphocyte cultures were established and harvested after 72 hr as previously described [3] with the exception that the fetal bovine serum supplement to the culture medium was reduced to 5%. Various culture media (as indicated in the results section) were used. These were purchased from Commonwealth Serum Laboratories, Melbourne, Australia, either as single strength media or as concentrates. All media were standard commercial products except for a special batch of Eagle's minimal essential medium (MEM-FA) for suspension cultures from which folic acid had been omitted (table 1). Some medium was buffered with bicarbonate, whereas for other experiments 20 mM HEPES was used.

MEM-FA with various concentrations of thymidine (Sigma, St Louis, Mo.) and folic acid (Abbotts Laboratories, Chicago, Ill.) were used for studying the effects of these components. The effect of the time of addition of folic acid and thymidine was studied by adding these at concentrations of 1.0 and 5.0 mg/l respectively at varying times during culture. Cultures set up in Ham's F10 (bicarbonate buffered) had 10 mg/l of methotrexate (Lederle, Pearl River, N.Y.) added at varying times during culture. HEPES buffered MEM-FA was set up over a pH range from approximately 6.8–8.0. The pH of each culture was measured at the time of addition of colchicine using a Corning 175 automatic blood pH and gas analysis meter. Fifty cells per culture were scored to determine the frequency of expression of the fragile sites.

## RESULTS

### *Effect of Folic Acid*

The effect of addition of folic acid on the frequency of lesions at the fragile sites is shown in figure 1*a* and *b*. Some sites require higher concentrations of folic acid to inhibit their expression than others. The majority are almost completely inhibited by 0.2 mg/1, although the sites on 2q and 10q usually required 0.5–1.0 mg/1 for inhibition. The site on 16q appears to be independent of folic acid concentration over the range shown and showed no sensitivity up to 80 mg/1. The frequency of the

TABLE 1

COMPOSITION OF MEM-FA MEDIUM USED

| Component | Amount/liter | Component | Amount/liter |
|---|---|---|---|
| NaCl | 6.8 g | 1-Methionine | 15 mg |
| KCl | 0.4 g | 1-Phenylalanine | 32 mg |
| $MgCl_2$, $6H_2O$ | 0.2 g | 1-Threonine (allo-free) | 48 mg |
| $NaH_2PO_4.2H_2O$ | 1.6 g | 1-Tryptophane | 10 mg |
| Glucose | 1.0 g | 1-Valine | 46 mg |
| $NaHCO_3$ | 1.13 g | 1-Cystine | 24 mg |
| Choline dihydrogen citrate | 2.2 mg | 1-Tyrosine | 36 mg |
| i-Inositol.$2H_2O$ | 2.4 mg | 1-$\alpha$-Alanine | 8.9 mg |
| Nicotinamide | 1.0 mg | 1-Asparagine.$H_2O$ | 15.0 mg |
| Calcium-d-pantothenate | 1.0 mg | 1-Aspartic acid | 13.3 mg |
| Pyridoxal.HCl | 1.0 mg | 1-Glutamic acid | 14.7 mg |
| Riboflavin | 0.1 mg | 1-Proline | 11.5 mg |
| Thiamine.HCl | 1.0 mg | 1-Serine | 10.5 mg |
| 1-Arginine.HCl | 105 mg | Glycine | 7.5 mg |
| 1-Histidine.HCl.$H_2O$ | 31 mg | 1-Glutamine | 292 mg |
| 1-Isoleucine (allo-free) | 52 mg | Phenol red | 10 mg |
| 1-Leucine | 52 mg | Penicillin | 100,000 U |
| 1-Lysine.HCl | 58 mg | HEPES | 20 mM |

satellited appearance of chromosome 17 in one sample of blood was independent of folic acid concentration over the range from 0–80 mg/1.

The time of addition of the folic acid is important (fig. 1*c* and *d*). For those sites which are folic acid sensitive, expression of the fragile sites is almost totally inhibited if the folic acid is added 24 hr prior to harvest. (The site on 16p has not been studied this way.) The expression is progressively inhibited if the folic acid is added during the last 24 hr of culture. Even when added with the colchicine (2 hr prior to harvest) the frequency of expression is usually reduced compared to cultures which remain folic acid free (shown as time = 0 in fig. 1*c* and *d*).

Folinic acid has a similar affect to folic acid. At a concentration of 5 mg/1 it almost totally inhibited expression of sites on 2q, 10q, 11q, 16p, and Xq. (The other folic acid sensitive sites were not studied.) The constriction on 17p was not effected by 40 mg/1 of folinic acid. (The site on 16q was not studied.)

*Effect of Thymidine*

The effect of the addition of thymidine on the frequency of expression of the fragile sites is shown in figure 2*a* and *b*. The inhibitory effect on the expression of the fragile sites is less rapid and less complete with increasing concentration than for folic acid. The frequency of lesions at the sites is reduced to less than 10% by 3 mg/1 of thymidine for all sites studied except 16q, which is independent of thymidine concentrations up to 20 mg/1. This site (subject B, [2]) was in the same cell as one on Xq; while the site on Xq was inhibited by folic acid and thymidine, the one on 16q was not. The satellited chromosome 17 showed no inhibition by concentrations of thymidine up to 40 mg/1.

The time of addition of thymidine is also important (fig. 2*c* and *d*). For thymidine sensitive sites, (the site on 16p was not studied in this way) maximum inhibition is 24 hours prior to harvest. As with folic acid, addition of thymidine continues to be inhibitory up to the time of colchicine addition. Preliminary experiments have shown that bromodeoxyuridine (BrdU) at 10 mg/1 inhibits expression of the sites almost completely for the sites on 2q and Xq, but not the one on 16q. Cytidine and uridine at 10 mg/1 did not inhibit sites on 2q, 16q, or Xq.

The effects of thymidine and folic acid are somewhat additive. Figure 3 shows the effect of varying both folic acid and thymidine concentrations simultaneously on expression of the fragile site at 2q1. This was done in MEM-FA buffered with bicarbonate. This is the reason for the apparent increase in frequency of lesions when the folic acid concentration is 0.01 mg/1 compared with zero. The folic acid was in an alkaline solution which raised the pH of the culture and hence the frequency of expression of the sites, especially at the lowest folic acid concentrations (see later). Similar curves have been obtained for the other folic acid and thymidine sensitive fragile sites. The site on 16q did not show inhibition by folic acid (10 mg/1) and thymidine (10 mg/1) in combination.

*Effect of Methotrexate*

In view of the sensitivity of fragile sites to folic acid, the effect of methotrexate was studied on lymphocytes cultured in Ham's F10. This medium contains 1.32 mg/1 of folic acid and 0.73 mg/1 of thymidine and almost totally inhibits expression of fragile

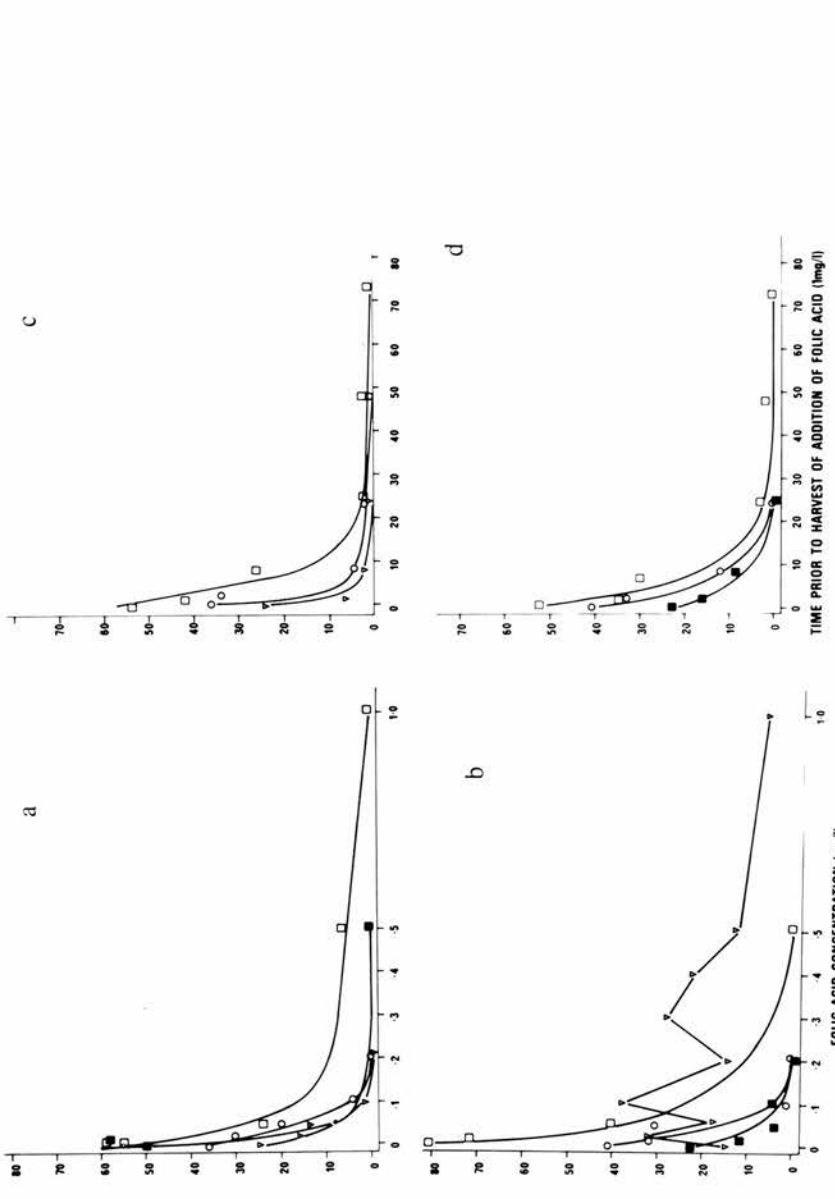



FIG. 1.—*a*, *b*, Effect of addition of folic acid at various concentrations on frequency of lesions at fragile site for sites at: *a*, 2q1 (□ – □), 16p12(■ – ■), 20p11(0–0), and Xq27 or 28(▽ – ▽) from subject B [2], and *b*, 10q23(□ – □), 11q13(■ – ■), 16q22(▽–▽), and Xq27 or 28(0–0) in a male with only this site being present. MEM-FA buffered with HEPES to pH 7.35 used for all sites except 2q11, 16p12, and 10q23 which were in the same medium, but with bicarbonate buffer. *c*, *d*, Effect of addition of 1 mg/l folic acid during period of culture on frequency of lesions at fragile sites; time in hours; *c* sites identified as in *a*; *d* sites identified as in *b*. All results in MEM-FA buffered with HEPES except site at 10q23 which was in bicarbonate buffered medium 199.

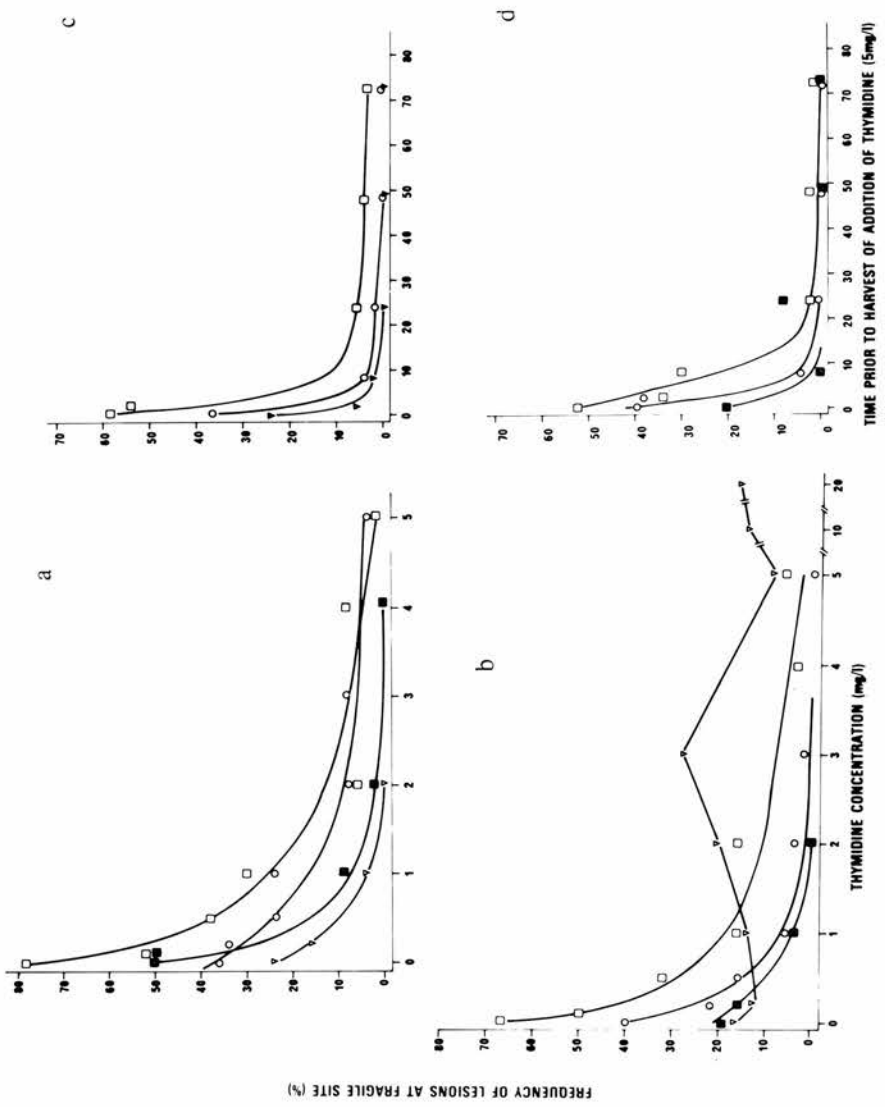



FIG. 2.—*a*, *b*, Effect of addition of thymidine at various concentrations on frequency of lesions at fragile sites. Sites and media as in figure 1*a* and *b* respectively. *c*, *d*, Effect of addition of thymidine (5 mg/l) during culture on frequency of lesions at fragile sites—sites and media as in figure 1*c* and *d* respectively, except 2q1 was in bicarbonate buffered medium 199. Time in hours.

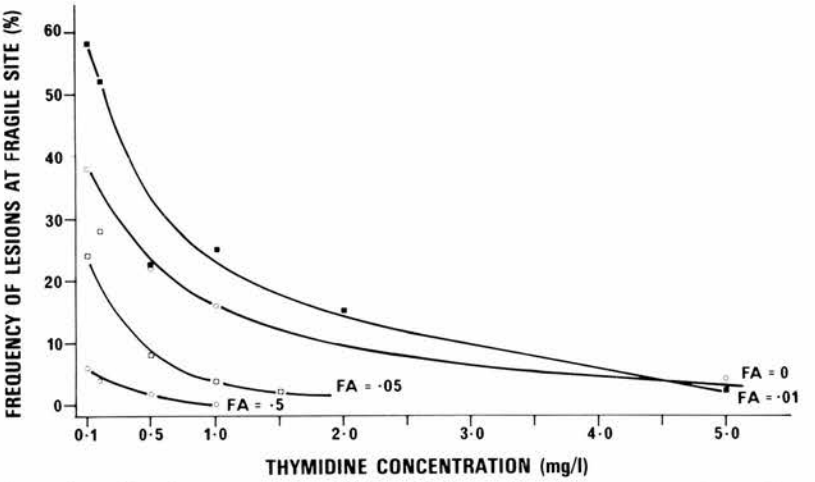



FIG. 3.—Effect of simultaneous variation of folic acid (*FA*) and thymidine concentrations on frequency of lesions at 2q1. *FA* concentration is in mg/1; in MEM-FA with bicarbonate buffer.

sites. Methotrexate caused poor growth of the cultures which led to some problems. At a concentration of 10 mg/1, sufficient growth could usually be obtained to allow 50 metaphases to be scored. Figure 4*a* and *b* show the effect of addition of methotrexate (10 mg/1) throughout the period of culture. Note that methotrexate induces lesions at the fragile site if added 24 hr or more before harvest, but that its effect diminishes during the last 24 hr of culture. Methotrexate is not as effective or consistent at inducing lesions under these conditions as a folic acid-deficient medium. Table 2

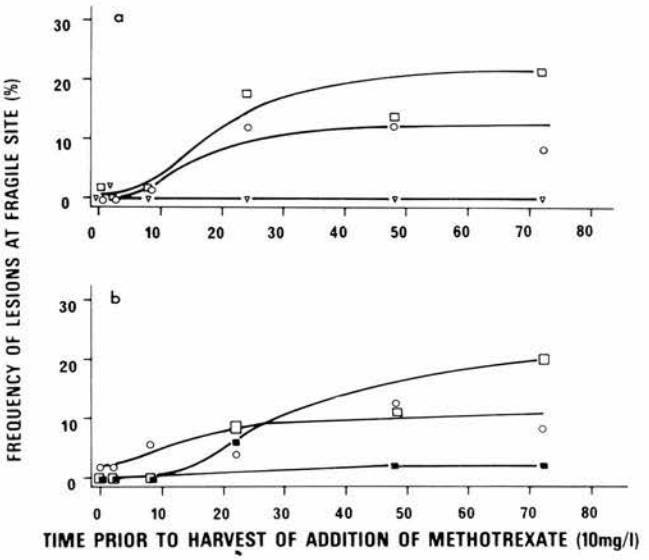



FIG. 4.—Effect of addition of methotrexate (10 mg/1) in inducing lesions at fragile sites in lymphocytes cultured in Ham's F10. Sites as in figure 1*a* and *b* respectively. Time in hours.

TABLE 2

EFFECT OF ADDITION OF 10 mg/l OF MT ON EXPRESSION OF FRAGILE SITES COMPARED WITH GROWTH IN FA DEFICIENT CULTURE MEDIUM

| SITE | CULTURE MEDIUM | | |
|---|---|---|---|
| | Ham's F10 | Ham's F10 and Mt | MEM-FA (pH 7.35) |
| 2q11 | 3/100 | 27/100 | 47/100 |
| 10q23 | 1/100 | 19/100 | 60/100 |
| 11q13 | 1/100 | 5/100 | 22/100 |
| 16p124 | 0/ 50 | 6/ 50 | 20/ 40 |
| 16q22* | 8/100 | 15/ 89 | 8/ 50 |
| 20p11 | 0/100 | 14/100 | 36/100 |
| Xq27 or 8* | 0/100 | 2/ 89 | 12/ 50 |
| X27 or 8 | 0/100 | 8/100 | 40/100 |

* Both fragile sites in the same patient, subject B [2].

shows the frequency of expression of the fragile sites in MEM-FA and in Ham's F10 with and without methotrexate. Allowing for this difference in frequency of lesions at the sites, it can be seen that the shape of the curve in figure 4*a* and *b* is approximately the inverse of that in figure 1*c* and *d*. Methotrexate did not effect the expression of the site on 16q, and the satellited 17 was not studied in this manner.

*Effect of pH*

The experiments with folic acid showed that its addition to the medium caused a pH rise, hence a control experiment was conducted to ensure that the inhibition of fragile sites was not due to pH. Increasing the pH caused the frequency of lesions at the fragile sites to increase rather than decrease. This pH effect was studied, and the results are shown in figure 5*a* and *b*. The effect was highly significant for the sites on 2q, 20p, and Xq, but not for those on 10q, 11q, or 16q. Preliminary data suggests that the pH effect may not be significant for the satellited 17, but for the site on 16p the results were equivocal.

## DISCUSSION

Based on data in this paper and in [2], the following definition of a fragile site is proposed: A fragile site is a specific point on a chromosome which is liable to show the following features: (1) a non-staining gap of variable width which usually involves both chromatids, (2) the site is always at exactly the same point on the chromosome in cells examined from any individual patient or kindred, (3) the site is inherited in a Mendelian dominant fashion, and (4) fragility must be evident by the production (under appropriate in vitro conditions) of acentric fragments, deleted chromosomes, triradial figures, and the like. In accord with the Paris Conference [5], fragile sites will be described by the triplet *fra* such that the correct designation of the site on 2q would be fra(2)(q11). The practice of recording them as *h*+ should be discontinued as there is no evidence that heterochromatin is involved.

The fragile sites which suit the proposed definition are those indicated in figure 9 of the following paper [2], by the solid arrows. The satellited chromosome 17 does not fit

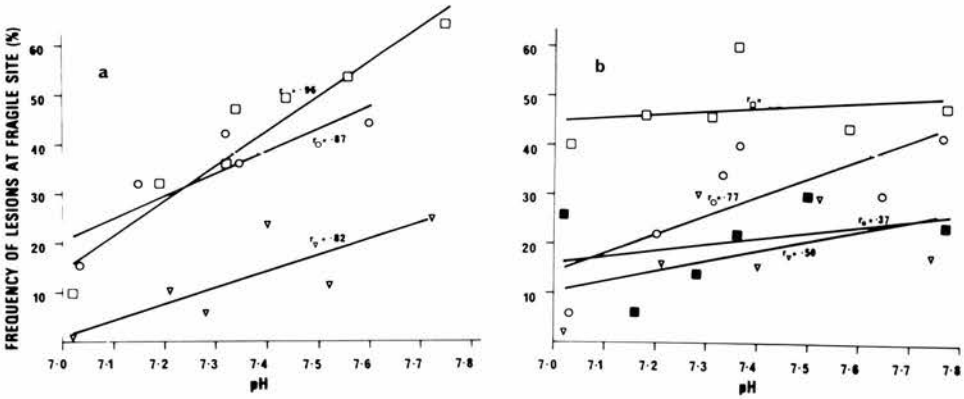



FIG. 5.—Effect of pH on frequency of lesions at fragile sites (identified as in fig. 1*a* and *b* respectively). Linear regression lines are shown. The correlation coefficients in *a* (2q, r=.96, p<.001; 20p11, r=.87, p<.01; Xq27 or 8 from subject B [2], r=.82, p<.001) are all statistically significant. In *b* the correlation coefficients (10q24, r=.250, N.S.; 11q13, r=.37, N.S.; 16q22, r=.50, N.S.) are not significant except for the site at Xq27 or 28 from a retarded male with no other fragile site (r=.77; p<.01).

this definition, since the constriction which gives rise to this has not been shown to be fragile. A classification of fragile sites can be made on the basis of their in vitro behavior. A proposed classification is shown in table 3. This must be regarded as tentative, as some fragile sites have been studied only in a single individual and in most cases, in a single kindred. The only established fragile site which has not been available for study is that at 12q13. This must await classification, but there is no doubt that it fits the proposed definition of a fragile site (H. R. McCreanor, personal communication, 1977). The site at 11q13 is certainly a fragile site, but it has yet to be shown to be heritable.

Fragile sites had been regarded as rare variants, and their dependence on conditions of culture was not known prior to an earlier report from this laboratory [3]. Bühler et al. [6] had investigated the effect of different techniques of harvesting cultures on expression of a fragile site at 2q1 but reached no conclusions. The reduction of expression of the fragile site in their autoradiography preparations was not commented upon. It was presumably due to the use of thymidine as a radioactive label. However, the addition of tritiated thymidine has been reported to have reduced the frequency of lesions at a site at 2q1 [7]. Noël et al. [8] reported a much lower frequency of triradial

TABLE 3

CLASSIFICATION OF HERITABLE FRAGILE SITES ACCORDING TO THEIR EXPRESSION UNDER DIFFERENT TISSUE CULTURE CONDITIONS. RESPONSE TO FOLIC ACID AND THYMIDINE

| | SENSITIVE | |
|---|---|---|
| RESISTANT | pH correlated | pH independent |
| 16q22, (??17p) | 2q11, 20p11, Xq27 or 28 | 10q23, 11q13*, 16p124 |

* Not yet shown to be heritable.

figures in blood cultures from a patient with a fragile site at 2q1 when BrdU had been added compared with BrdU free cultures. These studies are the only corroboration available in the literature for the findings in the present report.

In most cells in which fragile sites are expressed, the appearance is that of an isochromatid gap. In only a small proportion of cells has there been a break at the gap giving rise to an acentric fragment, triradial, or other manifestation of fragility. Chaudhuri [9] examined the nature of chromatid gaps (although not those associated with fragile sites) and concluded that gaps result from extreme despiralization of the DNA due to failure of compact folding in the metaphase chromosome. He considered four factors to be involved in spiralization of chromosomes: the DNA itself, histones, non-histone proteins, and divalent cations. Increasing the calcium ion concentration in culture medium does not effect the expression of fragile sites (Sutherland, unpublished results). Since fragile sites could be heritable isochromatid gaps, they are probably manifestations of DNA coded information.

Sporadic chromatid gaps are thought to be due to some process occurring in $G_2$, but occurring in $G_1$ for isochromatid gaps. The frequency of expression of fragile sites can be altered by changes to the composition of the culture medium at the time of addition of colchicine (2 hr prior to harvest). Therefore, expression of the sites must be influenced directly at the time of spiralization of the chromatids in late $G_2$ or early prophase. This process would have to be a reversible one. The nature of this process, the disruption of which could be involved in the expression of the fragile sites, remains unclear.

An alternative explanation is suggested by the nature of the inhibitors and inducer of fragile sites indicating that the process of expression is more likely to be operating during DNA synthesis. The area of metabolism involved could be the following reaction catalyzed by thymidylate synthetase [10, 11]: Uridine monophosphate (dUMP) + 5,10-methylene tetrahydrofolate (5,10-meTHFA) → thymidine monophosphate (dTMP) + dihydrofolate (DHFA). If this reaction were slower than normal, or if more than a normal amount of dTMP were required, there could be a relative deficiency of dTMP available for DNA synthesis. That this might be the area of metabolism involved is evidenced by the inhibition of fragile sites directly by thymidine and probably by its analogue, BrdU. Furthermore, an increase in folic acid concentration could result in an increase in THFA and 5,10-MeTHFA, leading to increased production of dTMP. Induction of the fragile sites by methotrexate can be explained in terms of the above reaction, since this blocks the conversion of DHFA back to THFA by inhibition of dihydrofolate reductase. This would inhibit production of dTMP.

This alternative explanation implies that the fragile site is due to a section of thymidine rich DNA (or DNA which cannot complete synthesis when the thymidine supply is restricted) and consequently does not compact normally for mitosis. If the chromosomes come through metaphase intact, this could be repaired during $G_1$ or early in the next S period. DNA synthesis studies with tritiated thymidine are being done to see if expression of fragile sites is determined during S or $G_2$.

Fragile sites could also be sites of viral DNA modification. If so, the virus may only be able to modify specific DNA sequences. Adenovirus 12 causes specific lesions on chromosomes 1 and 17 [12, 13, 14] which superficially resemble the lesions seen at

fragile sites. Study of individuals with fragile sites for abnormal DNA may be rewarding.

The effect of the culture medium's pH on the expression of some fragile sites could result from almost any area of metabolism affected by this factor [15]. For example, folic acid has different ionic forms at different pHs [16]; these may have different rates of transport into the cells. Furthermore, Ceccarini [17] has shown that the cellular uptake of thymidine is affected by the pH of the culture medium. This pH effect is of practical value in that the culture medium can be modified to maximize expression of fragile sites. Apparently the best formula for this purpose is MEM-FA buffered to about pH 7.6 with HEPES. Detection of fragile sites is important in the case of the clinically relevant site on Xq; no clinical cytogenetic report on a retarded male can be regarded as complete without positive exclusion of this fragile site.

ACKNOWLEDGMENTS

I thank Lynene Day, Helen Eyre, Trudy Hocking and Erica Woollatt for technical assistance and Dr. D. L. Hayman for helpful discussions during the preparation of this report. Mr. Andrew MacGregor of Commonwealth Serum Laboratories provided useful information about tissue culture medium and the special MEM-FA.

REFERENCES

1. DEKABAN A: Persisting clone of cells with an abnormal chromosome in a woman previously irradiated. *J Nucl Med* 6:740–746, 1965
2. SUTHERLAND GR: Heritable fragile sites on human chromosomes II. Distribution, phenotypic effects and cytogenetics. *Am J Hum Genet* 31:136–148, 1979
3. SUTHERLAND GR: Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. *Science* 197:265–266, 1977
4. LITTLEFIELD JW, BASILICO C: Infection of thymidine kinase-deficient BHK cells with polyoma virus. *Nature* 211:250–252, 1966
5. PARIS CONFERENCE (1971) SUPPLEMENT (1975). Standardization in human cytogenetics. *Birth Defects: Orig Art Ser* 11(9), 1975
6. BÜHLER EM, LUCHSINGER U, BÜHLER UK, MÉHES K, STADLER GR: Non-condensation of one segment of a chromosome no. 2 in a male with otherwise normal karyotype (and severe hypospadias). *Humangenetik* 9:97–104, 1970
7. FRACCARO M, LINDSTEN J, TIEPOLO L, RICCI N: Instability of the paracentric region and selective reduplication of chromosome 2 in man. *Chromosomes Today* 3:138–146, 1972
8. NOËL B, QUACK B, MOTTET J, NANTOIS Y, DUTRILLAUX B: Selective endoreduplication or branched chromosome? *Exp Cell Res* 104:423–426, 1977
9. CHAUDHURI JP: On the origin and nature of achromatic lesions. *Chromosomes Today* 3:147–151, 1972
10. ERBE RW: Inborn errors of folate metabolism. *New Engl J Med* 293:753–757, 1975
11. O'DONOVAN GA, NEUHARD J: Pyrimidine metabolism in microorganisms. *Bacteriol Rev* 34:278–343, 1970
12. ZUR HAUSEN H: Induction of specific chromosomal aberrations by adenovirus type 12 in human embryonic kidney cells. *J Virol* 1:1174–1185, 1967
13. MCDOUGALL JK: Adenovirus induced chromosome aberrations in human cells. *J Gen Virol* 12:43–51, 1971
14. MCDOUGALL JK, KUCHERLAPATI R, RUDDLE FH: Localisation and induction of the human thymidine kinase gene by adenovirus 12. *Nature (New Biol)* 245:172–175, 1973
15. EAGLE H: The effect of environmental pH on the growth of normal and malignant cells. *J Cell Physiol* 82:1–8, 1973
16. KALLEN RG, JENCKS WP: The dissociation constants of tetrahydrofolic acid. *J Biol Chem*

241:5845–5850, 1966
17. CECCARINI C: Effect of pH on plating efficiency, serum requirement and incorporation of radioactive precursors into human cells. *In Vitro* 11:78–86, 1975

*Am J Hum Genet* 31:136–148, 1979

# Heritable Fragile Sites on Human Chromosomes II. Distribution, Phenotypic Effects, and Cytogenetics

GRANT R. SUTHERLAND[1]

SUMMARY

Individuals and families have been documented in which there are a number of fragile sites on chromosomes. These include sites at 2q11, 10q23, 11q13, 16p124, 16q22, 20p11, and Xq27 or 28. Fragile sites reported in the literature are compiled. The cytogenetics of the sites is discussed. The phenotypic effects of the sites are considered, and it is speculated that homozygosity of the autosomal sites might be deleterious as is hemizygosity of the site on Xq. These sites are used in the previous report which documents the effect of tissue medium components on their expression.

Since Dekaban [1] reported the first fragile site on the long arm of a C-group chromosome in 1965, there have been numerous reports of fragile sites on a variety of chromosomes. Lejeune [2] was the first to show that such sites were heritable when he described a site at 2q1 in a woman and her daughter. Most fragile sites have been regarded as normal variants. One, however, on the end of the long arm of the X chromosome has been shown to be a marker for one form of X-linked mental retardation [3, 4, 5].

Sutherland [6, 7] showed that the expression of fragile sites in lymphocyte culture was dependent upon the composition of the tissue culture medium used. The main factor necessary for expression of these sites is that the culture medium be deficient in folic acid and thymidine. This report documents families and individuals with fragile sites located at 2q11, 10q23, 11q13, 16p124, 16q22, 20p11, and Xq27 or 28. Lymphocytes from individuals in these families were used for the studies reported in the preceeding paper [7].

Received August 29, 1978; revised November 1, 1978.

[1] Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A., 5006, Australia.

## MATERIALS AND METHODS

All lymphocyte cultures were set up and harvested as previously described [6]. In some cases Eagle's minimal essential medium (MEM) was used rather than medium 199, and the fetal bovine serum concentration was reduced to 5% in most cultures in the latter part of this study. Some cultures were grown in a special folic acid free MEM (MEM-FA), [7]. Bone marrow was collected directly in either MEM or medium 199 containing 1 $\mu$g/ml colchicine and harvested 2 hr later using the same method as for blood lymphocyte cultures (except that the exposure time to .075M KCl was 20 min). Lymphoblastoid cultures were established by the method of Pope et al. [8] except that they were cultured in medium 199 supplemented with 10% fetal bovine serum.

Skin fibroblast cultures were grown in medium 199 and harvested for chromosome studies using a standard trypsin method.

Until 1973 this Cytogenetics Unit used medium 199 for routine diagnostic work, and some families in this report had been detected prior to that time. Since late 1976, all diagnostic lymphocyte cultures have been grown in medium 199 and Ham's F10, and additional families ascertained. Institutionalized retarded males were screened by lymphocyte culture to detect individuals with a fragile site at Xq27 or 28. In routine diagnostic cultures 30 metaphases have been examined. At least 50 metaphases have been examined looking for fragile sites on the X. All data presented on the frequency of fragile sites are based on examination of at least 50 cells.

## FAMILY STUDIES

### *Family F*

This family (fig. 1*a*) has a fragile site at 2q1. The propositus was a severely retarded boy referred for chromosome study at 12 years as part of investigation of his retardation. He has no major physical malformations and no satisfactory explanation for his retardation has been found. Cytogenetic results have been described [6]. The chromosomal expressions of the fragile site are shown in figure 2.

### *Family Ay*

This family (fig. 1*b*) has a fragile site on 10q23. The propositus was a 31-year-old retarded schizophrenic male referred for chromosome study as part of an investigation of his severe mental retardation of unknown origin. Cytogenetic results have been previously summarized for the propositus [6]. Different forms resulting from this fragile site are shown in figure 3.

### *Subject At*

This girl has a fragile site at 11q13. At 11 years she was retarded, had epilepsy, and spastic quadriplegia. Her retardation was thought to be due to a combination of prematurity, maternal pre-eclamptic toxemia, and a neonatal convulsion. Initial cytogenetic studies in 1973 showed a fragile site to be present in 20% of cells examined. In 1977 the site was seen in 44% of cells cultured in medium 199. Extensive studies of her parents revealed no evidence of the fragile site. The appearances of this site are shown in figure 4.

### *Family D*

This family (fig. 1*d*) has a fragile site at 16p124. The proposita was a 7-year-old girl with Laurence-Moon-Biedl syndrome and an ill-defined lymphoreticular malignancy. Her parents were nonconsanguineous. An older sister had died at 17 years

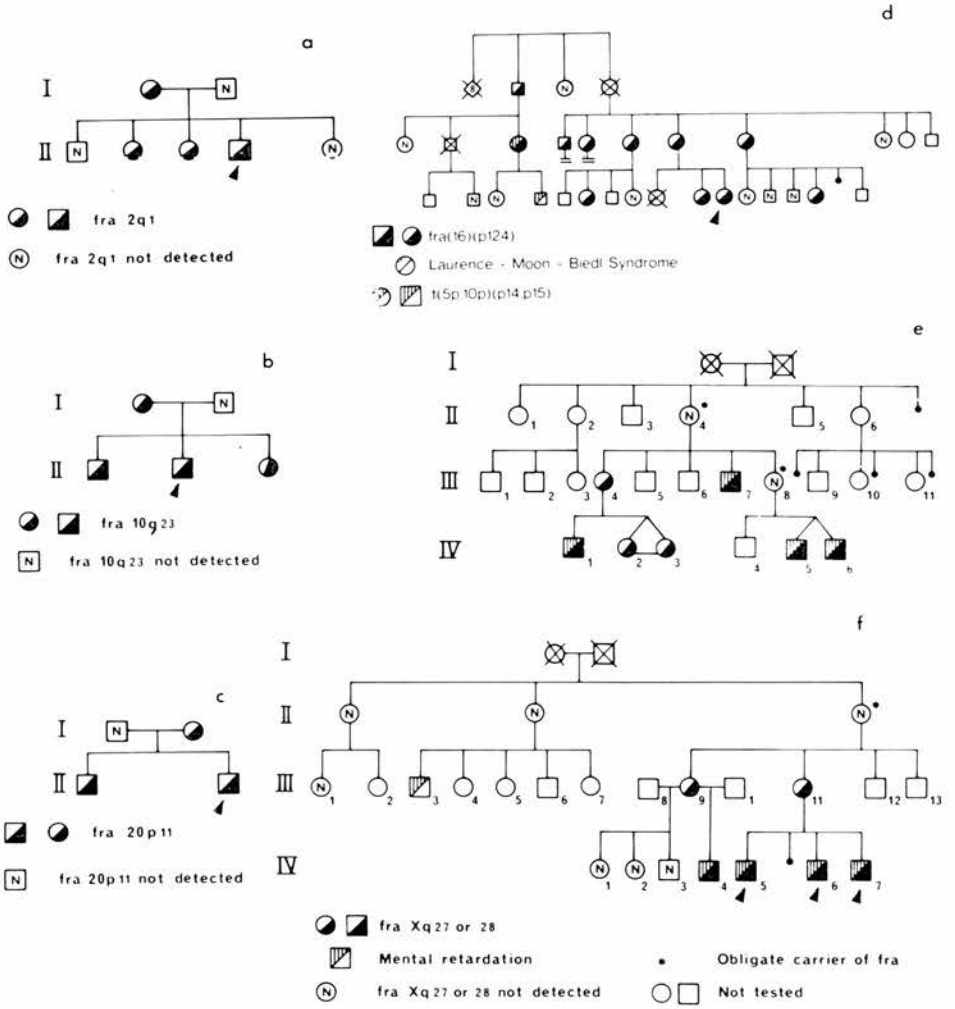



FIG. 1.—Family pedigrees: *a*, Family F; *b*, Family Ay; *c*, Family Mi; *d*, Family D, *e*, Family E; *f*, Family Ma. Note—symbols for *e* and *f* are the same.

from what appeared to be the same condition. The proposita is of normal intelligence and has responded well to standard leukemia remission induction therapy. The appearances of the fragile site are shown in figure 5.

*Subject B*

This 29-year-old mildly retarded male was detected in an institutional survey. He has fragile sites at 16q22 and Xq27 or 28. The cause of his retardation was unknown; he has no physical abnormalities. Family studies have not yet been carried out. The appearances of his fragile sites are shown in figure 6.

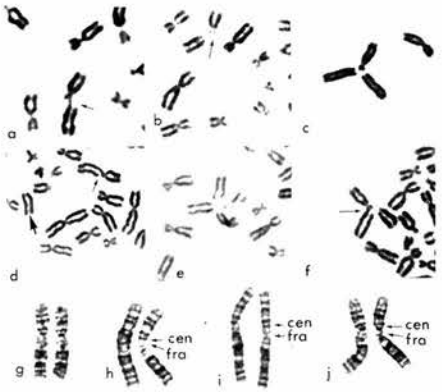



FIG. 2.—Appearances of the fragile site on 2q; *a*, single chromatid break; *b*, chromosome break; *c*, triradial configuration; *d*, single chromatid break (*small arrow*) and acentric fragment (*large arrow*) of $2q_1 \rightarrow 2qter$; *e*, quadriradial configuration; *f*, lesion at the fragile site from a skin fibroblast metaphase; *g*–*j*, G-banded chromosomes showing the break point for the site at 2q at the distal end of 2q11; *g*, no lesion at fragile site; *h*–*j*, chromosome gaps at the fragile site.

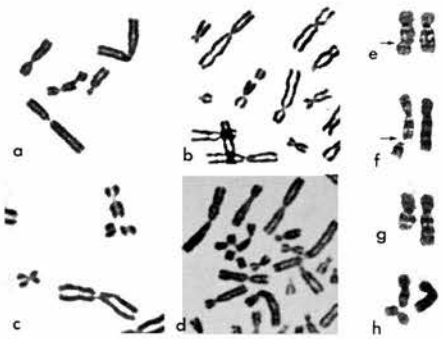



FIG 3.—Appearances of the fragile site on 10q; *a*, single chromatid break; *b*, chromosome break; *c*, triradial configuration; *d*, pentaradial configuration; *e* and *f*, G-banded chromosomes showing break point at the distal end of band 10q23; *g*, G-banded chromosome showing deletion of the chromosome distal to the fragile site; *h*, G-banded triradial configuration.

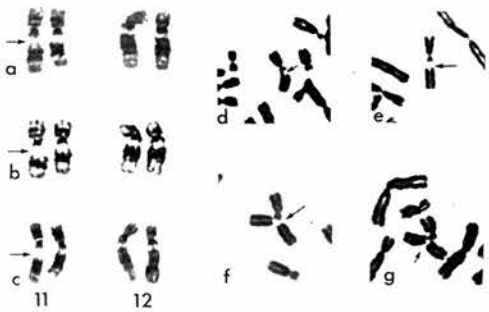



FIG 4.—Appearances of the fragile site at 11q13; *a*–*c*, G-banded chromosomes 11 and 12 showing the site in the proximal part of 11q13; *d*, single chromatid break at the site; *e*, chromosome break at the site; *f* and *g*, triradial configurations.

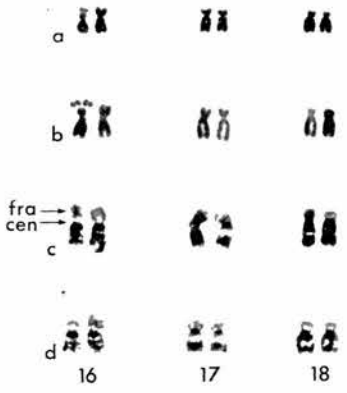



FIG. 5.—E-group chromosomes from cells with a lesion on 16p; *a*, chromosome gap; *b*, triradial configuration; *c*, G-banded chromosomes showing fragile site at band 16p124; *d*, G-banded chromosomes showing deletion of material distal to the fragile site.

*Family Mi*

This family (fig. 1*c*) has a fragile site on 20p11. The propositus was a profoundly retarded 6-year-old boy who was referred for chromosome studies. His retardation is presently regarded as being due to CNS degeneration of unknown cause despite intensive investigation. Results of cytogenetic studies on the propositus and his normal brother have been recorded [6]. The appearances of the fragile site are shown in figure 7.

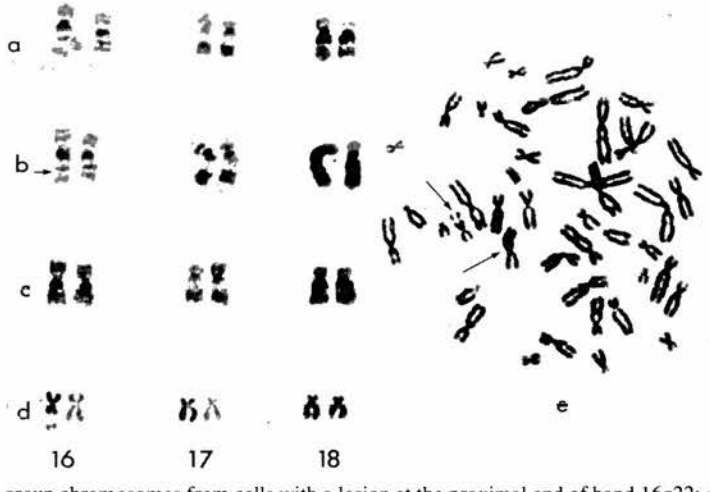



FIG. 6.—E-group chromosomes from cells with a lesion at the proximal end of band 16q22; *a*, G-banded triradial configuration; *b*, G-banded chromosome showing lesion (*arrow*) at 16q22; *c*, C-banded chromosomes from a metaphase not showing a lesion; *d*, unbanded chromosomes showing chromosome break at the fragile site; *e*, metaphase showing lesions at fragile sites at 16q22 and Xq27 or 28.

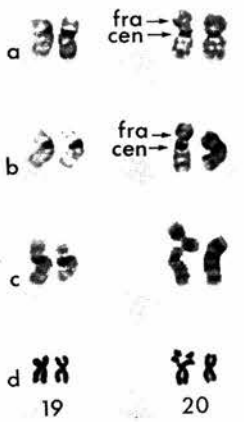



FIG. 7.—Appearance of the fragile site on 20p; *a–c*, G-banded chromosomes showing the site at band 20p11; *d*, unbanded F-group chromosomes showing triradial configuration resulting from the fragile site.

*Family Ma*

This family (fig. 1*f*) has a fragile site at Xq27 or 28. The propositi were three mildly retarded boys who are their parents only children. They were ascertained as part of a search for families with possible X-linked mental retardation. Cytogenetic data on this family have been given in [6] and are shown in more detail in table 1. Appearances of the fragile site are shown in figure 8.

*Family E*

This family (fig. 1*e*) also has a fragile site at Xq27 or 28. The propositi were mildly retarded twin boys. They were referred for chromosome study because the family was thought to be one in which there was X-linked mental retardation. The appearance of the fragile site is identical to that in family Ma. Cytogenetic data are shown in table 1.

## DISTRIBUTION OF FRAGILE SITES

The fragile site at 2q1 was the first to be shown to be heritable [2]. Since then there have been numerous reports of individuals and families with this site which have recently been reviewed [9]. Conen and Erkman [10] recorded a child with Down syndrome and leukemia who showed breakage in the short arm of chromosome 2 near the centromere in a number of cells. Parental chromosomes were not studied; a fragile site at this location must therefore await confirmation. Similarly, Tartaglia et al. [11] reported a patient with congenital erythroid hypoplasia who had an achromatic lesion in the middle of one arm of chromosome 1. The lesion appeared to affect only one chromatid, and the published karyotypes show its position to vary. Parental chromosomes were not studied. It is unlikely that they had a patient with a heritable fragile site as previously defined [7]; Bühler et al. [12] were also of this opinion.

Brøgger [13] reported a mentally retarded boy with a gap in the middle of one arm of chromosome 3 which appeared to be fragile. This appeared to affect one chromatid

TABLE 1

FREQUENCY OF FRAGILE SITE AT Xq27 OR 28 IN LYMPHOCYTE METAPHASES

| Subject | Culture medium | Proportion of cells with fragile sites |
|---|---|---|
| Family Ma: | | |
| II 2 | 199 | 0/30 |
| II 3 | 199 | 0/100 |
| | MEM-FA | 0/100 |
| III 9 | 199 | 7/50 |
| | MEM-FA | 30/200 |
| III 10 | 199 | 5/50 |
| IV 4 | 199 | 18/50 |
| | MEM-FA | 51/100 |
| IV 5 | 199 | 7/50 |
| IV 6 | 199 | 1/50 |
| IV 7 | 199 (1st culture) | 8/50 |
| | 199 (2nd culture) | 5/50 |
| | MEM-FA | 15/50 |
| Family E: | | |
| II 4 | 199 | 0/50 |
| III 4 | 199 | 1/40 |
| III 7 | 199 | 5/50 |
| III 8 | 199 | 0/50 |
| IV 1 | 199 | 4/50 |
| IV 2 | 199 | 8/100 |
| IV 3 | 199 | 9/50 |
| IV 5 | 199 (1st culture) | 10/50 |
| | 199 (2nd culture) | 16/50 |
| IV 6 | 199 (1st culture) | 0/50 |
| | 199 (2nd culture) | 10/50 |

more often than both and in some cells was present on both number 3 chromosomes. Parental chromosomes were normal. It is unlikely that this report concerned a heritable fragile site.

A number of C-group chromosomes with fragile sites reported prior to chromosome banding have been reviewed by Giraud et al. [4]. They were the first to specifically identify such C-group chromosomes and reported fragile sites at 10q242 and 12q13. The present report of a site at 10q23 probably involves the site Giraud et al. [4] reported to be at 10q242. They used R-banding, whereas G-banding has been used in this report suggesting that the site is near the distal end of band 10q23. Savage [14] has drawn attention to discrepancies in breakpoint localization when different banding methods are employed.

There have been reports of abnormal fragility in the C-band heterochromatin of chromosome 9 [15, 16, 17]. The karyotypes published by Fraccaro et al. [16] suggest that chromosome 11 or 12 is more likely to be involved. Two other reports are more convincing about identification of chromosome 9, although in the absence of banding, this remains uncertain. Neither case showed multiradial chromosomes resulting from the fragility in chromosome 9. Further study is necessary before the possibility of a fragile site in or adjacent to the C-band on chromosome 9 can be confirmed. The fragile site at 11q13 is the first recorded at this location. There are few positively identified paracentric fragile sites on C-group chromosomes. Giraud et al. [4] recorded two cases

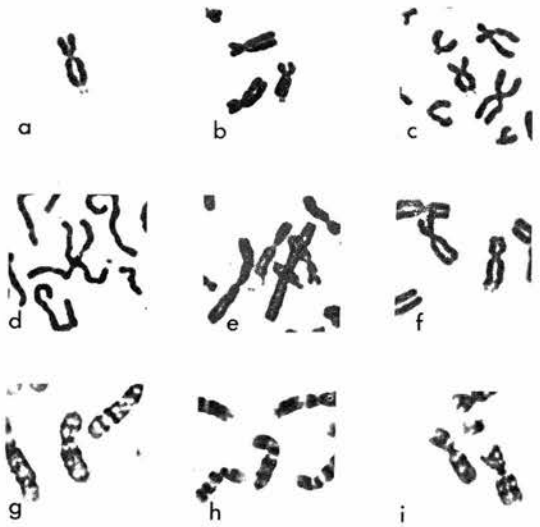



FIG. 8.—Appearances of the fragile site on Xq; *a–c*, usual "satellited" appearance in metaphase chromosomes; *d*, appearance in prophase; *e* and *f*, "double satellited" appearance equivalent to triradial configuration; *g–i*, G-banded chromosomes show the fragile site on the very distal end of Xq.

involving chromosome 12, and McCreanor (personal communication, 1977) has studied a family in which such a fragile site is segregating. This site at 12q13 is the only well established fragile site which has not been available for study in this laboratory. There is only one previous report [18] concerning a site on 16p in a man who also appeared to have a translocation involving chromosome 18; his parents were not studied. The present report shows that a fragile site on 16p is certainly heritable. A large kindred in which a chromosome with a fragile site at 16q22 was segregating without apparent phenotypic effect has been documented [19]. A further six individuals, of whom two were father and son, with this fragile site have been recorded [4]. Drets et al. [20] recorded a family in which several members had one chromosome 16 replaced in a proportion of their cells by what appeared to be a chromosome 16 with a greatly lengthened long arm, and a fragile site near its mid-point. This fragile site is certainly heritable, but without chromosome banding its origin and location remain unknown.

The site on 20p11 has not been previously described. Apart from the family in this report, another unrelated and less well-documented family is known with the same site.

The site on the distal end of Xq was first described by Lubs [3], shown to be heritable, and its association with mental retardation recorded. Harvey et al. [5] confirmed this site as a marker for one form of X-linked mental retardation. The two families recorded here further document this association. Other retarded males with this site, such as subject B, have been detected, and other families not included in this report are known.

The well-documented and potential fragile sites are summarized in figure 9. Chromosome 17 is not included in this summary and requires further discussion. This chromosome undoubtedly contains a heritable constriction on the short arm which gives rise to its so-called satellited appearance in some families [21, 22]. It has also been reported in homozygous form in a normal woman [23]. This chromosome behaves differently in several ways from all the fragile sites which have been examined. It appears not to be fragile in that the satellites do not appear to separate from the chromosome as a minute fragment, nor are double satellited chromosomes (equivalent to the triradials of the fragile site chromosomes) produced. This satellited appearance is not dependent upon conditions of culture as are all the other fragile sites studied (except for that at 16q22 [7]). These satellited chromosomes are undoubtedly heritable variants, but they do not have a fragile site as this term applies to the other chromosomes discussed [7].

There is virtually nothing known about the frequency of fragile sites in the population. Most neonatal surveys aimed at establishing frequencies of chromosome abnormalities and variants have not used culture medium suitable for the demonstration of fragile sites. Furthermore, such surveys have been based on the examination of a very small number of cells, usually two, per individual. Consequently, even if fragile sites were expressed, they would not always be detected. The only fragile site detected in a neonatal survey [24] was one in a C-group chromosome (probably an 11 or 12) in one infant out of 3,543 studied. None of the infants in this survey had any phenotypic abnormality.

Fragile sites have not been reported in other species, possibly due to the relatively few individuals usually studied. White [25] reported a type of fragile site in a meiotic study of an undescribed species of morabine grasshopper. Breakage occurred at a specific locus during first premetaphase in all cells examined from a single individual but not in the remaining members of the species studied. Fragile sites in man have not been studied in meiosis.

## CYTOGENETICS

The cytogenetics of fragile sites has been discussed in some detail [26, 27, 28]. The most striking appearance of the sites is the multiradial configurations. These were

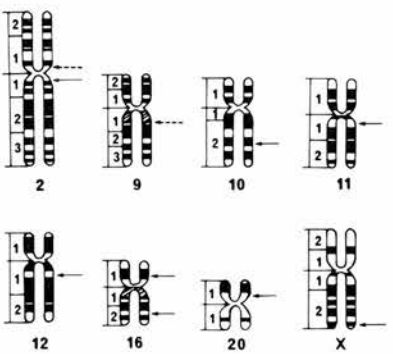



FIG. 9.—Known fragile sites. *Solid arrows* indicate well established fragile sites, and *broken arrows* indicate sites for which definitive evidence is lacking.

originally claimed by Lejeune [2] to be the result of selective endoreduplication. Others [26, 27] have suggested that they could be due to nondisjunction of an acentric chromatid following breakage at the site. This suggestion has been confirmed [28, 29] using BrdU incorporation and differential sister chromatid staining. The term "branched chromosomes" has been used to refer to such multiradials [27].

Lubs [3] studied DNA replication by autoradiography in a female with a site at Xq27 or 28 and found that the X with the fragile site did not appear to be selectively inactivated. Fraccaro et al. [30] similarly studied a site at 2q1 and noted that in most cells there was no detectable asynchrony in DNA synthesis between homologs. In one cell they found a triradial with the whole section distal to the fragile site late labeling and despiralized. Nöel et al. [28] recorded a number of such chromosomes with despiralization distal to the fragile site. Such chromosomes have been seen occasionally in the present study but not at the frequency recorded by Nöel et al. [28].

### TISSUE OF ORIGIN OF CHROMOSOMES

Fragile sites in the present study were found almost exclusively in blood lymphocyte cultures. There was limited opportunity to examine bone marrow chromosomes. Bone marrow from a male with a site at Xq27 or 28 did not show a single fragile site in 200 metaphases. Blood lymphocytes cultured at the same time expressed the site in 10% of cells in medium 199, and 30% in MEM-FA. Similarly, bone marrow from a carrier of a site at 16p12 showed no evidence of the site in 60 metaphases, but it was present in 46% of metaphases from blood lymphocytes collected at the same time and cultured in medium 199. Magenis et al. [19] found the site on 16q in two of 41 metaphases from bone marrow. Dr. H. R. McCreanor (personal communication, 1977) found the site on 2q in 36 out of 168 bone marrow metaphases. Other reports do not mention the study of bone marrow chromosomes.

Skin fibroblast cultures were established from individuals with the following fragile sites: 2q1, 10q23, 11q13, 16q22, 20p11 and Xq27 or 28. Fragile sites were rarely identified in these fibroblast cultures even when grown in medium 199, as shown in table 2. Fraccaro et al. [16] could not detect a fragile site at 2q1 in fibroblast cultures, and Magenis et al. [19] could not detect the site on 16q in 165 metaphases from fibroblast culture. Ferguson-Smith [26] found the site at 2q1 in fibroblast culture but at

TABLE 2

FREQUENCY OF FRAGILE SITES IN SKIN FIBROBLAST CULTURES, MEDIUM 199

| Site | Proportion of cells with fragile sites |
|---|---|
| 2q1 | 2/50 |
| 10q23 | 0/50 |
| 11q13 | 2/50 |
| 16q22 (Subject B) | 0/100 |
| 20p11 | 1/50 |
| Xq27 or 28 (Family Ma) | 0/50 |
| Xq27 or 28 (Family E) | 0/50 |
| Xq27 or 28 (Subject B) | 0/100 |

a lower frequency than in lymphocyte cultures. Dekaban [1] presented data on skin fibroblast culture which suggested that the site was not present in this material. The reasons why some authors can detect fragile sites in fibroblast cultures and others cannot remain unknown. However, in view of the strong dependence of their expression in lymphocyte cultures on the culture conditions, this is not surprising.

Transformed lymphocyte cultures were established from individuals with fragile sites at 2q, and Xq27 or 28. No expression of the fragile site was seen in either case in 50 cells examined. No other authors have examined such cultures from individuals with fragile sites.

## PHENOTYPIC EFFECTS OF FRAGILE SITES

No abnormal phenotype is associated with the autosomal fragile sites. Many of these sites were detected in abnormal individuals, but probably this only reflects the type of person undergoing chromosome analysis. Williams and Howell [9] suggested that breakage at the fragile site in vivo could give rise to a variety of aneuploid cell lines leading to abnormal development. While this appears possible, there is little evidence that fragile sites are expressed in vivo; indeed, they may be artefacts of tissue culture. The variety of phenotypic abnormalities associatied with the sites suggests that they are without phenotypic effect in the heterozygote. By analogy with mutant reciprocal translocations which can be associated with phenotypic abnormality [31], when a fragile site newly arises it might produce an abnormal phenotype. There is no reliable evidence in the literature regarding fragile sites which are new mutants. Several authors found no fragile sites in the parents of their index cases, but because of the previously unknown dependence of these sites on culture conditions, such findings cannot be regarded as definite evidence of mutation. The retarded girl described in this report with a fragile site at 11q13 appears to be a mutant, although paternity has not been checked, and the cause of her mental retardation is largely conjectural.

The fragile site on Xq is undoubtedly a marker for one form of X-linked mental retardation. This was first shown by Lubs [3] for one family and subsequently by Harvey et al. [5] in four more families; this group has since detected several additional such families (Weiner, personal communication, 1977). Giraud et al. [4] described a number of retarded males with this fragile site. Two families are documented in the present report, and several others are currently being studied as a result of screening 203 institutionalized retarded males for fragile sites. Among these, five (two are brothers) with fragile sites at Xq27 or 28 were identified. This condition is apparantly not rare, but because the appearance of the fragile site is not spectacular, and in some instances is expressed in only a small proportion of metaphases, and because lymphocytes must be cultured in specific types of culture medium, it has gone largely unrecognized. The nature of the association between the fragile site and the mental retardation remains obscure. Not all families with X-linked mental retardation show the fragile site. It may be that demonstration of the site in some families is more difficult than in others. Even in those retarded males studied, the proportion of metaphases in which the site is expressed ranged from less than 5% up to more than 30%. There is, however, no reason why X-linked mental retardation could not be a group of different conditions, only one of which is associated with the fragile site.

The fragile site on Xq is associated with mental retardation in the hemizygote but not the heterozygote. This would allow the speculation that homozygosity for the autosomal folic acid sensitive fragile sites would lead to an abnormal phenotype. Such homozygosity has not been reported, although a normal female homozygous for a "satellited" chromosome 17 has been recorded. Rare autosomal recessive disorders should be reexamined chromosomally as some may be due to homozygosity for fragile sites.

ACKNOWLEDGMENTS

I thank Lynene Day, Helen Eyre, Trudy Hocking, and Erica Woollatt for technical assistance and Dr. D. L. Hayman for helpful discussions during the preparation of this report.

REFERENCES

1. DEKABAN A: Persisting clone of cells with an abnormal chromosome in a woman previously irradiated. *J Nucl Med* 6:740–746, 1965
2. LEJEUNE J, DUTRILLAUX B, LAFOURCADE J, BERGER R, ABONYI D, RETHORÉ MO: Endoréduplication sélective du bras long du chromosome 2 chez une femme et sa fille. *C R Acad Sci (Paris)* 266:24–26, 1968
3. LUBS HA: A marker X chromosome. *Am J Hum Genet* 21:231–244, 1969
4. GIRAUD F, AYME S, MATTEI JF, MATTEI MG: Constitutional chromosomal breakage. *Hum Genet* 34:125–136, 1976
5. HARVEY J, JUDGE C, WIENER S: Familial X-linked mental retardation with an X chromosome abnormality. *J Med Genet* 14:46–50, 1977
6. SUTHERLAND GR: Fragile sites on human chromosomes: Demonstration of their dependence on the type of tissue culture medium. *Science* 197:265–266, 1977
7. SUTHERLAND GR: Heritable fragile sites on human chromosomes I. Effect of composition of culture medium on expression. *Am J Hum Genet* 31:125–135, 1979
8. POPE JH, HORNE MK, SCOTT W: Transformation of foetal human leukocytes *in vitro* by filtrates of a human leukaemic cell line containing Herpes-like virus. *Int J Cancer* 3:857–866, 1968
9. WILLIAMS AJ, HOWELL RT: A fragile secondary constriction on chromosome 2 in a severely mentally retarded patient. *J Ment Defic Res* 21:227–230, 1976
10. CONEN PE, ERKMAN B: Combined mongolism and leukaemia. *Am J Dis Child* 112:429–443, 1966
11. TARTAGLIA AP, PROPP S, AMAROSE AP, PROPP RD, HALL CA: Chromosome abnormality and hypocalcaemia in congenital erythroid hypoplasia (Blackfan-Diamond syndrome). *Am J Med* 41:990–999, 1966
12. BÜHLER EM, LUCHSINGER U, BÜHLER UK, MÉHES K, STADLER GR: Non-condensation of one segment of a chromosome no. 2 in a male with otherwise normal karyotype (and severe hypospadias). *Humangenetik* 9:97–194, 1970
13. BRØGGER A: Virus som arsak til kromosomaberrasjoner hos menneske. *Tidsskr Nor Laegeforen* 88:1741–1747, 1968
14. SAVAGE JRK: Assignment of aberration breakpoints in banded chromosomes. *Nature* 270:513–514, 1977
15. SCHMID W, VISCHER D: Spontaneous fragility of an abnormally wide secondary constriction region in a human chromosome no. 9. *Humangenetik* 7:22–27, 1969
16. FRACCARO M, HANSSON K, LINDSTEN J, TIEPOLO L: Specific instability of the paracentric region of chromosome number 9 in a normal woman and her mongoloid son. *Ann Génét* 14:149–152, 1971
17. REEVES BR, LAWLER SD: Preferential breakage of sensitive regions of human chromosomes. *Humangenetik* 8:295–301, 1970
18. DAY EJ, MARSHALL R, MACDONALD PAC, DAVIDSON WM: Deleted chromosome 18 with

paternal mosaicism. *Lancet* 2:1307, 1967
19. MAGENIS RE, HECHT F, LOVRIEN EW: Heritable fragile site on chromosome 16: probable localization of haptoglobin locus in man. *Science* 170:85–87, 1970
20. DRETS ME, CARDOSO JH, DELFINO AH, CARRAU J: Familial normal/partial trisomy 16 with selective endoreduplication in malformed proband. *Cytogenetics* 9:333–350, 1970
21. EMERIT I, DEGROUCHY J, VERNANT P, CORONE P: Chromosomal abnormalities and congenital heart disease. *Circulation* 36:886–905, 1967
22. SCHMID E, BAUCHINGER M: Structural polymorphism in chromosome 17. *Nature* 221:387–388, 1969
23. BERG JM, FAUNCH JA, PENDREY MJ, PENROSE LS, RIDLER MAC, SHAPIRO A: A homozygous chromosomal variant. *Lancet* 1:531, 1969
24. GERALD PS, WALZER S: Chromosome studies of normal newborn infants, in *Human Population Cytogenetics*, edited by JACOBS PA, PRICE WH, LAW P, Edinburgh Univ Press 1970, pp 143–151
25. WHITE MJD: A case of spontaneous chromosome breakage at a specific locus occurring at meiosis. *Aust J Zool* 14:1027–1034
26. FERGUSON-SMITH MA: Inherited constriction fragility of chromosome 2. *Ann Génét* 16:29–34, 1973
27. FORD CE, MADAN K: Branched Chromosomes. An alternative to the hypothesis of selective endoreduplication, in *Chromosome Identification, Nobel Symposium 23*, edited by CASPERSSON T, ZECH L, New York and London, Academic Press, 1973, pp 98–103
28. NOËL B, QUACK B, MOTTET J, NANTOIS Y, DUTRILLAUX B: Selective endoreduplication or branched chromosome? *Exp Cell Res* 104:423–426, 1977
29. FERGUSON-SMITH MA: Human chromosome polymorphism. *Rec Adel Child Hosp* 1:278–286, 1977
30. FRACCARO M, LINDSTEN J, TIEPOLO L, RICCI N: Instability of the paracentric region and selective reduplication of chromosome 2 in man. *Chromosomes Today* 3:138–146, 1972
31. JACOBS PA: Correlation between euploid structural chromosome rearrangements and mental subnormality in humans. *Nature* 249:164–165, 1974

Hum. Genet. 48, 117—120 (1979)

Human Genetics



# X-Linked Mental Retardation With Macro-Orchidism and the Fragile Site at Xq27 or 28

G. R. Sutherland* and P. L. C. Ashforth

Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia

**Summary.** Data are presented suggesting that the form of X-linked mental retardation with macro-orchidism and the form associated with a fragile site at Xq27 or 28 are the same entity.

## Introduction

Mental retardation with macro-orchidism in males, with probably X-linked inheritance, has been described by Turner et al. (1975) and Cantú et al. (1976, 1978). It has now been established that one form of X-linked mental retardation is associated with the fragile site on the distal end of the X chromosome (Harvey et al., 1977; Sutherland, 1977, 1979b). Data are presented suggesting that these two forms of mental retardation are the same entity.

## Materials and Methods

Thirteen males with mental retardation and the fragile site at Xq27 or 28, identified from family histories and chromosomal surveys of retarded males (Sutherland, 1979b), were available for study. Measurements of testicular length ($l$) and width ($w$) were carried out with a ruler. The volume was calculated from the formula $\pi/6.l.w.^2$ (Cantú et al., 1976). Control values were obtained from the literature and by measurement of ten retarded males with normal karyotypes. Penile length (stretched) was measured with a ruler, and midshaft circumference with a measuring tape. Control values were obtained as for testicular volume.

## Results

The ages and genital measurements of the males with the fragile site at Xq27 or 28 are shown in Table 1. All measurements are in centimetres, and the estimated accuracy of each measurement is $\pm 0.2$ cm. The control group of retarded males

* To whom offprint requests should be sent

0340-6717/79/0048/0117/$ 01.00

**Table 1.** Genital measurements (cm) of males with fra(X)(q27 or 28)

| Subject | Age (years) | Penis | | Testes | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Length | Circumference | Right | | | Left | | |
| | | | | Length | Width | Volume (ml) | Length | Width | Volume (ml) |
| Family E[a] | | | | | | | | | |
| $III_7$ | 26 | 13.0 | 9.0 | 6.5 | 4.5 | 69 | 6.0 | 4.0 | 50 |
| $IV_5$ | $3^{7}/_{12}$ | 5.0 | 4.5 | 2.5 | 1.7 | 3.8 | 2.5 | 1.5 | 2.9 |
| $IV_6$ | $3^{7}/_{12}$ | 6.0 | 5.0 | 2.5 | 1.5 | 2.9 | 2.5 | 1.5 | 2.9 |
| Family Ma[a] | | | | | | | | | |
| $IV_5$ | $10^{5}/_{12}$ | 6.0 | 5.0 | 2.5 | 2.0 | 5.2 | 2.7 | 2.0 | 5.6 |
| $IV_6$ | $6^{10}/_{12}$ | 6.5 | 4.5 | 2.5 | 1.0 | 1.3 | 2.5 | 1.2 | 1.8 |
| $IV_7$ | $5^{6}/_{12}$ | 5.0 | 4.0 | 2.5 | 1.5 | 2.9 | 3.0 | 1.5 | 3.5 |
| P | $13^{7}/_{12}$ | 12.0 | 7.5 | 5.5 | 3.5 | 35 | 5.0 | 3.0 | 24 |
| Fe | 18 | 9.0 | 10.0 | 5.5 | 3.5 | 35 | 5.5 | 3.0 | 31 |
| Fi | 23 | 9.0 | 9.0 | 5.0 | 3.5 | 32 | 5.0 | 3.5 | 32 |
| Family B | | | | | | | | | |
| N | 27 | 12.5 | 9.5 | 5.5 | 3.5 | 35 | 5.0 | 3.5 | 32 |
| A | 28 | 10.0 | 11.0 | 6.0 | 3.5 | 39 | 5.0 | 3.0 | 24 |
| Mi | 65 | 13.0 | 11.0 | 3.0 | 2.5 | 10 | 4.5 | 2.5 | 15 |
| E | 16 | 14.0 | 8.0 | 7.0 | 3.5 | 45 | 7.0 | 4.0 | 59 |
| B[a] | 30 | 10 | 7.0 | 6.5 | 4.0 | 54 | 7.0 | 4.0 | 59 |

[a] From Sutherland (1979b); other subjects have not been published. Individuals from family B are brothers

ranged in age from 23 to 35 years (mean 29.0); penile length was 8—15 cm (mean 12.5 cm), penile circumference 8—12 cm (mean 9.2 cm), right testicular volume 9—24 ml (mean 17.8 ml), and left testicular volume 9—24 ml (mean 16.2 ml). Figure 1 compares the mean of the right and left testicular volumes of all those measured with normal values taken from the literature.

## Discussion

The data in the literature regarding genital size are conflicting, due to various methods of measurement and possible population differences (Farkas, 1972). Prader (1974) gives 10—17 cm as the normal length of the stretched penis after puberty; the penile length of both groups of postpubertal males described in the present report approximates to this range, but this measurement is significantly affected by the degree of stretching during measurement. Prader (1974) gives the length of the stretched penis as 4—8 cm and the circumference as 3—6 cm before puberty. The prepubertal males in this report appear to have penises of normal

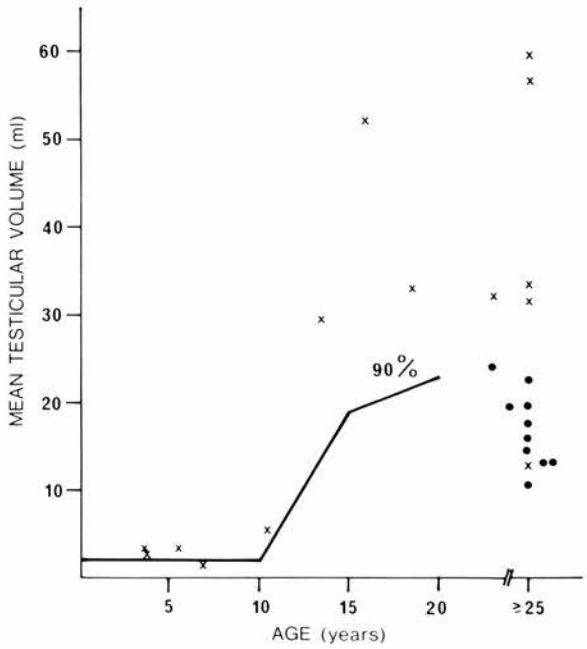



**Fig. 1.** Average (left and right) testicular volume of males with the fragile site (X) and control group (•). The 90% line is the percentile given by Prader (1974) from ages 10—20 and his upper limit of normal up to the age of 10 years. All males aged 25 years or more are shown as ≥25

size. Farkas (1972) gives the mean penile circumference, measured similarly to in the present study, as 9.6 cm, with a standard deviation of 0.83 cm and a range of 7.7—12.0 cm. The penile circumference of the present groups of postpubertal males is within this range.

Testicular volume can only be estimated, either by measurement of length and width or by the use of an orchidometer and comparative palpation. Using the orchidometer, Prader (1974) gives a prepubertal testicular volume range of 0.75 ml to a maximum of 2 ml. Zachmann et al. (1974) give a mean volume of about 19 ml in postpubertal males, with a volume of about 23 ml as the 90th percentile. Rundle and Sylvester (1962) reported larger volumes calculated from length and width measurements, but used a formula that would have given volumes about 35% greater than those calculated from the formula of Cantú et al. (1976).

The control group of retarded males in the present study had testicular volumes well within the range of Zachmann et al. (1974). The testicular volume of the postpubertal males with the fragile site ranged from 32 to 69 ml for the right, and from 24 to 59 ml for the left, if subject Mi is excluded. Subject Mi is 65 years old and may have undergone some testicular atrophy. These volumes are not as great as those recorded by Cantú et al. (1976, 1978), which ranged from 68 to 128 ml in adults, but are all above the 90th percentile. In three subjects in particular (Family E, $III_7$; D; and B) the testicular volume is far greater than that

of normal males. The volumes in the present study are more in accord with those found by Turner et al. (1975), which ranged from 25 ml in a 13-year-old up to approximately 45 ml in adults.

All the prepubertal males, except for one, have testicular volumes above the upper limit of normal (Prader, 1964). Cantú et al. (1976) indicated that testicular size was probably increased from birth, and the mother of one of the present subjects said that his genitals had always been larger than those of other males in the family. It would appear that increased testicular size is present in this form of retardation from birth and not only after puberty.

The results presented suggest that the X-linked mental retardation with macro-orchidism described by Turner et al. (1975) and by Cantú et al. (1976, 1978) is the same entity as X-linked mental retardation associated with the fragile site at Xq27 or 28. Reports of normal chromosomes in males with macro-orchidism by Turner et al. (1975) and by Cantú et al. (1976, 1978) cannot be regarded as definitive, since the need to use culture medium deficient in folic acid and thymidine for the demonstration of fragile sites (Sutherland, 1979a) was then unknown. Chromosome studies on the patients described by these authors should be repeated if possible, to determine whether they have the fragile site on Xq.

## References

Cantú, J.-M., Scaglia, H. E., Medina, M., González-Diddi, M., Morato, T., Moreno, M. E., Pérez-Palacios, G.: Inherited congenital normofunctional testicular hyperplasia and mental deficiency. Hum. Genet. **33**, 23—33 (1976)

Cantú, J.-M., Scaglia, H. E., González-Diddi, M., Hernández-Jáuregui, P., Morato, T., Moreno, M. E., Giner, J., Alcántar, A., Herrera, D., Pérez-Palacios, G.: Inherited congenital normofunctional testicular hyperplasia and mental deficiency. Hum. Genet. **41**, 331—339 (1978)

Farkas, L. G.: Basic morphological data of external genitals in 177 healthy central European men. Am. J. Phys. Anthropol. **34**, 325—328 (1972)

Harvey, J., Judge, C., Wiener, S.: Familial X-linked mental retardation with an X chromosome abnormality. J. Med. Genet. **14**, 46—50 (1977)

Prader, A.: Clinical endocrinology. Theory and practice, A. Labhart (ed.), pp. 1036. Berlin-Heidelberg-New York: Springer 1974

Rundle, A. T., Sylvester, P. E.: Measurement of testicular volume. Arch. Dis. Child. **37**, 514—517 (1962)

Sutherland, G. R.: Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. Science **197**, 265—266 (1977)

Sutherland, G. R.: Heritable fragile sites on human chromosomes. I. Effect of composition of culture medium on expression. Am. J. Hum. Genet. (in press, 1979a)

Sutherland, G. R.: Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects and cytogenetics. Am. J. Hum. Genet. (in press, 1979b)

Turner, G., Eastman, C., Casey, J., McLeay, A., Procopis, P., Turner, B.: X-linked mental retardation associated with macro-orchidism. J. Med. Genet. **12**, 367—371 (1975)

Zachmann, M., Prader, A., Kind, H. P., Häfliger, H., Budliger, H.: Testicular volume during adolescence. Helv. Paediatr. Acta **29**, 61—72 (1974)

Received November 27, 1978

Hum. Genet. 53, 23–27 (1979)

Human Genetics



# Heritable Fragile Sites on Human Chromosomes

## III. Detection of fra(X)(q27) in Males with X-Linked Mental Retardation and in Their Female Relatives

G. R. Sutherland

Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide S. A., 5006 Australia

**Summary.** The fragile site at Xq27 which is associated with X-linked mental retardation with macro-orchidism has been studied in 21 retarded males. These males were from 12 families, and studies of nine of the families were possible. Detection of carrier females is difficult, especially with increasing age. The fragile site was demonstrated in only five of 13 obligate carrier females. It is concluded that using present methods, cytogenetic detection of carriers is fairly reliable in females aged less than 20—25 years but unreliable in older females.

### Introduction

It is now established that the fragile site at Xq27 is associated with, and may even be the cause of, the form of X-linked mental retardation with macro-orchidism (Harvey et al., 1977; Turner et al., 1978a, b; Sutherland and Ashforth, 1979; Sutherland et al., 1979). Nevertheless, demonstration of the fragile site is not easy and has been the subject of controversy (Mulcahy, 1978; Sutherland, 1978; Turner et al., 1978a; Wiener and Judge, 1979); in particular, the determination of carrier status in females remains difficult and unreliable. This paper records studies of the fragile site in 21 mentally retarded males, 13 obligate carrier females and 26 potential carrier females.

### Materials and Methods

Peripheral blood cultures were established and harvested as previously described (Sutherland, 1977a). The culture media used were TC 199 and Eagles Minimal Essential Medium specially formulated (Sutherland, 1979a) without folic acid (MEM-FA). Fetal bovine serum supplement concentration was 5%. First studies were usually carried out using TC 199 with bicarbonate buffer. When the first study yielded a low frequency of fragile sites the study was repeated using TC199 or MEM-FA with 20 mM Hepes buffer and a pH greater than 7.5.

Males with fra(X)(q27) were detected by studying families with suspected X-linked mental retardation, retarded males referred for clinical cytogenetic analysis and screening institutionalised retarded males.

### Results

*The Patients.* Fig. 1 shows the family trees of the index cases where relatives have been studied. Families Ma and E are shown in Fig. 1 of Sutherland (1979b); family Ma is represented here, since results of additional studies have been added.

*Cytogenetics.* The individuals studied fall into three groups—retarded males, obligate carriers and potential carriers. The frequency of observation of the fragile sites when first studied in the retarded males (Table 1) ranged from zero in two cases up to 44%. Repeat studies usually yielded a higher frequency of fragile sites, ranging from 4 to 56%. One male (T.K.) who had a retarded brother with the fragile site had been studied in another laboratory and had not been found to have fra(Xq) was re-studied in this laboratory after both brothers had been found to have macro-orchidism (Sutherland et al., 1979). He was found to have the fragile site in 4% of his lymphocytes.

Results of studies on obligate carriers have been disappointing. Table 2 shows that of 13 obligate carriers studied only five showed any evidence of the fragile site

0340-6717/79/0053/0023/$ 01.00

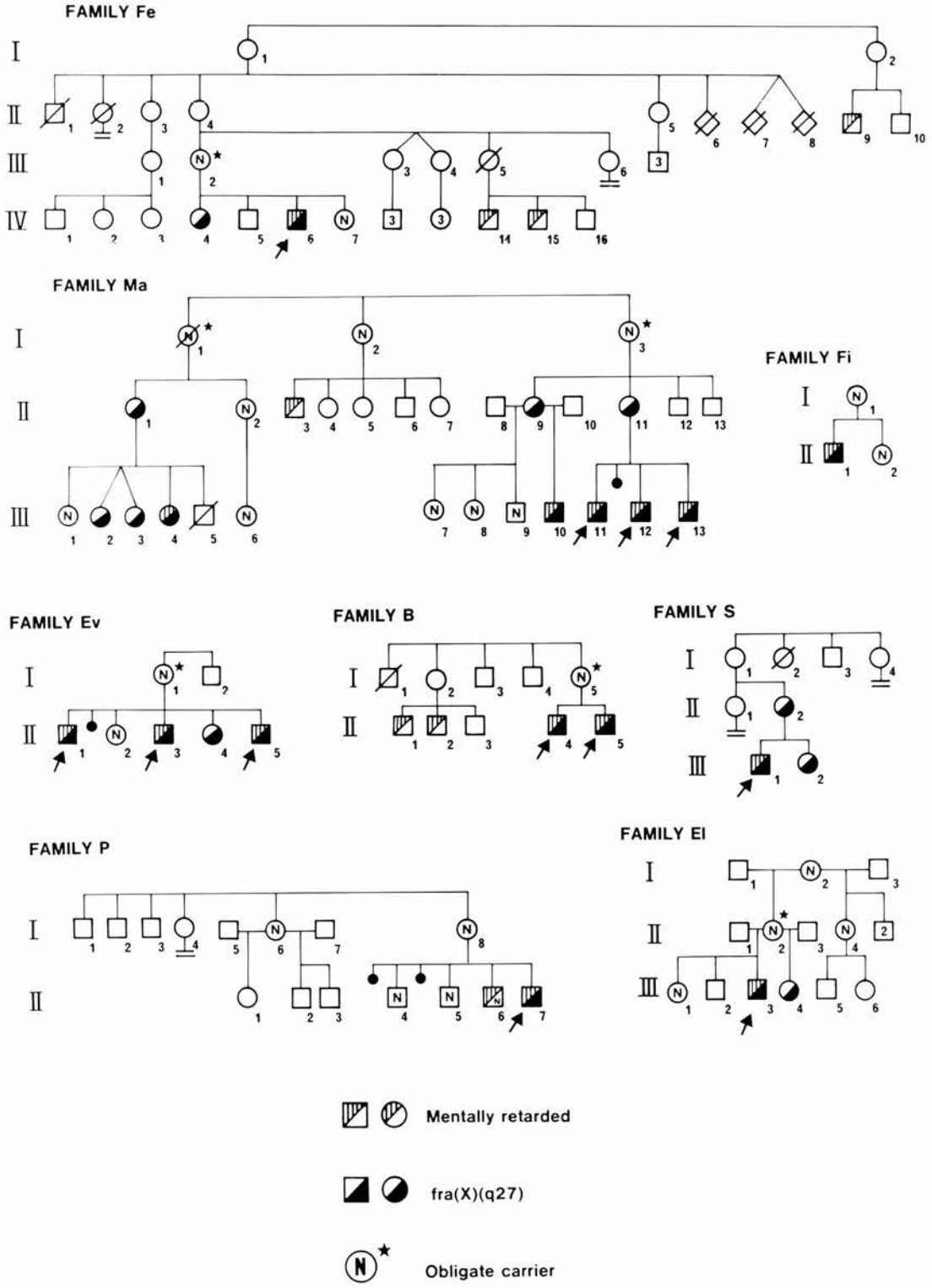



**Fig. 1.** Family trees of index cases where relatives have been studied

**Table 1.** Frequency of observation of fra(X)(q27) in retarded males

| Subject | | Age (years) | First culture | Maximum frequency seen |
|---|---|---|---|---|
| Family E[a] | III.7 | 24 | 5/50 | — |
| | IV.1 | 9 | 4/50 | — |
| | IV.5 | 1½ | 10/50 | 16/50 |
| | IV.6 | 1½ | 0/50 | 10/50 |
| Family Ma | III.10 | 4 | 18/50 | 28/50 |
| | III.11 | 7 | 7/50 | 26/100 |
| | III.12 | 5 | 1/50 | 30/100 |
| | III.13 | 3 | 8/50 | 17/50 |
| Family P[b] | II.7 | 12 | 6/50 | 10/50 |
| Family Fe[b] | IV.6 | 18 | 7/50 | 22/50 |
| Family Fi[b] | II.1 | 22 | 13/50 | 21/50 |
| Family B[b] | II.4 | 27 | 6/50 | — |
| | II.5 | 28 | 3/50 | 6/50 |
| Family El[b] | III.3 | 16 | 9/50 | 23/50 |
| Family Ev[e] | II.1 | 20 | 2/50 | — |
| | II.3 | 17 | 12/50 | 16/50 |
| | II.5 | 13 | 8/50 | — |
| Family S[e] | III.1 | 4½ | 22/50 | — |
| Subject B[b] | | 30 | 1/50 | 12/50 |
| Subject Mi[b] | | 65 | 1/50 | 5/50 |
| Subject T.K.[c] | | 15 | 0/50[d] | 6/150 |

[a] From Sutherland (1979b)
[b] Same identification as in Sutherland and Ashforth (1979), except E is now El
[c] From Sutherland et al. (1979)
[d] Studied in another laboratory
[e] No data on these previously published

**Table 2.** Frequency of observation of fra(X)(q27) in obligate carrier females

| Subject | | Age at study (years) | Culture medium | Frequency |
|---|---|---|---|---|
| Family E | II.4 | 56 | 199 | 0/50 |
| | III.4 | 31 | 199 | 1/40 |
| | | 33 | 199 | 0/50 |
| | | | MEM-FA | 0/50 |
| | III.8 | 26 | 199 | 0/50 |
| | | 28 | 199 | 0/50 |
| | | | MEM-FA | 0/50 |
| Family Ma | I.1 | ≈65 | 199 | 0/30 |
| | I.3 | 58 | 199 | 0/100 |
| | | | MEM-FA | 0/150 |
| | II.1 | 35 | 199 | 12/150 |
| | | | MEM-FA | 16/82 |
| | II.9 | 34 | 199 | 7/50 |
| | | | MEM-FA | 45/300 |
| | II.11 | 30 | 199 | 9/100 |
| | | 32 | 199 | 0/50 |
| | | | MEM-FA | 0/100 |
| Family Fe | III.2 | 44 | 199 | 0/50 |
| | | | MEM-FA | 0/50 |
| Family B | I.1 | 60+ | MEM-FA | 0/50 |
| Family El | II.2 | 43 | 199 | 0/60 |
| Family Ev | I.1 | 40 | 199 | 0/50 |
| | | | MEM-FA | 0/50 |
| Family S | II.2 | 23 | 199 | 0/150 |
| | | | MEM-FA | 3/50 |

and in one of these only one cell in 140 examined had the site.

Of the potential carriers, there were 17 (including one set of identical twins) who were daughters of obligate carriers (Table 3). Nine of these, including the twins, were shown to carry the fragile site; another (Ma I.2) is a probable carrier, since she has a retarded son who has not been studied and was about 60 years old when her chromosomes were examined. Of the other potential carriers, there were two mothers of retarded males who had the fragile site but there was no other known retarded male in the family. It should be noted that some of the obligate carriers were originally in this group until results from other female members of their families showed that they must have been carriers. A daughter of one of the mothers of a sporadic case, two nieces of an obligate carrier and one sister and one half-sister of an obligate carrier and a mother and a granddaughter of an obligate carrier completed the group of possible carriers. None of these were shown to carry the fragile site.

Age at the time of study appears to be a relevant factor in the demonstration of carrier status in females but not in the frequency of observation in males. Of the obligate carrier females, the fragile site was demonstrated in five of the six aged less than 35 years, but in none of the seven older than 35. In subject Ma II.11 the site was demonstrated readily at age 30 but could not be shown 2 years later.

## Discussion

Other authors who have been able to demonstrate the fragile site in retarded males have had trouble doing so in carrier females. Lubs (1969) studied two obligate carrier females: the older one aged 59 years did not express the fragile site, but in her niece aged 24 years it was present in 28% of cells. In the younger sister of the niece, aged 22 years, it was present in only 2% of cells.

Giraud et al. (1976) studied five retarded males who had the fragile site in 20—50% of their cells. In only one

**Table 3.** Frequency of observation of fra(X)(q27) in potential carrier females

| Subject | | Age (years) | Frequency | Remarks |
|---|---|---|---|---|
| Family E | II.4 | 56 | 0/ 50 | Sister of obligate carrier |
| | III.3 | 20 | 0/100 | Niece of obligate carrier |
| | III.11 | 18 | 0/100 | Niece of obligate carrier |
| | IV.2 | 7 | 8/100 | Identical twins of obligate carrier |
| | IV.3 | 7 | 8/100 | |
| Family Ma | I.2 | ≃60 | 0/ 50 | Daughter of obligate carrier, has retarded son |
| | II.2 | 27 | 0/150 | Daughter of obligate carrier |
| | III.1 | 14 | 0/150 | Daughter of obligate carrier |
| | III.2 | 13 | 16/ 98 | Daughter of obligate carrier |
| | III.3 | 13 | 12/106 | Daughter of obligate carrier |
| | III.4 | 11 | 12/150 | Daughter of obligate carrier, mentally retarded |
| | III.6 | 7/12 | 0/150 | Grand-daughter of obligate carrier |
| | III.7 | 12 | 0/ 50 | Daughter of obligate carrier |
| | III.8 | 13 | 0/ 50 | Daughter of obligate carrier |
| Family Fe | IV.4 | 21 | 13/ 50 | Daughter of obligate carrier |
| | IV.7 | 13 | 0/100 | Daughter of obligate carrier |
| Family El | I.2 | ≃60 | 0/150 | Mother of obligate carrier |
| | II.4 | ≃40 | 0/200 | Half-sister of obligate carrier |
| | III.1 | 22 | 0/350 | Daughter of obligate carrier |
| | III.4 | 2½ | 6/100 | Daughter of obligate carrier |
| Family Ev | II.2 | 19 | 0/ 50 | Daughter of obligate carrier |
| | II.4 | 15 | 10/100 | Daughter of obligate carrier |
| Family S | III.2 | 3½ | 13/150 | Daughter of obligate carrier |
| Family Fi | I.1 | ≅50 | 0/230 | Mother of ? sporadic retarded male |
| | II.2 | 17 | 0/200 | Sister of ? sporadic retarded male |
| Family P | I.8 | 44 | 0/350 | Mother of ? sporadic retarded male |

of these was the mother studied (age not given), and was found to have the fragile site in 2% of the cells examined. Similarly, a non-retarded 3-month-old girl had the site in 20% of her cells but her mother had it in only 2%. Turner et al. (1978a) demonstrated the fragile site in four of 11 obligate carriers and five of 16 possible carriers. Howard-Peebles et al. (1979) did not detect the fragile site in an obligate carrier, however they stated that the technical quality of their cultures was poor.

Harvey et al. (1977) have been the most successful in demonstrating the fragile site in carriers. They studied six obligate carrier females, and only in two aged 59 and 35 years were they unable to demonstrate the site. The ages (Judge, personal communication, 1978) and the frequencies of detection of the fragile sites were (pedigree reference numbers are for Harvey et al., 1977) for AI.6, greater than 70 years, 2%; for AII.9, 44 years, 8%; for BI.4, 82 years, 6%; and for BII.1, approximately 50 years, 24%. Harvey et al. also detected one other carrier aged about 50 years (AII.3) with the site in 7% of cells examined and a younger carrier female (AIII.10) with the site in 17% of cells examined. In the present study the oldest female who has been shown to be a carrier is 35 years old. Amongst younger daughters of obligate carriers, about half have also been shown to be carriers. This suggests that carrier detection may be fairly reliable below the age of about 20—25 years. However, for obligate carriers above this age cytogenetic carrier detection is highly unreliable.

Sutherland (1979b) has reported that the fragile site on Xq is not detectable in skin fibroblast or lymphoblastoid cell cultures from males with this fragile site. At present, this probably precludes the specific prenatal diagnosis of the associated form of mental retardation by amniotic fluid cell culture (Sutherland, 1977b). If fetal blood samples were available, such diagnosis might be possible.

The diagnosis of X-linked mental retardation with macro-orchidism remains difficult. Not all the retarded males with the fragile site have macro-orchidism (Sutherland et al., 1979). Furthermore, the fragile site can often be demonstrated in only a small proportion of metaphases from some retarded males even when the diagnosis is virtually certain on clinical grounds, and from a study of the family history and other affected relatives. Despite the methods developed for manipulation of the composition of the culture medium to

increase the frequency of fragile site expression in males (Sutherland, 1979a) this remains totally inadequate for females and less than satisfactory for males.

Further experiments are required to induce expression of the fragile site in a higher proportion of lymphocytes, particularly from females, and in other cell types.

*Acknowledgments.* I thank the physicians who referred some of the index cases for chromosome study and who helped with family studies, and Helen Eyre, Lynene Hinton, Trudy Hocking, Peter Leonard and Erica Woollatt for technical assistance.

## References

Giraud, F., Ayme, S., Mattei, J. F., Mattei, M. G.: Constitutional chromosomal breakage. Hum. Genet. **34**, 125—136 (1976)

Harvey, J., Judge, C., Wiener, S.: Familial X-linked mental retardation with an X chromosome abnormality. J. Med. Genet. **14**, 46—50 (1977)

Howard-Peebles, P. M., Stoddard, G. R., Mims, M. G.: Familial X-linked mental retardation, verbal disability, and marker X chromosome. Am. J. Hum. Genet. **31**, 214—222 (1979)

Lubs, H. A.: A marker X chromosome. Am. J. Hum. Genet. **21**, 231—244 (1969)

Mulcahy, M. T.: Carrier detection in X-linked mental retardation. Med. J. Aust. **2**, 489 (1978)

Sutherland, G. R.: Fragile sites on human chromosomes: Demonstration of their dependence on the type of tissue culture medium. Science **197**, 265—266 (1977a)

Sutherland, G. R.: Marker X chromosomes and mental retardation. N. Engl. J. Med. **296**, 1415 (1977b)

Sutherland, G. R.: Carrier detection in X-linked mental retardation. Med. J. Aust. **2**, 624 (1978)

Sutherland, G. R.: Heritable fragile sites on human chromosomes. I. Effect of composition of culture medium on expression. Am. J. Hum. Genet. **31**, 125—135 (1979a)

Sutherland, G. R.: Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects and cytogenetics. Am. J. Hum. Genet. **31**, 136—148 (1979b)

Sutherland, G. R., Ashforth, P. L. C.: X-linked mental retardation with macro-orchidism and the fragile site at Xq27 or 28. IIum. Genet. **48**, 117—120 (1979)

Sutherland, G. R., Judge, C. G., Wiener, S.: Familial X-linked mental retardation with an X-chromosome abnormality and macro-orchidism. J. Med. Genet. (in press, 1979)

Turner, G., Gill, R., Daniel, A.: Marker X chromosomes, mental retardation and macro-orchidism. N. Engl. J. Med. **299**, 1472 (1978a)

Turner, G., Gill, R., Daniel, A.: Carrier detection in X-linked mental retardation. Med. J. Aust. **2**, 624 (1978b)

Wiener, S., Judge, C.: Carrier detection in X-linked mental retardation. Med. J. Aust. **1**, 128 (1979)

Received July 4, 1979

Hum. Genet. 53, 29–30 (1979)

Human Genetics



# Heritable Fragile Sites on Human Chromosomes

## IV. Silver Staining

G. R. Sutherland* and P. Leonard

Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A., 5006 Australia

**Summary.** The chromosomal gaps associated with fragile sites at 2q11, 10q23, 11q13, 16q22, 20p11 and Xq27 do not stain with silver nitrate as do NOR regions of the acrocentric chromosomes.

### Introduction

The nucleolus organizer regions (NOR) of human chromosomes are the satellite stalks of the acrocentrics, as has been shown using N-banding (Hayata et al., 1977) and silver staining (Goodpasture et al., 1976). Fragile sites on human chromosomes have a region similar in appearance to satellite stalks (indeed the site near the distal end of Xq gives rise to a satellited appearance of this chromosome) but these do not take part in satellite associations with the acrocentric chromosomes (Harvey et al., 1977; Sutherland, 1979b).

Oliver et al. (1978) have shown that the variant of chromosome 17p, often referred to as a satellited marker, does not exhibit silver staining. Sutherland (1979a) has demonstrated that this variant 17 is not due to the presence of a fragile site of the type seen on other autosomes and the X chromosome. Silver staining for NOR has been carried out on the folic acid- and thymidine-sensitive fragile sites at 2q11, 10q23, 11q13, 12p11 and Xq27 and the resistant site at 16q22.

### Materials and Methods

Individuals with the fragile sites studied have been previously documented (Sutherland, 1979b). Lymphocytes cultured in medium 199 or MEM-FA (Sutherland, 1979a), which allow expression of fragile sites, were used for chromosome preparations.

Silver staining was carried out using the Ag-I method of Bloom and Goodpasture (1976) modified to the extent that slides covered with 50% $AgNO_3$ were incubated for 5 h at 75°C.

### Results

None of the fragile sites exhibited any silver staining over the non-staining gap which characterises such sites (Fig. 1). There was silver staining of the NOR of the acrocentrics indicating that the technique was working well and suggesting that if the fragile sites contained material capable of causing silver deposition, staining would have occurred.

### Discussion

Oliver et al. (1978) concluded from their finding of the absence of silver staining of the satellited 17p that its appearance was not due to translocation of satellites from one of the acrocentrics. A similar conclusion can be drawn regarding the fragile sites, that is, that they are not due to insertion of a satellite stalk from an acrocentric chromosome. It has been shown that a satellite stalk involved in a translocation can retain its silver staining property (Neu et al., 1976).

The specificity of Ag staining of NOR's remains unclear. Miller et al. (1977) concluded that Ag probably stained a protein rather than DNA or RNA. The regions of silver deposition correspond to the loci

* To whom offprint requests should be sent

0340-6717/79/0053/0029/$ 01.00

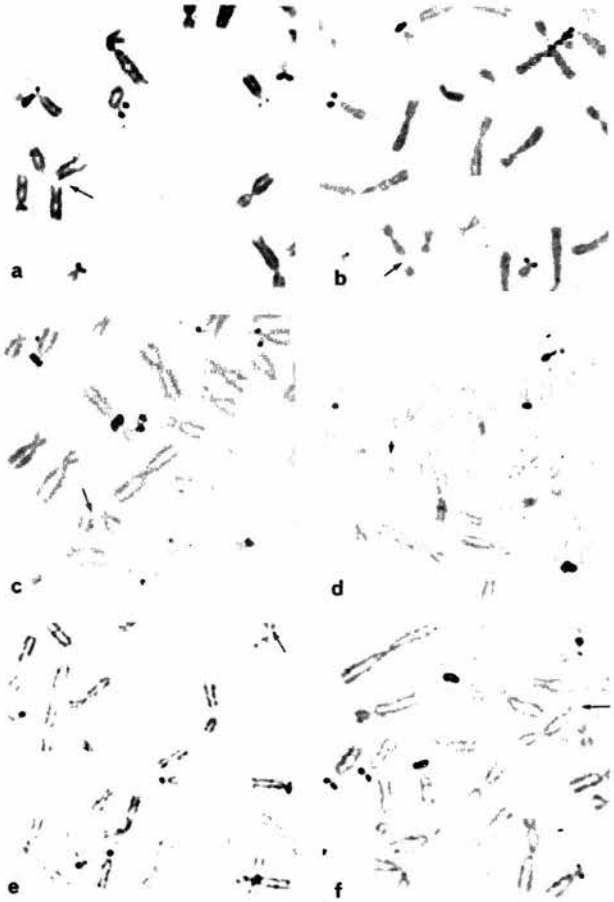



**Fig. 1.** NOR-stained partial metaphases showing the following fragile sites: (**a**) 2q11; (**b**) 10q23; (**c**) 11q13; (**d**) 16q22; (**e**) 20p11; (**f**) Xq27

which contain rDNA, but probably indicate activity of the rRNA genes rather than just their location (Miller et al., 1976). Whether other gene products, either RNA or protein, could be stained with silver in metaphase preparations is not known. The nature of the non-staining gaps produced at fragile sites remains speculative.

## References

Bloom, S. E., Goodpasture, C.: An improved technique for selective silver staining of nucleolar organiser regions in human chromosomes. Hum. Genet. **34**, 199—206 (1976)

Goodpasture, C., Bloom, S. E., Hsu, T. C., Arrighi, F. E.: Human nucleolus organisers: The satellites of the stalks? Am. J. Hum. Genet. **28**, 559—566 (1976)

Harvey, J., Judge, C., Wiener, S.: Familial X-linked mental retardation with an X-chromosome abnormality. J. Med. Genet. **14**, 46—50 (1977)

Hayata, I., Oshimura, M., Sandberg, A. A.: N-Band polymorphism of human acrocentric chromosomes and its relevance to satellite association. Hum. Genet. **36**, 55—61 (1977)

Miller, D. A., Tantravahi, R., Dev, V. G., Miller, O. J.: Frequency of satellite association of human chromosomes is correlated with amount of Ag-staining of the nucleolus organiser region. Am. J. Hum. Genet. **29**, 490—502 (1977)

Neu, R. L., Ortega, C. C., Barg, G. A., Pinto, W., Gardner, L. I., Howell, W. M., Denton, T. E.: Inclusion of satellites in an 18/21 translocation chromosome shown by ammoniacal-silver staining (sat-banding) in a case of partial trisomy 18. J. Med. Genet. **13**, 520—522 (1976)

Oliver, N., Francke, U., Taylor, K. M.: Silver staining studies on the short arm variant of human chromosome 17. Hum. Genet. **42**, 79—82 (1978)

Sutherland, G. R.: Heritable fragile sites on human chromosomes I. Factors affecting expression in lymphocyte culture. Am. J. Hum. Genet. **31**, 125—135 (1979a)

Sutherland, G. R.: Heritable fragile sites on human chromosomes II. Distribution, phenotypic effects and cytogenetics. Am. J. Hum. Genet. **31**, 136—148 (1979b)

Received July 4, 1979

*Am J Hum Genet* 32:542–548, 1980

# Heritable Fragile Sites on Human Chromosomes. V. A New Class of Fragile Site Requiring BrdU for Expression

GRANT R. SUTHERLAND,[1] ELIZABETH BAKER,[1] AND R. S. SESHADRI[2]

SUMMARY

A new fragile site at 10q25 is described, representing a new class of fragile site that requires bromodeoxyuridine (BrdU) in the culture medium for expression. This new fragile site is present in approximately one in 30 of the Australian population; it has only been observed in heterozygotes.

## INTRODUCTION

Heritable fragile sites on human chromosomes have been defined and provisionally classified according to their expression under different conditions of tissue culture [1, 2]. A new fragile site at 10q25 is described which requires BrdU in the culture medium for expression and which is present as a polymorphism in the population studied.

## MATERIALS AND METHODS

Sister chromatid exchange (SCE) studies were carried out on several groups of individuals as part of another project. These individuals were selected for a variety of reasons, such as the presence of neoplastic disease; exposure to possible environmental mutagens or teratogens during pregnancy, in which cases maternal blood and cord blood pairs were studied; and controls for these groups. Whole blood lymphocyte cultures were established in Ham's F10 (Commonwealth Serum Laboratories, Melbourne, Australia), supplemented with 20% fetal bovine serum to which 10 mg/liter of BrdU (Sigma, St. Louis, Mo.) had been added. The folic acid and thymidine-free culture medium used was that described by Sutherland [1]. Cultures were set up and harvested as described [3] after 2 hrs exposure to colchicine. Differential chromatid staining was by a modification of the method of Korenberg and Freedlender [4]. Unbanded chromosomes

Received October 25, 1980.

This work was supported in part by a grant from the Channel 10 Children's Medical Research Foundation of South Australia.

[1] Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia.

[2] Haematology Department, Flinders Medical Centre, Bedford Park, S.A. 5062, Australia.

were stained with Leishman stain and G-banded chromosomes by a trypsin-Leishman method. Silver staining was carried out as described [5]. All estimates of the proportion of metaphases within a culture showing lesions at the fragile site are based on the examination of 50 metaphases unless otherwise stated.

## RESULTS

There have been 12 independent ascertainments of the fragile site at 10q25. The fragile site appears to be in the middle of band q25 (fig. 1). Five families have been studied (fig. 2) and have shown that the fragile site is inherited as a Mendelian codominant. Titration of BrdU shows that 5–10 mg/liter of it is essential for maximum expression of the fragile site (fig. 3). The effect of the time prior to harvest of the addition of BrdU is shown in figure 4. From this, it can be seen that if BrdU is present from late S or earlier, the maximum fragile-site expression is achieved. Fragile sites could not be suppressed by addition of up to 20 mg/liter of folic acid nor induced in the absence of BrdU by methotrexate (10 mg/liter). Culture in folic acid and thymidine-free medium did not induce fragile-site expression in the absence of BrdU. The frequency of fragile-site expression was not related to culture medium pH over the range 7.2–7.7. Addition of high concentrations of thymidine (greater than 20 mg/liter) did inhibit the fragile site, although this effect was minimal.

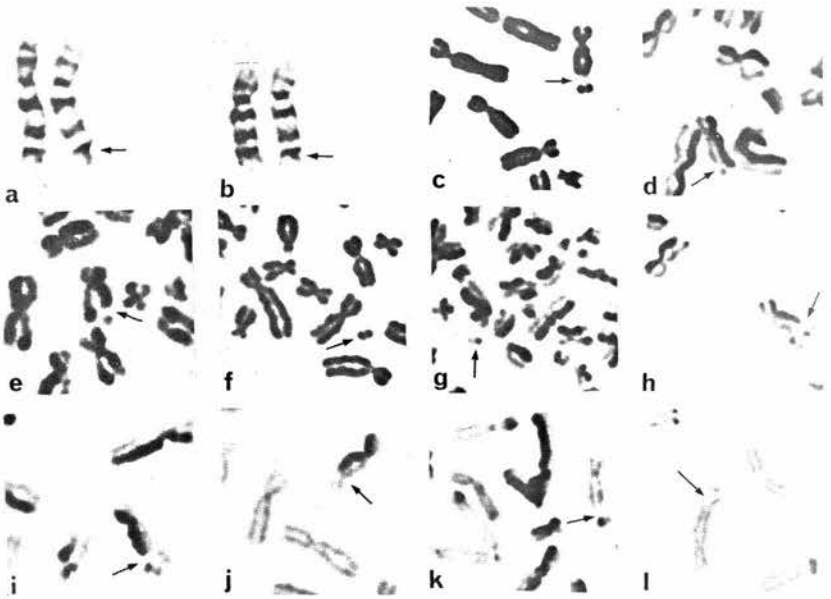



FIG. 1.—Appearances of the fragile site: *a* and *b*, G-banded chromosome pair 10 showing a chromatid gap in middle of band 10q25; *c*, chromosome break; *d*, gap in light-staining chromatid; *e*, chromatid gap with SCE at fragile site; *f* and *g*, isolated fragment 10q25-10qter; *h* and *i*, triradial configuration at fragile site; and *j* and *k*, third-division metaphases confirming SCE at fragile site in previous divisions; and *l*, silver-NOR-stained metaphase showing fragile site to be negative for this stain.

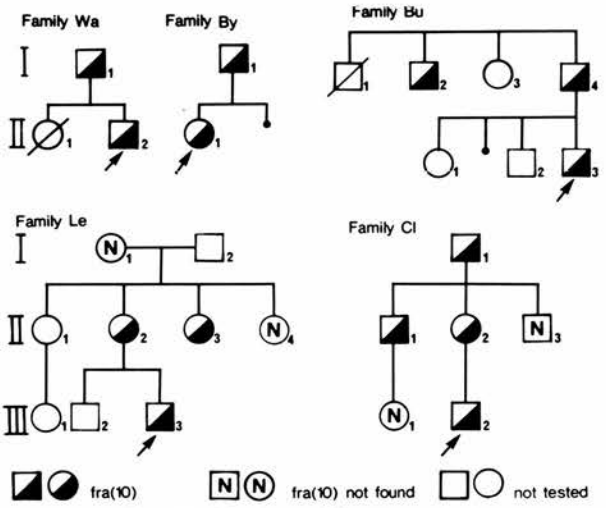



FIG. 2. —Pedigrees of families studied

## CYTOGENETICS

Lesions at the fragile site have a variety of appearances in a variable proportion of cells. Table 1 shows the frequency of the different fragile-site appearances in randomly selected metaphases from II.2 in family Le. Table 2 lists the more common appearances and their frequencies in the propositi; these data relate to metaphases showing differential chromatid staining; hence, they apply to cells which have undergone two rounds of replication in BrdU.

Metaphases showing two rounds of replication in BrdU and only a single chromatid break have only been seen in the bifilarly (light-staining) substituted chromatid. If the break is in both chromatids, then an SCE has often occurred at or very close to the fragile site. Some metaphases show only a single chromatid break, but SCE has occurred at the fragile site. In metaphases in the third division after exposure to BrdU, it can be confirmed that SCE has occurred at the fragile site (four out of 41 in table 1). There is an impression that SCE is more common at the fragile site than would be expected if SCE points were distributed at random. Figure 1 shows a variety of different appearances of the fragile site, which is Ag-NOR stain negative.

## INCIDENCE

The presence of this fragile site was unrelated to the reasons for the original study. Out of 276 individuals and 60 mother-child pairs studied, the fragile site was ascertained 12 times. Detection of the site in a mother-child pair was counted as only a single ascertainment. This gives a carrier frequency of approximately 1/30 and a "gene" frequency of approximately 1/60. No other BrdU-requiring fragile sites have been detected.

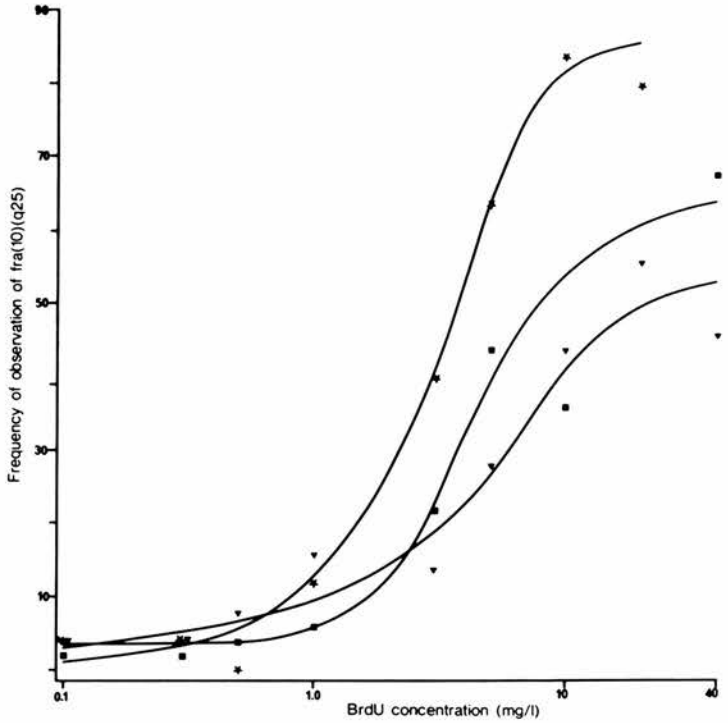



FIG. 3.—Effect of concentration of BrdU on frequency of expression of fragile site. Three curves represent studies on three unrelated individuals.

## DISCUSSION

The behavior of this fragile site at 10q25 fulfills the definition of a heritable fragile site previously put forward [1]. The major difference between this fragile site and those previously documented lies in the tissue culture conditions required for expression. The main requirement is that BrdU be present with a concentration of 5–10 mg/liter for the maximum expression frequency. The site is not inhibited by folic acid concentrations up to 20 mg/liter. High concentrations of thymidine tend to inhibit the site, but the most probable explanation for this is that excess amounts of thymidine swamp the BrdU and compete with it for a fixed number of incorporation sites which can utilize either thymidine or BrdU [6]. The pH of the medium had no effect on the frequency of lesions at the fragile site, although this factor is important in the expression of other fragile sites which have been described [1].

Many workers (e.g., [7, 8]) have shown BrdU to affect chromosome morphology, such as in the enhancement of secondary constrictions and the elongation of the centromere regions. Hsu and Somers [9] studied the effect of BrdU on Chinese hamster ovary cells and reached two conclusions: "(1) with BrdU concentration at 25 $\mu$g/ml, breakages occurred as early as 12 hours after treatment, and (2) the great majority (86%) of the breakages was found at the secondary constriction" (of the number one

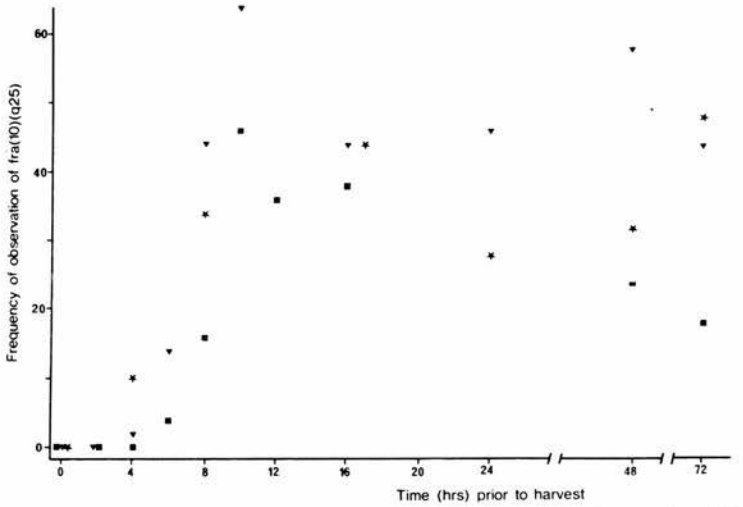



FIG. 4. —Effect of time prior to harvest of addition of BrdU on frequency of expression of fragile site in three unrelated individuals.

chromosomes). This report [9] is the only reference to breakage induced by BrdU at a specific locus. We found that there was no more nonspecific breakage than is seen in cultures without BrdU.

The action mechanism of BrdU in inducing the fragile site is unclear. Mazrimas and Stetka [6] have shown that unincorporated BrdU can cause SCE when present at concentrations of about $10^{-4}$ M. The fact that BrdU can cause expression of this fragile site when present during late S, but not after this stage, suggests that incorporation of BrdU into the DNA is a requirement for expression. Band 10q25 is fairly early replicating as shown by BrdU incorporation studies [10, 11]; however, since only a specific locus in the middle of this band is involved in the fragility, this could possibly be incorporating BrdU in late S without making the whole band appear late replicating. It would seem that for expression, the chromatid must have at least unifilarly substituted DNA. However, the cytological appearance of chromatid breaks indicates

TABLE 1

DIFFERENT FORMS OF FRAGILE-SITE EXPRESSION IN UNSELECTED METAPHASES FROM ONE INDIVIDUAL

| | NO. ROUNDS REPLICATION IN BRDU | | |
|---|---|---|---|
| FORM OF EXPRESSION | 1 | 2 | 3 |
| Not expressed | 7 | 43 | 16 |
| Chromosome gap or break | 5 | 7 | 10 |
| Chromatid gap or break (in light chromatid if differentially stained) | 4 | 20 | 8 |
| Chromatid gap or break with SCE | ··· | 3 | ··· |
| Chromosome break or gap with SCE | ··· | 1 | 4 |
| Triradial configuration | ··· | ··· | 2 |
| Additional fragment 10q25→10qter | ··· | 3 | 1 |
| Total cells examined | 16 | 77 | 41 |

TABLE 2

DIFFERENT FORMS OF FRAGILE-SITE EXPRESSION IN THE PROPOSITI AT THE TIME OF ASCERTAINMENT

| FORM OF EXPRESSION | SUBJECT OR FAMILY | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cl III.2 | Wa II.2 | Le III.3 | Ta | By II.1 | Li | Gr | Bu II.3 | Ha | Su | Cw | Ro |
| Not expressed | 42/100 | 9/100 | 15/50 | 12/50 | 39/50 | 37/50 | 8/20 | 35/50 | 18/50 | 41/50 | 46/50 | 82/100 |
| Chromatid break or gap | 41/100 | 61/100 | 23/50 | 12/50 | 11/50 | 11/50 | 7/20 | 11/50 | 16/50 | 8/50 | 4/50 | 15/100 |
| Chromosome break or gap | 10/100 | 17/100 | 9/50 | 16/50 | ... | 2/50 | 3/20 | 3/50 | 12/50 | 1/50 | ... | 3/100 |
| Isolated fragment 10q25→10qter | 5/100 | 3/100 | 3/50 | 10/50 | ... | ... | 1/20 | ... | 3/50 | ... | ... | ... |
| Miscellaneous, including triradials | 2/100 | 10/100 | ... | ... | ... | ... | 1/20 | 1/50 | 1/50 | ... | ... | ... |
| % cells expressing the fragile site | 58 | 91 | 70 | 76 | 22 | 26 | 60 | 30 | 64 | 18 | 8 | 18 |

that bifilarly substituted chromatids are more fragile. Another possible mechanism may lie in the suggestions that BrdU can alter protein binding or protein synthesis [11]. The knowledge that fragile sites on chromosomes require specific tissue-culture conditions for expression [3] has led to the confirmation of one of the fragile sites as a marker for one form of X-linked mental retardation, and the speculation that homozygosity for the folic-acid-sensitive autosomal fragile sites may be deleterious [2]. There is no base from which to extrapolate when considering any potential phenotypic effects of the BrdU-requiring fragile site. There is no consistent phenotypic abnormality in individuals who are heterozygous for the site at 10q25, suggesting that this fragile site is without phenotypic effect in heterozygotes, as are the other autosomal fragile sites [2].

The frequency of this fragile site, being present on approximately 1/60 number 10 chromosomes, is much higher than that of other fragile sites [12], and can be regarded as a polymorphism. Approximately one in 4,000 conceptions would be expected to be homozygous for the fragile site at 10q25. Provided such homozygosity is not lethal or semilethal in the fetus, then roughly the same frequency of neonates should also be homozygous for this site. If this homozygous state is associated with any disease, then that disease would mimic an autosomal recessive disorder which would be common in the population. Efforts should be made to detect homozygotes for this fragile site.

## ACKNOWLEDGMENTS

We thank Helen Eyre, Lynene Hinton, Trudy Hocking, and Erica Woollatt for technical assistance.

## REFERENCES

1. SUTHERLAND GR: Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. *Am J Hum Genet* 31:125–135, 1979
2. SUTHERLAND GR: Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects, and cytogenetics. *Am J Hum Genet* 31:136–148, 1979
3. SUTHERLAND GR: Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. *Science* 197:265–266, 1977
4. KORENBERG J, FREEDLENDER E: Giemsa technique for detection of sister chromatid exchanges. *Chromosoma* 48:355–360, 1974
5. SUTHERLAND GR, LEONARD P: Heritable fragile sites on human chromosomes. IV. Silver staining. *Hum Genet* 53:29–30, 1979
6. MAZRIMAS JA, STETKA DG: Direct evidence for the role of incorporated BUdR in the induction of sister chromatid exchanges. *Exp Cell Res* 117:23–30, 1978
7. PALMER CG: 5-Bromodeoxyuridine-induced constrictions in human chromosomes. *Can J Genet Cytol* 12:816–830, 1970
8. ZAKHAROV AF, BARANOVSKAYA LI, IBRAIMOV AI, BENJUSCH VA, DEMINTSEVA VS, OBLAPENKO NG: Differential spiralization along mammalian mitotic chromosomes. II. 5-Bromodeoxyuridine and 5-bromodeoxycytidine-revealed differentiation in human chromosomes. *Chromosoma* 44:343–359, 1974
9. HSU TC, SOMERS CE: Effect of 5-bromodeoxyuridine on mammalian chromosomes. *Proc Natl Acad Sci USA* 47:396–403, 1961
10. LATT SA: Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. *Proc Natl Acad Sci USA* 70:3395–3399, 1973
11. DUTRILLAUX B, FOSSE A: Sur le mécanisme de la segmentation chromosomique induite par le BUDR (5-bromodeoxyuridine). *Ann Genet (Paris)* 17:207–211, 1974
12. HECHT F, KAISER-MCCAW B: The importance of being a fragile site. *Am J Hum Genet* 31:223–225, 1979

*Journal of Medical Genetics*, 1980, 17, 73-74.

## Familial X-linked mental retardation with an X chromosome abnormality and macro-orchidism

SIR,

Two forms of X-linked mental retardation have been described, one associated with an X chromosome which has a fragile site at Xq27 or 28,[1 2] and the other associated with macro-orchidism.[3 4 5] As a result of measuring the external genitals and calculating testicular volumes of retarded males with the fragile site at Xq27 or 28, Sutherland and Ashforth[6] have suggested that these two forms of mental retardation are the same entity.

We have recently measured the genitals and calculated testicular volumes of some of the retarded males with fragile sites at Xq27 or 28 who were originally described by Harvey *et al.*[1] Of the seven males examined (figure, table), six had testicular volumes greater than the 90th centile of Prader.[7]

Turner *et al*[8] have independently re-examined the chromosomes, under conditions appropriate for demonstration of fragile sites,[9] of the males originally described with mental retardation and macro-orchidism. They found that these males do have the fragile site at Xq27 or 28. That finding, in conjunction with the report of Sutherland and Ashforth[6] and the data presented here, confirm that the two forms of X-linked mental retardation recorded in published reports are the same. Hence, it is now possible to add large testes, as well as small ones, to the list of indications for chromosome studies.

GRANT R SUTHERLAND*, C G JUDGE†, AND S WIENER‡

**Cytogenetics Unit, Adelaide Children's Hospital, North Adelaide, South Australia 5006; †Children's Cottages, Kew, Victoria 3101; and ‡Chromosome Laboratory, St Nicholas Hospital, Carlton South, Victoria 3053, Australia*

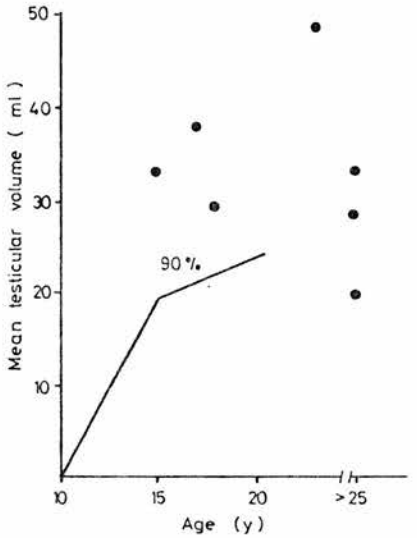



FIGURE *Average (left and right) testicular volume of retarded males examined. The 90% line is the centile given by Prader[7] for ages 10 to 20 years. All males aged 25 years or more are shown as >25.*

TABLE *Genital measurements of males with mental retardation and fra (X) (q27 or 28).*

| *Subject* | *Age* | *Penis* | | *Testicular volume (ml)* | |
|---|---|---|---|---|---|
| | | *Length* | *Circum-ference* | *Right* | *Left* |
| Family A | | | | | |
| II.4 | 54 | 11·0 | 8·0 | 42 | 24 |
| II.10 | 46 | 9·0 | 8·0 | 24 | 32 |
| II.11 | 44 | 13·0 | 7·5 | 18 | 21 |
| III.9 | 17 | 10·0 | 8·5 | 38 | 38 |
| Family B | | | | | |
| III.1 | 23 | 10·0 | 9·0 | 39 | 58 |
| Subject TK | 15 | 11·0 | 8·5 | 37 | 29 |
| Subject NK | 18 | 12·0 | 10·0 | 30 | 29 |

Family identification as in Harvey *et al.*[1] Subjects TK and NK are brothers from another family mentioned in their addendum.

### References

1 Harvey J, Judge C, Wiener S. Familial X-linked mental retardation with an X chromosome abnormality. *J Med Genet* 1977;14:46–50.
2 Sutherland GR. Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects and cytogenetics. *Am J Hum Genet* (in press).
3 Turner G, Eastman C, Casey J, McLeay A, Procopis P, Turner B. X-linked mental retardation associated with macro-orchidism. *J Med Genet* 1975;12:367–71.
4 Cantu JM, Scaglia HE, Medina M, *et al.* Inherited congenital normofunctional testicular hyperplasia and mental deficiency. *Hum Genet* 1976;33:23–33.
5 Cantu JM, Scaglia HE, Gonzalez-Diddi M, *et al.* Inherited congenital normofunctional testicular hyperplasia and mental deficiency. *Hum Genet* 1978;41:331–8.
6 Sutherland GR, Ashforth PLC. X-linked mental retardation with macro-orchidism and the fragile site at Xq27 or 28. *Hum Genet* (in press).
7 Prader A. In: Labhart A, ed. *Clinical endocrinology, theory and practice.* Berlin: Springer-Verlag, 1974:1036.
8 Turner G, Gill R, Daniel A. Marker X chromosomes, mental retardation and macro-orchidism. *N Engl JMed* 1979;299:1472.
9 Sutherland GR. Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. *Am J Hum Genet* (in press).

Hum Genet (1981) 57:217–219



Human Genetics



# Heritable Fragile Sites on Human Chromosomes

## VI. Characterization of the Fragile Site at 12q13

Grant R. Sutherland and Lynene Hinton

Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia

**Summary.** A kindred is described in which six members have a fragile site at 12q13. This fragile site was found to be suppressed by folic acid and thymidine in lymphocyte culture. An updated classification of known fragile sites is presented.

### Introduction

In a report of the factors affecting expression of fragile sites in lymphocyte culture Sutherland (1979) was able to study all the existing documented fragile sites except the one at 12q13. This fragile site has been recorded by Giraud et al. (1976), Donti et al. (1979), and McCreanor (personal communication 1977), but its response in lymphocyte culture to folic acid, thymidine, and other culture medium variables has not been previously documented.

### Materials and Methods

Methods of chromosome study and tissue culture medium composition have been previously described (Sutherland 1979). Silver staining was performed according to the method described by Sutherland and Leonard (1979) and BrdU replication banding as described in Sutherland and Eyre (to be published). All frequencies of expression of the fragile site are based upon the study of 50 metaphases from lymphocyte cultures.

The fragile site was found in a family (Fig. 1) studied because several members had autosomal dominant Köbner-type epidermolysis bullosa.

### Results

The appearance of fra(12)(q13) was similar to that of other fragile sites (Fig. 2a–c). There was a lesion at 12q13 in about 30% of metaphases in cultures grown in MEM-FA for three days. The lesion was mainly a chromosome or chromatid gap, but deletions, triradials etc., were seen in 2–5% of metaphases. G-banding (Fig. 2e and f) and bromodeoxyuridine (BrdU) replication banding (Fig. 2g and h) showed the fragile site to be in the middle of band 12q13. The fragile site was NOR-silver stain negative (Fig. 2d).

The effect of the addition of folic acid and thymidine to medium MEM-FA is shown in Fig. 3. The fragile site is highly sensitive to folic acid being completely suppressed by 0.1 mg/l. Sensitivity to thymidine is present but not so marked; 5 mg/l was required for complete suppression. The fragile site was not affected by the pH of the culture medium over the range 7.09 to 7.90. The fragile site was affected by BrdU added at the time cultures were initiated, 5 mg/l suppressed fragile site expression from 30% of metaphases to 4% and 10 mg/l to 2%. The time in culture prior to harvest (Fig. 4) was important in that the maximum frequency of expression was not reached until day 4.

### Discussion

The response of the fragile site at 12q13 to variations in culture medium composition is very similar to that of the fragile sites at 10q23, 11q13, and 16p12 and differs from the other folic acid sensitive sites only by its expression being unrelated to the pH of the culture medium (Sutherland 1979).

Jacobs et al. (1979) have indicated that the frequency of expression of the fragile site at Xq27 is influenced by the time in culture. Similar behaviour by other fragile sites has been observed (Sutherland, unpublished observations) and is documented here for fra(12)(q13). In particular, the difference in frequency of expression from day 2 to day 3 is considerable; attempting to study or detect fragile sites in two-day cultures is

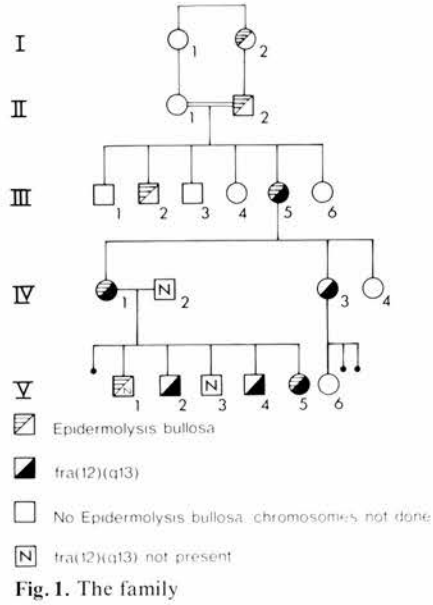



**Fig. 1.** The family

*Offprint requests to:* G. R. Sutherland

0340-6717/81/0057/0217/$ 01.00

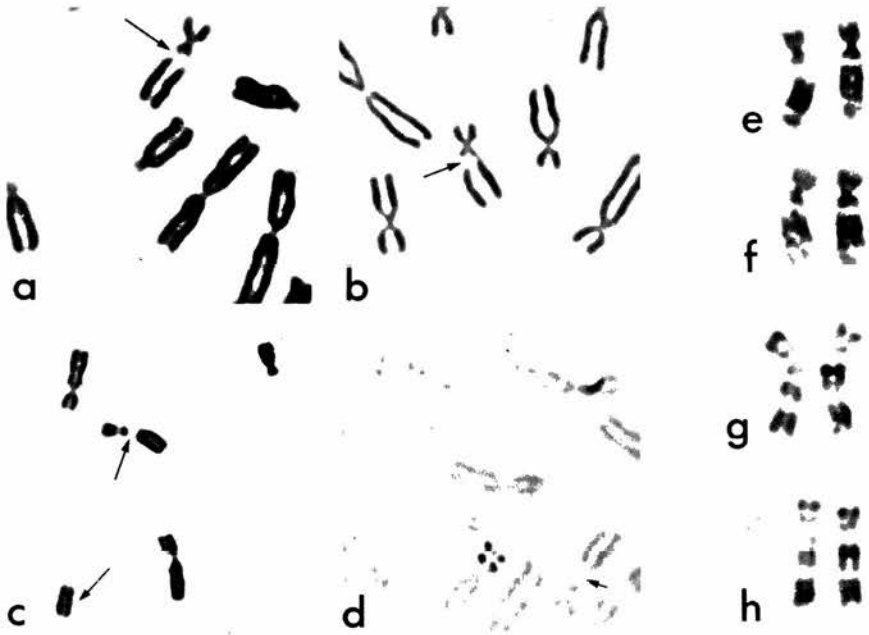



**Fig. 2.** Appearances of the fragile site, **a** chromosome gap, **b** chromatid gap, **c** additional fragment 12q13→12qter, **d** NOR-silver stained preparation, **e, f** G-banded number 12 chromosomes from two metaphases, the chromosome expressing the fragile site is on the right, **g, h** BrdU replication banded number 13 chromosomes from two metaphases

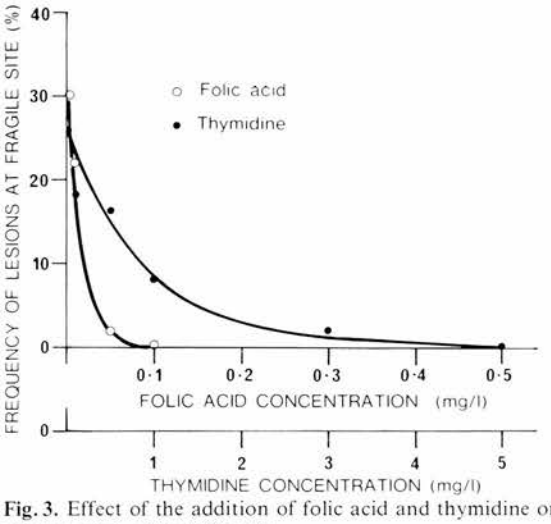



**Fig. 3.** Effect of the addition of folic acid and thymidine on frequency of expression of fra(12)(q13)

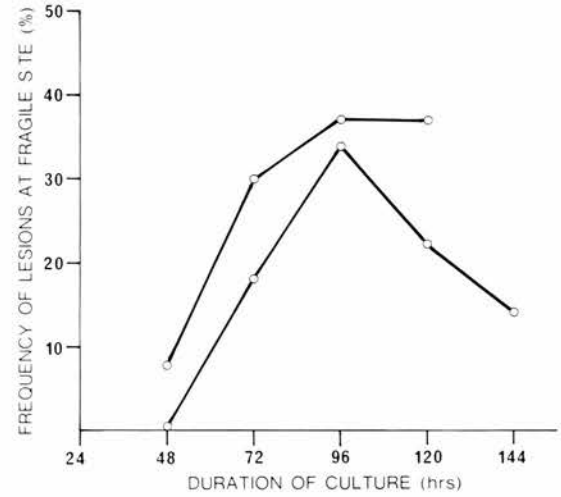



**Fig. 4.** Effect of duration of lymphocyte culture prior to harvest on frequency of expression of fra(12)(q13)

unrewarding. Furthermore, the maximum frequency of expression is usually reached after four days when it may remain stable but usually declines (along with the quality of the chromosome preparation).

Cytologically, fra(12)(q13) behaves in a similar way to the other fragile sites, being expressed mainly as a chromosome or chromatid gap with a small proportion of cells having deletions or duplications of chromosome material distal to the fragile site. The associations between the fragile site and satellites of the acrocentric chromosomes described by Donti et al. (1979) in their case of fra(12)(q13) were not seen in any members of the present family with this fragile site. As with other fragile sites, fra(12)(q13) is NOR-silver stain negative (Sutherland and Leonard 1979).

**Table 1.** Classification of heritable fragile sites according to expression under different tissue culture conditions

| BrdU requiring | | | |
|---|---|---|---|
| Yes | No | | |
| | Folic acid and thymidine sensitive | | |
| | Yes | | No |
| | pH correlated | | |
| | Yes | No | |
| 10q25 | 2q11, 20p11, Xq27 | 10q23, 11q13[a], 12q13, 16p12 | 16q22 |

[a] Not yet shown to be heritable

The documentation of fra(12)(q13) as being sensitive to folic acid and thymidine, and independent of pH completes classification of the well established fragile sites (Sutherland 1979). There are now at least three types of fragile site known (Table 1), the majority being sensitive to folic acid and thymidine but the one at 16q22 being resistant and the common one at 10q25 requiring BrdU for expression (Sutherland et al. 1980; Scheres and Hustinx 1980). Fragile sites other than those shown in Table 1 have not been documented although a probable folic acid sensitive fragile site at 9p21 has been partly characterised (Manuel, personal communication 1980).

*Acknowledgements.* We thank Dr. T. W. Turner for referring the family and Elizabeth Baker for help with the cytogenetics. This work was supported by a grant from the Adelaide Children's Hospital Research Trust.

## References

Donti E, Venti G, Bocchini V, Rosi G, Armellini R, Trabalza N (1979) The constitutional fragility of chromosome 12 in a case of 46,XX, var(12)(qh', RGH, GAG, CBG). Hum Genet 48:53–59

Giraud F, Aymé S, Mattei JF, Mattei MG (1976) Constitutional chromosome breakage. Hum Genet 34:125–136

Jacobs PA, Mayer M, Rudak E, Gerrard T, Ives EJ, Shokeir MHK, Hall J, Jennings M, Hoehn H (1979) More on marker X chromosomes, mental retardation and macro-orchidism. N Engl J Med 300:737–738

Scheres JM, Hustinx TW (1980) Heritable fragile sites and lymphocyte culture medium containing BrdU. Am J Hum Genet 32:628–629

Sutherland GR (1979) Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. Am J Hum Genet 31:125–135

Sutherland GR, Baker E, Seshadri RS (1980) Heritable fragile sites on human chromosomes. V. A new class of fragile site requiring BrdU for expression. Am J Hum Genet 32:542–548

Sutherland GR, Leonard P (1979) Heritable fragile sites on human chromosomes. IV. Silver staining. Hum Genet 53:29–30

Received October 20, 1980

*Am J Hum Genet* 33:946–949, 1981

# Heritable Fragile Sites on Human Chromosomes. VII. Children Homozygous for the BrdU-Requiring fra(10)(q25) Are Phenotypically Normal

GRANT R. SUTHERLAND[1]

SUMMARY

A brother and sister have been detected who are homozygous for the bromodeoxyuridine (BrdU)-requiring fragile site at 10q25. The children are phenotypically normal, indicating that homozygosity for this fragile site is harmless, at least during childhood.

## INTRODUCTION

A fragile site at 10q25 requiring BrdU for expression was reported independently by Sutherland et al. [1] and by Scheres and Hustinx [2] and shown to be polymorphic in an Australian Caucasian population [1]. Since the fragile site at Xq27 produces mental retardation in hemizygous males, the possibility that fra(10)(q25) would be deleterious in homozygotes was investigated. Two homozygous siblings have been detected and are phenotypically normal children.

## MATERIALS AND METHODS

All clinical case referrals to the Cytogenetics Unit and a series of unselected neonates studied by cord blood lymphocyte culture were examined for the presence of fra(10)(q25) as described [1]. When an individual with the fragile site was ascertained, family studies were undertaken when possible, including chromosome studies of the spouses of individuals with fra(10)(q25).

## RESULTS

Fra(10)(q25) has been ascertained 49 times, and family studies have produced 35 couples of which only one spouse had this fragile site. Both of the 36th couple,

Received February 17, 1981; revised April 7, 1981.

This work was supported by a grant from the Adelaide Children's Research Trust.

[1] Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia.

studied as the result of finding the fragile site in a male neonate, were found to be carriers of this fragile site. The couple, neither of whom are related to each other, has four children (fig. 1), two homozygous and two heterozygous for the fragile site. One of the homozygotes is a 12-year-old girl who is phenotypically normal and doing well at school. The other is the neonatal male who, at six weeks, is developmentally normal.

*Cytogenetics*

The cord blood study on the propositus did not work well, and only 35 cells were available for examination (table 1). Two of these apparently showed the fragile site on both no. 10 chromosomes. On repeat culture by heel prick, 19% of cells showed the fragile site on both chromosomes (fig. 2). The homozygous sister of the propositus expressed the fragile site on both no. 10 chromosomes in 25% of her metaphases.

## DISCUSSION

In heterozygotes, the fragile site at 10q25 is usually seen in 15%–80% of metaphases, although it is occasionally seen at frequencies outside this range [1]. The forms of expression are variable, and in one heterozygote a single metaphase showed the site apparently expressed on both chromosomes. A similar observation in a heterozygote was recorded by Scheres and Hustinx [2]. In the family studies, there was no difficulty in differentiating the homozygotes from the heterozygotes.

The only fragile site for which homozygosity has probably been demonstrated is the one at 16q22 [3]. This fragile site is not sensitive to folic acid, thymidine, or

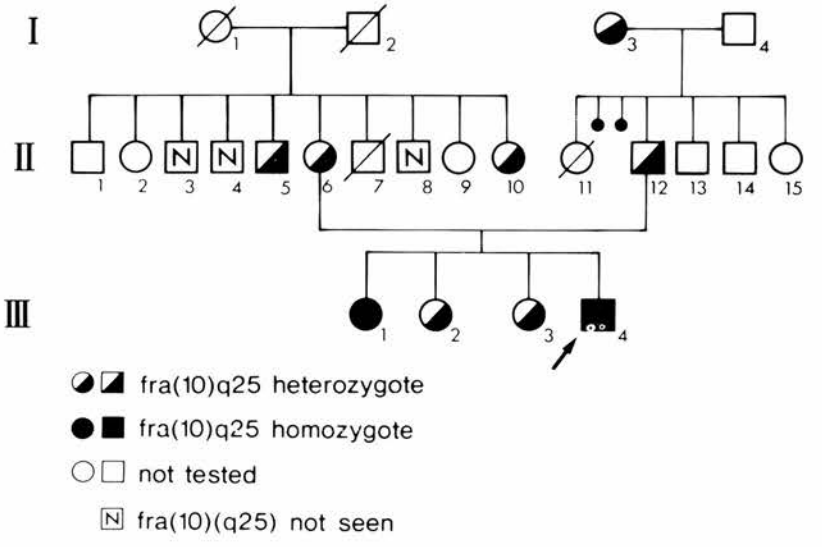



FIG. 1.—The family pedigree

TABLE 1

EXPRESSION OF fra(10)(q25) IN FAMILY MEMBERS

| PEDIGREE NO. | HOMOLOGUES 0 | 1 | 2 | TOTAL CELLS |
|---|---|---|---|---|
| $II_6$ | 38 | 12 | 0 | 50 |
| $II_{12}$ | 36 | 9 | 0 | 45 |
| $III_1$ | 18 | 27 | 15 | 60 |
| $III_2$ | 31 | 19 | 0 | 50 |
| $III_3$ | 25 | 25 | 0 | 50 |
| $III_4$: Cord blood | 24 | 9 | 2 | 35 |
| Capillary blood | 50 | 69 | 26 | 145 |

BrdU, but can be induced by distamycin A [3]. The homozygote described was a normal male. Whether he is truly homozygous is uncertain since Schmid et al. [3] divided the fra(16)(q22) into two types: one a true fragile site, and the second, more like a secondary constriction or nonstaining gap that did not show fragility. The homozygote had one of each of these types of fragile site. Similarly, homozygosity for the "satellited" 17p, which is somewhat similar in appearance to a fragile site but is not regarded as one [4], has been described in a normal woman [5].

Since the folic acid-sensitive fragile site at Xq27 produces mental retardation in hemizygous males, and mild mental deficiency in some heterozygous females [6], it

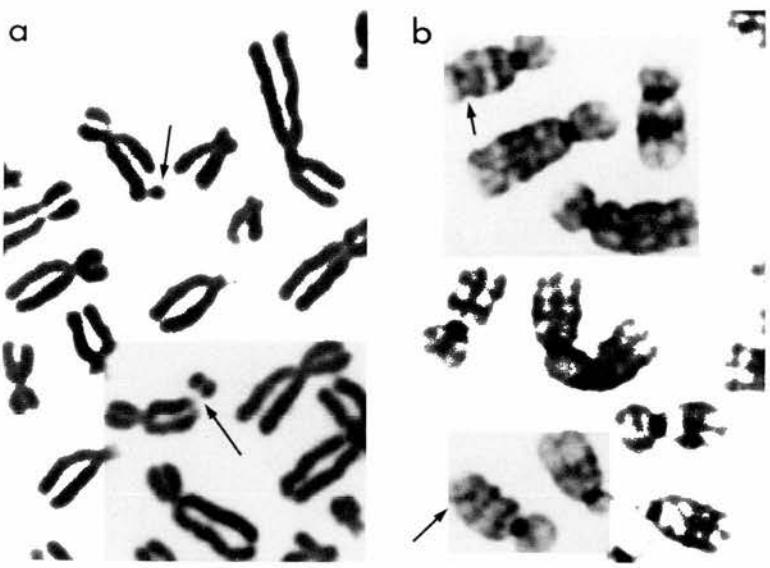



FIG. 2.—Two metaphases showing homozygous expression of the fragile site. *(a)* Unbanded cell in which one chromosome 10 has a chromatid gap and the other a chromosome gap; *(b)* G-banded chromosomes showing the fragile site at 10q25.

has been speculated that homozygosity for the other folic acid-sensitive fragile sites could be deleterious [4]. Since the fragile site at 10q25 appears to be unique in that it is the only one known to require BrdU for expression in humans and is present in polymorphic frequencies [1], there is no basis for speculation about its effects on homozygotes.

The family reported here demonstrates that if homozygosity has any effect, it is not apparent in children. Homozygosity for this fragile site could result in some problem, however, in older individuals.

## ACKNOWLEDGMENTS

I thank Helen Eyre, Lynene Hinton, Trudy Hocking, and Erica Woollatt for assistance with cytogenetics, Dr. Keith Lovell for clinically examining the homozygous neonate, and the family for their help and cooperation.

## REFERENCES

1. Sutherland GR, Baker E, Seshadri RS: Heritable fragile sites on human chromosomes. V. A new class of fragile site requiring BrdU for expression. *Am J Hum Genet* 32:542–548, 1980
2. Scheres JMJC, Hustinx TWJ: Heritable fragile sites and lymphocyte culture medium containing BrdU. *Am J Hum Genet* 32:628–629, 1980
3. Schmid M, Klett C, Niederhofer A: Demonstration of a heritable fragile site in human chromosome 16 with distamycin A. *Cytogenet Cell Genet* 28:87–94, 1980
4. Sutherland GR: Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects, and cytogenetics. *Am J Hum Genet* 31:136–148, 1979
5. Berg JM, Faunch JA, Pendrey MJ, Penrose LS, Ridler MAC, Shapiro A: A homozygous chromosomal variant. *Lancet* 1:531, 1969
6. Turner G, Brookwell R. Daniel A, Selikowitz M, Zilibowitz M: Heterozygous expression of X-linked mental retardation and X-chromosome marker fra(X)(q27). *N Engl J Med* 303:662–664, 1980

*Am J Hum Genet* 34:452–458, 1982



# Heritable Fragile Sites on Human Chromosomes. VIII. Preliminary Population Cytogenetic Data on the Folic-Acid-Sensitive Fragile Sites

GRANT R. SUTHERLAND[1]

SUMMARY

The incidence of the autosomal folic-acid-sensitive fragile sites in 524 institutionalized retardates (.0095) was found to be significantly higher than in 1,019 unselected neonates (.00098), suggesting that heterozygosity for these fragile sites may not be as harmless as previously thought. When one of the parents of an index case was found to carry the fragile site, that parent was always the mother. The fragile site at Xq27 was not found among the neonates studied, but was present in 1.6% of the institutionalized retarded males examined; if this fragile site occurs in normal males, then it does so rarely. Further cytogenetic studies of fragile sites are required on both normal and abnormal populations.

## INTRODUCTION

There are now nine known folic-acid-sensitive fragile sites on the human karyotype [1, 2]. The frequencies of these in normal and abnormal populations are unknown. Chromosomal surveys of randomly selected neonates that have established the frequencies of other constitutional chromosomal anomalies have not provided data on fragile sites because they were mostly carried out using unsuitable culture medium and were based upon the examination of small numbers (usually two) of cells. The only such survey to record a fragile site [3] was a two-cell study that found one child with a fragile site on C group chromosome among 3,543 phenotypically normal infants studied. This was almost certainly an incomplete detection of the fragile sites in that population.

Received July 28, 1981; revised August 25, 1981.

This work was supported by a grant from the Adelaide Children's Hospital Research Trust.

[1] Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia.

Three groups within the South Australian population have been examined for folic-acid-sensitive fragile sites. These were unselected neonates, patients referred for diagnostic chromosome study, and institutionalized retardates.

## MATERIALS AND METHODS

### *Neonates*

Cord blood was collected by the nursing staff from the first 20 infants born after 8 P.M. Sunday each week in an obstetric hospital in which there were approximately 3,000 deliveries per year. As these criteria were not rigorously adhered to, some minor selection may have been exercised. Cultures were established in MEM-FA supplemented with 5% fetal bovine serum as described [4, 5], and harvested after 72 hrs using standard methods. Two unbanded cells were fully analyzed microscopically, and a further 48 examined for fragile sites.

### *Patients*

Since late 1976, most patients referred for diagnostic chromosome study have had their lymphocytes cultured in medium suitable for the expression of fragile sites. Initially, this was medium 199, but more recently MEM-FA has been used. Venous blood was studied in the majority of cases, but in neonates and small children, capillary blood collected by heel or finger prick was used. Thirty unbanded metaphases per person were examined microscopically, a further two or three G-banded metaphases were also examined, and a banded karyotype prepared. In retarded males suspected clinically of having the fra(X)(q27), a further 20 metaphases were examined for fragile sites. The patients were not all retarded, but covered the spectrum seen in a general cytogenetics unit (e.g., retardates, couples with reproductive problems, and dysmorphic children). When families were studied, only the index case of any family is shown in table 1.

TABLE 1

FOLIC-ACID-SENSITIVE FRAGILE SITES IN THE GROUPS STUDIED

| GROUP | NO. STUDIED | FRAGILE SITES | |
|---|---|---|---|
| | | Xq27 | Autosomal |
| Neonates: | | | |
| Males | 522 | 0 | 0 |
| Females | 497 | 0 | 1 |
| Patients | 2,237 | 7 | 10 |
| Minda Home: | | | |
| Males | 298 | 5 | 1 |
| Strathmont: | | | |
| Males IQ > 36 | 98 | 2 | 2 |
| Other males | 6 | 0 | 0 |
| Females | 35 | 0 | 0 |
| Ru Rua: | | | |
| Males | 42 | 0 | 2 |
| Females | 45 | 0 | 0 |

*Retardates*

All the male residents with otherwise normal chromosomes from Minda Home, a residential institution for the retarded, were studied by capillary blood culture in the first instance. If a fragile site was detected or suspected, then a repeat culture from venous blood was performed. All residents of Ru Rua, an institution for the totally dependent mentally retarded, with otherwise normal karyotypes, were studied from venous blood. Strathmont, a residential institution for the retarded that has been previously studied for major chromosome anomalies [6] was restudied in part. Initially, all males with an otherwise normal karyotype and I.Q.s greater than 36 were studied from venous blood; thereafter, both males and females with otherwise normal karyotypes who were venipunctured for other reasons were studied. Institutional residents' lymphocytes were cultured in MEM-FA, and at least 50 unbanded metaphases per person were examined for fragile sites.

## RESULTS

The number of individuals in each of the populations studied and the fragile sites found are shown in table 1. The numbers in the patient and neonate groups are those in which the requisite number of metaphases could be scored and not the total number studied. The incidence of the autosomal fragile sites in the retardates is significantly greater than that in the neonates ($\chi^2_1 = 3.87$, $P < .05$) but not different from that in the patient group ($\chi^2_1 = 1.6$, not significant). Similarly, the difference between the retardate and patient groups is not significant ($\chi^2_1 = 1.19$, not significant).

Details of the 16 propositi with the autosomal folic-acid-sensitive fragile sites are shown in table 2. The most common fragile site seen in this group was that at 10q23, which accounted for seven ascertainments. All the other fragile sites detected have been documented previously except for the one at 9q31. This showed the typical behavior of a folic-acid-sensitive fragile site with regard to response to culture medium composition, although medium pH was not studied. Cytogenetically, the appearance was typical, showing chromosome and chromatid breaks at 9q31 and, more importantly for confirmation as a fragile site, triradial figures and deletions [2].

Family studies showed the mothers of the index cases to have the fragile site in all but two instances when it could not be detected in either parent. In these two instances, genetic marker studies showed a high probability of correct paternity. In a further two cases, family studies could not be carried out.

All the index cases with fra(X)(q27) were retarded males, with one exception in the patient group being a borderline retarded female. Family studies of a number of these have been reported [7]; in most cases, the fra(X)(q27) was found in other family members, although in three instances, extensive studies suggested that the index case may have been the result of a new mutation. One of the males with fra(X)(q27) also had fra(16)(q22), but family studies in this instance were not achieved (subject B [5]).

## DISCUSSION

The detection of fragile sites is still not without difficulties; hence the data presented is for a minimum incidence of fragile sites. Even using the best-known

TABLE 2

DATA ON PROPOSITI WITH FOLIC-ACID-SENSITIVE AUTOSOMAL FRAGILE SITES

| Patient | Group | Age | fra | Sex | Maximum frequency of lesions at site (%) | Parental origin | Maximum frequency of lesions at site in parent | Clinical status |
|---|---|---|---|---|---|---|---|---|
| 559/80..... | Neonate | Newborn | 10q23 | F | 7 | Maternal | 34 | Normal neonate |
| 797/79..... | Patient | 8 yrs | 2q1 | M | 56 | Maternal | 6 | Benign ovarian teratoma |
| 463/80..... | Patient | 1 yr | 10q23 | M | 14 | Maternal | 14 | Retarded |
| 852/77..... | Patient | 7 yrs | 16p12 | M | 82 | Maternal | 72 | Family D [10], leukemia |
| 485/80..... | Patient | 2 yrs | 12q13 | F | 16 | Maternal | 30 | Epidermolysis bullosa [1] |
| 302/80..... | Patient | 12 yrs | 10q23 | F | 28 | Not done | ... | Short stature |
| 833/80..... | Patient | 20 yrs | 11q13 | F | 26 | Maternal | 12 | Multiple miscarriages |
| 169/80..... | Patient | 2 yrs | 10q23 | M | 40 | Maternal | 20 | Failure to thrive, ? retarded |
| 91/81..... | Patient | 11 yrs | 9q31 | M | 21 | ? Mutant | 0 | Gynecomastia |
| 208/81..... | Patient | 6 days | 10q23 | M | 28 | Maternal | 2 | Severe hypospadias |
| 185/81* ..... | Patient | 14 yrs | 10q23 | F | 24 | Maternal | 19 | Mildly retarded |
| 591/77..... | Minda | 20 yrs | 20p11 | M | 46 | Not done | ... | Retardate |
| 360/72..... | Strathmont | 12 yrs | 2q1 | M | 78 | Maternal | 42 | Family F [10], retardate |
| 558/72..... | Strathmont | 31 yrs | 10q23 | M | 86 | Maternal | 45 | Family Ay [10], retardate |
| 505/73..... | Ru Rua | 6 yrs | 20p11 | M | 66 | Maternal | 2 | Family Mi [10], retardate |
| 587/73..... | Ru Rua | 19 yrs | 11q13 | M | 36 | ? Mutant | 0 | Totally dependent retardate |

* Also has the BrdU-requiring fragile site at 10q25 on the same chromosome.

conditions for lymphocyte culture, the site at Xq27 is not detectable in some females who are obligate carriers of it and is seen in a very low percentage of metaphases in some males [7]. The section of this work carried out using capillary blood is probably less reliable than that carried out using venous blood. One of the males in Minda Home had the fra(X)(q27) in only 2% of cells from capillary blood but in 10% from venous blood. A possible reason for this difference is the observation that microbial contamination of lymphocyte cultures renders them useless for fragile-site detection, even if adequate metaphases are present to permit karyotyping; such low-grade contamination is more common in capillary blood than in venous blood cultures.

Demonstration of the autosomal fragile sites can also be a problem in some families, and presumably in some individuals. Table 2 shows that the fragile site was detected in less than 5% of metaphases in some family members. Two of the autosomal fragile sites detected seemed to be new mutants in that they were not detected in either of the parents of the index cases. However, a better explanation may be that these apparent new mutants were familial cases but that the fragile sites were not detected in the carrier parents; it is not possible to be certain about this. If a new mutant fragile site is harmless, then it is most unlikely that two such rare events would be detected in this small series. There is often morbidity associated with new mutant translocations [8]; perhaps the same could hold for new mutant fragile sites, in which case their detection in abnormal individuals would not be unexpected. Three of the retarded males with fra(X)(q27) appear to be new mutants; indeed, if the reproductive fitness of these males is zero (and the mutation rates in the two sexes are equal), then one-third of new cases would be expected to be mutants [9].

The finding of a higher incidence of autosomal fragile sites in retardates than in neonates was unexpected since it had been thought that heterozygosity for the autosomal fragile sites was without phenotypic effect [10]. The findings from the present study require close examination. The significance may be due to chance or possibly to bias. Only one fragile site was found in the neonates; if one more had been found in this group, then the significance would disappear. Since there may have been some minor selection in the neonates studied, it could be argued that if heterozygosity for the fragile sites was deleterious then such infants may not have been studied. This is possible but unlikely as all infants born in the hospital during the period of the survey who had clinical indications for chromosome studies were scored for fragile sites and none were found. Further bias comes from the fact that some of the retardates were known to have fragile sites prior to the survey; indeed, it was the availability of such individuals that first stimulated the author's interest in fragile sites some years ago. The possibility that expression of fragile sites in cord blood lymphocytes is different from that in lymphocytes of older individuals could also account for these results. There is no evidence available on this point; however, there is usually no special difficulty in demonstrating fragile sites in young children; indeed, the fragile site at Xq27 becomes increasingly difficult to detect with advancing age in females [7]. This work needs to be repeated on other

retarded populations, a more rigorously selected group of neonates, and a group of age-matched controls for the retardates.

If the difference in incidence of fragile sites in retardates and normal individuals is confirmed, the mechanism of the effect is worth considering. Williams and Howell [11] suggested that breakage of the fragile sites at critical stages of development could lead to monosomic cell lines that might persist and have a deleterious effect either genetically or through poor viability. In individuals with fragile sites, 5%–10% of metaphases expressing the fragile site are aneuploid as a result of breakage at this site. If this breakage occurs in vivo, even to a lesser extent, it could explain why some but not all carriers of fragile sites are phenotypically abnormal and why there is no consistent pattern to the abnormalities.

In all instances in which family studies yielded a parent of an index case with the autosomal fragile site, this parent was the mother. While the probability of this occurring by chance is small, it is difficult to attribute any biological significance to it since males readily transmit fragile sites. Could it be that if heterozygosity for the autosomal recessive fragile sites can be deleterious then this deleterious effect is more likely to occur if the fragile site is inherited from the mother?

In the retarded populations in Minda and Strathmont, 1.7% of males had the fra(X)(q27). In these institutions, about 12%–15% of the males had Down syndrome [6, 12]. Most of the males with fra(X)(q27) function at about the same intellectual level as Down syndrome patients. Hence, if the calculation of Herbst and Miller [13] of an incidence of fra(X)(q27) in males of .92 per 1,000 is correct, then almost the same number of fra(X)(q27) males as Down syndrome males would have been expected, especially since there is no evidence of the increased infant mortality in fra(X)(q27) males that is seen in Down syndrome. Even with the limitations of technique in detecting fra(X)(q27), there is no doubt that in the institutions studied there are many more Down syndrome individuals than individuals with fra(X)(q27). It would appear that the estimate of Herbst and Miller [13] may be a very considerable overestimate. Not enough retarded females have been studied to allow comment upon the finding [14] that 7% of mildly retarded females without physical stigmata have the fra(X)(q27).

This preliminary study of the population cytogenetics of fragile sites has asked more questions than it has answered. The incidence of the folic-acid-sensitive fragile sites in the general population remains unknown. The possibility that these fragile sites might be deleterious in some heterozygotes, particularly if inherited maternally, has been raised. The fragile site at Xq27 was not detected among the neonates studied; however, all that can be concluded from this is that it is much less common in such a population than it is in retarded populations and that if it does occur in normal males [15], then it does so rarely. The fra(X)(q27) was recognized in about 2% of institutionalized retarded males with otherwise normal karyotypes. It would seem that its incidence is considerably less than that estimated by Herbst and Miller of .92/1,000 males [13]. Further population cytogenetic studies of normal and retarded populations are required to help elucidate the biological significance of fragile sites.

## ADDENDUM

Further studies on the family of 587/73 (table 2) have indicated that the subject's fragile site is familial since it was found in his siblings; hence, it was undetected in an obligate carrier parent. Another male with fra(X)(q27) was identified in Minda Home (total now six) after his mildly retarded sister was independently ascertained to have the fragile X. This male had been studied by capillary blood culture when the institution was surveyed but was not identified at that time.

## ACKNOWLEDGMENTS

I thank the many people who have assisted in this work: the management and staff of the institutions involved for allowing patients under their care to be studied; the Labour Ward staff at the Queen Victoria Hospital for collecting blood samples; Elizabeth Baker, Helen Eyre, Lily Gowans, Lynene Hinton, Trudy Hocking, Barbara Menzies, and Erica Woollatt for their excellent help with the cytogenetics; and John Mulley for the paternity testing.

## REFERENCES

1. Sutherland GR, Hinton L: Heritable fragile sites on human chromosomes. VI. Characterization of the fragile site at 12q13. *Hum Genet* 57:217–219, 1981
2. Manuel A, Sutherland GR, Molesworth J: A new fragile site at 9p21. Paper presented at 5th Annual Scientific Meeting of the Human Genetics Society of Australasia, Canberra, 1981
3. Gerald PS, Walzer S: Chromosome studies of normal newborn infants, in *Human Population Cytogenetics*, edited by Jacobs PA, Price WH, Law P, Edinburgh, Edinburgh Univ. Press, 1970, pp 143–151
4. Sutherland GR: Fragile sites on human chromosomes: demonstration of their dependence on the type of tissue culture medium. *Science* 197:265–266, 1977
5. Sutherland GR: Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. *Am J Hum Genet* 31:125–135, 1979
6. Sutherland GR, Murch AR, Gardiner AJ, Carter RF, Wiseman C: Cytogenetic survey of a hospital for the mentally retarded. *Hum Genet* 34:231–245, 1976
7. Sutherland GR: Heritable fragile sites on human chromosomes. III. Detection of fra(X)(q27) in males with X-linked mental retardation and their female relatives. *Hum Genet* 53:23–27, 1979
8. Jacobs PA: Correlation between euploid structural chromosome rearrangements and mental subnormality in humans. *Nature* 249:164–165, 1974
9. Haldane JBS: The rate of spontaneous mutation of a human gene. *J Genet* 31:317–326, 1935
10. Sutherland GR: Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects and cytogenetics. *Am J Hum Genet* 36:136–148, 1979
11. Williams AJ, Howell RT: A fragile secondary constriction on chromosome 2 in a severely mentally retarded patient. *J Ment Defic Res* 21:227–230, 1976
12. Sutherland GR, Clisby SR, Bloor G, Carter RF: Down's syndrome in South Australia. *Med J Aust* 2:58–61, 1979
13. Herbst DS, Miller JR: Non-specific X-linked mental retardation. II. The frequency in British Columbia. *Am J Med Genet* 7:461–469, 1980
14. Turner G, Brookwell R, Daniel A, Selikowitz M, Zilibowitz M: Heterozygous expression of X-linked mental retardation and X-chromosome marker fra(X)(q27). *N Engl J Med* 303:662–664, 1980
15. Daker MG, Chidiac P, Fear CN, Berry AC: Fragile X in a normal male: a cautionary tale. *Lancet* 1:780, 1981

*Am J Hum Genet* 34:753–756, 1982

# Heritable Fragile Sites on Human Chromosomes.

## IX. Population Cytogenetics and Segregation Analysis of the BrdU-Requiring Fragile Site at 10q25

GRANT R. SUTHERLAND[1]

SUMMARY

The frequencies of the bromodeoxyuridine (BrdU)-requiring fragile site at 10q25 in 1,026 unselected neonates, 901 patients referred for chromosome studies, and 87 institutionalized retardates were not significantly different from each other. The gene frequency was .013, and the population was in Hardy-Weinberg equilibrium. Segregation analysis confirmed that the fragile site followed codominant inheritance. This fragile site and its nonfragile allelomorph can be considered to constitute the first true chromosomal polymorphism to be described in man.

## INTRODUCTION

A BrdU-requiring fragile site at 10q25 was independently reported by Scheres and Hustinx [1] and Sutherland et al. [2]. This fragile site was subsequently shown to be common in the Australian population and to be without recognizable phenotypic effect in homozygous children [3]. Several groups within the population have now been studied for the presence of this fragile site that can be regarded as the first true chromosomal polymorphism to be described in man.

## MATERIALS AND METHODS

Three groups have been studied: unselected newborn infants; patients referred for diagnostic chromosome studies since late 1979; and the residents of Ru Rua, an institution for the totally dependent mentally retarded. Details of these three groups have been published [4]. The neonates were studied from cord blood lymphocyte culture, the Ru Rua residents

Received November 25, 1981; revised January 20, 1982.

This work was supported by grants from the Adelaide Children's Hospital Research Trust and the Channel 10 Children's Medical Research Foundation.

[1] Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia.

from venous blood samples, and most of the patients from venous blood samples, although some of the youngest patients were studied from capillary blood collected by heel or finger-prick. All lymphocyte cultures were established in Ham's F10 culture medium supplemented with 20% (more recently 5%) fetal bovine serum and 10 mg/l of BrdU. Cultures were harvested after 72 hrs using standard methods. Twenty unbanded metaphases per individual were examined for fragile sites, and the location of the fragile site was confirmed in each individual using trypsin-Leishmann G-banding.

When any individual was found to have the fragile site, family studies were carried out when possible. To avoid ascertainment bias in the calculation of segregation ratios, the index cases and their direct ancestors in each generation were eliminated from consideration, as were unkaryotyped individuals. Not all parents of individuals used in the calculations had been karyotyped: when only one parent had been karyotyped and was found to have the fragile site, the other parent was assumed not to have it; similarly, when the one karyotyped parent was found not to have the fragile site, the other was presumed to have it. In some sibships, neither parent had been studied, and it was assumed that only one had the fragile site.

## RESULTS

The numbers of individuals studied in each group and the numbers with fra(10)(q25) are shown in table 1. The differences in the frequency of the fragile site between the three groups studied are not significant. The gene frequency is .013, and the heterozygote incidence is 1 in 40 persons. The population is in Hardy-Weinberg equilibrium (table 2). No other BrdU-requiring fragile sites have been detected. All the individuals found to carry the fragile site were of British or other northern European origin. The numbers in other racial groups studied were too small to attach significance to this finding.

Both parents of the index cases were studied on 27 occasions. In 18 instances, the mother was found to carry the fragile site; in seven, the father; in one, neither; and in the case of the homozygous neonate, both parents [3]. Paternity testing in the family in which neither parent was found to have the fragile site after repeated chromosome studies in which a total of 85 metaphases were examined from the father and 75 from the mother showed that there was a 96.6% probability of correct paternity.

The segregation ratios derived from 21 families in which known or presumed carriers were married to known or presumed noncarriers are shown in table 3. The

TABLE 1

GROUPS STUDIED

| GROUP | NO. | CARRIERS OF fra(10)(q25) | |
|---|---|---|---|
| | | Homozygotes | Heterozygotes |
| Neonates | 520 males* | 1 | 10 |
| | 506 females | 0 | 14 |
| Patients | 901 | 0 | 22 |
| Ru Rua | 42 males | 0 | 1 |
| | 45 females | 0 | 2 |
| Total | 2,014 | 1 | 49 |

TABLE 2

GENOTYPES OBSERVED AND EXPECTED ON THE BASIS OF HARDY-WEINBERG EQUILIBRIUM

| Genotype | Observed | Expected |
|---|---|---|
| Homozygous N..... | 1,964 | 1,963.3 |
| Heterozygotes ..... | 49 | 50.4 |
| Homozygous fra ..... | 1 | 0.3 |
| Total ..... | 2,014 | 2,014.0 |

ratio of carriers to noncarriers is not significantly different from 1:1 (54:49). In the only known mating of two fragile-site carriers, there were two homozygotes and two heterozygotes for the fragile site, one of the homozygotes being the index case [3].

## DISCUSSION

Detection of the fragile site at 10q25 has usually presented no difficulties with the frequencies of expression ranging from 8% to 91% of cells examined among individuals who carry it. However, the finding that neither parent of one individual with the fragile site appeared to carry it suggests that the technique may not always detect the fragile site when it is present. Since this fragile site is common and apparently innocuous, the alternative explanation that this case represents a new mutation is unlikely.

The segregation analysis, which showed a nonsignificant excess of carriers over noncarriers, indicates that if the fragile site follows Mendelian codominant inheritance there has not been any significant problem in the detection of carriers. Conversely, if it is accepted that the detection of individuals with the fragile site is virtually complete, then the segregation ratios obtained are compatible with codominance. Although the population is apparently in Hardy-Weinberg equilibrium, little significance may be attached to this because the frequency of the fragile site was estimated primarily from the number of heterozygotes. For example, the

TABLE 3

SEX AND KARYOTYPE OF OFFSPRING OF CARRIERS OF fra(10)(q25) MARRIED TO NONCARRIERS

| | OFFSPRING | | | |
|---|---|---|---|---|
| | fra CARRIER | | fra NOT PRESENT | |
| MATING TYPE | Males | Females | Males | Females |
| Mother carrier ..... | 11 | 12 | 14 | 7 |
| Father carrier ..... | 15 | 8 | 9 | 10 |
| Carrier parent unknown ..... | 4 | 4 | 5 | 4 |
| Total ..... | 30 | 24 | 28 | 21 |

population would still appear to be in equilibrium even if only half the fragile site heterozygotes had been detected.

Polymorphism is defined as the occurrence of two or more distinct forms in such proportions that the rarest cannot be maintained by mutation alone [5]. It is generally accepted that the rare form must be more common than 1%–2% to qualify as a polymorphism [6, 7]. Most chromosomal variation in man does not fit the definition of a polymorphism since those variants that are distinct are too rare, and those that are common are the result of continuous variation such as that seen in the C-band regions of some autosomes. Consequently, the fragile site at 10q25 and its nonfragile allelomorph can be considered to constitute the first true chromosomal polymorphism to be described in man. The biological significance of this polymorphism is unknown; homozygotes for the fragile site are apparently phenotypically normal [3]. It will be of interest to see whether or not the frequency of the fragile site at 10q25 differs between various ethnic groups.

## ACKNOWLEDGMENTS

I thank Elizabeth Baker, Helen Eyre, Lynene Hinton, Trudy Hocking, Barbara Menzies, and Erica Woollatt for assistance with cytogenetics, Maria Zadoroznyj for assisting with the family studies, and John C. Mulley for the paternity testing.

## REFERENCES

1. Scheres JMC, Hustinx TWJ: Heritable fragile sites and lymphocyte culture medium containing BrdU (Letter to the editor). *Am J Hum Genet* 32:628–629, 1980
2. Sutherland GR, Baker E, Seshadri RS: Heritable fragile sites on human chromosomes. V. A new class of fragile site requiring BrdU for expression. *Am J Hum Genet* 32:542–548, 1980
3. Sutherland GR: Heritable fragile sites on human chromosomes. VII. Children homozygous for the BrdU-requiring fra(10)q25 are phenotypically normal. *Am J Hum Genet* 33:946–949, 1981
4. Sutherland GR: Heritable fragile sites on human chromosomes. VIII. Preliminary population cytogenetic data on the folic acid-sensitive fragile sites. *Am J Hum Genet* 34:452–458, 1982
5. Ford EB: The genetics of polymorphism in the lepidoptera. *Adv Genet* 5:43–87, 1953
6. Sheppard PM: *Natural Selection and Heredity*. London, Hutchinson, 1958, p 62
7. Vogel F, Motulsky AG: *Human Genetics. Problems and Approaches*. Berlin, Springer-Verlag, 1979, p 373

# The Autosomal Fragile Sites

Grant R. Sutherland

Fragile sites on chromosomes have attracted much attention recently, mainly because the one on the X chromosome is now known to be associated with a common mental retardation syndrome. However, there are at least 14 other fragile sites in addition to the one on the X. All these fragile sites, with the exception of the one at 10q25, are rare and their significance remains to be determined.

Fragile sites have been known since the mid-60s and have been the subject of many case reports but it was not until the 1970s that Giraud et al. (1976) and Harvey et al. (1977) showed that the fragile site on the X chromosome was associated with one form of X-linked mental retardation and was not an isolated rare finding as it had been considered since it was first described by Lubs in 1969. The two papers from the mid-70s kindled interest in fragile sites and this combined with the finding of the essential method for detecting fragile sites in lymphocyte cultures by Sutherland (1977) opened up a new area of cytogenetics.

## Definition

A fragile site (Sutherland, 1979) is a specific point on a chromosome which is liable to show the following features: 1) a non-staining gap of variable width which usually involves both chromatids; 2) is always at exactly the same point on the chromosome in cells examined from any individual patient or kindred; 3) *fragility* must be evident by the production of acentric fragments, deleted chromosomes, triradial figures, etc. The hallmark of a fragile site is the triradial (or multiradial) figure since this is evidence that breakage at the fragile site has occurred in the mitosis prior to the metaphase in which it is seen (Ferguson-Smith, 1973) and is not an artefact of chromosome preparation. Fragile sites are almost never seen in all metaphases, the proportion expressing them ranging from only a few percent up to greater than 80% depending upon the individual and the conditions of lymphocyte culture.

The heritable fragile sites must be differentiated from several other phenomena which can be confused with fragile sites. There is a heteromorphic form of chromosome 17 which can have a satellited appearance and cytologically can resemble a fragile site. This is not regarded as a fragile site at present since it has not been shown to be fragile; its expression is unrelated to culture medium composition. The multibranched chromosomes seen in some immunodeficiency states (Tiepolo et al., 1979) are not currently considered to be heritable fragile sites as these appear to be products of the diseases in which they occur.

All chromosomes can occasionally show isochromatid gaps which are not fragile sites and should not be called fragile sites. In particular, there are loci at 6q26 and 3p14 at which these gaps occur more frequently than would be expected by chance, indeed the one at 6q26 can be confused with the fragile site at Xq27 (Leversha et al., 1980). These gaps at 6q26 and 3p14 have not been shown to be heritable, are present in only a small portion of metaphases (usually $< 5\%$) and do not show the multiradial configurations characteristic of fragile sites.

## What is a Fragile Site?

The nature of fragile sites remains speculative. It would appear that the fragile site represents a region of chromosome which fails to compact normally as the chromosome goes through mitosis. All that is known about fragile sites suggests that this is due to a problem with the DNA in this region rather than with histone or protein. However, further research is needed to help define the exact nature of the lesion.

## Classification of Fragile Sites

The known fragile sites are shown in Figure 1. These can be classified into three groups.

Group 1. This group contains the fragile sites which are folate sensitive and includes the one on the long arm of the X which is of great clinical importance. This group can be subdivided according to the relationship between expression of the fragile sites and pH of the medium, however, not all fragile sites have been studied for this effect; this subdivision probably has little practical value. For these fragile sites to be expressed the apparently essential condition of tissue culture is that a state of thymidine starvation exists. This can be achieved by depleting endogenous thymidine synthesis by having low or absent folic acid and thymidine present in the medium, by blocking folate metabolism with methotrexate, or by inhibiting the formation of thymidine monophosphate by blocking the enzyme thymidylate synthetase with fluorodeoxyuridine (FUdR).

Group 2. This group contains only the fragile site at 16q22 which is apparently independent of the conditions of lymphocyte culture for expression although this is reportedly enhanced by distamycin A. A probable homozygote for this fragile site has been reported to be phenotypically normal.

Group 3. This group also contains only one fragile site, that at 10q25 which is the only common fragile site, being present in one in 40 members of the Australian population. This fragile site is the only true chromosomal polymorphism in man and is only seen when bromodeoxyuridine (BrdU) has been present in the culture medium for the last 8-12 hours before harvest. Bromodeoxycytidine is as effective as BrdU in inducing this fragile site. Children homozygous for this fragile site are phenotypically normal.

## Cytogenetics

The appearance of some of the fragile sites are shown in Figure 2. There is usually a non-staining gap, which mostly affects both chromatids but may only affect one. In 2%-5% of metaphases there is usually evidence of fragility such as separation within the metaphase of the parts of the chromosome distal and proximal to the fragile site following breakage, or deletion or duplication of the chromosome distal to the fragile site.

The proportion of metaphases in which the fragile site is expressed can be highly variable. Since their expression is so dependent upon conditions of culture and possibly other unknown factors, comparison of frequencies of expression between laboratories, and even within one laboratory over time, is unreliable. Fragile sites have been reported in 100% of metaphases examined (Anneren, 1981) but in the author's laboratory are usually found in 20%-50% of metaphases, although they have been seen in more than 80% of metaphases. However, in some individuals who are obligate carriers they can be found in a very small proportion of cells and even not detected on occasions. Detection of the autosomal fragile sites is not as difficult as detection of the fragile X in females.

Since any diagnostic cytogenetics laboratory should be looking for the fragile site on the X chromosome if retarded individuals are karyotyped, why not look for the autosomal sites as well?

If any laboratory is not currently capable of detecting the folate sensitive fragile sites then a change in methodology is overdue and simple to implement.

The current best method to detect fragile sites is as follows:

1. Use a folate deficient culture medium. Standard TC199 is adequate if one of the new folate-free media is not available.
2. Buffer strongly (e.g. 20mM Hepes) to a pH of 7.5 - 7.6. The pH of the medium should be greater than 7.3 at the time of harvest.
3. Use only 5% serum supplement.
4. Harvest after 96 hours if possible, 72 hours suffices but 48 hours is inadequate.
5. Use venous rather than capillary blood since any microbial contamination of the culture will render it useless for fragile site detection.
6. Examine unbanded chromosomes. Fragile sites are easier to see on these than banded chromosomes, particularly if a trypsin banding technique is used.

This methodology is easy to incorporate into any diagnostic laboratory. There is also no need to run parallel cultures in other media since this method consistently results in good chromosome preparations suitable for other cytogenetic techniques. In any individual in whom a fragile site is suspected an additional culture in a folate rich medium which is deficient in thymidine (e.g. RPMI 1640) to which 0.01 mg/l of FUdR is added 24 hours before harvest might help if the fragile site is not being adequately detected by the above technique.

### Clinical Significance

The fragile sites at 16q22 and 10q25 are probably of no clinical significance; homozygotes for both of them have been found and were normal.

The full significance of the folate sensitive fragile sites remains to be determined. Since hemizygosity for the fragile site on the X chromosome produces a mental retardation syndrome it is reasonable to speculate that homozygosity for the autosomal folate sensitive fragile sites would be deleterious. No such homozygote has been described. If such homozygosity were compatible with survival to birth and was deleterious then the resultant problem would follow apparent autosomal recessive inheritance. Rare recessive disorders should be looked at for the presence of fragile sites. On the other hand, if homozygosity were lethal it might result in sub-fertility or recurrent abortion. Couples being karyotyped for these problems should be looked at for fragile sites.

The only population cytogenetic data on autosomal folic acid sensitive fragile sites (Sutherland, 1982) showed a tenfold increase in these amongst the

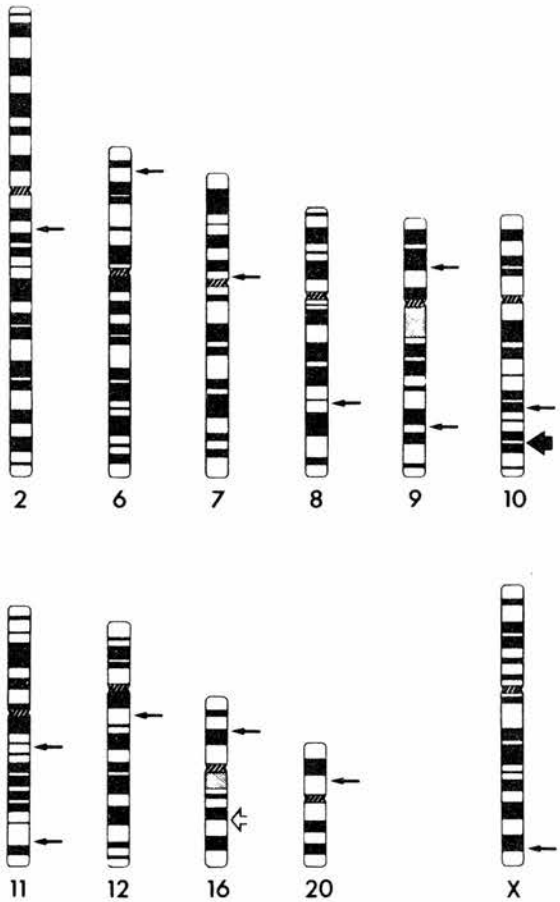



Figure 1. The known fragile sites. Group 1, small arrows, at 2q13, 6p23, 7p11, 8q22, 9q21, 9q32, 10q23, 11q13, 11q23, 12q13, 16p12, 20p11 and Xq27; Group 2, open arrow, 16q22; Group 3, broad arrow, 10q25.

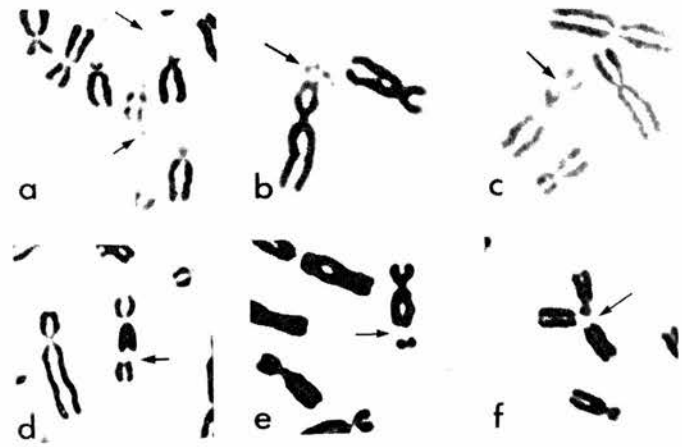



Figure 2. Appearance of some of the fragile sites: a, despiralisation of chromosome material distal to 2q13; b, duplication of chromosome distal to 6p23; c, chromosome gap at 9p21; d, chromosome gap at 10q23; e, chromosome gap at 10q25; f, duplication of chromosome distal to 11q13.

mentally retarded compared with unselected newborn infants. However, these population cytogenetic data are very limited and much more information is required before any claim is made that heterozygosity can result in mental retardation. If heterozygosity does indeed result in mental retardation then the mechanism suggested by Williams and Howell (1976) may be responsible. Their suggested mechanism was based upon the observation that a small percentage of metaphases in lymphocyte cultures from fragile site heterozygotes are monosomic or trisomic for the segment of chromosome distal to the fragile site. If such aneuploid cells arose early in development they could generate clones of aneuploid cells which either because of their aneuploidy or poor viability would result in abnormal development. This rather non-specific mechanism could account for the reason why phenotypically abnormal heterozygotes are not clinically similar to each other.

### Incidence

The only fragile site for which there is reliable incidence data is the one at 10q25. This is present in one in 40 members of the Australian population. All the other autosomal fragile sites are rare, some being the subject of only single case reports. An examination of reported cases suggests that the two commonest fragile sites are those at 2q13 and 10q23, possibly the one at 16q22 being the next commonest and the relative frequencies of the others not able to be assessed at present. The relative frequencies of the fragile sites may vary from population to population making the above assessment unreliable. In the author's laboratory the site at 10q23 accounts for approximately half all autosomal Group 1 fragile site ascertainments.

The absolute incidence of fragile sites in unknown. Most of the cytogenetic surveys of randomly selected newborn infants have been performed using media unsuitable for fragile site detection and have been based on the examination of a small number of cells, usually two, which would be unlikely to detect fragile sites expressed in small proportions in metaphases.

The only neonatal survey to specifically look for fragile sites (Sutherland, 1982) found only one amongst 1,019 neonates but found five amongst 524 institutionalized retardates. This difference is statistically significant but this work needs to be repeated before this difference can be regarded as established. Much more population cytogenetic data on fragile sites are needed.

### Other Cell Types

All the above discussion of fragile sites pertains to their behaviour in short term PHA stimulated lymphocyte cultures. Apart from the fragile X, very little work has been done on attempting to demonstrate these in other cell types. The folate sensitive fragile sites are difficult to demonstrate in fibroblast and lymphoblastoid cell cultures but, by analogy with the fragile X, can probably be induced in these cell types with FUdR or methotrexate (Brookwell et al., 1982; Jacobs et al., 1982). The fragile site at 10q25 can be induced in fibroblasts by the addition of BrdU prior to harvest but has not been studied in lymphoblastoid cells. The 16q22 fragile site has not been seen in cells other than lymphocytes; attempts to demonstrate it in fibroblasts have been unsuccessful but it may be worth trying to induce it with distamycin A.

### Conclusions

Unfortunately none of the methods of detection of fragile sites are completely satisfactory. The propensity for a fragile site to be expressed must be present in every cell otherwise Mendelian inheritance would not occur. The reasons why fragile site expression cannot be induced in all metaphases remains unclear. Why is it that in some metaphases the fragile site is expressed on one chromatid and not the other? Until some of these problems have been solved and it becomes possible to induce fragile site expression in the majority of metaphases the current methods need to be persevered with. The fact that the methods for fragile site detection are not perfect should not inhibit their widespread use for they will detect the majority of individuals with fragile sites and contribute to a better understanding of the significance of these chromosomal aberrations.

### Summary

There is still much to be learned about the exact nature of fragile sites and their expression in metaphase chromosomes. There are no meaningful data on the incidence of folate sensitive fragile sites. The clinical significance of the folate sensitive fragile sites remains to be determined in both heterozygotes and homozygotes. The detection of the fragile site on the X chromosome should be part of any diagnostic cytogenetic assessment and since the same methodology will also detect the autosomal fragile sites this presents a great opportunity to collect further data on this fascinating phenomenon.

### References

Brookwell, R. Daniel, A, Turner, G and Fishburn, J (1982) Fragile (X)-linked mental retardation. FUdR as an inducing agent for fra(X)(q27) expression in lymphocytes, fibroblasts and amniocytes. Am. J. Med. Genet. (in press).

Ferguson-Smith, MA (1973) Inherited constriction fragility of chromosome 2. Ann. Genet. 16: 29-34.

Jacobs, PA, Hunt, PA, Mayer, M, Wang, J-C, Boss, G and Erbe, RW (1982) Expression of the marker (X)(q28) in lymphoblastoid cell lines. Am. J. Hum. Genet. (in press).

Leversha, MA, Webb, GC and Pavey, SM (1981) Chromosome banding required for studies on X-linked mental retardation. Lancet i: 49.

Sutherland, GR (1977) Fragile sites on human chromosomes: Demonstration of their dependence on the type of tissue culture medium. Science 197: 63-67.

Sutherland, GR (1979) Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. Am J. Hum. Genet. 31: 125-135.

Sutherland, GR (1982) Heritable fragile sites on human chromosomes. VIII. Preliminary population cytogenetic data on the folic acid senstive fragile sites. Am J. Hum. Genet. (in press).

Tiepolo, L, Maraschio, P, Gimelli, G, Cuoco, C, Gargani, GF and Romano, C (1979) Multibranched chromosomes 1, 9, and 16 in patients with combined IgA and IgE deficiency. Hum. Genet. 51: 127-137.

Williams, AJ and Howell, RT (1976) A fragile secondary constriction on chromosome 2 in a severely mentally retarded patient. J. Ment. Defic. Res. 21: 227-230.■

**Grant R. Sutherland, PhD**
**Chief Cytogeneticist**
**Cytogenetics Unit**
**Department of Histopathology**
**The Adelaide Children's Hospital Inc.**
**North Adelaide**
**South Australia 5006**■

# The Fragile X Chromosome



GRANT R. SUTHERLAND

*Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, Australia*



## I. Introduction

The fragile X chromosome has recently been recognized to be associated with, if not the cause of, a common form of X-linked mental retardation in males and possibly females. While the exact contribution of this chromosome aberration to mental retardation remains to be determined, it is at least of the same magnitude as Down syndrome and possibly more important genetically as it is usually familial (Gerald, 1980).

This chromosomal fragile site at Xq27, which has now become known as the fragile X chromosome, was first described by Lubs in 1969 in association with mental retardation. His discovery had become no more than another rare chromosomal curiosity confined to a single family until the independent reports of Giraud *et al.* (1976) and Harvey *et al.* (1977) demonstrated the probable association of this fragile site with one common form of X-linked mental retardation. The key to the widespread confirmation of these reports was the discovery that the fragile site was expressed only under highly specific conditions of lymphocyte culture which were not those employed by most cytogenetics laboratories in the late 1970s.




ISBN 0-12-364481-X

The fragile X and its associated syndrome of mental retardation in males have now been found in most countries where this has been sought and in many racial groups. However, there are still problems with the demonstration of the fragile X in some males and many females, and controversy about the clinical syndrome which the fragile X produces in males and the extent of mental handicap it is responsible for in females. Very little is known about the population cytogenetics of the fragile X and the nature of its association with mental retardation remains obscure, especially since some allegedly normal males have been reported to have the fragile X.

## II. What Is the Fragile X?

The fragile X is so called because it has a fragile site at the distal end of the long arm. Fragile sites are morphological features of chromosomes which were defined by Sutherland (1979a) as specific points which are liable to show the following features:

1. A nonstaining gap of variable width which usually involves both chromatids.
2. Is always at exactly the same point on the chromosome in an individual or kindred.
3. Is inherited in a Mendelian codominant fashion.
4. Exhibits fragility by the production of acentric fragments, deleted chromosomes, triradial figures, etc.

The triradial (or multiradial) figure is the most spectacular cytogenetic manifestation of the fragile site (Fig. 1) and also an essential manifestation for confirmation as a fragile site rather than some other phenomenon causing chromosomal damage. While the triradial was originally considered to be due to selective endoreduplication (Lejeune *et al.*, 1968), it was proposed by Ferguson-Smith (1973) and subsequently confirmed by Ferguson-Smith (1977) and Noël *et al.* (1977) that the mechanism for production of triradials was breakage at the fragile site followed by nondisjunction (Fig. 2).

While the nature of a fragile site, in terms of chromosome structure, is unknown it presumably represents a segment of chromosome which does not undergo normal compaction for mitosis. Rarely, cells are seen in which all the chromosome material distal to the fragile site is not normally compacted at mitosis (Fraccaro *et al.*, 1972). Fragile sites have not been studied in meiotic cells hence it is unknown whether they are expressed in them.

To date 15 fragile sites have been detected and these are shown in the partial ideogram (Fig. 3) and partial karyotype (Fig. 4). These fragile sites fall into three

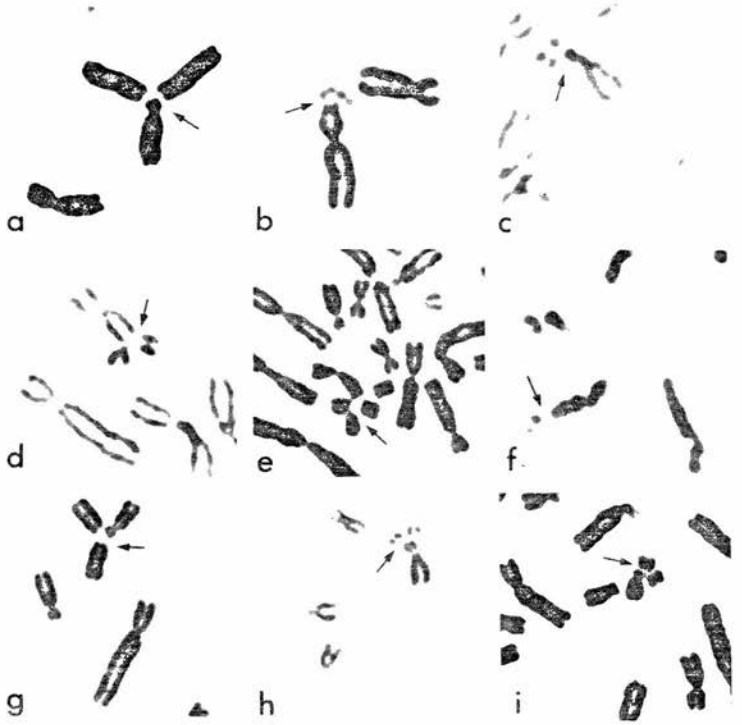



FIG. 1. Multiradial figures produced by fragile sites. (a) Triradial at 2q13; (b) triradial at 6p23; (c) triradial at 9p21; (d) triradial at 9q23; (e) pentaradial at 10q23; (f) triradial at 10q25; (g) triradial at 11q13; (h) triradial at 16p12; (i) triradial at 20p11.

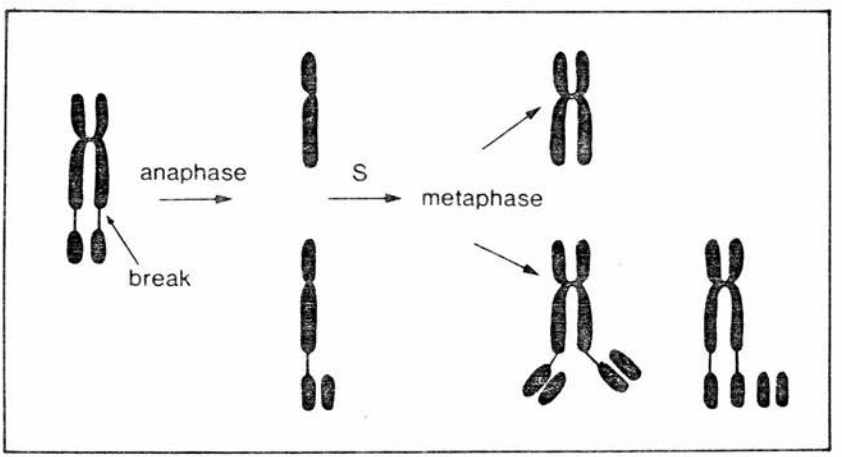



FIG. 2. Mechanism of production of triradial figures and deleted chromosomes by breakage at the fragile site followed by nondisjunction. (After Ferguson-Smith, 1973.).

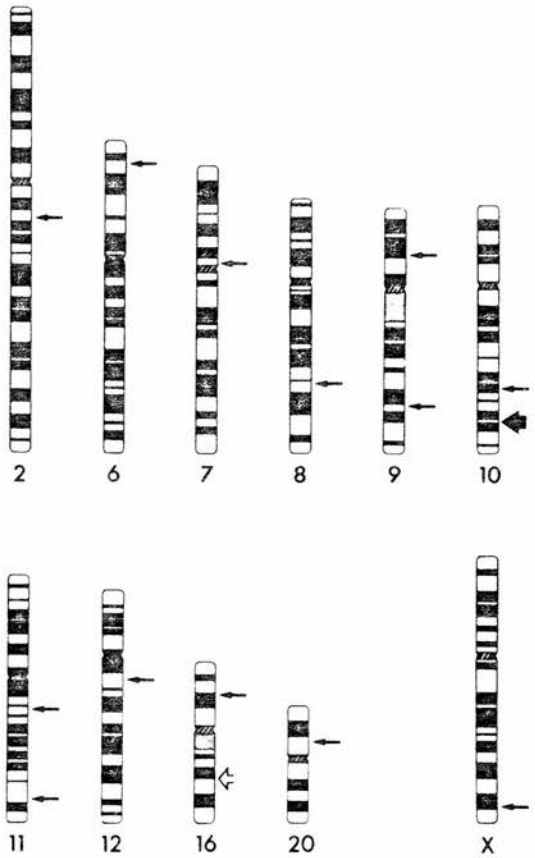



FIG. 3. The known fragile sites. Group 1, small arrows, at 2q13, 6p23, 7p11, 8q22, 9p21, 9q32, 10q23, 11q13, 11q23, 12q13, 16p12, 20p11, and Xq27; Group 2, open arrow, 16q22; Group 3, broad arrow, 10q25. Reprinted from Sutherland *et al.*, *American Journal of Human Genetics*, May 1983, by permission of the University of Chicago Press.

groups. The first group, the folate-sensitive fragile sites, contains all except two of the known sites which each comes into separate categories. The multibranched chromosomes formed by various combinations of the arms of chromosomes 1, 9, and 16 seen in some immunodeficiency states (Tiepolo *et al.*, 1979; Hultén, 1978) are not currently considered to be heritable fragile sites since they appear to be products of the diseases involved.

*Group 1.* The folate-sensitive fragile sites are those at 2q13, 6p23, 7p11, 8q22, 9p21, 9q32, 10q23, 11q13, 11q23, 12q13, 16p12, 20p11, and Xq27. They are termed folate sensitive because removal of folic acid (and thymidine)

from culture medium was the first factor found to be essential for their demonstration in lymphocyte culture.

*Group 2*. This group contains only the fragile site at 16q22. This fragile site is not dependent upon conditions of tissue culture (Sutherland, 1979a) but its expression is reportedly enhanced by the addition of distamycin A to lymphocyte

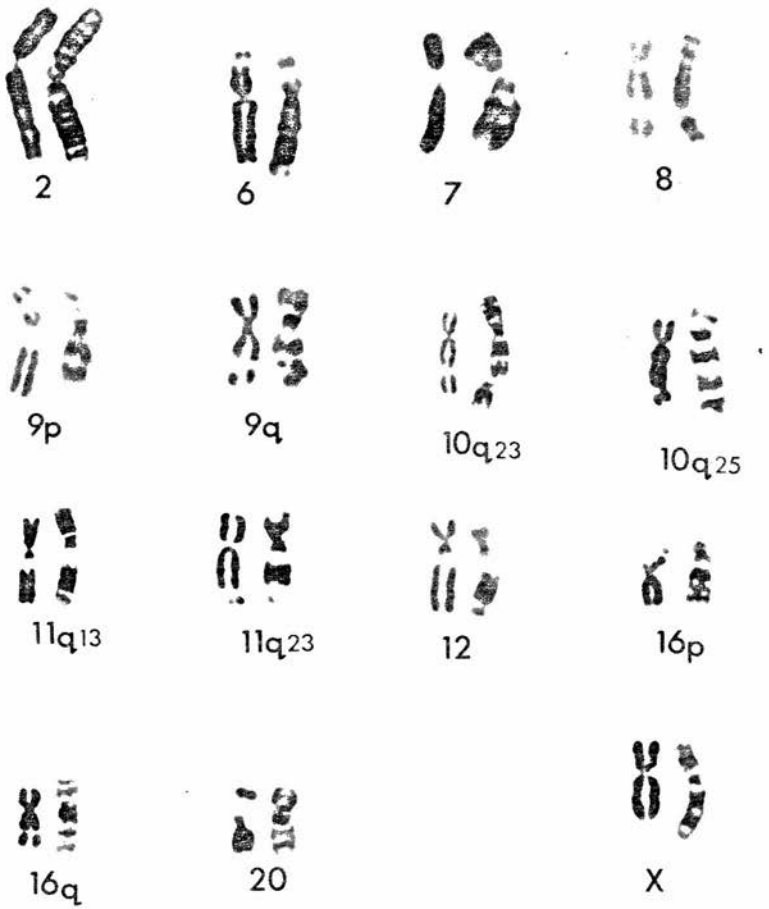



FIG. 4. Partial karyotype showing the known fragile sites. The left chromosome of each pair is unbanded and the right G-banded. (Pair number 8 courtesy Dr. N. B. Kardon, pair number 7 courtesy Dr. G. C. Webb.)

cultures (Schmid *et al.*, 1980). If the variant chromosome 17, sometimes referred to as a satellited 17, is shown to be fragile and is accepted as a fragile site then it might be classified within this group.

*Group 3.* This group contains only the fragile site at 10q25. This fragile site was independently discovered by Scheres and Hustinx (1980) and Sutherland *et al.* (1980a) and shown to be a polymorphism present in 1 in 40 members of the Australian population sample studied (Sutherland, 1982b). This fragile site is unique in that its expression is dependent upon the presence of BUdR or BCdR in lymphocyte cultures some hours prior to harvest. This is the only common fragile site (Sutherland, 1982b).

Hence, while the fragile site on the X chromosome may have a number of features which distinguish it from the other fragile sites, any attempts to elucidate the nature of this fragile site should not ignore the fact that it is one of only a number of folate-sensitive fragile sites.

The correct nomenclature for the fragile X remains to be finalized. Lubs (1969) called it a marker X chromosome so this form certainly has priority and is preferred by some (Jacobs *et al.*, 1980; Turner and Opitz, 1980). However, in 1969 it was not known that the marker X was due to a fragile site, now a well-documented phenomenon, on the distal end of the long arm and the more specific term fragile X is preferable to the nonspecific term, marker X. Every structurally changed X chromosome is a marker X until the structural change is specified. The triplet *fra* was suggested by Sutherland (1979a) and has gained fairly wide acceptance. The exact location of the fragile site is yet to be finalized. The rather cumbersome Xq27 or 28 was originally used to indicate this uncertainty, but evidence from Turner *et al.* (1978) suggested that Xq27 was correct.

Others have opted for Xq28, and it will not be until good prophase banding of this area of chromosome is produced that the matter will be finalized. Most of the prophase banding methods are incompatible with a high level of fragile site expression and Jennings *et al.* (1980) found that the position of the fragile site was unresolved using this technology. Until the matter is resolved, fra(X)(q27) will be used to specify the fragile X.

## III. Tissue Culture Conditions

Fragile sites have mainly been demonstrated in PHA-stimulated lymphocyte cultures and their expression in other cell types is discussed below. To elicit expression of fragile sites, including the fragile X, Sutherland (1977b) showed that it was necessary to culture lymphocytes in medium 199 rather than a range of other culture media examined. The essential feature of medium 199 was subsequently found to be its deficiency in folic acid and thymidine (Sutherland,

1979a). Addition of folic acid or thymidine to medium 199 inhibited expression of the fragile sites, as did the biologically related compounds folinic acid and BUdR. Dose–response curves were produced for the effect of folic acid and thymidine on expression of the fragile X (Sutherland, 1979a). These effects of folic acid, thymidine, and BUdR on inhibition of fragile site expression have been fully confirmed by Glover (1981).

When folic acid deprivation was found to be important for fragile site expression, methotrexate, an inhibitor of folate metabolism, was found to induce expression of fragile sites in medium which contained folate (Ham's F10), although not to the extent that absence of folic acid did (Sutherland, 1979a). Unfortunately this work was carried out in Ham's F10 which also contains thymidine. Fonatsch (1981b) using skin fibroblasts, has induced the fragile X in a high proportion of cells using methotrexate in Dulbecco's medium which does not contain thymidine.

The time of deprivation of folic acid and thymidine is critical for expression of fragile sites. Deprivation during the last few hours of culture time is necessary for expression (Sutherland, 1979a) indicating that expression of fragile sites is determined either late in S or early in $G_2$.

Another factor involved in expression of some fragile sites, including the fragile X, is the pH of the culture medium. A significant positive correlation was found between the pH of medium 199 or MEM-FA at the time of harvest and fragile X expression (Sutherland, 1979a). Others (e.g., Gustavson *et al.*, 1981; Howard-Peebles and Pryor, 1981) have confirmed this pH effect, although using "M" medium, essentially Ham's F10 without folic acid, thymidine, or hypoxanthine, Jacobs *et al.* (1980) considered pH to be unimportant.

In lymphocyte cultures the duration of the culture before harvesting might be important in maximizing the frequency of expression of the fragile X. Jennings *et al.* (1980) considered that 96- and 120-hour cultures were better than 72-hour cultures, whereas Gustavson *et al.* (1981) observed no difference between 72- and 94-hour cultures. Howard-Peebles and Pryor (1981) found that increasing the length of culture time enhanced fragile X expression. Jacobs *et al.* (1980) preferred 96-hour cultures for their studies of the fragile X. Harvey *et al.* (1977) found only half the frequency of expression in 2-day cultures compared with 3-day cultures. In the author's laboratory 2-day cultures have been found to be unsatisfactory for studying fragile sites because of very low frequencies of expression. The frequency of expression at 4 days is usually greater than at 3 days, but the difference is small, whereas in 5-day or longer cultures the frequency of expression declines along with the quality of the preparation. The reasons for the apparent increase in frequency of expression with time in culture are not clear but depletion of media components which inhibit expression may account for it.

Howard-Peebles and Pryor (1979, 1981) have claimed that serum concentration in the culture medium is an important factor in fragile X expression, with

TABLE I

EFFECT OF METHIONINE CONCENTRATION ON EXPRESSION OF FRAGILE SITES

| Methionine[a] concentration (mg/liter) | Fragile sites and proportion of cells expressing them | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9p21[b] | 10q23[c] | 10q23[d] | 10q23[e] | 11q13[f] | 12q13[g] | Xq27[h] | Xq27[i] | 10q25[j] | 10q25[k] | 10q25[l] |
| 0 | 9/50 | 10/50 | 14/50 | 1/50 | 2/50 | 1/50 | 0/50 | 0/27 | 15/40 | 32/50 | 2/28 |
| 0.05 | 14/50 | — | — | 4/50 | 0/42 | 2/11 | 2/50 | 1/26 | 15/50 | 17/23 | 3/33 |
| 0.1 | 17/50 | 19/50 | 17/50 | — | 2/50 | 2/50 | 3/50 | 0/10 | 11/50 | 30/41 | 3/50 |
| 0.5 | 11/50 | 23/50 | 29/50 | 14/50 | 10/50 | 7/50 | 9/50 | 12/50 | 19/36 | 8/13 | 11/50 |
| 1 | 11/33 | 22/50 | 15/50 | — | 9/50 | 7/50 | 12/50 | 8/35 | — | 45/50 | — |
| 3 | — | 23/50 | 21/50 | — | — | 8/50 | 23/50 | 7/50 | — | — | — |
| 5 | 19/50 | 28/50 | 8/50 | — | 12/50 | 15/50 | 20/50 | 9/50 | 21/40 | 44/50 | 12/50 |
| 10 | — | 25/30 | 17/50 | — | — | — | 10/50 | — | — | — | — |
| 15 | 11/50 | 21/30 | 18/50 | 24/50 | 10/50 | 5/50 | 10/50 | 5/50 | 27/50 | 42/50 | 17/50 |

[a]Studied in MEM-FA (Sutherland, 1979b) without methionine, supplemented with 5% fetal bovine serum to which the stated concentrations of L-methionine were added when cultures were established. For the fragile site at 10q25 MEM-FA-methionine was used to which was added 1 mg/liter folic acid and 10 mg/liter BUdR when cultures were established.

[b]Manuel *et al.* (1981).

[c–e]Sutherland (1979b) family AY $II,_2$, $I,_1$, and $II,_3$.

[f]Unpublished.

[g]Sutherland and Hinton (1981) $IV,_1$.

[h]Sutherland (1979c) $IV,_6$.

[i]Unpublished, mildly retarded female.

[j–l]Unpublished unrelated individuals.

higher levels of expression at lower serum concentrations. This effect has not been documented by detailed study. The widespread use of only 5% fetal bovine serum in fragile site work is probably more determined by cost than any proven need for low serum concentrations. Indeed, Webb *et al.* (1982) found that increasing the fetal calf serum concentration from 5 to 14% gave an improved yield of metaphases without affecting fragile X expression.

The next factor in culture medium composition to be regarded as important was the need for methionine. Howard-Peebles *et al.* (1980) and Howard-Peebles and Pryor (1981) demonstrated that even under conditions of folic acid and thymidine deprivation methionine was essential for fragile X expression, although their results were not clear-cut. One black male expressed the fragile X in the absence of methionine. Attempts to confirm this role of methionine have been inconclusive because lymphocytes grow poorly in the absence of methionine. The limited data produced (Table I) have tended to support the observations of Howard-Peebles and Pryor (1981) although again these results are not unequivocal. Gardner *et al.* (1982) claimed that high levels of methionine (115 mg/liter) added to medium 199 produced a 2- to 4-fold increase in expression of the fragile X in skin fibroblast cultures. Glover and Howard-Peebles (1981) have shown that the need for methionine can be overcome when FUdR is used to induce fragile X expression.

Glover (1981) and Tommerup *et al.* (1981b) showed that FUdR would induce the expression of the fragile X even in the presence of normally inhibiting concentrations of folic acid. FUdR is a powerful inhibitor of thymidylate synthetase and this finding supported and added to the original suggestion of Sutherland (1979a) that the area of metabolism involved is that shown in Fig. 5, and that fragile site expression is dependent upon a deficiency of thymidine monophosphate which leads to impaired DNA synthesis (Tommerup *et al.*, 1981b). Brookwell *et al.* (1982), in a major study of FUdR on fragile X expression in lymphocytes and fibroblasts, found that the level of fragile X expression induced in lymphocytes by FUdR was not dependent upon the medium used. This agrees with Glover (1981) who recommended raising folic acid levels when FUdR is used to help combat its toxicity. Brookwell *et al.* (1982) also claimed that FUdR induction of the fragile X resulted in an enhancement of expression by about 30%, however, they did not separate their freshly cultured bloods from those in which delays in culture occurred where they claimed enhancement of up to 500%. There is a need to determine whether FUdR will induce a higher level of fragile site expression in freshly cultured lymphocytes than occurs in folic acid and thymidine-free medium and to confirm that any drop in expression with delay in culturing can be overcome by FUdR induction.

A variety of other studies on the conditions of lymphocyte culture have been reported. Fonatsch (1981a) reported a marked decrease in the frequency of expression of the fragile X in blood which had been stored for 5 days prior to

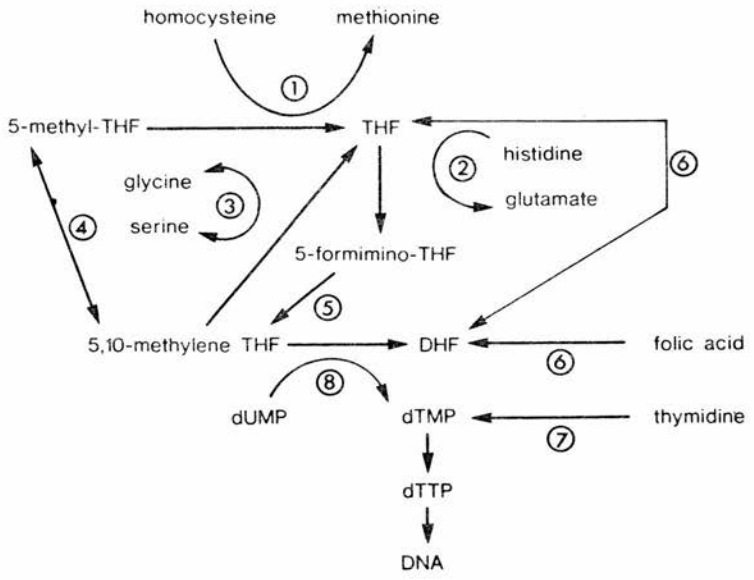



FIG. 5. The area of folate metabolism involved in fragile site expression (after Erbe, 1975; Scott and Weir, 1981). The enzymes controlling the various reactions are (1) methionine synthetase, (2) glutamate formiminotransferase, (3) serine hydroxymethyltransferase, (4) methylene-THF reductase, (5) formimino-THF cyclodeaminase, (6) dihydrofolate reductase, (7) thymidine kinase, (8) thymidylate synthetase. THF, Tetrahydrofolate; DHF, dihydrofolate; dUMP, uridine monophosphate, dTMP, thymidine monophosphate.

culture compared with freshly cultured blood. Jacobs *et al.* (1980) generally found lower levels of fragile X expression in Hawaii from blood which was in transit for up to 5 days than in Saskatoon where it was studied while fresh, however, to draw any conclusions from a comparison between laboratories is hazardous. Brookwell *et al.* (1982) found that delay in culturing lymphocytes decreased fragile X expression but that the addition of FUdR to the cultures enhanced expression to the level which might have been expected had the delay not occurred. Gustavson *et al.* (1981) regarded time between sampling and culture to be unimportant in fragile X expression. Eberle *et al.* (1981) claimed that cocultivation of male fragile X lymphocytes with female fragile X or normal lymphocytes resulted in a decrease in fragile X expression in the male but did not enhance expression in the female lymphocytes.

All available evidence suggests that the folate-sensitive fragile sites will be expressed if there is a relative deficiency of thymidine monophosphate available for DNA synthesis during late S. (The possibility that the fragile site could be repaired during $G_2$ cannot be discounted and requires investigation.) The action

of methotrexate can be explained by its inhibition of the dihydrofolate reductase-controlled conversion of DHF to THF, an essential cofactor for thymidylate synthetase to produce thymidine monophosphate. This also accounts for the action of FUdR which specifically inhibits thymidylate synthetase in the presence of folate. It is more difficult to see how the need for methionine and some of the other amino acids (claimed to effect fragile X expression, Lejeune, 1980) involved in one carbon transfers fit into this scheme. It is difficult to see why methionine is apparently essential for fragile site expression. According to Scott and Weir (1981) methionine deficiency will depress DNA synthesis and cell division and this is seen in lymphocyte cultures which grow very poorly under such conditions. Since methionine deficiency also leads to intracellular folate deficiency it would be expected that methionine deficiency would enhance fragile site expression rather than depress it.

Even though it now seems clear that the area of metabolism shown in Fig. 5 is involved it is not clear how it is involved. Why is it that in some cells the fragile site is expressed only on one chromatid? Analogously, in two homozygotes for the fragile site at 10q25 the fragile site was more often expressed on one chromosome than on both (Sutherland, 1981) even though environmental conditions which surrounded the homologous chromosomes during DNA synthesis must be more similar to each other than that in different cells. Two possible explanations for these phenomena can be suggested. First, that in conditions appropriate for expression of fragile sites they are expressed in close to 100% of chromosomes at the completion of S but are gradually repaired during $G_2$. This could account for expression in a single chromatid since presumably the repair events would be independent for each chromatid but expression, if it does occur during S, would not be. Second, fragile sites may be expressed, under appropriate conditions, in virtually 100% of metaphases but that some physical stretching of the chromosome during the process of harvesting may be required to separate the two segments of chromosome across the fragile site (P. B. Jacky, personal communication), thus making it visible.

Effects of harvesting techniques on fragile site expression have been found and give some support to the concept that physical forces on the chromosome might be important. Jacky (1980) found that Na citrate used as a hypotonic agent resulted in a higher frequency of expression of the fragile X in fibroblast cultures than if KCl was used; Gardner *et al.* (1982) supported the use of Na citrate as the preferred hypotonic agent for fibroblast cultures. Bühler *et al.* (1970) investigated the effects of harvesting on the expression of the fragile site at 2q13 and found a higher level of expression when Na citrate was the hypotonic solution used in harvesting than when KCl or a combination of the two was used. Howard-Peebles and Pryor (1981) reported a higher frequency of fragile X expression if slides were air dried rather than flame dried.

## IV. Cytogenetics

### A. Lymphocytes

Some of the morphological appearances of the fragile X are shown in Fig. 6. It will be noted that the appearance is more distinctive in the unbanded preparations than in the G-banded ones. When G-banding methods which employ trypsin are used the segment of chromosome distal to the fragile site becomes merely a fuzziness which is not as readily detected as the striking appearance of the fragile X on unbanded chromosomes. Buckton (1981) (in Hecht *et al.*, 1982) reports that if the ASG banding technique of Sumner *et al.* (1971) is used then the fragile X is easier to score microscopically than from unbanded preparations.

Cytogenetic investigations of the nature of fragile sites have not been very fruitful. In good quality preparations a fine strand of chromosome material can be seen across the gap when the fragile site is expressed as a chromatid or

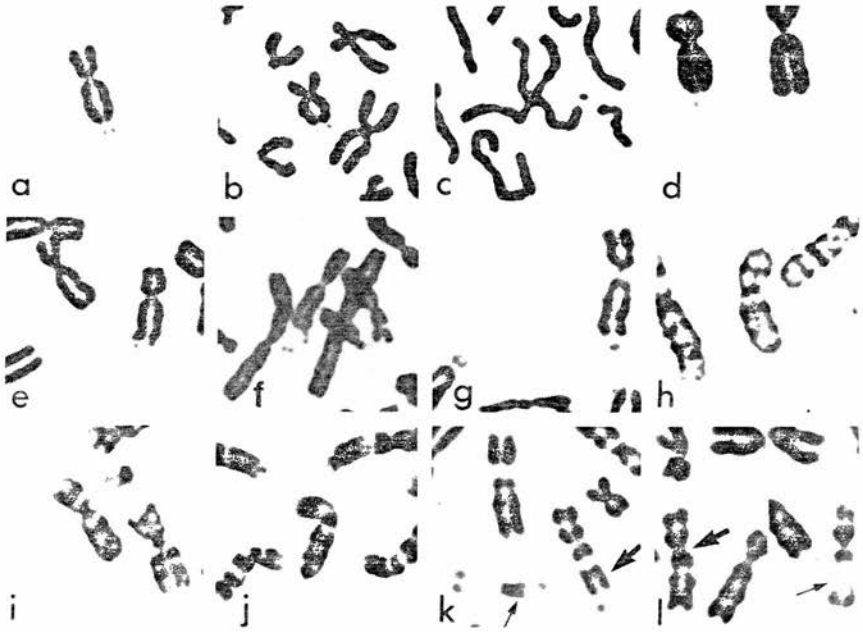



Fig. 6. The fragile X. (a–c) Expression on chromosomes at different stages of compaction; (d) despiralization of the chromosome material distal to the fragile site; (e,f) double satellited appearance equivalent to triradial; (g) expression in skin fibroblast metaphase (courtesy Dr. P. B. Jacky); (h–j) G-banded appearance; (k) BUdR labeling showing early replicating fragile X (broad arrow) and late replicating normal X (small arrow); (l) similar to k but with fragile X late replicating.

chromosome gap. None of the fragile sites stains with Ag-NOR stain (Sutherland and Leonard, 1979; Howard-Peebles and Howell, 1979). Fragile sites have not been examined by electron microscopy due to the technical problem of examining a specific area of a specific chromosome. The nature of the material across the gap in fragile sites remains to be determined. Howard-Peebles and Howell (1981) studied the chromosome core with silver stain and showed that the core ends were fused in the centric portion of the chromosome in a proportion of metaphases from individuals with the fragile X and fra(16)(q22). These fusions were not visible when the chromosomes were stained with Giemsa. This interesting observation has not been repeated by others and its significance remains unclear.

Lubs (1969) reported that X-inactivation was random with respect to the fragile X in females. Similar studies using BUdR labeling rather than tritiated thymidine and autoradiography by Jacobs *et al.* (1980) and Martin *et al.* (1980) support Lubs (1969) but there is still a paucity of data in this area. Technical difficulties arise in that both BUdR and thymidine inhibit expression of the fragile X and, when BUdR labeling is used, the fragile site may be more difficult to see on the late labeling X because it is pale staining (Fig. 6). More data in this area are needed in view of its relevance to explaining the clinical findings in females with the fragile X.

One problem which arises when examining chromosomes for fragile sites is the occurrence of chromatid gaps and breaks which occur at a higher frequency in all cultures grown under the conditions required for expression of fragile sites than when cells are grown in complete medium. The occurrence of these gaps and breaks appears to be nonrandom and there are a number of "hot spots" where they are seen more frequently than would be expected by chance. These hot spots include bands 3p14, 6q26, and 16q23.1 and gaps or breaks in these regions will be referred to as autosomal lesions. These are differentiated from fragile sites because they rarely occur in more than 3–4% of metaphases from any individual, inheritance has not been demonstrated, and the classical triradial configuration produced by fragile sites has not been reported. The factors involved in expression of autosomal lesions appear to be similar to those involved in fragile site expression (Glover, 1981).

The autosomal lesions can cause problems when they mimic the known fragile sites (Leversha *et al.*, 1981). The 6q26 lesion can resemble the fragile X on unbanded preparations; Leversha *et al.* (1981) have observed this lesion in up to 3.5% of metaphases and Soudek and McGregor (1981) recorded it in 1.2% of metaphases from 31 individuals. In this group they also recorded 0.9% of cells with other C-group chromosomes mimicking the fragile X. If doubt exists as to whether unbanded preparations have a low frequency of the fragile X or the 6q26 lesion then G- or Q-banding of scored slides will allow resolution of this uncertainty. There appears to be a fragile site and an autosomal lesion (Sutherland,

1979b; Jennings *et al.*, 1980) at 16q22 and the resolution of confusion between the two could be difficult (Coté and Katsantoni, 1980) but might be resolved by culture of cells in a range of medium types, the use of distamycin A (Schmid *et al.*, 1980), and family studies.

In view of the many factors which influence the expression of fragile sites it is perhaps pointless to compare frequencies at which they are detected in different laboratories. In general, fragile sites are seen in less than 100% of metaphases, indeed there is only one report of a fragile site (at 2q13) being seen in all cells (Annerén and Gustavson, 1981). The fragile X has been reported in up to 56% of lymphocyte metaphases (Jacobs *et al.*, 1980; Brookwell *et al.*, 1982; Webb *et al.*, 1982) and 71% of fibroblast metaphases (Fonatsch, 1981b) but is more usually seen in less than 50% of metaphases. In a 3-year-old girl 82% of lymphocyte metaphases were found to express the fragile X (A. Daniel and R. Brookwell, personal communication). Jacobs *et al.* (1982) have suggested that the maximum frequency of expression possible might be 50%.

There is sometimes difficulty in inducing expression of fragile sites. In obligate carriers of the autosomal fragile sites they are occasionally not detected or found at such low frequencies that it is difficult to be certain that they are actually present. This problem appears to be greater for the fragile X and the situation in males and females differs.

In males the problems associated with expression of the fragile X are only beginning to be appreciated. There is usually no difficulty in detecting the fragile X which is usually present in 10–30% of metaphases if lymphocytes are grown under the currently known optimal conditions. There are, however, reports of individuals who on clinical or family history grounds would appear to be certain to have this chromosome in which it cannot be detected at all or barely detected. For example, only one of a pair of brothers originally studied by Harvey *et al.* (1977) showed the fragile X. After repeated study the second brother was found to have this in only 3% of cells (Sutherland, 1979c). Jacobs *et al.* (1980) have suggested that the fragile X should be detected in 4% or more of metaphases before an individual is considered to have it, although Rhoads *et al.* (1982) reported a probable obligate carrier female with 3% of her cells expressing a fragile X on one occasion and only 1 in 125 cells on another. This 4% rule would appear to be conservative since an apparent fragile X (as distinct from the 6q26 autosomal lesion) has not been seen in normal individuals in spite of extensive study in the author's laboratory. Herbst *et al.* (1981) suggested 1% might be a better cut off point to use than 4%. Proops and Webb (1981) reported the fragile X in up to 3% of lymphocytes of individuals who probably did not have this chromosome but it is not clear whether they excluded the 6q26 lesion from their data. No fragile X was seen in seven normal males studied in detail by Jennings *et al.* (1980). Jacobs *et al.* (1980) recorded a control female with fragile X in 5/389 cells. In the author's experience any individual in whom even one fragile

X chromosome is seen should be regarded as probably having the chromosome until this can be disproved by extensive and repeated chromosome studies or an improvement in technology in this area. This holds particularly when there is good family or clinical suspicion. Soudek *et al.* (1981) have claimed that the proportion of cells showing the fragile X is a familial character and that most members of any one family will have similar frequencies of expression. However, Proops and Webb (1981) remarked upon the inconsistency within the same family. Rhoads *et al.* (1982) noted individuals to have similar frequencies of expression when sampled over a period of several months.

In females, expression of the fragile X appears to be a different problem from that in males. In many females who are obligate carriers of the fragile X it has not been demonstrated. This problem has been encountered and verified by almost all those who have studied females who are obligate or potential carriers of this chromosome. It appears that the proportion of metaphases showing the fragile X decreases with advancing age. Many obligate carriers aged 30 years and above either do not express the fragile X or do so in a very low proportion of metaphases. On the other hand, in females who are potential carriers and aged 20 years or less (these are usually the sisters of young retarded males with the fragile site) the proportion found to have the fragile site is close to 50% (Sutherland, 1979c). Howard-Peebles (1980) has suggested that there may be two types of families with the fragile X, those in which the females are dull and readily express it regardless of age, and those in which they are of normal intelligence and in which expression decreases with age.

Jacobs *et al.* (1980) have claimed that there is a correlation between the frequency of expression of the fragile X in females and their IQ, as well as their age, with the duller females having a higher frequency of expression. They suggest that it is the brighter females (and consequently those with absent or very low frequencies of expression) who are most likely to reproduce and be encountered in family studies as obligate carriers possibly making the apparent age affect either an illusion resulting from ascertainment bias or possibly the result of two different factors affecting expression. Rhoads *et al.* (1982) found the correlation between IQ and frequency of expression in several carrier females in their Japanese family contrary to the suggestion of Howard-Peebles (1980) that there are two types of family in this regard. Herbst *et al.* (1981) were also unable to support this suggestion of Howard-Peebles (1980). In those young carrier females reported by Sutherland (1979c) there was only one who was regarded as dull so this does not support the IQ as opposed to age association with expression at least in younger females.

Jacobs *et al.* (1980) put forward two hypotheses to account for problems of expression of the fragile X in females. The first is that there is selection against cells in which the active X is fragile and that if the fragile site is more easily demonstrated when it is on an active X chromosome there will be a decrease in

the proportion of X chromosomes expressing the fragile X as age increases. This hypothesis depends upon the easier demonstration of the fragile X on active X chromosomes and there is no evidence to suggest that this is so and some to suggest otherwise.

The second hypothesis of Jacobs *et al.* (1980) is that the chromosome material distal to the fragile site may be lost from some cells and that if it is lost from the active X, the cell dies but if from the inactive X the cell survives. This would lead to selection of cells in which the active X did not have the fragile site, the deleted inactive X not being able to be detected cytologically would appear normal and there would be an apparent decrease in fragile sites with increasing age. Since carriers with lower intelligence are presumably the result of differential X inactivation they would start out with a higher proportion of active X fragile chromosomes than carriers of normal intelligence.

Both the above hypotheses are speculative but are the only ones put forward to date to help explain the puzzling and frustrating behavior of the fragile X chromosome in females. Unfortunately FUdR induction of the fragile X does not enhance expression in female carriers where this is low or absent (Rhoads *et al.*, 1982; Brookwell *et al.*, 1982). Much more data on females who are carriers or potential carriers are required.

## B. Other Cell Types

Almost all the above discussion of fragile X chromosomes has pertained to their expression in PHA-stimulated lymphocyte cultures. Most studies on fragile sites have used these cells and only limited studies have been performed on other cell types.

### 1. *Bone Marrow*

Only two studies of bone marrow chromosomes on individuals with folate sensitive fragile sites have been reported. The late Dr. H. R. McCreanor (personal communication) found the site at 2q13 in 36 out of 168 bone marrow metaphases but the technique used was not recorded. Sutherland (1979b) examined 200 metaphases from a boy with the fragile X but did not detect it in the bone marrow. This study employed an *in vitro* time of only 2 hours. It would have been of interest to culture the bone marrow for longer in folate-free medium or in the presence of FUdR and so determine whether the fragile X could be induced. These very limited data do not indicate whether fragile sites are expressed as fragile sites *in vivo*, a question of critical importance to the above hypotheses relating to expression of the fragile X in females and possible deleterious effects of other fragile sites (Sutherland, 1982b) in heterozygotes.

2. *Lymphoblastoid Cell Lines (LCL)*

LCL from individuals with fra(2)(q13) and the fragile X were examined by Sutherland (1979b) and under conditions of folic acid and thymidine deprivation were not found to express the fragile sites. Subsequently it has been confirmed that the fragile X is not seen in these cells under such conditions but its expression can be induced by FUdR (Jacobs *et al.*, 1982). The use of LCL, with induction of fragile sites by FUdR, may provide a useful system for studying fragile sites, the study of which until now has been hampered by the need to collect fresh blood samples from patients whenever experimental work was done. Furthermore, the expression of the fragile X in these lines derived from female carriers may provide insight into this difficult area and possibly provide a reliable means of carrier detection.

3. *Fibroblasts*

Fibroblasts have been used to prepare chromosomes to be examined for fragile sites without success by Fraccaro *et al.* (1971) and Magenis *et al.* (1970). Ferguson-Smith (1973) found the fragile site at 2q13 in fibroblasts. Sutherland (1979b) examined fibroblasts cultured under conditions suitable for the demonstration of fragile sites in lymphocytes from carriers of the fragile sites at 2q13, 10q23, 11q13, 16p22, 20p11, and Xq27. Some of the autosomal fragile sites were seen in up to 4% of metaphases but the fragile X was not seen in fibroblasts from three individuals.

It was not until Jacky and Dill (1980) demonstrated the fragile X in fibroblasts by severe restriction of folic acid and thymidine that the possibility of using these cells for studies of fragile sites became feasible. Unfortunately the method of Jacky and Dill (1980) has proved difficult to reproduce consistently (Jacobs *et al.*, 1980; Turner *et al.*, 1980a; Mattei *et al.*, 1981).

The development of reliable methods for the demonstration of fragile sites in fibroblasts has proceeded slowly. Glover (1981) reported inducing the fragile X in 20 and 25% of fibroblasts using FUdR at concentrations of 0.05 or 0.1 $\mu M$, 24 or 48 hours prior to harvest. Tommerup *et al.* (1981a) reported generally lower frequencies of expression in two males and one female studied using a range of FUdR concentrations added 24 hours prior to harvest. Fonatsch (1981b), although only having studied one male, reported having induced the fragile X in up to 71% of metaphases in fibroblasts cultured in Dulbecco's medium with the folate antagonists methotrexate and aminopterine. Mattei *et al.* (1981) reported inducing the fragile X reliably in fibroblasts using medium 199 and methotrexate. Several groups (Tommerup *et al.*, 1981a; A. Daniel, R. Brookwell, G. Turner, and J. Fishburn, 1981, in Hecht *et al., 1982; Jenkins et al.*, 1981) have reported demonstration of the fragile X in the fibroblasts of female carriers who did not express it in their lymphocytes. If this finding can be confirmed and

extended then a reliable method of carrier detection may become possible. Jenkins *et al.* (1981) have used FUdR to induce expression of the fragile X in cultured amniotic fluid cells thus making prenatal diagnosis of the fragile X possible. However, in view of the difficulties involved in this area Sutherland and Jacky (1982) have suggested that this be approached with great caution.

4. *Somatic Cell Hybrids*

The only report of the fragile X in somatic cell hybrids is that of Bryant *et al.* (1981) who attempted to determine whether the normal genome could suppress fragile site expression by complementation. Expression of the fragile X was not suppressed by the normal genome.

## V. Genetics

All the known fragile sites appear to behave in a Mendelian codominant fashion. Only one has been subjected to segregation analysis and no distortion of expected segregation ratios was observed (Sutherland, 1982b). There has been the suggestion that the fragile X may not follow expected ratios and be preferentially transmitted (Harvey *et al.*, 1977). The same possibility has been raised for X-linked mental retardation without the fragile X (Renpenning *et al.*, 1962). There is great difficulty in trying to establish preferential transmission of either form of retardation because of problems of ascertainment. Families in which there are multiple affected individuals are much more likely to be ascertained than those with small numbers of or single affected individuals. Indeed, many of the families which have been studied to date were ascertained because of mental retardation which followed an X-linked pattern of inheritance. Such families are likely to include more affected males and more carrier females than families identified via a single individual. Howard-Peebles *et al.* (1979) found 51% of the males in their families to have the fragile X but this was without any correction for ascertainment bias. If preferential transmission of the fragile X does occur this needs to be confirmed because it has major implications for genetic counselling. Perhaps the best way of studying this problem would be prospectively. There should be no bias in a recording of all the offspring born (or prenatally diagnosed when this becomes routine) to known carrier females. Such data should finally determine the segregation pattern shown by the fragile X.

The racial origin of most fragile X males has been European (Herbst, 1980) but they have been identified in American blacks (Howard-Peebles and Stoddard, 1980a), Japanese and Filipino (Rhoads *et al.*, 1982), several of the racial groups in South Africa (Venter *et al.*, 1981), and Australian aboriginals (Turner, 1981). Hence it would appear that the fragile X will be found to be present in most racial groups where it is sought.

There have been anecdotal reports of a possible association between fragile sites and two separate phenomena, the first being an increased incidence of the fragile X in individuals with other major chromosome abnormalities such as XXY (Wilmot *et al.*, 1980), XYY (McCarthy, 1981), Down syndrome (Jacobs *et al.*, 1980; J. Lafourcade, personal communication to H. Riveira *et al.*, 1981), and Trisomy 8 (de Grouchy, 1981; Turleau *et al.*, 1979). In the Dunn *et al.* (1963) family a Down syndrome child was produced by a carrier female. Kaiser-McCaw and Hecht (1980) reported a female with a deleted fragile X, del(X)(q22q26), the presence of the fragile site proving that the deletion was interstitial. Shabtai *et al.* (1980) recorded an XXY male and his mother with the fragile site at 16q22. Second, there might be an increased incidence of other chromosome abnormalities in families in which chromosomes with fragile sites are segregating (Coté *et al.*, 1978; Sørensen *et al.*, 1979). This latter phenomenon is most likely the result of ascertainment bias but the former one is difficult to assess since it is also undoubtedly influenced by selective case reporting. Only further data will determine whether female carriers of the fragile X are more liable to produce aneuploid gametes, with or without the fragile X, than are noncarriers.

In an X-linked disease where the reproductive fitness of affected males is zero and the mutation rates in the two sexes equal, one-third of affected males should be new mutants. The reproductive fitness of males with the fragile X is certainly not zero (G. C. Webb *et al.*, 1981; Nielsen *et al.*, 1981b) but probably closely approaches it. Because of the difficulty in excluding the fragile X from mothers of sporadic fragile X males it is difficult to be certain that any sporadic males are new mutants. Several have been found where this is the most probable explanation of their origin. On the other hand, there is no convincing example of a new mutant for other fragile sites and such claims are most probably due to failure to detect the fragile site in one parent (Sutherland, 1982a).

There has been only one reported demonstration of linkage between a fragile site and another genetic marker; this was the fragile site at 16q22 and *HPA* (Magenis *et al.*, 1970). The fragile site at 6p23 has been formally shown to be linked to *HLA* (Mulley and Sutherland, unpublished). Fried and Sanger (1973) found possible linkage between a form of X-linked mental regardation and *XG* but with the localization of *XG* to the opposite end of the X chromosome from the fragile site there can be no linkage between these markers. The fragile X status of Fried's (1972) family is unknown. Linkage has been formally demonstrated between the two fragile sites on 10q and a map distance of 11 female cM between them calculated (Sutherland *et al.*, 1982). Since fragile sites are manifested as defects in chromosome compaction for mitosis it is possible that if such defects were present during meiosis they could affect crossing-over and distort the genetic length of the chromosome near them. Only accurate gene localization near fragile sites, by means other than segregation with fragile sites, together with information from segregation analysis will determine this.

The genes *HPRT* and *G6PD* have been mapped closely to the fragile site at Xq27 although no linkage studies with the *G6PD* locus have been reported. Carroll and Howard-Peebles (1981) found normal levels and electrophoretic mobilities of G6PD in erythrocytes and fibroblasts of fragile X males. Similar findings for G6PD were made by Mareni and Migeon (1981) who also measured mutation rate at the *HPRT* locus and found no difference between fragile X males and controls. This work of Mareni and Migeon (1981) suggests that the difficulty in obtaining expression of the fragile X in some cells is not because the chromosomal material distal to the fragile site has been lost.

There is very little information on the population cytogenetics of any of the fragile sites except for the one at 10q25 which is polymorphic in the Australian population (Sutherland, 1982b). There is a suggestion, based on inadequate numbers, that the autosomal folic acid-sensitive fragile sites may be more common among the mentally retarded than unselected neonates (Sutherland, 1982a), but this work needs to be extended before this suggestion can be confirmed or refuted. The available data on the population cytogenetics of the fragile X are shown in Table II. This shows that the fragile X is not common, is not usually found in other than retarded males and their female relatives, and that apparently normal males with the fragile X must be rare.

TABLE II
POPULATION CYTOGENETIC DATA ON THE FRAGILE X

| Series | Group | Number studied | Number fra(X) |
|---|---|---|---|
| Sutherland (1982b) | Male neonates | 522 | 0 |
| | Female neonates | 497 | 0 |
| | Patients referred for diagnostic chromosome studies | 2237 | 7 |
| | All males in Minda Home (institutionalized retardates) | 298 | 6 |
| | Strathmont (institutionalized retardates) | | |
| | Males with $36 < IQ < 70$ | 98 | 2 |
| | Other males | 6 | 0 |
| | Females | 35 | 0 |
| | Totally dependent profoundly mentally retarded residents of Ru Rua | | |
| | Males | 42 | 0 |
| | Females | 45 | 0 |
| Turner *et al.* (1980b) | Girls with $55 < IQ < 75$ | | |
| | Normal phenotype | 72 | 5 |
| | Abnormal phenotype | 56 | 0 |
| Soudek and Gorzny (1980) | Normal adult males | 57 | 0 |

The incidence of the fragile X and its contribution to mental handicap in males and females are not known. Turner and Turner (1974) estimated that the prevalence of all forms of X-linked mental retardation resulting in an IQ in the range 30–55 (moderate mental retardation) was 0.53/1000 males (Turner and Opitz, 1980). In a more recent examination of the same population Fishburn *et al.* (1982) estimated this prevalence as 0.55/1000 males. If approximately one-half of X-linked mental retardation is due to the fragile X (Herbst, 1980; Brookwell *et al.*, 1982) and if two-thirds of affected males come into the IQ range 35–55, then the incidence of the fragile X would be approximately 0.40/1000 males. Fishburn *et al.* (1982) estimated the prevalence of fragile X males with moderate mental retardation to be 0.19/1000 males. Herbst and Miller (1980) have estimated that 0.92/1000 live born males in British Columbia would have the fragile X. This latter estimate is probably an overestimate since, after the increased infant mortality in Down syndrome this would make the fragile X a more common cause of mental retardation than Down syndrome in males. Enough work has been done to know that this is not so. Sutherland (1982a) has shown that in an institution in which 10–15% of the inmates have Down syndrome only 1.6% had the fragile X. This would indicate that mental retardation due to the fragile X is less common than Down syndrome but since Turner (1981) and Fishburn *et al.* (1982) claim that fragile X males are less liable to institutionalization because of their relatively normal appearance and amicable nature than other males with the same degree of retardation the fragile X males may be underrepresented in the institutional sample. All estimates of the incidence of the fragile X are unreliable to a greater or lesser extent because the standard assumptions used when making estimates involving X-linked conditions, that the condition is lethal (reproductively) in the male and fully recessive in the female, just do not apply to the fragile X.

The contribution of the fragile X to mental handicap in females remains uncertain. Turner *et al.* (1980b) and Fishburn *et al.* (1982) have suggested that as many as 30% of carriers are borderline or mildly retarded and their studies remain the only ones which have examined a population of retarded girls. Again this aspect of fragile X work is bedevilled by the problem of ascertainment and this question is in urgent need of an answer for genetic counselling.

## VI. Clinical Aspects

The autosomal fragile sites have usually been considered to be without phenotypic effect (Sutherland, 1979b). Homozygotes for the folate-sensitive autosomal fragile sites have not been identified but Sutherland (1979b) has speculated that such homozygosity could be deleterious. Homozygotes for the BUdR requiring fragile site at 10q25 are phenotypically normal (Sutherland, 1981) as are

probable homozygotes for fra(16)(q22) (Schmid *et al.*, 1980). Sutherland (1982a) found the autosomal fragile sites to be 10 times more common among institutionalized retardates than unselected newborns; this difference was statistically significant although the groups studied were small and this work needs repeating on other groups. If this finding is confirmed then heterozygotes for the autosomal fragile sites may be at some increased risk of being mentally retarded although the majority will not be. The only fragile site which is unequivocally associated with a mental retardation syndrome is the one on the X chromosome.

X-linked mental retardation has been recognized for many years and achieved respectability as a result of the publications of Lerke (1972, 1974). The main publications prior to this were those of Martin and Bell (1943), Dunn *et al.*, (1963), and Renpenning *et al.* (1962). The name of the senior author of this last paper had come into eponymous use to describe "nonspecific" X-linked mental retardation. However, since the family described by Renpenning *et al.* (1962) does not have the fragile X chromosome (Fox *et al.*, 1980) the term Renpenning syndrome should be restricted to one of those forms of X-linked mental retardation which does not have the fragile X chromosome. Indeed there would appear to be at least four categories of X-linked mental retardation which have now been recognized clinically (Turner and Opitz, 1980; Fishburn *et al.*, 1982) and Herbst and Miller (1980) have estimated that there could be between 7 and 19 X-linked genes responsible for X-linked mental retardation. Good reviews of the development of X-linked mental retardation as a clinical concept have been published (Turner and Opitz, 1980; Herbst, 1980) and only the form associated with the fragile X will be considered further.

The form of X-linked mental retardation associated with the fragile site has no generally accepted succinct name. Turner and Opitz (1980) suggested MOMX syndrome for *m*acro*o*rchidism-*m*arker *X* but this is inappropriate since macroorchidism is not a constant or the only clinical feature of the condition and is certainly inappropriate to apply to those females who are retarded as a consequence of carrying the fragile X. Fragile X-linked mental retardation was suggested by Kaiser-McCaw *et al.* (1980) and modified to fragile (X)-linked mental retardation by Brookwell *et al.* (1982) and this is preferred since it can be applied to either sex.

### A. The Phenotype in Males

Before the fragile X had been recognized as a common entity there were a number of reports of a syndrome of mental retardation associated with macroorchidism (Cantú *et al.*, 1976, 1978; Turner *et al.*, 1975; Ruvalcaba *et al.*, 1977) which was thought to be X-linked. After the discovery of the conditions necessary to demonstrate the fragile X it was shown by Sutherland and Ashforth (1979) Turner *et al.* (1978), and Rivera *et al.* (1981) that the form of X-linked

mental retardation with macroorchidism and that with the fragile X were the same entity. It now appears that X-linked mental retardation with macroorchidism and other features of fragile (X)-linked mental retardation can exist without the fragile X (Herbst *et al.*, 1981; Fishburn *et al.*, 1982).

Apart from widespread interest in the association of macroorchidism with the fragile X there have been few detailed clinical studies of a series of males with this chromosome. The first such study was that of Turner *et al.* (1980a) who examined 25 males from 7 families. They proposed a clinical syndrome in males with the fragile X composed of the following features:

1. Mental retardation which was usually moderate but varied from severe to mild.
2. Speech delay greater than motor development delay. The speech tended to be narrative and compulsive and was referred to as litany speech.
3. Behavior problems in some cases which had been labeled as autistic and hyperactive.
4. Macroorchidism, generally present in adults and possibly in prepubertal males.
5. Birth weight greater than that of siblings, the mean of 22 males was on the seventieth percentile.
6. Increased head circumference before puberty returning to normal in adulthood.
7. Normal but characteristic facial appearance due to (a) large forehead with supraorbital fullness, (b) prominent chin, (c) large ears, and (d) pale irides (in 22 of 25 males examined).

This concept of a syndrome comprising any more than mental retardation and possibly macroorchidism associated with the fragile X has been challenged (Kaiser-McCaw and Hecht, 1980; Kaiser-McCaw *et al.*, 1980). Each of the components of this syndrome will be examined.

Mental retardation is almost always present. Proops and Webb (1981) recorded IQs ranging from 20 to 65 in 11 males who unequivocally had the fragile X. In the original family of Lubs (1969) one male had a measured IQ of 70 but others in the family were more severely retarded. The males described by Jennings *et al.* (1980) were all severely retarded. Martin *et al.* (1980) reported brothers with IQs of 57 and 67. Gustavson *et al.* (1981) reported males with IQs from severely retarded to 70. There are numerous other reports which taken together indicate that most of the males are moderately retarded, however, it would appear that there is a broad range of IQs shown by fragile X males with a small proportion coming into the normal range. Some of the mildly retarded males have reproduced (Jacobs *et al.*, 1980), as have some of the normally intelligent ones (G. C. Webb *et al.*, 1981; Nielsen *et al.*, 1981b).

The spectrum of intellectual development in fragile X males raises the possibility of this chromosome being present in normal males. Daker *et al.* (1981) reported brothers of normal intelligence who appear to have the fragile X but full family studies were not carried out and folic acid sensitivity of the fragile X was not demonstrated. Rhoads *et al.* (1982) recorded a probable hemizygote of normal intelligence who on pedigree data has transmitted the fragile X to a retarded grandson; strangely, the fragile X could not be demonstrated in this normal man. Nielsen *et al.* (1981b) described a family in which three nonretarded males apparently transmitted the fragile X to retarded grandchildren. G. C. Webb *et al.* (1981) were the first to document the fragile X in a normal male, his daughter and retarded grandson. At present it is reasonable to conclude that the great majority of fragile X males will be retarded although in some instances only mildly, with a small number having normal intellectual function. A practical consequence of this is that the fragile X can be transmitted from normal males to their retarded grandsons (G. C. Webb *et al.*, 1981) and this pattern of segregation should always be considered when carrying out family studies. Furthermore, nonretarded male siblings of fragile X males should have their chromosomes studied.

The question of a specific developmental problem in regard to speech has been considered by Herbst (1980) and Herbst *et al.* (1981) who concluded that there was no association between language abilities and the fragile X. This conclusion is in the face of many reports summarized by Herbst (1980) in which a verbal problem has been either noted clinically or documented by a difference between verbal and performance IQ scores. Unfortunately most of this work was done before the fragile X was recognized to be present in a proportion of cases of X-linked mental retardation and is based on a heterogeneous group with this diagnosis. Jacobs *et al.* (1980) reported speech patterns similar to the litany speech of Turner *et al.* (1980a) and suggested it was characteristic enough to suspect the fragile X on this basis alone. Rhoads *et al.* (1982) found a relative verbal disability among the affected males in their Japanese family and the only one fully assessable had litany speech. Howard-Peebles *et al.* (1979) and Howard-Peebles and Stoddard (1979) examined the verbal abilities of individuals with X-linked mental retardation with and without the fragile X and found no distinctive pattern. The verbal disabilities of the two groups were not different from each other or from other retarded groups studied, however, the numbers studied were small and the authors indicated that further studies of males with X-linked mental retardation were required. Further documentation of the verbal abilities of males with the fragile X is required before a deficit in this area can be regarded as proven.

Turner *et al.* (1980a) reported behavior problems in only three of the males they studied. Rhoads *et al.* (1982) recorded a Japanese male with autistic behavior and hyperactivity. Herbst (1980) considered behavior problems in her series,

and although they had been recorded in more than half the males concluded that they may be no more a feature of X-linked mental retardation (the heterogeneous group) than of other forms of mental retardation. Brown *et al.* (1982) recorded four fragile X males with autism. Most authors have studied adults with the fragile X and there is little doubt that these rarely have behavior problems and are not difficult to manage either in institutions or in the community (Turner *et al.*, 1980a). Giraud *et al.* (1976) noted behavior problems in two of five fragile X boys. Lejeune (1982) noted severe behavior problems, including psychotic complication of mental retardation and autism, in 8 out of 16 fragile X males. The author's impression from dealing with a number of children with the fragile X is that apart from mental retardation, behavior problems are the main feature of the condition which causes parental complaint. The problems have mostly been related to hyperactivity but some of the children had been labeled as autistic prior to diagnosis and others have psychotic features to their behavior. In general, these problems appear to have improved with age. Again, more data are required to document a possible association between the fragile X and childhood behavior problems.

There still appears to be confusion about the relationship between macroorchidism and the fragile X. Turner *et al.* (1978), Sutherland and Ashforth (1979), Sutherland *et al.* (1980a), and Rivera *et al.* (1981) concluded that the form of X-linked mental retardation associated with macroorchidism and that associated with the fragile X were the same entity. Jacobs *et al.* (1979) claimed that there was no correspondence between the two forms of mental retardation and that either could exist separately but later (Jacobs *et al.*, 1980) withdrew this claim emphasizing the point made by Howard-Peebles and Stoddard (1980b) that clinical impressions of macroorchidism were useless and that actual measurement of testicular volume was required. It would now seem that X-linked mental retardation with macroorchidism [and the other features of fragile (X)-linked mental retardation] can exist without the fragile X being present (Herbst *et al.*, 1981; Fishburn *et al.*, 1982). Such a family was described by Ruvalcaba *et al.* (1977) where Jennings *et al.* (1980) were unable to demonstrate the fragile X. This condition will be considered later in Section VII.

Testicular volume is a difficult parameter to measure in the living male and can be approached using the Prader orchidometer which has limitations in that the largest bead on the standard model is 25 ml. For testes larger than this (and for smaller ones) length and width can be measured and the volume calculated from the formula $\Pi/_6 lw^2$ where $l$ is the length and $w$ the width of the testis (Cantú *et al.*, 1976). Normal data have been provided for Caucasian males by Prader (1974), Farkas (1972), and Zachmann *et al.* (1974) and it has been suggested (Rhoads *et al.*, 1982) that different criteria of normality may be required for other racial groups. Benign macroorchidism has been described by Padron *et al.* (1979) who found 15 out of 202 normal Cuban males to have testes greater than

25 ml in volume. Of these, six had volumes calculated from measurement to be greater than 2 SD above the mean and ranged from 58 to 74 ml. Semen analysis was normal but the volume of ejaculate and sperm counts were much greater than in males with normal testicular volumes.

An analysis of the published and unpublished testicular volumes of retarded adult males of Caucasian origin with the fragile X, ascertained via mental retardation rather than macroorchidism, shows that the mean testicular volumes of 83 such males is 48 ml. The range is from 15 to 127 ml (Jacobs *et al.*, 1980). The distribution (Fig. 7) is skewed such that the modal volume is in the range 35–40 ml. Hence it would appear that the fragile X chromosome has the effect of increasing the mean testicular volume in males from about 19 ml (Zachmann *et al.*, 1974) in normal males to 48 ml and about 80% of fragile X males will have a mean testicular volume of greater than 30 ml. There can be little doubt that macroorchidism is a feature of most males with the fragile X, and in all families with the fragile X at least some of the males with this chromosome have macroorchidism.

Histological studies of enlarged testes from fragile X males have been carried out by Cantú *et al.* (1976, 1978), Ruvalcaba *et al.* (1977), Bowen *et al.* (1978),

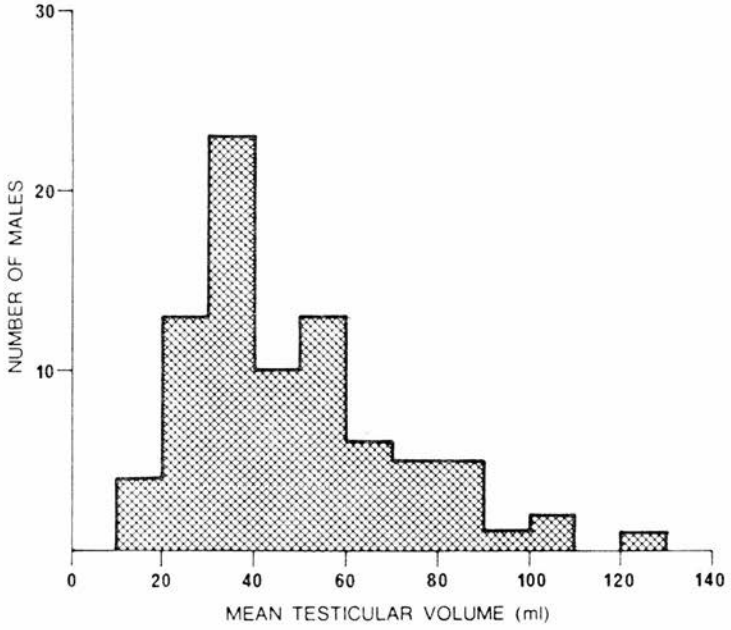



FIG. 7. Distribution of mean testicular volume of 83 adult caucasian fragile X males in whom macroorchidism was not a factor in ascertainment. Mean volume is the average of the right and left testicular volume.

and Turner *et al.* (1975) and the only consistent finding seems to be a relatively normal testis with interstitial edema. Ruvalcaba *et al.* (1977) concluded that macroorchidism was due to an increase of water content in the testis. Normal semen analysis was reported by Cantú *et al.* (1976).

The use of macroorchidism as a means of screening males to identify those with the fragile X has produced conflicting results. Brown *et al.* (1981) screened 15 males with nonspecific mental retardation and found five with one or both testicles having a volume of greater than 25 ml; four were shown to have the fragile X. Nielsen *et al.* (1981a) screened 178 retarded males and, using the same criteria as Brown *et al.* (1981), identified 10 with macroorchidism of whom only one had the fragile X. On the other hand, Pozsonyi *et al.* (1981) screened 818 retarded males, again using the same criteria as Brown *et al.* (1981), and found 190 with macroorchidism of whom only nine had the fragile X. This latter finding would suggest that macroorchidism is either very common among the retarded group studied or that the criteria for determining it were wrong. Some of the males identified in this way presumably had X-linked mental retardation with macroorchidism but without the fragile X. In the survey of Fishburn *et al.* (1982), of 18 families with X-linked mental retardation and macroorchidism, 12 had the fragile X and 6 did not.

There are only limited data available on birth weight of fragile X males. Those studied by Turner *et al.* (1980a) had an average birth weight on the seventieth percentile and in 12 of 16 their birth weight was on average greater than that of their unaffected siblings. In the two families of Ruvalcaba *et al.* (1977) birth weights were noted to be above the nintieth percentile for gestational age. Herbst *et al.* (1981) recorded birth weights of nine fragile X males and only one had a birth weight greater than all his siblings. Hence the information on birth weights is equivocal and more data are needed on this aspect of development.

There is not much data on head circumference in children with the fragile X other than that presented by Turner *et al.* (1980a). Jennings *et al.* (1980) reported the head circumference on a 5-year-old boy to be greater than the nintieth percentile. Jacobs *et al.* (1980) recorded head circumferences on three children and two of these are at the nintieth percentile. Herbst *et al.* (1981) recorded head circumferences of two boys and seven adult fragile X males and all were normal. The limited data available indicate probable increased head circumference in children but not adults (Turner *et al.*, 1980a), although Rhoads *et al.* (1982) recorded an increased head circumference in adult Japanese males with the fragile X.

The characteristic facial appearance described by Turner *et al.* (1980a) has been confirmed by others but challenged by some. Jacobs *et al.* (1980) referring to this remarked they appear "to be cast in the same mould." Jennings *et al.* (1980) state that facial appearance is unique and describe it as mid-facial hypoplasia with large prominent ears and prognathism. Fox *et al.* (1980) found the

characteristic fragile X syndrome in members of the original Dunn *et al.* (1962) family which has the fragile X but not in that described by Renpenning *et al.* (1962) which does not have the fragile X. Herbst *et al.* (1981) noted normal faces in many of their fragile X males but suggested a tendency toward long narrow faces, prominent jaws, and large or lop ears. Kaiser-McCaw and Hecht (1980) have challenged the existence or at least the constancy of the phenotype after study of a number of retarded males in a large Spanish kindred.

One feature of the fragile X syndrome not mentioned by Turner *et al.* (1980a) is the hypogonadal appearance of some of the adult fragile X males. This can include sparse body hair with a female pubic hair distribution and gynecomastia. In their original description of the syndrome Turner *et al.* (1975) reported one male to have minimal beard growth, female pubic hair distribution, gynecomastia, and striae on the buttocks, abdomen, and axillae; this man's brother was similarly affected in this way. Ruvalcaba *et al.* 1977) commented upon a male having lack of secondary sexual development at the age of 16.7 years. Bowen *et al.* (1978) recorded gynecomastia in one male, as did Webb *et al.* (1982). Although the endocrinological investigations of fragile X males have been normal (Cantú *et al.*, 1976; Bowen *et al.*, 1978; Ruvalcaba *et al.*, 1977) this is an area which may reward further study. Numerous other clinical findings have occasionally been mentioned by various authors but the only others to occur frequently are clumsiness with poor fine motor control, large hands and feet, and an increased incidence of seizures. Fishburn *et al.* (1982) report exaggerated reflexes without clinical spasticity to be common in fragile X males.

It would seem that from the literature and the author's experience that there is indeed a syndrome associated with the fragile X in males (Fig. 8) but that it is variable although the variation is probably no greater than that seen in other dysmorphic syndromes, especially those associated with chromosome abnormalities. Not every fragile X male will be diagnosed clinically but as a retarded male will be worthy of chromosome study.

### B. The Phenotype in Females

No female homozygous for the fragile X has been described so that all clinical data on the fragile X in females are derived from heterozygotes. The main study of heterozygotes is that of Turner *et al.* (1980b) who karyotyped 128 mentally retarded girls of whom 72 were regarded as being physically phenotypically normal; among the 72 there were five who had the fragile X chromosome. This study suggested that 7% of girls with an IQ in the range 55 to 75 and who have no physical abnormality may be retarded because they carry the fragile X. In the families of these girls 18 heterozygotes were identified (excluding the index cases) of whom six were at least educationally retarded. This would suggest that one-third of fragile X carriers may be mildly retarded or worse. Fishburn *et al.*

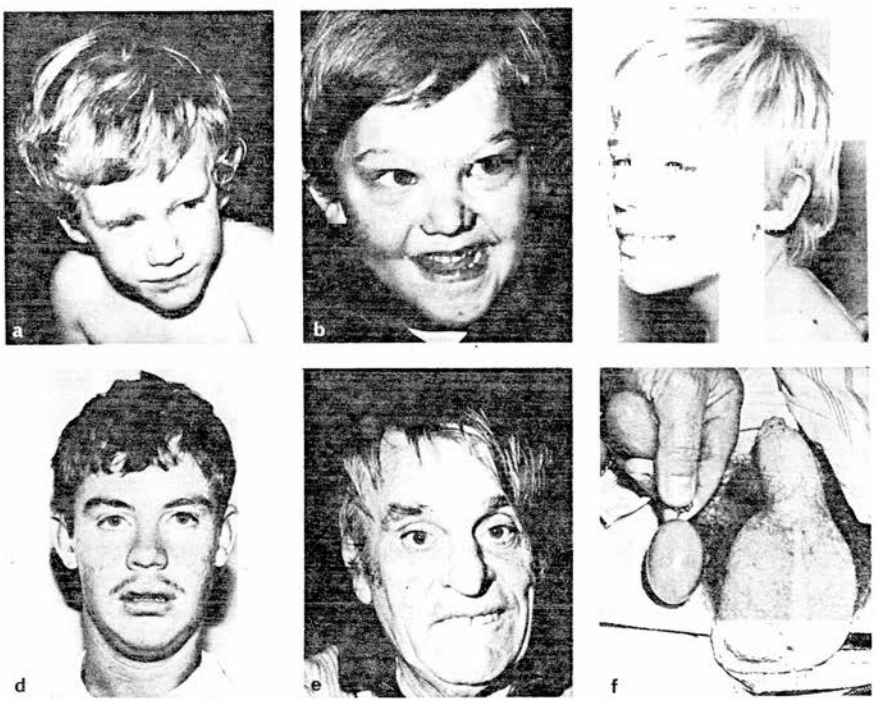



FIG. 8. Facial appearances of five retarded fragile X males (a) aged 3 years, (b) aged 5 years, (c) aged 11 years, (d) aged 16 years, (e) aged 62 years. Macroorchidism shown by a fragile X male, note size of testes in relation to 25 ml orchidometer bead (f).

(1982), from a more extensive study of 40 families, confirm that one-third of female heterozygotes are borderline or mildly mentally retarded but have no physical abnormality.

Physical examination of the index cases identified by Turner *et al.* (1980b) showed no abnormality other than obesity in four of the five, and pale irides in two. Webb *et al.* (1982) reported a severely retarded heterozygote (IQ less than 30) with a moderately retarded niece; neither had dysmorphic features although both were mildly obese, one had bright blue irides and the other hazel irides. Many authors have recorded retarded heterozygotes but have not commented upon their physical phenotype and there is a need for further study in this area.

### C. TREATMENT

Lejeune (1982) has claimed that treatment of fragile X males with oral or intramuscular folate derivatives has resulted in an improvement in the behavior

of seven out of eight who had psychotic behavior. The basis of this treatment is entirely empirical since no abnormality of folate metabolism has been demonstrated in fragile X males (Jennings *et al.*, 1980; Popovich *et al.*, 1980). No other treatment trials have been reported and there is need for a blind trial of folate treatment to determine whether the claim of Lejeune (1982) can be substantiated. A vitamin treatment study of the mentally retarded was reported by Harrell *et al.* (1981) in which small increases in IQ were recorded for most of those studied except for one boy whose IQ at age 7 years was 25 to 30, but after treatment at the age of 9 years was about 90. Did this child have the fragile X?

### D. Genetic Counselling and Prenatal Diagnosis

Genetic counselling in fragile X families is very difficult for a number of reasons. The inability to detect the fragile X in many obligate carriers makes carrier detection uncertain, even when females are studied at an early age. It is not possible to be absolutely certain that males with the fragile X will be retarded although the degree of uncertainty here is so small that for practical purposes this problem can be ignored. Another problem is the possibility that normal males in these families could have the fragile X and not manifest it (Rhoads *et al.*, 1982) although again the probability of this is very low. The risks to a carrier female of having a child who has the fragile X are still uncertain and could be greater than 50% (see Section V). If this is so then some carrier females may be reluctant to embark upon prenatal diagnosis if their aim is to have a child (male or female) who does not have the fragile X. If prenatal diagnosis is offered to carrier females then there is a problem when a female fetus is found with the fragile X. Her probabilities of being a bit dull, mildly or moderately retarded, or worse are not known although Turner *et al.* (1980b) and Fishburn *et al.* (1982) have estimated that one-third of such females are retarded to some degree. Most couples undertaking prenatal diagnosis would not wish to continue with a female pregnancy if there was a one in three chance that the child was going to be retarded and for this reason the usual practice of fetal sexing in X-linked conditions, with abortion of male fetuses, will be of limited use in fragile (X)-linked mental retardation.

The concept of prenatal diagnosis has been mooted for a long time and was suggested by Lubs (1969) in his original description of the fragile X. Harvey *et al.* (1977) also proposed this as an option for carrier females. These suggestions were made before the difficulties in obtaining fragile X expression in fibroblasts were appreciated (Sutherland, 1977a). Jenkins *et al.* (1981) were the first to demonstrate the fragile X in cultured amniotic fluid cells by induction with FUdR and Shapiro *et al.* (1982) achieved a prospective prenatal diagnosis using the same approach. T. Webb *et al.* (1981) used lymphocyte culture from fetal blood sampling via fetoscopy to show that a male fetus had the fragile X. Either of these approaches should be successful in prenatally diagnosing the fragile X if

they are only utilized by those with considerable laboratory experience in demonstrating this chromosome in a variety of cell types (Sutherland and Jacky, 1982).

## VII. Karyotype–Phenotype Relationship

There is little doubt that the fragile X chromosome is associated with a mental retardation syndrome in most males who have it. It is also clear that a proportion of females who carry the fragile X are mildly retarded or worse. Furthermore, the mental retardation syndrome seen in males with the fragile X can exist in males in whom the fragile X cannot be demonstrated (Fishburn *et al.*, 1982).

The nature of the association between the fragile X and its associated syndrome is unclear. The possibility of a locus for the mental retardation syndrome being so closely linked to the fragile site that recombination will not usually be seen within any family could explain some of the observed facts. It could certainly account for the finding of the syndrome associated with the fragile site in some families and not in others and with the finding of normal males with the fragile X (Daker *et al.*, 1981). It cannot however readily account for normal males transmitting the fragile X and mental retardation to their grandsons (Rhoads *et al.*, 1982; G. C. Webb *et al.*, 1981; Nielsen *et al.*, 1981a). This would, on a linkage hypothesis, require such males to have married females who were carriers of the locus and for recombination to have occurred in their daughters before the recombinant chromosome was transmitted—a series of events so improbable that it can be discounted. The most likely explanation for apparently normal fragile X males is that either the genetic effect of the fragile X is nonpenetrant or only very mildly expressed. There is a suggestion in the family described by Nielsen *et al.* (1981b) that the normal fragile X males were less intelligent than their brothers without the fragile X who were of superior intelligence. Presumably the deleterious effect of the fragile X is imposed upon the genetic component of intellectual endowment and where this is very high even substantial impairment of it could result in normal intelligence. No fragile X male of superior intelligence has been reported.

Until any further evidence to the contrary is presented it is probably safe to conclude that the fragile site at Xq27 is a cytological manifestation of the gene responsible for the mental retardation syndrome seen in association with it. The gene may not be 100% penetrant and its expression can certainly vary both clinically and cytologically. Mental retardation seen in female carriers of the fragile X is presumably the result of differential X-inactivation.

There is still the problem of those males without the fragile X but with the clinically indistinguishable syndrome of X-linked mental retardation with its associated physical findings including macroorchidism. There are several possible explanations for this phenomenon and it is not possible to choose between them at this time. There is still a paucity of clinical information about these males

and it might be that they will be found eventually to be a separate clinical entity. If this is not so then two main possibilities arise. First, all such males have the fragile X but under conditions of culture it is not expressed. Soudek *et al.* (1981) suggested that fragile X families had a characteristic frequency of expression of the fragile site in lymphocyte culture and that on average males in some families had a very low frequency of expression. If Soudek's suggestion is true, then in some families expression may be so difficult to elicit using current technology that males in these families may appear not to have the fragile X. Attempts to demonstrate a fragile X in fibroblast or lymphoblastoid cultures in these males may help resolve this matter, or further technical advances in the demonstration of fragile sites may be needed before it can be finally resolved. Second, there may be an allelic mutation which will not result in expression as a fragile site but which results in an abnormal gene product with the same phenotypic consequences as the mutation which is expressed as a fragile site, such mutation could be in the form of a small deletion. Genetic heterogeneity is commonly found in well-studied genetic diseases and there is no reason to think fragile (X)-linked mental retardation will be any different.

## VIII. Conclusions

The fragile X chromosome is a common cause of mental retardation in males and females. Much more information is required on the nature and effects of the fragile X in virtually every area where it has been examined from chromosome structure, the biochemistry involved in expression in different cell types, the incidence in various populations and its contribution to mental retardation, the nature of its association with abnormal phenotypes in most males and some females, its possible role in chromosome segregation of both the X chromosome carrying it and other chromosomes, and its linkage relationships with other genes on the X chromosome to its relationship to the autosomal fragile sites. Perhaps some of these questions will not be answered until recombinant DNA technology has been applied to fragile sites (Gerald, 1980); nevertheless even at this present state of rudimentary knowledge study of the fragile X chromosome is an essential part of clinical cytogenetics where its contribution to diagnosis, prevention, and possibly treatment of mental handicap is just beginning.

## IX. Apologia

Gerald (1981) has expressed the hope that the circumstances surrounding my finding that fragile sites depend upon conditions of tissue culture for expression (Sutherland, 1977b) would be documented. In 1975 shortly after I had taken up my present appointment in Adelaide I attempted to restudy some of the autoso-

mal fragile sites which had been found in the mid-1960s in patients studied in the Unit. This was only intended to be a short project to occupy my time until I settled into my new job and decided upon some more worthwhile activity. Between the time these patients were ascertained and my arrival the laboratory (along with many others around the world) had switched from using TC199 for lymphocyte culture and was using RPMI 1640 which I soon changed to Ham's F10, which I had been using in Edinburgh, so that we could use a single medium for culture of lymphocytes, fibroblasts, and amniotic fluid cells. My early attempts to study fragile sites used Ham's F10, and, needless to say, were quite unsuccessful. In late 1975 I visited my old laboratory in Melbourne and Jill Harvey, who was my successor there, showed me her work on the fragile X which was subsequently published (Harvey *et al.*, 1977).

I guessed that this marker X chromosome (as it then was) probably had a fragile site similar to the autosomal ones I was attempting to restudy (mainly because of the double satellited appearance occasionally seen) and guessed that I would not be able to demonstrate it. On returning to Adelaide I briefly tried a few changes to my culturing technique attempting to induce the vanished autosomal sites by reproducing the Melbourne conditions. Initially I tried steptomycin in my Ham's F10 because this was in use in Melbourne, whereas I was using only penicillin. Eventually I decided to try and duplicate the Melbourne conditions as faithfully as I could and purchased some TC199. Under these conditions my vanished autosomal fragile sites miraculously reappeared and study of a few families suspected of having X-linked mental retardation soon allowed me to confirm the common occurrence of the fragile X and provided material for further studies. To my chagrin, one of the fragile X males had been studied and pronounced to have a normal karyotype by me during my period in the Melbourne laboratory. Jill Harvey assured me that checking of the preparation I had examined showed it to be of fairly poor quality but this was probably just Jill being nice to me. Had I been observant enough at the time, Lubs' initial discovery would well have been confirmed shortly after it had been made.

ACKNOWLEDGMENTS

I thank Dr. P. B. Jacky for helpful discussion during the preparation of this article and many authors who sent me preprints of work in press. This work has been supported by grants from the National Health and Medical Resource Council of Australia, The Channel 10 Children's Medical Research Foundation, and The Adelaide Children's Hospital Research Trust.

REFERENCES

Annerén, G., and Gustavson, K. H. (1981). *Hereditas* **95,** 63–67.
Bowen, P., Biederman, B., and Swallow, K. A. (1978). *Am. J. Med. Genet.* **2,** 409–414.

Brookwell, R., Daniel, A., Turner, G., and Fishburn, J. (1982). *Am. J. Med. Genet.* **13,** 139–148.
Brown, W. T., Mezzacappa, P. M., and Jenkins, E. C. (1981). *Lancet* **2,** 1055.
Brown, W. T., Friedman, E., Jenkins, E. C., Brooks, J., Wisniewski, K., Raguthu, S., and French, J. H. (1982). *Lancet* **1,** 100.
Bryant, E. M., Hoehn, H., and Martin, G. M. (1981). *Am. J. Hum. Genet.* **33,** 99A.
Bühler, E. M., Luchsinger, U., Bühler, U. K., Méhes, K., and Stalder, G. R. (1970). *Hum. Genet.* **9,** 97–104.
Cantú, J. M., Scaglia, H. E., Medina, M., González-Diddi, M., Morato, T., Moreno, M. E., and Pérez-Palacios, G. (1976). *Hum. Genet.* **33,** 23–33.
Cantú, J. M., Scaglia, H. E., González-Diddi, M., Hernández-Jáuregui, P., Morato, T., Moreno, M. E., Giner, J., Alcántar, A., Herrera, D., and Pérez-Palacios, G. (1978). *Hum. Genet.* **41,** 331–339.
Carroll, A. J., and Howard-Peebles, P. N. (1981). *Am. J. Hum. Genet.* **33,** 826–828.
Côté, G. B., and Katsantoni, A. (1980). *Ann. Génét.* **23,** 241–243.
Côté, G. B., Papadakou-Lagoyanni, S., and Pantelakis, S. (1978). *Ann. Génét.* **21,** 209–214.
Daker, M. G., Chidiac, P., Fear, C. N., and Berry, A. C. (1981). *Lancet* **1,** 780.
de Grouchy, J. (1981). *Int. Congr. Hum. Genet., 6th, Israel.*
Dunn, H. G., Renpenning, H., Gerrard, J. W., Miller, J. R., Tabata, T., and Federoff, S. (1963). *Am. J. Ment. Defic.* **67,** 827–848.
Eberle, G., Zankl, H., and Zankl, M. (1981). *Lancet* **1,** 557.
Erbe, R. W. (1975). *N. Engl. J. Med.* **293,** 753–757.
Farkas, L. G. (1972). *Am. J. Phys. Anthropol.* **34,** 325–328.
Ferguson-Smith, M. A. (1973). *Ann. Génét.* **16,** 29–34.
Ferguson-Smith, M. A. (1977). *Rec. Adelaide Child. Hosp.* **1,** 278–286.
Fishburn, J., Turner, G., Daniel, A., and Brookwell, R. (1983). *Am. J. Med. Genet.* (in press).
Fonatsch, C. (1981a). *Lancet* **1,** 494.
Fonatsch, C. (1981b). *Hum. Genet.* **59,** 186.
Fox, P., Fox, D., and Gerrard, J. W. (1980). *Am. J. Med. Genet.* **7,** 491–495.
Fraccaro, M., Lindsten, J., Tiepolo, L., and Ricci, N. (1972). *Chromosomes Today* **3,** 138–146.
Fried, K. (1972). *Clin. Genet.* **3,** 258–263.
Fried, K., and Sanger, R. (1973). *J. Med. Genet.* **10,** 17–18.
Gardner, A. P., Howell, R. T., and McDermott, A. (1982). *Lancet* **1,** 101.
Gerald, P. S. (1980). *New Engl. J. Med.* **303,** 696–697.
Gerald, P. S. (1981). *Pediatrics* **68,** 594–595.
Giraud, F., Ayme, S., Mattei, J. F., and Mattei, M. G. (1976). *Hum. Genet.* **34,** 125–136.
Glover, T. W. (1981). *Am. J. Hum. Genet.* **33,** 234–242.
Glover, T. W., and Howard-Peebles, P. N. (1981). *Am. J. Hum. Genet.* **33,** 104A.
Gustavson, K.-H., Holmgren, G., Blomquist, H. G., and Mikkelsen, M. (1981). *Clin. Genet.* **19,** 101–110.
Harrell, R. F., Capp, R. H., Davis, D. R., Peerless, J., and Ravitz, L. R. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 574–578.
Harvey, J., Judge, C., and Wiener, S. (1977). *J. Med. Genet.* **14,** 46–50.
Hecht, F., Jacky, P. B., and Sutherland, G. R. (1982). *Am. J. Med. Genet.* **11,** 489–495.
Herbst, D. S. (1980). *Am. J. Med. Genet.* **7,** 443–460.
Herbst, D. S., and Miller, J. R. (1980). *Am. J. Med. Genet.* **7,** 461–469.
Herbst, D. S., Dunn, H. G., Dill, F. J., Kalousek, D. K., and Krywaniuk, L. W. (1981). *Hum. Genet.* **58,** 366–372.
Howard-Peebles, P. N. (1980). *Am. J. Med. Genet.* **7,** 497–501.
Howard-Peebles, P. N., and Howell, W. M. (1979). *Cytogenet. Cell Genet.* **23,** 277–278.
Howard-Peebles, P. N., and Howell, W. M. (1981). *Cytogenet. Cell Genet.* **31,** 115–119.

Howard-Peebles, P. N., and Pryor, J. C. (1979). *New Engl. J. Med.* **301,** 166.
Howard-Peebles, P. N., and Pryor, J. C. (1981). *Clin. Genet.* **19,** 228–232.
Howard-Peebles, P. N., and Stoddard, G. R. (1979). *Hum. Genet.* **50,** 247–251.
Howard-Peebles, P. N., and Stoddard, G. R. (1980a). *Am. J. Hum. Genet.* **32,** 629–630.
Howard-Peebles, P. N., and Stoddard, G. R. (1980b). *Clin. Genet.* **17,** 125–128.
Howard-Peebles, P. N., Stoddard, G. R., and Mims, M. G. (1979). *Am. J. Hum. Genet.* **31,** 214–222.
Howard-Peebles, P. N., Pryor, J. C., and Stoddard, G. R. (1980). *Am. J. Hum. Genet.* **32,** 73A.
Hultén, M. (1978). *Clin. Genet.* **14,** 294.
Jacky, P. B. (1980). Ph.D. thesis, University of British Columbia.
Jacky, P. B., and Dill, F. J. (1980). *Hum. Genet.* **53,** 267–269.
Jacobs, P. A., Mayer, M., Rudak, E., Gerrard, J., Ives, E. J., Shokeir, M. H. K., Hall, J., Jennings, M., and Hoehn, H. (1979). *New Engl. J. Med.* **300,** 737–738.
Jacobs, P. A., Glover, T. W., Mayer, M., Fox, P., Gerrard, J. W., Dunn, H. G., and Herbst, D. S. (1980). *Am. J. Med. Genet.* **7,** 471–489.
Jacobs, P. A., Hunt, P. A., Mayer, M., Wang, J-C., Boss, G., and Erbe, R. W. (1982). *Am. J. Hum. Genet.* **34,** 552–557.
Jenkins, E. C., Brown, W. T., Duncan, C. J., Brooks, J., Yishay, M. B., Giordano, F. M., and Nitowsky, H. M. (1981). *Lancet,* **2,** 1292.
Jennings, M., Hall, J. G., and Hoehn, H. (1980). *Am. J. Med. Genet.* **7,** 417–432.
Kaiser-McCaw, B., and Hecht, F. (1980). *Am. J. Hum. Genet.* **32,** 114A.
Kaiser-McCaw, B., Hecht, F., Cadien, J. D., and Moore, B. C. (1980). *Am. J. Med. Genet.* **7,** 503–505.
Lehrke, R. (1972). *Am. J. Ment. Defic.* **76,** 611–619.
Lehrke, R. G. (1974). *Birth Defects Orig. Art Ser.* **12,** 1–100.
Lejeune, J. (1980). *C.R. Acad. Sci. Paris Ser. D.* **290,** 1075–1077.
Lejeune, J. (1982). *Lancet* **1,** 273–274.
Lejeune, J., Dutrillaux, B., Lafourcade, J., Berger, R., Abonyi, D., and Rethoré, M. O. (1968). *C.R. Acad. Sci. Paris Ser. D.* **266,** 24–26.
Leversha, M. A., Webb, G. C., and Pavey, S. M. (1981). *Lancet* **1,** 49.
Lubs, H. A. (1969). *Am. J. Hum. Genet.* **21,** 231–244.
McCarthy, C. M. T. (1981). *Annu. Sci. Meet. 5th. Hum. Genet. Soc. Aust., Canberra.*
Magenis, R. E., Hecht, F., and Lovrien, E. W. (1970). *Science* **170,** 85–87.
Manuel, A., Sutherland, G. R., and Molesworth, J. (1981). *Annu. Sci. Meet. 5th. Hum. Genet. Soc. Aust., Canberra.*
Mareni, C., and Migeon, B. R. (1981). *Am. J. Hum. Genet.* **33,** 752–761.
Martin, J. P., and Bell, J. (1943). *J. Neurol. Psychiatr.* **6,** 154–157.
Martin, R. H., Lin, C. C., Mathies, B. J., and Lowry, R. B. (1980). *Am. J. Med. Genet.* **7,** 433–441.
Mattei, M. G., Mattei, J. F., Vidal, I., and Giraud, F. (1981). *Hum. Genet.* **59,** 166–169.
Nielsen, K. B., Tommerup, N., Dyggve, H., and Schou, C. (1981a). *N. Engl. J. Med.* **305,** 1348.
Nielsen, K. B., Tommerup, N., Poulsen, H., and Mikkelsen, M. (1981b). *Hum. Genet.* **59,** 23–25.
Noël, B., Quack, B., Mottet, J., Natois, Y., and Dutrillaux, B. (1977). *Exp. Cell Res.* **104,** 423–426.
Padron, R. S., Hung, S., Licea, M., Pérez-Plaza, M., and Arce, B. (1979). *Int. J. Androl.* **2,** 1–5.
Popovich, B. W., Rosenblatt, D. S., Vekemans, M., and Cooper, B. A. (1980). *Am. J. Hum. Genet.* **32,** 84A.
Pozsonyi, J., Sergovich, F., Kirkilionis, A., and Sheridan, G. (1981). *Int. Congr. Hum. Genet., 6th, Israel.*

Prader, A. (1974). *In* "Clinical Endocrinology. Theory and Practice" (A. Labhart, ed.), p. 1036. Springer-Verlag, Berlin and New York.
Proops, R., and Webb, T. (1981). *J. Med. Genet.* **18,** 366–373.
Renpenning, H., Gerrard, J. W., Zaleski, W. A., and Tabata, T. (1962). *Can. Med. Assoc. J.* **87,** 954–956.
Rhoads, F. A., Oglesby, A. C., Mayer, M., and Jacobs, P. A. (1982). *Am. J. Med. Genet.* **12,** 205–217.
Rivera, H., Hernandez, A., Plascencia, L., Sanchez-Corona, J., Garcia-Cruz, D., and Cantu, J. M. (1981). *Ann. Génét.* **24,** 220–222.
Ruvalcaba, R. H. A., Myhre, S. A., Roosen-Runge, C., and Beckwith, J. B. (1977). *J. Am. Med. Assoc.* **238,** 1646–1650.
Scheres, J. M. J. C., and Hustinx, T. W. J. (1980). *Am. J. Hum. Genet.* **32,** 628–629.
Schmid, M., Klett, C., and Niederhofer, A. (1980). *Cytogenet. Cell Genet.* **28,** 87–94.
Scott, J. M., and Weir, D. G. (1981). *Lancet* **2,** 337–340.
Shabtai, F., Bichacho, S., and Halbrecht, I. (1980). *Hum. Genet.* **55,** 19–22.
Shapiro, L. R., Wilmot, P. L., Brenholz, P., Leff, A., Martino, M., Harris, G., Mahoney, M. J., and Hobbins, J. C. (1982). *Lancet* **1,** 99–100.
Sørensen, K., Nielsen, J., Holm, V., and Haahr, J. (1979). *Hum. Genet.* **48,** 131–134.
Soudek, D., and Gorzny, N. (1980). *Clin. Genet.* **19,** 140–141.
Soudek, D., and McGregor, T. (1981). *Lancet* **1,** 556–557.
Soudek, D., Partington, M. W., and McGregor, T. (1981). *Int. Congr. Hum. Genet., 6th, Israel* Abstr P.17.15, p. 251.
Sumner, A. T., Evans, H. J., and Buckland, R. A. (1971). *Nature (London) New Biol.* **232,** 31–32.
Sutherland, G. R. (1977a). *New Engl. J. Med.* **296,** 1415.
Sutherland, G. R. (1977b). *Science* **197,** 265–266.
Sutherland, G. R. (1979a). *Am. J. Hum. Genet.* **31,** 125–135.
Sutherland, G. R. (1979b). *Am. J. Hum. Genet.* **31,** 136–148.
Sutherland, G. R. (1979c). *Hum. Genet.* **53,** 23–27.
Sutherland, G. R. (1981). *Am. J. Hum. Genet.* **33,** 946–949.
Sutherland, G. R. (1982a). *Am. J. Hum. Genet.* **34,** 452–458.
Sutherland, G. R. (1982b). *Am. J. Hum. Genet.* **34,** 753–756.
Sutherland, G. R., and Ashforth, P. L. C. (1979). *Hum. Genet.* **48,** 117–120.
Sutherland, G. R., and Hinton, L. (1981). *Hum. Genet.* **57,** 217–219.
Sutherland, G. R., and Jacky, P. B. (1982). *Lancet* **1,** 100.
Sutherland, G. R., and Leonard, P. (1979). *Hum. Genet.* **53,** 29.
Sutherland, G. R., Baker, E., and Seshadri, R. S. (1980a). *Am. J. Hum. Genet.* **32,** 542–548.
Sutherland, G. R., Judge, C. G., and Wiener, S. (1980b). *J. Med. Genet.* **17,** 73.
Sutherland, G. R., Baker, E., and Mulley, J. C. (1982). *Science* **217,** 373–374.
Tiepolo, L., Maraschio, P., Gimelli, G., Cuoco, C., Gargani, G. F., and Romano, C. (1979). *Hum. Genet.* **51,** 127–137.
Tommerup, N., Nielsen, K. B., and Mikkelsen, M. (1981a). *Am. J. Med. Genet.* **9,** 263–264.
Tommerup, N., Poulsen, H., and Brøndum-Nielsen, K. (1981b). *J. Med. Genet.* **18,** 374–376.
Turleau, C., Czernichow, R., Royer, P., and de Grouchy, J. (1979). *Ann. Génét.* **22,** 205–209.
Turner, G. (1981). *Int. Congr. Hum. Genet., 6th, Israel.*
Turner, G., and Opitz, J. M. (1980). *Am. J. Med. Genet.* **7,** 407–415.
Turner, G., and Turner, B. (1974). *J. Med. Genet.* **11,** 109–113.
Turner, G., Eastman, C., Casey, J., McLeay, A., Procopis, P., and Turner B. (1975). *J. Med. Genet.* **12,** 367–371.
Turner, G., Gill, R., and Daniel, A. (1978). *N. Engl. J. Med.* **299,** 1472.
Turner, G., Daniel, A., and Frost, M. (1980a). *J. Pediatr.* **96,** 837–841.

Turner, G., Brookwell, R., Daniel, A., Selikowitz, M., and Zilibowitz, M. (1980b). *N. Engl. J. Med.* **303,** 662–664.

Venter, P. A., Gericke, G. S., Dawson, B., and Op't Hof, J. (1981). *S. Afr. Med. J.* **60,** 807–811.

Webb, G. C., Rogers, J. G., Pitt, D. B., Halliday, J., and Theobald, T. (1981). *Lancet* **2,** 1231–1232.

Webb, G. C., Halliday, J. L., Pitt, D. B., Judge, C. G., and Leversha, M. (1982). *J. Med. Genet.* **19,** 44–48.

Webb, T., Butler, D., Insley, J., Weaver, J. B., Green, S., and Rodeck, C. (1981). *Lancet* **2,** 1423.

Wilmot, P. L., Shapiro, L. R., and Duncan, P. A. (1980). *Am. J. Hum. Genet.* **32,** 94A.

Zachmann, M., Prader, A., Kind, H. P., Häfliger, H., and Budliger, H. (1974). *Helv. Paediatr. Acta* **29,** 61–72.

Reprint Series
23 July 1982, Volume 217, pp. 373–374

SCIENCE

# Genetic Length of a Human Chromosomal Segment Measured by Recombination Between Two Fragile Sites

Grant R. Sutherland, Elizabeth Baker, and John C. Mulley

# Genetic Length of a Human Chromosomal Segment Measured by Recombination Between Two Fragile Sites

Abstract. *Two families were studied in which the same homolog of chromosome pair 10 expressed both the fragile sites on the long (q) arm at 10q23 and 10q25. Recombination between the fragile sites was observed in 3 of the 27 offspring in whom it could occur. The genetic length of chromosome between the fragile sites was 11 female centimorgans within a 95 percent probability interval of 4 to 28 centimorgans. This estimate of genetic length is comparable to those obtained with other methods.*

Fragile sites are morphological features of human chromosomes that can be recognized cytologically when lymphocytes are cultured under appropriate conditions (*1*). Since any fragile site is always expressed at the same locus in individuals from the same kindred, kindreds in which fragile sites are segregating can be used for gene mapping (*2*). Two fragile sites have been described on chromosome 10. One, in band 10q23, is expressed only under conditions of folic acid and thymidine deprivation (*1*), and the other, in band 10q25, is expressed only if bromodeoxyuridine or bromodeoxycytidine is present in the tissue culture medium for some hours before harvest (*3*). The discovery of two families in which both of these fragile sites were present on one chromosome (Fig. 1) provided an opportunity to measure the genetic length of the segment between the fragile sites by segregation analysis.

The two families are not known to be related to each other, although both are of British origin. The families were ascertained through mildly retarded girls, but other members of these families who have the fragile sites on chromosome 10 were normal. Examples of number 10 chromosomes expressing both the fragile sites are shown in Fig. 2. On G-banded preparations, one fragile site is at the distal end of 10q23 (*1*), probably at 10q23.32 or 10q23.33, and the other in the middle of 10q25 (*3*), probably at 10q25.2 (*4*).

The frequency of expression of the fragile sites is shown in Table 1. One member of family Ho, III-12, has been omitted from consideration because in the small number of cells available for examination, it was not possible to be sure that fra(10)(q25) was absent. Fragile sites were present on the same homolog of chromosome 10 in several individuals, including I-6 from family Ho and I-1

from family Ch. In those individuals in whom the fragile sites were not found, at least 50 metaphases were usually examined for each fragile site.

Three recombinants were detected in family Ho, but none were seen in family Ch. Recombination occurred in 3 of the 27 offspring of females who expressed the fragile sites on the same homolog of chromosome 10. The recombination fraction is 11 percent (3/27), and since double crossovers are unlikely for such a small segment, the estimate of the genetic length of chromosome between the fragile sites in females is 11 centimorgans (cM) with a 95 percent probability interval of 4 to 28 cM (*5*). In general, the female map length is approximately twice that of males (*6*). On this basis the length of segment between the fragile sites in males would be about 6 cM with 95 percent probability interval of 2 to 21 cM (*5*), provided the general relationship between male and female recombination rates is applicable to this chromosomal segment.

An estimate of the genetic length of the segment of chromosome between the fragile sites from meiotic studies in males is 3.8 cM, with a possible maximum length of 13.5 cM, according to Hultén's model (*7*). Direct measurement of the segment between the bands expressing the fragile sites from prometaphase chromosomes (*4*) shows that the segment is about 18 percent of mitotic length of the long arms of chromosome 10 and that the approximate position of the segment is the proximal half of the distal third of the long arm of the mitotic chromosome. The meiotic map of the human genome put forward by Cook *et al.* (*6*) indicates that the distal third (in mitotic length) of the long arm of chromosome 10 is approximately 36 cM for males. The segment between the fragile sites corresponds to a male length of approximately 19 cM with Cook's map.

Table 1. Frequency of expression of the fragile sites in family members.

| Family member | 10q23 | 10q25 |
|---|---|---|
| | *Family Ch* | |
| I-1 | 25/50 | 15/50 |
| II-1 | 14/50 | 13/67 |
| II-3 | 14/50 | 5/40 |
| | *Family Ho* | |
| I-6 | 4/20 | 4/20 |
| II-4 | 11/58 | 14/45 |
| II-8 | 0/50 | 7/73 |
| II-11 | 6/20 | 4/20 |
| II-13 | 9/40 | 3/20 |
| II-16 | 6/50 | 5/40 |
| II-18 | 12/20 | 5/50 |
| III-2 | 16/50 | 19/25 |
| III-4 | 27/90 | 8/44 |
| III-7 | 7/20 | 6/20 |
| III-9 | 3/38 | 7/20 |
| III-11 | 9/30 | 0/50 |
| III-12 | 0/50 | 0/8 |
| IV-1 | 7/25 | 0/65 |

There is good agreement between the estimates of the genetic length of the chromosomal segment between the fragile sites from segregation analysis and meiotic observation. Furthermore, the 95 percent probability interval of the estimate from segregation analysis encompasses the estimate from Cook's meiotic map, but Hultén's maximum estimate is shorter than that obtained from Cook's map. Cook *et al.* (*6*) have indicated they believe that Hultén's model underestimates genetic length.

The possibility that fragile sites could affect crossing-over should be considered. Crossing-over occurs in chromosomes in which the DNA is compacted, although not to the degree seen in C-metaphases. Fragile sites are defects in chromosome compaction for mitosis. If this defect in compaction also holds for meiosis, the fragile sites could in some way inhibit or possibly even facilitate recombination. This could lead to biased estimates of genetic length when fragile sites are used as genetic markers for linkage studies. The level of recombination observed between the fragile site at 16q22 and the haptoglobin locus *Hp* (*2*) may help to clarify this issue when *Hp* is precisely localized by methods other than linkage with the fragile site.

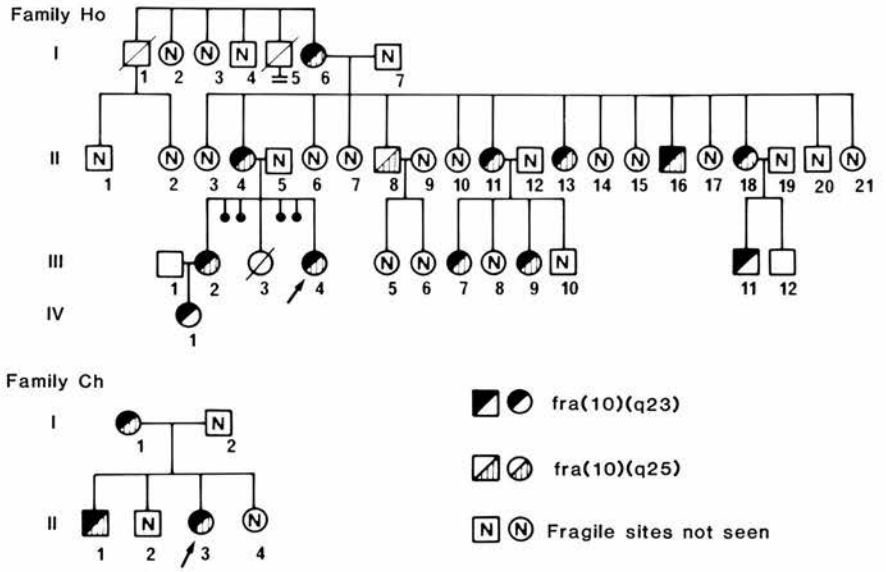



Fig. 1. Pedigrees of the two families.

Fig. 2. Chromosome 10 from four metaphases showing both fragile sites expressed. The fragile site at 10q23 is indicated by the broad arrow and the one at 10q25 by the small arrow. To induce simultaneous expression of both fragile sites, lymphocytes were cultured in MEM-FA (*1*), and bromodeoxycytidine (75 mg/liter) was added 6 hours before harvest.


GRANT R. SUTHERLAND
ELIZABETH BAKER
JOHN C. MULLEY

*Cytogenetics Unit,*
*Department of Histopathology,*
*Adelaide Children's Hospital,*
*North Adelaide, S.A. 5006, Australia*


### References and Notes


1. G. R. Sutherland, *Science* **197**, 265 (1977); *Am. J. Hum. Genet.* **31**, 125 and 136 (1979).
2. R. E. Magenis, F. Hecht, E. W. Lovrien, *Science* **170**, 85 (1970); F. Hecht and B. Kaiser-McCaw, *Am. J. Hum. Genet.* **31**, 223 (1979).
3. G. R. Sutherland, E. Baker, R. Seshadri, *Am. J. Hum. Genet.* **32**, 542 (1980).
4. An International System for Human Cytogenetic Nomenclature, *Cytogenet. Cell Genet.* **31**, 1 (1981).
5. A. H. E. Emery, *Methodology in Medical Genetics. An Introduction to Statistical Methods* (Churchill Livingstone, Edinburgh, 1976), p. 70.
6. P. J. L. Cook *et al.*, *Ann. Hum. Genet.* **44**, 61 (1980).
7. M. Hultén, *Hereditas* **76**, 55 (1974); personal communication (1981).


8. We thank B. Menzies and L. Maland for technical assistance. Supported by grants from the National Health and Medical Research Council of Australia and the Channel 10 Children's Medical Research Foundation.

15 March 1982

American Journal of Medical Genetics 11:489–495 (1982)

# The Fragile X Chromosome: Current Methods

**Frederick Hecht, Peter B. Jacky, and Grant R. Sutherland**

*The Genetics Center of Southwest Biomedical Research Institute, Tempe, Arizona (F.H.); MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh, Scotland (P.B.J.); and Cytogenetics Unit, The Adelaide Children's Hospital, N. Adelaide, South Australia (P.B.J., G.R.S.)*

At the International Congress of Human Genetics (Jerusalem, September, 1981) a Workshop was held on the fragile X chromosome; it was entitled "The Fragile X Chromosome: Current Methods." It was to focus on laboratory methods required to detect or to enhance the detection of the fragile X.

The fragile site on the X chromosome is one of 13 proven fragile sites known in humans. (A diagram of these sites is provided.) The fragile site on the X is sensitive to the concentration of folic acid in the medium in which cells are cultured. Cytologically, gaps and breaks are increased at the X fragile site, which is located at band Xq27-28. To enhance expression in lymphocytes, prolongation of culture time to 96 hours, elevation of the pH, diminution of the colcemid effect, and air drying of slides are helpful. The need for methionine in low-folate media can be overridden by the addition of fluorodeoxyuridine (FUdR). Detection of the fragile X in males requires meticulous attention to methods of lymphocyte culture and metaphase preparation and then the examination of a sufficient number of mitoses, eg, 50–100 metaphases per individual. Detection of the fragile X in female carriers is often more difficult. Uniform detection of all obligate female carriers has not been achieved. Difficulty may correlate with increasing age or intelligence of females.

Key methodologic advances with the fragile X include the addition of methotrexate, trifluorothymidine or, especially of FUdR to the culture medium. FUdR, for example, is helpful in demonstrating the fragile X in lymphoblastoid cell lines and fibroblasts. Both of these cell types now represent an opportunity to study the biochemistry of the fragile X.

The success of the FUdR technique with skin fibroblasts heralds the feasibility of demonstrating the fragile X chromosome in cultured amniocytes. Since the Workshop, it has been reported that with FUdR the fragile X could in fact be detected in 46,XY amniotic fluid cells.

Received for publication November 28, 1981; revision received December 19, 1981.

Address reprint requests to F. Hecht, MD, The Genetics Center of Southwest Biomedical Research Institute, 123 E. University Drive, Tempe, AZ 85281.

Key words: X chromosome, fragile sites on chromosomes, fragile X chromosome, mental retardation, lymphocytes, lymphoblastoid cell lines, fibroblasts, amniocytes, folic acid, thymidine, fluorodeoxyuridine (FUdR), prenatal genetic diagnosis

## PREAMBLE

The program for an International Congress on a subject such as Human Genetics has, by necessity, to be developed long in advance of the meeting. This was the case with the most recent International Congress of Human Genetics. Between the time the Congress was conceived and the time it was held, certain topics emerged as of unusual interest. Among these topics was the "fragile X chromosome." No session wholly devoted to the fragile X had been contemplated at first, but due to a rising tide of interest, Peter Jacky initiated a workshop on the fragile X with the cooperation of the organizing committee and program commitrtee. The Workshop was chaired by Frederick Hecht. This summary was prepared by the authors with the generous help of many of the participants in the Workshop.

This synopsis is intended to be in the general style utilized by *Nature*, so formal references to research in print or in press are not given.

## INTRODUCTION

The "fragile X chromosome" is currently an active interest for many investigators in human genetics.

Clinical interest stems from the intimate association between the fragile X and X-linked mental retardation; hemizygous males are usually (perhaps always) mentally retarded. Further clinical interest derives from the association between the fragile X and the tendency to intellectual dullness in female carriers.

Laboratory interest in the fragile X jumped in 1977 when Sutherland discovered that the fragile X phenomenon was "folic acid sensitive," ie, invisible in "complete" media but can be seen in all affected males and a proportion of carrier females whose lymphocytes have been cultured in "deprived" media (such as medium 199) deficient in folic acid and thymidine.

Many other crucial investigations such as those by Lubs (1969) have contributed to our current knowledge of the fragile X. The evolution of past work on X-linked mental retardation and the fragile X is outlined in the December, 1980 issue of the *American Journal of Medical Genetics*, which contains a set of articles on it and allied topics.

## MAP OF FRAGILE SITES

Sutherland has noted, and the Workshop agreed, that the fragile X is one of 13 proven "fragile" sites in the human genome. "Fragile" meaning that chromosomes tend to break preferable at these sites.

The X fragile site is now firmly established as being near the end of Xq. There is still some uncertainty whether the site is at band Xq27 or band Xq28 or at the interface of these 2 bands.

The known fragile sites now include locations on chromosomes 2, 6, 7, 9–12, 16, 20, and the X (Fig. 1). There are two fragile sites on chromosomes 9, 10, and 16; and one on the rest. In addition, there are "look-alike" sites on other chromosomes such as

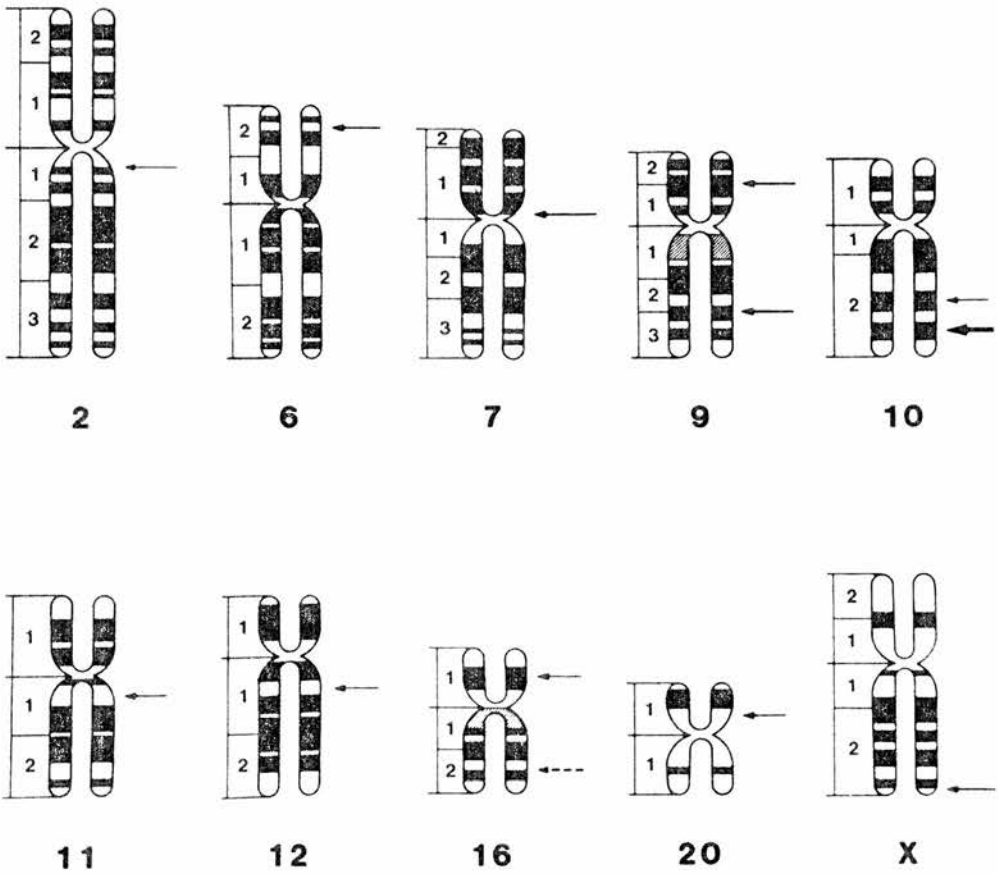



Fig. 1. Current map of heritable human fragile sites. The thin arrows point to folic acid sensitive sites including that on Xq. The thick arrow points to the BrdU inducible site on chromosome 10 and the dashed arrow indicates the location of the constitutive fragile site on chromosome 16 linked by genetic family studies to the $\alpha$-haptoglobin locus.

on chromosome 6. There was agreement that the "look-alike" sites such as that on 6q are *not* true fragile sites, since they do not appear to be *heritable*.

Only two of the fragile sites that have been fully studied are *not* sensitive to the concentration of folic acid in the culture medium. Sutherland suggested that in the long run virtually all fragile sites may prove to be folic acid sensitive. Alternatively Hecht suggested that the current prevalence of folic acid sensitive sites may reflect the contemporary utilization of folic acid deficient media in the search for fragile sites. Clearly, time will tell.

## CYTOLOGIC BEHAVIOR OF FRAGILE SITES

The cytologic behavior of fragile sites, including that on the X, is now established. The chromosome at these sites tends to elongate and display a negatively heterochromatic gap. Sutherland has found that unbanded chromosome spreads are best for

analysis of fragile sites; however, Karen Buckton (Edinburgh, United Kingdom) and others employ banded preparations for reading fragile sites. Although gaps are most common, single chromatid and chromosome breaks at fragile sites are clearly increased in frequency. The portion of the chromosome distal to the fragile site can sometimes be seen in multiple copies within the cell. Although it was originally proposed that this was due to selective endoreduplication, Ferguson-Smith (Glasgow, United Kingdom) ascribed it to nondisjunction and he and Noël subsequently proved this was so, using BrdU labelling of chromatids.

## GENETICS OF FRAGILE SITES

Fragile sites are heritable cytologic markers. Aside from the fragile X, all proven fragile sites are on autosomes and are inherited in a codominant pattern.

Fragile sites provide an exciting addition to the formidable armory of available markers for linkage and mapping purposes. The demonstration by Magenis, Lovrien and Hecht in 1970 that a fragile site on chromosome 16 was linked to the alpha-haptoglobin gene locus helped chart the "geography" of this chromosome. Sutherland's group is studying a family segregating for two fragile sites on chromosome 10; both sites are on 10q (Fig. 1) and cytologically are not far from one another; a rough recombination frequency of 16 centimorgans has been found.

## FRAGILE X IN LYMPHOCYTES

To optimize the expression of the fragile X in lymphocytes, Patricia Howard-Peebles (Birmingham, Alabama and Dallas, Texas) stressed the need to prolong time in culture to 4 days (96 hours), to raise the pH of the culture medium to pH 7.2 or above (particularly toward the time of final harvest), to decrease colcemid concentration (or time of exposure to colcemid) and to air-dry (not flame-dry) slides. Howard-Peebles noted that numerous factors do *not* enhance fragile X frequency. For example, the addition of 5-azacytidine, distamycin A, betaine, or ethionine does not facilitate fragile X expression.

Methionine has been found by Howard-Peebles to be required for fragile X expression. Thomas Glover (Tempe, Arizona) and Howard-Peebles have shown that this effect of methionine is a secondary one that can be overridden by the addition of FUdR, a potent inhibitor of thymidylate synthetase. With FUdR, it no longer matters whether methionine is present or absent. The simplest explanation of these data is that inhibition of DNA synthesis is involved in the expression of the fragile X. It would appear from the literature that males with the fragile X are always easy to detect in the laboratory. This is incorrect. Their lymphocytes must be cultured with meticulous attention to the fastidious requirements of the fragile X. Metaphases must be prepared appropriately and, equally important, if the fragile X is not readily seen, the sample of good metaphases must be sufficiently large (eg, 50–100) to ensure reliable results. For example, Barbara Kaiser-McCaw and Glover (Tempe, Arizona) have on occasion needed to examine 50–100 metaphases from a suspect male to score him as having the fragile X.

## THE CASE OF THE "VANISHING" CARRIER

By pedigree analysis, it is clear that some females are obligate carriers of the fragile X. They may have an affected brother and an affected son, so they must carry the fragile X. Yet it appears that every laboratory with experience has had the frustration of utterly failing to detect the fragile X in one or more obligate carriers.

Why is fragile X expression in carriers so inconstant? The answer generally given is that it is a matter of age; ie, in older carriers the fragile X vanishes. However, it should be noted that no one has yet demonstrated this phenomenon by serial studies over time in a carrier. Thus, at the moment this is a supposition.

Could it be that the fragile X does not vanish with age? Might another factor be responsible for the situation? This question was raised and discussed by Patricia Jacobs (Honolulu, Hawaii). Jacobs proposed that intelligence might be the key factor or, at least, another factor.

The idea is that some females are more intelligent because they are favorable Lyonization mosaics. The smaller the "mutant" cell line; the smarter the woman is, and the greater is the chance that she will be ascertained *late* for reproductive genetic counseling. By contrast, other females with the fragile X may be dull because they are unfavorable Lyonization mosaics. The larger the "mutant" cell line, the less intelligent is the female. And the duller she is, the greater is the chance that she will be ascertained and studied *early*.

The problem—age per se or intelligence—is clearly amenable to study and should soon, we hope, be solved.

## OTHER MYSTERIES SURROUNDING THE FRAGILE X

Lest we convey the mistaken impression that the fragile X phenomenon is well understood, questions about it abounded in the Workshop. For example, can the fragile X be seen in at most 50% of cells from an individual, as Patricia Jacobs (Honolulu, Hawaii) suggested? Or, is there a significant familial correlation in the proportions of cells showing the fragile X, as was suggested by D. Soudek (Ontario, Canada)?

Does the fragile X occur in normal males? This is, of course, a critical question. A brief letter reporting two "normal" males allegedly with the fragile X appeared in the *Lancet*. The general reaction of the Workshop was one of considerable doubt. This doubt was deepened by Sutherland's examination of a thousand normal neonates (both sexes) for folic acid sensitive sites and his failure to find the fragile X in any normal newborn. More work is needed on this point before we accept the idea that the fragile X can be seen in normal males.

Does the fragile X occur more commonly in association with other chromosome abnormalities than would be expected? There were at the Workshop numerous anecdotal reports of its association with XXY, XYY, trisomy 8, etc. Together with Barbara Kaiser-McCaw (Tempe, Arizona), Hecht observed the fragile X in an intellectually normal female with 46,X,del(Xq) Ullrich-Turner syndrome; the apparent fragile site was at or near the end of the abbreviated Xq.

Conversely, does the fragile X predispose to other chromosome abnormalities? For example, does it predispose to sporadic translocations, to trisomy, etc.?

## NEWER METHODS FOR ENHANCING EXPRESSION

J.-F. Mattei (Marseille, France) observed that the addition of methotrexate (10 mg/l) for the last 24 hours is useful with lymphocytes cultured for a total of 96 hours in TC 199. The same two factors – prolongation of culture time in TC 199 and addition of methotrexate – gave satisfactory results in fibroblasts.

The observation that folic acid and thymidine inhibit and methotrexate induces the fragile site suggests that the biochemical reaction dUMP → dTMP catalyzed by thymidylate synthetase might be involved in this process. Recently further evidence for this was presented independently by Glover and by Niels Tommerup (Glostrup, Denmark): FUdR, a specific inhibitor of thymidylate synthetase, was shown to be an effective inducer of the X fragile site in both lymphocytes and fibroblasts. With K. Brøndum-Nielsen and M. Mikkelsen, Tommerup also found FUdR able to induce the folic acid dependent heritable fragile sites at 2q13, 10q24, 11q11, and 12q13. Preliminary evidence suggests one possibility: that the induction of these fragile sites is due to specific inhibition of dTMP formation, and not due to inhibition of DNA synthesis per se. Trifluorothymidine, another agent inhibiting thymidylate synthetase, was found to induce the X fragile site, but hydroxyurea and deoxyadenosine, which inhibit DNA synthesis by other means, did not. By addition during the last 24 hours of the culturing period of FUdR to the culture medium (TC 199 + 20% pooled human serum + 4% bovine embryo utrafiltrate extract), the fragile X was induced in fibroblasts from seven males and one female, all mentally retarded and showing the fragile X in their lymphocytes. Likewise, the fragile X was induced in fibroblasts from two obligate carriers and one female at risk (50%) in whose lymphocytes fra(X) was not expressed despite repeated attempts. (The optimal concentration of FUdR is that which ensures both a reasonable number of mitoses and a high frequency of fragile sites.) Two control cultures treated with FUdR did not express the X fragile site. These data suggest to Tommerup and coworkers that fibroblasts might be a more reliable cell-system for carrier-detection than lymphocytes.

Arthur Daniel (Randwick, Australia and Helena, Montana) reported work done with R. Brookwell, G. Turner, and J. Fishburn on the effect of FUdR on the expression of fra(X) in lymphocytes and/or fibroblasts in 15 affected males and 3 females from 9 families. Six different culture media were used: Ham's F10/5% serum/pH 7.3 (37°C), Medium 199/5% serum/pH 7.6 (37°C), Folate-free 199/5% serum pH 7.6 (37°C), and these three media with FUdR (0.0125 $\mu$M). In lymphocytes there was no significant difference in the percentage of expressing cells between any of the FUdR containing media. The maximum percentage of expressing cells seen in lymphocyte cultures with FUdR was 62%. The average enhancement with FUdR in the 199 and folate-free 199 media was 26%. This relative enhancement with FUdR was very much higher in a few blood specimens delayed in transit. FUdR may prevent some of the false-negative results obtained from mailed specimens. FUdR did not induce the marker in cultures from three obligate carriers with previously negative results. In skin fibroblasts from affected males, cells were grown in the six specific media for the final 48 hours. Two of the six culture media yielded reproducibly positive results. These were 199-FUdR and folate-free 199-FUdR with mean percentages of expressing cells of 12.4 ± 6.8% and 11.3 ± 6.1%, respectively. Ham's FUdR (which contains thymidine) did not permit expression of the marker in fibroblasts but was as effective as the thymidine-free medium for lymphocytes. Daniel and coworkers concluded that FUdR shows promise as an agent to enhance the detection of the marker in cultured fibroblasts and lymphocytes.

## LYMPHOBLASTOID CELL LINES WITH THE FRAGILE X

Lymphocytes were the first type of cells shown to express the fragile X. Next came skin fibroblasts. The newest cell system was announced by Richard Erbe (Boston, Massachusetts). Erbe and Jacobs have transformed fragile X carrying lymphocytes into permanent lymphoblastoid cell lines. Using FUdR, they were able to detect the fragile X in lymphoblasts. Lymphoblasts now represent an enticing cell type for study of the biochemistry of the fragile X.

## PRENATAL DIAGNOSIS OF THE FRAGILE X

It was noted by Tommerup, Daniels, and other participants in the Workshop that FUdR is so successful with fibroblast cultures that prenatal diagnosis of the fragile X with the Glover–Tommerup method should soon be feasible. Since the Workshop ended (and during the revision of this paper), it was announced in a press conference in Albany by Governor Carey of New York that this had been accomplished. Subsequently it was reported in the *Lancet* of December 6, 1981 by H. Wisniewski, W.T. Brown, and A. Jenkins (Staten Island, New York) that, using FUdR, they had indeed found the fragile X in amniocytes cultured from a male conceptus.

Clearly this feat will need to be repeated before there is full agreement that the fragile X can be readily and definitely demonstrated in male amniocytes. (The test metaphases should ideally be read blindly by two or three microscopists, looking also at suitable nonfragile-X male control amniocytes.) Further, the consistent prenatal demonstration of the fragile X in carrier female amniocytes may be horrendously difficult, even with FUdR. Time again will tell.

## TREATMENT

This is an area of controversy. Jérôme Lejeune (Paris, France) and coworkers have administered folic acid to nine males with the fragile X. In six of nine patients "mental improvement" was noted with "alleviation of the psychotic-like component of behavior." However, this will need to be confirmed.

More to the point, can the mental retardation associated with the fragile X be prevented? If so, how? With folic acid? And when does the treatment need to be instituted? Before birth? At birth? Before a month of age?

## MECHANISMS AND THE FUTURE

The mechanisms of the fragile X and its associated mental retardation are still obscure. Much is now known about the fragile X. And much, it is clear, remains to be learned in the years ahead.

## ACKNOWLEDGMENTS

The Workshop could not have been held were it not for the assistance of our colleagues in Israel who arranged the Congress. Contributors to the Workshop and to key advances on the fragile X have been cited in some instances above by name; others whom we have failed to name have unquestionably made crucial contributions. The valuable parts of this synopsis were made possible by our colleagues. The errors are our doing.

*Am J Hum Genet* 35:000–000, 1983

# Heritable Fragile Sites on Human Chromosomes. X. New Folate-Sensitive Fragile Sites: 6p23, 9p21, 9q32, and 11q23

GRANT R. SUTHERLAND,[1] PETER B. JACKY,[1] ELIZABETH BAKER,[1] AND ANNE MANUEL[2]

SUMMARY

Four new folate-sensitive fragile sites are documented at 6p23, 9p21, 9q32, and 11q23. These have all been shown to be heritable except for the one at 9p21, which has been seen only in a single individual. As with the other autosomal fragile sites, these appear to be innocuous in heterozygotes.

## INTRODUCTION

Known heritable fragile sites on human chromosomes have been summarized by Sutherland and Hinton [1]. At that time, seven folate-sensitive fragile sites had been documented, although the one at 11q13 had not been shown to be heritable. Pavey and Webb [2] have since documented a folate-sensitive fragile site at 7p11 in several members of a family, and Kochen and Kardon [3] described an apparent folate-sensitive fragile site at 8q22 in siblings, but not in their parents. Previously undocumented fragile sites at 6p23, 9p21, 9q32, and 11q23 are described.

## MATERIALS AND METHODS

Lymphocyte cultures were established and harvested using MEM-FA and Ham's F10, and folic acid and thymidine sensitivities of the fragile sites were tested as described [4]. Fluorodeoxyuridine (FudR)-induced expression of fragile sites was after Glover [5].

Received July 12, 1982; revised September 27, 1982.

This work was supported by grants from the Adelaide Children's Hospital Research Trust and the Channel 10 Children's Medical Research Foundation.

[1] Cytogenetics Unit, Adelaide Children's Hospital, North Adelaide, S.A. 5006, Australia.

[2] Foundation 41, The Women's Hospital (Crown St.), Sydney, N.S.W. 2000, Australia.

## RESULTS

The fragile site at 6p23 was found in a 2-year-old boy with developmental delay and dysmorphic features. Family studies (fig. 1) revealed the same fragile site in others, none of whom were mentally or physically abnormal. The appearance of the fragile site is shown in figure 2; on G-banding, it appears to be near the distal end of p23. The frequency of expression of this fragile site under various conditions of tissue culture is shown in table 1.

TABLE 1

FREQUENCY OF EXPRESSION OF THE FRAGILE SITES UNDER DIFFERENT CONDITIONS OF CULTURE

| Fragile site and subject | F10 | MEM-FA |
|---|---|---|
| 6p23, I.2 | · · · | 4/50 |
| II.3 | 0/50 | 5/75 |
| II.12 | 0/20 | 7/50 |
| III.3 | · · · | 8/100 |
| III.5 | 0/50 | 44/100 |
| 9p21 | 0/50 | 22/50 |
| 9q32, S.B. | 0/50 | 21/100 |
| 9q32, II.1 | 0/50 | 10/50 |
| III.1 | 0/50 | 17/50 |
| 11q23, I.1 | · · · | 1/75 |
| I.2 | · · · | 0/100 |
| II.2 | 0/50 | 15/50 |
| II.6 | 0/50 | 15/50 |

The fragile site at 9q32 has been ascertained twice. The first time was in an 11-year-old boy (S. B.) with gynecomastia. This fragile site was not detected in either of his parents or in four of his siblings who were studied. The second ascertainment was in an 8-year-old boy with mild mental retardation and minor dysmorphic features. Studies on the rather small family (fig. 1*b*) showed his normal mother to also have the fragile site. The appearance of the fragile site is shown in figure 2; on G-banding, it appears to be in the proximal part of q32. The frequency of expression of this fragile site is shown in table 1.

The fragile site at 9p21 was found in the mother of a neonate with Down syndrome. The infant had karyotype 46,XX −21, +t(21q21q). The mother's parents were deceased, and studies on her only sibling, a paternal aunt, and a maternal uncle have not shown the fragile site [6]. The appearance of the fragile site is shown in figure 2 and its expression under various conditions of tissue culture in table 1. On G-banding, the fragile site was shown to be in the proximal part of p21.

The fragile site at 11q23 was found in the father of a neonate studied because the child was homozygous for the bromodeoxyuridine (BrdU)-requiring fragile site at 10q25 [7]. The father thus has both fragile sites. Results of family studies are shown in figure 1*c*, and the appearance of the fragile site in figure 2. G-banding studies showed the fragile site to be near the distal end of q23.3. The frequency of expression of this fragile site is shown in table 1.

Complete dose-response curves for frequency of expression of the fragile sites

to concentrations of thymidine and folic acid were obtained for the fragile sites at 6p23 and 9p21 (fig. 3), confirming their status as folate-sensitive fragile sites. For 9q32, complete dose-response curves were not obtained, but in III.1, expression of the fragile site was fully suppressed by either 0.5 mg/l of folic acid or 5 mg/l of thymidine added to MEM-FA. Dose-response data were not obtained for fra(11)(q23); however, although this fragile site was not expressed in Ham's F10 or RPMI, it was expressed in TC199 and MEM-FA and elicited with FUdR in Ham's F10 and RPMI. Hence, although fra(11)(q23) has not been formally proven to be folate sensitive, there is overwhelming evidence that it is.

## DISCUSSION

Documentation of the four fragile sites in this report brings to 15 the number of known fragile sites (fig. 4). All of these fragile sites have now been shown to be heritable except for the one at 9p21, which, in view of its behavior as a typical fragile site, will probably eventually be found to be heritable. Two families have been studied in which the fragile site at 11q13, which had not previously been shown to be heritable [8, 9], is segregating.

All the known fragile sites, with the exception of those at 7p11, 8q22, and 9p21, have been ascertained at least once in the Adelaide laboratory. Pavey and Webb [2] ascertained the 7p11 fragile site twice and showed it to be both heritable and folate sensitive. Kochen and Kardon [3] demonstrated the 8q22 site in siblings and in one in 50 of their father's cells examined and showed that fragility of 8q22 was enhanced in medium 199 (N. Kardon, personal communication, 1981). While it is probable that this fragile site is folate sensitive, further characterization is required.

The problem in failing to demonstrate fra(9)(q32) in either parent of subject S. B. is one that has been occasionally encountered with other autosomal folate-sensitive fragile sites. Similarly, there remains some uncertainty as to which member of generation I carries fra(11)(q23). Other kindreds have been studied in which obligate carriers of autosomal folate-sensitive fragile sites have not expressed the fragile site using either methods of folate deprivation or of FUdR induction [5, 10]. One possible explanation for this finding is that the propositus represents a new mutation. However, it is much more likely that current methods are inadequate for fragile site detection in some individuals. A similar situation is the well-documented failure to detect fra(X)(q27) in some obligate carrier females [11].

The fact that all the folate-sensitive fragile sites appear to behave similarly in response to variations in culture medium composition suggests that they all share a common underlying mechanism of expression. A relative deficiency of thymidylate late in the S period of the cell cycle appears to be the essential factor imposed by all the methods used to elicit fragile site expression [11], although it remains to be determined why this is important.

The increasing number of folate-sensitive fragile sites that have been reported from very few sources suggests that as more laboratories begin to routinely employ

culture conditions suitable for fragile site detection many more will likely be found. It is important that these be documented with full family studies and, in particular, that the search for individuals homozygous for folate-sensitive fragile sites continues so that more information may be gained about the clinical significance of the autosomal folate-sensitive fragile sites in both heterozygotes and homozygotes. Because of the rarity of the individual fragile sites (and there are no data on their incidence other than that even the most common one is probably present in less than one in 1,000 individuals [11].), homozygotes will be exceedingly rare, probably less than one in $10^6$ live births if homozygosity is compatible with live birth. If homozygosity is lethal, then study of couples with repeated abortion may yield some cases in which both are heterozygous for the same fragile site. If homozygosity is deleterious but not lethal, then the abnormal phenotype should be recognized to follow apparent autosomal recessive inheritance. Individuals with rare autosomal recessive disorders should be studied for fragile sites.


## ACKNOWLEDGMENTS

We thank Sharon Bain, Lily Gowans, Louise Maland, Jim Molesworth, and Cathy Orell for technical assistance with the cytogenetics, and Wendy James for help with the family studies.


## REFERENCES


1. SUTHERLAND GR, HINTON L: Heritable fragile sites on human chromosomes. VI. Characterization of the fragile site at 12q13. *Hum Genet* 57:217–219, 1981
2. PAVEY SM, WEBB GC: A fragile site at 7p11. Presented to the Human Genetics Society of Australia, Adelaide, May 1982
3. KOCHEN K, KARDON N: Burkitt lymphoma (BL): chromosome findings in siblings. Presented to the American Society for Pediatric Research, May 1981
4. SUTHERLAND GR: Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. *Am J Hum Genet* 31:125–135, 1979
5. GLOVER TW: FUdR induction of the X chromosome fragile site: evidence for the mechanism of folic acid and thymidine inhibition. *Am J Hum Genet* 33:234–242, 1981
6. MANUEL A, SUTHERLAND GR, MOLESWORTH J: A new fragile site at 9q21. Presented to the Human Genetics Society of Australasia, Canberra, May 1981
7. SUTHERLAND GR: Heritable fragile sites on human chromosomes. VII. Children homozygous for the BrdU-requiring fra(10)(q25) are phenotypically normal. *Am J Hum Genet* 33:946–949, 1981
8. SUTHERLAND GR: Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects and cytogenetics. *Am J Hum Genet* 31:136–148, 1979
9. SUTHERLAND GR: Heritable fragile sites on human chromosomes. VIII. Preliminary population cytogenetic data on the folic-acid-sensitive fragile sites. *Am J Hum Genet* 34:452–458, 1982
10. TOMMERUP N, POULSEN H, BRONDUM-NIELSEN K: 5-fluoro-2′-deoxyuridine induction of the fragile site on Xq28 associated with X linked mental retardation. *J Med Genet* 18:374–376, 1981
11. SUTHERLAND GR: The fragile X chromosome. *Int Rev Cytol.* 81:107–43 1983

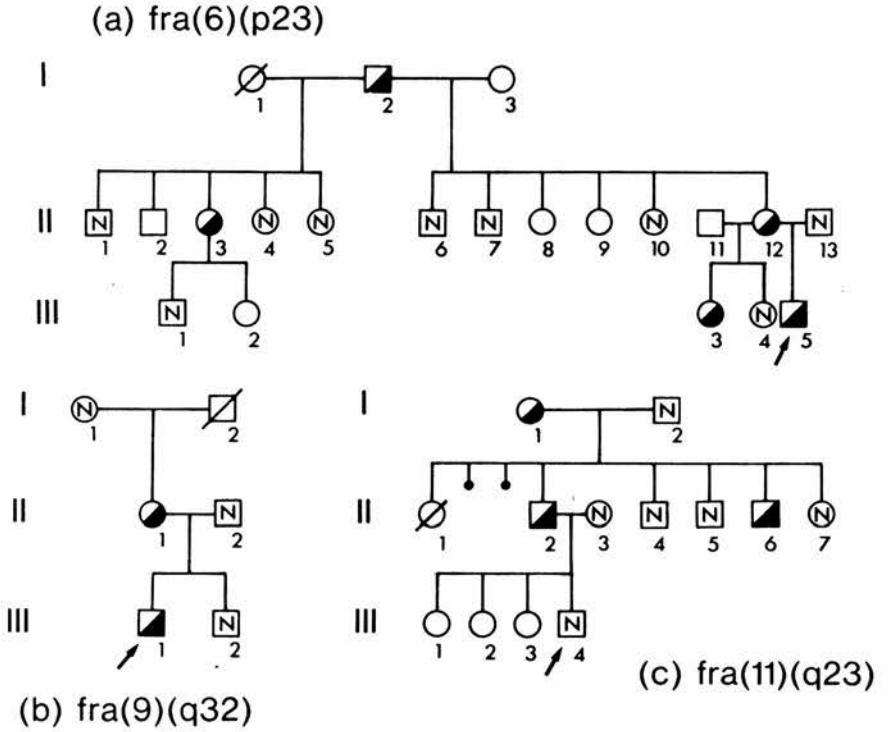



FIG. 1.—The families. *Half-shaded symbols* indicate carriers of the fragile sites; *N* indicates that the fragile site was not detected.

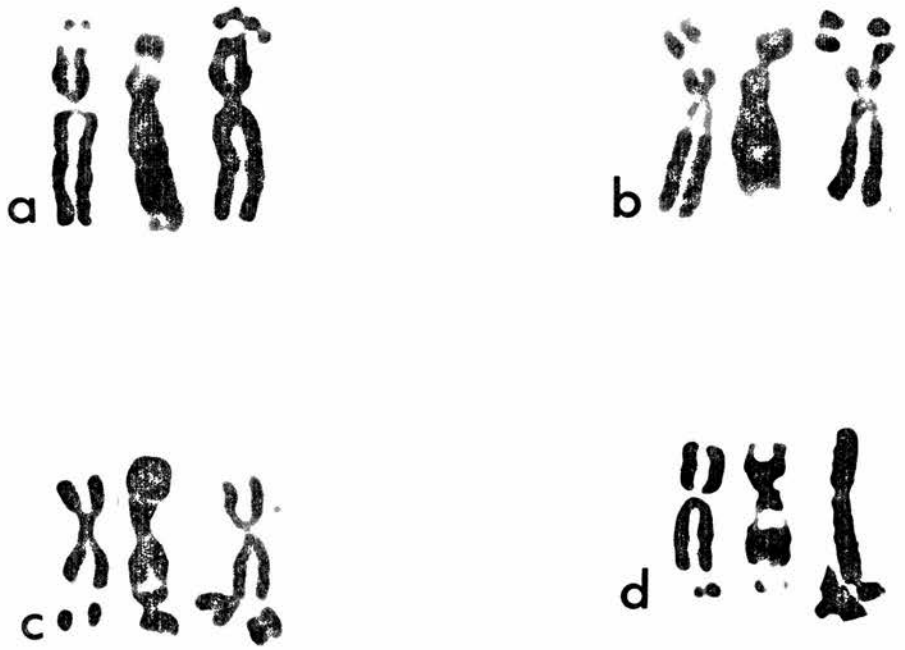



FIG. 2.—The new fragile sites: *a*, 6p23; *b*, 9p21; *c*, 9q32; *d*, 11q23. *Chromosome to the left of each group* is unbanded; *the middle one*, G-banded; and *the right one* shows duplication of chromosome material distal to the fragile site.

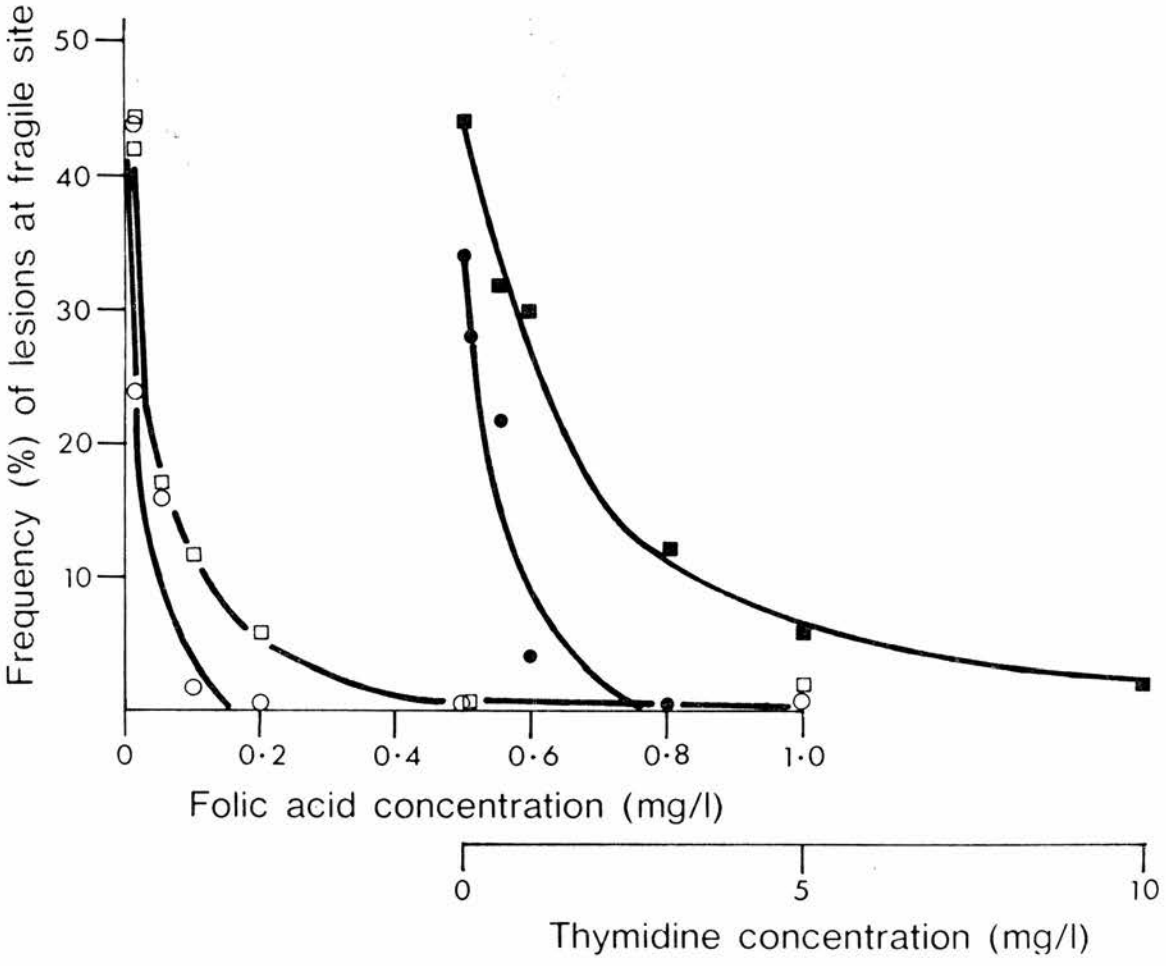



FIG. 3.—Effect of various concentrations of folic acid (*open symbols*) and thymidine (*solid symbols*) added to MEM-FA on frequency of expression of the fragile sites at 9p21 (*circles*) and 6p23 (*squares*).

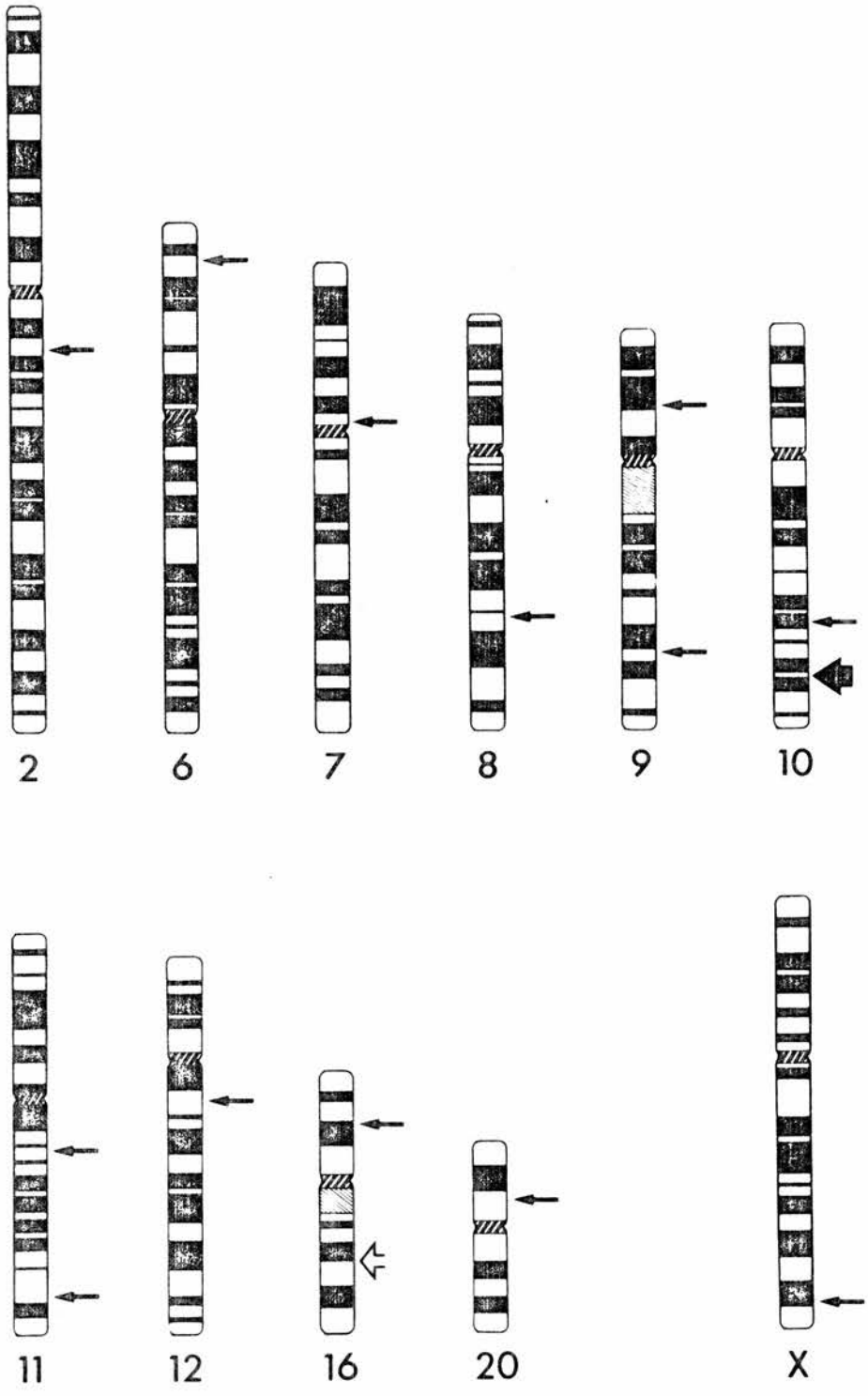



FIG. 4.—The known fragile sites. *Small arrows* indicate the folate-sensitive fragile sites at 2q13, 6p23, 7p11, 8q22, 9p21, 9q32, 10q23, 11q13, 11q23, 12q13, 16p12, 20p11, and Xq27; *open arrow* shows the fragile site at 16q22; *broad arrow* shows the BrdU-requiring fragile site at 10q25.

In 'Nutritional Factors in the Induction and Maintenance of Malignancy', C.E. Butterworth, Editor, Academic Press. (In Press).



# FRAGILE CHROMOSOMES

Grant R. Sutherland

Cytogenetics Unit

Adelaide Children's Hospital

North Adelaide S.A. 5006

Australia

## I. INTRODUCTION

Chromosome abnormalities are seen in most malignant cells, indeed chromosomal damage or breakage and subsequent rearrangement is probably one of the primary events in the induction of some malignancies (Radman et al., 1982). An understanding of the mechanisms involved in chromosome breakage could provide insights into cellular processes of malignant transformation. Chromosomal fragility occurs, apparently non-specifically and sporadically at a very low level, in all individuals. It is seen at higher levels and with some specificity in the chromosome instability syndromes and at much higher levels at a specific locus in carriers of heritable fragile sites. The chromosomal fragility which occurs at fragile sites is the only type for which a biochemical basis is at least partly known.

## II. THE HERITABLE FRAGILE SITES

Fragile sites are morphological features of chromosomes which were defined by Sutherland (1979a) as specific points which are liable to show the following features :

(1) A non-staining gap of variable width which usually involves both chromatids.

(2) Is always at exactly the same point on the chromosome in an individual or kindred.

(3) Is inherited in a Mendelian co-dominant fashion.

(4) Exhibits fragility by the production of acentric fragments, deleted chromosomes, triradial figures, etc.

The triradial (or multiradial) figure is the most spectacular cytogenetic manifestation of the fragile site (Fig.1) and also an essential manifestation for confirmation of a lesion as a

fragile site rather than some other phenomenon causing chromosome damage. Whilst the nature of a fragile site, in terms of chromosome structure, is unknown it presumably represents a segment of chromosome which does not undergo normal compaction for mitosis.

To date 15 fragile sites have been detected and these are shown in the partial ideogram (Fig.2) and partial karyotype (Fig.3). These fragile sites can be classified into three groups. The first group, the folate sensitive fragile sites, contains all except two of the known sites which each come into separate categories.

Group 1

The folate sensitive fragile sites are those at 2q13, 6p23, 7p11, 8q22, 9p21, 9q32, 10q23, 11q13, 11q23, 12q13, 16p12, 20p11 and Xq27. They are termed folate sensitive because removal of folic acid (and thymidine) from culture medium was the first factor found to be essential for their demonstration in lymphocyte culture.

Group 2

This group contains only the fragile site at 16q22. This fragile site is not dependent upon conditions of tissue culture (Sutherland, 1979a) but its expression is reportedly enhanced by the addition of distamycin A to lymphocyte cultures (Schmid et al., 1980). If the findings of Croci (1982) are confirmed then this fragile site may be regarded as a BrdU requiring fragile site similar to the one in Group 3.

Group 3

Contains only the fragile site at 10q25. This common fragile site was independently discovered by Scheres and Hustinx (1980) and Sutherland et al. (1980) and shown to be a polymorphism present in one in 40 members of the Australian population sample studied (Sutherland, 1982a). This fragile site is unique in that its expression is dependent upon the presence of BrdU or BrdC in lymphocyte cultures some hours prior to harvest.

Of all the fragile sites only the one at Xq27 (the fragile X) has been proven to be of clinical significance in that it is associated with, if not the cause of, one form of X-linked mental retardation (Sutherland, 1982b). However, none of the folate sensitive fragile sites have been seen in homozygotes and it has been suggested that they could be deleterious in this situation (Sutherland, 1979b). On the other hand, heterozygotes for the autosomal folate sensitive fragile sites have been found to be 10 times more common amongst the mentally retarded than amongst neonates but this data is very limited and more information is required before it can be asserted that such heterozygotes (most of whom are intellectually normal) are at increased risk of mental retardation (Sutherland, 1982b).

## III. THE BIOCHEMISTRY OF FRAGILE SITE EXPRESSION

A number of factors have been shown to be important in fragile site expression since Sutherland (1977) showed that they were expressed in lymphocyte cultures only if the cells were grown in medium 199 and not in a range of other commercially available culture media. Sutherland (1979a) subsequently demonstrated that fragile site expression will only occur if the culture medium is free of folic acid and thymidine, that it is enhanced by a culture medium pH of greater than 7.3, that in the presence of folic acid it can be induced by the folate antagonist methotrexate, and that the folic acid precursor folinic acid or the thymidine analogue BrdU will suppress it in folate free medium (Sutherland, 1979a). Addition of fluorodeoxyuridine (FudR), an inhibitor of the enzyme thymidylate synthetase will induce expression in the presence of folic acid but not thymidine (Glover, 1981). Methionine has been shown to be essential for fragile site expression even in folic acid free medium (Howard-Peebles and Pryor, 1981; Sutherland, 1982b) but the reasons for this may be indirect in that cell cycle times are greatly increased in the absence of methionine and the effect of methionine deprivation can be overcome by FudR induction (Glover and Howard-Peebles, 1982).

All available data suggest the areas of metabolism involved is that shown in Fig.4 and that any pertubation of conditions of tissue culture which lead to a relative deficiency of thymidine monophosphate towards the end of S will result in fragile site expression. Even though it now seems clear that this is the

area of metabolism involved it is not clear how it is involved. Why is it that in some cells the fragile site is only expressed on one chromatid? Analogously, in two homozygotes for the fragile site at 10q25 the fragile site was more often expressed on one chromosome than on both (Sutherland, 1981) even though environmental conditions which surrounded the homologous chromosomes during DNA synthesis must be more similar to each other than in different cells. One possibility is that in conditions appropriate for expression of fragile sites they are expressed in close to 100% of cells at the completion of S but are gradually repaired during G2. This could account for expression in a single chromatid since presumably the repair events would be independent for each chromatid, but expression, if it does occur during S, would not be.

## IV. 'SPONTANEOUS' CHROMOSOMAL FRAGILITY

Chromosome breakage in lymphocyte cultures has been a recognised phenomenon for many years and some genetic toxicology testing is based upon increases of particular types of chromosome damage after exposure to potentially toxic agents. Chromosome breakage is difficult to quantitate cytogenetically, partly because of difficulties in distinguishing between chromosomal gaps and breaks. Chromosomal breakage in proliferating cell systems, which include PHA-stimulated lymphocyte cultures, is followed by the formation of micronuclei which can be easily scored in interphase cells and provide a reliable approach to quantitation of chromosome breakage (Heddle et al., 1978; Beek et al., 1980; Obe and Beek, 1982).

In proliferating lymphocyte cultures micronucleus frequency has been found to reach a peak after five days (Fig.5) and fragile site carriers have been found to have higher frequencies of micronucleus formation (Table I) than do appropriate controls without fragile sites (Beek et al., 1982) when the cultures are grown under conditions of folic acid and thymidine deprivation suitable for fragile site expression. When these components are present in the culture medium only low micronucleus frequencies are seen in cultures from fragile site carriers and controls (Table I). This difference in micronucleus frequency seen for all individuals studied, whether fragile site carriers or controls, between cells cultured in the absence or presence of folic acid or thymidine suggests that the same mechanisms which control fragile site expression might also be involved in the manifestation of 'spontaneous' chromosomal breakage. This means that the study of fragile sites may be able to be used as a model for the study of spontaneous chromosome breakage which is the primary event in at least some forms of carcinogenesis.

## V. THE CHROMOSOME INSTABILITY SYNDROMES

There are a number of rare genetic diseases, mostly inherited in an autosomal recessive manner, in which there is an increase in various types of chromosomal breakage (Hecht and McCaw, 1977). In all these disorders there is a marked predisposition to develop different types of cancers.

In Bloom syndrome, which predisposes mainly to non-lymphocytic leukaemias, the chromosome breakage is non-random and is usually shown by the presence of quadriradials involving homologous chromosomes. The rate of spontaneous sister chromatid exchange is also markedly increased. Patients with Fanconi anaemia have an increased risk of developing leukaemia, squamous cell carcinomas and hepatic adenomas and have an increase in apparently random chromatid breakage (Fig.6) : sister chromatid exchange is normal. In ataxia telangiectasia there is a marked increase in malignancies of the lymphoreticular system and increased breakage of the chromosome, rather than chromatid, type which is apparently at random although clones of cells with a translocation involving a break at 14q11-12 are frequently seen in such patients. There are also a number of other chromosome instability syndromes which predispose to cancer but which are not as well documented as the ones mentioned here.

None of the chromosome instability syndromes, or other syndromes involving defects of DNA repair which have been reported not to show chromosomal instability, have been studied to détermine whether the type of chromosome breakage which they manifest can be affected by varying the concentrations of folic acid and thymidine in the medium in which the cells are cultured for chromosome studies. This should be done and could be approached directly by chromosome examination or indirectly by determination of micronucleus frequencies.

## VI. CONCLUSIONS

Various types of chromosome fragility have been reviewed and two of them, the one associated with heritable fragile sites and at least one form of 'spontaneous' chromosome breakage, have been shown to be greatly influenced by concentrations of folic acid and thymidine in tissue culture medium. Since chromosome breakage is probably the primary event in the genesis of some forms of cancer it is not improbable that these compounds may be found to be important in protecting against malignant transformation at the cellular level.

## ACKNOWLEDGEMENTS

Financial support for this work has been provided by grants from the National Health and Medical Research Council of Australia, The Channel 10 Children's Medical Research Foundation and the Adelaide Children's Hospital Research Trust. I thank Dr. P.B. Jacky for helpful discussions during the preparation of this paper.

REFERENCES

Beek, B., Klein, G., and Obe, G. (1980). Biol. Zbl., 99, 73-84.

Beek, B., Jacky, P.B., and Sutherland, G.R. (1982). Ann. Génét. "in press".

Croci, G. (1982). Am. J. Hum. Genet. "in press".

Erbe, R.W. (1975). N. Engl. J. Med., 293, 753-757.

Glover, T.W. (1981). Am. J. Hum. Genet., 33, 234-242.

Glover, T.W., and Howard-Peebles, P.N. (1982). Am. J. Hum. Genet., "in press".

Hecht, F., and McCaw, B.K. (1977). In "Progress in Cancer Research and Therapy, Vol.3. Genetics of Human Cancer" (J.J. Mulvihill, R.W. Miller and J.F. Fraumeni, eds.), pp. 105-123. Raven Press, New York.

Heddle, J.A., Benz, R.D., and Countryman, P.J. (1978). In "Mutagen Induced Chromosome Damage in Man" (H.J. Evans and D.C. Lloyd, eds.), pp. 191-200. Edinburgh University Press, Edinburgh.

Howard-Peebles, P.N., and Pryor, J.C. (1981). Clin. Genet., 19, 228-232.

Jacky, P.B., Beek, B., and Sutherland, G.R. (1982). Science "in press".

Obe, G., and Beek, B. (1982). In "Chemical Mutagens - Principles and Methods for Their Detection" (A. Hollaender and F.J. de Serres, eds.), Vol.7, pp.337-400. Plenum Press, New York and London.

Radman, M., Jeggo, P., and Wagner, R. (1982). Mutation Res., 98, 249-264.

Scheres, J.M., and Hustinx, T.W. (1980). Am. J. Hum. Genet., 32, 628-629.

Scott, J.M., and Weir, D.G. (1981). Lancet, ii, 337-340.

Schmid, M., Klett, C., and Niederhofer, A. (1980). Cytogenet. Cell Genet., 28, 87-94.

Sutherland, G.R. (1977). Science, 197, 265-266.

Sutherland, G.R. (1979a). Am. J. Hum. Genet., 31, 125-135.

Sutherland, G.R. (1979b). Am. J. Hum. Genet., 31, 136-148.

Sutherland, G.R. (1981). Am. J. Hum. Genet., 33, 946-949.

Sutherland, G.R. (1982a). Am. J. Hum. Genet., 34, 753-756.

Sutherland, G.R. (1982b). Int. Rev. Cytol. "in press".

Sutherland, G.R., Baker, E., and Seshadri, R.S. (1980). Am. J. Hum. Genet., 32, 542-548.

TABLE I

Frequency of micronuclei in 5-day lymphocyte cultures in medium deficient in and supplemented with folic acid and thymidine or in Ham's F10 from fragile site carriers and controls. (Data from Beek et al., 1982 and Jacky et al., 1982.)

| Fragile Site | Medium Supplement | | | Ham's F10 |
|---|---|---|---|---|
| | Nil | 5 mg/l Folic Acid | 10 mg/l Thymidine | |
| fra(2)(q13) | 11.7 | | | 2.4 |
| fra(6)(p23) | 12.2 | | | 2.3 |
| fra(9)(p21) | 14.4 | | | |
| fra(12)(q13) | 13.8 | | | |
| fra(X)(q27) | 9.2 | 1.8 | 1.2 | |
| Control 1 | 6.6 | 1.7 | 0.9 | |
| 2 | 6.5 | 0.2 | 0.6 | |
| 3 | 4.4 | 0.4 | 0 | |
| 4 | 1.0 | 0 | 0 | |
| 5 | 3.6 | 0.2 | 0.6 | |

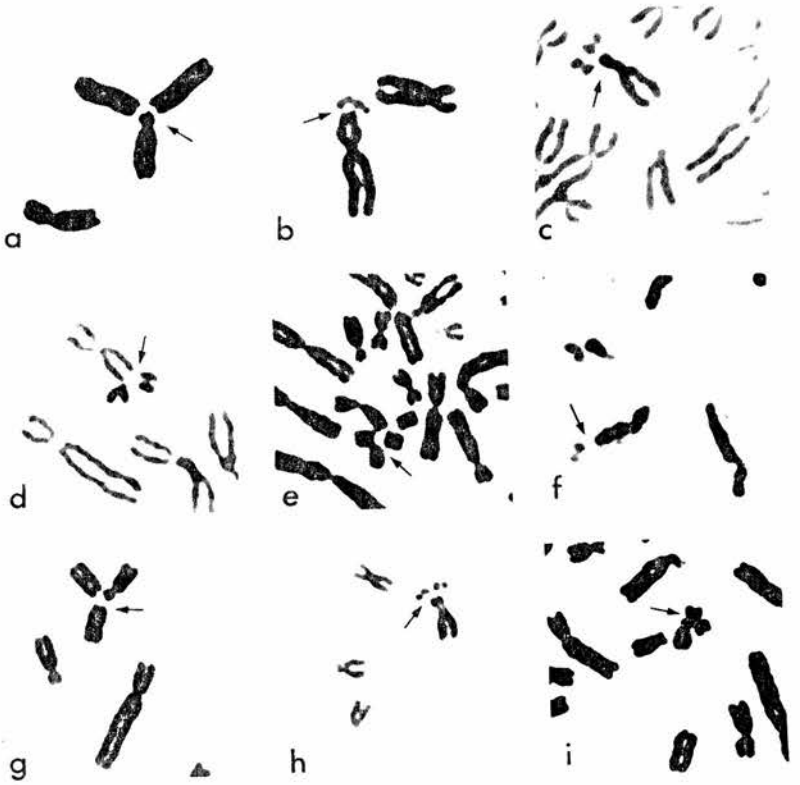



Fig. 1 Multiradial figures produced by fragile sites.

a, triradial at 2q13; b, triradial at 6p23;

c, triradial at 9p21; d, triradial at 9q23;

e, pentaradial at 10q23; f, triradial at 10q25;

g, triradial at 11q13; h, triradial at 16p12;

i, triradial at 20p11.

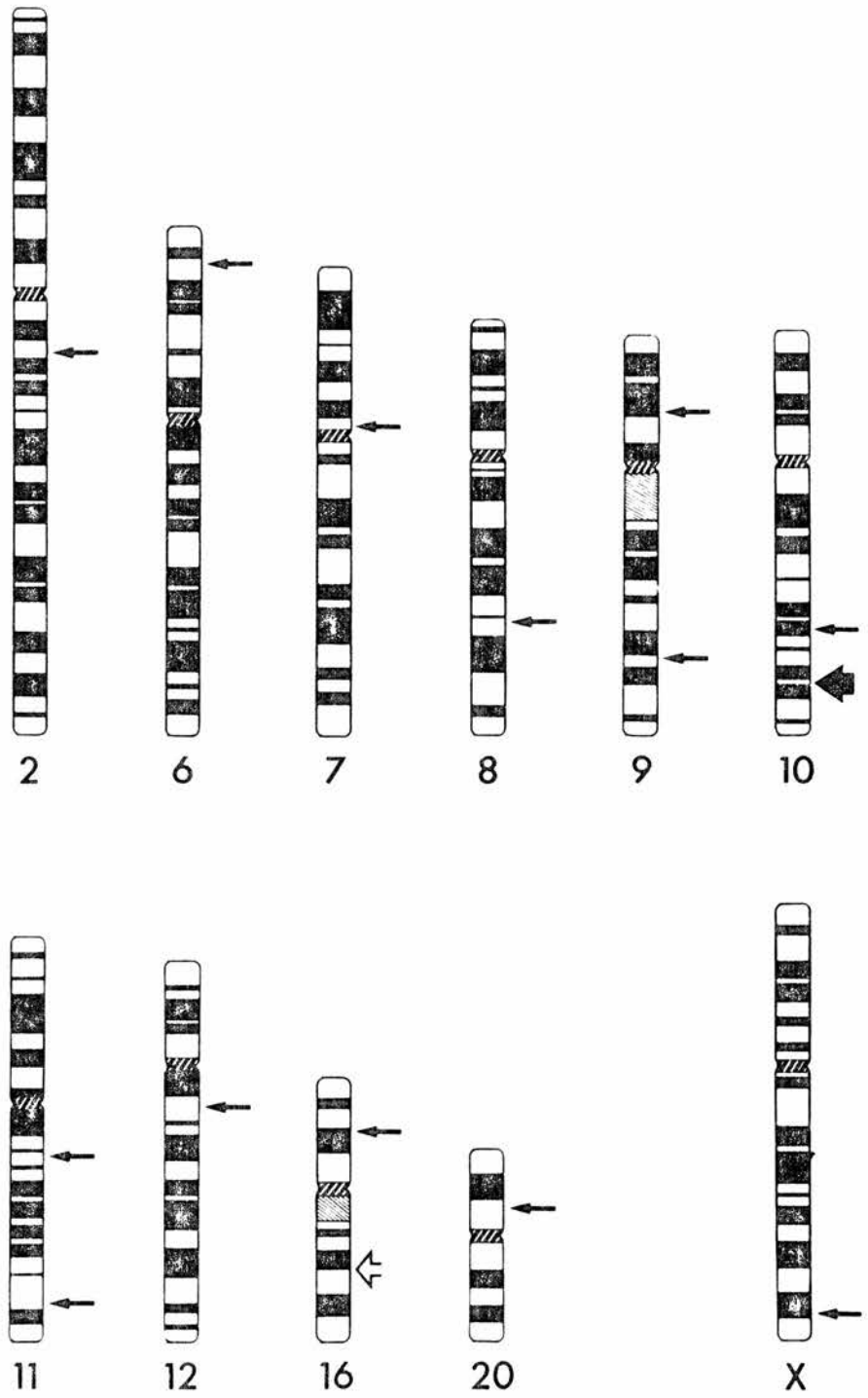



Fig.2 The known fragile sites. Group 1, small arrows, at 2q13, 6p23, 7p11, 8q22, 9p21, 9q32, 10q23, 11q13, 11q23, 12q13, 16p12, 20p11 and Xq27; Group 2, open arrow, 16q22; Group 3, broad arrow, 10q25.

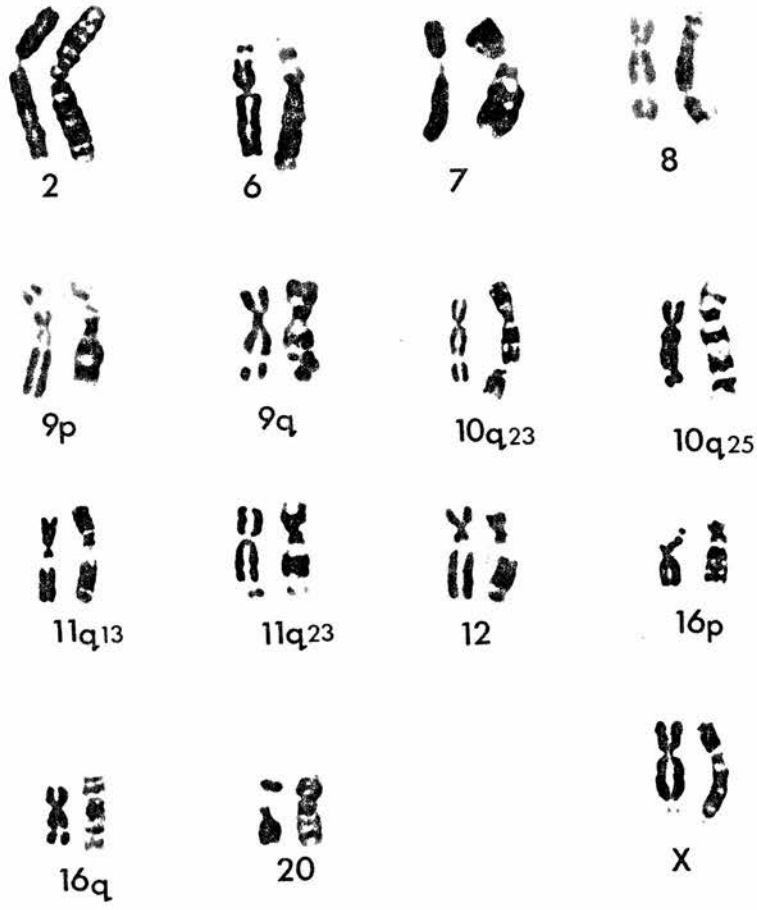



Fig.3 Partial karyotype showing the fragile sites.
The left chromosome of each pair is unbanded and the right G-banded. (Pair number 8 courtesy Dr. N.B. Kardon, pair number 7 courtesy Dr. G.C. Webb.)

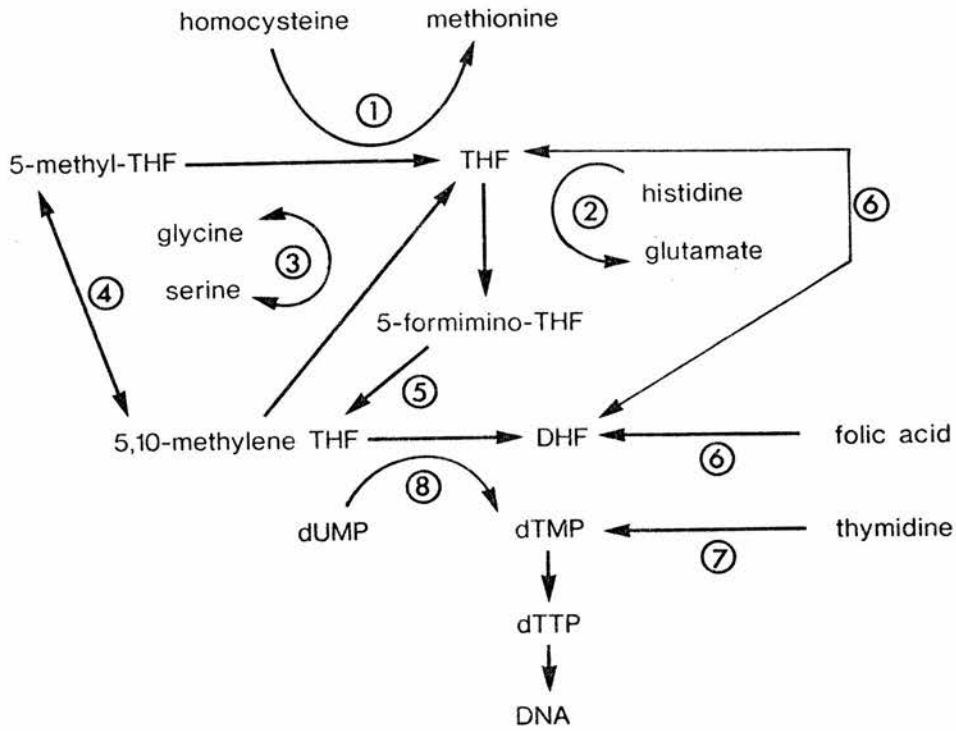



Fig.4 The area of folate metabolism involved in fragile site expression (after Erbe, 1975; and Scott and Weir, 1981). The enzymes controlling the various reactions are

1. methionine synthetase, 2. glutamate formiminotransferase, 3. serine hydroxymethyltransferase, 4. methylene-THF reductase, 5. formimino-THF cyclodeaminase, 6. dihydrofolate reductase, 7. thymidine kinase, 8. thymidylate synthetase.

Abbreviations used : THF - tetrahydrofolate, DHF - dihydrofolate, dUMP - uridine monophosphate, dTMP - thymidine monophosphate.

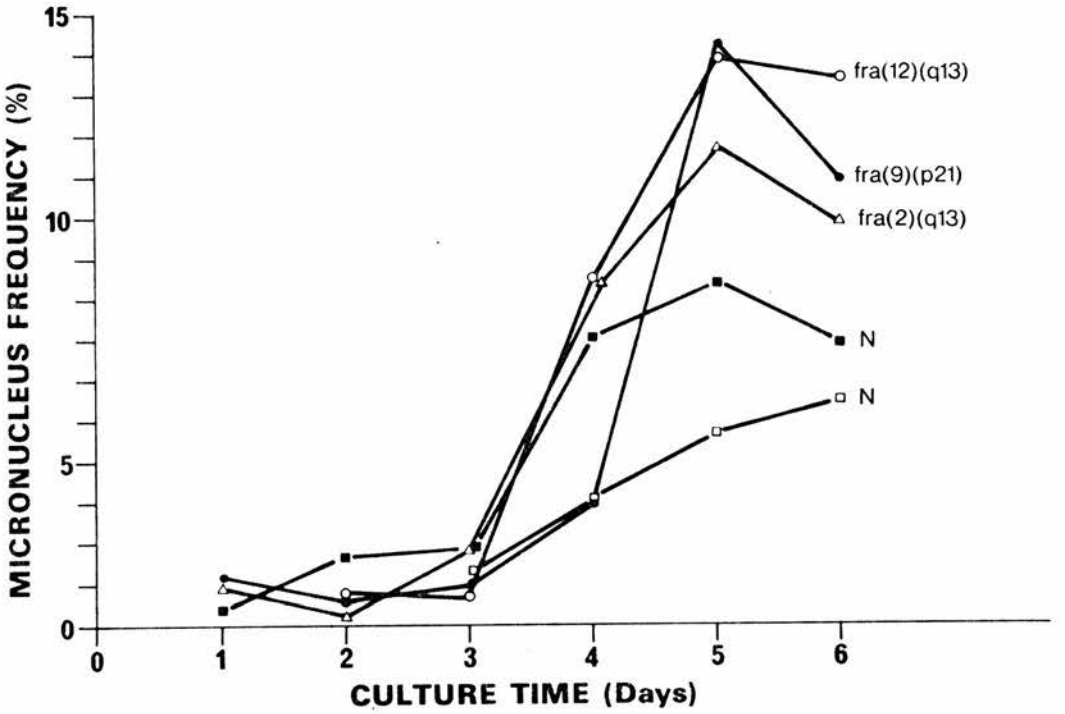



Fig.5 Micronucleus frequencies in lymphocytes from carriers of fragile sites (fra) and controls (N) cultured in folic acid and thymidine deficient medium and harvested at different times (data from Beek et al., 1982).

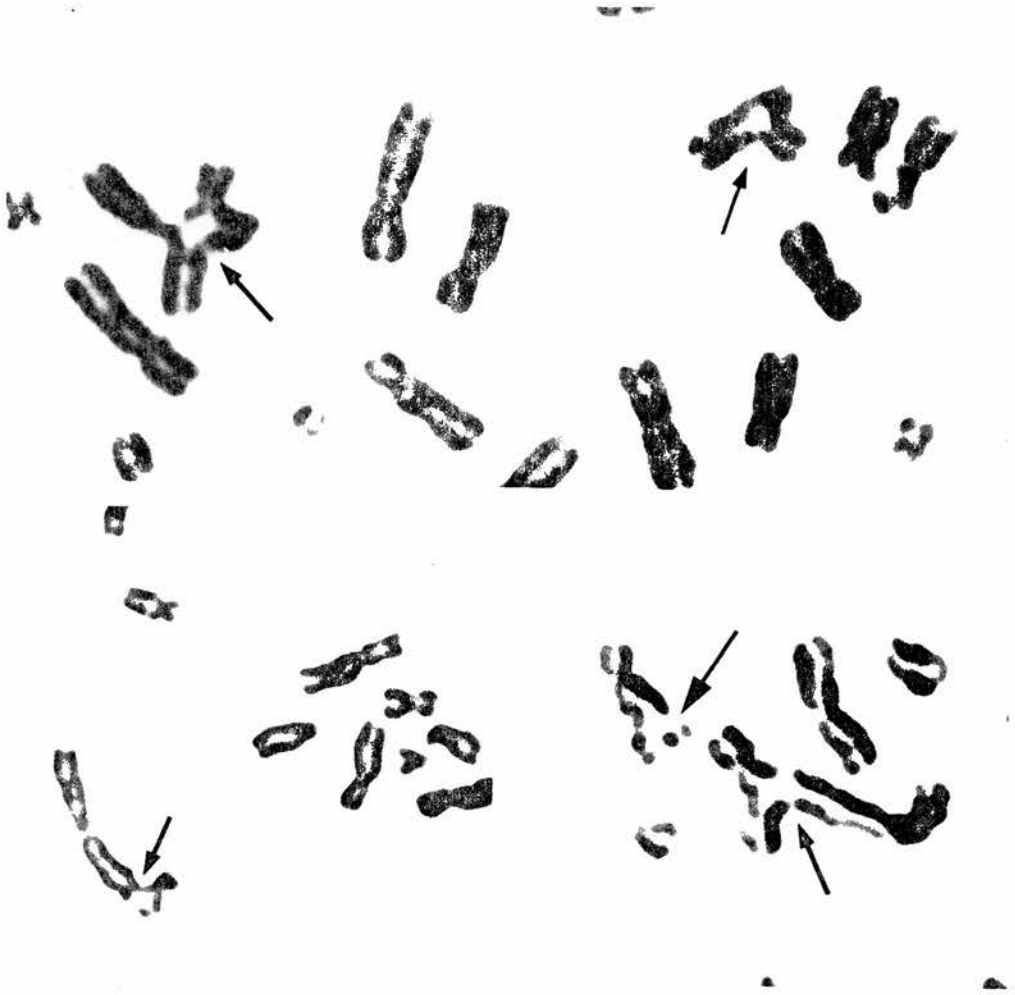


Fig.6 Partial metaphases from a patient with Fanconi anaemia and coincidently, a fragile site at 10q25 (large arrow). Note assymetric chromatid exchange figures (small arrows) which are typical of the chromosome damage seen in this disorder.

in press Ann. Genet.

D 79

# HERITABLE FRAGILE SITES AND MICRONUCLEUS FORMATION

Bernd Beek[1,2], Peter B. Jacky[2] and Grant R. Sutherland[2]

[1] Institut für Genetik
Freie Universität Berlin
Arnimallee 507
1000 Berlin 33
West Germany

[2] Cytogenetics Unit
Department of Histopathology
Adelaide Children's Hospital
North Adelaide, S.A. 5006,
Australia

SUMMARY

Human leukocytes from individuals carrying different fragile sites, including the fragile X, show elevated frequencies of micronucleus formation in interphase cells after cultivation in media deficient in folic acid and thymidine. Less elevated frequencies of micronucleus formation were also observed in two control subjects studied using comparable culture conditions, indicating the occurrence of a high frequency of "spontaneous" chromosomal breakage under culture conditions suitable for the expression of fragile sites. This effect may be suppressed by the addition of the thymidine analogue bromodeoxyuridine (BUdR) or by cultivation in Ham's F10, a medium containing folic acid and thymidine. These results point to a possible common link between a particular type of "spontaneous" chromosomal breakage and the expression of folate sensitive fragile sites.

## INTRODUCTION

Heritable fragile sites on human chromosomes have attracted much attention recently, especially because of the association of the fragile X chromosome with a common form of mental retardation. Fragile sites are generally characterised by an achromatic gap at exactly the same location on a particular chromosome in an individual, by their inheritance in a Mendelian co-dominant manner, and by an actual fragility as indicated by the formation of acentric fragments, deleted chromosomes and triradials (Sutherland, 1982). Whilst chromosomal breakage can occur at fragile sites its exact quantification in metaphase preparations is very difficult, especially because of the known problems in discriminating between achromatic chromosome lesions and true chromosome breaks.

The occurrence of chromosomal breakage in proliferating cell systems, such as phytohaemagglutinin (PHA)-stimulated human leukocyte cultures, is followed by the formation of micronuclei which can be easily scored in interphase cells and provide a reliable approach to the quantification of chromosomal breakage (Heddle et al., 1978; Beek et al., 1980; Obe and Beek, 1982a). In this communication we report on micronucleus formation in individuals with different heritable fragile sites and control subjects whose leukocytes were grown under different culture conditions relevant to the expression of most fragile sites (Sutherland, 1979a).

## MATERIALS AND METHODS

Human leukocyte cultures (5 ml) were set up with heparinised blood (0.2 ml per culture) from two laboratory staff members as controls, and from five individuals carrying six different fragile sites (Fig.1). The carrier of fra(16)+fra(X) is subject B of Sutherland (1979b), the one with fra(12) is $IV_1$ of Sutherland and Hinton (1981), the one with fra(9) is documented by Sutherland et al. (1982) and the one with fra(6) is $II_{12}$ of Sutherland et al. (1982). Culture medium was Ham's F10 or TC199 (pH 7.6, 20 mM HEPES) with or without the addition of bromodeoxyuridine (BUdR, 10 µg/ml) added at culture initiation, or Eagle's MEM-FA (Sutherland, 1979a). All cultures contained 5% fetal calf serum, 0.1 ml PHA, and 100 U/ml penicillin. BUdR-containing cultures were incubated in the dark, and colchicine was added to all cultures one hour prior to chromosome harvest carried out using standard techniques which included hypotonic treatment with 0.075 M KCl for five minutes and fixation in methanol:acetic acid (3:1). The slides were stained with Leishman stain. Fifty metaphases were scored in determining the frequency of expression of a fragile site and at least 1,000 interphases were scored from coded and randomised slides in determining the frequency of micronucleus formation.

RESULTS

Fig.2 shows a comparison of the frequency of expression of fragile sites at metaphase with the frequencies of interphase micronuclei at different culture times (1-6 days), scored from the same slide. Maximum frequencies of fragile site expression were reached either on day 3 or day 4 of culture, and declined at later culture times. An increase in the micronucleus frequency is seen from day 4 of culture onwards, reaching a maximum of about 12-14% either on day 5 or day 6 of culture. It appears that maximum frequencies of expression of fragile sites are followed by increases in micronucleus frequency with a delay of one or two days.

Figs.3 and 4 show results from two independent but similar experiments. Leukocytes from either a fra(6) carrier and a control (Fig.3) or a fra(2) carrier and a different control (Fig.4) were grown in TC199 with and without BUdR. Additionally, in each of the experiments leukocytes from the respective fragile site carriers were grown in Ham's F10. The micronucleus frequency was determined over the period of culture up to six days. While in both experiments low micronucleus frequencies predominate up to culture times of three days, increases in the micronucleus frequencies are seen with increasing culture time, followed in most cases by a slight decrease by day 6. The most pronounced increases in micronucleus frequencies in both experiments were found in cultures set up with blood from the fragile site carriers using TC199 without BUdR, but increases were also observed with control leukocytes under the same culture conditions (Fig.4).
The addition of BUdR to leukocyte cultures grown in TC199

clearly suppressed micronucleus formation in cultures from the two fragile site carriers, and from one of the control subjects (Fig.4). A more marked suppression of micronucleus formation however was found after cultivation of the fragile site leukocytes in Ham's F10 medium in comparison with cultivation in TC199.

During the scoring of metaphases for fragile sites micronucleus derived premature chromosome condensation (MN-PCC) was seen (Obe and Beek, 1982b). In the 4 day culture from the fra(2) carrier MN-PCC were observed in 12 of 359 metaphases scored. Both $G_2$- and S-type PCC were seen (Fig.6).

## DISCUSSION

The expression of most heritable fragile sites on human chromosomes is dependent on culture conditions, namely a deficiency in folic acid and thymidine (Sutherland, 1979a). Consequently, the folate sensitive fragile sites can be demonstrated only by the use of culture media deficient in these compounds, such as TC199 or MEM-FA, and not with culture media containing normal levels of folic acid and thymidine, such as Ham's F10. Addition of folic acid, folinic acid, thymidine or its analogue BUdR to deficient media inhibits the expression of the folate sensitive fragile sites. Similarly, the addition of inhibitors of folate and thymidine metabolism to medium containing normal concentrations of these substances will elicit the expression of the folate sensitive fragile sites (Hecht et al., 1982). Culture time also seems to play an important role in that maximum expression is usually found at day 3 or 4 of culture,

being lower at earlier or later culture times (Sutherland, 1982).

Micronuclei in interphase cells are the consequence of induced or "spontaneous" chromosomal damage in a proliferating cell system (Obe and Beek, 1982b). As shown in Figs.2-4, high micronucleus frequencies were found in human leukocytes cultivated under conditions necessary for the expression of fragile sites from about day 3 of culture onwards, indicating a high frequency of chromosomal aberrations in these cells. From the results presented in Fig.2 it seems likely that the expression of fragile sites can be followed by chromosomal breakage. Although the maximum frequencies of micronuclei reached during leukocyte cultivation of the various fragile sites are higher than those found with control leukocytes, the control material is inadequate to decide whether fragile site leukocytes exhibit a higher frequency of chromosomal damage than controls under the conditions of culture studied. Further investigations are necessary to determine this. A higher sensitivity of fragile site cells to an increase in "spontaneous" chromosomal breakage under conditions of folate and thymidine deprivation offers a possible explanation for the known or suspected deleterious effects of fragile sites. No homozygote for a folate sensitive fragile site has yet been recorded (Sutherland, 1982).

Medium composition in human leukocyte culture is known to have an influence on chromosomal breakage, both "spontaneous" (Obe et al., 1976) and induced (Beek and Obe, 1974). However, the effect of folate and thymidine deprivation seems to be surprisingly large in fragile site leukocytes, and also in controls (Fig.4). Moreover, the results shown in Figs.3 and 4 indicate that this effect may be compensated for by the addition of the thymidine

analogue BUdR, at a concentration that has been reported to be without any influence on cell cycle progression (Tice et al., 1976), or by cultivation in folic acid and thymidine rich Ham's F10. Hence, the area of DNA precursor metabolism which appears to be related to the expression of fragile sites could also be involved in formation of "spontaneous" chromosomal damage, possibly pointing to a common underlying mechanism.

In fragile site leukocytes the part of the chromosome distal to the fragile site (the presumptive fragment) could be expected to contribute to the pool of lagging chromatin leading to the formation of micronuclei. However, it seems clear that an unknown but probably high proportion of micronuclei may be derived from chromosomal damage that has occurred at other sites in the genome. This would appear to be so from the findings in the control cultures and also by the observation during micronucleus scoring that there was considerable size variation of micronuclei irrespective of the fragment size attributable to the particular fragile site under investigation (Fig.5). Investigations of the distribution of micronuclei sizes (Yamamoto and Kikuchi, 1980) in leukocytes with different fragile sites may lead to an elucidation of the contribution of breakage at the fragile site to the pool of lagging chromatin. Breakage at the fragile site could be at least partly responsible for the reduced frequencies of fragile site expression seen when culture time is extended beyond four days (Fig.2).

The formation of micronuclei probably represents part of a selection mechanism against cells with unstable chromosomal damage in proliferating cell systems, both in vivo and in vitro. Micronucleus formation is followed by the occurrence of MN-PCC

irrespective of the origin of chromosomal damage (Obe and Beek, 1982b). This type of PCC was observed in the course of the present study (Fig.6).

More questions have been raised than answered by the results of this study but the micronucleus technique may provide a useful approach to the study of heritable fragile sites, and their relation to a particular type of spontaneous or perhaps DNA precursor deprivation-induced chromosomal breakage.

REFERENCES

BEEK B., OBE G. - The effect of lead acetate on human leukocytes in vitro. Experientia 1974, 30, 1006-1007.

BEEK B., KLEIN G., OBE G. - The fate of chromosomal aberrations in a proliferating cell system. Biol Zbl., 1980, 99,73-84.

HECHT F., JACKY P.B., SUTHERLAND G.R. - The fragile X chromosome. Current Methods, 1982, 11, 489-495.

HEDDLE J.A., BENZ R.D., COUNTRYMAN P.J. - Measurement of chromsomal breakage in cultured cells by the micronucleus technique. In: Evans H.J., Lloyd D.C. (eds) Mutagen-induced Chromosome damage in Man. Edinburgh University Press, Edinburgh, 1978, pp 191-200.

OBE G., BEEK B. - The human leukocyte test system. In: Hollaender A., Serres F.J. de (eds) Chemical Mutagens - Principals and Methods for Their Detection, vol. 7. Plenum Press, New York and London, 1982a, pp 337-400.

OBE G., BEEK B. - Premature chromosome condensation in micronuclei. In: Rao P.N., Johnston R.T., Sperling K. (eds) Premature Chromosome Condensation: Application in Basic, Mutation and Clinical Research. Academic Press, New York, 1982b, in press, pp113-130.

OBE G., BEEK B., SLACIK-ERBEN R. - The use of the human leukocyte test system for the evaluation of potential mutagens. In: European Society of Toxicology, 17th Meeting, Montpellier, June 16-18, 1975, Int Congr Ser No 376, Excerpta Medica, Elsevier/North Holland, Amsterdam, 1976, pp 118-126.

SUTHERLAND G.R. - Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. Am.J.Hum. Genet., 1979a, 31, 125-135.

SUTHERLAND G.R. - Heritable fragile sites on human chromosomes. II. Distribution,phenotypic effects and cytogenetics. Am.J.Hum. Genet., 1979b, 31, 136-148.

SUTHERLAND G.R. - The fragile X chromosome. Int. Rev. Cytol., 1982, in press.

SUTHERLAND G.R., HINTON L. - Heritable fragile sites on human chromosomes. VI. Characterisation of the fragile site at 12q13. Hum. Genet., 1981, 57, 217-219.

SUTHERLAND G.R., JACKY P.B., BAKER E., MANUEL A. - Heritable fragile sites on human chromosomes. X. New folate sensitive fragile sites : 6p23, 9p21, 9q32, 11q23. Am.J.Hum.Genet., 1982, in press.

TICE R., SCHNEIDER E.L.,RARY J.M. - The utilization of bromodeoxyuridine incorporation into DNA for the analysis of cellular kinetics. Exp. Cell. Res., 1976, 102, 232-236.

YAMAMOTO K.J., KIKUCHI Y. - A comparison of diameters of micronuclei induced by clastogens and by spindle poisons. Mutat. Res., 1980, 71, 127-131.

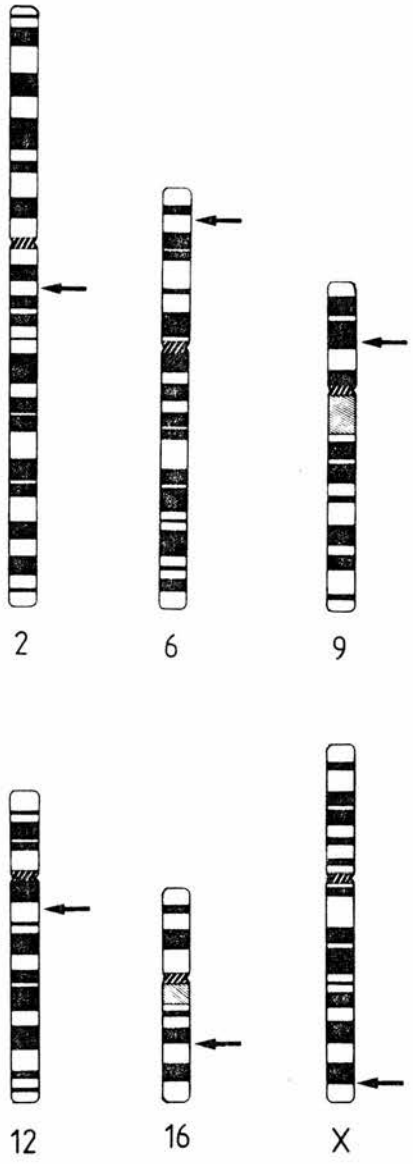



Fig.1 The fragile sites in this study (arrows) : 2q13, 6p23, 9p21, 12q13, 16q22, Xq27. These fragile sites are all folate sensitive except for 16q22.

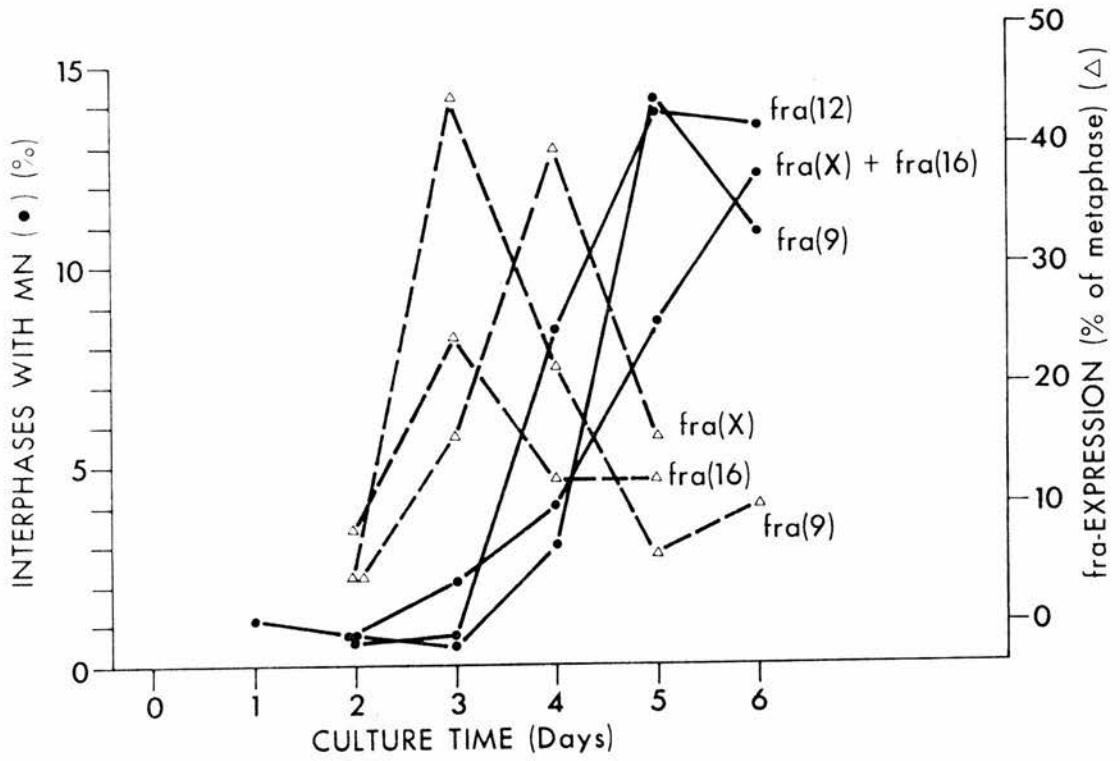



Fig.2 Frequencies (%) of expression of fragile sites in metaphases (fra; right ordinate, Δ ) and of interphases with micronuclei (MN; left ordinate, • ) in human leukocyte cultures harvested at different culture times. Cultures were set up in MEM-FA.

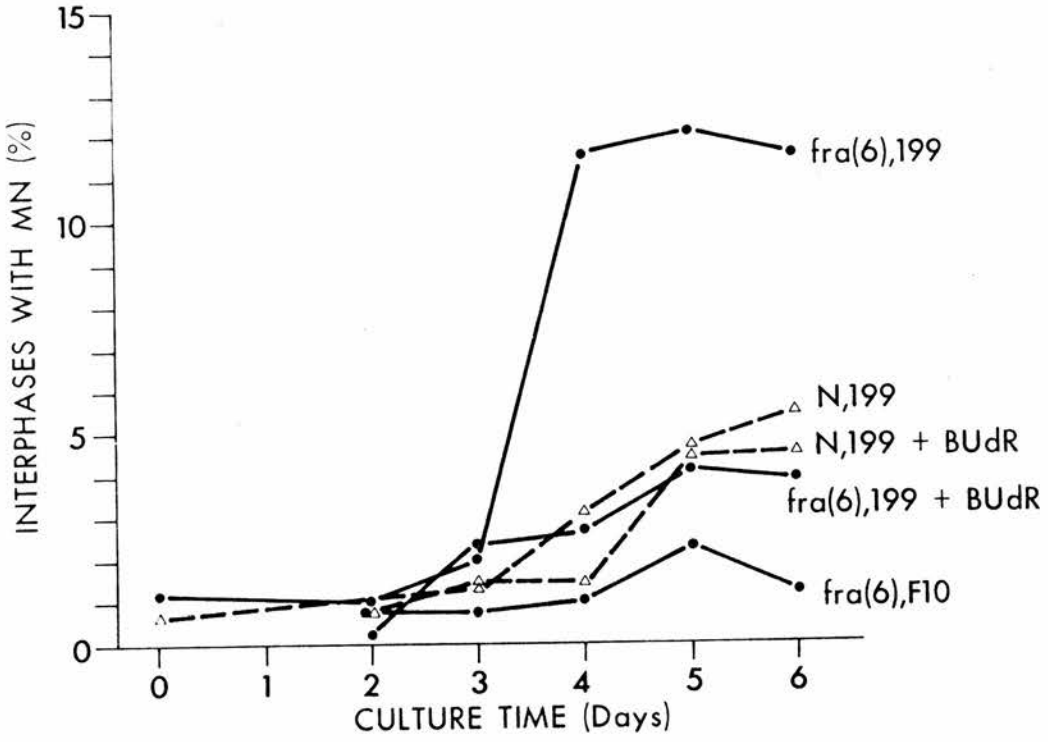



Fig.3 Micronucleus (MN) frequencies (%) in human leukocytes cultured in TC199 or Ham's F10 from a fra(6) carrier and a control (N) harvested at different culture times. BUdR : Addition of BUdR, 10 µg/ml at culture initiation.

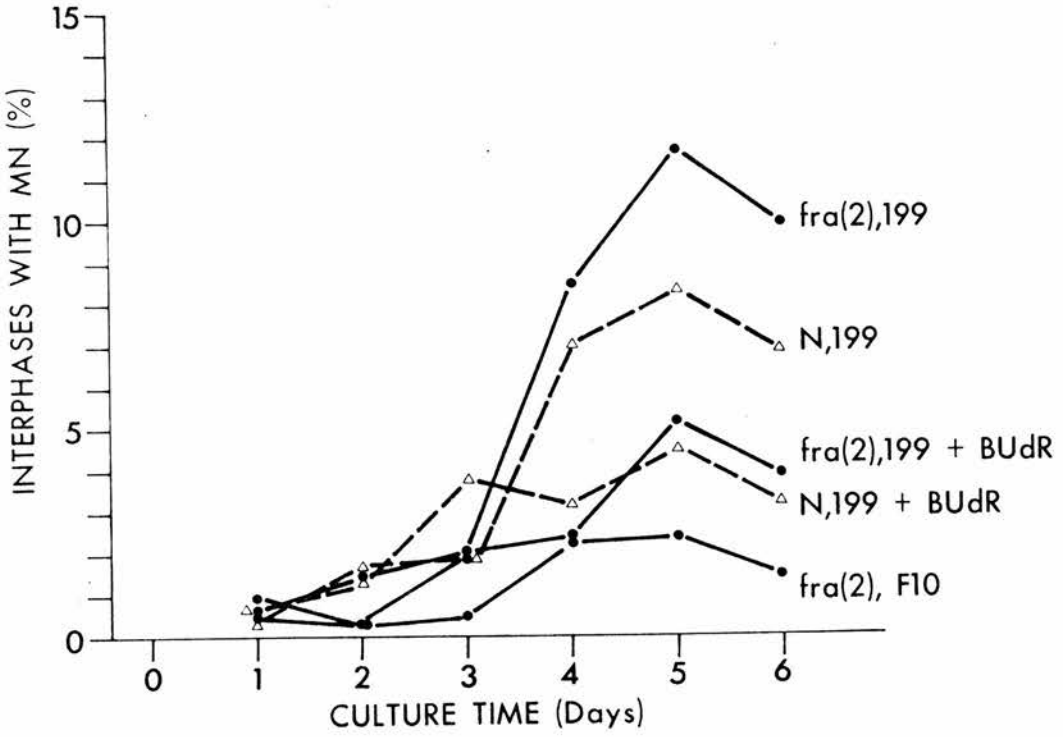



Fig.4 Micronucleus (MN) frequencies (%) in human leukocytes cultured in TC199 or Ham's F10 from a fra(2) carrier and a control (N) harvested at different culture times. BUdR : Addition of BUdR, 10 µg/ml at culture initiation.

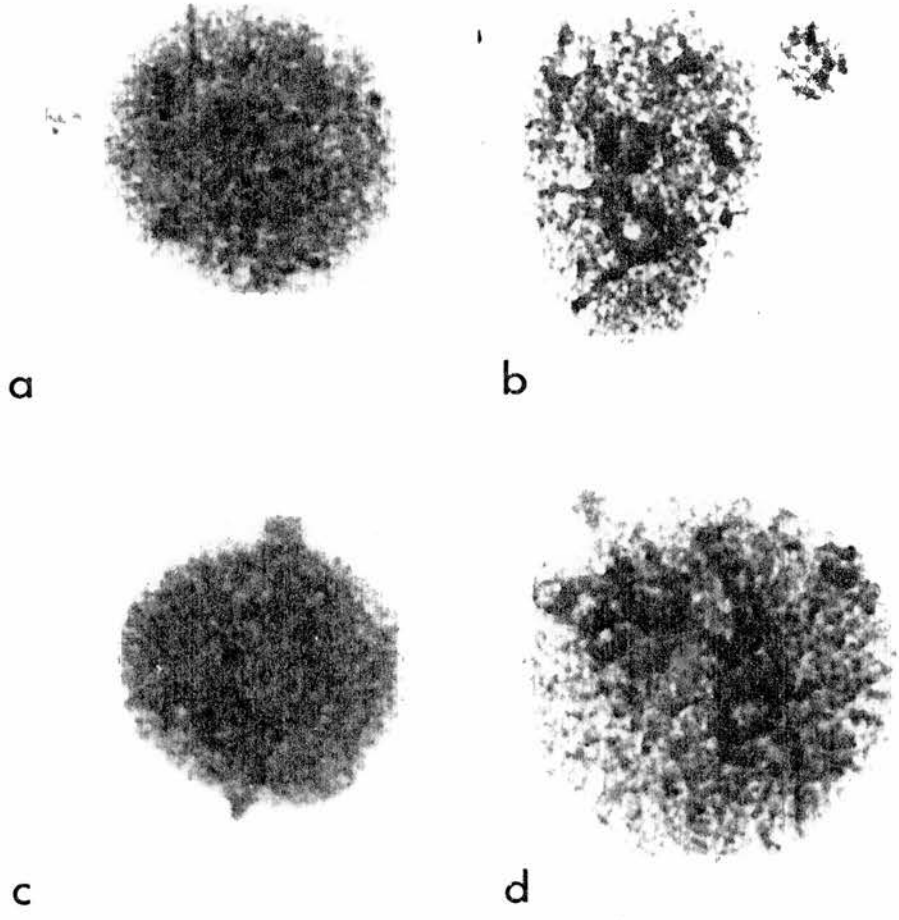



Fig.5 Examples of main nuclei with micronuclei in interphase from human leukocytes set up with blood from a fra(2) carrier. a, b, d : interphases with one micronucleus; c : interphase with two micronuclei. Note the variable size of the micronuclei.

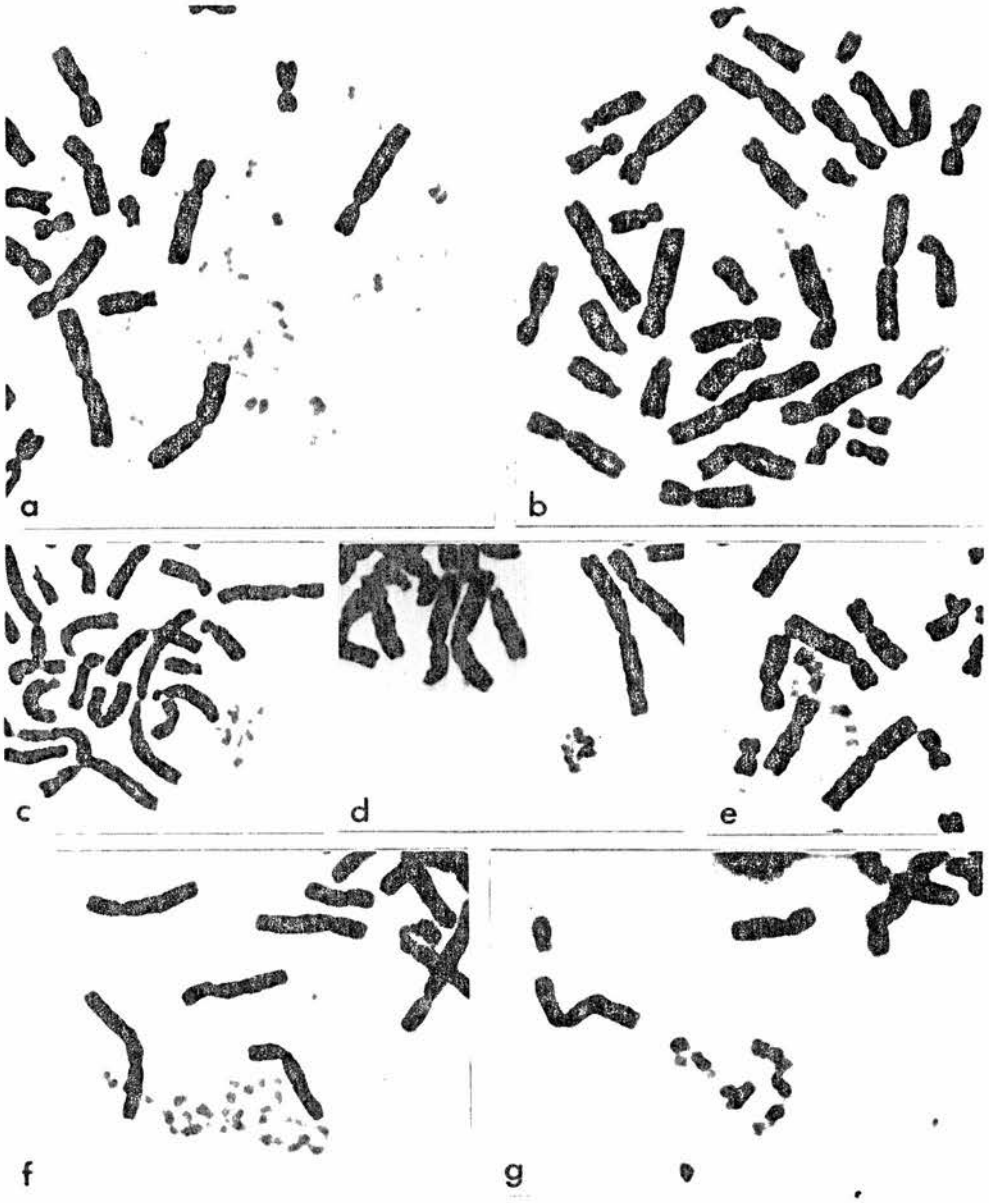



Fig.6 Examples of MN-PCC in human leukocyte metaphases from the fra(2) carrier. a-d : pulverised S-type PCC; e-g : broken ladder-like double-stranded $G_2$-type PCC.

tracks (Fig. 1) show that the character of the magnetic anomalies is generally smoother and of longer wavelength for the approximately east-west trending tracks than for the north-south trending tracks.

The Mesozoic sequence of magnetic anomalies from M9 (~ 121 million years old) through M25 (~ 153 million years old) are symmetric about a ridge formed at the time of anomaly M9. These segments appear to be offset left-laterally from east to west and to be normal to the Davie fracture zone, but more magnetic data is required to confirm this and to determine if oblique sea-floor spreading occurred. The Jurassic magnetic quiet zone is observed on both sides of the ridge axes landward of magnetic anomaly M25. We identified a large amplitude magnetic anomaly on both landward sides of the Jurassic quiet zone. This anomaly may represent the ocean-continent boundary, as has been proposed for similar magnetic anomalies bordering other passive continental margins [for example, the magnetic anomaly off eastern North America (*18*) or anomaly G bordering the South Atlantic margin (*19*)]. The magnetic anomalies are disturbed in places by seamounts and islands in the southern part of the basin. Some of the islands have experienced volcanism since the Miocene (*20*).

The magnetic data point to the motion of Madagascar relative to Africa being from the north, with the Africa-Madagascar separation beginning during the time of the Jurassic quiet zone (~ 165 million years ago) and ending at a time of formation of anomaly M9 (~ 121 million years ago). The Africa-Madagascar separation thus began at about the same time as the breakup of Gondwanaland and the separation of North America from Africa. We used the Mesozoic time scales (*21*) for dating these anomalies, and derived half-spreading rates of 1.7 to 1.8 cm year$^{-1}$. We note that the results of recent deep sea drilling in the North Atlantic on the older parts of the Mesozoic sequence may reduce the age assigned to magnetic anomaly M25 and the Jurassic quiet zone (*22*) and hence increase the spreading rates.

Deep Sea Drilling Project (DSDP) site 241 (*23*) is located on the lower continental rise off northeastern Kenya and southeastern Somalia in the Jurassic quiet zone and just landward of magnetic anomaly M25. A multichannel seismic section from our recent *Vema* cruise across this site (*24*) is shown in Fig. 3.

Relative motion between Madagascar and Africa ceased ~ 121 million years ago, about 25 million years before creation of the purple reflector [Early Senonian (Fig. 3), corresponding to the age of the oldest sediment recovered during drilling]. Schlich *et al.* (*25*) and Simpson *et al.* (*23*) conclude, primarily from the drilling results, that the continental margin off northeastern Kenya and southeastern Somalia has been evolving passively for at least the last 90 million years and argue that it is unlikely that Madagascar occupied a position adjacent to that part of Africa during those 90 million years. Our marine magnetics data and seismic analysis are in good agreement with these drilling results (*23*, *25*).

PHILIP D. RABINOWITZ
*Department of Oceanography, Texas A & M University, College Station 77843*, and *Lamont-Doherty Geological Observatory, Columbia University, Palisades, New York 10964*
MILLARD F. COFFIN
*Lamont-Doherty Geological Observatory, Columbia University*
DAVID FALVEY*
*University of Sydney, New South Wales 2006, Australia*

### References and Notes

1. D. P. McKenzie and J. G. Sclater, *Geophys. J. R. Astron. Soc.* **25**, 437 (1971).
2. I. O. Norton and J. G. Sclater, *J. Geophys. Res.* **84**, 6803 (1979).
3. A. L. DuToit, *Our Wandering Continents* (Oliver & Boyd, Edinburgh, 1937).
4. A. G. Smith and A. Hallam, *Nature (London)* **225**, 139 (1970).
5. J. R. Heirtzler and R. H. Burroughs, *Science* **174**, 488 (1971); J. Segoufin and P. Patriat, *C. R. Acad. Sci.* **291**, 85 (1980).
6. P. D. Rabinowitz, *J. Geophys. Res.* **76**, 7107 (1971).
7. R. A. Scrutton, *Earth Planet. Sci. Lett.* **39**, 84 (1978); _____, W. B. Heptonstall, J. H. Peacock, *Mar. Geol.* **43**, 1 (1981).
8. E. T. Bunce and P. Molnar, *J. Geophys. Res.* **82**, 5305 (1977).
9. M. W. McElhinny, *Nature (London)* **228**, 977 (1970); _____ and B. J. J. Embleton, *Earth Planet. Sci. Lett.* **31**, 101 (1976); _____, L. Daly, J. P. Pozzi, *Geology* **4**, 455 (1976); B. J. J. Embleton, J. J. Veevers, B. D. Johnson, C. M. Powell, *Tectonophysics* **61**, 381 (1980); C. M. Powell, B. D. Johnson, J. J. Veevers, *ibid.* **63**, 13 (1980); B. J. J. Embleton and M. W. McElhinny, *Earth Planet. Sci.*
10. R. T. Cannon, W. M. [illegible] Karanja, *Earth Planet. Sc*
11. P. D. Rabinowitz, M. F. Coffin, D. Falvey, *Science* **215**, 663 (1982).
12. A. G. Green, *Nature (London)* **236**, 19 (1972).
13. G. Flores, *Trans. Geol. Soc. S. Afr.* **73**, 1 (1970).
14. J. B. Wright and P. McCurry, *Earth Planet. Sci. Lett.* **8**, 267 (1970); E. J. Barron, C. G. A. Harrison, W. W. Hay, *Eos* **59**, 436 (1978); E. J. Barron and C. G. A. Harrison, in *Mechanisms of Continental Drift and Plate Tectonics*, P. A. Davies and S. K. Runcorn, Eds. (Academic Press, New York, 1980), p. 89; _____, J. L. Sloan, W. W. Hay, *Eclogae Geol. Helv.* **74**, 443 (1981).
15. P. E. Kent, *Nature (London)* **238**, 147 (1972); in *Implications of Continental Drift to the Earth Sciences*, D. H. Tarling and S. K. Runcorn, Eds. (Academic Press, New York, 1973), pp. 873 and 949; M. F. J. Flower and D. F. Strong, *Earth Planet. Sci. Lett.* **7**, 47 (1969); D. H. Tarling, *Nature (London)* **229**, 17 (1971); *ibid.* **238**, 92 (1972); _____ and P. E. Kent, *ibid.* **261**, 304 (1976); B. W. Darracott, *Earth Planet. Sci. Lett.* **24**, 282 (1974); F. Förster, *Palaeogeog. Palaeoclimatol. Palaeoecol.* **17**, 267 (1975).
16. T. J. Francis, D. Davies, M. N. Hill, *Phil. Trans. R. Soc. London Ser. A* **259**, 240 (1966).
17. J. Segoufin, *C. R. Acad. Sci. Ser. D* **287**, 109 (1978); E. S. W. Simpson, J. G. Sclater, B. Parsons, I. Norton, L. Meinke, *Earth Planet. Sci. Lett.* **43**, 260 (1979).
18. C. L. Drake, J. I. Ewing, H. Stockard, *Can. J. Earth Sci.* **5**, 993 (1968); K. O. Emery, E. Uchupi, J. D. Phillips, C. O. Bowin, E. T. Bunce, S. T. Knott, *Bull. Am. Assoc. Petr. Geol.* **54**, 44 (1970); P. D. Rabinowitz, in *The Geology of Continental Margins*, C. A. Burk and C. L. Drake, Eds. (Springer-Verlag, New York, 1974), p. 67; K. D. Klitgord and J. C. Behrendt, U.S. Geological Survey Miscellaneous Field Studies Map MF 913 (1977).
19. P. D. Rabinowitz and J. LaBrecque, *J. Geophys. Res.* **84**, 5973 (1973).
20. T. L. Vallier, *Init. Rep. Deep Sea Drill. Proj.* **25**, 515 (1974).
21. R. L. Larson and T. W. C. Hilde, *J. Geophys. Res.* **80**, 2586 (1975); S. C. Cande, R. L. Larson, J. L. LaBrecque, *Earth Planet. Sci. Lett.* **41**, 434 (1978).
22. F. Gradstein *et al.*, *Init. Rep. Deep Sea Drill. Proj.*, in press; J. E. T. Channell, J. G. Ogg, W. Lowrie, in preparation.
23. E. S. W. Simpson *et al.*, *Init. Rep. Deep Sea Drill. Proj.* **25**, 884 (1974).
24. M. F. Coffin and P. D. Rabinowitz, in *1982 Proc. Offshore Technol. Conf.* **2**, 421 (1982).
25. R. Schlich, E. S. W. Simpson, T. L. Vallier, *Init. Rep. Deep Sea Drill. Proj.* **25**, 743 (1974).
26. We thank the officers, crew, and scientists on the R.V. *Vema* for assistance in gathering the data and S. Cande and J. LaBrecque for critically reviewing the manuscript. Supported by grant OCE 79-19389. This is Lamont-Doherty Geological Observatory contribution 3447.

* Present address: Division of Mineral Geosciences and Petroleum Geology, Bureau of Mineral Resources, Canberra 2601, Australia.

9 August 1982; revised 1 November 1982

D 80

## Fragile Sites in Chromosomes: Possible Model for the Study of Spontaneous Chromosome Breakage

Abstract. *The tissue culture condition that is required for the type of chromosome breakage seen at most fragile sites, namely, the absence of folic acid and thymidine in the medium, greatly enhanced micronucleus formation in proliferating lymphocyte cultures from normal individuals. This suggests that chromosome breakage at fragile sites and the apparently spontaneous damage that gives rise to micronuclei are controlled by the same mechanism.*

Fragile sites are heritable points on human chromosomes. Expressed as nonstaining gaps during metaphase, they are places where the chromosomes are very susceptible to breakage (*1*). The best evidence for this phenomenon is the formation of multiradial figures at metaphase, which arise from breakage and malsegregation of the chromosome fragment distal to the fragile site (*2*). Fragile

sites can be detected in preparations of lymphocyte chromosomes if the lymphocytes are grown in a tissue culture medium that is deficient in folic acid and thymidine (*1*).

Chromosome breakage in proliferating cells gives rise to micronuclei (*3*). The finding that micronuclei may appear more often in individuals with fragile sites than in individuals without them suggests that chromosome fragments resulting from breakage at such sites are eliminated by micronucleus formation (*4*). We report that, in lymphocytes from normal individuals, more micronuclei are formed when the cells are grown in medium lacking folic acid and thymidine than when they are grown in medium containing either of these compounds.

Blood from healthy volunteers was used to establish lymphocyte cultures. Each culture contained blood (0.2 ml), phytohemagglutinin (0.1 ml), penicillin (100 U/ml), and 5 ml of minimum essential medium free of folic acid and thymidine and supplemented with 5 percent fetal bovine serum (*1*). Replicate cultures were grown in this medium or in the same medium containing folic acid (5 mg/liter) or thymidine (10 mg/liter). After 5 days micronucleus frequency reached a maximum (*4*) and the cells were harvested by standard techniques. Slides were randomized and coded and micronucleus frequency was determined by examining 500 interphase nuclei per culture. Only nuclei that showed evidence of blastic transformation were counted.

The average frequency of micronuclei in lymphocytes grown in medium without folic acid and thymidine was 4.4 percent, compared to 0.4 percent in medium with folic acid or thymidine (Table 1). Micronucleus frequency varied in accordance with the concentration of folic acid and thymidine in the culture medium (Fig. 1). The dose-response relation is very similar to that seen for the expression of fragile sites under identical culture conditions (*1*).

Table 1. Frequency of occurrence of micronuclei in cultured lymphocytes from normal volunteers. The lymphocytes were cultured for 5 days in minimum essential medium containing folic acid (FA; 5 mg/liter) or thymidine (T; 10 mg/liter), or neither. Values are percentages.

| Donor | Age | Sex | FA | T | Neither |
|---|---|---|---|---|---|
| 1 | 12 | F | 1.7 | 0.9 | 6.6 |
| 2 | 23 | M | 0.2 | 0.6 | 6.5 |
| 3 | 32 | F | 0.4 | 0.0 | 4.4 |
| 4 | 40 | F | 0.0 | 0.0 | 1.0 |
| 5 | 18 | F | 0.2 | 0.6 | 3.6 |
| 6 | 22 | F | 0.0 | 0.4 | 3.4 |
| 7 | 55 | F | 0.4 | 0.2 | 2.9 |
| 8 | 37 | F | 0.6 | 0.4 | 5.8 |
| 9 | 35 | M | 0.2 | 0.6 | 5.0 |
| Mean | | | 0.4 | 0.4 | 4.4 |

The influence of culture medium composition on spontaneous (*5*) and induced (*6*) chromosome breakage has been documented but has not generally been recognized to be of the magnitude reported here. An increase in the frequency of random and nonrandom gaps and breaks has been reported in chromosomes of lymphocytes cultured under conditions suitable for fragile site expression (*7–9*). Some of the more common nonrandom damage includes gaps and breaks at 1qter, 2q23, 3p14, 6q26, 9q13, and 13qter. These regions can mimic true fragile sites but have not been shown to be heritable or to give rise to the triradial figures that characterize fragile sites, and they are usually seen in only a small portion of chromosomes in metaphase (*9*). Investigators of chromosomal aberrations or micronucleus formation will need to take the concentration of folic acid and thymidine in the culture medium into account.

The suppression of chromosome damage in lymphocytes cultured under the same conditions that suppress the expression of fragile sites suggests that a similar mechanism underlies both the chromosome damage resulting from fragile sites and the apparently spontaneous damage exhibited by normal individuals. Expression of fragile sites probably depends on a reduced amount of thymidine monophosphate during the late stages of DNA synthesis. This can be achieved by reducing the concentration of folic acid and thymidine in the culture medium to virtually zero (*1*); by adding methotrexate, an inhibitor of folate metabolism, to folic acid–containing medium (*10*); or by adding fluorodeoxyuridine, an inhibitor of thymidylate synthetase, which converts uridine monophosphate to thymidine monophosphate (*11, 12*). This inhibition can be overcome by adding excess thymidine but not excess folic acid (*11*). Perhaps there is a general class of chromosome breakage, of which the heritable fragile sites are special cases, that results from a deficiency of DNA precursor substances. Further studies of the mechanism by which heritable fragile sites are expressed may lead to a wider understanding of the mechanisms of spontaneous chromosome damage.

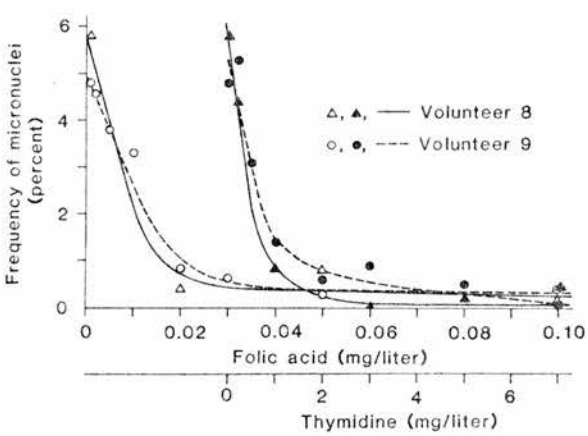



Fig. 1. Relation between micronucleus frequency in 5-day-old lymphocyte cultures and concentration of folic acid and thymidine in the medium for volunteers 8 and 9.

PETER B. JACKY
*Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital, North Adelaide, South Australia 5006, Australia*
BERND BEEK
*Institut für Genetik, Freie Universität Berlin, 1000 Berlin 33, Federal Republic of Germany*, and *Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital*
GRANT R. SUTHERLAND
*Cytogenetics Unit, Department of Histopathology, Adelaide Children's Hospital*

### References and Notes

1. G. R. Sutherland, *Am. J. Hum. Genet.* **31**, 125 (1979).
2. ———, *Int. Rev. Cytol.* **81**, 107 (1983).
3. J. A. Heddle, R. D. Benz, P. J. Countryman, in *Mutagen-Induced Chromosome Damage in Man*, H. J. Evans and D. C. Lloyd, Eds. (Edinburgh Univ. Press, Edinburgh, 1978), pp. 191–200.
4. B. Beek, P. B. Jacky, G. R. Sutherland, *Ann. Genet.*, in press.
5. G. Obe, B. Beek, R. Slacik-Erben, *Eur. Soc. Toxicol. Int. Congr. Ser. No. 376* (1976), pp. 118–126.
6. B. Beek and G. Obe, *Experientia* **30**, 1006 (1974).
7. M. Jennings, J. G. Hall, H. Hoehn, *Am. J. Med. Genet.* **7**, 417 (1980).
8. R. H. Martin, C. C. Lin, B. J. Mathies, R. B. Lowry, *ibid.*, p. 433.
9. M. A. Leversha, G. C. Webb, S. M. Pavey, *Lancet* **1981-I**, 49 (1981).
10. C. Fonatsch, *Hum. Genet.* **59**, 186 (1981).
11. T. W. Glover, *Am. J. Hum. Genet.* **33**, 234 (1981).
12. N. Tommerup, H. Poulsen, K. Brøndum-Nielsen, *J. Med. Genet.* **18**, 374 (1981).

29 September 1982

# REGIONAL LOCALISATION FOR HLA BY RECOMBINATION WITH A FRAGILE SITE AT 6p23

J.C. Mulley[1]

Judith Hay[2]

L.J.Sheffield[3]

and

G.R.Sutherland[1]

1 Department of Histopathology,
Adelaide Children's Hospital,
North Adelaide, S.A. 5006,
Australia.

2 Australian Red Cross Society,
Blood Transfusion Service,
Adelaide, S.A. 5000,
Australia.

3 Medical Genetics and Epidemiology Unit,
Adelaide Children's Hospital,
North Adelaide, S.A. 5000,
Australia.

SUMMARY

A family with a fragile site on chromosome 6 at band p23 was examined for recombination between the fragile site and HLA. Recombination was observed in four of the 20 offspring in whom it could occur. The estimate of the genetic length of chromosome between the fragile site and HLA is 20 centimorgans with a lower 95 percent probability limit of 8.5 centimorgans. The likely regional localisation for HLA is proximal to about the midpoint of 6p22. The basis for exclusion of 6p23 and the distal half of 6p22 is that fragile sites do not measurably disrupt recombination frequency and that the "allele" predisposing to the expression of a fragile site is situated at the fragile site. Conversely, the validity of these assumptions is reinforced given the general agreement of this HLA localisation with methods that do not involve recombination with the fragile site.

## INTRODUCTION

The regional localisation for HLA on chromosome 6 between p21.2 and p23 is well established (1). There is evidence from exclusion mapping for its elimination from the distal portion of this interval (2,3,4). A family segregating for a fragile site in the distal region of band p23 on chromosome 6 provided an opportunity to localise HLA by measuring the genetic distance between these markers. Given a proximal limit for HLA at 6p21.2, the distance between HLA and the fragile site must be less than 25 map units according to the male meiotic map presented by Cook et al (5). Hence, HLA and this fragile site can be assumed to be linked.

## MATERIALS AND METHODS

The family was examined for folate sensitive fragile sites by standard procedures. Part of this family and the fragile site have been previously described (6). At least 50 metaphases were routinely scored for the presence of fragile sites. HLA haplotypes at the A, B and C loci were determined by the microcytotoxicity test.

RESULTS

Four generations were available for study (Fig.1). The fragile site at 6p23 was detected without difficulty in every generation. Eight carriers were detected and one untested individual (II.11) was an obligate carrier. It is highly improbable that the other untested, unrelated individuals (I.3, II.4, II.12) would carry the fragile site since this is the only family in which this fragile site has been recorded. The frequency of expression was 44% from the propositus (II.13), and ranged from seven to 30 percent of cells for the other carriers. For the purpose of linkage analysis the fragile site in this family may be regarded as a heritable condominant variant.

The inheritance of the fragile site and HLA haplotypes are shown in Fig.1. HLA haplotypes have been coded as indicated in Table 1. The HLA phenotypes could be inferred in the untested individuals as 7/8 in I.3, 6/? in II.4, 2/8 in II.11 and 11/12 in II.12. There were three definite non-crossovers (NCO) in generation IV. There were seven definite NCO and two definite crossovers (CO) in generation III. Given prior knowledge of linkage, it is highly probable that there are five NCO and two CO in generation II rather than five CO and two NCO.

The combined male and female recombination frequency ($\Theta$) is 20% (4/20), well within the maximum expected male recombination frequency of less than 25%. The recombination frequency is equivalent to map distance expressed as centimorgans (cM) because the relationship between map distance and $\Theta$ is approximately linear up to $\Theta = 0.25$. The lower 95% probability limit for this interval is 8.5 cM and was determined by subtracting 5% of the area under the truncated relative probability curve from one end of the curve. The curve was truncated at 6p21.2.

Given that the female meiotic map is about twice the length of the male meiotic map, the estimate of 20 cM for the interval between HLA and the fragile site at 6p23 is probably an over estimate for comparison with a map expressed in male cM. The maximum lod score was 1.7 at $\Theta = 0.2$. These results suggest a probable regional localisation for HLA between about the mid-point of 6p22 (near to 6p22.2) and the previously known proximal limit for HLA at 6p21.2. HLA is not tightly linked to the fragile site and is unlikely to lie within 6p23 or the distal half of 6p22 (Fig.2).

## DISCUSSION

Considerable information already exists for the regional localisation of HLA. Family study with a translocation t(6;21) (p22;q11) suggested the probable localisation of HLA proximal to 6p22 (2). A translocation family with t(6;20) (p21;p13) demonstrated close linkage ($\Theta = 0.05$) between the breakpoint (in 6p21 near 6p22) and HLA (3). While the effect of reciprocal translocations on recombination is not definitely known there is unlikely to be any major disruption (7). Ferrando et al. (4) reported the absence of a duplicated set of parental HLA antigens in a child partially trisomic for 6p22.2→pter derived from a balanced translocation t(6;10) (p22.2;pter). This excluded HLA from 6p22.2→pter. These results suggest a regional localisation for HLA proximal to 6p22.2 which agrees very closely with the distal 95% probability limit derived from recombination with the fragile site at 6p23. Berger et al. (8) (and personal communication) proposed a more precise localisation for HLA:

suggesting that a chromosomal break at 6p21.2 or at the interface of 6p21.2 and 6p21.3 was within the HLA cluster.

There is no evidence for any major disruption to recombination in the chromosomal segments near the loci of expression of fragile sites. The intervals between the fragile sites at 10q23 and 10q25 in families with both fragile sites (9) and now between HLA and the fragile site at 6p23, have been estimated by recombination and fall within limits established by other procedures. HP and the fragile site at 16q22 are probably as close to each other as is indicated by the low frequency of recombination between them (10).

The alleles responsible for the expression of fragile sites are probably situated on the chromosomes at or very near to the fragile site. This has been verified for the fragile sites at 10q23, 10q25 (9) and now 6p23. The interval between genes responsible for the expression of fragile sites at 10q23 and 10q25 was estimated by recombination and is consistent with that expected from their locations on mitotic chromosomes. A positive lod score between HLA and the fragile site at 6p23 is consistent with existing knowledge of the location of HLA and the position of the fragile site at 6p23. Control of fragile site expression must be within the homologue expressing the fragile site since confirmed heterozygous carriers do not express fragile sites in both homologues.

The rapid rate of discovery of fragile sites (6) indicates that many more of these are likely to exist and be used for linkage studies. Fragile sites have advantages over other chromosomal variants because they are not restricted to paracentromeric regions.

Because they are precisely mapped by banding, fragile sites are ideal both for use in searching for linkages with unassigned markers in chromosome regions devoid of polymorphic genetic markers, and for the regional localisation of markers shown to be near regions of fragile site expression. The fragile site at 6p23 confirms a more exact regional localisation for HLA and consequently the cluster of genes that are known to be linked to HLA. Confirmation that the location of DNA responsible for a fragile site is at the locus of expression may now permit the characterisation of this DNA by study of the relevant chromosomal segments.

ACKNOWLEDGEMENTS

We wish to thank Sharon Bain, Elizabeth Baker, Helen Eyre, Lily Gowans, Louise Maland, Cathy Orell and Erica Woollatt for technical assistance with the cytogenetics, and Greg Bennett and Ann Sheldon for technical assistance with HLA typing. The National Health and Medical Research Council of Australia and the Channel 10 Children's Medical Research Foundation of Australia provided support.

REFERENCES

Human Gene Mapping 6 (1981). Sixth International Workshop on Human Gene Mapping. Cytogenet. Cell Genet. 32: Nos. 1-4, 1982. 341 pages.

Borgaonkar, D S and Bias, W B (1974). HLA loci and chromosome 6. Cytogenet. Cell Genet. 13: 67-68.

Breuning, M H, van den Berg-Loonen, E M, Bernini, L F, Bijlsma, J B, van Loghem E, Meera Khan, P and Nijenhuis L E (1977). Localisation of HLA on the short arm of chromosome 6. Hum. Genet. 37: 131-139.

Ferrando, P, San Román, C, Rodriguez de Cordoba, S and Arnaiz - Villena, A (1981). Partial trisomy 6p:46, XX, -10, der(10), t(6;10)(p22;q26)pat and HLA localisation.

Cook, P J L, Noades J E, Loman C G, Buckton K E and Robson, E B (1980). Exclusion mapping illustrated by the MNSs blood group. Ann. Hum. Genet. Lond. 44: 61-73.

Sutherland, G R, Jacky, P B, Baker E and Manuel, A (1983). Heritable fragile sites on chromosomes. X. New folate sensitive fragile sites : 6p23, 9p21, 9q32, 11q23. Amer. J. Hum. Genet. (In press).

Cook, P L J, Robson E B, Buckton K E, Jacobs, P A and Polani, P E (1974). Segregation of genetic markers in families with chromosome polymorphisms and structural rearrangements involving chromosome 1. Ann. Hum. Genet. Lond. 37: 261-274.

Berger, R, Bernheim, A, Sasportes, M. Hauptmann, G, Hors, J, Legrand L and Fellous, M. (1979). Regional mapping of the HLA on the short arm of chromosome 6. Clin. Genet. 15: 245-251.

References continued.....

Sutherland, G R, Baker, E and Mulley, J C (1982). Genetic length of a human chromosomal segment measured by recombination between two fragile sites. Science. 217: 373-374.

Magenis, R E, Hecht, F and Lovrien, E W (1970). Heritable fragile site on chromosome 16: Probable localisation of haptoglobin locus in man. Science.170: 85-87.

TABLE 1

HLA HAPLOTYPE CODE

| CODE | HAPLOTYPE |
|---|---|
| 1 | Aw31, Bw57, Bw4, Cw6 |
| 2 | A1, Bw49, Bw4 |
| 3 | A1, B8, Bw6 |
| 4 | Aw32, B27, Bw4, Cw1 |
| 5 | A2, Bw44, Bw4, Cw5 |
| 6 | A11, Bw22, Bw6, Cw3 |
| 7 | A29, B7, Cw6 |
| 8 | A3, B7, Bw6 |
| 9 | A26, Bw41, Bw6 |
| 10 | Aw33, B14 |
| 11 | Aw30, B18, Bw6, Cw5 |
| 12 | A2, Bw35, Bw6, Cw4 |
| 13 | A28, B27 |

Fig.1. Inheritance of HLA and the fragile site at 6p23. See text for probable phenotypes of untested individuals.

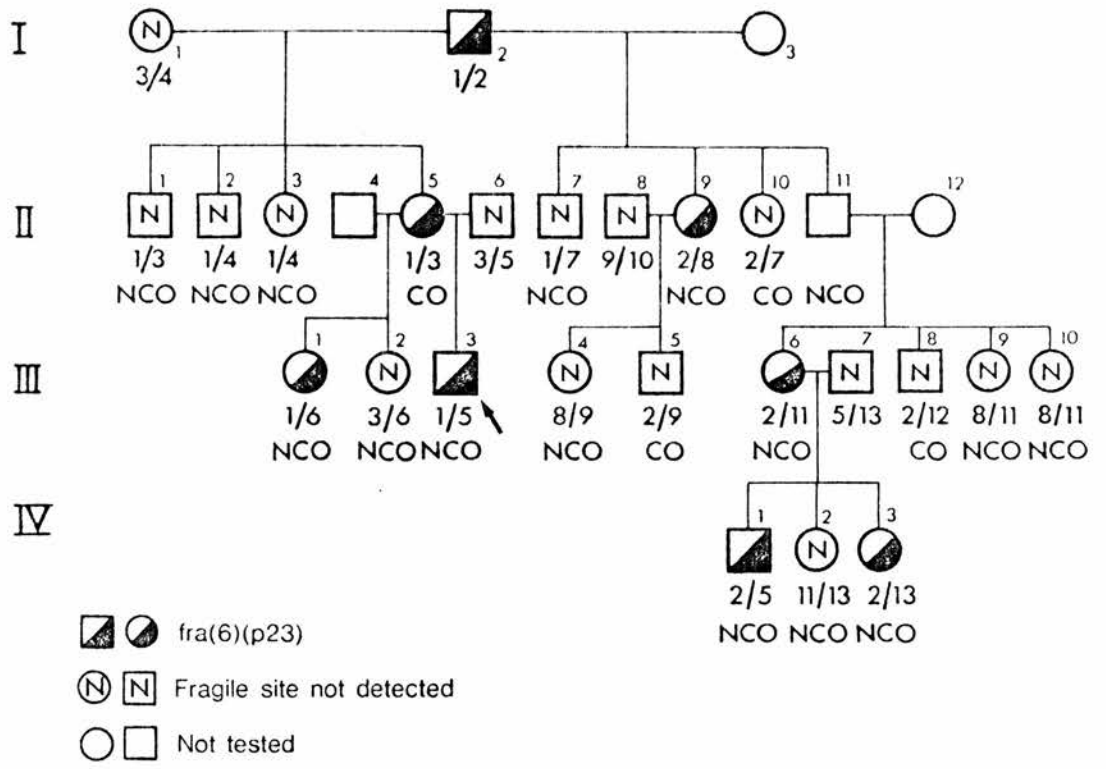
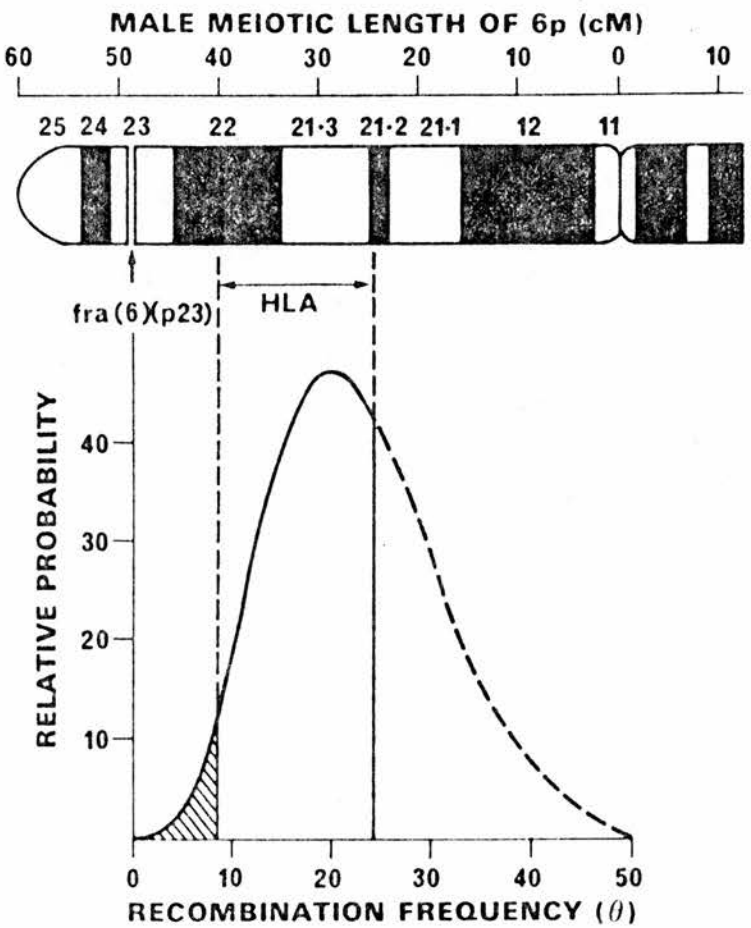


Fig.2 Probable localisation of HLA by recombination with the fragile site at 6p23.



In Press : Hum. Genet.

D 82

# A SEARCH FOR LINKAGE IN FAMILIES WITH FRAGILE SITES

John C. Mulley, Catherine Nicholls

and

Grant R. Sutherland

Department of Histopathology

Adelaide Children's Hospital

North Adelaide S.A. 5006

Australia

SUMMARY

Linkage relationships to unassigned and provisionally assigned genetic markers were examined from 53 families segregating for various fragile sites. Fragile sites were at Xq27, 2q13, 6p23, 9p21, 9q32, 10q23, 10q25, 11q13, 11q23, 12q13 and 16p12. No new assignments were made but extensive exclusion data is presented for the markers F13A, F13B, CHE2, K, TF, GPT, C3 and Lu arising from linkage comparisons with fragile sites and other genetic markers. Exclusions of at least 10cM involving fragile site markers were made for F13A (6p23, 9q32, 10q25), F13B (10q23, 10q25, 16p12), CHE2 (16p12), K (10q23), TF (10q23, 10q25, 11q13), GPT (10q23, 10q25, 11q13), C3 (10q23, 10q25) and Lu (10q25, 11q13).

## INTRODUCTION

Linkage analysis using a heritable chromosomal marker was responsible for the first autosomal gene assignment in man (Donahue et al., 1968). The common heteromorphisms of C-band chromatin and fluorescence intensity have since been used extensively for linkage analysis, but have not provided any new assignments (Ferguson-Smith and Aitken, 1982). Linkage data are now presented for families in which fragile sites are segregating.

## MATERIALS AND METHODS

Fifty-three families with fragile sites were analysed for linkage by the computer program LIPED (Ott, 1976). The fragile sites (number of families in parentheses) were at Xq27 (12), 2q13 (1), 6p23 (1), 9p21 (1), 9q32 (2), 10q23 (7), 10q25 (26), 11q13 (2), 11q23 (1), 12q13 (1) and 16p12 (2). Three families were segregating for more than one fragile site. The conditions necessary for expression of fragile sites have been described previously (Sutherland, 1979; Sutherland et al., 1980) and their G-band positions demonstrated (Sutherland, 1983). In those individuals in whom the fragile sites were not detected at least 50 metaphases were usually examined for fragile sites. Genetic marker nomenclature is in accord with Shows and McAlpine (1982).

Penetrance of the autosomal fragile sites is apparently complete in most instances, however minor anomalies were observed in five families. The 9q32 site was present in a single individual and could not be detected in either of his parents. The two 11q13 families did not

show the fragile site in an obligate carrier parent at three and four generations back, respectively. Among offspring of these non-expressing parents were four carriers from eight, and six carriers from 12, suggesting that non-expression is not a major problem. The 10q25 fragile site could not be demonstrated in parents of a child who carried the fragile site in one instance among 26 pedigrees with this fragile site. The site at 16p12 was not detected in parents of a child who carried the fragile site in one two-generation family but there was no difficulty in detecting this fragile site from another large kindred of three generations.

## RESULTS

Results of linkage tests with fragile sites are given in Table 1. Lod scores are presented for the comparisons between fragile sites and unassigned or provisionally assigned test loci. Lod scores for comparison between the test loci of Table 1 and all other informative markers are given in Table 2. This data was derived from families in which fragile sites were detected irrespective of whether the family was informative for linkage with the fragile site.

## DISCUSSION

No measurable disruption occurs to recombination in chromosomal segments adjacent to the fragile sites at 10q23, 10q25 and 6p23 (Sutherland et al., 1982; Mulley et al., 1983). There is unlikely to be any disruption to recombination anywhere else in the genome of individuals in whom these fragile sites have been demonstrated because the genetic material

responsible for fragile site expression is at the fragile site locus (Mulley et al., 1983). It is reasonable to assume that fragile sites do not affect recombination and that lod scores are unbiased from all families in which fragile sites are segregating.

The loci F13A, F13B, CHE2, K and TF are unassigned (Human Gene Mapping 6, 1982). C3 and Lu are provisionally placed on chromosome 19 and GPT is inconsistently assigned to chromosomes 8 and 16 (Shows and McAlpine, 1982). There is no evidence of an assignment for any of these loci (Tables 1 and 2), but the information presented represents a considerable addition to the exclusion map. Exclusion data was only presented where lod scores were less than -2 at $\theta = 0.05$, representing an exclusion from at least 10cM of chromosome. Such data were not presented where previous exclusion data (Keats, 1981; Keat et al., 1979) had already provided an exclusion to at least $\theta = 0.2$. The value of exclusion data to gene mapping has been clearly demonstrated by Cook et al. (1980) and by many instances of regional localisation following gene asssignment.

Few lod scores were positive and these were only presented if they reached +1. The F13A:C3 comparison almost reached +1 at $\theta = 0.1$. The CHE2:HP comparison reached +1 at $\theta = 0.05$, however the provisional status given to the CHE2:HP linkage (Shows and McAlpine, 1979) is no longer recognised (Shows and McAlpine, 1982). The GPT:PGP comparison reached +1.7 at $\theta = 0.2$. These observations are not sufficient evidence to suggest linkage.

In contrast to many polymorphic markers, the locations of fragile sites on banded chromosomes are precisely defined. Herein lies their value as markers for use in linkage tests. Fragile sites are not commonly used in linkage analysis because of their rarity. Their potential for gene assignment is largely restricted to the mapping of loci not expressed in somatic cells.

ACKNOWLEDGEMENTS

We wish to thank Sharon Bain, Elizabeth Baker, Helen Eyre, Trudy Hocking, Louise Maland, Cathy Orell and Erica Woollatt for technical assistance with the cytogenetics.
The National Health and Medical Research Council of Australia and the Channel 10 Children's Medical Research Foundation provided support.

REFERENCES

Cook, P.J.L., Noades, J.E., Lomas, C.G., Buckton, K.E. and Robson, E.B. (1980). Exclusion mapping illustrated by the MNS blood group. Ann. Hum. Genet. Lond. 44: 61-73.

Donahue, R.P., Bias, W.B., Renwick, J.H. and McKusick, V.A. (1968). Probable assignment of the Duffy blood group locus to chromosome 1 in man. Proc. Natl. Acad. Sci. USA 61: 949-955.

Ferguson-Smith, M.A. and Aitken, D.A. (1982). The contribution of chromosome aberrations to the precision of human gene mapping. Cytogenet. Cell Genet. 32: 24-42.

Human Gene Mapping 6 (1982). Oslo Conference (1981). International Workshop on Human Gene Mapping. Birth Defects: Original Article Series Vol. 18, No. 2.

Keats, B.J.B. (1981). Linkage and chromosome mapping in man. University Press of Hawaii, Honolulu.

Keats, B.J.B., Morton, N.E., Rao, D.C. and Williams, W.R. (1979). A Source Book for Linkage in Man. Johns Hopkins University Press, Balitmore and London.

Mulley, J.C., Hay, J., Sheffield, L.J. and Sutherland, G.R. (1983). Regional localisation for HLA by recombination with a fragile site at 6p23. Am. J. Hum. Genet. (in press).

Ott, J. (1976). Computer program for linkage analysis of general human pedigrees. Am. J. Hum. Genet. 28: 528-529.

Shows, T.B. and McAlpine, P.J. (1979). The 1979 catalog of human genes and chromosome assignments. Cytogenet. Cell Genet. 25: 117-127.

Shows, T.B. and McAlpine, P.J. (1982). The 1981 catalog of assigned human genetic markers and report of the nomenclature committee. Cytogenet. Cell Genet. 32: 221-245.

Sutherland, G.R. (1979). Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. Am. J. Hum. Genet. 31: 125-135.

Sutherland, G.R. (1983). The fragile X chromosome. Int. Rev. Cytol. 81: 107-143.

Sutherland, G.R., Baker, E. and Mulley, J.C. (1982). Genetic length of a human chromosomal segment measured by recombination between two fragile sites. Science 217: 373-374.

Sutherland, G.R., Baker, E. and Seshadri, R.S. (1980). Heritable fragile sites on human chromosomes. V. A new class of fragile site requiring BrdU for expression. Am. J. Hum. Genet. 32: 542-548.

TABLE 1

LOD SCORES BETWEEN SELECTED MARKERS AND FRAGILE SITES

| TEST LOCUS | MARKER LOCUS | θ | | |
|---|---|---|---|---|
| | | .05 | .1 | .2 |
| F13A | 6p23 | -3.8 | -2.2 | -0.9 |
| | 9q32 | -2.0 | -1.4 | -0.8 |
| | 10q25 | -6.4 | -3.5 | -0.9 |
| F13B | 10q23 | -2.0 | -1.2 | -0.5 |
| | 10q25 | -4.4 | -2.4 | -0.7 |
| | 16p12 | -2.5 | -1.4 | -0.6 |
| CHE2 | 16p12 | -1.9 | -1.1 | -0.5 |
| K | 10q23 | -2.8 | -2.0 | -1.2 |
| TF | 10q23 | -2.6 | -1.4 | -0.4 |
| | 10q25 | -3.6 | -1.8 | -0.4 |
| | 11q13 | -5.1 | -2.9 | -1.0 |
| GPT | 10q23 | -7.1 | -4.7 | -2.2 |
| | 10q25 | -11.2 | -6.5 | -2.3 |
| | 11q13 | -4.2 | -2.4 | -0.9 |
| C3 | 10q23 | -4.8 | -2.6 | -0.8 |
| | 10q25 | -7.2 | -3.7 | -0.9 |
| Lu | 10q25 | -1.9 | -1.2 | -0.6 |
| | 11q13 | -2.6 | -1.6 | -0.7 |

TABLE 2

LOD SCORES BETWEEN SELECTED MARKERS AND NON-FRAGILE SITE MARKERS

| TEST LOCUS | MARKER LOCUS | θ | | | TEST LOCUS | MARKER LOCUS | θ | | |
|---|---|---|---|---|---|---|---|---|---|
| | | .05 | .1 | .2 | | | .05 | .1 | .2 |
| F13A | F13B | -2.0 | -0.8 | -0.1 | TF | GPT | -14.4 | -9.2 | -4.4 |
| | TF | -4.5 | -3.0 | -1.6 | | Lu | -2.8 | -1.5 | -0.5 |
| | PI | -11.7 | -6.9 | -2.9 | | PI | -16.2 | -9.1 | -3.0 |
| | GPT | -7.6 | -4.0 | -1.1 | | PGP | -4.1 | -1.9 | -1.0 |
| | C3 | 0.3 | 0.9 | 0.8 | | AK1 | -2.7 | -1.4 | -0.4 |
| | Jk | -2.6 | -1.3 | -0.4 | | ADA | -2.2 | -1.3 | -0.5 |
| | MNS | -9.6 | -5.3 | -1.9 | | ESD | -4.8 | -2.8 | -1.0 |
| | PGP | -2.3 | -1.4 | -0.6 | | GALT | -6.3 | -3.8 | -1.6 |
| | ACP1 | -3.8 | -1.5 | 0.1 | | GLO1 | -5.6 | -2.0 | -0.4 |
| | ESD | -2.0 | -0.6 | 0.3 | CHE2 | PI | -1.9 | -1.2 | -0.6 |
| | PGM1 | -2.7 | -1.6 | -0.7 | | HP | 1.5 | 1.4 | 1.0 |
| | GALT | -2.0 | -1.3 | -0.6 | | GC | -2.3 | -1.4 | -0.5 |
| | GLO1 | -2.8 | -0.8 | 0.4 | | | | | |
| | HP | -7.7 | -4.1 | -1.2 | GPT | PGP | -1.9 | 1.2 | 1.7 |
| | GC | -4.8 | -1.6 | 0.3 | | ESD | -2.8 | -0.9 | 0.4 |
| | ABO | -4.3 | -2.7 | -1.3 | | GALT | -7.1 | -4.0 | -1.6 |
| | Rh | -4.0 | -2.0 | -0.6 | C3 | PI | -11.0 | -6.0 | -2.0 |
| F13B | TF | -3.1 | -1.8 | -0.7 | | Jk | -3.3 | -2.0 | -0.7 |
| | GPT | -7.3 | -4.2 | -1.5 | | Lu | -2.6 | -1.5 | -0.5 |
| | C3 | -3.2 | -2.0 | -0.9 | | PGP | -4.4 | -2.0 | -0.3 |
| | CHE2 | -2.5 | -1.7 | -0.9 | | ESD | -7.6 | -3.2 | -0.6 |
| | PI | -11.1 | -6.6 | -2.7 | | GALT | -1.9 | -0.8 | 0.0 |
| | Jk | -2.4 | -1.6 | -0.8 | | GLO1 | -4.1 | -2.1 | -0.5 |
| | Lu | -2.2 | -1.4 | -0.7 | Lu | PI | -5.0 | -3.3 | -1.7 |
| | MNS | -12.5 | -7.3 | -2.9 | | Jk | -3.8 | -2.4 | -1.0 |
| | ACP1 | -6.9 | -4.3 | -1.9 | | ACP1 | -4.8 | -2.7 | -1.0 |
| | PGM1 | -5.6 | -3.3 | -0.9 | | GLO1 | -2.0 | -1.2 | -0.5 |
| | GLO1 | -4.6 | -2.3 | -0.8 | | GC | -4.4 | -2.6 | -1.0 |
| | HP | -2.8 | -1.0 | 0.0 | | | | | |
| | GC | -13.0 | -7.4 | -2.7 | | | | | |
| | ABO | -3.6 | -2.0 | -0.7 | | | | | |
| | Rh | -3.0 | -1.7 | -0.6 | | | | | |
| | Fy | -2.7 | -1.7 | -0.8 | | | | | |
| K | GLO1 | -2.0 | -1.2 | -0.6 | | | | | |
| | GPT | -1.9 | -1.1 | -0.4 | | | | | |

In Press : Am J Hum Genet

D 83

# THYMIDYLATE SYNTHETASE INHIBITORS AND FRAGILE SITE EXPRESSION IN LYMPHOCYTES

Peter B. Jacky and Grant R. Sutherland

Cytogenetics Unit

Department of Histopathology

Adelaide Children's Hospital

North Adelaide S.A. 5066

Australia

SUMMARY

The ability of three thymidylate synthetase inhibitors, fluorodeoxyuridine, fluorodeoxycytidine and trifluorothymidine, to induce the expression of eight different folate sensitive fragile sites has been investigated in 22 patients and compared with the efficacy of simple folate deprivation for inducing fragile site expression. Fluorodeoxyuridine and fluorodeoxycytidine were equal in their ability to elicit fragile site expression but fluorodeoxycytidine proved less cytotoxic under comparable culture conditions. Both fluorodeoxyuridine and fluorodeoxycytidine were found to be more efficient than trifluorothymidine at comparable concentrations but less efficient than simple folate deprivation in eliciting fragile site expression in lymphocytes. Since the three inhibitors induced expression of eight different folate sensitive fragile sites it is likely that all folate sensitive fragile sites have a common underlying mechanism of expression. The practical application of thymidylate synthetase inhibitors in the routine detection of heritable fragile sites is discussed.

## INTRODUCTION

The fragile X chromosome and other heritable folate sensitive fragile sites are expressed in lymphocytes cultured under conditions which lead to a relative deficiency of thymidine monophosphate for DNA synthesis. This can be achieved by culture in the absence of folic acid and thymidine or by the presence of inhibitors of folate metabolism such as methotrexate [1]. More recently it has been shown [2,3] that the thymidylate synthetase (TS) inhibitor 5-fluorodeoxyuridine (FUdR) can induce fragile sites in the presence of folic acid but not in the presence of thymidine. The ability of other TS inhibitors, 5-fluorodeoxycytidine (FCdR) and trifluorothymidine (F3TdR), to induce fragile sites has been investigated and compared with the efficacy of simple folate deprivation for the induction of fragile sites.

## MATERIALS AND METHODS

Patients and Lymphocyte Culture. Lymphocyte cultures were established from 22 individuals each carrying a folate sensitive fragile site (Table 1). For each individual whole blood cultures were established in Eagle's Minimal Essential Medium without folic acid (FA) and thymidine (MEM-FA) [1] and RPMI 1640 medium (GIBCO) containing 1.0 mg/l FA but lacking thymidine, and Ham's F10 medium (CSL) containing 1.32 mg/l FA and 0.73 mg/l thymidine. Culture media were routinely supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 2% M-PHA (GIBCO) at culture initiation. Cultures were harvested after 72 or 96 hours using standard methods following exposure to 1 mg/l colchicine for one hour. Where possible 50 unbanded metaphases per culture were scored for fragile sites.

TS Inhibitors. Three specific TS inhibitors were studied, FUdR (Sigma) and FCdR (Calbiochem-Behring) were tested at concentrations from 0.001 to 1.0 mg/l, and F3TdR (Sigma) was tested from 0.005 to 1.0 mg/l. All substances were dissolved in distilled water and added to the cultures 24 hours prior to chromosome harvest in keeping with the earlier recommendations of Glover [2] and Tommerup et al. [3,4] for FUdR. Initial studies indicated that there was no difference in frequency of expression between cultures harvested 72 or 96 hours after initiation. Thus most of this work was on 72 hour cultures.

## RESULTS

In order to determine an optimal concentration and culture medium each TS inhibitor was first tested for six patients over a range of concentrations in different culture media. For three patients (Patients B, G and M) any of the TS inhibitors, even at relatively low concentrations, studied in MEM-FA were found to be too cytotoxic for chromosome analysis. Results for each of the substances at varying concentrations in RPMI 1640 were presented in Table 2. When less than 50 metaphases were available for scoring this was an indicator of the toxicity of the inhibitor. While FUdR and FCdR proved equally effective in their ability to elicit fragile site expression, FCdR was generally found to be more useful because of its lower toxicity (Table 2). F3TdR was found to be less cytotoxic than either FUdR or FCdR at similar concentrations but also less effective in eliciting fragile site expression. There was no difference in the ability of the inhibitors to induce fragile sites in either RPMI 1640 or Ham's F10 in five patients tested with both media.

Results comparing maximum frequencies of fragile site expression obtained for each patient in MEM-FA and RPMI 1640 plus FUdR or FCdR are presented in Table 1. Maximum frequencies of expression were generally obtained at inhibitor concentrations of 0.01 to 0.05 mg/l (Table 2). Eight of the 22 patients studied showed differences in frequencies of expression when comparing MEM-FA with RPMI 1640 + FUdR (Patients C, D, E, F, K, O, R, S). With the exception of Patient K, these frequencies were all higher in MEM-FA. In comparisons between FUdR and FCdR frequencies of expression were considered to be different in four of the eight patients studied. Patients K and L showed higher frequencies with FUdR while Patients B and E showed higher frequencies with FCdR. In five patients with the fra(X) and one with the fra(11)(q13) studied with F3TdR, frequencies of expression were consistently lower (maximum frequency of expression seen was 10%) using this TS inhibitor than those obtained either with FUdR or FCdR.

Lymphocytes from three fra(X) patients (N, P, T) were also tested using RPMI 1640 plus 10 mg/l methotrexate (Lederle) according to the recommended protocols of Sutherland [1] and Fonatsch [5] for eliciting fragile site expression. Frequences of expression for all three patients (N, 29%; P, 13%; T, 16%) using this culture condition were comparable to those obtained with MEM-FA.

In an attempt to confirm the report of Brookwell et al. [6] of enhanced frequencies of fragile site expression using FUdR for blood samples delayed in transit, samples from five fragile site patients were stored at 4°C for up to nine days after venepuncture and periodic cultures were initiated in MEM-FA and RPMI 1640 plus 0.01 mg/l FUdR (Table 3). For two of these five patients (A, T) the frequencies of expression

under either culture condition were not different for cultures initiated as much as five days after venepuncture. Patient P showed a decrease in frequencies of expression for both culture conditions with increasing storage time, and Patient Q showed little change in the frequency of expression in MEM-FA and a decrease in the frequency of expression in RPMI 1640 + FUdR over seven days in storage. For a similar period of storage Patient M showed a decrease in frequency of expression in MEM-FA and an increase in frequency of expression using FUdR (Table 3). Study of this problem is complicated by a deterioration in the proliferative potential of lymphocytes with extended periods in storage.

## DISCUSSION

The use of a TS inhibitor to induce fragile sites on chromosomes was proposed and demonstrated independently by Glover [2] and Tommerup et al. [3]. The basis of this was that the conditions found by Sutherland [1] to be essential for fragile site expression led to thymidylate depletion in tissue culture. The first TS inhibitor used was FUdR [2-4] and Tommerup also reported using F3TdR [7]. FCdR was studied after the suggestion of Kalman (personal communication and [8]) that it was an effective TS inhibitor which was less cytotoxic than FUdR. The cytotoxicity of FUdR is probably due to its partial degradation to 5-fluorouracil which directly interferes with cellular RNA metabolism [8,9]. Finding no difference in the ability of TS inhibitors to induce fragile sites in either RPMI 1640 or Hams' F10 is not consistent with earlier reported methods stressing the use of thymidine free medium (i.e. RPMI 1640) with TS inhibitors for eliciting folate sensitive fragile sites [2]. However, varying amounts of folic acid and thymidine in culture media probably result in different concentrations of inhibitors required to induce fragile sites and result in differences in inhibitor cytotoxicity in different tissue culture media.

The TS inhibitor FUdR has proved essential for the demonstration of the fragile X in lymphoblastoid cell lines [10]. It may be more reliable than earlier reported methods [1] for demonstration of the fragile X in fibroblasts [2,4] and it has proved useful for demonstration of the fragile X in cultured amniotic fluid cells [12,13]. FCdR has not previously been shown to induce folate sensitive fragile sites and in view of its lower toxicity may be more useful than FUdR for fragile site induction in lymphoblastoid and fibroblast cell lines. This is the first demonstration that TS inhibitors will induce folate sensitive fragile sites other than the fragile X, fra(11)(q13) and fra(12)(q13) [7] and is further evidence that all the folate sensitive fragile sites likely share a common underlying mechanism of expression.

We were unable to support the findings of Brookwell et al. [6] that FUdR when added to TC199, folate free TC199 or Ham's F10 enhanced fragile site expression by about 30% compared to simply culturing cells in TC199 or in folate free TC199. A possible explanation for this could be that in the present study RPMI 1640 medium was the culture medium in which TS inhibitors were used from fragile site induction whilst folate free conditions were provided by MEM-FA, MEM-FA in combination with TS inhibitors was found unsatisfactory for adequate culture growth and chromosome analysis. However, MEM-FA and TC199 have been found to work equally well for fragile site induction [14] and others have used various folate free media with equal satisfaction (e.g. "M" medium, [15]). Similarly, we were unable to substantiate the claim of Brookwell et al. [6] that TS inhibitors produce a fragile X frequency of expression five times greater than folate free medium for blood samples delayed in transit. The present study did not involve bloods delayed in transit and

storage at 4°C may not adequately simulate an in transit delay. However, the data of Brookwell et al. [6] pertained only to four samples of blood. This potentially useful application of TS inhibitors requires further examination.

Little has been reported in the way of direct comparisons between frequencies of fragile site expression induced by TS inhibitors and frequencies possible with other folate antagonists such as methotrexate or aminopterin. These latter substances actively inhibit conversion of dihydrofolate to tetrahydrofolate by competitively inhibiting dihydrofolate reductase (DHFR) and metabolically affect a folate deficiency [1,14,15]. For the limited number of patients tested with methotrexate in this study, frequencies of fragile site expression were all comparable to those obtained using either MEM-FA or TS inhibitor culture conditions. Sutherland [1] found frequencies of expression of seven different fragile sites to be lower when induced with methotrexate than by folate deprivation with MEM-FA. Unfortunately he cultured cells in Ham's F10 for induction with methotrexate and since this medium contains thymidine it would be expected to result in lower frequencies of fragile site expression than whan a thymidine free medium such as RPMI 1640 or Dulbecco's Modified Eagle medium [5] is used. Cytotoxicity problems similar to those associated with TS inhibitors have also been described using DHFR inhibitors [16,17] and further comparison between such substances and TS inhibitors or simple folate deprivation are required.

There is no doubt that TS inhibitors will induce expression of the folate sensitive fragile sites and that it would appear that FCdR is the inhibitor of choice, at least in lymphocyte cultures, because of its lower toxicity. Nevertheless the use of simple folate deficient culture medium is equally, if not more, effective in demonstrating folate sensitive fragile sites, both in terms of maximising frequencies of expression and for yielding sufficient metaphases for chromosome analysis, and from a practical point of view this provides for an easier laboratory routine. These new findings with FCdR are consistent with earlier reported findings concerning the metabolic requirements for fragile site expression, namely in creating a relative deficiency in thymidylate.
The mechanism operating between meeting such a tissue culture requirement and the actual expression of the lesion on the chromosome remains to be elucidated.

ACKNOWLEDGEMENTS

We thank Elizabeth Baker, Sharon Bain, Helen Eyre, Trudy Hocking, Louise Maland, Cathy Orell and Erica Woollatt for assistance with the cytogenetics. This work was suppored by grants from The Adelaide Children's Hospital Research Trust, the Anti-Cancer Foundation of the Universities of South Australia and the National Health and Medical Research Council of Australia.

REFERENCES

1. Sutherland GR: Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. Am J Hum Genet 31: 125-135, 1979.

2. Glover TW: FUdR induction of the X chromosome fragile site : Evidence for the mechanism of folic acid and thymidine inhibition. Am J Hum Genet 33: 234-242, 1981.

3. Tommerup N, Poulsen H, Brøndum-Nielsen K: 5-fluoro-2'deoxyuridine induction of the fragile site on Xq28 associated with X-linked mental retardation. J Med Genet 18: 374-376, 1981.

4. Tommerup N, Nielsen KB, Mikkelsen M: Marker X chromosome induction in fibroblasts by FUdR. Am J Med Genet 9: 263-264, 1981.

5. Fonatsch C: A simple method to demonstrate the fragile X chromosome in fibroblasts. Hum Genet 59: 186, 1981.

6. Brookwell R, Daniel A, Turner G, Fishburn J: The fragile X(q27) form of X-linked mental retardation : FUdR as an inducing agent for fra(X)(q27) expression in lymphocytes, fibroblasts, and amniocytes. Am J Med Genet 13: 139-148, 1982.

7. Hecht F, Jacky PB, Sutherland GR: The fragile X chromosome : Current methods. Am J Med Genet 11: 489-495, 1982.

8. Kalman TI, Goldman D: Inactivation of thymidylate synthetase by a novel mechanism-based enzyme inhibitor : 1-(β-D-2'-deoxyribofuranosyl) 8-azapurin-2-one 5'-monophosphate. Biochem Biophysic Res Comm 102: 682-689, 1981.

9. Newman EM, Santi DV: Metabolism and mechanism of action of 5-fluorodeoxycytidine. PNAS 79: 6419-6423, 1982.

10. Jacobs PA, Hunt PA, Mayer M, Wang J-C, Boss GR, Erbe RW: Expression of the marker (X)(q28) in lymphoblastoid cell lines. Am J Hum Genet 34: 552-557, 1982.

11. Jacky PB, Dill FJ: Expression on fibroblast culture of the satellited-X chromosome associated with familial sex-linked mental retardation. Hum Genet 53: 267-269, 1980.

12. Jenkins EC, Brown WT, Duncan CJ, Brooks J, Ben-Yishay M, Giordano FM, Nitowsky HM: Feasibility of fragile X chromosome prenatal diagnosis demonstrated. Lancet ii: 1292, 1981.

13. Shapiro LR, Wilmot PL, Brenholz P, Leff A, Martino M, Harris G, Mahoney MJ, Hobbins JC: Prenatal diagnosis of fragile X chromosome. Lancet i: 99-100, 1982.

14. Sutherland GR: The fragile X chromosome. Int Rev Cytol 81: 107-143, 1983.

15. Erbe RW: Genetic aspects of folate metabolism. Adv Hum Genet 9: 293-354, 1979.

16. Jennings M, Hall JG, Hoehn H: Significance of phenotypic and chromosomal abnormalities in X-linked mental retardation (Martin-Bell or Renpenning syndrome). Am J Med Genet 7: 417-432, 1980.

17. Mattei MG, Mattei JF, Vidal I, Giraud F: Expression in lymphocyte and fibroblast culture of the fragile X chromosome : A new technical approach. Hum Genet 59: 166-169, 1981.

TABLE 1

MAXIMUM FREQUENCIES OF FOLATE SENSITIVE FRAGILE SITE EXPRESSION OBTAINED FOR 22 PATIENTS STUDIED COMPARING FOLATE DEPRIVATION (MEM-FA) WITH SPECIFIC INHIBITION OF THYMIDYLATE SYNTHETASE WITH 5-FLUORODEOXYURIDINE (FUdR) AND 5-FLUORODEOXYCYTIDINE (FCdR).

| PATIENT | FRAGILE SITE | SEX | AGE (yrs) | MEM-FA | RPMI 1640 + FUdR[a] | RPMI 1640 + FCdR[a] |
|---|---|---|---|---|---|---|
| A | 2q13 | F | 11 | 5/50 (10%) | 3/50 (6%) | - |
| B | | F | 29 | 13/52 (25%) | 10/45 (22%) | 20/50 (40%) |
| C | 6p23 | M | 3 | 44/100 (44%) | 18/75 (24%) | - |
| D | 8q22 | F | 8 | 18/50 (36%) | 9/50 (18%) | 10/50 (20%) |
| E | | F | 26 | 21/45 (47%) | 6/50 (12%) | 14/50 (28%) |
| F | 9q32 | F | 35 | 10/50 (20%) | 2/71 (3%) | - |
| G | 11q13 | M | 21 | 18/100 (18%) | 8/50 (16%) | 12/50 (24%) |
| H | | F | 22 | 23/150 (15%) | 17/100 (17%) | - |
| I | 11q23 | M | 24 | 14/113 (12%) | 11/75 (15%) | - |
| J | 12q13 | F | 23 | 15/100 (15%) | 5/50 (10%) | 2/50 (4%) |
| K | Xq27 | M | 6 | 16/70 (23%) | 8/23 (35%) | 13/50 (26%) |
| L | | M | 9 | 25/100 (25%) | 14/50 (28%) | 6/50 (12%) |
| M | | M | 18 | 63/165 (38%) | 14/40 (35%) | 15/50 (30%) |
| N | | M | 20 | 31/93 (33%) | 21/59 (36%) | - |
| O | | M | 21 | 9/26 (35%) | 16/136 (12%) | - |
| P | | M | 30 | 15/91 (16%) | 8/92 (9%) | 3/19 (16%) |
| Q | | M | 49 | 17/74 (23%) | 15/57 (26%) | 12/50 (24%) |
| R | | F | 11 | 16/100 (16%) | 5/92 (5%) | - |
| S | | F | 17 | 31/80 (39%) | 3/33 (9%) | - |
| T | | F | 17 | 14/91 (15%) | 18/100 (18%) | - |
| U | | F | 19 | 20/194 (10%) | 13/126 (10%) | 8/50 (16%) |
| V | | F | 36 | 9/50 (18%) | 12/48 (25%) | 12/50 (24%) |

a = For concentration of TS inhibitor see Table 2 and text.

TABLE 2

COMPARISON OF FREQUENCIES OF FRAGILE SITE EXPRESSION FOR SIX PATIENTS STUDIED USING MEM-FA AND RPMI 1640 PLUS VARIOUS CONCENTRATIONS OF THREE INHIBITORS OF THYMIDYLATE SYNTHETASE : 5-FLUORODEOXYURIDINE (FUdR), 5-FLUORODEOXYCYTIDINE (FCdR) AND TRIFLUOROTHYMIDINE (F3TdR).

| PATIENTS | B | | G | | | K | | | L | | M | | | V | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RPMI + TS Inhibitor (mg/l) | FUdR | FCdR | FUdR | FCdR | F3TdR | FUdR | FCdR | F3TdR | FUdR | FCdR | FUdR | FCdR | F3TdR | FUdR | FCdR |
| 0.0 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 |
| .001 | 1/50 | 3/50 | - | 1/50 | - | 0/50 | 2/50 | - | 1/50 | 0/50 | - | 0/50 | - | 0/50 | - |
| .005 | 6/50 | 10/50 | 3/50 | 0/50 | 1/50 | 1/50 | 2/50 | 0/50 | 1/50 | 3/50 | 5/50 | 2/50 | 0/50 | 5/50 | 3/50 |
| .01 | 10/45 | 20/50 | 8/50 | 6/50 | 3/50 | 3/50 | 13/50 | 0/50 | 2/50 | 5/50 | 12/50 | 2/50 | - | 12/48 | 12/50 |
| .05 | 5/10 | 19/53 | 5/50 | 6/50 | 4/50 | 8/23 | 9/50 | - | 14/50 | 5/50 | 14/40 | 8/41 | 1/50 | 2/26 | 10/50 |
| .1 | 2/15 | 8/16 | 8/50 | 5/50 | 2/50 | 7/50 | 9/50 | 0/50 | 5/50 | 6/50 | 5/22 | 15/50 | 2/50 | 3/26 | 5/27 |
| .3 | 0/5 | 1/11 | 5/50 | 10/50 | - | 2/9 | 11/50 | 0/50 | 4/45 | 3/50 | 1/12 | 10/50 | 1/50 | 2/20 | 7/23 |
| .5 | n.g. | 2/5 | 2/50 | 12/50 | 4/50 | 4/20 | 6/50 | 5/50 | 0/40 | 3/23 | 3/30 | 9/18 | 4/50 | 5/26 | 5/35 |
| 1.0 | n.g. | 3/10 | n.g. | 4/29 | 3/50 | 2/8 | 5/30 | 1/19 | 0/23 | 2/44 | n.g. | n.g. | 2/50 | 4/27 | 1/13 |
| MEM-FA[a] | 13/52 (25%) | | 18/100 (18%) | | | 16/70 (23%) | | | 25/100 (25%) | | 63/165 (38%) | | | 9/50 (18%) | |

a = Frequency of fragile site expression in MEM-FA from Table 1.

n.g. = Scored as no growth.

TABLE 3

FREQUENCIES OF FRAGILE SITE EXPRESSION FOR CULTURES INITIATED FROM BLOOD SAMPLES STORED FOR VARIOUS PERIODS FOR FIVE PATIENTS.

| PATIENT | FRAGILE SITE | BLOOD STORAGE (Days) | MEM-FA | | RPMI + .01 mg/l FUdR | |
|---|---|---|---|---|---|---|
| A | 2q13 | 0[a] | 5/50 | (10%) | 3/50 | (6%) |
| | | 3 | 4/50 | (8%) | 1/50 | (2%) |
| | | 5 | 5/50 | (10%) | 1/50 | (2%) |
| M | Xq27 | 0 | 63/165 | (38%) | 14/40 | (35%) |
| | | 3 | 20/50 | (40%) | 12/50 | (24%) |
| | | 5 | 6/50 | (12%) | 10/50 | (20%) |
| | | 7 | 8/50 | (16%) | 20/50 | (40%) |
| P | | 0 | 15/91 | (16%) | 8/92 | (9%) |
| | | 3 | 4/50 | (8%) | 1/50 | (2%) |
| | | 5 | 3/50 | (6%) | - | |
| Q | | 0 | 17/74 | (23%) | 15/57 | (26%) |
| | | 3 | 7/50 | (14%) | 4/50 | (8%) |
| | | 5 | 13/50 | (26%) | 4/50 | (8%) |
| | | 7 | 11/50 | (22%) | 3/50 | (6%) |
| T | | 0 | 14/91 | (15%) | 18/100 | (18%) |
| | | 3 | 5/22 | (23%) | 10/90 | (11%) |
| | | 5 | 9/50 | (18%) | 12/100 | (12%) |

a = Frequency of fragile site expression at storage day zero from Table 1.

In Press : Ann. Génét.

D 84

# PROTEASE INHIBITOR (PI) PHENOTYPE OF INDIVIDUALS WITH CHROMOSOMAL FRAGILE SITES

John C. Mulley and Grant R. Sutherland

Cytogenetics Unit,
Department of Histopathology,
Adelaide Children's Hospital,
North Adelaide, S.A. 5006
Australia.

SUMMARY

One hundred and four fragile sites were ascertained from 95 unrelated families. In each family the ancestry of the fragile site was traced as far as possible and the PI phenotype of the carrier in the earliest generation determined. Mildly deficient PI phenotypes were more prevalent than expected among earliest carriers of folate sensitive fragile sites, but not for BrdU dependent fragile sites. The significance of this finding is unclear and is based upon relatively small numbers. Another series of fragile site carriers would need to be studied and similar results found before the question of any relationship between PI phenotype and mutagenesis to fragile sites could be raised.

INTRODUCTION

Fragile sites are non-staining gaps in chromosomes inherited as codominant markers. The folate sensitive fragile site at Xq27 is associated with mild to severe mental retardation in males, and mild mental retardation in some females (Sutherland, 1983 [9]). The significance of the autosomal folate sensitive fragile sites is unclear at present, but in some carriers could possibly be associated with mental retardation (Sutherland, 1982 [8]). The common BrdU dependent site at 10q25 is phenotypically harmless even when homozygous (Sutherland, 1983 [9]). Similarly, the fragile site at 16q22, which in some individuals has no special tissue culture requirements for expression, appears to be phenotypically harmless in both heterozygous and homozygous carriers (Schmid et al. 1980 [6]).

The PI locus (protease inhibitor system) has a major influence upon levels of alpha-1-antitrypsin ($\alpha_1$AT), the predominant regulator of proteolytic activity in body fluids. The common alleles PI*M1, PI*M2 and PI*M3 are associated with normal levels of $\alpha_1$AT, while some of the less frequent alleles, PI*S, PI*Z, PI*I, PI*F and others, are associated with reduced levels of $\alpha_1$AT. Mildly deficient PI phenotypes have been associated with some chromosomal aberrations: but the evidence is unconvincing (Fagerhol and Cox, 1981 [2]).
The nature of the mutations which give rise to fragile sites are unknown and this investigation examines the possibility of an association between mildly deficient PI phenotypes and mutations to fragile sites.

MATERIALS AND METHODS

This investigation is unusual in that the chromosomal lesion is a heritable characteristic and any association between mutation to the fragile site and PI phenotype is directly testable in families only at the generation in which the mutation arose. Evidence for such association would be eroded in successive generations by

Fig.1 independent assortment (Fig. 1a) and non PI*M alleles could easily associate with fragile sites by chance (Fig.1b). For this reason only the carrier in the earliest available generation of each family was examined for statistical association to eliminate as far as possible effects of independent assortment and association by chance. This provided a sample of carriers as close as possible to the mutational event required for the expression of fragile sites.

Table 1 The earliest carrier, whether propositus or otherwise (Table 1), was determined prior to PI typing. The number of generations available for study depended on the availability and co-operation of family members.

All the fragile sites ascertained fit the criteria for classification as true fragile sites (Sutherland, 1979 [7]). PI typing was carried out as previously described for the determination of allele frequencies in the normal population (Mulley, 1980; 1982 [3,4]). Fragile site acertainments were grouped for statistical analysis into three classes : folate sensitive at Xq27, folate sensitive at autosomal loci and BrdU dependent at 10q25. There were insufficient fragile sites at 16q22 for meaningful statistical analysis.

Of the families with folate sensitive fragile sites, 18 of the 40 ascertained had been examined for the presence of the BrdU dependent fragile site. All BrdU dependent fragile site ascertainments had been examined for folate sensitive fragile sites.

RESULTS

A total of 104 fragile sites were ascertained from 95 unrelated families. There were 17 ascertainments for the fragile site at Xq27, 23 for various folate sensitive autosomal sites, 59 for the BrdU dependent site and 5 for the site at 16q22. More than one fragile site was found in 8 families. One family had three fragile sites, two at 10q25 (homozygous in two individuals) and one at 11q23. The remaining seven families were all double ascertainments : an Xq27 with 16q22, two cases of 10q23 with 10q25, three cases of 10q25 with 16q22 and a 20p11 with 16q22. In all 8 families the earliest carrier for one fragile site was also the earliest carrier for another fragile site. The earliest carrier was the propositus for three families, the parent of the propositus in two families and the grandparent of the propositus in three families.

The distribution of PI phenotypes determined from the earliest carrier of all fragile site ascertainments is shown in Table 2. For example, the phenotypes given for the pedigrees in Fig.1 are presented in Table 2 as M1Z (Fig.1a) and M1 (Fig.1b). Comparisons of the fragile site groups with those in a random sample from the population (phenotypes classified as either PI M or non PI M) are given in Table 3. Non PI M phenotypes have at least one allele other than PI*M1, PI*M2 or PI*M3. The distribution of PI phenotypes

was similar to that of the general population for the BrdU dependent fragile site sample ($\chi^2_1 = 0.36$, $0.50 < P < 0.75$). The corresponding comparison for the autosomal folate sensitive fragile sites is significant ($\chi^2_1 = 4.34$, $P < .05$). The data derived from the limited sample of 17 ascertainments with the fragile site at Xq27 also detected an association ($\chi^2_1 = 8.41$, $P < .01$).

## DISCUSSION

There is strong selection against the folate sensitive fragile site at Xq27, which in terms of reproductive fitness, is virtually lethal in affected males and in some females. A significant proportion of independent Xq27 fragile site ascertainments are expected to result from new mutations and many of the remaining ascertainments are likely to be of relatively recent mutational origin. This group of fragile site families is therefore likely to provide the most sensitive test of any hypothetical relationship between PI phenotype and mutagenesis to fragile sites. An association was indeed found in this study. Unfortunately, the identification of new mutants is uncertain given the difficultyin detecting this fragile site in some female carriers (Sutherland, 1983 [9]).

If a relation between PI phenotype and mutation to the fragile site at Xq27 does exist, then a similar relationship may also exist for the other fragile sites. While it is not inconceivable that recent mutations are represented amongst the autosomal folate sensitive fragile site sample, the mutational origin would be expected to be more remote than for the fragile site at Xq27. Most heterozygotes for the autosomal folate sensitive fragile sites are normal individuals and any selection pressure against them is small.

Whilst these sites might prove harmful in homozygotes, by analogy with known clinical effects of hemizygosity for the fragile site at Xq27, no such homozygote has been recorded. In view of the probable long period of elapsed time in generations between mutation and ascertainment, any relationship that might exist between PI phenotype and mutation to the autosomal folate sensitive fragile sites would be unlikely to be detected. Surprisingly, an association was found in the present study.

A third class of fragile site at 10q25 is a harmless polymorphism. Lack of selection against this fragile site implies that virtually all ascertainments are far removed in generations from the actual mutational events, and genetic drift might be responsible for its common occurrence. No association was found between PI phenotype and this fragile site as expected.

The molecular mechanism underlying fragile site expression is unclear. The chromosomal phenotype is probably associated with a mutant gene given the heritable nature of fragile sites. Linkage between the fragile sites at 10q23 and 10q25 (Sutherland, et al. 1982 [10]) and between the fragile site at 6p23 and HLA (Mulley, et al. 1983 [5]) confirms that the genes responsible are at the position of fragile site expression. They may affect either condensation of DNA, DNA repair or DNA synthesis. Penetrance and expressivity at the cellular level is largely dependent upon the culture environment.

The statistical association between mildly deficient PI phenotypes and carriers of the folate sensitive fragile sites may be only coincidental because the numbers analysed were low. This could be

confirmed by an independent investigation of a separate series, but folate sensitive fragile sites are extremely rare and a separate series will not be available to the present investigators in the immediate future.

Another matter for speculation arising from the series studied concerns the apparent high rate of double ascertainment. The observed rate is a lower limit because 22 families known to have folate sensitive fragile sites were not examined with BrdU for the presence of the fragile site at 10q25. The detection of the fragile site at 16q22 can be difficult and in some patients requires the addition of distamycin A to lymphocyte cultures 24 hours prior to harvest (Schmid, et al. 1980 [6]) or high (30mg/l) levels of BrdU 6-8 hours prior to harvest (Croci, 1983 [1]). Consequently the significance of double ascertainment involving the site at 16q22 is unknown as culture conditions for expression of this fragile site were suboptimal in the great majority of individuals examined.

ACKNOWLEDGEMENTS

We wish to thank Elizabeth Baker, Helen Eyre, Lily Gowans, Louise Maland and Erica Woollatt for technical assistance with the cytogenetics, Dr. Peter Jacky for useful discussions on the nature of fragile sites and the National Health and Medical Research Council of Australia, the Channel 10 Children's Medical Research Foundation and the Adelaide Children's Hospital Research Trust for support.

REFERENCES

1. Croci, G. BrdU - sensitive fragile site on long arm of chromosome 16. Am J Hum Genet, 1983, (in press).

2. Fagerhol, MK and Cox, DW. The PI polymorphism : Genetic, biochemical, and clinical aspects of human $\alpha_1$-antitrypsin. IN: Advances in Human Genetics, Vol.11, 1981, pp.1-62 (Eds. Harris, H and Hirschhorn, K).

3. Mulley, JC. Distribution of the six $\alpha_1$-antitrypsin (PI) subtypes from a sample of blood donors. Aust J Exp Biol Med Sci, 1980, 58, 409-414.

4. Mulley, JC. Distribution of $\alpha_1$-antitrypsin (PI) subtypes from newborns. Aust J Exp Biol Med Sci, 1982, 60, 207-209.

5. Mulley, JC, Hay, J, Sheffield, LJ and Sutherland, GR. Regional localisation for HLA by recombination with a fragile site at 6p23. Am J Hum Genet, 1983 (in press).

6. Schmid, M, Klett, C and Niederhofer, A. Demonstration of a heritable fragile site in human chromosome 16 with distamycin A. Cytogenet Cell Genet, 1980, 28, 87-94.

7. Sutherland, GR. Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. Am J Hum Genet, 1979, 31, 125-135.

8. Sutherland, GR. Heritable fragile sites on human chromosomes. VIII. Preliminary population cytogenetic data on the folic acid-sensitive fragile sites. Am J Hum Genet, 1982, 34, 452-458.

9. Sutherland, GR. The fragile X chromosome. Int Rev Cytol, 1983, 81, 107-143.

10. Sutherland, GR, Baker, E and Mulley, JC. Genetic length of a human chromosomal segment measured by recombination between two fragile sites. Science, 1982, 217, 373-374.

Fig.1 Pedigrees showing segregation of fragile sites and PI alleles to demonstrate the need to consider only the earliest carrier.

a) The true relationship is eroded in generations II and III by independent assortment,

b) Association developed in generations II and III is spurious.

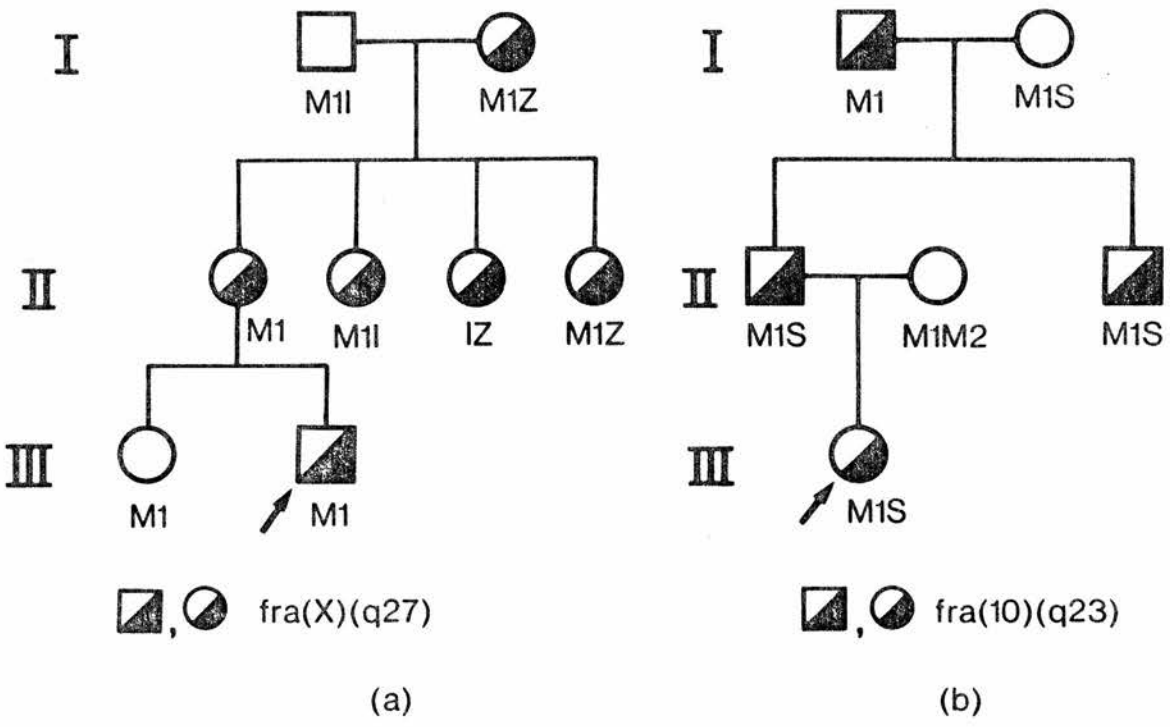


TABLE 1

PORTION OF PEDIGREE FROM WHICH THE EARLIEST CARRIER WAS EXAMINED.

| Fragile Site | Propositus | Carrier Parent | Carrier Grandparent | Carrier Great-Grandparent | Total |
|---|---|---|---|---|---|
| Xq27 | 3 | 11 | 3 | 0 | 17 |
| Autosomal - Folate sensitive | 10 | 9 | 3 | 1 | 23 |
| 10q25 | 25 | 20 | 13 | 1 | 59 |
| 16q22 | 3 | 1 | 1 | 0 | 5 |
| | 41 | 41 | 20 | 2 | 104 |

TABLE 2

PI PHENOTYPE IN THE EARLIEST CARRIER

OF EACH FRAGILE SITE ASCERTAINMENT

| Fragile Site | No. | PI M | | | | Non PI M | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M1 | M1M2 | M1M3 | M2M3 | M1S | M2S | M3S | M1Z | M1F | M2F | M1I |
| Xq27 | 17 | 6 | 4 | 1 | 0 | 2 | 0 | 3 | 1 | 0 | 0 | - |
| Autosomal - Folate Sensitive | | | | | | | | | | | | |
| 2q13 | 2 | - | 1 | - | - | 1 | - | - | - | - | - | - |
| 6p23 | 1 | - | - | - | - | - | - | - | - | - | - | 1 |
| 9p21 | 1 | - | - | - | - | - | - | - | - | 1 | - | - |
| 9q32 | 2 | 2 | - | - | - | - | - | - | - | - | - | - |
| 10q23 | 9 | 5 | 2 | 1 | 1 | - | - | - | - | - | - | - |
| 11q13 | 2 | - | - | 1 | 1 | - | - | - | - | - | - | - |
| 11q23 | 1 | - | - | 1 | - | - | - | - | - | - | - | - |
| 12q13 | 1 | 1 | - | - | - | - | - | - | - | - | - | - |
| 16p12 | 2 | - | 1 | - | - | - | - | - | 1 | - | - | - |
| 20p11 | 2 | - | - | - | - | 1 | - | - | 1 | - | - | - |
| Total | 23 | 8 | 4 | 3 | 2 | 2 | 0 | 0 | 2 | 1 | 0 | 1 |
| 10q25 | 59 | 31 | 12 | 6 | 2 | 5 | 1 | 0 | 0 | 1 | 1 | - |
| 16q22 | 5 | 3 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | - |

In Press : Am J Hum Genet



# HERITABLE FRAGILE SITES ON HUMAN CHROMOSOMES.

# XI. FACTORS AFFECTING EXPRESSION OF FRAGILE SITES AT 10q25, 16q22 and 17p12

by

Grant R. Sutherland

Peter B. Jacky

Elizabeth R. Baker

Cytogenetics Unit

Department of Histopathology

Adelaide Children's Hospital

North Adelaide S.A. 5006

Australia

SUMMARY

The fragile sites at 10q25, 16q22 and 17p12 can all be induced in lymphocyte culture by BrdU or BrdC added 6-12 hours prior to harvest. Without induction fra(10)(q25) is rarely expressed spontaneously whereas fra(16)(q22) is frequently expressed spontaneously. Fra(17)(p12) is frequently expressed spontaneously but it is unknown whether it will only be expressed after induction in some individuals. Distamycin A, Netropsin and Hoechst 33258 induced high levels of expression of fra(16)(q22) and fra(17)(p12) but did not enhance expression of fra(10)(q25). The mechansims of induction of fra(16)(q22) by BrdU and Distamycin A appear to be different since the time of action of BrdU reaches a maximum about 12 hours prior to harvest whereas induction by Distamycin A requires much longer exposure. The fragile sites at 10q25 and 16q22 were both induced in fibroblast culture by BrdU. Fra(17)(p12) is accepted as a fragile site because preliminary studies show that it behaves similarly in lymphocyte culture to fra(16)(q22), however there is only limited evidence for fragility at 17p12.

## INTRODUCTION

Of the known heritable fragile sites on human chromosomes only two are not suppressed by the addition of folic acid to lymphocyte culture [1] . The first of these, at 16q22, was originally considered to be insensitive to conditions of tissue culture [2] but it has been shown that its expression can be greatly enhanced by the addition of either Distamycin A [3] or BrdU [4] to lymphocyte culture. Expression of the fragile site at 10q25 has been shown to require BrdU to be present in lymphocyte cultures for some hours prior to harvest [5] . Since both these fragile sites can be induced with BrdU a detailed study was undertaken to determine whether they behave in similar ways under a variety of conditions of tissue culture in lymphocytes and fibroblasts. The variant chromosome 17, the so-called satellited 17, was not accepted by Sutherland [1] as a fragile site although Shabtai et al. [6] have proposed that it is one. Preliminary studies have suggested that it is a fragile site which has properties very similar to those of fra(16)(q22).

## MATERIALS AND METHODS

The fragile site at 16q22 has been studied in seven individuals, one of whom also has the fragile X (Subject B [7]), two are brothers JE and TE, two are mother and daughter, Mrs. L. and PL, land there were two unrelated women (Cl and Co) who also have the BrdU requiring fragile site at 10q25 (Fig.1). The fragile site at 10q25 has been ascertained more than 75 times and more than 200 individuals with it have been studied [8]. Data relating to eight individuals (numbered 1-8) are presented in the results. One individual with fra(17)(p12) has been available for study.

Lymphocytes have been cultured in a variety of media including TC199, RPMI 1640, Ham's F10 and Eagle's MEM without folic acid (MEM-FA [2]). The media were supplemented with 5% fetal bovine serum and methods of culture and harvesting for chromosome studies have been previously described [2]. Expression of the fragile sites was studied in media used without additives and with Distamycin A, Netropsin, Bromodeoxyuridine (BrdU), Bromodeoxycytidine (BrdC), Hoechst 33258 and thymidine added at different concentrations and at different times prior to harvest as shown in the results.

Fibroblast cultures obtained from Subject B and several individuals with fra(10)(q25) were grown in Ham's F10 with 10% fetal bovine serum. The chromosome preparations from fibroblasts were obtained using an in situ method [11] except that 0.075 M KC1 was used as the hypotonic solution. The effects of BrdU, BrdC and Distamycin A at various concentrations and for various times, as shown in the results, on the expression of the fragile sites was studied.

Once the location of the fragile site in any individual had been confirmed by chromosome banding all other studies were carried out on unbanded material and frequencies of expression were determined from the examination of at least 50 metaphases.

## RESULTS

### Spontaneous expression in lymphocytes

Frequencies of expression of the fragile site at 16q22 in various media without any additives are shown in Table I. There were no consistent differences in the frequency of expression according to the type of media used, but there were considerable fluctuations for any individual studied. The fragile site at 10q25 has not been detected under these conditions of culture in more than 200 individuals studied who had been shown to carry it following BrdU induction. Rarely, in fra(10)(q25) carriers, one or two metaphases out of 50 examined from cultures to which BrdU or another inducing agent had not been added did appear to be expressing the fragile site. It is difficult to be sure from unbanded chromosomes that this finding of a very low frequency of expression is any more than would be seen in individuals who do not carry this fragile site. Fra(17)(p12) has been ascertained nine times and seen in parent-child pairs twice. All these ascertainments resulted from spontaneous expression of the fragile site.

Induction in lymphocytes by BrdU and BrdC

The effects of BrdU and BrdC on expression of 16q22 are shown in Table II for several individuals and from this it can be seen that addition of either of these substances at the time of culture initiation had little effect on the frequency of expression of the fragile site. Either substance, when added closer to the time of harvest usually caused a large increase in the frequency at which this fragile site was expressed. A more detailed study of the action of these compounds for Subject B is shown in Table III. The frequency of expression is maximised by addition of the compounds 6-12 hours prior to harvest. Dose response curves for BrdU and BrdC added eight hours prior to harvest to cultures from Subject TE are shown in Fig.2 and it can be seen that maximum induction of the fragile site had not been reached even when 200 mg/l of the inducer was used.

Table IV shows the effect of BrdU and BrdC upon expression of fra(10)(q25) in five individuals. Both compounds are effective in inducing this fragile site although slightly higher frequencies of expression were usually seen when the less toxic BrdC was used. BrdC was present at a higher molar concentration and this may explain the difference. Further data on the response of fra(10)(q25) to BrdU has been published [5] and the dose response and time course for the induction of this fragile site by BrdC are shown in Figs. 3 and 4. The maximum induction of this fragile site had not apparently been reached even by the use of 200 mg/l of BrdC. At higher concentrations toxicity became a problem and resulted in low numbers of metaphases suitable for examination. The time course data show that with

50 mg/l BrdU maximal induction occurred about 12 hours prior to harvest but with the lower concentration (15 mg/l) a longer exposure was required for this.

Induction in lymphocytes by DNA binding substances

Data obtained with Distamycin A is somewhat limited due to the current unavailability of this compound. Table V shows the response of six individuals with fra(16)(q22) to 100 mg/l Distamycin A added 21 or 24 hours prior to harvest, two of these individuals also have fra(10)(q25) and one also has fra(X)(q27). One individual with fra(10)(q25) alone is also included in Table V. From this table it can be seen that Distamycin A greatly increased the frequency of expression of fra(16)(q22) but had no effect upon fra(10)(q25) or fra(X)(q27). The time of addition of Distamycin A is shown for Subject Co in Table III, in two other individuals addition of Distamycin A at culture initiation or 21 hours prior to harvest greatly increased the frequency of expression (from 5% to 54% and 58% respectively in Subject PL and from 25% to 85% and 80% respectively in her mother) but had no effect when added at eight hours prior to harvest (5% to 4% and 25% to 24%). A dose response curve for expression of fra(16)(q22) to Distamycin A is shown in Fig.5. A dose of 75 mg/l appeared to result in maximal induction and the decreased induction at 100 mg/l in Subject B may reflect toxicity of this compound. Netropsin greatly enhanced expression of fra(16)(q22) in Subject B. The frequency of expression of this fragile site was 40% in RPMI but addition of 100 mg/l or 200 mg/l of Netropsin gave frequencies of 66% and 60% respectively.

Hoechst 33258 was found to induce fra(16)(q22) and a dose response curve for this induction is shown in Fig.6. Maximum induction was achieved by the addition of 50-150 mg/l of this compound 24 hours prior to harvest. The time prior to harvest of maximum induction is not certain, but in Subject C1 where spontaneous expression of fra(16)(q22) was seen in 4% of metaphases in RPMI 1640, addition of 50 mg/l of Hoechst 33259 ten hours prior to harvest increased expression to 40% of metaphases and at 36 and 48 hours prior to harvest to 50% of metaphases. Hoechst 33258 did not induce fra(10)(q25) in Subject C1 when added 24 hours prior to harvest at concentrations up to 150 mg/l.

The results of preliminary studies on fra(17)(p12) are given in Table VI. These show a low but consistent spontaneous expression of the fragile site (6% to 14%) in the various culture media used without addition of an inducing substance. Addition of BrdU or BrdC greatly enhanced expression (24% to 74%) and even further enhancement was seen in the presence of Distamycin A and Netropsin (up to 100% of metaphases examined). Hoechst 33258 induced the lesion in 68% of metaphases but the concentration was low (10 mg/l) and further induction may have occurred if higher concentrations had been used.

Fragile site induction in fibroblasts

Fragile sites at 10q25 and 16q22 were induced in fibroblasts. There were no fibroblasts from any individual with fra(17)(p12) available for study. Table VII shows a comparison of the maximum frequency of expression of the fragile sites induced with BrdU in fibroblasts and lymphocytes from the same individual. Generally the frequency of expression was found to be higher in

lymphocytes than fibroblasts. Fig.7 shows the dose response curve for the induction of fra(10)(q25) with BrdU added 48 hours prior to harvest. It can be seen from this figure that maximum induction of the fragile site was not achieved since increasing the concentration of BrdU was too toxic. Furthermore, it was generally seen that addition of BrdU to cultures 48 hours prior to harvest resulted in higher frequencies of expression than at 24 hours prior to harvest. Preliminary experiments suggested that Distamycin A would not induce fra(10)(q25) in fibroblasts but this study could not be pursued due to the unavailability of Distamycin A. Similarly, induction of fra(16)(q22) in fibroblasts with this substance could not be tested.

## DISCUSSION

The tissue culture conditions for expression of the folate sensitive fragile sites and the area of metabolism involved in this are known, if not completely understood [1]. There are only three fragile sites which are not folate sensitive and this study has attempted a detailed examination of the tissue culture requirements for the expression of these in the hope of defining the area of metabolism involved or the chromosomal mechanism underlying fragile site expression. The results indicate that although these fragile sites have several features in common, there are differences between fra (10)(q25) which is only induced by BrdU or BrdC, and fra(16)(q22) and fra(17)(p12) which are very similar and are also induced by the DNA binding compounds studied. Table VIII briefly summarises the properties of each of these fragile sites.

The fragile sites at 16q22 and 17p12 are spontaneously expressed in a variable proportion of metaphases from lymphocyte culture. The fragile site at 10q25 is rarely expressed spontaneously and is usually only seen after induction by BrdU. There are two reports [12,13] of spontaneous expression of this fragile site in families in which expression was greatly enhanced by BrdU, and there is one report [14] of familial spontaneous expression which was not enhanced by BrdU. It is possible that there is no real difference in the ability of these three fragile sites to spontaneously express themselves. All the fra(10)(q25) individuals in Adelaide were found as a result of specific induction of this fragile site with BrdU and none were seen to spontaneously express it whereas the fra(16)(q22) and fra(17)(p12) individuals were selected on the basis that they spontaneously expressed these fragile sites. Schmid et al. [3] recorded several members of two families in which fra(16)(q22) was not expressed spontaneously. There is no information available on the induced versus spontaneous expression of fra(17)(p12) since this is the first report indicating that it can be induced and presumably all previous reports related to its spontaneous expression.

Croci [4] was the first to show that fra(16)(q22) could be induced with BrdU and he increased the frequency of expression from less than 5% of metaphases up to 50% of metaphases by the addition of 30 mg/l of BrdU to lymphocyte cultures 6 to 6½ hours prior to harvest and suggested that the reason Sutherland [2] had not found such an effect was that he added 10 mg/l of BrdU at initiation of culture. This suggestion appears to be correct in part since the results of the present study show that the

induction of the fragile site is maximised by addition of BrdU 6 to 8 hours prior to harvest and confirms that the addition of 10 mg/l at culture initiation has little effect on the expression of this fragile site. The three fragile sites in this study were all induced by BrdU and BrdC.

BrdC has been found to be a very useful alternative to BrdU. Unpublished data shows that it is less toxic, and particularly at high concentrations or after longer exposure times, the quality of chromosomes is much better and the number of usable metaphases per slide (either for SCE determiantion of fragile site scoring) is higher than when equimolar concentrations of BrdU have been used. BrdC has been used as an alternative to BrdU for SCE determiantion [15,16] and in reporting in vivo studies with this compound in grasshoppers Pijnacker and Ferwerda [17] noted that it appeared to be less toxic than BrdU. BrdC has been found to be as effective as BrdU in induction of fragile sites. In this discussion wherever BrdU is reported to have had an effect it can be assumed that a similar effect was obtained with BrdC unless otherwise stated.

Since BrdU is an analog of thymidine, exposure of lymphocyte cultures to excess thymidine was performed but this did not induce the fragile sites (data not shown). Sutherland et al. [5] showed that addition of increasing amounts of thymidine in the presence of BrdU gradually inhibited expression of fra(10)(q25). Similar data (not shown) were obtained for fra(16)(q22). Such findings are in line with the suggestion [18] that BrdU and thymidine compete for available incorporation sites and that the effect of BrdU can be swamped by excess thymidine. This is in contrast to

the mechanism of BrdU induced mutagenesis in mammalian cells [19] where the effect of BrdU is enhanced by thymidine. Presumably the mechanism of BrdU induction of fragile sites is not the same as that of BrdU mutagenesis. The chromosomal mechanism responsible for expression of both the BrdU inducible and folate sensitive fragile sites remains poorly understood.

There exists some doubt and controversy as to whether the undoubted morphological variant of the short arm of chromosome 17 is really a fragile site. There have been numerous reports (see [20] for collation) of this chromosome variant, which shows many features of a fragile site. It is usually seen in only a proportion of metaphases, although it has been reported to be in all of them in some cases (e.g. [21]). It is undoubtedly heritable and constant in position, however it has only been rarely reported to be fragile. Cytogenetically this fragile site is different from all the others. The appearance is usually that of an incomplete gap, not unlike that described by Schmid et al. [3] for their type 2 Distamycin A inducible lesion at 16q22. Complete gaps are uncommon and breaks even more uncommon. Shabtai et al. [6] published "triradial-like figures" thought to result from this fragile site, but a convincing triradial of the type described for all the other fragile sites has not been documented. Since the behaviour of this chromosomal variant in response to manipultion of conditions of tissue culture is apparently identical to that shown by the fragile site at 16q22 it is tentatively accorded the status of a fragile site, although the evidence for fragility at 17p12 is minimal.

The specific induction of the fragile site at 16q22 by Distamycin A was first reported by Schmid et al. [3]. They found that this fragile site was not expressed spontaneously in members of two families studied but was seen if 100 mg/l of Distamycin A was added to the culture medium 24 hours prior to harvest. Indeed this fragile site was undoubtedly missed on some occasions in the Adelaide laboratory. The finding of 2% to 4% of metaphases with this fragile site was not rare and erroneously lead Sutherland [1] to conclude that there was an autosomal lesion as well as a fragile site at this locus. Subsequent re-study of some of these individuals using either BrdU or Distamycin A induction has confirmed that they have the fragile site.

Since the oligopeptide antibiotic Distamycin A was found to induce the fragile sites at 16q22 and 17p12, the effects of a similar antibiotic, Netropsin, which also binds to A-T rich regions of DNA [10], and Hoechst 33258 which also binds to such regions [22] were studied. These three compounds all strongly induced both fragile sites. Repeated attempts to induce the fragile site at 10q25 with Distamycin A and one attempt to induce it with Netropsin and Hoechst 33258 at different concentrations have been unsuccessful. It is reasonable to conclude that none of these compounds will induce fra(10)(q25).

Both Distamycin A and Netropsin are oligopeptide antibotics which bind in the minor groove of DNA at A-T rich regions [10] and Hoechst 33258 probably binds to the major groove in such regions [22,23]. The ability of these compounds to induce the fragile sites at 16q22 and 17p12 might suggest that these fragile sites are A-T rich regions of DNA and with the compound bound to the DNA,

chromosomal compaction for mitosis is inhibited and results in expression of a fragile site.

It is of interest to note that the times of action of Distamycin A and BrdU in inducing fra(16)(q22) are different. At 12 hours prior to harvest Distamycin A has no effect as a fragile site inducer yet the maximal effect of BrdU on induction occurs about this time. This would suggest that the two types of inducer operate via different mechanisms. Jacky and Dill [24] found that Ethidium bromide, Hoechst 33258, Olivomycin, Netropsin and Actinomycin D had no effect on expression of the fragile X in lymphocyte cultures and this presumably holds for the other folate sensitive fragile sites. These substances and others which bind to DNA should be tested for their ability to induce expression of the folate insensitive fragile sites.

Whilst this study has not shed much light on the mechanism of expression of fragile sites or revealed the area of metabolism involved in their expression it is perhaps reasonable to conclude that since BrdU induction can be achieved so close to harvest that the fragile sites are late replicating regions of DNA, and since fra(16)(q22) and fra(17)(p12) can be induced with DNA ligands that these are regions of A-T rich DNA.

ACKNOWLEDGEMENTS

We thank Helen Eyre, Louise Maland and Cathy Orell for assistance with the cytogenetics and Dr. Christoph Zimmer for his gift of the Netropsin. This work was supported by grants from the National Health and Medical Research Council of Australia and the Channel 10 Children's Medical Research Foundation.

REFERENCES

1. Sutherland GR: The fragile X chromosome. Int Rev Cytol 81: 107-143, 1983.

2. Sutherland GR: Heritable fragile sites on human chromosomes. I. Factors affecting expression in lymphocyte culture. Am J Hum Genet 31: 125-135, 1979.

3. Schmid M, Klett C, Niederhofer A: Demonstration of a heritable fragile site in human chromosome 16 with distamycin A. Cytogenet Cell Genet 28: 87-94, 1980.

4. Croci G: BrdU sensitive fragile site on the long arm of chromosome 16. Am J Hum Genet (in press).

5. Sutherland GR, Baker E, Seshadri RS: Heritable fragile sites on human chromosomes. V. A new class of fragile site requiring BrdU for expression. Am J Hum Genet 32: 542-548, 1980.

6. Shabtai F, Klar D, Halbrecht I: Chromosome 17 has a real fragile site at p12. Hum Genet 61: 177-139, 1982.

7. Sutherland GR: Heritable fragile sites on human chromosomes. II. Distribution, phenotypic effects and cytogenetics. Am J Hum Genet 31: 136-148, 1979.

8. Sutherland GR: Heritable fragile sites on human chromosomes. IX. Population cytogenetics and segregation analysis of the BrdU requiring fragile site at 10q25. Am J Hum Genet 34: 753-756, 1982.

9. Zimmer C, Marck C, Schneider C, Guschlbauer W: Influence of nucleotide sequence on dA-dT specific binding of netropsin to double stranded DNA. Nucleic Acids Res 6: 2831-2837, 1979.

10. Van Dyke MW, Hertzberg RP, Dervan PB: Map of distamycin, netropsin, and actinomycin binding sites on heterogeneous DNA: DNA cleavage - inhibition patterns with methidiumpropyl-EDTA.Fe(II). Proc Natl Acad Sci USA 79: 5470-5474, 1982.

11. Jacky PB, Dill FJ: Expression in fibroblast culture of the satellited-X chromosome associated with familial sex-linked mental retardation. Hum Genet 53: 267-269, 1980.

12. Ledbetter DH, Bock HO, Caskey CT: Cytogenetic studies of a BrdU-sensitive but not BrdU-dependent fra(10)(q25) in a patient with multiple anomalies. Am J Hum Genet 34: 132A, 1982.

13. Taylor AMR, Bundey S: Spontaneous expression of the chromosome fragile site fra(10)(q25). Am J Hum Genet 35: 123-125, 1983.

14. Petit P, Fryns JP: Expression of fragile site at 10q25 in normal culture conditions. Am J Hum Genet 35: 126-127, 1983.

15. Connor MK, Boggs SS, Turner JH: Comparisons of in vivo BrdU labelling methods and spontaneous sister chromatid exchange frequencies in regenerating murine liver and bone marrow cells. Chromosoma (Berl) 68: 303=311, 1978.

16. Zakharov AF, Bairanjan TL: 5-Bromodeoxycytidine in the study of sister chromatid exchanges in human lymphocytes. Hum Genet 55: 259-263, 1980.

17. Pijnacker LP, Ferwerda MA: Normal and BrdC-substituted chromosomes during spermatogenesis with an endomeiotic chromosome reduplication in Carausius morosus Br'. Chromosoma (Berl) 85: 67-82, 1982.

18. Mazrimas JA, Stetka DG: Direct evidence for the role of incorporated BUdR in the induction of sister chromatid exchanges. Exp Cell Res 117: 23-30, 1978.

19. Ashman CR, Davidson RL: Bromodeoxyuridine mutagenesis in mammalian cells is related to deoxyribonucleotide pool imbalance. Molec Cell Biol 1: 254-260, 1981.

20. Guichaoua M, Matteï MG, Matteï JF, Giraud F: Aspects genetiques des sites fragiles autosomiques. A propos de 40 cas. J Génét Hum 30: 183-197, 1982.

21. Court Brown WM, Buckton KE, Jacobs PA, Tough IM, Kuenssberg EV, Knox JDE: Chromosome studies on adults. Eugenics Lab Memoirs XL11 Cambridge University Press, London, 1966.

22. Comings DE: Mechanisms of chromosome banding. VIII. Hoechst 33258-DNA interaction. Chromosoma (Berl) 52: 229-243, 1975.

23. Prantera G, Pimpinelli S, Rocchi A: Effects of distamycin A on human leukocytes in vitro. Cytogenet Cell Genet 23: 103-107, 1979.

24. Jacky PB, Dill FJ: Fragile X chromosome and chromosome condensation. Ann Génét (in press).

TABLE I

Proportion of metaphases showing expression of fra(16)(q22) in different culture media without the addition of any specific inducing substances.

| SUBJECT AND DATE OF STUDY | | MEDIUM | | | |
|---|---|---|---|---|---|
| | | 199 | MEM-FA | F10 | RPMI |
| B. | 4/4/78 | 21/100 | 8/50 | 8/100 | |
| | 12/6/78 | 10/50 | 6/41 | 19/50 | |
| | 21/2/83 | 20/50 | 19/50 | 10/50 | 20/50 |
| P.L. | | | | | 5/100 |
| Mrs. L. | | | 17/100 | | 25/100 |
| T.E. | 2/8/82 | | 12/50 | | 21/100 |
| | 25/3/83 | 10/50 | 13/50 | 17/50 | 18/50 |
| J.E. | | | 4/50 | | |
| Co | | | 3/50 | | 10/100 |

TABLE II

Percentage of metaphases expressing fra(16)(q22) when 10 mg/l of BrdU (or 15 mg/l of BrdC) was added at culture initiation (h-72) or 50 mg/l when either was added 21 (h-21) or 8 (h-8) hours prior to harvest, compared to a control culture without BrdU.

| SUBJECT AND DATE OF STUDY | MEDIUM | CONTROL | TIME OF ADDITION OF BrdU (or BrdC) | | |
|---|---|---|---|---|---|
| | | | h-72 | h-21 | h-8 |
| B. 12/6/72 | F10 | 38 | 24 | | |
| 21/2/83 | RPMI 1640 | 40 | 42 | | 62 (80) |
| P.L. | RPMI 1640 | 5 | 24 (22) | 28 (30) | 42 (24) |
| Mrs. L. | RPMI 1640 | 25 | 26 (38) | 60 (68) | 60 (62) |
| T.E. | RPMI 1640 | 21 | | | 50 (68) |
| Co | RPMI 1640 | 10 | 10 (13) | 22 (6) | 24 (12) |

TABLE III

Expression of fra(16)(q22) induced by BrdU and BrdC in Subject B and by Distamycin A in Subject Co. Effect of time of induction on frequency of expression in RPMI 1640.

| TIME OF INDUCTION PRIOR TO HARVEST (HOURS) | INDUCER | | | | |
|---|---|---|---|---|---|
| | BrdU (10 mg/l) | BrdU (50 mg/l) | BrdC (15 mg/l) | BrdC (50 mg/l) | Distamycin A (75 mg/l) |
| 72 | 21/50 | | | | |
| 48 | 20/50 | | 22/50 | | |
| 24 | 19/50 | 21/50 | 36/50 | 35/50 | 22/50 |
| 16 | 19/50 | 30/50 | 30/50 | 30/50 | 16/50 |
| 12 | | 24/50 | | 39/50 | 5/50 |
| 8 | 31/50 | 36/50 | 29/50 | 40/50 | 0/50 |
| 6 | | 37/50 | | 39/50 | |
| 4 | 27/50 | 17/50 | 22/50 | 24/50 | 2/50 |
| Control | 40/100 | 40/100 | 40/100 | 40/100 | 10/100 |

TABLE IV

Comparison of BrdU (10 mg/l) and BrdC (15 mg/l) in induction of fra(10)(q25) in Ham's F10 (Subjects 1-4) or RPMI 1640 (Subject Co).

| SUBJECT | CONTROL | BrdU | BrdC |
|---|---|---|---|
| 1 | 0/50 | 6/25 | 13/25 |
| 2 | 0/50 | 3/50 | 10/50 |
| 3 | 0/50 | 8/21 | 4/30 |
| 4 | 0/50 | 24/50 | 35/50 |
| Co | 0/50 | 9/50 | 14/50 |

TABLE V

The effect of addition of 100 mg/l of Distamycin A to RPMI 1640 on expression of the fragile site.

| SUBJECT | | CULTURE MEDIUM | |
|---|---|---|---|
| | | RPMI 1640 | RPMI 1640 + DA |
| B. | fra(16) | 20/50 | 36/50 |
| | fra(X) | 0/50 | 0/50 |
| P.L. | fra(16) | 5/100 | 27/50 |
| Mrs. L. | fra(16) | 25/100 | 43/50 |
| T.E. | fra(16) | 21/100 | 30/50 |
| Co | fra(10) | 0/50 | 0/50* |
| | fra(16) | 10/100 | 33/50 |
| Cl | fra(10) | 0/50 | 0/50* |
| | fra(16) | 2/50 | 32/50 |
| 2 | fra(10) | 0/50 | 0/50* |

* The fragile site at 10q25 was induced in 9/50 metaphases in Subject Co and 11/50 metaphases in Subject Cl by 10 mg/l BrdU added at culture initiation and in 20/50 metaphases in Subject 2 by 100 mg/l BrdC added at culture initiation. The fragile X in Subject B was seen in 17/50 metaphases in MEM-FA.

TABLE VI

Effect of various additives on expression of fra(17)(p12).

All additions were made 24 hours prior to harvest.

| MEDIUM | ADDITIVE | CONCENTRATION (mg/l) | FREQUENCY OF FRA(17) |
|---|---|---|---|
| F10 | Nil | | 3/50 |
| MEM-FA | Nil | | 5/50 |
| 199 | Nil | | 7/50 |
| RPMI 1640 | Nil | | 7/50 |
| " | BrdU | 10 | 20/50 |
| " | BrdU | 50 | 12/50 |
| " | BrdC | 15 | 19/50 |
| " | BrdC | 50 | 37/50 |
| " | Distamycin A | 50 | 43/50 |
| " | Distamycin A | 100 | 50/50 |
| " | Netropsin | 200 | 47/50 |
| " | Netropsin | 300 | 46/50 |
| " | Hoechst 33258 | 5 | 31/50 |
| " | Hoechst 33258 | 10 | 34/50 |

TABLE VII

Comparison of expression of fragile sites induced with BrdU in lymphocytes and fibroblasts from the same subject.

| SUBJECT | FRA | LYMPHOCYTES | FIBROBLASTS |
|---|---|---|---|
| 5 | 10q25 | 30/50 | 22/50 |
| 6 | 10q25 | 35/50 | 14/50 |
| 7 | 10q25 | 16/50 | 2/50 |
| 8 | 10q25 | 32/50 | 12/50 |
| B | 16q22 | 31/50 | 3/100 |

TABLE VIII

Summary of properties of the fragile sites at 10q25, 16q22 and 17p12.

| PROPERTY | 10q25 | 16q22 | 17p12 |
|---|---|---|---|
| Spontaneously expressed | Very rarely | Yes | Yes |
| Induced by 10 mg/l BrdU at culture initiation | Yes | Minor effect | Not known |
| Induced by 50 mg/l BrdU 8 hours prior to harvest | Yes | Yes | Yes |
| Time of maximum induction by BrdU | 6-12 hours | 6-12 hours | 6-12 hours |
| Induced by Distamycin A | No | Yes | Yes |
| Induced by Netropsin | No | Yes | Yes |
| Induced by Hoechst 33258 | No | Yes | Yes |
| Time of maximum induction by Distamycin A | Not relevant | 24+ hours | ? 24+ hours |

Fig.1 Partial metaphase showing simultaneous expression of fra(10)(q25) (large arrow) and fra(16)(q22) (small arrow).

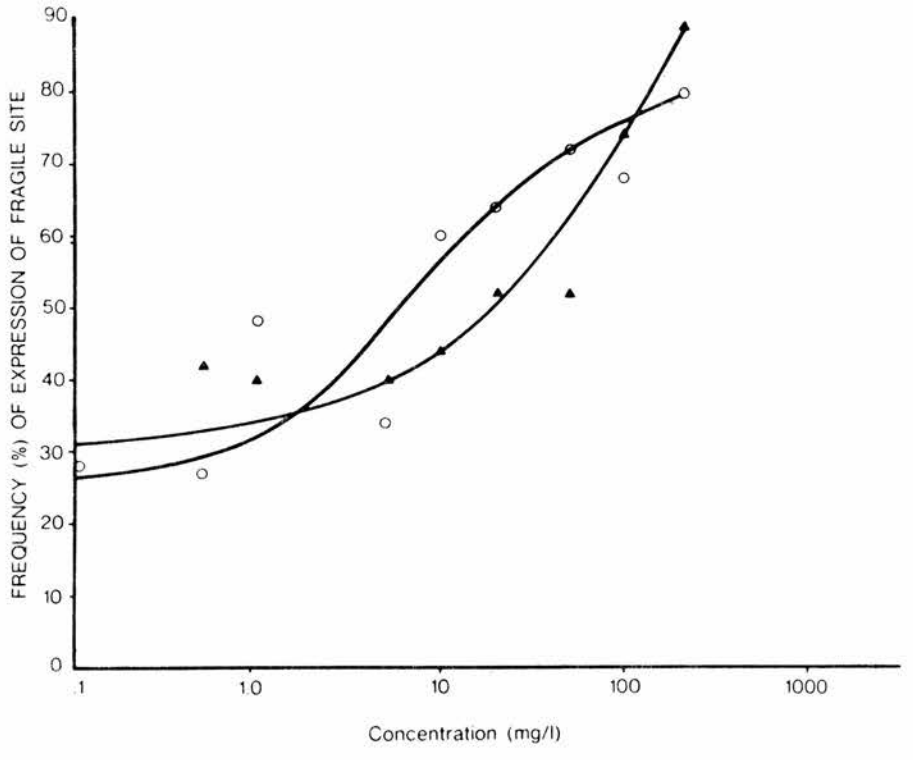



Fig.2 Dose response curves for BrdU (O) and BrdC (Δ) added eight hours prior to harvest on expression of fra(16)(q22) in lymphocyte cultures.

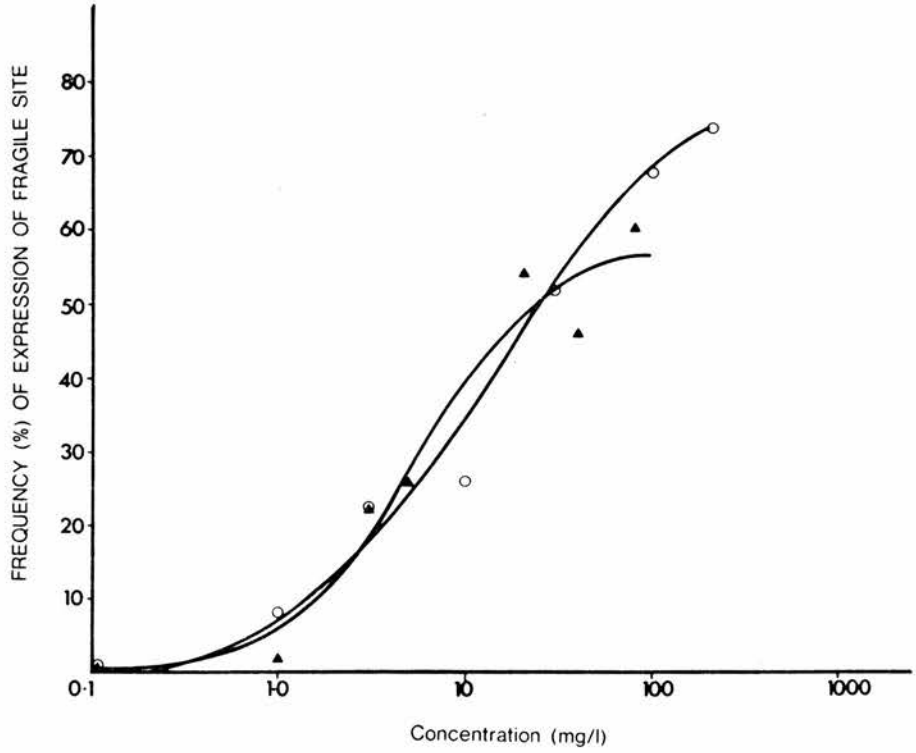



Fig.3 Dose response curves for expression of fra(10)(q25) in lymphocyte culture with concentration of BrdC, in two individuals (O, Δ). The BrdC was added 24 hours prior to harvest.

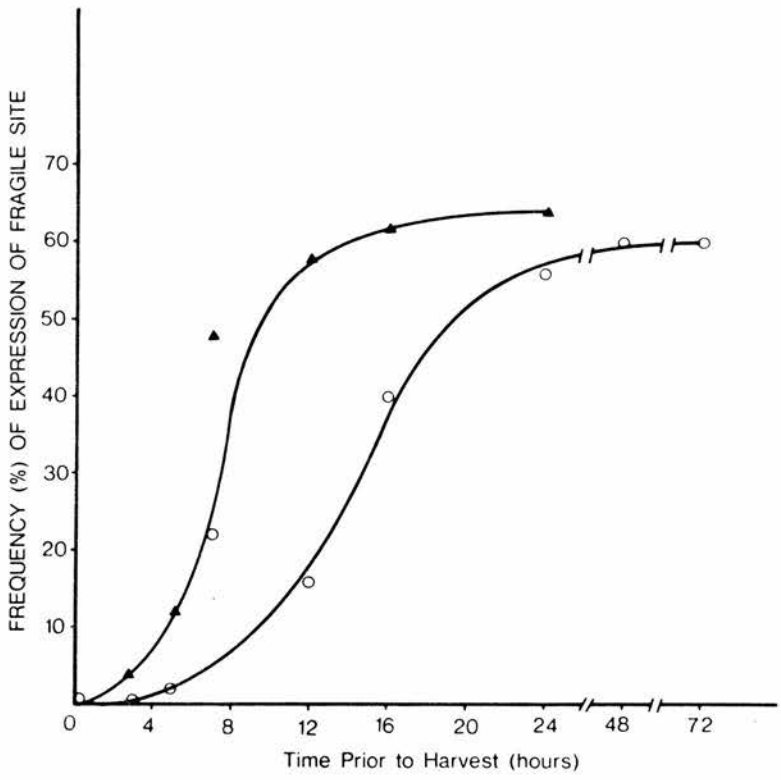



Fig.4 Effect of time prior to harvest of addition of BrdC at concentrations of 50 mg/l (Δ) and 10 mg/l (O) upon expression of fra(10)(q25).

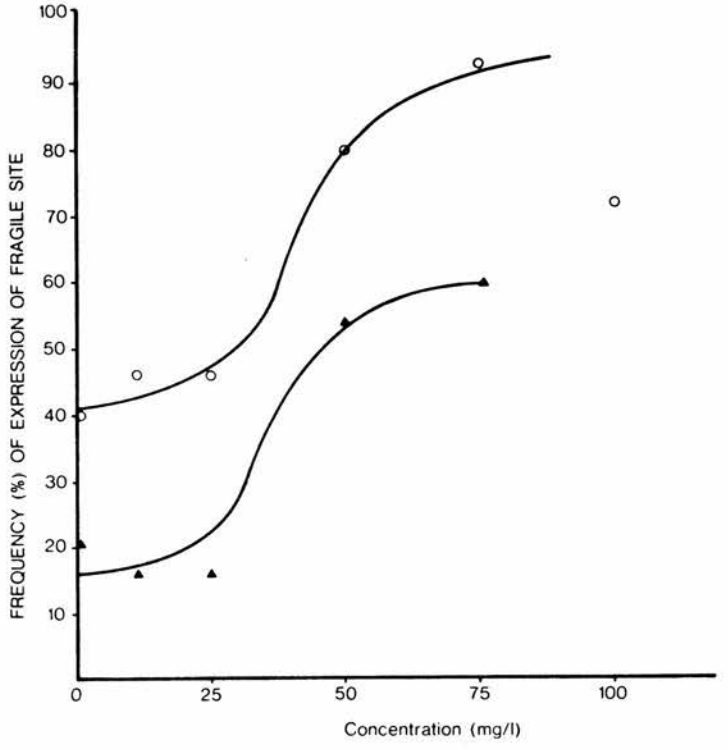



Fig.5 Dose response curves for expression of fra(16)(q22) in lymphocyte cultures from two individuals (O, Δ) with concentration of Distamycin A.

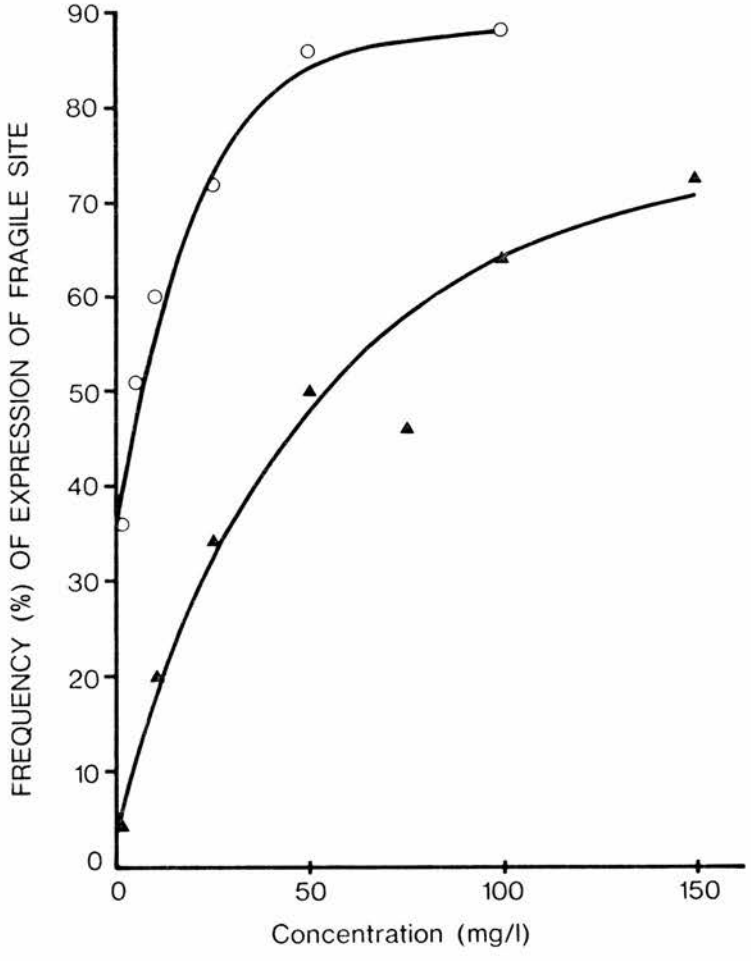



Fig.6 Dose response curves for expression of fra(16)(q22) in lymphocyte cultures from two individuals (O, Δ) with concentration of Hoechst 33258.

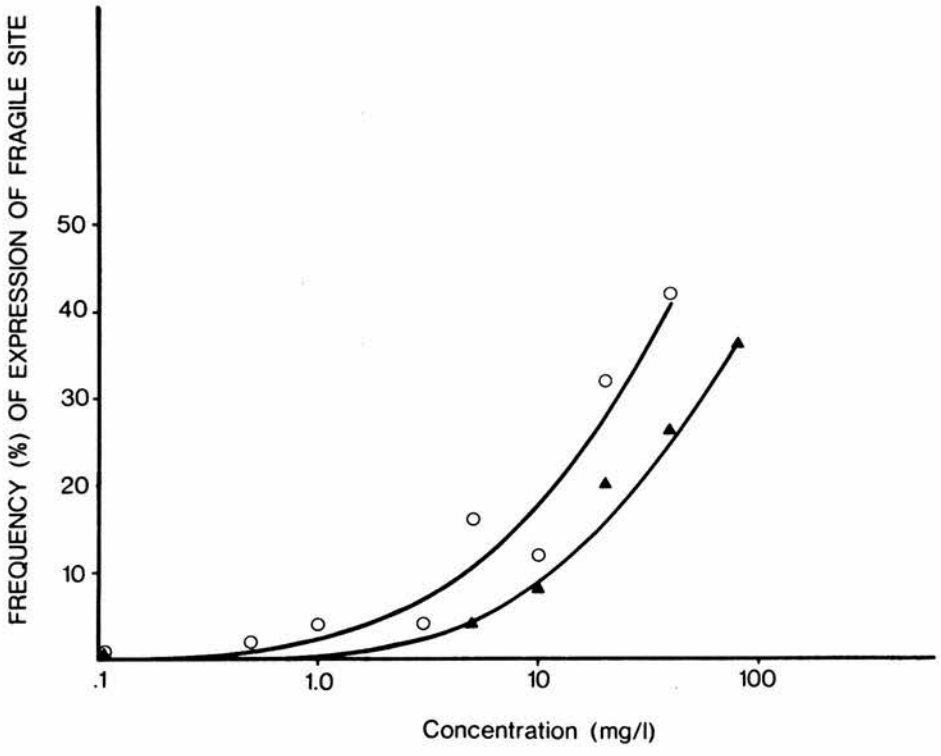



Fig.7 Dose response curves for expression of fra(10)(q25) in fibroblast cultures with concentration of BrdU from two individuals.