IMMUNOGLOBULIN SYNTHESIS IN HUMAN LYMPHOID CELL LINES

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PhD
University of Edinburgh
1981

The work included in this thesis has not been submitted for any other degree or professional qualification.

This thesis has been composed by myself and the work in it is my own.

CONTENTS

ABSTRACT

As an initial step in the investigation of immunoglobulin synthesis in human lymphoid cell lines over one hundred of these lines were screened for the secretion of immunoglobulins using a sensitive haemagglutination inhibition assay. The vast majority of cell lines showed a monoclonal pattern of immunoglobulin secretion with IgM as the predominant class of secreted immunoglobulin. All the cell lines derived from cord blood secreted only IgM. No polyclonal immunoglobulin secretion was observed in established cell lines which had been in culture for some time. Several cell lines were identified which secreted no detectable immunoglobulin or secreted only a single type of immunoglobulin polypeptide (presumptive variants).

In order to establish variant clones from a known immunoglobulin secreting cell line for comparison, cloning and mutagenesis experiments were carried out. A semi-solid agarose cloning technique was developed and used in conjunction with "Melphalan", a human anti-tumour drug which is an established mutagen of immunoglobulin synthesis in mouse myeloma cells. No variant patterns of immunoglobulin synthesis were detected in over two thousand clones of untreated and Melphalan-treated cells analysed for the secretion of both heavy and light chains, nor were any variants detected in over one hundred previously established clones from cultures of other cell lines which had been treated with the mutagens EMS and MNNG.

Further analysis of the immunoglobulins secreted by normal B lymphoblastoid cell lines and the presumptive variant cell lines were undertaken. SDS PAGE profiles, under reducing and non-reducing conditions, of unfractionated biosynthetically radiolabelled culture supernatants suggested that some immunoglobulin polypeptides could be directly identified and might be present in different forms among the secreted proteins. However this analysis also demonstrated that, in general, a large number of diverse proteins were synthesised so that fractionation of the secreted products was necessary to study further the immunoglobulins present.

Serological and SDS PAGE analyses of radiolabelled culture supernatants from an IgM-synthesising cell line fractionated by sucrose gradient velocity sedimentation in the presence of a detergent

demonstrated that the IgM was secreted as a 19S pentamer which could be readily isolated from the other secreted proteins. Similar analyses of the secreted proteins of other IgM-synthesising cell lines demonstrated that; immunoglobulin can account for a very large proportion of the secreted proteins; there is type specific migration of human light chains in SDS gels; in addition to intact immunoglobulin molecules excess free light chains are secreted in varying amounts by different lines. The application of sucrose gradient velocity sedimentation to the investigation of secreted immunoglobulins from the presumptive variant cell lines showed that the single detectable polypeptides secreted by these lines were not present as components of larger otherwise unidentified molecules. This also suggested however, that isolation, and so further investigation, of these polypeptides was unlikely to be achieved in this way.

As an alternative to purification by physical means such as velocity sedimentation, immunoglobulins were isolated from cell culture supernatants by immunoprecipitation. Several methods and reagents were evaluated for immunoprecipitation using isolated 19S IgM for comparison to give optimal results. SDS PAGE analysis of immunoprecipitates from the radiolabelled culture supernatants of presumptive variant cell lines showed that the light chains secreted by these cell lines were of normal molecular weight with the exception of three cell lines which synthesised apparently structurally abnormal light chains.

Normal and presumptive variant cell lines were serologically analysed for intracellular and cell surface immunoglobulin. The normal cells contained, expressed on the cell surface and secreted the same single heavy chain class and light chain type whereas the presumptive variant lines showed an array of different combinations of heavy and light chain synthesis and expression.

The experimental observations outlined above are discussed and the principal conclusions formed can be summarised as follows: The pattern of immunoglobulin synthesis shown by established human lymphoblastoid cell lines suggests that they are derived from a subpopulation of blood lymphocytes which is principally characterised by the active secretion of immunoglobulin. The production of light chains in relative excess of heavy chains in human lymphoblastoid cell lines derived from normal B lymphocytes is consistent with this being a feature of normal immunoglobulin synthesis. In contrast to the

labile properties attributed to immunoglobulin genes in other systems the lack of cloned mutants from human lymphoid cell lines suggests that instability is not a feature of immunoglobulin genes per se but of the system in which they are expressed. The patterns of immunoglobulin synthesis and expression in the presumptive variant lines suggest that some of these may be mutants of immunoglobulin synthesis but others represent early stages of B lymphocyte differentiation and indicate that μ chain synthesis precedes light chain synthesis in B lymphocyte ontogeny. The implications of these conclusions for our current understanding of immunoglobulin synthesis are also discussed.

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INTRODUCTION

OVERVIEW

One of the most remarkable features of the vertebrate immune system is the ability to recognise an enormous range of different antigenic molecules. Antibodies, which belong to a class of proteins known as immunoglobulins, are a major component in the molecular recognition system of the immune response and over the past two decades or so there has been intense study towards understanding their structure, synthesis and genetics.

The biochemical and serological characterisation of immunoglobulin molecules from blood serum has been a source of extensive information about antibodies. However these studies have been superseded in the investigation of the molecular and genetic basis of immunoglobulin production by the use of cultured immunoglobulin synthesising cells. In this field there are two major systems, the mouse myeloma tumours (together with the cell lines which are derived from them) and human lymphoid cell lines. To date, studies have been almost exclusively limited to the former of these sources yet human lymphoid cell lines offer several advantages in studying immunoglobulins and the cells which synthesise them.

IMMUNOGLOBULINS AND THE IMMUNE RESPONSE

Antibodies are not isolated components in the immune system but participate in a continuum of cellular and molecular events. To illustrate this, several features of the immune response are mentioned here. A more complete and detailed account can be obtained from recent immunology text books (See Golub 1977, Roitt 3rd Edition 1977).

The introduction into the vertebrate body of an antigenic determinant, usually a macromolecule or composite of macromolecules, in immunogenic form and dose provokes a specific immune response. This response involves a complex series of events which can be loosely divided into those aspects involving antibody formation and those involving tissue reactions. These two types of response are termed

"humoral" and "cell-mediated" immunity. As a rule, free soluble antigen stimulates humoral immunity while membrane and tissue bound antigens promote cell-mediated immunity. There are however a great many exceptions to this generalisation and prediction must wait until the molecular basis of immunogenicity and antigenicity are better understood. The antibody molecules synthesised in a humoral response to an antigenic determinant belong to a group of proteins called immunoglobulins and bind specifically to that determinant or to similar structures. The combination of antibody and antigen is the first step in the elimination of that complex from the organism by the phagocytic action of macrophages and/or specific enzymic destruction. Both mechanisms involve the interaction of the antibody component in the antigen/antibody complex with one or a number of sequentially acting protein components of the blood serum collectively called "complement".

Although in vivo it requires the co-operation of at least two types of cells, the thymus derived T-lymphocyte and the bone marrow or bursa-derived B lymphocyte to produce immunoglobulin, the synthesis and secretion of large amounts of immunoglobulin is the exclusive property of the B cell lineage (Fig. 1). Constitutive receptor immunoglobulin molecules on the outer membrane of a circulating peripheral B-lymphocyte recognise an antigen and subsequent binding of that antigen to the B-lymphocyte cell surface immunoglobulin receptors induces the B-lymphocyte to proliferate and differentiate into a plasma cell rapidly synthesising and secreting immunoglobulin. Each plasma cell in the clone derived from a given antigen-sensitised B-lymphocyte produces a single molecular species of antibody which binds that antigen.

The complete role of T-lymphocytes is less well understood but as a class they have several subsets which can have effector or regulator functions. Effector T-lymphocytes are the agents of cell-mediated immune reactions and regulator subsets of T-lymphocytes modulate the response to most antigens controlling, among other things, immunoglobulin production by B-lymphocytes (Fig. 1). T-lymphocytes are also known to be antigen specific in their responses and there is evidence demonstrating that the T-lymphocyte receptor which mediates antigen recognition is infact a partial immunoglobulin molecule

Fig 1

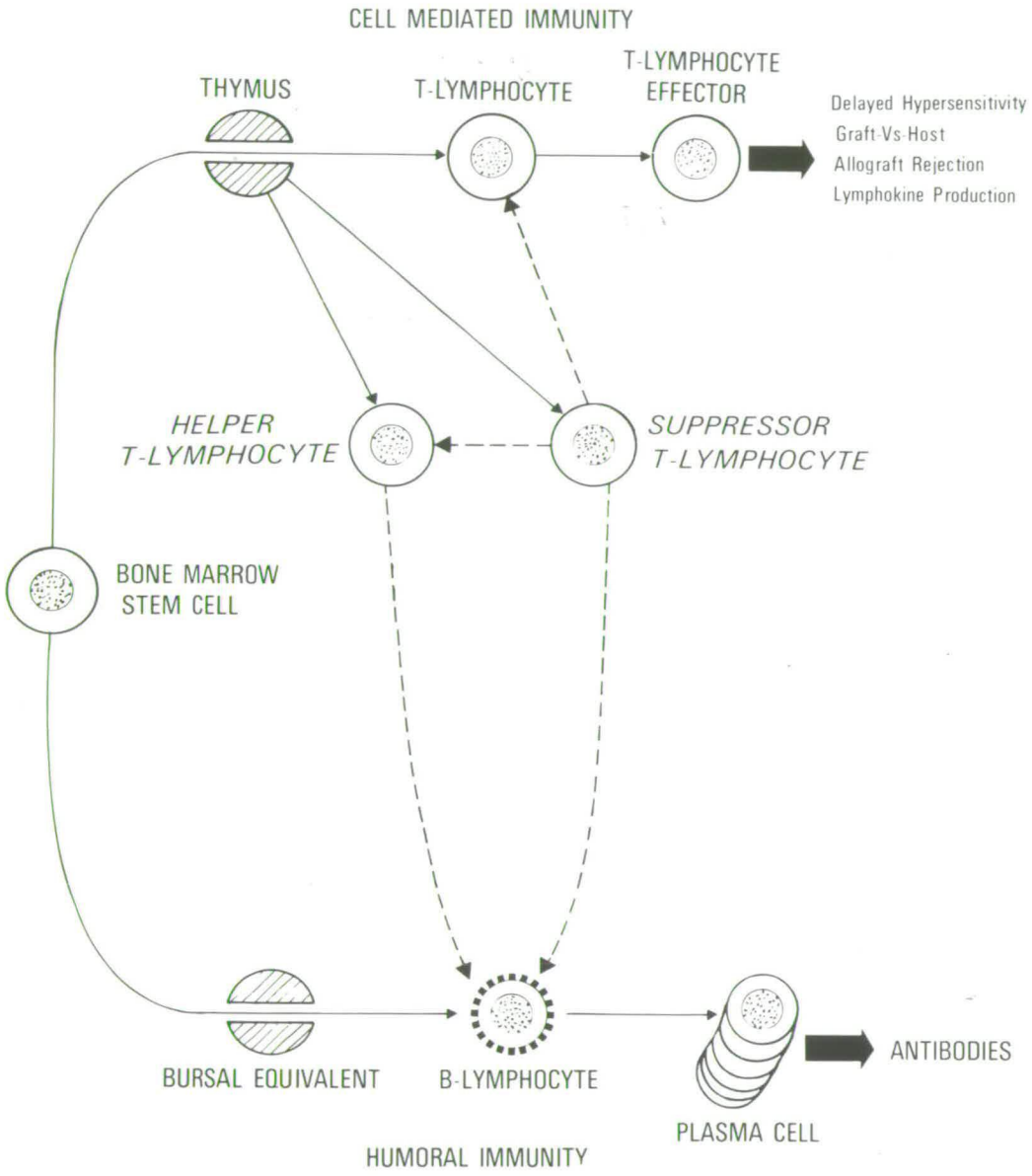

CELL MEDIATED IMMUNITY
THYMUS
T-LYMPHOCYTE
T-LYMPHOCYTE EFFECTOR
Delayed Hypersensitivity
Graft-Vs-Host
Allograft Rejection
Lymphokine Production
HELPER T-LYMPHOCYTE
SUPPRESSOR T-LYMPHOCYTE
BONE MARROW STEM CELL
BURSAL EQUIVALENT
B-LYMPHOCYTE
ANTIBODIES
PLASMA CELL
HUMORAL IMMUNITY

Fig. 1 A diagramatic overview of T and B lymphocyte development and function

(Adapted from Broder et al., 1978)

"Bursal Equivalent" refers to the Avian Bursa of Fabricius which is the organ of B lymphocyte development in that species. No similar organ has been found in other vertebrates but historically a "Bursal Equivalent" is inferred in non-Avian B lymphocyte ontogeny.

Ag : antigen

——— : infers a pathway of cellular development

------ : indicates cell interaction

synthesised by the T-lymphocyte itself (Krawinkel et al., 1979) and that regulator molecules produced by T-lymphocytes may also be in some part immunoglobulin-like (Sy et al., 1980). In addition, another family of immunologically important molecules, the classical transplantation or histocompatibility antigens have some structural similarities with the immunoglobulins (Orr et al., 1979). However, while accepting the importance of these related molecules, the immunoglobulins themselves are central to the functioning of the immune system.

THE STRUCTURE OF IMMUNOGLOBULINS

There is an extensive background of information on immunoglobulin structure, which has been described in detail in many reviews and texts (see for example Secher 1979 and Nisonoff et al., 1975). However a brief account of the principal aspects of immunoglobulin structure and nomenclature together with some reference to their relationship to function is appropriate here.

Malignant disorders of B-lymphocytes such as multiple myeloma in humans lead to abnormally high levels of homogeneous immunoglobulin molecules in the serum and urine of affected individuals. The study of these "myeloma proteins" together with the earlier analysis of pooled normal rabbit immunoglobulin (Porter 1959) established the basic polymeric composition of immunoglobulin molecules. Furthermore, detailed comparisons of the aminoacid sequences of polypeptides from various myeloma proteins yielded the characteristic primary structural organisation of the major immunoglobulin polypeptides (Edelman et al., 1969).

The major polypeptides of immunoglobulins; heavy chains and light chains

The basic immunoglobulin model for all vertebrates studied to date is the symmetrical four chain polypeptide unit shown in Fig. 2. (Nisonoff et al., 1975). This unit occurs singly in most immunoglobulins but can be present as a dimer or pentamer in some immunoglobulin classes (See Table 1). However, in every immunoglobulin the monomer

Fig. 2 A generalised IgG molecule showing the basic tetrapeptide chain structure of the monomeric immunoglobulin unit

(Adapted from Milstein and Svasti 1971)

The left hand part of the diagram shows the various regions within the molecule; the right hand side shows the orientation of the heavy and light chains and the arrangement of disulphide bridges.

-S-S- : disulphide bridge

CHO : carbohydrate

H : hinge

Fig 2

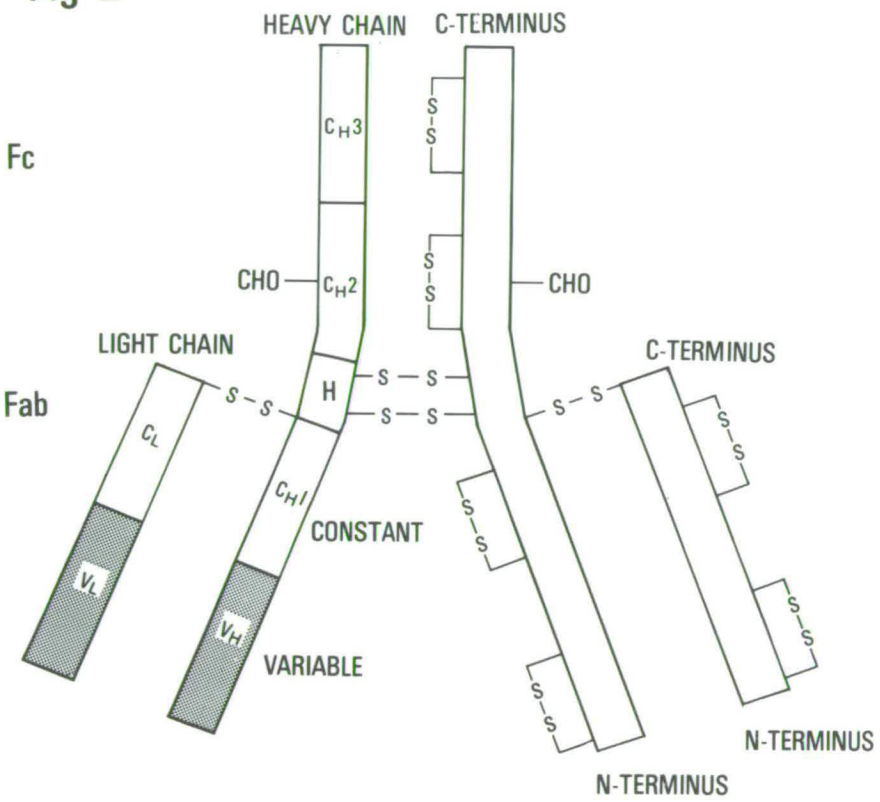

HEAVY CHAIN
C-TERMINUS
Fc
C_H3
CHO
C_H2
CHO
LIGHT CHAIN
C-TERMINUS
Fab
H
C_L
C_H1
CONSTANT
V_L
V_H
VARIABLE
N-TERMINUS
N-TERMINUS

Table 1 Some properties of human immunoglobulins

Adapted from Nisonoff et al., (1975) and Turner (1977)

TABLE 1

Immunoglobulin	Heavy chain	Structure	Sedimentation coefficient	Molecular weight ($x10^{-3}$)	Heavy chain molecular weight ($x10^{-3}$)	Number of heavy chain domains	Carbohydrate content (%)	Normal serum concentration (mg/ml)
IgG_1	γ_1	monomer	7	146	51	4	2 - 3	9
IgG_2	γ_2	monomer	7	146	51	4	2 - 3	3
IgG_3	γ_3	monomer	7	165	60	4	2 - 3	1
IgG_4	γ_4	monomer	7	146	51	4	2 - 3	0.5
IgM	μ	Pentamer + J chain	19	970	72	5	9 - 12	1.5
IgA_1	α_1	monomer	7	160	52 - 56	4	7 - 11	3
IgA_2	α_2	monomer	7	160	52 - 58	4	7 - 11	0.5
sIgA	α_1 or α_2	dimer + J chain + secretory component	11	380 - 390	52 - 58	4	7 - 11	0.05
IgD	δ	monomer	7	172 - 200	60 - 69	4 or 5	9 - 11	0.03
IgE	ϵ	monomer	8	188 - 196	72 - 76	4	12	0.0003

unit consists of two identical heavy chains (51,000 - 76,000 mol. wt.) and two identical light chains (21,600 mol. wt.). On the basis of comparisons of exact aminoacid sequences of immunoglobulin polypeptides, both heavy and light chains have been divided into two regions, an N-terminal "variable" region of about 110 - 120 aminoacid residues and a C-terminal "constant" region which comprises the remainder of each polypeptide. (Milstein et al., 1967, Putman 1974).

Constant regions and variable regions

This constant (C) regions of immunoglobulin polypeptides do not vary in aminoacid sequence except at a number of residues reflecting isotypic and allotypic variation (Milstein and Munro 1973). In humans there are five isotypes or classes of heavy chain α δ ε γ and μ, with subclasses among the α and γ chains and, as throughout the vertebrates, there are two light chain types; κ and λ. Immunoglobulin molecules are differentiated from each other by their heavy chain class, an α heavy chain containing molecule belonging to the IgA class of immunoglobulins, a γ heavy chain the IgG class and so on (See Table 1). An Ig molecule of any class may have light chains of either κ or λ type though not a hybrid mixture.

Immunoglobulin C region allotypes are alternative forms of isotypes present in only some individuals of a species. These serologically detected markers are of great significance when studying immunoglobulin genetics (Natvig and Kunkel 1973). However despite correlation of many of these markers with variations in aminoacid sequence, allotypes are not a major source of classification in immunoglobulin structure.

The variable (V) regions of immunoglobulin heavy and light chains are not uniformly variable but have distinct groups of "hypervariable" residues. There are three such hypervariable segments in the V regions of light chains (Wu and Kabat, 1970) and three or four in the V regions of heavy chains. The hypervariable regions of a heavy chain together with the corresponding sequences of the associated light chain fold together in the antibody molecule to form an antigen combining site (Poljak 1974). This gives the antibody molecule, in its monomeric form, two identical combining sites. In contrast to the hypervariable residues, the non-antigen binding portions of an immunoglobulin chain V region are conserved and are referred to as the V region "framework" sequences (Wu and Kabat 1970, Capra and Kehoe 1975).

Immunoglobulin domains

Immunoglobulin molecules show a high degree of internal homology in primary structure (Edelman 1972). Both heavy and light chains can be divided into contiguous regions of homology of about 110 - 120 aminoacids in length: Two in the light chains, V_L (variable light) and C_L (constant light) and four or five depending upon class in heavy chains, V_H (variable heavy), C_H1 (constant heavy 1), C_H2 (constant heavy 2) etc. (See Fig. 2 and table 1). The V region sequences are homologous with each other but not with the C regions; each of the C_H regions are homologous to each other and to C_L. Intrachain disulphide bonds are located, one in each homology region, enclosing a peptide loop of about 60 - 70 aminoacid residues in each case.

The linear periodicity in primary sequence and disulphide bond arrangement shown by immunoglobulin molecules underlies a similarly ordered tertiary and quaternary structure. The "domain" hypothesis proposed by Edelman (1970) suggested that each homologous region is folded into a compact globular structure (domain) and linked to neighbouring domains by more loosely folded stretches of polypeptide. Electron microscopic and X-ray crystalographic evidence have confirmed the existance of domains as independant three-dimentional entities (Pojak 1975) and in addition it has been shown that there is a characteristic "immunoglobulin fold" which, to date, has been found with only minor modifications in every immunoglobulin domain studied (Feinstein & Beale 1977). Edelman (1970, 1972) has further suggested that each homologous region has evolved as an active site mediating a function of the immunoglobulin molecule. There is evidence to support this concept in the function of the V region domains being antigen binding and in the location of certain effector functions of immunoglobulin molecules within specific heavy chain C region domains e.g. $C1_q$ (the first complement factor) binding to the C_H2 domain in human IgM and IgG classes and monocyte membrane binding by the C_H3 domain of human IgG (Turner 1977).

The hinge region

The interdomain segment known as the "hinge region" (See Fig. 2) occurs in humans in the centre of the linear sequence of α, δ, and γ

heavy chains (Edelman 1970, Liu et al., 1976, Spiegelberg 1977) and contains most or all of the interchain disulphide bonds. Immunoglobulin sequence homology is at its lowest in the hinge region (Michealson et al., 1977) which is rich in proline residues and is particularly susceptible to enzymic cleavage. It has been suggested that the role of the hinge may be in providing a flexible or specific angle between the two binding sites of the monomer molecule which, when antigen is bound, co-ordinates the action of the antigen binding (Fab) and effector (Fc) portions of the molecule (Huber et al., 1976).

The terms Fab and Fc (Fig. 2) are used in describing all immunoglobulins but originate from the proteolysis of rabbit IgG by the plant protease papain (Porter 1959): Fab - Fragment antigen binding; Fc - Fragment crystalline which was subsequently shown to be associated with the effector functions of the molecule.

Other polypeptide components

In addition to heavy chains and light chains there are two other polypeptides found in certain immunoglobulins: The J chain is a 15,000 Dalton polypeptide synthesised by antibody forming cells but which has no structural homology to other immunoglobulin polypeptides. It is invariably found in association with IgM pentamers and IgA dimers (Koshland 1975). Although no role other than its structural one has been forwarded for the human J chain, it has been proposed that the J chain has a controlling influence in the polymerisation of murine IgM (Della Corte and Parkhouse 1973). The secretory component (SC) is a 60,000 Dalton polypeptide structurally unrelated to other immunoglobulin polypeptides (Mestecky et al., 1971) and associated with sIgA which is the IgA dimer of external secretions (gut, saliva and milk). The secretory component is not synthesised by antibody forming cells but by epithelial cells and is attached during the secretion process (Poger and Lamm 1974).

Carbohydrate moiety

Besides polypeptides, immunoglobulins also contain oligosaccharides; infact all immunoglobulins are glycoproteins. The carbohydrate content in immunoglobulins varies between the different classes (See table 1)

and there is a wide variation in the sugar groups present (Clamp and Johnson 1972). Heavy chains always have covalently attached carbohydrate; in human α, δ and γ chains there is only one carbohydrate side chain in each case attached immediately N-terminal to the C_H2 domain (Edelman 1970, Pink 1970, Spiegelberg 1977), but μ chains can have up to five oligosaccharide units all at different positions in the C region (Putman 1973).

The secretory component and the J chain are also glycopeptides but light chains in general have no carbohydrate content. However oligosaccharide attachment to light chains has been reported at aspartic acid or asparagine residues in the light chain V region. In each case the light chains have been free Bence-Jones proteins of murine and human origin (Knope et al., 1975, Keifer et al., 1980). No satisfactory explanation has yet been offered as to the biological role of the oligosaccharide substituants of immunoglobulin molecules. It has been suggested that the attachment of carbohydrate facilitates the secretion of certain classes of immunoglobulin in murine myelomas but this observation excludes mouse IgG which can be secreted with or without its oligosaccharide groups (Hickman and Kornfield 1978). A structural role for immunoglobulin carbohydrate has been put forward in a human IgG myeloma protein where it prevents close contact of the C_H1 and C_H2 domains which is important in the interaction of the Fab and Fc regions of the molecule (Silverton 1977).

Although the oligosaccharides of immunoglobulins are interesting, such relationships between carbohydrates and proteins are common, however, the nature of immunoglobulin polypeptide chains especially in terms of the relationship between their structure and the genes which encode them is far from common and perhaps unique to those proteins.

THE GENETICS OF IMMUNOGLOBULINS

This is another well reviewed topic especially from the point of view of the classical genetics of polypeptide allotypes and sequences (Milstein and Munro 1973, Sogn and Kindt 1977) but also more recently

at the nucleic acid level (Rabbitts 1979). The latter of these two approaches using new DNA technology continues to make rapid inroads on immunoglobulin genetics and it is this field which forms the greater part of the following short account.

There are two crucial observations from immunoglobulin structure which have influenced the study of immunoglobulin genetics - The striking variable/constant nature of heavy chains and light chains and the extensive non-random diversity of V region sequences. To resolve the genetic problem of two structurally distinct regions within one polypeptide Dreyer and Bennet (1965) hypothesised that separate genes were required to code for the V and C regions of each chain. Furthermore, their hypothesis offered an explanation of antibody diversity by proposing that a single C region gene can be expressed in conjunction with any one of a battery of V region genes, each coding for a different V region, by somatic recombination. These arguments have been shown to be relevant to several features of antibody gene organisation and expression.

Separate V and C genes and their integration

Sequence studies and genetic analysis of the V and C regions of both heavy and light chains of myeloma and normal serum immunoglobulins have been consistant with the "two genes one polypeptide" argument of Dreyer and Bennet (1965). For example aminoacid sequence analysis of several human κ light chains has revealed three basic groups of V region sequences (Milstein 1967) defined by characteristic residues and shared sequence insertions or deletions. It is generally accepted that if the groups of sequences are well defined, as in the case of human κ chains V regions, they represent the expression of multiple non-allelic genes (for a more detailed explanation see the section on V region diversity). Since, in addition, the allotypes of human κ chain C regions segregate in a true allelic fashion (Mage et al., 1973) suggesting that there is only one κ C gene per haploid set of chromosomes separate V and C genes and their integration must be postulated. Analysis of aminoacid sequences and of the segregation of markers in other human immunoglobulin polypeptides and the immunoglobulins of the mouse and rabbit, where V region markers are also available (Milstein and Munro 1973, and Sogn and Kindt 1977) have given similar results and shown the following general lay-out of antibody genetics; The immunoglobulin

genes reside in three unlinked groups, one codes for the heavy chains another for the κ chains and the third for λ chains. Within each family there are separate clusters of V and C genes (the number of V genes being greater than the number of C genes) which are closely linked to each other but are clearly distinct since several recombinants have been detected in mice and rabbits (Mage et al., 1973, Leiberman et al., 1974).

Having established that for heavy chains and each of the light chain types there are separate V and C coding sequences the question then arises of the stage at which the information from the separate genes is combined to produce a complete heavy or light polypeptide chain. Evidence against this happening at either the transcriptional or translational levels comes from the contiguous translation of V and C regions from the same light chain messenger RNA isolated from a mouse myeloma tumour cell line grown in tissue culture (Milstein et al., 1974) and from the absence of V and C region scrambling in the immunoglobulins synthesised by hybrid myeloma cells (Kohler and Milstein 1975). Both these observations conform with a hypothesis that the V and C genes are rearranged and joined together at the DNA level.

Direct evidence for separate V and C genes in undifferentiated DNA (representing the germline) and their subsequent rearrangement or translocation in differentiated tissue has been presented by Hozumi and Tonegawa (1976). They demonstrated that the treatment of mouse embryonic DNA with a restriction endonuclease produced two fragments which showed significant hybridisation with a labelled κ light chain mRNA whereas the similar treatment of mouse myeloma DNA produced only one such fragment. In the same way a similar rearrangement was shown for the mouse λ V and C genes (Tonegawa et al., 1976). However although these experiments demonstrated the rearrangement of light chain V and C genes such that they could be detected on a single DNA fragment, they had not shown that the V and C genes were joined into a continuous coding sequence in that fragment. In fact the susceptibility of nucleic acid hybrids between κ cDNA (Rabbitts and Forster 1978) and κ mRNA (Mathyssens and Tonegawa 1978) to a single-strand specific nuclease suggested that the rearranged V and C genes were not contiguous. Furthermore, duplexes between mouse λ mRNA and a cloned myeloma V+C gene seen under the electron microscope formed

loops of intervening sequences between the V and C coding segments (Brack and Tonegawa 1977). The non-contiguous nature of the rearranged λ chain V and C genes was confirmed by the complete nucleotide sequence of a mouse myeloma λ gene which together with the sequence of the corresponding germline gene (Bernard et al., 1978) also revealed other interruptions in the gene structure and identified the site of integration of the V and C genes (See Fig. 3a). The germline sequence consisted of 45 base pairs (b.p.) coding for the leader (L) or signal peptide (an N-terminal peptide present in the unsecreted λ chain precursor), 93 b.p. of untranslated intervening sequence (I_1), 306 b.p. coding for the V region up to aminoacid residue 102 (V), about 4,500 b.p. of undefined DNA, a 39 b.p. sequence coding for the remainder of the V region (J), an untranslated intervening sequence of about 1,200 b.p. (I_2) and finally the uninterrupted C region coding sequence (C). In the myeloma DNA the two partial V region coding sequences were covalently linked together eliminating the large undefined segment of DNA from the gene; the rest of the gene structure remaining unchanged. A comparison of the germline and myeloma sequences shows that integration of the mouse λ chain coding segments does not occur at the V/C junction but between a foreshortened V region gene and a segment of DNA (which has been termed the J or joining segment) encoding the final V region sequences. Siedman and colleagues (1979) have reported a similar gene structure and analogous joining mechanism in a mouse κ light chain. Further sequence analysis (Max et al., 1979, Sakano et al., 1979) has shown the κ arrangement to be more complicated than the single λ J segment revealing a cluster of five J segments identified by homology to κ chain aminoacid sequences.

Murine immunoglobulin heavy chain genes are also interrupted by non-coding sequences and show somatic rearrangement. The hybridisation of mouse V_H and C_H cDNA with embryonic and myeloma DNA has shown the V genes to be distant from the cluster of C genes in the germline and rearranged in the differentiated tissue (Maki et al., 1980, Gough et al., 1980).

Comparisons of the DNA sequence of a germline V_H gene and the corresponding rearranged sequence expressed in an α chain (Early et al., 1980a)and a γ_{2b} (subclasses of the mouse γ chains are 1, 2a, 2b and 3) chain (Sakano et al., 1980) have shown that the germline V_H sequence

Fig. 3 Arrangement, rearrangment and transcription of murine immunoglobulin genes

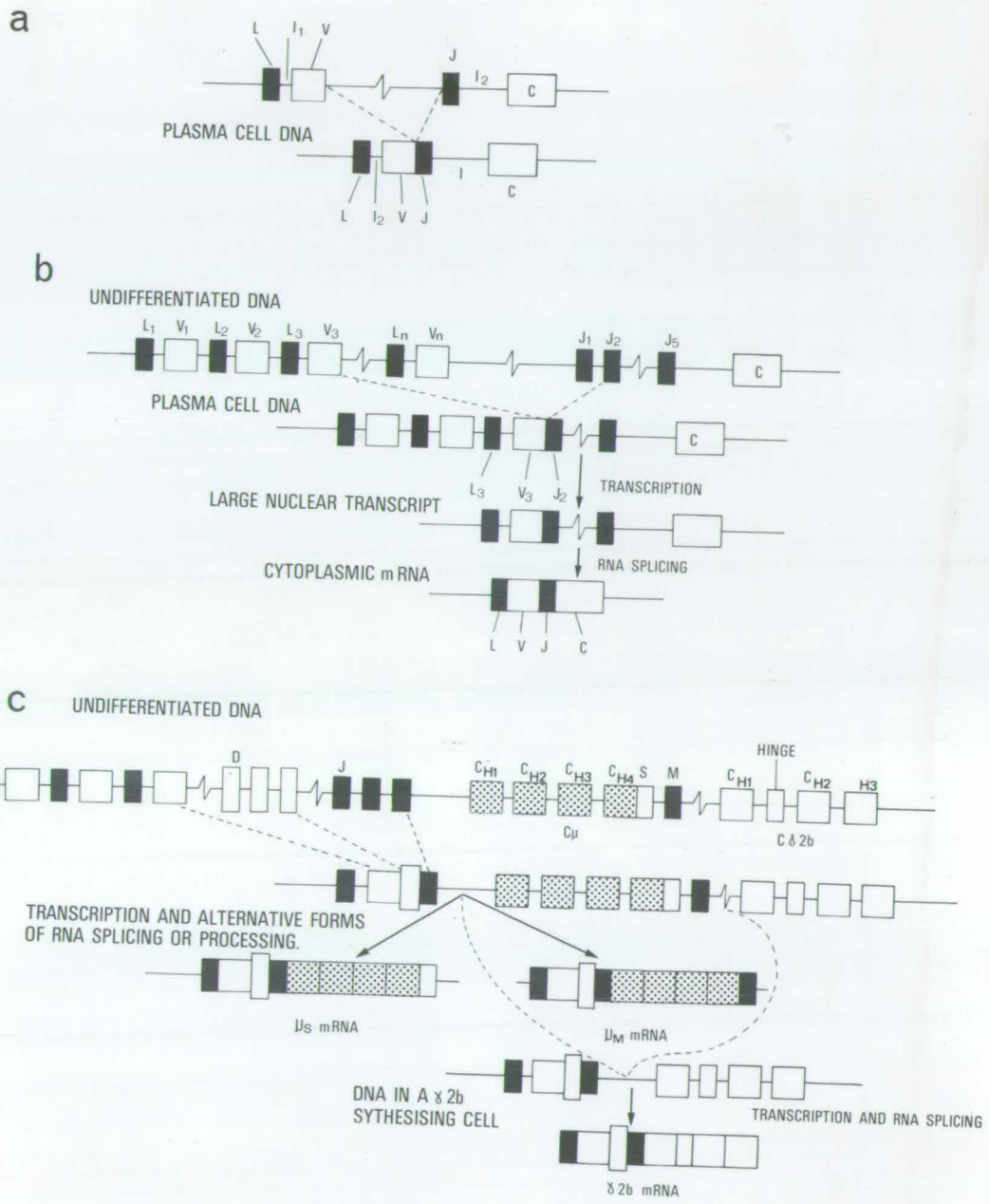
Horizontal lines are introns or non-transcribed sequences in DNA and untranslated portions of RNA; raised boxes are exons in DNA and translated sequences in RNA.

(a) Murine λ light chain genes in embryonic or germline (undifferentiated DNA) and plasma cell DNA.

(b) Murine κ light chain genes in embryonic or germline (undifferentiated DNA) and plasma cell DNA showing also the transcription of the plasma cell gene and the splicing of that transcript.

(c) Murine heavy chain genes in embryonic or germline (undifferentiated DNA) and in the DNA of μ and γ2b synthesising cells as a demonstration of the heavy chain switch; this diagram also shows the transcription of μs or μm mRNA.

Fig 3


a
L
I1
V
J
I2
C
PLASMA CELL DNA
I
C
L
I2
V
J
b
UNDIFFERENTIATED DNA
L1
V1
L2
V2
L3
V3
Ln
Vn
J1
J2
J5
C
PLASMA CELL DNA
C
L3
V3
J2
TRANSCRIPTION
LARGE NUCLEAR TRANSCRIPT
RNA SPLICING
CYTOPLASMIC m RNA
L
V
J
C
c
UNDIFFERENTIATED DNA
D
J
CH1
CH2
CH3
CH4
S
M
HINGE
CH1
CH2
H3
Cμ
C δ 2b
TRANSCRIPTION AND ALTERNATIVE FORMS
OF RNA SPLICING OR PROCESSING.
μS mRNA
μM mRNA
DNA IN A δ 2b
SYTHESISING CELL
TRANSCRIPTION AND RNA SPLICING
δ 2b mRNA

ends at the sequence coding for the third hypervariable region i.e. before the end of the expressed V_H sequence. By analogy with the light chain genes this suggested that the missing sequences should be found in the J segments associated with a C gene and in both cases the authors sought and found, by hybridisation, a cluster of J segments close to the Cμ gene. However, sequencing of the J segments revealed that in neither case did the sum of the V_H and J_H germline sequences equal that of the intact expressed V gene. In the case of the V region expressed in an α chain, the coding gap coincided with 14 aminoacid residues of the third hypervariable region and with the nucleotides encoding 6 aminoacid residues constituting the centre of the third hypervariable region in the case of the V region expressed in an γ2b chain. These observations have prompted the conclusion that the missing nucleotides originate from one or more of a third type of germline V gene segment termed the "D" or diversity segment, the complete gene being assembled by recombination of V, D and J segments. The existence of D segments in mouse heavy chain gene construction has also been predicted on the basis of aminoacid sequence data (Rao et al., 1979, Schilling et al., 1980).

Each coding region specifies a domain

The rearranged and expressed murine heavy chain V gene, in common with its light chain counterpart, is continuously coded but is itself separated from the constant region by a non-coding sequence (Early et al., 1980a, Sakano et al., 1980, Gough et al., 1980). In the case of murine light chains the constant region coding DNA is also uninterrupted (Bernard et al., 1978, Seidman et al., 1979) so that the entire rearranged light chain gene can be seen as being constructed from separate segments of DNA coding for each homology region or domain. Mouse heavy chain genes show the same fundamental structure as, in addition to the separately encoded V region domain, DNA sequence and hybridisation studies have shown that in the α (Early et al., 1979), γ_1 (Sakano et al., 1979, Honjo et al., 1979), γ2b (Kataoka et al., 1979), μ (Gough et al., 1980, Calame et al., 1980, Kawakami et al., 1980) and δ (Tucker et al., 1980) C region genes the individual domains (and hinge regions where present) are each encoded in separate segments of DNA (exons) divided from one another by non-coding intervening sequences (introns).

The Heavy Chain Switch

The existence of a number of different heavy chain C regions in any individual (e.g. nine in man, eight in mice) sharing the same set of V regions complicates the expression of heavy chain genes. Several studies in different species have shown that at an early stage of B lymphocyte differentiation an individual B lymphocyte initially expresses the IgM class of immunoglobulin, (Cooper et al., 1976). Later, although light chain expression remains unaltered, there is a "heavy chain switch" when the same B lymphocyte, or its clonal progeny changes the class of heavy chain it secretes while retaining the same heavy chain V region, (Gearhart et al., 1975, Wang et al., 1977, Abney et al., 1978).

In the mouse germline DNA the heavy chain J segments which mark the site of V gene integration in the heavy chain gene complex are located next to the $C\mu$ gene which lies at the start of a cluster containing all the heavy chain C region genes (Early et al., 1980a, Sakano et al., 1980). It has been suggested that from this germline configuration two separate somatic rearrangements are required to generate complete immunoglobulin heavy chains other than those of the μ class (See Fig. 3). The first joins the V, D and J segments of the V coding sequences and the second replaces the $C\mu$ coding sequences with those of another heavy chain class or subclass i.e. mediates the heavy chain switch. Direct evidence that DNA rearrangement is the mechanism of C_H switching has been presented from the examination by hybridisation and sequence studies of rearranged α (Davis et al., 1980a), γ_1 (Kataoka et al., 1980) and γ_{2b} (Sakano et al., 1980) genes from mouse myeloma tumours expressing those respective classes of heavy chain. In each of these cases the expressed gene contains, in addition to a fused V gene and the appropriate C_H gene, a portion of the sequences which flank the $C\mu$ gene on the J segment side. This suggests that the V_H gene was initially associated with the $C\mu$ gene and that a later DNA rearrangement then replaced the $C\mu$ gene with the subsequent C_H gene by linking together their respective flanking sequences. The point at which the flanking sequences join in the rearranged gene and the corresponding breakpoints in the germline DNA are termed the "switch sites". The sequence analysis of three different $C\mu$ - $C\alpha$ switching sites (Davis et al., 1980b) has shown that the sequences surrounding those sites are highly conserved blocks of thirty

nucleotides which may serve as a recognition sequence. These sequences are unlike those thought to mediate V-D-J joining in V_H genes (Sakano et al., 1979, Early et al., 1980a, Max et al., 1979) and they lack homology with the sequences reported for the $C\gamma_1$ (Kataoka et al., 1980) and $C\gamma_{2b}$ (Sakano et al., 1980), switching sites which in turn suggests that heavy chain switching is mediated by class specific recognition sequences. The γ_1 gene sequence of mouse myeloma MC101 (Kataoka et al., 1980) as analysed by Davis et al., (1980b) contains $C\mu$, $C\alpha$ and $C\gamma_1$ flanking sequences in that order between the V_H and $C\gamma_1$ gene segments indicating that switching progressed from $C\mu$ to $C\alpha$ and subsequently from $C\mu$ to $C\gamma_1$ and so it may be a multistep process. Alternatively, successive complex switching may be an aberration characteristic of myeloma cell lines.

In any case, the order of events contrasts with that of the simple deletion model for switching proposed by Honjo and Kataoka (1978). They employed hybridisation kinetics to determine the number of C_H genes in mouse myeloma tumours producing different classes of immunoglobulin and concluded that a V_H gene is combined with a second C_H gene by deletion of the intervening DNA between the V_H and second C_H genes. Based on this model their data fit a heavy chain gene order of $C\mu$-$C\gamma_3$-$C\gamma_1$-$C\gamma_{2b}$-$C\gamma_{2a}$-$C\alpha$. The use of cloned cDNA C_H probes to demonstrate that $C\mu$ genes were not present in the fractionated DNA of IgG and IgA secreting mouse myelomas although each retains a full complement of $C\alpha$ genes (Cory and Adams 1980) and similar experiments using different combinations of C_H probes and myeloma lines expressing different immunoglobulins (Cory et al., 1980, Coleclough et al., 1980, Rabbitts et al., 1980a) have supported Honjo and Kataoka's mechanism and order of C_H switching. However, in contrast to Honjo and Kataoka (1978) these later reports have shown that C_H switching was often not confined to one allelic set of heavy chain genes and even took different forms within a single cell line, presumably varying on different homologous chromosomes. That different gene arrangements in the same cell do not result in the synthesis of two different heavy chain polypeptides is due to "allelic exclusion" in immunoglobulin gene expression.

Allelic Exclusion

Each normal antibody secreting cell and its progeny from an individual heterozygous for immunoglobulin C region markers expresses only

one of either of the two available allotypes (Pernis et al., 1965, Davie et al., 1971) and this phenomenon has been termed "allelic exclusion". Despite having been established for some time, mechanisms have not readily been put forward to explain allelic exclusion in immunoglobulins. However during the studies of immunoglobulin gene structure allelic exclusion is another property of immunoglobulins which has been linked with the rearrangement of their genes.

Several authors have suggested that allelic exclusion operates by the expressed allele being rearranged while the immunoglobulin genes remain in their germline configuration on the excluded chromosome. This argument has been complicated by the frequent failure to detect unrearranged heavy chain (Cory and Adams 1980, Coleclough et al., 1980) and light chain (Lenhard-Schuller et al., 1978, Perry et al., 1980) genes in mouse myeloma cells. However, only one rearranged gene is expressed, others are non-functional or "abortive" rearrangements which may result, for example, in a reading frame shift producing a stop codon (Altenberger et al., 1980) or an erroneous splicing pattern of the primary transcript (Siedman and Leder 1980, Choi et al., 1980). Following these observations the first argument has been modified to suggest that allelic exclusion may be the result of a multistep process (Perry et al., 1980, Coleclough et al., 1980) with different V gene rearrangements taking place until one functional V-J or V-D-J integration has been formed, after which further rearrangements are inhibited by an unknown mechanism. The occurrence of κ mRNA's and rearranged κ genes in λ producing mouse myelomas has prompted the proposal that the same mechanism that mediates allelic exclusion is responsible for ensuring the synthesis of only one light chain type by each B lymphocyte and its progeny (Alt et al., 1980a) i.e. genic or isotypic exclusion. This would be achieved by exclusion of the formation of complete λ chain genes in cells already expressing κ chains while, following a "pecking order", λ genes would only be rearranged if the joining of κ gene segments failed to produce a functional light chain gene. In the case of heavy chains, more than one class is expressed in the same B lymphocyte during differentiation but these will all originate from the same allelic set of heavy chain genes, as the successful integration of a V_H gene will inhibit the same event on the other allele or that allele will previously have formed a non-functional V_H gene which will prevent the expression of C_H genes from that allelic cluster.

Alternatively the mechanism of allelic exclusion may be, as first thought based simply on the joining of immunoglobulin genes on one homologous chromosome only, for, in contrast to the reports on mouse myelomas in which more than one allele is rearranged, Joho and Weissman (1980) have reported that in normal mouse spleen cells expressing κ chains about 50% of the κ alleles are unchanged, indicating that non-functional rearrangements may occur rarely or not at all in normal lymphoid cells. If this is the case non-functional rearrangements can be explained as an abnormal feature related to the malignant nature of the mouse myeloma cells. However, the question has been raised whether the technique of Joho and Weissman (1980) has sufficient resolution to detect cells with both chromosomes rearranged if they are in a minority (Altenberger et al., 1980, Molgaard 1980). Therefore it is still open to question whether both myeloma cells and normal B lymphocytes have more than one V gene joining event per expressed heavy chain or light chain. However in addition to being thought of as a mechanism of allelic/genic exclusion, non-functional immunoglobulin gene rearrangements have been seen as part of the process of generating diversity in variable region genes.

V region Diversity

The hypothesis of Dreyer and Bennet (1965) on the molecular basis of antibody formation explained V region diversity in terms of each V region being coded for by a different V gene, all such genes being present in the germline DNA and this argument has become known as the "germline theory" of antibody diversity. Alternatively, somatic models (Gally and Edelman 1972) proposed that V region variation could be generated from a small V gene pool by somatic mutation or recombination during the development of the individual. As the germline theory predicts that all V regions are coded for in the germline whereas according to somatic models only a few basic V genes will be present, an evaluation of the number of germline V genes should determine which hypothesis is likely to be correct. The aminoacid sequences of V regions and nucleic acid hybridisation data have both been used to estimate the number of germline V genes.

The analysis of spontaneous human myeloma proteins established the classification of V region aminoacid sequences in to distinct groups

in both light chains and heavy chains (Hood and Talmage 1970, Capra and Kehoe 1975). There are four defined groups of human κ light chain V region aminoacid sequences and the same number in the λ light chain V regions, while among the human heavy chains there are three reasonably well defined groups of V region sequences. It has been argued that if a single gene coded for all the V regions belonging to a given immunoglobulin chain, multiple identical somatic mutations would be required to produce the observed ordered array of aminoacid sequences. As this was considered an unlikely event, it was inferred that the different basic sequences of a given V region are coded for by separate germline genes. (Milstein et al., 1967, Hood et al., 1967). Furthermore, the presence of different V region groups in all individuals implies that they do not represent alleles of a single locus, for if they did they would segregate (Milstein et al., 1969, Tischendorf et al., 1971, Wang et al., 1971). These studies on human immunoglobulins supported the idea that both heavy chain and light chain V regions are coded for by tandem non-allelic genes. Aminoacid studies in the mouse myeloma system have extended and refined these initial conclusions and in the same system the study of V region diversity has been pursued using immunoglobulin mRNA and cDNA probes.

Mouse κ chains, which constitute 95% of light chains in the mouse, show extensive variation in V region aminoacid sequences in both the hypervariable and framework residues. This pattern of variability can be explained in part by multiple germline genes since, from partial N-terminal aminoacid sequence data, the number of κ V region groups (and so the minimum number of V genes) has been estimated to be about fifty for either Balb/c or NZB mice (Hood et al., 1976).

The extensive study of one κ V region group V21 has stressed the importance of multiple germline V genes but has also suggested that antibody diversity may be increased by somatic means. The V21 group, on the basis of almost complete aminoacid sequences, may be further divided into six subgroups by related sequence patterns (McKean et al., 1978, Weigert et al., 1978) indicating that within that single group there are at least six germline genes. It is possible that additional V21 subgroups may be found but nucleic acid hybridisation data (Valbuenna et al., 1978) have suggested that the number of V21 germline genes is the same as the number of subgroups. This would favour

there being a somatic source of variation in this group as the entire group consists of nearly thirty different sequences.

In contrast to κ light chains the pattern of variability in the V regions of mouse λ light chains (which make up only 5% of mouse light chains) has been shown to be a simple one. (Weigert and Riblet 1976). In twenty one λ chains produced by myelomas induced in inbred Balb/c and NZB mice, twelve V region sequences were identical and the remaining nine differed by only one - three aminoacid substitutions all of which were present in hypervariable regions. Such a simple pattern of diversity is consistent with there being one germline V λ gene and variants arising by a somatic mechanism. This interpretation is supported by nucleic acid hybridisation studies which suggest that mouse λ V regions are coded for by one or a few germline genes (Tonegawa 1976).

The aminoacid sequence data on mouse heavy chain V regions are neither as extensive nor as revealing as the light chain sequences. The available sequences can be divided into four groups with the largest of them being further divisible into at least 5 subgroups (Barstard et al., 1974). This pattern of diversity suggests that the variable regions of mouse heavy chains are under multigene control encoded in at least eight germline genes. Hybridisation of mouse germline DNA using V_H probes has shown that there are at least 50 and probably many more V_H genes (Cory and Adams 1980, Rabbitts et al., 1980b). These estimates of the total number of germline V genes on their own do little to explain the generation of antibody diversity. However, together with the same information from other immunoglobulin chains it is possible, assuming that most light chains may combine with most heavy chains, to calculate the number of different antibody molecules that could be formed using germline sequences only. For example, using estimates of 350 V_L germline genes and 200 V_H germline genes compounded from mouse protein and nucleic acid data (Weigert et al., 1978), one can propose that 7×10^4 different antibody molecules could be formed. However, as estimates of the mammalian antibody repertoire are currently running at about 2×10^7 (Kohler et al., 1976, C an ro 1978) the difference between these two figures suggests that there must be some somatic mechanism involved in the generation of diversity.

Several authors have suggested the possibility that V-J joining in light chains and V-D-J joining in heavy chains may generate V region

sequence diversity by random combination of the coding segments involved (Weigert et al., 1978, Seidman et al., 1979) and codon variation at the joins between the segments (Bernard and Gough 1980, Max et al., 1979, Sakano et al., 1979). If the flexibility in the exact position of V gene joining necessary for this latter mechanism is accompanied by the generation of missence genes (Max et al., 1980), such a mechanism could account for the large number of non-functional gene rearrangements in myeloma cells.

Certainly V gene somatic rearrangement is a defined molecular mechanism which does lead to V region sequence differences and could generate considerable diversity. Nevertheless not all the variations in V region aminoacid sequence attributed to somatic diversity can be accounted for by these mechanisms. None of the sequence differences in mouse λ V-regions can be explained by V-J joining and there are 20 - 30 κ V-region aminoacid residues which show more variation than residue 96 whose codon coincides with the V-J join (Dunnick 1979). In general the antigen complementarity of an antibody molecule depends on all the hypervariable regions and not just those in the vicinity of V-J, V-D-J joining. The significance, in terms of antigen specificity of aminoacid sequence changes which can be attributed to joining and the mechanism(s) responsible for non-germline V region diversity in parts of immunoglobulin molecules not concerned with V region joining has yet to be demonstrated.

Human immunoglobulin gene structure; evidence from variant immunoglobulin polypeptides

Analysis of aminoacid sequences and the segregation of markers have shown that separate V and C genes code for both heavy chains and light chains in man and other species in addition to mice. However, there are, at the moment, only murine immunoglobulin nucleic acid probes and the greater detail of immunoglobulin gene structure and expression which has come from the study of immunoglobulin genes at the nucleic acid level has been confined to that species. Nevertheless there are several deletion mutants which occur in both murine and human immunoglobulins whose structure can be correlated with the known organisation of mouse immunoglobulin genes and so imply a similar organisation of human immunoglobulin genes.

The human immunoglobulin chains with internal deletions for which enzymic degradation can be excluded and a genetic basis implied in their origin have been summarised by Franklin (1979). In these variant immunoglobulin polypeptides an entire domain or hinge region is often deleted and it is an interesting feature of the deletions that they begin or end at or near what would be a germline exon/intron boundary if human immunoglobulin genes were similarly organised to those of the mouse. The majority of human heavy chain variants have been isolated from the serum of individuals with a rare lymphoproliferative disorder called "heavy chain disease" (Franklin 1964) and are referred to as heavy chain disease (H.C.D.) proteins; the remainder are from myeloma proteins. (See Fig. 4a). Five γ_1 and one γ_2 H.C.D. variants show a deletion which encompasses part of the variable region and all of the C_H1 domain of the constant region with resumption of the normal sequence at the beginning of the hinge region. A further three γ_1 variants have an exact and complete deletion of the hinge region itself. In three γ_3 variants, one shows a deletion of part of the V region and all of the C_H1 domain similar to those variants in the γ_1 and γ_2 class; a second shows the same deletion but extended into the hinge region (which in γ_3 chains is replicated three times) ending exactly before the final replicate of the hinge; the third has all of the C_H1 domain and a large internal portion of the V region missing but has retained, between the two deletions, a last part of the V region sequence which could be analogous to the murine heavy chain J segment. Similarly in an abnormal λ chain from a human myeloma the internal V region deletion ends at residue 99 which corresponds exactly to the position of V-J joining in murine λ light chains (Fig. 4b). A second abnormal human λ type myeloma light chain has a V region deletion which ends at a position (aminoacid residue 110) corresponding to the start of the murine J-C intron (Fig. 4b).

There are murine and human immunoglobulins whose heavy chains have hybrid C regions, that is they are composed of domains from different heavy chain classes. (Birshtein et al., 1980, Tsuzukida et al., 1979). These cases have been used to argue that there are separately encoded domains in immunoglobulin genes as shuffling of individual domain coding regions, for example by recombination, is a likely mechanism to account for such hybrid molecules. Human and mouse

Fig. 4 Variant human immunoglobulin polypeptides together with the corresponding normal chain

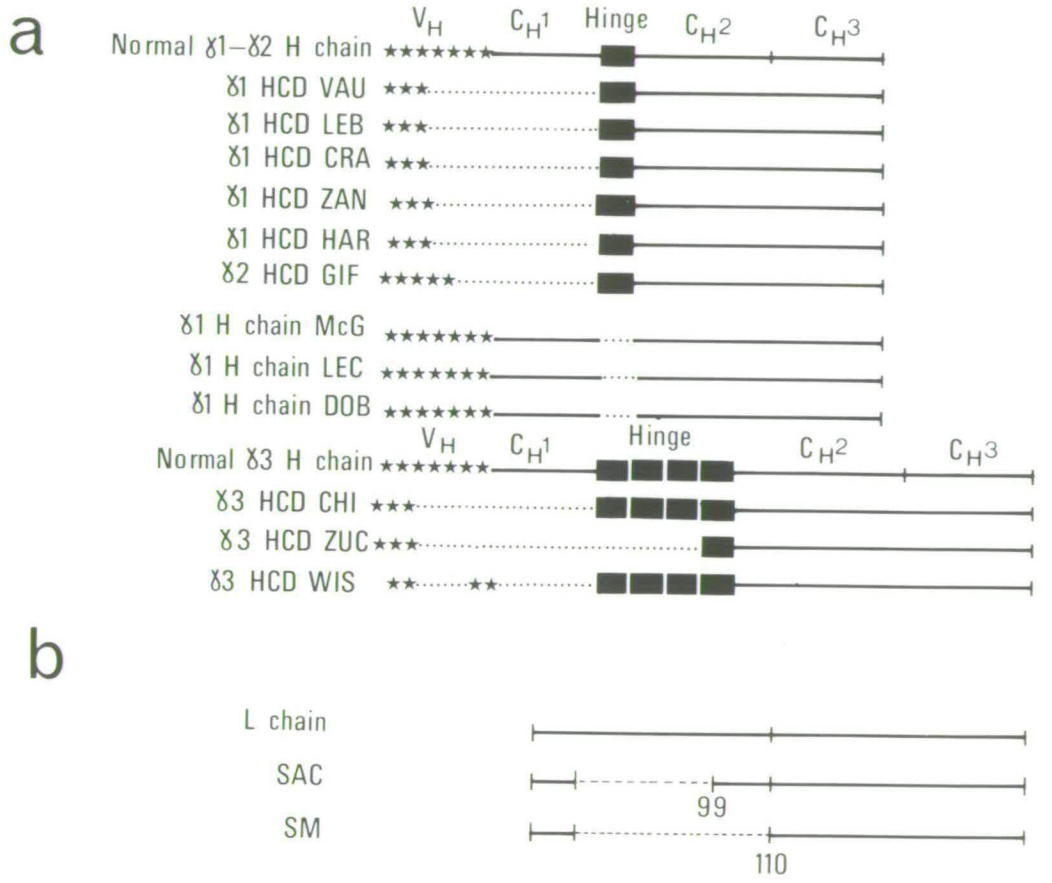
(a) Heavy chains

(b) Light chains

---------- Deletion

(Adapted from Franklin et al., 1979)

Fig 4


a
V_H
C_H1
Hinge
C_H2
C_H3
Normal γ1–γ2 H chain
γ1 HCD VAU
γ1 HCD LEB
γ1 HCD CRA
γ1 HCD ZAN
γ1 HCD HAR
γ2 HCD GIF
γ1 H chain McG
γ1 H chain LEC
γ1 H chain DOB
Normal γ3 H chain
γ3 HCD CHI
γ3 HCD ZUC
γ3 HCD WIS
b
L chain
SAC
99
SM
110

immunoglobulin gene structure have also been equated by comparison of a peptide spanning the V/C junction in human δ chains with the homologous region in other human heavy chain classes and subclasses which indicated the presence of a short J type sequence. (Milstein and Dèverson 1980).

In summary one might propose that, from the characterisation of variant human immunoglobulin polypeptides, human light chain C regions, heavy chain C region domains and hinge regions are separately encoded as exons in an immunoglobulin transcription unit and furthermore that the V regions of both heavy chains and light chains in humans are formed by the fusion of at least two germline segments.

THE BIOSYNTHESIS OF IMMUNOGLOBULINS

A complete account of the biosynthesis of a polymeric glycoprotein, which all immunoglobulins are, must consider transcription, translation, glycosylation, assembly and secretion. All these topics will be briefly considered here together with the regulation of immunoglobulin production. For a further account the reader is directed to extensive reviews by Williamson (1973) and Parkhouse (1977) although these do not include an account of the processing of an immunoglobulin primary RNA transcript nor of the role of the N-terminal signal peptide in immunoglobulin secretion.

Transcription of Immunoglobulin genes

Even in their integrated forms, active immunoglobulin genes are not contiguous in the genome. Recent studies on other genes of eukaryotes and their viruses have revealed several more cases of this split gene structure where coding segments of DNA (exons) alternate with non-coding intervening sequences (introns). In general the introns are excised from a large nuclear transcript to give a continuously coding mRNA, (for a review see Crick 1979). The immunoglobulins are no exception to this and Rabbitts (1978) and Gilmore-Hebert and Wall (1978) have demonstrated in mouse myeloma

cells that the smallest light chain coding nuclear RNA fragment is of a similar size to the cytoplasmic message. They concluded that light chain mRNA is processed from a larger nuclear transcript. (See Fig. 3b).

In both mouse myeloma and human lymphoid B cells membrane-bound and secretory IgM μ heavy chains have different carboxytermini which is reflected in their being coded for by separate species of mRNA that differ at their 3' ends, (Alt et al., 1980b, Singer et al., 1980). Although there is a single μC gene in the mouse (Cory and Adams 1980) apparently there are two separate 3' terminal sequences for μ RNA encoded in the genome so that mRNAs coding for either membrane bound μ chains (μm) or secretory μ chains (μs) may be created by alternative nuclear RNA processing (Early et al., 1980b) as shown in Fig. 3c. A similar system would appear to operate in the δ chain of IgD molecules the other major class of membrane bound immunoglobulin (Tucker et al., 1980).

However, despite differential processing of a primary transcript operating at this level the lack of detectable μ or α transcripts in the nuclei of γ producing mouse myeloma cells (Marcu et al., 1979) and the absence of V and C region scrambling in hybrid mouse myeloma cells producing two different classes of heavy chain (Schulman and Kohler 1978) argue against such a mechanism in heavy chain class determination.

Translation of immunoglobulin mRNA

There are no mechanisms peculiar to immunoglobulin translation and in common with all other proteins immunoglobulin polypeptides are synthesised in an N-terminal to C terminal direction from a single initiation point (Fleishman 1967, Lennox et al., 1967) which is the codon for methionine $tRNA_F$ (Milstein et al., 1972) the general initiator for eukaryotic proteins.

The heavy chains and light chains of mouse myeloma immunoglobulins are synthesised on separate polyribosomes on the membrane of the endoplasmic reticulum (Williamson and Askonas 1967, Cioli and Lennox 1973). The membrane bound character of the heavy and light chain synthesising polyribosomes ensures that transport of the nascent polypeptides into the cisternae of the endoplasmic reticulum which predetermines the addition of carbohydrate and ultimate secretion of the completed immunoglobulin molecules.

Glycosylation of immunoglobulins

The addition of sugars to immunoglobulin chains and the subsequent modification of those sugars (as studied in mouse B lymphoid cells) occurs in three main steps which are common to glycosylated proteins in general. First, the addition of core sugars to the nascent polypeptides as they emerge into the cisternae of the endoplasmic reticulum (Bergman and Kuehl 1977), second the partial trimming of these oligosaccharides in the endoplasmic reticulum (Tabas et al., 1978) and finally the addition of terminal sugars in the Golgi complex (Melchers 1973). Carbohydrate addition is not required for monomer assembly, polymerisation (when it occurs) or secretion since completely unglycosylated immunoglobulins can be assembled and secreted (Hickman and Kornfield 1978, Tartakoff and Vassalli 1979).

The Assembly of Immunoglobulin molecules

The nascent heavy and light chain polyptides are assembled into H_2L_2 immunoglobulin monomers in the cisternae of the endoplasmic reticulum. Assembly occurs between nascent heavy chains and a pool of free light chains (Williamson and Askonas 1968) initially by noncovalent interactions prior to covalent linkage by interchain disulphide bonds. There are several pathways of interchain disulphide bond formation but the two major intermediates are H_2 and HL (Bevan et al., 1972). Completion of the fourchain monomer then takes place by addition of light chains to H_2 or by dimerisation of HL.

Several studies on the assembly of murine polymeric immunoglobulins have shown that the conversion of (H_2 L_2) subunits into J chain containing polymers occurs just before or simultaneously with secretion from the cell (Buxbaum and Scharff 1973, Bargellesi et al., 1972, Parkhouse 1973). However, Tartakoff and Vassalli (1979) have found that IgM pentamers are rapidly assembled while still in transport through the endoplasmic reticulum before entry into the Golgi complex and so not immediately prior to secretion. Furthermore Howell-Saxton and Wettstein (1978) have demonstrated an intracellular pool of 19S (pentameric) IgM in a human lymphoblastoid cell line and Buxbaum et al., (1971) have shown that whereas some human lymphocytes and plasmacytes may accumulate intracellular pentameric IgM others may not.

The Secretion of Immunoglobulins

Normally, completely assembled immunoglobulin molecules are either secreted from the cell or incorporated into the plasma membrane. Both these events are predestined by the site of synthesis of immunoglobulin polypeptides being on membrane bound polyribosomes. The discussion of a mechanism to determine the secretory fate of immunoglobulins and other proteins raises the question of how certain mRNA molecules come to be selectively translated as part of a membrane bound polyribosome complex. One explanation postulated a non-translated nucleotide sequence in mRNAs coding for secretory proteins which had an affinity for membranes. However, the finding that immunoglobulin light chains are synthesised in a precursor form favoured an alternative idea which invoked the nascent polypeptide in the formation of membrane bound polyribosomes. Milstein and co-workers (1972), during a study of the cell-free translation of κ light chain mRNA isolated from mouse myeloma cells, observed that the light chain was synthesised in precursor form with an additional N terminal oligopeptide. They proposed that the extra sequence provided a "signal" for attaching the growing chain to the membrane of the endoplasmic reticulum. Later, the same group demonstrated that during protein synthesis membrane fragments can convert the precursor to the ultimate product, (Cowan et al., 1973). Several other murine and human immunoglobulin polypeptides have been shown to be synthesised in cell free systems as large precursors (Tonegawa and Baldi 1973, McCune et al., 1980) and exons coding for the precursor or signal peptide have been identified in the DNA sequences of both murine heavy chains and light chains. The primary structure of the N-terminal extra pieces of several mouse κ and λ chains have been shown to differ extensively (Burstein and Schechter 1978) yet they all have a high percentage of hydrophobic aminoacids; a property that would fit the proposed attachment of the sequence to the membrane. Many other secreted proteins, in both eukaryotes and prokaryotes, have been shown to be synthesised as a large precursor with an additional hydrophobic N terminal signal sequence that is cleaved by a membrane bound enzyme (Davis and Tai 1980), establishing the "Signal hypothesis".

Although the signal hypothesis has suggested a mechanism for the selection of secretory proteins and their passage across intracellular

membranes into the lumen of the endoplasmic reticulum in eukaryotic cells, the way in which immunoglobulins are ultimately transported via the Golgi apparatus to the outside of the cell or into the plasma membrane remains poorly understood. In general when proteins are secreted from the Golgi apparatus they are packaged into secretion vesicles or granules which move to the plasma membrane, fuse with it and release their contents to the outside of the cell. However, in the case of immunoglobulin secretion there is no apparent dense packaging of the molecules prior to secretion which in contrast appears to be a steady continuous process (Palade 1975).

As well as passing through the E.R. the eventual insertion or secretion of immunoglobulins at the plasma membrane may be controlled by membrane/peptide interaction. For example, membrane-bound immunoglobulins in both mice and humans have a different C terminal from their secreted counterparts (Alt et al., 1980b, McCune et al., 1980). and structurally mutant mouse myeloma λ light chains which possess a cleavable signal remain unsecreted in hybrids where the normal light chain of the other parent is secreted (Mossman and Williamson 1980).

Regulation of Immunoglobulin Synthesis

There are several events which can influence immunoglobulin synthesis at different stages. At the transcription level the manner in which immunoglobulin coding DNA segments are joined determines whether that immunoglobulin coding DNA can function as a transcription unit or not. The mechanisms of allelic exclusion and the heavy chain switch are both thought to function in this way (see section on immunoglobulin genetics) and as both the initial constitutive production of immunoglobulin and the maturation of the humoral immune response from IgM production to immunoglobulin of another class are important events in B lymphocyte differentiation they must be subject to developmental regulation.

Another event under similar control occurs at the RNA processing level where, during differentiation, B lymphocytes express predominantly membrane bound IgM or secreted IgM, the mRNAs for which are transcribed from a single μ gene and produced by alternative nuclear RNA processing pathways.

Little is known about the transport of processed RNA from the nucleus to the cytoplasm and even less about the regulation of such

events. Some attention, however has been paid to the control of the translation of immunoglobulin mRNAs as translational control is thought to be important in regulating the intracellular concentrations of heavy chains, light chains and assembled immunoglobulin molecules (Stevens and Williamson 1973). However, the orthodox view, from studies of normal murine lymphoid tissue and myeloma tumour has been that balanced intracellular concentrations of heavy chains and light chains are maintained by the rapid turnover of a pool of light chains which, due to their shorter length compared to heavy chains, are completed and released from their polyribosomes faster than heavy chains (Askonas and Williamson 1967).

In cases of unbalanced production of heavy chains and light chains the tendancy has always been found to be towards an increased light chain to heavy chain ratio. In fact, although Askonas and Williamson (1967) concluded that excess light chain synthesis is a consequence of neoplasia, Baumal and Scharff (1973) argued that while the synthesis of heavy chains and light chains can be balanced in normal lymphoid tissue there is almost invariably some excess light chain produced. In addition the consistant finding of excess light chain synthesis in immature human B-lymphocytic neoplasms has led Gordon et al., (1978) to suggest that this is a pattern of immunoglobulin synthesis related to normal cells. Kohler (1980) has also suggested that this may be the case because free heavy chain is toxic to immunoglobulin-synthesising cells. His evidence comes from the pattern of loss of heavy chain or light chain expression in hybrid mouse myeloma cells; chain loss was random only in lines with an excess of active light chain genes over heavy chain genes and in all other combinations preferential heavy chain loss was observed. The subsequent conclusion, that heavy chain synthesis is dependant upon prior light chain synthesis, is consistent with the findings that spontaneous loss of heavy chain expression but with maintenance of light chain expression is a frequent event in mouse myeloma cell lines (Milstein et al., 1975, Morrison et al., 1974) whereas the opposite is a very rare occurrence. Where heavy chains have been found to be the only expressed immunoglobulin polypeptide of a mouse myeloma cell line they appear always to be defective (Cowan et al., 1974, Wilde and Milstein 1980) as is the case in the cells which synthesise the variant

human heavy chain disease proteins in the absence of light chain synthesis (Buxbaum et al., 1978). Variant clones of a mouse myeloma cell line which synthesise apparently normal heavy chains and no light chains have been isolated after mutagenesis (Morrison and Scharff 1975) but all of the variant clones produced the lone heavy chain at a vastly reduced level. Recently some authors have reported free μ chain synthesis by pre-B lymphocytes (Burrows et al., 1979, Levitt and Cooper 1980) however, it is not known whether the heavy chains are variant or normal and if they are synthesised at a low level or not.

CULTURED CELL LINES; MODELS FOR THE STUDY OF IMMUNOGLOBULIN SYNTHESIS

The analyses of human serum immunoglobulins together with those from other species provided important insights into the structure synthesis and genetics of immunoglobulins. However, as the published work cited throughout this introduction has shown, mouse myeloma tumours and the cell lines derived from them have provided an excellent source of material for the direct study of immunoglobulins at the level of the gene and in synthesis, assembly and secretion. During this intense study of mouse myeloma tumours and cell lines there has been no similar development of any other system although it is well known that immunoglobulin synthesis is a stable characteristic of human lymphoma, lymphoblastoid and myeloma cell lines. In fact human lymphoid cell lines (as these lines are collectively known) are the only other major source of immunoglobulin synthesising cells established in culture. Furthermore and regardless of the species difference, human lymphoid cell lines differ from mouse myeloma cell lines in several potentially important ways.

The cells of mouse myeloma lines are malignant and abnormal so great care must be taken in relating immunoglobulin synthesis in these cells to the same process in normal lymphocytes. One example of contention in this respect is excess production of light chains frequently observed in mouse myeloma cell lines which, it has been argued, is either

a normal feature of lymphoid tissue or a consequence of neoplasm (Parkhouse 1977). Concern has also been expressed that the frequent absence of unrearranged immunoglobulin genes in mouse myeloma lines may be a consequence of the maintenance or re-expression of the gene-rearranging enzymes after the malignant transformation of the cells (Max et al., 1980). In addition the malignancy process in mouse myeloma selects in a non-random manner the lymphocytes to be transformed. Hapten binding and sequence analysis of myeloma proteins (Loh et al., 1979) have shown that mouse myeloma cell lines represent only a subset of the total lymphocyte repertoire. This has an obvious bearing on estimates of V region diversity from myeloma aminoacid sequence data although this is less important now that such estimates can be tackled at the nucleic acid level. However, this may still influence studies of the mode of expression of different V region genes and in the somatic generation of diversity. It should also be noted that having once demonstrated that the myeloma process in mice is selective, several features may in fact be selected for.

Among the human lymphoid cell lines the leukaemia, lymphoma and myeloma derived lines are of undoubted malignant descent however those lymphoblastoid lines established from normal B lymphocytes by the use of Epstein-Barr virus (EBV) are of presumed non-neoplastic origin (Nilsson and Pontein 1975). As EBV is thought to be a polyclonal B lymphocyte activator (Bird and Britton 1979) this suggests that it is not selective in its transformation of B lymphocytes. Even in cases where it has been argued that EBV preferentially transforms a subpopulation of B lymphocytes (Steel et al., 1977) that subpopulation has been outlined as those cells already synthesising immunoglobulin which is a developmental stage open at some time to all normal B lymphocytes.

The stages of B lymphocyte differentiation represented by the cells which comprise mouse myeloma lines and human lymphoid lines show important differences between the two systems. The cells of the mouse myeloma lines represent plasma cells which are the terminally differentiated state of an immunoglobulin producing cell. The comparisons which have been made to elucidate the events of immunoglobulin gene expression have been made at the gene level using DNA from twelve-day-old mouse embryos or sperm. Clearly what is missing is a developmental context in which to place the differences observed between these two extremes. This may become more essential in a search for

somatic mechanism(s) generating V region diversity which cannot be accounted for by the somatic recombination of V region genes (see section on V region diversity) and for the signals which trigger such events in B lymphocyte differentiation. Intermediate stages of B lymphocyte differentiation are not easily accessible in the mouse although the use of Abelson virus transformed mouse cells (Baltimore 1979) or hybridoma technology (Burrows et al., 1979, Levitt and Cooper 1980) may solve this problem. Alternatively there is an abundant source of intermediate developmental stages among human lymphoid cell lines which represent clonal expansions of cells at several discrete stages of B lymphocyte and (for a smaller number of cell lines) T lymphocyte development (See Table 2). These lines should allow an analysis of the particular aspects of immunoglobulin gene rearrangement and expression that occur during lymphocyte ontogeny.

Another feature of mouse myeloma cells in culture which may prove a drawback in understanding the molecular basis of immunoglobulin synthesis is that their chromosome complements are abnormal, unstable and difficult to identify. (Moriwaki and Imai 1978). The changing nature of the DNA content of mouse myeloma cells could be related to the multiple non-functional rearranged genes (Perry et al., 1980) and deleted coding sequences (Cory and Adams 1980) which appear common in mouse myeloma cells and have been implicated in the normal processes of allelic exclusion, the generation of diversity and the heavy chain switch (see section on immunoglobulin genetics). In contrast to mouse myeloma cells the karyotypes of human lymphoid cell lines are often diploid or near diploid, stable and readily identifiable by chromosome banding (Steel et al., 1980) so that they provide an opportunity to study immunoglobulin gene expression in a stable, recognisable genetic background.

As human lymphoid lines and mouse myeloma cells are both mammalian in origin, one does not expect to see any fundamental differences emerge in the normal organisation and expression of their respective immunoglobulin genes. Therefore, any use of the species difference between the two systems in determining how immunoglobulin genes work will only arise when both have been studied to the same detailed level. The two light chain types, κ and λ, present a well documented difference

Table 2 A tentative morphological and functional classification of normal human B lymphocyte differentiation; its relationship to lymphoproliferative disorders and human B lymphoid cell lines

Adapted from Nilsson (1978)

TABLE 2

Normal B lymphocyte lineage	Multipotent stem cell	Lymphoid stem cell	Immature B lymphocyte	B lymphocyte	B lymphoblast	Lympho-plasmacyte	Plasma cell
Morphology		No polyribosomes. No endoplasmic reticulum. Minute golgi apparatus.	Abundant free polyribosomes. Sparse endoplasmic reticulum. Minute golgi apparatus.		Numerous free polyribosomes. Some endoplasmic reticulum. Moderately developed gorgi apparatus.	Abundant endoplasmic reticulum. Few free polyribosomes. Well developed golgi apparatus. Numerous mitochondria.	
Immunoglobulin expression							
Intracellular immunoglobulin	-	-	+	(+)	+	++	+++
Surface immunoglobulin	-	-	(+)	+++	++	+	(+)
Secreted immunoglobulin	-	-	-	-	+	++	+++
Representative human lymphoid cell line		Non T, Non B ("Null") lymphoid lines	(a) EBV positive B lymphoma (b) EBV negative B lymphoid		EBV positive B lymphoblastoid		Plasma cell lines
Origins of cell lines		Leukaemia and lymphoma	(a) Burkitt lymphoma (b) Burkitt lymphoma, leukaemia and lymphoma		EBV transformed normal blood lymphocytes		Myeloma

between the species which may be represented in their gene systems. In mice κ chains account for 95% of light chains and the greater part of light chain diversity whereas murine λ chains are far less diverse and constitute only 5% of light chains (Hood et al., 1976). A comparison of the gene systems of mouse light chains with the κ and λ systems of humans where the two light chain types are about equally represented and equally diverse (Secher et al., 1979) may clarify the role of V genes, J regions and their interaction in the generation of light chain diversity. In general the overall determination of the structure and organisation of human immunoglobulin genes together with the current knowledge of these genes in the mouse would allow a comparison in a gene system which has undergone rapid evolution over a relatively short period of time.

The belief also exists that in studying the complex nature of immunoglobulin synthesis a better understanding of gene organisation and expression in other eukaryotic systems will follow. This has, to some extent, already proved to be the case in that immunoglobulin genes were the first in eukaryotes for which somatic changes in gene rearrangement were characterised at the molecular level (Max et al., 1979, Sakano et al., 1979). The "transposable elements" in yeasts and Drosophila have also been detailed in their movements within the somatic DNA of those oganisms (Calos and Miller 1980) and it is thought that such transpositions in the higher eukaryotes may be important in the origin of cancer and in the developmental process. The rearranged mouse light chain genes were among the first genes to be seen to have a "split" structure which is now considered a general feature of gene organisation in eukaryotes and their viruses (Crick 1979). It was also the study of the expression of an immunoglobulin polypeptide which founded the signal hypothesis in protein secretion (Milstein et al., 1972). As the immunoglobulins are as yet far from fully understood work on this system should continue to have implications for all aspects of the biology of multicellular organisms.

One area to which a study of human immunoglobulin synthesis is particularly relevant is medical science. This is true not only generally in that the immune system is important in infectious and neoplastic diseases but also specifically in conditions effecting the immune system itself. In humans there is a wide variety of inherited disorders of the immune system (Stanworth 1977) for example

the class-specific immunodeficiency diseases. These conditions may provide an insight into the genetic elements and cellular events that are required for normal immunoglobulin expression and so an opportunity to study the aetiology of a genetic disease at the molecular level.

In addition to their specialist capacity to synthesise immunoglobulins, established human lymphoid lines are also of general biomedical interest. As both human fibroblasts (Hayflick 1965) and normal glia cells (Ponten and MacIntyre 1968) have a limited life in culture, human lymphoblastoid cells are the only major source of permanent cultures derived from normal human tissue. These lymphoblastoid cultures together with human lymphoid lines from neoplasms constitute a convenient system which can be used not only to study the biology of normal cells but also to compare that with similar material of malignant origin.

THE AIMS AND APPROACH OF THE RESEARCH PROJECT

Spontaneous and induced mutations have been used to investigate the sequence and control of events in the synthesis of many biologically important molecules. Although this is particularly true in prokaryotes a similar approach has been made possible with mammalian eukaryotic cells through developments in cell culture. (Siminovitch 1976). This project investigates immunoglobulin synthesis in human lymphoid cells which has a number of advantages as a system for this type of study: The structure and chemistry of immunoglobulins are well understood, there are several interesting features in the expression of immunoglobulin genes and the cells which make up these different lines represent various stages of differentiation.

To identify variation in the patterns of immunoglobulin synthesis in human lymphoid cell lines it was intended to begin by screening the large number of lines available in the laboratory for secreted immunoglobulin and to examine in a similar way clones of mutagenised and untreated cultures. The initial screening was to be based on simple rapid serological test systems such as the haemagglutination

inhibition technique of Evans et al., (1974) and the antibody overlay of Scharff et al., (1975). Any variants arising in this manner would have been identified serologically and so in order to confirm which polypeptides were being secreted and the nature of those that were it would be necessary to fractionate, characterise and identify the proteins produced by the lines in question and by normal cells. The amount of protein produced by cells in vitro is insignificant in comparison to the large concentration of proteins in the form of foetal calf serum required for cell survival and growth so that any detailed investigation of cell line products was envisaged as involving the biosynthetic radiolabelling of secreted cell line proteins.

The absence of secreted immunoglobulin does not imply that synthesis of immunoglobulin has ceased, thus it was intended to extend the analysis to any intracellular and surface immunoglobulin of cell lines or clones of lines shown to be deficient or otherwise abnormal in their secreted immunoglobulin, together with apparently normal immunoglobulin-synthesising cells.

In summary, the aims of the project are to identify and characterise normal and abnormal immunoglobulin synthesis in human lymphoid cell lines. The results of these studies may then be used to interpret and clarify certain aspects of our current understanding of immunoglobulin genetics and synthesis as described in the introduction to this work.

ABBREVIATIONS

Bis	NN'-Methylenebisacrylamide
BSA	Bovine Serum Albumin
Dimethyl POPOP	1, 4- [2-(4-methyl-5-phenyloxazolyl)] -benzene
EMS	Ethylmethane sulphonate
FCS	Foetal calf serum
g_{av}	Centrifugal force at the average rotor radius
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
MOPS	2(N-morpholino) propanesulphonic acid
NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonyl fluoride
PPO	2, 5-Diphenyloxazole
RNAase A	Ribonuclease A
Sarkosyl	N-Lauryl sarcosine
SDS	Sodium dodecyl sulphate
SRBC	Sheep red blood cells
TCA	Trichloroacetic acid

MATERIALS

These materials were obtained as follows:

Acrylamide	BDH Chemicals Ltd
Antisera	As several commercial antisera, raised in different animals were used, each is described as it occurs in the Methods or Results Section.
Bis	Bio Rad Laboratories
BSA	Sigma Chemical Company Ltd
Coomassie Blue	Sigma Chemical Company Ltd
^{14}C valine	The Radiochemical Centre Amersham
D19 developer	Kodak Ltd
FCS	Gibco Bio-Cult Ltd
Ham's F10 medium	Gibco Bio-Cult Ltd
Human IgA and IgM	The American National Red Cross Blood Research Laboratory
Human IgG	Miles Laboratories
I^{125} Protein A	The Radiochemical Centre Amersham
Melphalan	Burroughs Wellcome Ltd
Microtest Plates	Sterilin Ltd
MOPS	Hopkin and Williams Ltd
Nigrosin	BDH Chemicals Ltd
NP-40	Shell Chemicals Ltd
Ovalbumin	Sigma Chemical Company
PBS	Oxoid Ltd
Penicillin	Glaxo Laboratories Ltd
Phosphorylase b	Sigma Chemical Company
PMSF	Sigma Chemical Company
Protein A	Pharmacia Fine Chemicals
Protein A-Sepharose	Pharmacia Fine Chemicals
RNAase A	Sigma Chemical Company
RP X-Omat film	Kodak Ltd
Standard human serum	Behringwerke
Staphylococcus aureus bacterial immunoabsorbant	CAMR Microbial Products
Streptomycin	Glaxo Laboratories Ltd

All other chemicals were reagent grade

METHODS

The principal methods used in this project are described below. Exceptions and additions to these procedures are given in the Results section with the experiments to which they apply.

CELL CULTURES

Most of the human lymphoid lines used in this study had been established in the laboratory where this project was carried out and their establishment has been previously described (Steel 1972). Other lines, obtained from elsewhere had been cultured in this laboratory for some months or years.

All lymphoid cultures were maintained in Ham's F10 medium supplemented with 10% FCS, penicillin (100,000 u/l) and streptomycin (100mg/l) at 37^{o}C in a 5% CO_2 atmosphere, or without gassing and with the addition of 12.5 mM MOPS to the culture medium. Each culture was subcultured once a week to maintain a viable cell density of about 0.5 - 1 x 10^6 cells/ml. The viability and cell density of cultures were determined by exclusion of the vital dye nigrosin at a concentration of 0.2% (Kaltenbach et al., 1958). Lymphoid cultures were screened at regular intervals for mycoplasma as a service facility of the laboratory and any positive cultures detected were discarded.

Human embryonic skin fibroblasts, human embryonic lung fibroblasts and the human fibroblast cell line Wi38 (Hayflick and Moorhead 1961) were obtained from cultures routinely established and maintained in the laboratory. They were cultured under the same conditions as the lymphoid lines and were subcultured at confluence to a density of about 2.5 x 10^5 cells/ml.

THE HARVESTING OF CELL CULTURES AND THEIR PRODUCTS

Culture supernatants

The medium in which cells had been growing, referred to throughout as culture supernatant, was collected by centrifugation. Small volumes of culture were spun initially at $12,800g_{av}$ for 30 secs in an Eppendorf microcentrifuge (which has a fixed speed setting) at $4^{o}C$ and the supernatants carefully drawn off and centrifuged again under the same conditions for 5 mins to remove any remaining debris. The supernatant from this second spin was collected and NP-40 added to a final concentration of 0.5%. Large samples were collected in the same way but by successive centrifugation at $500g_{av}$ for 15 mins and at $3,000g_{av}$ for 15 mins at $4^{o}C$ in an M.S.E. Chilspin refrigerated bench centrifuge. If collected supernatants were not used immediately they were stored at $-20^{o}C$ and thawed rapidly at room temperature before use.

Cells

Cells were harvested immediately before use by centrifugation at $750g_{av}$ for 15 mins at $4^{o}C$ in an M.S.E. refrigerated bench centrifuge and washed 3x in cold PBS. Alternatively smaller cultures were harvested by centrifugation at $12,800g_{av}$ for 30 sec in an Eppendorf microcentrifuge at $4^{o}C$ and washed 3x in cold PBS.

Cell lysates

Collected cells were lysed by suspending a washed cell pellet in cold lysis buffer (PBS, 0.5% NP-40, 1 mM PMSF) using 0.5mls of buffer for up to 2.5×10^{6} cells and standing the suspension on ice for 5 mins. The resulting lysates were cleared by centrifugation at $12,800g_{av}$ for 15 mins at $4^{o}C$ in an Eppendorf microcentrifuge to remove cellular fragments and nuclei.

CLONING

Lymphoid cell lines were cloned in agarose after the technique originally outlined by MacPherson (1965). Cells were plated out at

250 per dish in a 1ml layer of 0.25% agarose over a 2.5ml base layer of 0.30% agarose, all in normal culture medium without MOPS, in 60mm diameter plastic petri dishes. (Several different commercial preparation of agarose were evaluated, see Results section). After plating, agarose cultures were incubated at 37°C in a humid 5% CO_2 atmosphere and were overlaid with 1ml of medium 24 hrs after plating and at weekly intervals thereafter.

Cells could be cloned in this way without a feeder layer but where a feeder layer was used the petri dishes were inoculated with 2.0×10^5 fibroblasts 24 hrs before plating. A variety of feeder layers were used (See Results section).

Under these conditions microscopic colonies were visible 2 - 3 weeks after plating, and at 4 - 6 weeks colonies were macroscopic. At this latter stage colonies were picked from the agar in a sterile manner in a volume of 5µl with a Gilson pipetman micropipette. Each colony was then dispensed into 300µl of culture medium in one well of a microculture plate.

THE TREATMENT OF CELLS WITH A MUTAGEN

Se veral cultures of a lymphoid line were exposed to the L-phenyl alanine mustard "Melphalan" for 24 hrs at concentrations of up to 10µg/ml by addition of aliquots of a stock solution of 1mg/ml in propylene glycol. These cultures together with others to which only propylene glycol had been added were examined for viable cells and the concentration of Melphalan giving about 10% cell survival after treatment (see Results) was used in further experiments.

THE SEROLOGICAL DETECTION OF IMMUNOGLOBULINS

Culture supernatants, cell lysates and sucrose gradient fractions

A passive haemagglutination inhibition assay was used to determine the presence of the major classes of human immunoglobulin

heavy chains (α , γ , μ) and both types of light chain in culture supernatants, cell lysates and sucrose gradient fractions. The technique used was that adapted for human lymphoid cell lines by Evans et al., (1974) with some modifications.

Antigen coated red blood cells were prepared in the following manner. Erythrocytes from defibrinated sheep blood were washed 3x in PBS and stored in Alsevers solution for up to one month before use. The sources of antigen, human immunoglobulin in this case, were sera from myeloma patients obtained from the Haematology Departments of the Western General Hospital, Edinburgh and The Royal Infirmary, Edinburgh. In order to coat SRBC with a sample of myeloma serum they were first fixed by mixing a 5% suspension of washed SRBC in PBS with gluteraldehyde to a final concentration of 0.25%. The gluteraldehyde was added to the SRBC suspension slowly with stirring over a 15 min period and stirring continued for a further 45 mins at room temperature. The fixed SRBC were then washed 3x in PBS and gluteraldehyde fixed for a second time in the same way but with the addition of 1ml of myeloma serum in every 10mls of SRBC/gluteraldehyde mixture. The antigen coated SRBC were then washed 3x in PBS and stored in PBS containing 0.1% NaN_3 at 4^oC. Only one myeloma serum was used for each aliquot of fixed SRBC so that each batch of coated SRBC was sensitised for one particular heavy chain and/or light chain.

To carry out the haemagglutination inhibition assay doubling dilutions of goat antihuman α, γ and μ antisera (Technicon) and rabbit antihuman κ and λ antisera (Behringwerke) were prepared in PBS containing 1.0% FCS and 0.1% NaN_3. Aliquotes of 25µl from each doubling dilution of an antiserum were dispensed into separate wells in a row on a V-section microtest plate with the exception of the final well in each row to which a 25µl aliquot of PBS 1% FCS containing no antiserum was added. The test range of serial dilutions used varied for each antiserum and to a lesser extent for each batch of myeloma serum-coated SRBC (See Results). An equal volume of the sample to be tested was added to each well of one row and into the wells in the last row of each plate a control sample was added to establish the end point of the uninhibited agglutination reaction. The control samples were unused fresh culture medium containing 0.5% NP-40, lysis buffer, or 10% sucrose in 0.5 PBS 0.5% NP-40

depending upon the source of the test samples. The dilutions of antiserum and aliquots of test or control samples were mixed together by shaking the plates for 2 mins on a microtest plate shaker and then leaving them to stand for at least 15 mins at room temperature. A 25µl aliquot of a 0.25% (V/V) suspension of the appropriate immunoglobulin coated SRBC was added to each well after which the plates were again shaken for 2 mins and then left at 4°C overnight before analysis. The presence of a particular immunoglobulin heavy chain or light chain was recognised when the test sample inhibited agglutination by more than 2 wells, that is greater than a 4 fold difference in end point compared with the control row.

Cell surface immunoglobulin

Cell surface immunoglobulin was identified by an I^{125} protein A assay of cell surface bound rabbit antihuman immunoglobulin antibodies. A sample of 1 x 10^6 cells from a culture to be tested were harvested and transferred into an Eppendorf microcentrifuge tube. The washed cell pellet was resuspended in 100µl of a 1 : 20 dilution in PBS of the relevant antihuman immunoglobulin antiserum (Behringwerke) and then incubated at 4°C for 30 mins with mixing. The cells were then washed 3x in cold PBS and resuspended in 100µl of a solution of Protein A at a concentration of 2.5µg/ml in PBS and containing I^{125} labelled protein A at 0.5µCi/ml. This mixture was incubated for a further 30 mins at 4°C with mixing when the cells were washed 3x in cold PBS and the final cell pellet counted in a Packard automatic gamma counter which has a 65% counting efficiency for I^{125}. Control assays without the addition of antiserum and with non-immune normal rabbit serum in place of antiserum were carried out for each cell culture tested.

THE BIOSYNTHETIC RADIOLABELLING OF PROTEINS PRODUCED BY CELLS IN CULTURE

Cell culture protein products were biosynthetically radiolabelled using uniformly ^{14}C-labelled valine added at 10µCi/ml to the normal culture medium. The ^{14}C valine was supplied as an aqueous solution

and the required amount was evaporated to dryness in an empty sterile culture vessel which was then re-sterilised by placing directly under a tissue culture room U.V. bench light for 15 mins. The appropriate volume of culture at 1 x 10^6 viable cells/ml, taken from a stock culture 24 hrs after the last medium change, was then dispensed into the culture vessel containing the dried ^{14}C valine. The radioactive culture was then incubated for 48 hrs under normal culture conditions and the supernatant or cells were harvested as described previously.

THE DETECTION OF RADIOACTIVITY IN CULTURE SUPERNATANTS AND SUCROSE GRADIENT FRACTIONS

The radioactivity incorporated into the polypeptides present in culture supernatants and sucrose gradient fractions was determined using the technique of Bollum (1966). Aliquots of the samples to be assayed were applied to 3mm filter paper discs which were then given 3 washes for 10 mins each in 5% TCA followed by 3 x 10 mins washes in ethanol/ether 1 : 1 and then air dried. The dried discs containing the precipitated protein were placed in vials in which 5mls of a solution consisting of 0.5% PPO and 0.03% dimethyl-POPOP in toluene was added. The radioactivity present in each vial was measured in a Packard "Tri-carb" liquid scintilation counting with a gain setting of 7% and a window setting of 50 - 1000 giving a counter efficiency of 56% for ^{14}C.

The presence of NP-40, SDS and sarkosyl (TCA precipitable compounds) did not effect the determination of incorported TCA precipitable radioactivity.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

The discontinuous buffer system and acrylamide/bis ratio of Laemmli (1970) were used. Electrophoresis was carried out on

15cm x 15cm x 0.15cm linear 3% - 20% gradient gels and uniform 12% gels of the same dimensions at either a constant 30mA for 6 hrs at room temperature or a constant 12mA for 16 hrs at room temperature for both types of gel. The different running conditions created some differences in mobility however each gel contained a track of several marker proteins of known molecular weight giving an internal measure of mobility on each gel.

Completed gels or portions of completed gels were stained for protein using 0.1% Coomassie blue in 30% (V : V) methanol, 10% (V : V) acetic acid, 60% H_2O and destained in the same solution without Coomassie blue. Those gels or portions of gels containing radioactive samples were prepared for fluorography by impregnation with PPO using the technique of Bonner and Laskey (1974). The resultant destained or PPO-impregnated gels were dried down under vacuum onto 3mm filter paper. Radioactive components in gels prepared for fluorography were visualised by exposing the dried gel to preflashed (Laskey and Mills 1975) RP X - Omat film at -70°C for 1 - 4 weeks. Exposed films were developed for 4 mins at room temperature in a 1 : 4 aqueous dilution of D19 developer then fixed, washed and dried.

THE PREPARATION OF UNFRACTIONATED CELL CULTURE PRODUCTS FOR SDS PAGE

Samples of culture supernatants and cell lysates were prepared for SDS PAGE by precipitation in 10x their own volume of acetone on ice for 15 mins. The precipitated proteins were pelleted by centrifugation at 12,800g_{av} for 2 mins in an Eppendorf microcentrifuge at 4°C and washed 2x under the same conditions. The final pellet was air dried and dissolved in SDS PAGE sample buffer.

SUCROSE GRADIENT VELOCITY SEDIMENTATION

Samples (300µl) were analysed on 4.5ml 5% - 20% linear sucrose gradients containing 0.5 PBS and 0.5% NP-40 (other detergents were also

used in sucrose gradients, see Results). The gradients were centrifuged at 200,000g_{av} for 4 hrs at 4°C in a Spinco model L ultracentrifuge using an SW 50 swing-out rotor at 45,000 r.p.m. or in a Sorvall OTD 65 ultracentrifuge using an AH 65 swing-out rotor at the same speed. After centrifugation the gradients were divided into fractions of approximately 150µl which were collected into the flat bottomed wells of a microtest plate.

Fractions which were to be analysed by the haemagglutination inhibition assay for immunoglobulin were diluted 1 : 1 with 0.5 PBS containing 0.5% NP-40 to give an adequate volume for analysis.

The distribution of radioactivity in sucrose gradients was determined by TCA precipitation using 10µl samples from each fraction. The contents of radioactive fractions were prepared for SDS PAGE by precipitation in 10x the fraction volume of ethanol on ice for 1 hr. The precipitated proteins were pelleted by centrifugation for 2 mins at 12,800g_{av} in an Eppendorf microcentrifuge, washed 3x in 1 ml of ethanol followed by a similar volume of acetone. The final acetone pellet was then air dried and dissolved in SDS PAGE sample buffer.

THE AMMONIUM SULPHATE FRACTIONATION OF CELL CULTURE SUPERNATANTS

Salt fractionation of cell culture supernatants (prepared without NP-40) was carried out by the addition of a 100% saturated solution at 0°C of $(NH_4)\ SO_4$ pH 7.2 to a sample of culture supernatant on ice to give the required final concentration of $(NH_4)_2\ SO_4$. Following the addition of $(NH_4)_2\ SO_4$ the mixture was allowed to stand on ice, with occasional mixing for 30 mins when the precipitate was pelleted by centrifugation at 80,000g_{av} for 15 mins at 4°C in a Spinco model L ultracentrifuge using a No. 4 fixed angle rotor at 35,000 r.p.m. The supernatant was discarded and the pellet dissolved in 300µl of distilled water containing 0.5% NP-40 for analysis by sucrose gradient velocity sedimentation. Pellets from large samples of culture supernatant contained too much salt to be layered directly onto a sucrose gradient. In this case the pellet was redissolved in 3mls of distilled water containing 0.5% NP-40 and the proteins repelleted

from solution by centrifugation under the same condition as above for 16 hrs. The supernatant was discarded leaving a salt-free pellet.

THE IMMUNOPRECIPITATION OF CELL CULTURE IMMUNOGLOBULINS

Several techniques and antisera were evaluated (see Results) the conditions described below gave optimal results.

Immunoprecipitation was carried out using rabbit antihuman heavy chain class specific and light chain type specific antisera (Behringwerke) together with formalin fixed, heat killed, protein A-bearing Staphylococcus aureus bacteria as an immunoadsorbant (Kessler 1975). A 0.4ml sample of radiolabelled culture supernatant was incubated with 5µl of the relevant antihuman immunoglobulin antiserum or a similar volume of a control serum at $4^{o}C$ with mixing in a microcentrifuge tube. After 15 mins 50µl of a 3x washed 10% (wet cell weight : volume) suspension of the Staphylococcal immunoadsorbant was added and the mixture incubated in the same way for a further 15 mins. The bacteria were then removed from the mixture by centrifugation at $12,800g_{av}$ for 1 min in an Eppendorf microcentrifuge and washed 3x in PBS 0.5% NP-40. Immunoprecipitated material was then eluted from the washed bacterial absorbant by suspension in 25µl of 2x SDS PAGE sample buffer and incubating in a boiling water bath for 3 mins. The elute was collected by centrifugation at $12,800g_{av}$ for 5 mins at room temperature to remove the bacteria. Before applying all or a portion of the eluted sample to a gel it was clarified by centrifugation under the same conditions for 5 mins.

RESULTS

THE DETECTION OF IMMUNOGLOBULINS USING THE HAEMAGGLUTINATION INHIBITION TEST

The serum coated SRBC described in the Materials and Methods Section on the serological detection of immunoglobulins were tested for their specificity by assaying them against serial dilutions of goat antihuman heavy chain antisera (Technicon) and rabbit antihuman light chain antisera (Berhingwerke). SRBC preparations coated with samples of human myeloma or macroglobulinaemia serum characteristically demonstrated high end points for agglutination reactions with a single antiheavy chain antiserum and a single antilight chain antiserum (Fig. 5) although occasionally SRBC coated with a particular myeloma serum would agglutinate only with an antilight chain antiserum. Similar preparations coated with normal human serum showed raised end points against anti γ and anti κ antisera. However, the agglutination titres of these preparations were considerably lower against every antiserum than those of myeloma or macroglobulinaemia serum coated SRBC.

Inhibition of agglutination of such high antisera titres allows the sensitive and specific detection of human immunoglobulin heavy and light chains in the supernatants of cultured human lymphoid cell lines (Fig. 6). The sensitivity of this system can be measured by assaying dilutions of standardised human serum. In this way γ chains can be detected in standardised serum (Berhingwerke Standard Human Serum batch no. A975) at dilutions of $1 : 10^6$ (V/V in unused culture medium) which is equivalent to an IgG concentration of 12.9ng/ml, μ chains at $1 : 6.4 \times 10^4$ (9.28ng IgM/ml) and α chains at $1 : 64 \times 10^4$ (20ng IgA/ml). κ chains can be detected at a $1 : 2.5 \times 10^5$ dilution of the same standard serum and λ chains at a $1 : 6.4 \times 10^4$ dilution. By calibrating the test in this way (for a given batch of immunoglobulin coated SRBC) one can quantify the amount of immunoglobulin secreted by human lymphoid cell lines. Fig. 7 and Table 3 show just such an analysis of the immunoglobulin produced in cultures of the cell lines RPMI 8866 and SMI_1. In both cultures, over a period of 5 days, their peak of production was about 10μg of immunoglobulin/10^6 cells/24hrs. This is a similar figure to that estimated in the same way for several other cell lines being maintained under normal conditions.

Fig. 5 The agglutination reactions of serum coated SRBC

Gluteraldehyde fixed SRBC coated with serum from a Waldenstroms Macroglobulinaemia patient (ODI), serum from a myeloma patient (SNE) and pooled normal human serum (PHS) were tested against doubling dilutions of antihuman heavy chain antisera and antihuman light chain antisera in the presence of unused culture medium in V section microtest plate wells.

Fig 5

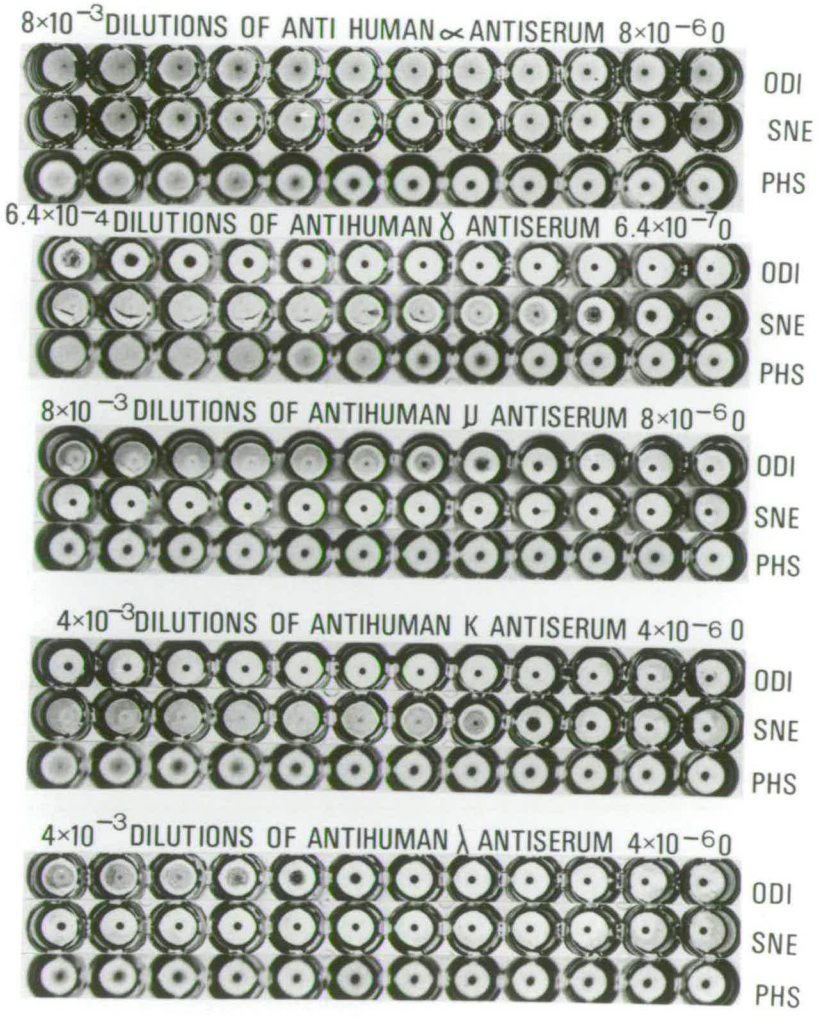

8×10⁻³ DILUTIONS OF ANTI HUMAN α ANTISERUM 8×10⁻⁶ 0
ODI
SNE
PHS
6.4×10⁻⁴ DILUTIONS OF ANTIHUMAN χ ANTISERUM 6.4×10⁻⁷ 0
ODI
SNE
PHS
8×10⁻³ DILUTIONS OF ANTIHUMAN μ ANTISERUM 8×10⁻⁶ 0
ODI
SNE
PHS
4×10⁻³ DILUTIONS OF ANTIHUMAN K ANTISERUM 4×10⁻⁶ 0
ODI
SNE
PHS
4×10⁻³ DILUTIONS OF ANTIHUMAN λ ANTISERUM 4×10⁻⁶ 0
ODI
SNE
PHS

Fig. 6 The haemagglutination inhibition assay

The appearance of microtest plates containing assays using the haemagglutination inhibition technique for the presence of μ heavy chains (a) and κ light chains (b) in the supernatants of cultures of seven human B lymphoid cell lines.

Fig 6

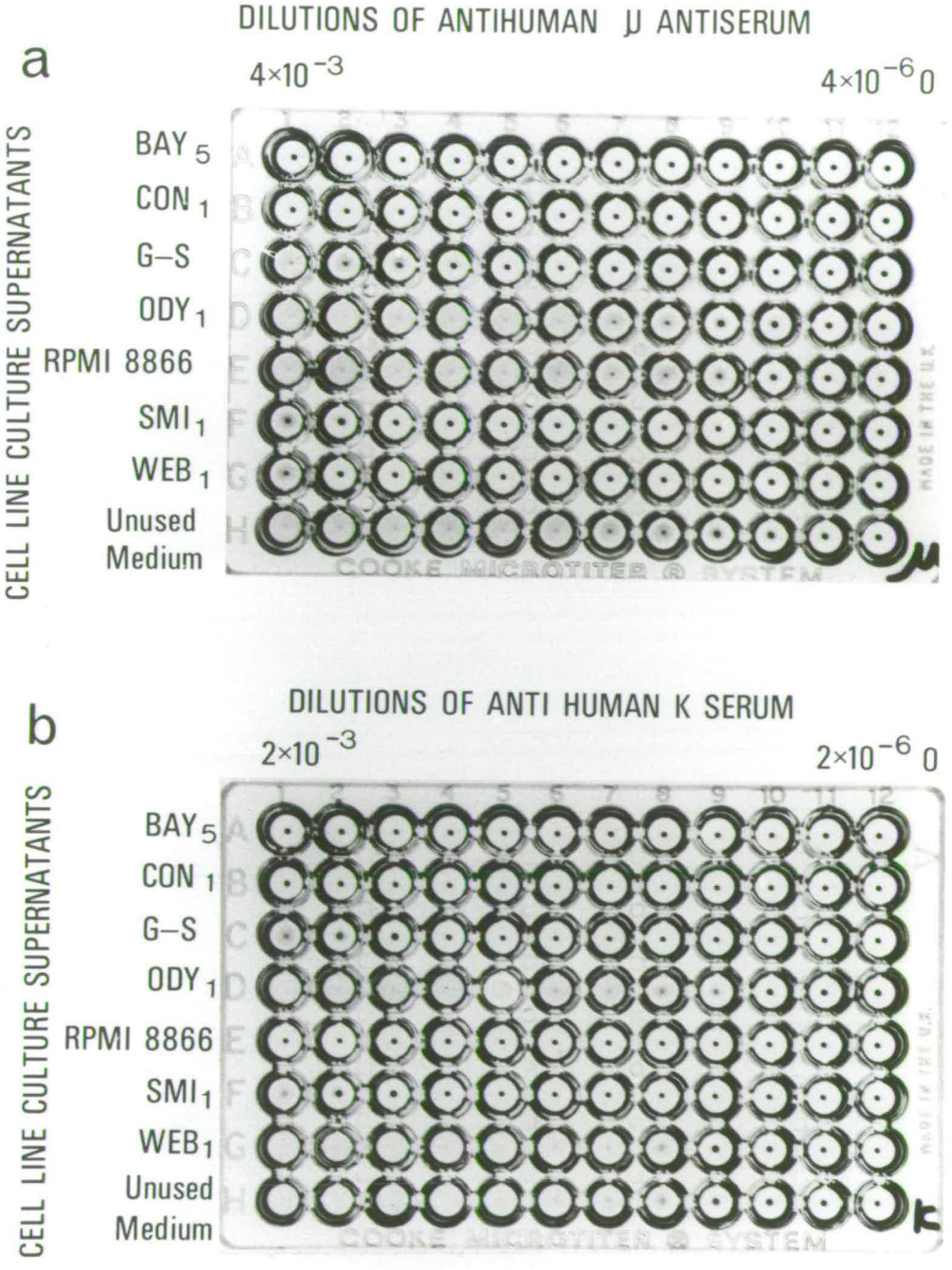

a
DILUTIONS OF ANTIHUMAN JJ ANTISERUM
4×10⁻³
4×10⁻⁶ 0
CELL LINE CULTURE SUPERNATANTS
BAY 5
CON 1
G–S
ODY 1
RPMI 8866
SMI 1
WEB 1
Unused Medium
b
DILUTIONS OF ANTI HUMAN K SERUM
2×10⁻³
2×10⁻⁶ 0
CELL LINE CULTURE SUPERNATANTS
BAY 5
CON 1
G–S
ODY 1
RPMI 8866
SMI 1
WEB 1
Unused Medium

Fig. 7 The accumulation of immunoglobulin in the supernatants of human B lymphoid cell lines

The amount of immunoglobulin present, with time, in the supernatants of cultures of (a) RPMI 8866 an IgG secretor and (b) SMI_1 an IgM secretor. The IgG and IgM present in the supernatants is plotted as the relative decrease in agglutination end point which is calibrated in Table 3. (immunoglobulin in supernatant ○ , live cell number ● , viability ▲)

Table 3 The quantification of immunoglobulin detected in the haemagglutination inhibition assay for IgM and IgG

Fig 7

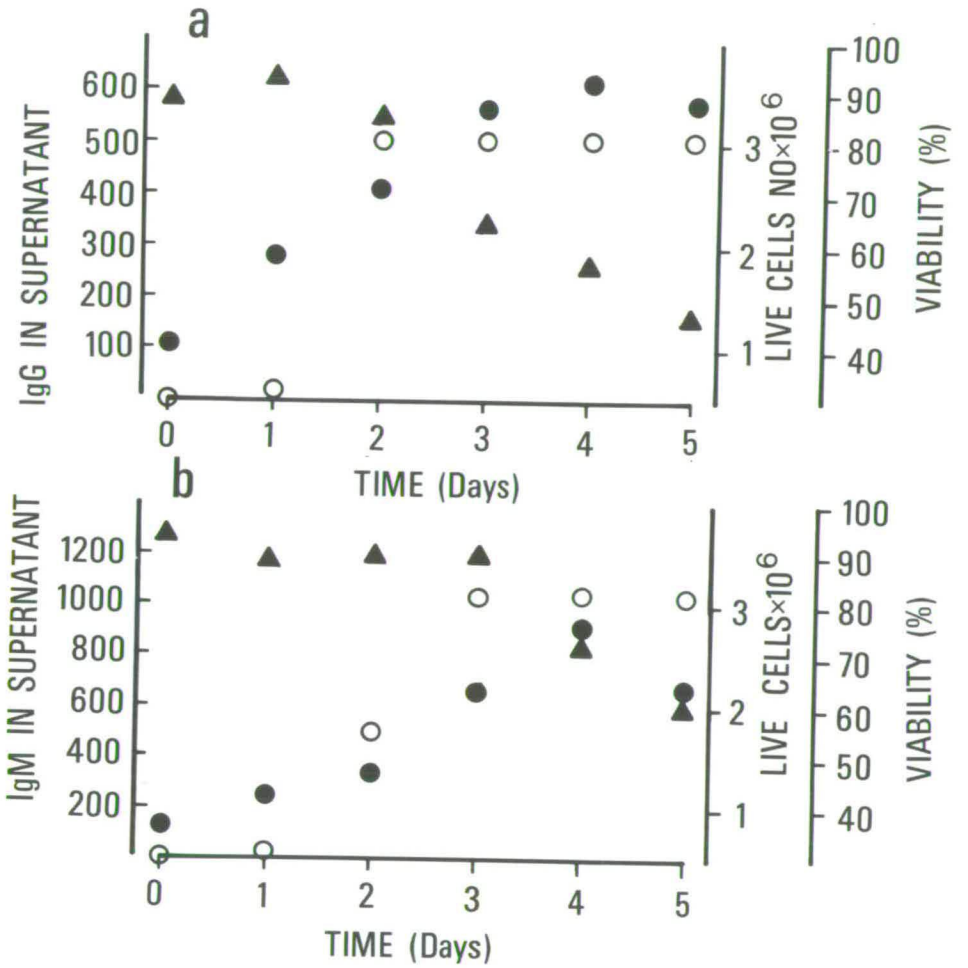


TABLE 3

Wells of inhibition	0	1	2	3	4	5	6	7	8	9	10
Relative decrease in agglutination end point	0	x2	x4	x8	x16	x32	x64	x128	x256	x512	x1024
Conc. of IgG showing equivalent inhibition in a γ test (ng/ml)	-	-	12.4	25	50	100	200	400	400	800	1600
Conc. of IgM showing equivalent inhibition in a μ test (ng/ml)	-	-	10	20	40	40	80	160	320	320	640

SCREENING OF HUMAN LYMPHOID CELL LINES FOR SECRETION OF IMMUNOGLOBULINS

A large number of established human lymphoid cell lines were assayed for the secretion of α, γ and μ heavy chains and κ and λ light chains by testing culture supernatant in the haemagglutination inhibition technique. Each line was assayed on more than one occasion and most at least three times. The majority of lines secreted a single heavy chain class together with a single light chain type and these are summarised in Table 4 (the details of whose contents are given in Appendix I). The most common class of immunoglobulin secreted by these cell lines is IgM. Although this distribution does not hold for the small number of cell lines established from individuals with infectious mononucleosis it is evident in all the lines established from other sources and is particularly striking in those lines from cord bloods where no examples of secretion of any other immunoglobulin class but IgM were found.

The proportion of κ and λ light chain secreting cell lines appeared similar for each group from which the lines were established and within each class of immunoglobulin secreted. The overall ratio of κ light chain secretors to λ chain secretors was 1.3 : 1 reflecting the relatively similar abundance of κ and λ chains found in sources of human immunoglobulin.

Several lines appeared to secrete more than one class of heavy chain and/or more than one type of light chain. However this pattern was mostly apparent in relatively "young" cell lines and in all cases gave way to secretion of a single heavy chain class and light chain type on more prolonged culture.

A second group of cell lines whose immunoglobulin secretion pattern differs from the majority of established lines are those which secrete no detectable immunoglobulin or only a single heavy chain or light chain. However, in contrast to those lines secreting more than one heavy chain or light chain, this group have been in culture for some time, are mainly derived from individuals with disorders affecting the lymphoreticular system and for the most part the lines have stable and reproducible patterns of immunoglobulin secretion. The most common pattern in this group, shown by eight lines, is a lack of detectable heavy chains in the presence of light chain secretion (see Table 5). Of these lines Jijoye has been previously described as secreting $\gamma\mu$ and κ chains and EB_3 as secreting

TABLE 4

The number of human B lymphoid cell lines secreting a single class of heavy chain and a single class of light chain

Source of blood lymphocytes	Immunoglobulin secretion					
	ακ	αλ	γκ	γλ	μκ	μλ
Cord blood	0	0	0	0	21	19
Adults*	4	3	10	8	20	16
Adults with infectious mononucleosis	3	2	4	2	1	1
Adults suffering from disorders of the lymphoreticular system	1	2	4	2	8	3

*This group consists of normal healthy individuals together with those suffering from genetic and chromosomal disorders or from malignant disease not affecting the lymphoreticular system

TABLE 5

ESTABLISHED HUMAN B LYMPHOID CELL LINES SHOWING APPARENTLY VARIANT PATTERNS OF IMMUNOGLOBULIN SECRETION

Cell line	Sex	Donor Condition	Immunoglobulin secretion				
			α	γ	μ	κ	λ
Daudi	M	Burkitt lymphoma	-	-	-	-	-
FAL_1	F	Healthy cord blood	-	-	-	-	-
F89	M	Subacute lymphatic leukaemia	-	-	-	-	-
Raji	M	Burkitt lymphoma	-	-	-	-	-
F137	M	Chronic lymphatic leukaemia	-	-	+	-	-
EB_2	F	Burkitt lymphoma	-	-	-	-	+
EB_3	M	Burkitt lymphoma	-	-	-	-	+
JIM_1	M	Healthy normal adult	-	-	-	-	+
Jijoye	M	Burkitt lymphoma	-	-	-	+	-
KAT_1	F	Infectious mononucleosis	-	-	-	+	-
U266BL	M	Multiple myeloma	-	-	-	-	+
RPMI 8226	F	Multiple myeloma	-	-	-	-	+

no detectable immunoglobulin chains as determined by immunoelectrophoresis (Van Furth et al., 1972). However, in common with Evans et al., (1974) and using a similar haemagglutination inhibition technique, this report classified Jijoye as secreting κ chains only and EB_3 λ chains only. The secretion of λ chains only by the human plasma cell line RPMI 8226 agrees with the results of Matsuoka et al., (1968) from the analysis of culture supernatant by immunodiffusion and immunoelectrophoresis. Although U266BL is described here as secreting λ chains alone this line is derived from the blood lymphocytes of an IgE myeloma patient and has been demonstrated, by immunodiffusion, to secrete ε heavy chains (Nilsson et al., 1970). However, as the assay system used in this project initially to screen for secreted immunoglobulin detects only α , γ and μ heavy chains, any lines secreting δ or ε heavy chains remain unidentified at this stage.

Among the secreted proteins of a single cell line (FB7) only a heavy chain (μ) is detectable (Table 5). The detection of μ chains was far less positive in the supernatants of FB7 cultures than the detection of light chains in light chain only secreting cell lines and the amount of heavy chain detected in FB7 supernatants was far smaller (of the order of 500x less) than that in other μ chain secreting cultures. Nevertheless, the result was consistantly reproducible and agrees with that of Evans et al., (1974).

Four human B lymphoid cell lines had no detectable heavy chains or light chains in their culture supernatants (Table 5). Of these Raji has been reported to secrete μ chains only and Daudi κ chains only (Evans et al., 1974). However, in my hands Raji secretes no immunoglobulin and Daudi culture supernatants have been positive for κ chains only a few times in a large number of assays and then just by a matter of 2 or 3 wells of inhibition. Both these lines have been classified here as non-secretors.

The cell lines CCRF, $MOLT_4$ and K562 also failed to secrete any detectable immunoglobulin, but, as CCFR and $MOLT_4$ are derived from T lymphocyte leukaemias (Foley et al., 1965, Minowada et al., 1973) and K562 is an erythroleukaemic cell line (Anderson et al., 1979) i.e. they are all non-B lymphoid lines, this result is to be expected.

It is appropriate to also mention here the lines BR18 and BR18T for, although they do not show abnormal patterns of immunoglobulin

secretion (see Appendix I), they represent a change in secreted immunoglobulin. BR18 secretes μ and κ chains and is the parent line of BR18T, a spontaneous tetraploid derivative which also secretes μ heavy chains but together with λ light chains. This alteration in light chain expression using what should be the same immunoglobulin genesmay have implications for genic and allelic exclusion in light chain synthesis.

The effect on immunoglobulin production of co-culturing human B lymphoid cell lines with T lymphocytes

Kishimoto et al., (1978) claimed that IgM production could be enhanced and IgG production induced in human lymphoblastoid cell cultures by normal human T cells. This report led to an attempt to repeat these results using the haemagglutination inhibition test to detect the immunoglobulin produced. If these observations were repeated this system could provide a model in human lymphoid cell lines of the heavy chain switch in immunoglobulin synthesis.

Five different IgM secreting human B lymphoblastoid cell lines (BAR, BR18, G-S, MON and RPMI 1788) were each co-cultured with fresh human T lymphocytes from three different donors under exactly those conditions described by Kishimoto et al., (1978). However, using the haemagglutination inhibition assay, no increase in IgM production over the control level of that produced by lymphoblastoid cells cultured on their own nor induction of IgG production was detected in those lines co-cultured with T lymphocytes. The levels of IgG production, of the order of 100μg/ml, claimed by Kishimoto et al., (1978) would be readily detected in the haemagglutination inhibition assay.

The screening of available human B lymphoid cell lines established a single heavy chain, single light chain "monoclonal" pattern of immunoglobulin secretion as the norm, with the predominant class of immunoglobulin secreted being IgM. A small number of cell lines have been identified which differ from the majority in their pattern of immunoglobulin secretion their synthesis of immunoglobulins. However, these are independently established cell lines from different clinical sources and may not represent true variants or mutants but, for example, the "fixing" in culture of cells representing separate stages of

B lymphocyte differentiation. In this case the availability of cultures from the same line which were respectively normal and variant in terms of immunoglobulin production would provide useful additional material for studying immunoglobulin synthesis in human lymphoid cell lines.

CLONING AND MUTAGEN TREATMENT

As indicated at the end of the last section of results a comparison of normal and variant immunoglobulin-synthesising cultures from the same cell line would be helpful in determining the events giving rise to variants and so hopefully to understanding immunoglobulin synthesis in general. So, in addition to identifying abnormal immunoglobulin secretion among established cell lines, an attempt was made to detect variant immunoglobulin synthesising cells within a cell line by experiments involving mutagen treatment and cloning.

The human B lymphoid cell line RPMI 8866 was used in initial experiments to determine the cloning conditions as this line has been shown previously to grow in semi-solid agarose (Lever and Seegmiller 1976). It was found that RPMI 8866 would form colonies in two of the commercial brands of agarose tested (Table 6) but with a low frequency. The low efficiency of colony formation in agarose led initially to the use of different forms of semisolid media. The most successful of these was collagen, but, although higher cloning efficiencies were obtained ($\simeq$ 25%) in a collagen matrix constructed according to Elsdale and Bard (1972), this proved a difficult medium from which to remove the cell colonies. Therefore, conditioned media and feeder layers were used in an attempt to increase the cloning efficiency in agarose. Conditioned media had little or no effect but the inclusion of a fibroblast feeder layer, in particular one of human embryonic lung fibroblasts, gave a dramatic increase in cloning efficiency (Table 7) which was maintained over a wide range of cell densities (Table 8). Using these conditions several human B lymphoid lines were cloned with a high efficiency, (Table 9).

Table 6 Colony formation in different types of agarose

The cloning efficiency (number of colonies formed expressed as a % of the number of cells plated) of RPMI 8866 in different commercial forms of agarose. Each result is the mean of 3 petri dishes containing 100 cells each in agarose.

Table 7 The variation in cloning efficiency with culture conditions

The cloning efficiency of RPMI 8866 in International Enzyme Grade A agarose under different culture conditions. Each result is the mean of 2 petri dishes containing 100 cells each in those conditions.

Table 8 The variation in cloning efficiency with cell number

The variation in cloning frequency of RPMI 8866 with the number of cells plated in International Enzyme Grade A agarose over a human embryonic lung fibroblast feeder layer. Each result is the mean of 2 petri dishes at that cell density.

TABLE 6

Agarose	Cloning efficiency (%)
May and Baker	0
Miles	0
Miles LGT	0
International Enzymes (Grade A)	5
Sigma (electrophoretic grade)	2

TABLE 7

Conditions	Cloning efficiency (%)
No feeder layer, fresh medium	6
No feeder layer, fibroblast conditioned medium	9
No feeder layer, lymphoblast conditioned medium	8
Human embryonic skin fibroblast feeder layer, fresh medium	21
Human embryonic lung fibroblast feeder layer, fresh medium	52
WC38 fibroblast feeder layer, fresh medium	20

TABLE 8

Cells per dish	Cloning efficiency (%)
10	25
50	45
100	48
250	47
500	42
1000	40

Table 9 The cloning efficiencies of several human B lymphoid cell lines

The conditions are those described in Table 8. Each line was plated in duplicate.

Table 10 Immunoglobulin secretion in clones of RPMI 1788, RPMI 8866 and a mixed culture of the two

The cultures were cloned in the conditions described in Table 8. In the cases of RPMI 1788 and RPMI 8866 samples of 10 clones each were tested and of the mixed culture clones 50 were sampled.

Table 11 The cytotoxic effect of Melphalan

The pooled results of 2 experiments on the viability of 1×10^6 cells/ml cultures of RPMI 8866 exposed for 24hrs to differing concentrations of Melphalan. The 0 represents the addition of only the solvent, propylene glycol, at a final concentration of 0.1% to the culture. Each result is a mean of duplicate cultures.

TABLE 9

Cell line	Cloning efficiency (%)
RPMI 8866	58
SMI_1	37
ORI_1	25
EB_4	30
RPMI 1788	35
RPMI 8226	71

TABLE 10

Cell line	Cloning efficiency	Immunoglobulin secreted by sample clones		
		IgG	IgM	IgG & IgM
RPMI 1788	25	0	10	0
RPMI 8866	41	10	0	0
RPMI 1788/ 8866 mix	28	12	38	0

TABLE 11

Melphalan (g/ml)	% viable cells after 24hrs exposure
1×10^{-7}	88
2×10^{-7}	93
4×10^{-7}	28
6×10^{-7}	11.7
8×10^{-7}	5.4
1×10^{-6}	0
1×10^{-5}	0
0	86

In order to detect immunoglobulin-secreting colonies in agarose it was intended to employ the "antibody overlay" technique of Scharff et al., (1975) used to identify immunoglobulin production by colonies of mouse myeloma cells in agar. However, goat antihuman γ chain antiserum (Technicon) at concentrations ranging from 1 : 10 to 1 : 100 in a 2ml 0.25% agarose overlay failed to detect immunoglobulin secretion in 1, 2 and 3 week colonies of RPMI 8866. As an alternative all colonies to be tested for immunoglobulin production were picked from agarose and assayed in microculture by haemagglutination inhibition using a reduced range of antisera concentrations. Although far more time consuming than antibody overlay this regime did have an advantage in that each clone could be tested for secretion of more than one immunoglobulin polypeptide. This facility was used to demonstrate the accuracy of the cloning technique in producing clones of single cell origin in that no clones producing both IgM and IgG were identified among 50 sample clones derived from a mixed culture of RPMI 1788 and RPMI 8866 (Table 10) in which both classes of immunoglobulin could be detected.

The effect of a 24hr exposure to increasing concentrations of Melphalan, a mutagen shown to effect immunoglobulin synthesis in mouse myeloma cells, on the viability of cultures of RPMI 8866 is shown in Table 11. Using a concentration from the middle of the range, which has a visible cytotoxic effect, a 5ml culture of RPMI at 1×10^6 cells/ml was exposed to 6×10^{-7} g/ml of Melphalan for 24hrs. The surviving cells were separated out by centrifugation on 11% hypaque at $500g_{av}$ for 10 mins at room temperature (J. Evans, personal communication) washed in medium and samples plated out in 10 petri dishes at 500 cells/dish. A similar sample from an untreated culture of the same cell line was plated out in a similar way. A total of 1117 clones were recovered from the plated sample of the mutagenised culture and 836 from the control culture. However, every one of the mutagen treated and control clones secreted both, apparently normal, γ and κ chains. In addition 130 cultures established during other experiments in this laboratory, by dilution cloning of the cell lines F137 and YAK_1 after treatment with MNNG or EMS in a manner which has produced several biochemical changes (Porey et al., 1973, Arthur et al., 1975, Gardiner et al., 1977) were assayed for secretion of α, γ, μ, κ and λ chains. Each culture showed no change in its immunoglobulin secretion pattern from their parent line.

In terms of generating mutant clones the mutagenesis and cloning experiments produced negative results. To repeat or extend such experiments would be time consuming and, in the light of these results, perhaps fruitless. Thus, it was decided to devote the time available to further examination of normal lines and the presumptive variant lines identified earlier.

THE ANALYSIS OF RADIOACTIVITY LABELLED PROTEINS SECRETED BY HUMAN LYMPHOID CELL LINES

Following the serological identification of immunoglobulins in culture supernatants a more detailed analysis of these molecules requires that the proteins synthesised by human B lymphoid cell lines can be distinguished from the FCS proteins in which the cells are cultured. This distinction can be made by the biosynthetic labelling of the cell products using a radioactive precursor. An initial assessment of the nature and quantity of proteins labelled in this way and secreted into the culture medium was made by the analysis of a selection of samples of biosynthetically radiolabelled culture supernatants from different cell lines by SDS PAGE. The samples were run under reducing conditions which break S-S bonds (Fig. 8) and under non-reducing conditions, (Fig. 9) on 3% - 20% gradient gels.

The more striking feature of these gels is the large number of protein bands shown by each cell line supernatant, especially in the reduced gel, and the high proportion of bands which appear common to all the cell lines including the T lymphoid line CCRF. However among the large number of components several are prominent. In the reduced gel (Fig. 8) the tracks from several of the immunoglobulin secreting cell lines have a conspicuous band corresponding to the size of a light chain (22,000 mol. wt.) but this band does seem to vary from line to line in its mobility. The reduced track of RPMI 8866 shows a large band at 50,000 mol. wt. which possibly represents the secreted γ chains of that line. The tracks in the same gel of SMI_1 and U266BL both show a band not present in other samples at about 70,000 mol. wt.

Fig. 8 SDS PAGE analysis of reduced cell line supernatants

This flurograph shows the SDS PAGE profiles of unfractionated ^{14}C valine biosynthetically labelled culture supernatants under reducing conditions (i.e. with 5% 2-mercaptoethanol in the sample buffer) on a linear 3% - 20% gradient gel. All the samples of culture supernatant contained an equal number of TCA precipitable counts and an equal amount of total protein. The molecular weights referred in the text are given ($x10^{-3}$) on the right and were estimated from the mobility of molecular weight markers shown ($x10^{-3}$) on the left. The molecular weight markers were; phosphorylase b (94,000), BSA (68,000), IgG heavy chain (51,000), IgG light chain (22,000) and RNAase A (13,000).

Fig. 9 SDS PAGE analysis of non-reduced cell line supernatants

This flurograph shows the same analysis as in Fig. 8 but without 2-mercaptoethanol in the sample buffer. The markers were; IgG (146,000), BSA (68,000) and RNAase A (13,000).

Fig 8

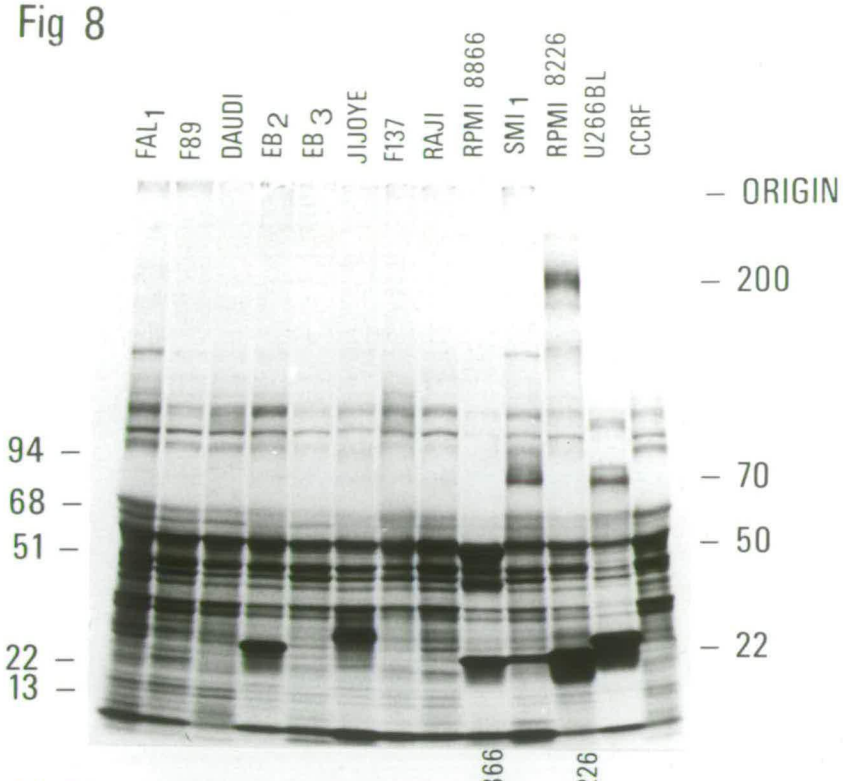
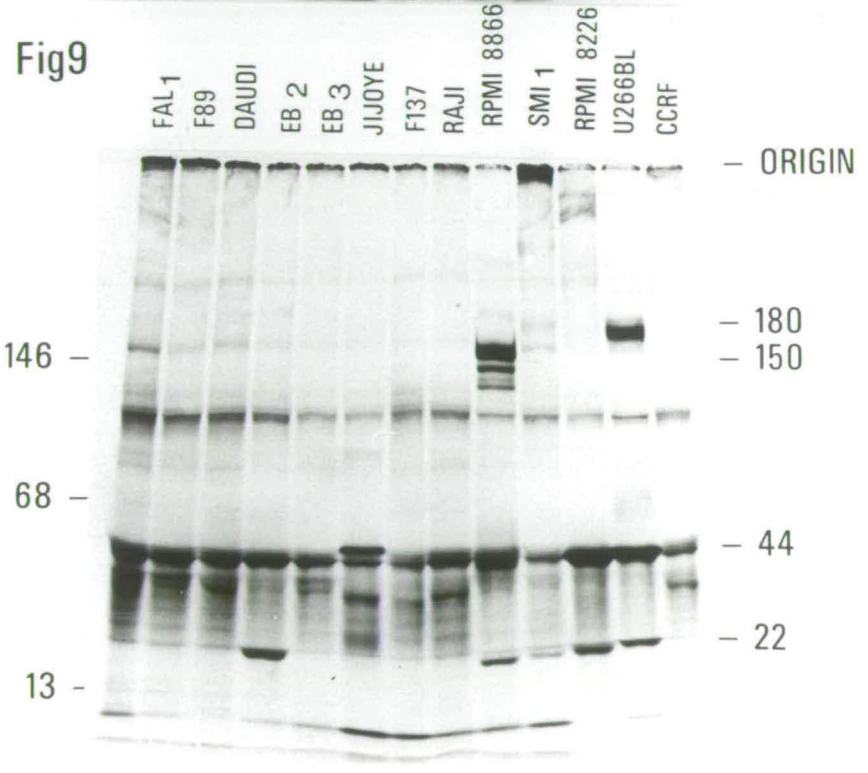

FAL1
F89
DAUDI
EB 2
EB 3
JIJOYE
F137
RAJI
RPMI 8866
SMI 1
RPMI 8226
U266BL
CCRF
ORIGIN
200
94
68
51
70
50
22
22
13

Fig9


FAL1
F89
DAUDI
EB 2
EB 3
JIJOYE
F137
RAJI
RPMI 8866
SMI 1
RPMI 8226
U266BL
CCRF
ORIGIN
180
146
150
68
44
22
13

As SMI_1 is a $\mu\kappa$ secretor and, although only λ chains were detected in U266BL cultures, it has been reported as secreting IgE (Nilsson et al., 1970) these bands may represent μ and ε chains respectively. The diffuse band of about 200,000 mol. wt. present only in the reduced track of RPMI 8226 cannot be explained in terms of its known immunoglobulin secretion pattern, but, as this is the only line among these samples which grows as an attached culture, this band may represent a large molecular weight peptide involved in cell adherence.

The non-reducing gel (Fig. 9) shows a similar group of bands around light chains size except in the sample from JIJOYE but here there is an additional band about double the light chain molecular weight indicating that the light chains may be secreted as dimers in this case. In fact similar dimerbands are present, in addition to those of single light chain size, in the tracks of other immunoglobulin secreting cell lines and RPMI 8226 has been reported as secreting light chains in a dimer form (Matsuoka et al., 1968). If these bands are light chains this indicates that they are free in the cell line supernatant yet there are no putative heavy chain bands visible in the non-reducing gel and the 150,000 mol. wt. band of RPMI 8866, the 180,000 mol. wt. band of U266BL and the large band at the origin in the SMI_1 track indicate the presence of entact IgG, IgE and IgM molecules respectively. This raises the possibility that these cell lines may secrete intact immunoglobulin molecules and free light chains.

However these points are only speculation at this stage. The overriding conclusion from this analysis is that human B lymphoid cell lines secrete a large number of proteins in addition to immunoglobulins and so some fractionation of their secreted proteins is required for any further examination of a particular component.

THE PURIFICATION OF IMMUNOGLOBULINS FROM SECRETED PROTEINS

The different immunoglobulin classes were originally defined by their sedimentation velocities and have well characterised sedimentation coefficients. Thus, it was considered that sucrose gradient velocity

sedimentation could be used in fractionating culture supernatants to analyse secreted immunoglobulins. Furthermore, it was thought unlikely that there would be many proteins among those secreted by human B lymphoid cell lines of the size of 19S pentameric IgM. Therefore, if the IgM secreted by human B lymphoid cells is in the pentameric form, IgM secreting cell line supernatants should provide useful starting material for this approach.

It was found that the biosynthetically radiolabelled secreted proteins of the IgM secreting cell line SMI_1 could be resolved into a fast sedimenting 19S component and a slower component of about 4S by sucrose gradient velocity sedimentation (Fig. 10a). Serological analysis of the gradient fractions demonstrated that μ heavy chains were present only in the 19S component (Fig. 10b), but κ light chains were present in both components of the secreted proteins (Fig. 10c). These results indicate that intact 19S IgM molecules are present in the proteins synthesised and secreted by SMI_1. The analysis of the radioactivity labelled proteins present in the 19S and slower components by SDS PAGE (Fig. 11) showed that the two most abundant polypeptides present in the 19S component have similar molecular weights to human μ chains and light chains confirming that 19S IgM is synthesised and secreted by this cell line. The presence of a polypeptide in the slower component with the molecular weight of a human light chain is consistent with the serological analysis of the gradient fractions from the same cell line showing the presence of light chains in slow components and supports the evidence cited earlier that in addition to intact 19S IgM, free light chains are secreted by SMI_1.

All other IgM-secreting human B lymphoid cell lines whose biosynthetically labelled secreted proteins were analysed by sucrose gradient velocity sedimentation showed a similar profile to SMI_1 that is a 19S and a slower sedimentating component. Several of these are shown in Fig. 13. In some of these lines the 19S component represents a substantial proportion ($>50\%$) of the secreted protein. The SDS PAGE analysis of the radiolabelled proteins present in these components from several lines also confirms the initial results from SMI_1 showing the μ and light chain polypeptides in the 19S components and an additional light chain band present, to a varying extent in different cell lines, in the slow component (Fig. 14).

Fig. 10 Sucrose gradient velocity sedimentation analysis of culture supernatant from an IgM secreting cell line

0.3mls of secreted proteins from SMI_1 (a μ κ secreting human B lymphoid cell line) were analysed on a 4.5ml 5% - 20% sucrose gradient containing 0.5% NP-40 at $200,000g_{av}$ for 4hrs at $4^{o}C$ as described in the Methods section.

(a) Profile of ^{14}C valine biosynthetically labelled proteins, 10μl of each fraction were TCA precipitated and counted. The remainder of each fraction was analysed for μ chains (b) and κ chains (c) by the haemagglutination inhibition test.

The sedimentation velocities were calibrated by analysing, during the same run, on an identical gradient a mixed sample of the same volume containing diluted serum from a macroglobulinaemia patient and human haemoglobin (d). The fractions were assayed for μ chains (shown in histogram form) to give the position of the 19S serum IgM and for their optical density (O.D.) at 415nm for the position of the 4S haemoglobin.

Fig 10

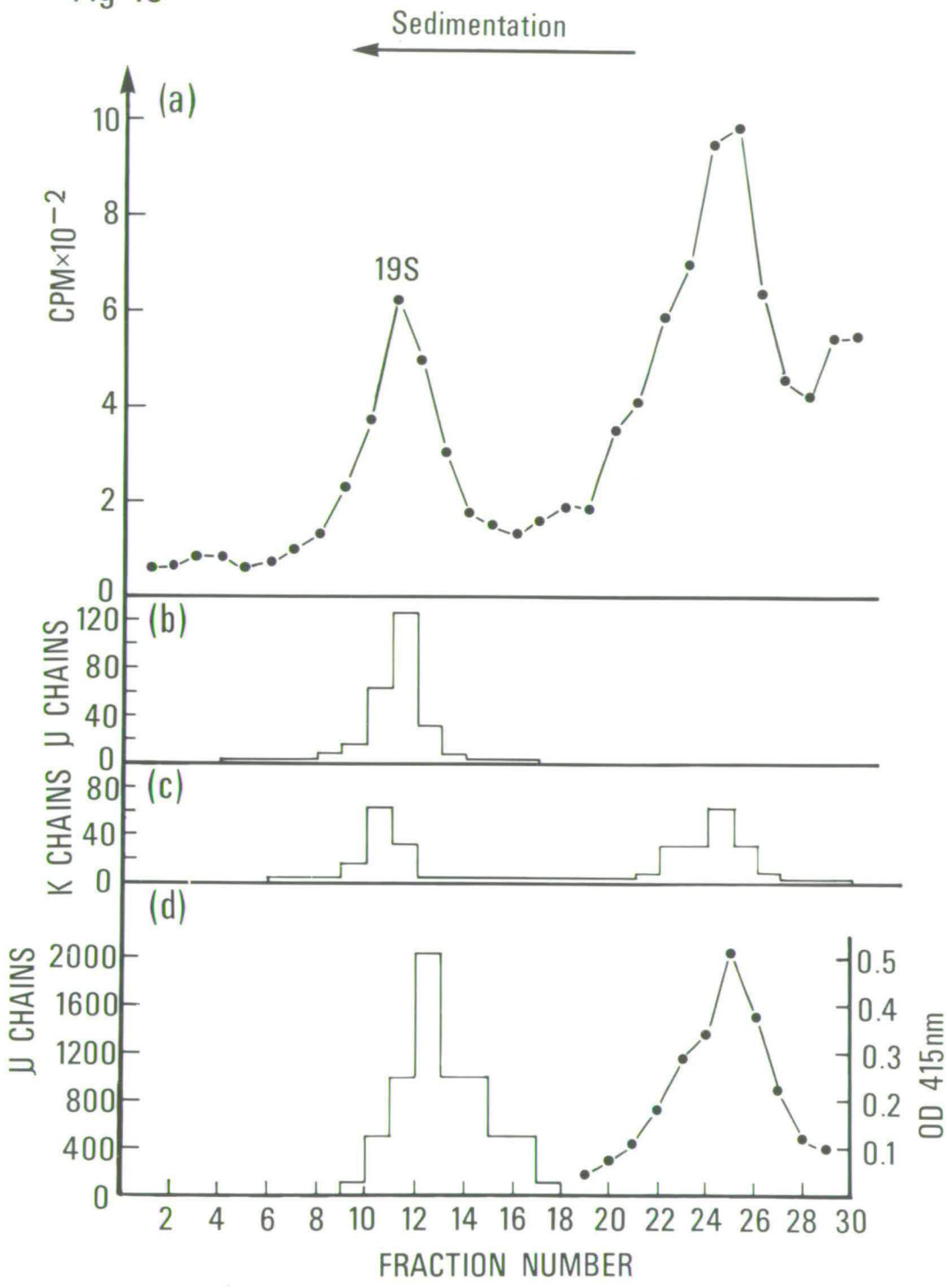

Sedimentation
(a)
CPM×10⁻²
19S
(b)
K CHAINS μ CHAINS
(c)
(d)
μ CHAINS
OD 415nm
FRACTION NUMBER

Fig. 11 SDS PAGE analysis of sucrose gradient fractions

A 30µl sample of unfractionated SMI_1 radiolabelled culture supernatant (UF) and the central fractions from each of the 19S and slow components (SC) isolated by the method described in Fig. 10 from SMI_1 secreted proteins were analysed by SDS PAGE on 12% gels. The flurograph of these gel tracks is shown here together with marker proteins run on the same gel. The Coomassie Blue stained portions of the gel containing the markers was swollen to the same size as that part which was prepared for flurography by incubating in 20X its volume of water for 1hr after destaining. The molecular weights of the markers are: IgM µ chain 72,000; IgG γ chain 51,000; light chains 22,000 (the differences in mobility are discussed later in the text); Phosphorylase b 94,000; BSA 68,000; ovalbumin 43,000; RNAase A 13,000.

Fig. 12 Comparative SDS PAGE analysis of purified immunoglobulin standards and myeloma, macroglobulinaemia and normal sera

Purified IgA, IgM and IgG, obtained as described in the Materials section, were analysed along with samples from two myeloma sera, GRE (α λ) and SNE (γ κ), a Waldenstrom's macroglobulinaemia serum ODI (µ λ) and a normal serum. The myeloma and macroglobulinaemia sera had been typed for their immunoglobulins in the haemagglutination inhibition test.

Fig 11

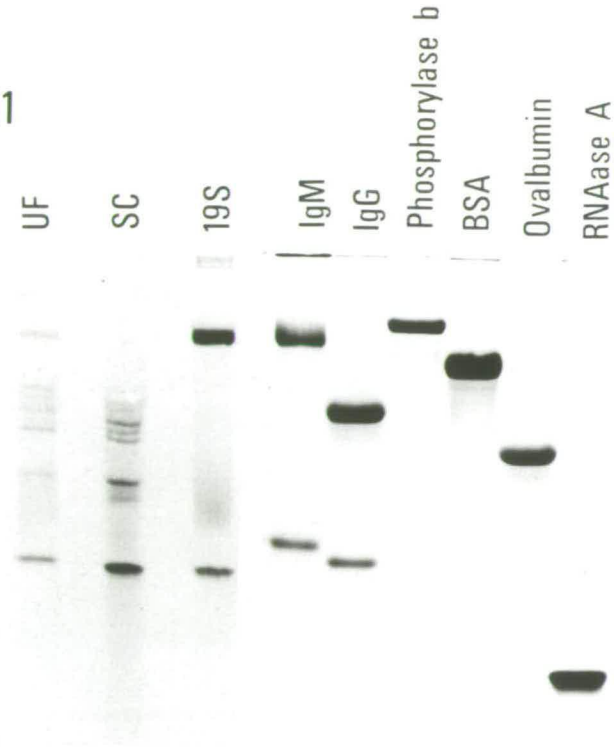

UF
SC
19S
IgM
IgG
Phosphorylase b
BSA
Ovalbumin
RNAase A

Fig 12

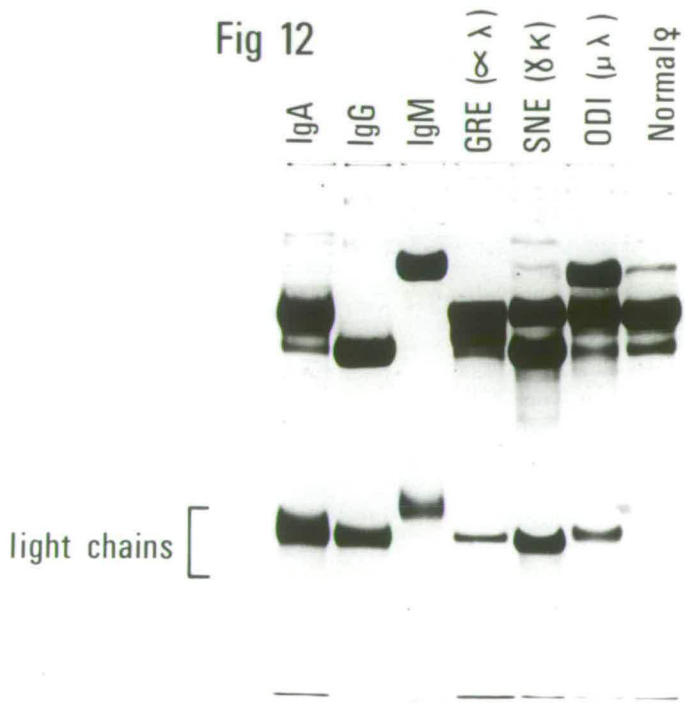

IgA
IgG
IgM
GRE (∝ λ)
SNE (δ κ)
ODI (μ λ)
Normal♀
light chains

Fig. 13 The velocity sedimentation analysis of the secreted proteins from different cell lines

300µl samples of biosynthetically radiolabelled cell culture supernatants from several different IgM synthesising human B lymphoid cell lines were analysed by sucrose gradient velocity sedimentation under the conditions described in Fig. 10. 10µl samples from each fraction were TCA precipitated and counted.

Fig 13

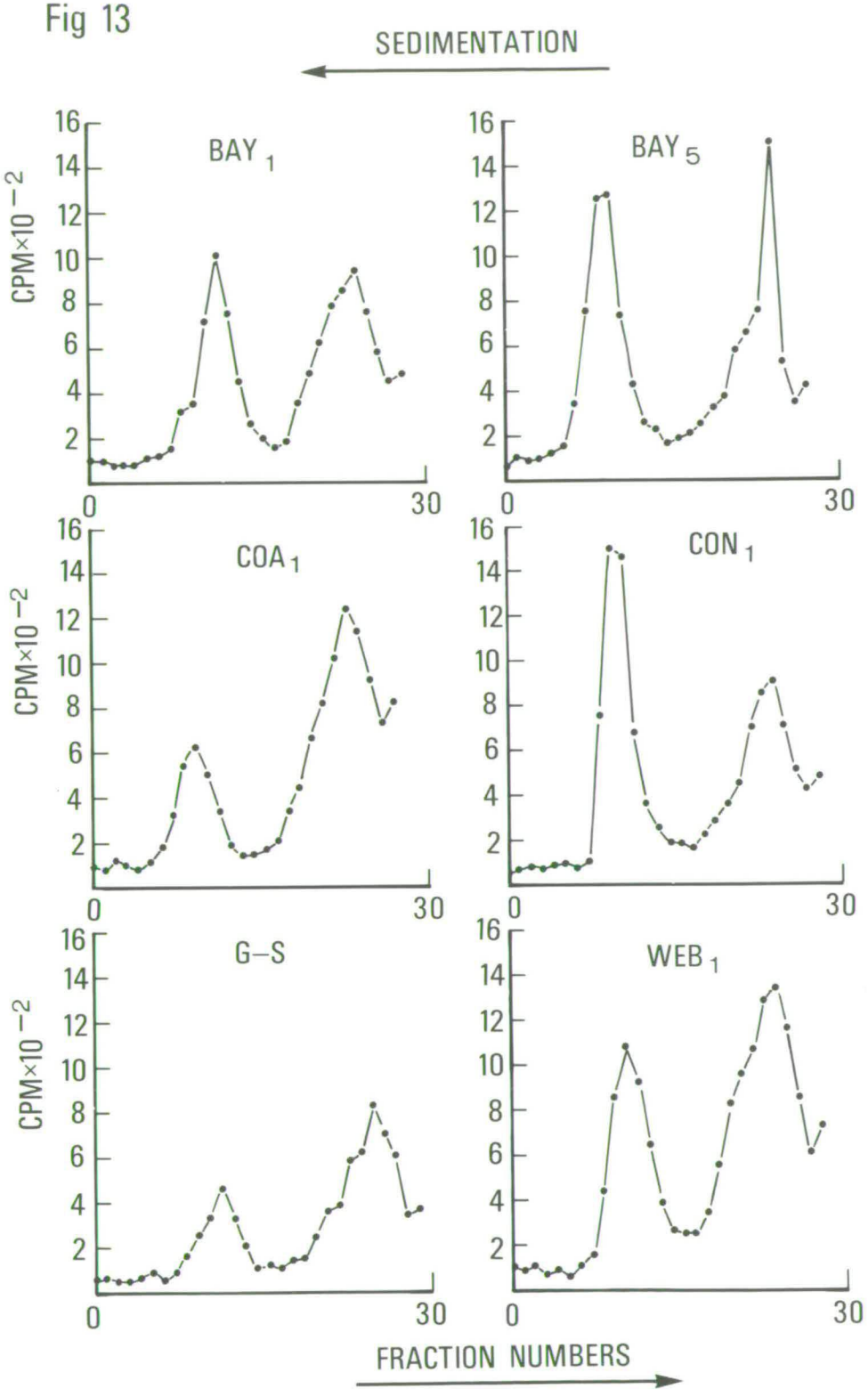
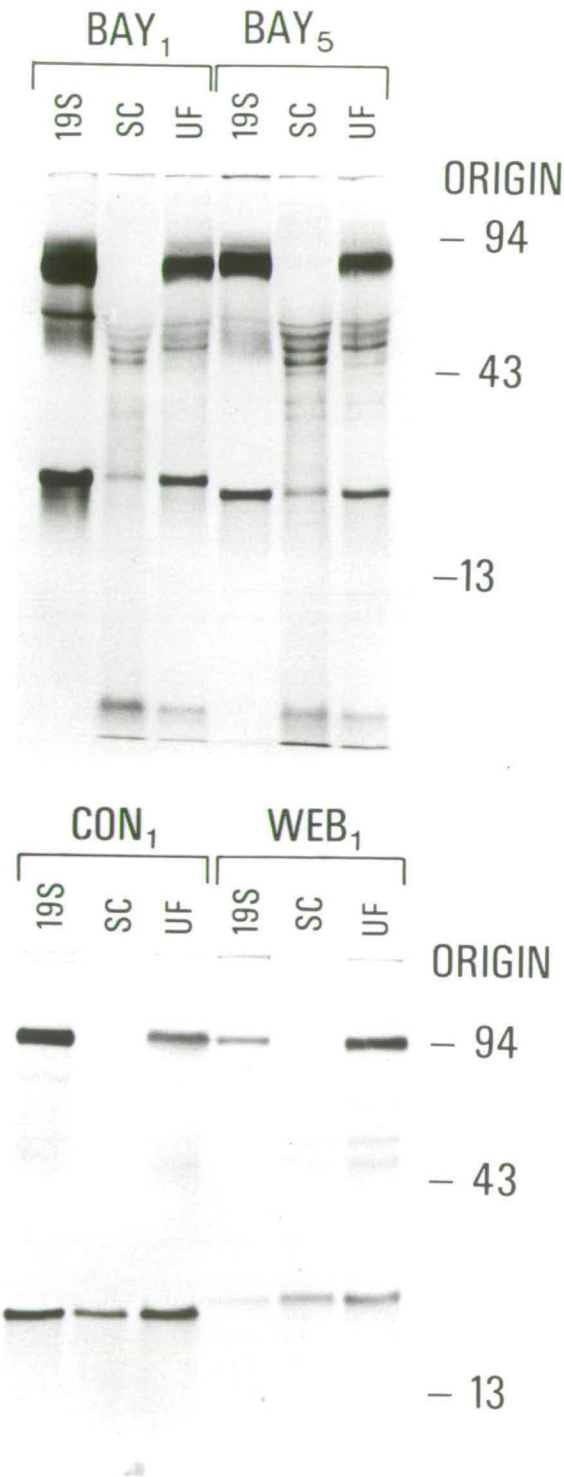

SEDIMENTATION
BAY 1
BAY 5
COA 1
CON 1
G–S
WEB 1
CPM×10 −2
0
30
2
4
6
8
10
12
14
16
FRACTION NUMBERS

Fig. 14 Comparative SDS PAGE analysis of radioactively labelled sucrose gradient components from different cell lines

The central fraction from each of the 19S and slow components (SC) from some of the sucrose gradient velocity sedimentation analyses of cell line supernatants shown in Fig. 13 together with 30µl samples of unfractionated radiolabelled culture supernatant from the respective cell lines were analysed on 12% SDS PAGE gels. Molecular weights are shown ($x10^{-3}$) on the right hand side; the markers were phosphorylase b (94,000) Ovalbumin (43,000 and RNAase A (13,000).

Fig 14


BAY1
BAY5
19S
SC
UF
19S
SC
UF
ORIGIN
– 94
– 43
–13
CON1
WEB1
19S
SC
UF
19S
SC
UF
ORIGIN
– 94
– 43
– 13

An additional feature of these gels where IgM isolated from more than one cell line are analysed together is that the electrophoretic mobility of the lightchains is not constant. Furthermore, this difference in mobility seems related to light chain type (BAY_1 and WEB_1 are $\mu\ \lambda$ secretors and BAY_5 and CON_1 $\mu\ \kappa$) so that κ chains migrate in SDS gels more rapidly than λ chains. This observation was also made in the SDS PAGE analysis of samples of myeloma and macroglobulinaemia sera which had been serologically typed using the haemagglutination inhibition test. Samples of these sera were analysed on an SDS gel. Purified human IgA, IgG and IgM were run in parallel with these samples since these immunoglobulins had shown a difference in their light chain mobilities when they were used as molecular weight standards in the analysis of the SMI_1 radiolabelled sucrose gradient fractions (Fig. 11). Fig. 12 confirms that the apparent molecular weight of the IgM light chain (identified as κ by the haemagglutination inhibition test) is larger than that of the light chain from the purified human IgG and shows that it is also larger than any of the other bands identified as light chains. Thus its mobility cannot be explained in terms of the difference in κ and λ chain mobility in SDS gels observed in the secreted IgM of human B lymphoid cell lines. Alternative explanations of this and other observed examples of variation in light chain mobility will be discussed later.

The use of detergents in sucrose gradients

Initial sucrose gradient runs showed very small 19S components or no components of this size at all in the analysis of IgM-containing cell line supernatants. In addition, the total TCA precipitable radioactivity recovered in the fractions of these gradients was considerably less than that in the sample of culture supernatant which had been applied to the gradient and a significant amount of TCA precipitable radioactivity could be detected by counting the empty used centrifuge tube. It was concluded that a portion of the radioactive proteins present had adhered to the polypropylene tube in which the gradient was run. Centrifuge tubes manufactured from different plastics were also found to bind large amounts of radioactive protein from the sample of culture supernatant. The use of a detergent was also

considered as a means of overcoming this problem. The inclusion of 0.5% NP-90 in the gradient prevented the binding of proteins to the centrifuge tube and dramatically altered the observed profile of radioactivity in the gradient fractions (Fig. 15). The apparent preferential binding of the 19S component to the centrifuge tube in the absence of detergent may be because the IgM which constitutes this component is a heavily glycosylated protein (see Introduction, section on immunoglobulin structure, Table 1) or as it moves further in the gradient the 19S component is exposed to a greater area of the tube and in a lower concentration of protein than the slower sedimenting component.

The use of other detergents such as SDS and sarkosyl also prevented the binding of proteins to the centrifuge tube (Table 12) and had the same effect on sucrose gradient analysis of culture supernatants as NP-40 (Fig. 16), although the 19S component in the sarkosyl gradient shown seems less well defined. However unlike the non-ionic detergent NP-40 the ionic detergents SDS and sarkosyl were both found to affect the haemagglutination inhibition test (presumably by disrupting hydrogen bonding) when present in sucrose gradient fractions or culture supernatants at concentrations which prevent binding of proteins to plastic surfaces. Thus NP-40 was the detergent of choice from those tested and was then used not only in gradients but in other preparations of culture supernatants to prevent loss of the secreted immunoglobulin.

Analysis of supernatants from presumptive variant cultures by sucrose gradient velocity sedimentation

The ability to detect heavy chains and light chains in sucrose gradients can be used to analyse the nature of the immunoglobulin secreted by those cell lines in which only a heavy chain or light chain can be identified serologically in the culture supernatant. The immunoglobulin polypeptides secreted by these cell lines could be part of a larger molecule whose other heavy or light chains have not been identified either because they are δ or ε chains which cannot be assayed using the haemagglutination inhibition technique in its current form or because they are altered chains which cannot

Fig. 15 The comparison of sucrose gradient velocity sedimentation in the presence and absence of a detergent

Two 300µl samples of biosynthetically radiolabelled culture supernatant from the same culture of SMI_1 were analysed by velocity sedimentation under the conditions described in Fig. 10 on identical gradients except that one contained 0.5% NP-40.

- ● Radioactivity in the absence of NP-40
- ○ Radioactivity in the presence of NP-40

Fig 15

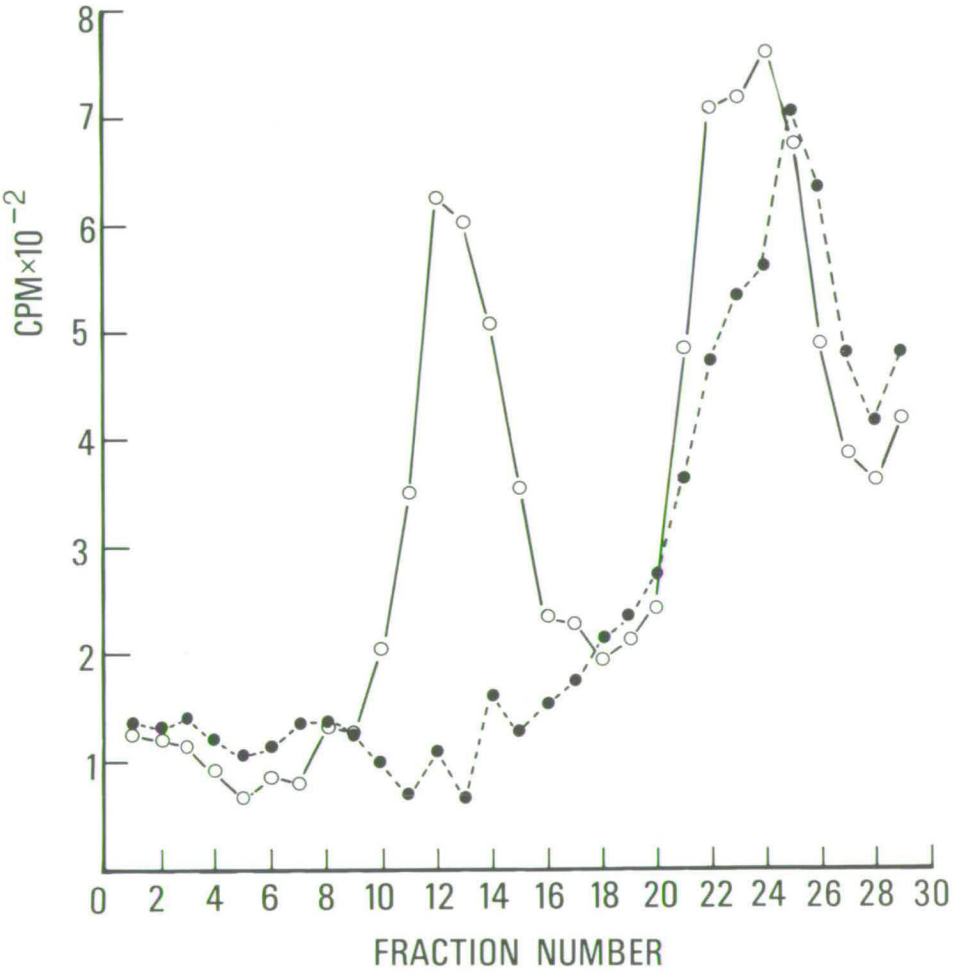

CPM×10⁻²
8
7
6
5
4
3
2
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
FRACTION NUMBER

Fig. 16 The effect of different detergents on the sucrose gradient velocity sedimentation of radiolabelled culture supernatants

Four 300µl samples of culture supernatant from the same biosynthetically radiolabelled culture of SMI_1 were analysed by velocity sedimentation under the conditions described in Fig. 10 in identical gradients except that one contained no detergent (a) and the remaining three contained different detergents (b) 0.1% sarkosyl (c) 0.1% SDS (d) 0.5% NP-40

Table 12 The adherence of cell culture secreted proteins to polypropylene centrifuge tubes in the presence of different detergents

The centrifuge tubes from the experiment described in Fig. 16 were washed out three times with 5% TCA to remove any remaining free label and a further three times with ethanol, dried, immersed in scintilation fluid in a counting vial and counted.

Fig 16

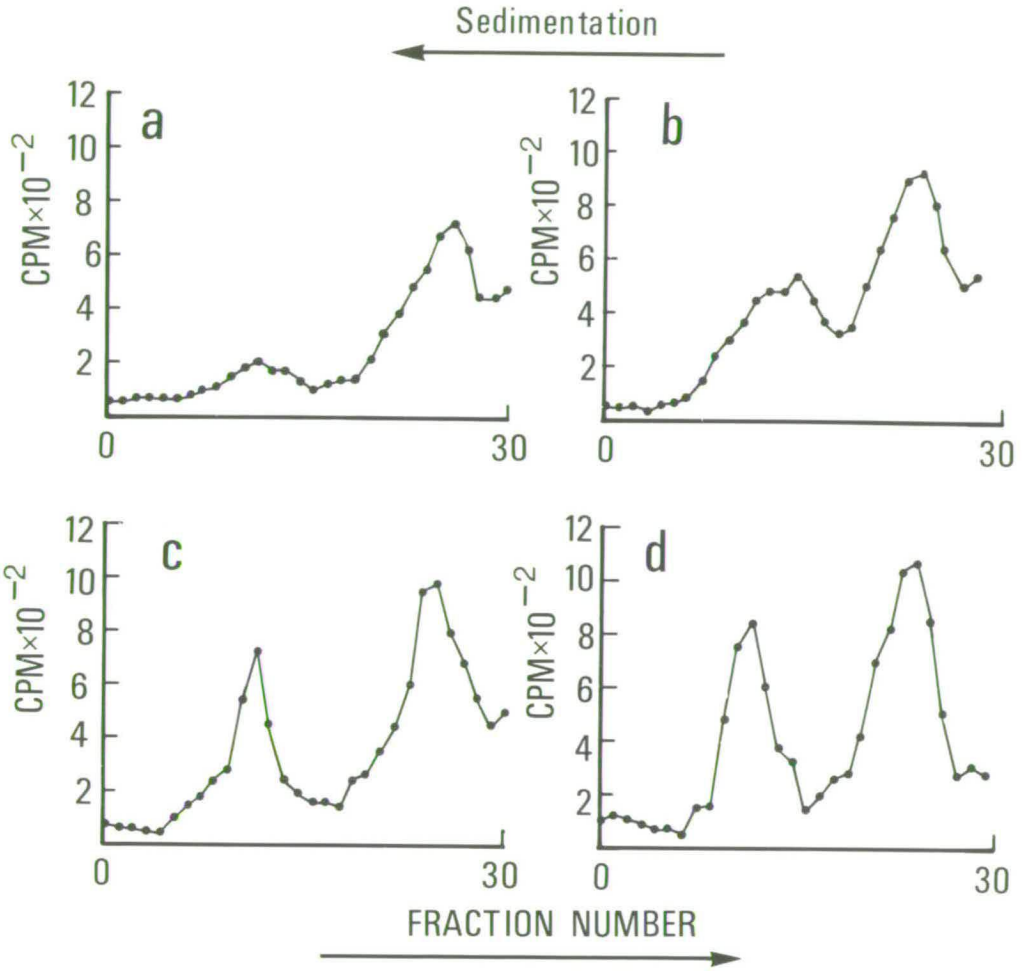


TABLE 12

Detergent content of gradient	TCA precipitable radioactivity adhering to the centrifuge tube (cpm)
0	13,211
0.1% Sarkosyl	1,504
0.1% SDS	397
0.5% NP-40	663

be recognised serologically. In these cases the detectable polypeptide would show a greater sedimentation velocity than that expected for single heavy or light chains.

In all the supernatants from cell lines apparently secreting light chains only which were examined in this way, their respective light chain types were all identified as poorly defined components present in the sample fractions and in the first one or two fractions at the top end of the gradient. A typical profile of this type (from the cell line EB_2) is shown in Fig. 17. This indicates that these light chains are not part of larger molecules and that the largest complex they represent would be a light chain dimer which has sedimented as far as the top four fractions of the gradient. The λ light chains detected in the culture supernatant of U266BL were present in a far greater number of fractions extending further into the gradient (Fig. 17). This is consistent with the light chains being present in a free of dimer form and in addition, also as part of a larger molecule which in turn agrees with the interpretation of the analysis of U266BL supernatant on SDS gradient gels. U266BL has been characterised as a secretor of ε chains and λ chains (Nilsson et al., 1970). ε chains cannot, at this stage, be identified by the haemagglutination inhibition test.

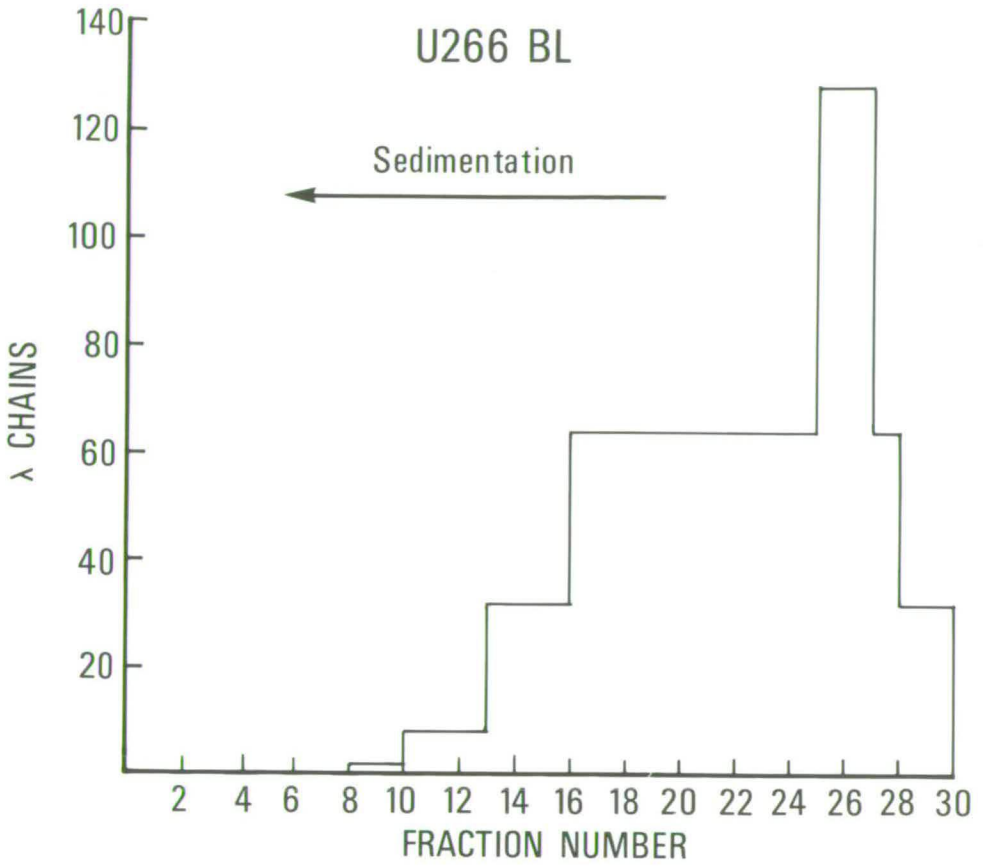
The cell line F137 has been identified in this project as secreting μ chains only but in the analysis of a sample of F137 supernatant by sucrose gradient velocity sedimentation no μ chains could be detected in any of the gradient fractions. This may be because the μ chains secreted by F137 are normally present at a low level in any case and samples are always subject to some dilution in the gradient. Normal and radiolabelled samples of F137 culture supernatant concentrated by ammonium sulphate precipitation also gave negative results when tested both for detectable μ chains in the haemagglutination inhibition test and for a radiolabelled 19S component by sucrose gradient sedimentation. These findings contrast with those for other μ secreting cell lines whose 19S components could be enriched by this procedure (Fig. 18). This indicates that the μ chains of F137 are not secreted as part of a large IgM-like molecule.

Sucrose gradient velocity, sedimentation analysis of culture supernatant has demonstrated that IgM is secreted by human B lymphoid

Fig. 17 The analysis of culture supernatants from presumptive variants of immunoglobulin secretion by velocity sedimentation

0.4ml samples of culture supernatants from the lines to be analysed were fractioned by velocity sedimentation as described in Fig. 10. Each fraction was analysed for the heavy or light chain normally detected in the culture supernatant. The resulting gradient profiles of U266BL and EB_2 are shown (the presence of λ chains is expressed as the relative decrease in agglutination end point).

Fig 17


U266 BL
Sedimentation
λ CHAINS
140
120
100
80
60
40
20
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
FRACTION NUMBER

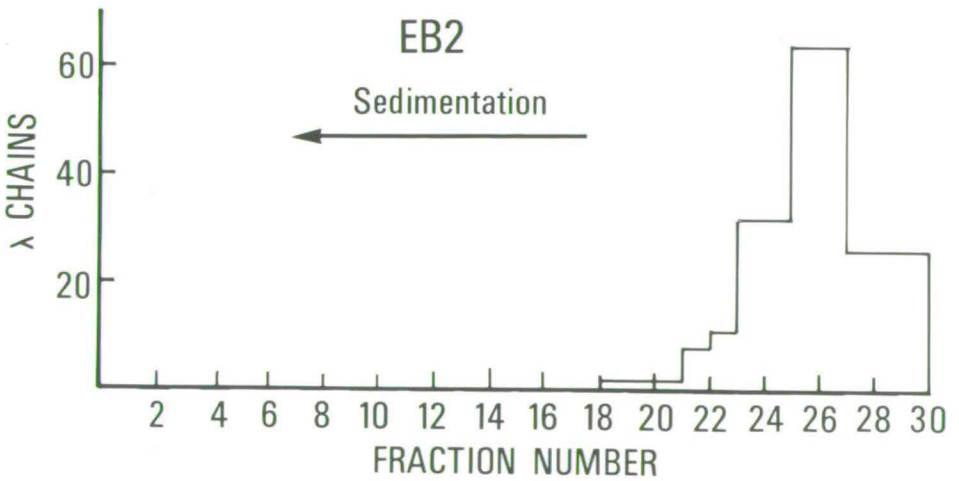
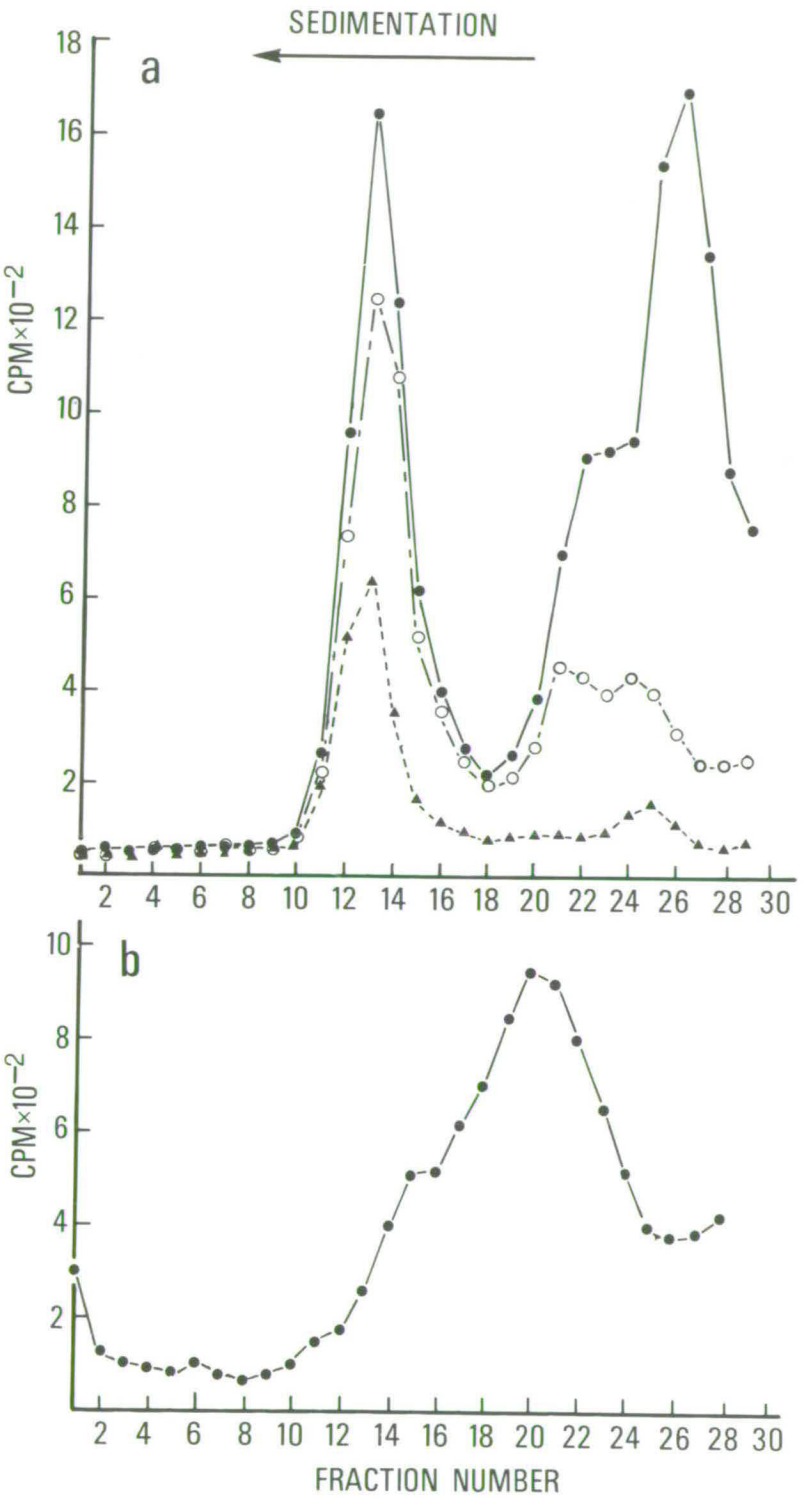

EB2
Sedimentation
λ CHAINS
60
40
20
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
FRACTION NUMBER

Fig 18



cell lines as complete 19S molecules which in some lines represents a large portion of the total secreted protein; this is revealed particularly when steps are taken to avoid losses of immunoglobulin on handling. This analysis has also shown that in addition to complete IgM molecules free excess light chains are also secreted to a varying extent in different cell lines. The investigation of presumptive variant immunoglobulin-synthesising cell lines using this technique has shown that with the exception of U266BL the single heavy chains or light chains identified in the supernatants of these cell lines are secreted as such and not as part of a larger molecule whose other constituant polypeptides are not recognised serologically. As this is the case, even although the resolution of their sedimentation velocities could be improved by running different gradients and under different conditions, it is unlikely that free heavy or light chain polypeptides could be isolated in this way as the bulk of the other secreted proteins sediment around the same 4S size. Thus, although sucrose gradient velocity sedimentation can provide information on the form in which the immunoglobulin chains of presumptive variant lines are secreted, this technique cannot be readily used in investigating the nature of the polypeptides themselves.

THE ISOLATION OF IMMUNOGLOBULINS FROM CELL LINE SUPERNATANTS BY IMMUNOPRECIPITATION

As the secreted immunoglobulins of human B lymphoid cell lines can be readily identified serologically in the haemagglutination inhibition test it was considered that it might be possible to purify them on a serological basis. Isolation of the secreted immunoglobulins of human B lymphoid cells in this way would be particularly useful in analysing the products of the presumptive variant lines which would be difficult to purify by physical means. The most popular technique in this field is immune- or immuno-precipitation. However, this technique has several limitations, the principal one being specificity, which depends largely on the antisera used. In many of the published results

using immunoprecipitation for the successful isolation of immunoglobulins from cell lines, antisera prepared and purified by the authors themselves have been used (see for example Singer and Williamson 1979). The time available does not permit the raising of antisera in this project and so commercial preparations must be employed. For this reason - and because there are several methods of immunoprecipitation - it was decided initially to evaluate this technique in the isolation of immunoglobulins from human B lymphoid cell line supernatants. It has been demonstrated that IgM can readily be isolated physically from cell culture supernatant, so this provides a useful standard against which to compare immunoprecipitated immunoglobulins from the same source.

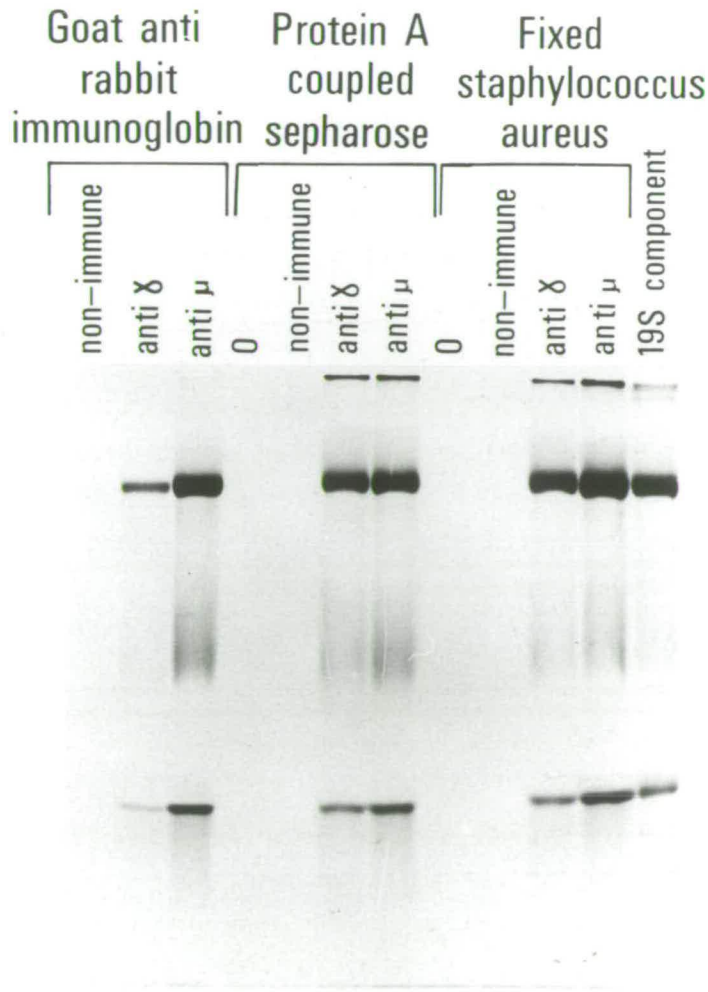
Fig. 19 compares, by SDS PAGE, immunoprecipitates formed by three different methods, all using the same primary antisera with IgM isolated by velocity sedimentation from the same radiolabelled sample of culture supernatant. All three methods show isolation of the relevant heavy chains and light chains in comparable purity to those from 19S component. However the classical indirect immunoprecipitation using a second anti-immunoglobulin antiserum uses more primary antiserum and takes longer to complete than the other two techniques where the second antiserum is replaced by two different protein A immunoabsorbants. In the two protein A-based techniques the staphylococcal immunoabsorbant forms a far smaller pellet on removal from the sample of culture supernatant by centrifugation than the much larger protein A coupled sepharose beads. This allows for a more efficient elution of precipitated material in a smaller volume of sample buffer making the fixed Staphylococcus aureus organisms a more convenient immunoabsorbant.

A striking feature of the analysis shown in Fig. 19 is that in each immunoprecipitation method, although the control non-immune sera precipitated nothing, anti γ and anti μ antisera both precipitated μ chains and light chains from the supernatant of a $\mu\ \kappa$ secreting cell line. This brings into question the specificity of the antisera. The more pronounced light chains in the immunoprecipitated samples in comparison to the 19S component, although both were isolated from the same volume of culture supernatant, suggests that the lack of specificity in this case was due to anti light chain activity in the antisera supplied as anti γ chain and anti μ chain specific.

Fig. 19 Evaluation of immunoprecipitation : A comparison of different methods

0.3ml aliquots from the same sample of radiolabelled SMI_1 culture supernatant were immunoprecipitated using non-immune rabbit serum, rabbit anti human γ chain (Miles) and rabbit anti human μ chain (Miles). These primary sera were precipitated using a second goat anti-rabbit immunoglobulin anti-serum (Miles) or either of two immunoadsorbants which were fixed staphylococcus aureus organisms or protein A coupled sepharose beads. Where a second precipitating anti-serum was used 10µl of the primary antiserum was incubated with the sample of supernatant for 1hr at $4^{o}C$ followed by 20µl of the precipitating antiserum and incubation overnight at $4^{o}C$. The precipitate was then spun down at $12,800g_{av}$ for 15 mins washed three times in PBS 0.5% NP-40 and dissolved in SDS PAGE sample buffer. The immunoprecipitation protocol described in the Methods section was used when an immunoadsorbant was employed in place of a second antiserum with the substitution of 15mg of protein A-Sepharose per sample for the staphylococcal immunoadsorbant. Where the immuno-adsorbants were used additional control samples containing only the immunoadsorbant were set up and processed as immunoprecipitates to detect any direct binding of secreted proteins to protein A. The immuno-precipitates and controls together with a 19S component, isolated as previously described, from an identical sample of supernatant were analysed on a 12% SDS PAGE gel, a flurograph of which is shown opposite. The primary serum or antiserum used is indicated directly above the relevant track and above that the second antiserum or immunoadsorbant.

Fig 19


Goat anti rabbit immunoglobin
Protein A coupled sepharose
Fixed staphylococcus aureus
non–immune
anti δ
anti μ
0
non–immune
anti δ
anti μ
0
non–immune
anti δ
anti μ
19S component

Following this observation, several different commercial antisera were evaluated for their specificity in this way. The SDS PAGE analysis of immunoprecipitates, from a radiolabelled μ κ secreting cell line supernatant, using different anti heavy chain and anti light chain antisera (Fig. 20) demonstrates that there is considerable variation in specificity among those antisera tested. The Behringwerke anti λ track is empty and the anti κ track shows the expected polypeptides whereas the anti γ antiserum from the same supplier appears to precipitate a small but detectable amount of the same protein recognised by its anti μ equivalent. The Technicon anti γ and anti μ antisera and the anti IgG Fc and anti IgM Fc each show results in keeping with their claimed specificities. However the anti γ and anti μ from the Scottish Antibody Production Unit both precipitate polypeptides with the characteristics of IgM constituents.

Protein A has a high affinity for rabbit antibodies and antisera raised in rabbits are generally used in protein A dependent techniques. However, the analysis shown in Fig. 20 uses antisera which are also derived from different species and demonstrates that, despite their reported lower affinity for protein A, antibodies from sheep and goats (sources of many commercial antisera) can also be successfully used in immunoprecipitation with Staphylococcus aureus protein A immunoadsorbent.

The analysis of secreted chains from the presumptive variant immunoglobulin synthesising cell lines by immunoprecipitation

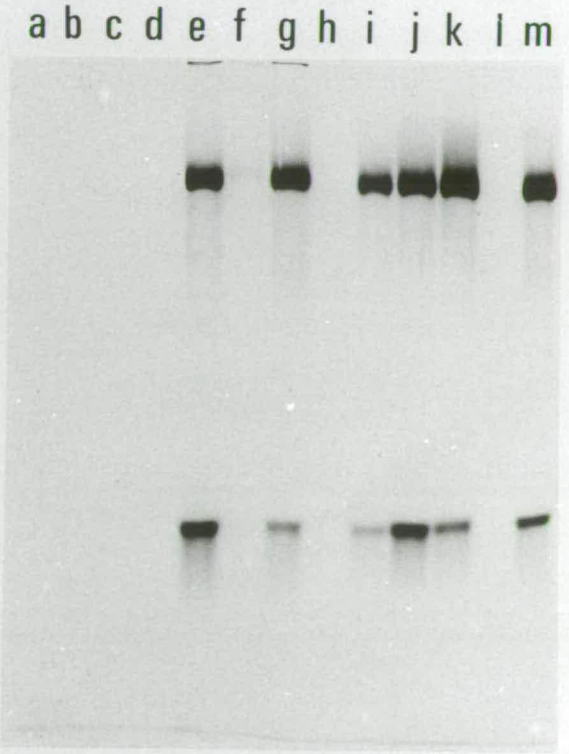
The SDS PAGE analysis of immunoprecipitates, formed with the relevant antisera, from the radiolabelled supernatants of EB_2, EB_3, JIM_1, and U266BL showed single components which all had the molecular weight (22,000) characteristic of light chains. This indicates that these light chains are secreted as such and, with the exception of U266BL, is consistant with the earlier analysis of the same supernatants by velocity sedimentation. The velocity sedimentation analysis of U266BL suggested that a portion of the secreted λ chains may be part of a larger molecule and this is in keeping with its description as an IgE secretor (Nilsson et al., 1970). The remaining λ chains by being present in excess and/or by having a greater affinity for the anti λ antibodies may account for the absence of an ε heavy chain in the immunoprecipitate.

Fig. 20 Evaluation of immunoprecipitation : A comparison of different antisera

0.3ml samples of SMI_1 radiolabelled culture supernatant were immunoprecipitated using the staphylococcal immunoadsorbant technique described in the Methods Section with a range of different antisera. The resulting immunoprecipitates were analysed on a 12% SDS PAGE gel a flurograph of which is shown opposite. The tracks relating to the use of the different antisera are as follows:

a	No serum
b	Rabbit non-immune serum
c	Rabbit anti BSA
d	Rabbit anti human λ (Behringwerke)
e	Rabbit anti human κ (Behringwerke)
f	Rabbit anti human γ (Behringwerke)
g	Rabbit anti human μ (Behringwerke)
h	Goat anti human γ (Technicon)
i	Goat anti human μ (Technicon)
j	Sheep anti human γ (SAPU)
k	Sheep anti human μ (SAPU)
l	Goat anti human IgG Fc (North east)
m	Goat anti human IgM Fc (North east)

Fig 20



The characteristic light chain molecular weight of the immunoprecipitated components from these cell lines indicates that the secreted light chain polypeptides have a normal structure. However, immunoprecipitated κ light chains from Jijoye radiolabelled supernatant have an apparently larger molecular weight of about 30,000 (Fig. 21) suggesting that they are structurally abnormal. Components of the same size are present in the anti κ immunoprecipitate from KAT_1 and the anti λ from RPMI 8226 which are also shown in Fig. 21. However these components are co-precipitated with a polypeptide of normal light chain molecular weight. It is unlikely that the larger component is derived from the apparently normal light chain polypeptide which has been so altered in its aminoacid structure as to show a large change in charge or number of residues as this would require the synthesis of two different light chains from the same cell or that the cultures concerned are biclonal with respect to their light chain synthesis. A more probable explanation would be that the light chains synthesised by KAT_1 and RPMI 8226 are homogeneous in each line with respect to their aminoacid sequence but they are differentially glycosylated. Efficient glycosylation of the secreted light chains of Jijoye could also explain their similar slow mobility. There are other explanations for variation in light chain size but these will be considered later. Alternatively of course, the co-precipitated larger component from KAT_1 and RPMI 8226 need not be a light chain at all but, for example, an N-terminal heavy chain fragment.

The immunoprecipitation with anti human μ chain antisera of a relatively large volume of F137 radiolabelled supernatant failed to demonstrate any μ chain polypeptides in analysis of the immunoprecipitate by SDS PAGE (Fig. 22). At the same time a 0.2ml radiolabelled sample of G-S (a relatively low producer of IgM, See Fig. 13) diluted to the same volume and treated in the same way could be shown to contain polypeptides characteristic of μ chains and light chains. This situation is in keeping with previous experiments where the constant but weak detection of μ chains in the supernatant of F137 cultures by haemagglutination inhibition has failed to yield to further analysis. This suggests that the initial detection of μ chains in the supernatant of F137 is an artefact, perhaps caused by the secretion of some other protein or the interaction of a secreted protein and components from the culture medium.

Fig. 21 Analysis of immunoprecipitates from radiolabelled supernatants of Jijoye, KAT$_1$ and RPMI 8226

The immunoprecipitates from 0.3ml samples of supernatants from radiolabelled cultures of the above cell lines together with untreated 30µl aliquotes from the same supernatant samples (UT) were analysed on a 12% SDS PAGE gel, a flurograph of which is shown opposite. The anti sera used are indicated directly above the relevant track and the origin of the supernatant samples above that. Molecular weight mobilities are shown on the right ($x10^{-3}$) and the markers were; phosphorylase b (94,000), ovalbumin (43,000) and RNAase A (13,000).

Fig 21

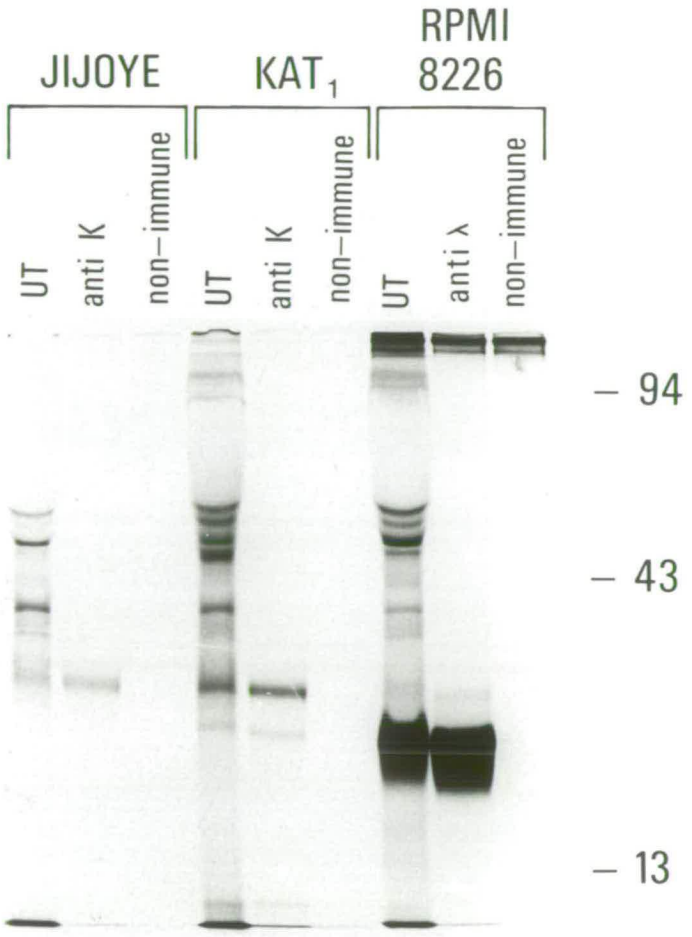
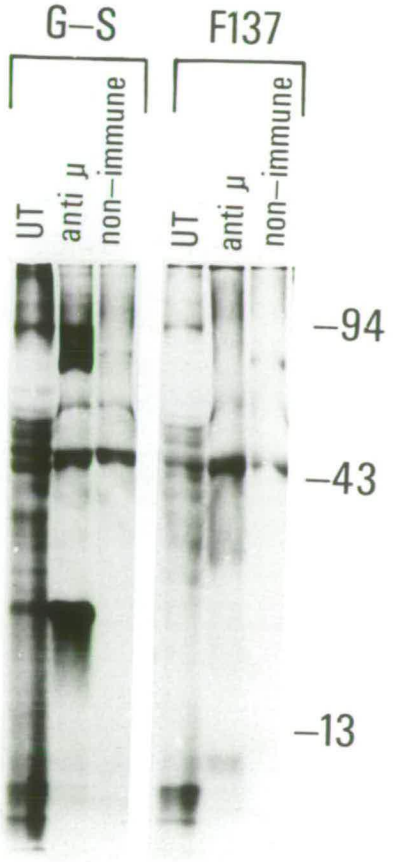

JIJOYE
KAT1
RPMI 8226
UT
anti K
non–immune
UT
anti K
non–immune
UT
anti λ
non–immune
– 94
– 43
– 13

Fig. 22 Analysis of immunoprecipitates from G-S and F137 radiolabelled culture supernatants

Immunoprecipitates from 2.5ml samples of radiolabelled supernatant from F137 and a 0.2ml radiolabelled supernatant sample from G-S diluted to the same volume in used medium were analysed together with 30µl samples of untreated radiolabelled culture supernatant (UT) from the same cell lines on a 12% SDS PAGE gel a flurograph of which is shown opposite. The antisera used are shown above each track and above that the origin of the supernatant samples. Molecular weight mobilities ($x10^{-3}$) are shown on the right, the markers were; phosphorylase b (94,000), ovalbumin (43,000) and RNAase A (13,000).

Fig 22



Alternatively the μ chains may in fact be present but derived from some source other than normal secretion such as the shedding of cell surface immunoglobulin or the spillage of intracellular immunoglobulin from dead or dying cells. In this case the proteins concerned may be unstable and so they are only readily detected in samples taken from continuous cultures where their extracellular concentration will be maintained.

THE SEROLOGICAL ANALYSIS OF INTRACELLULAR AND CELL SURFACE IMMUNOGLOBULIN IN HUMAN B LYMPHOID CELL LINES

Immunoglobulins are not only secreted proteins but can also be expressed on the surface of cells and their synthesis for either secretion or surface expression requires the intracellular presence of immunoglobulins. Lack of secretion of immunoglobulins, therefore, does not imply lack of their synthesis. This is particularly relevant to the study of those cell lines whose immunoglobulin synthesis appears deficient or abnormal on the basis of the heavy chains or light chains identified among their secreted proteins. Therefore experiments were conducted to determine whether intracellular or surface immunoglobulins were present in and on human B lymphoid cell lines. These experiments were directed particularly at those cell lines which secrete no detectable immunoglobulin or only a single type of immunoglobulin polypeptide but also included a selection of normal immunoglobulin secreting lines as controls.

The presence of intracellular immunoglobulin was determined simply by assaying a lysate of washed cells in the haemagglutination inhibition test (the details are described in the Methods section). The lack of any effect of NP-40 on the serological detection of immunoglobulins, discovered in the sucrose gradient velocity sedimentation experiments, and the ability of NP-40 to lyse cells but not their nuclei prompted this assay. However, the use of a detergent meant that cell surface proteins would also be present in the lysate. As the cell surface immunoglobulins were also being assayed, the true cellular distribution of the

immunoglobulins could be determined by a comparison of the two results. Despite contamination by cell surface immunoglobulin this adaptation of the haemagglutination inhibition assay represents a more convenient and rapid method of determining the presence of cellular immunoglobulin than conventional immunofluorescence microscopy.

Cell surface immunoglobulin was identified using an I^{125} protein A binding assay which was adapted from the method of Cohen (personal communication) as a measure of the binding of various antihuman heavy and light chain antisera to the cell surface and the details of which are described in the Methods section. Behringwerke class specific rabbit antihuman heavy chain antisera were used in this assay despite the previously discussed suspicion of their specificity in immunoprecipitation. This was because the goat antihuman heavy chain antisera which showed the greater specificity in immunoprecipitation appeared not to bind significant levels of protein A in the cell surface assay. This contrasts with the binding of these antisera by protein A bearing fixed Staphylococcus aureus organisms and indicates that the immunoglobulin binding properties of protein A vary depending upon its presence in the staphylococcal membrane or in free solution. However the specific positive binding of antisera in the cell surface immunoglobulin assay was clearly discernable by showing a five fold or greater increase in I^{125} protein A bound over control values and a four fold increase over negative experimental values. (Table 13). The assay results shown in Table 13 are summarised, together with those results from analysing cell lysates in the haemagglutination-inhibition test for the presence of immunoglobulin, in Table 14. In addition the previously established immunoglobulin secretion patterns are also included in Table 14 for comparison.

The T lymphoid line CCRF, as one might expect, does not synthesise immunoglobulin therefore secretes no immunoglobulin and has no cell surface immunoglobulin. The normal immunoglobulin-synthesising B lymphoid lines ODY_1, RPMI 8866 and SMI_1 each synthesise a single heavy chain class and light chain type, secrete the same heavy chains and light chains and have them present on the cell surface although whether they are incorporated into the structure of the membrane or merely "in passage" as part of the secretion process cannot be determined. However, the intracellular, surface and secreted heavy chains and light chains of the other cell lines analysed demonstrated several combinations of synthesis and expression

Table 13 The identification of cell surface immunoglobulins in human B lymphoid cell lines

Values for the binding of I^{125} protein A (cpm) to similar samples of cells from different cell lines in the presence of different rabbit antihuman immunoglobulin antisera (Behringwerke) are shown. The different cell lines are given at the left hand side of each row and the various human immunoglobulin chains against which the antisera were directed are indicated at the top of each column together with columns showing the results obtained when non-immune rabbit serum was used and when no serum or antiserum was present (0). These values represent the pooled results from three experiments; 2 covering together the heavy chains and a third the light chains. In each of the heavy chain experiments non-immune and 0 controls were present but in the light chain assays only non-immune controls were used having established that none of the cell lines spontaneously bound large amounts of I^{125} protein A. The non-immune values, therefore, a mean from the heavy chain and light chain assays.

Those results accepted as positive for the purposes of discussion are underlined.

TABLE 13

	α	γ	μ	κ	λ	Non-immune	0
Daudi	775	218	4181	5276	632	274	267
EB_2	238	157	2269	470	2201	232	202
EB_3	195	197	462	453	8085	314	218
FAL_1	264	291	279	440	512	288	213
F89	173	196	275	385	412	248	156
F137	715	388	2568	428	882	229	212
Jijoye	185	199	277	3200	522	236	153
JIM_1	286	189	1851	559	2604	228	191
KAT_1	352	183	1735	1881	470	194	188
Raji	404	215	1314	353	659	252	215
RPMI 8226	284	188	272	637	1665	228	170
ODY_1	2329	230	915	464	1412	331	528
8866	223	1504	347	2636	604	257	167
SMI_1	417	219	1553	1829	664	293	204
CCRF	250	239	166	368	545	171	162

Table 14 Intracellular, surface and secreted immunoglobulins of Human B lymphoid cell lines

The results of assaying cell lysates from different lines in the haemagglutination inhibition test for the presence of α γ and μ heavy chains and κ and λ light chains are shown. In addition a summary of the radio binding assay results for cell surface immunoglobulin is given together with the previously determined pattern of secreted immunoglobulins for comparison.

TABLE 14

	Lysate	Surface	Secreted
FAL_1	μ	-	-
F89	μ	-	-
F137	μ	μ	(μ)
Raji	μ	μ	-
Daudi	μκ	μκ	-
EB_2	μλ	μλ	λ
JIM_1	μλ	μλ	λ
KAT_1	μκ	μκ	κ
EB_3	λ	λ	λ
Jijoye	κ	κ	κ
RPMI 8226	λ	λ	λ
ODY_1	αλ	αλ	αλ
SMI_1	μκ	μκ	μκ
RPMI 8866	γκ	γκ	γκ
CCRF	-	-	-

of immunoglobulins: FAL_1 and F89 synthesise only a heavy chain which is not secreted nor expressed on the cell surface; Raji and F137 synthesise a heavy chain which is expressed on the cell surface but not secreted; Daudi synthesises a heavy chain and light chain which are both expressed on the cell surface but not excreted; EB_2, JIM_1 and KAT_1 synthesise a heavy chain and a light chain both of which are expressed on the surface but only the light chain is secreted; EB_3, Jijoye and RPMI 8226 synthesise only a light chain which can be detected on the cell surface and is secreted. In these "variant" combinations of immunoglobulin synthesis and expression, μ chains are the only heavy chains present and where they are synthesised in the absence of light chains they are not secreted. In contrast to this pattern of heavy chain secretion in all cases where light chains are synthesised they are secreted whether heavy chains are present or not. This might suggest that although there is no requirement of heavy chains for light chain secretion there is a dependence on light chains for heavy chain secretion. However, EB_2, JIM_1 and KAT_1 synthesise heavy chains and light chains but secrete only the light chains showing that heavy chain secretion is not dependent only upon light chain synthesis.

Other possible mechanisms underlying these observations and their relevance to immunoglobulin synthesis and expression in B lymphocytes will be discussed later.

DISCUSSION

The pattern of immunoglobulin synthesis in established human B lymphoblastoid cell lines

The overall pattern of immunoglobulin expression seen in this survey of human B lymphoid cell lines is "monoclonal" that is one heavy chain class and a single light chain type. This is the established format in these cell lines (Nilsson 1971, Evans et al., 1974) as it is in B lymphoid lines from other species and in normal lymphocytes. Occasionally reports have been made of human lymphoblastoid cell lines which have the capacity to produce two different heavy chains. Takashi et al., (1969) claimed to have demonstrated α chain and γ chain production in the same cells of a lymphoblastoid cell line by immunofluorescence and Bloom et al., (1971) reported the secretion of both γ and μ chains in two lymphoblastoid cell lines detected by immunodiffusion of concentrated culture supernatant. Cloned cultures of human lymphoid cell lines have been reported as expressing multiple heavy chain classes (Hinuma and Grace 1967) detected by cell surface immunofluorescence. The same technique has also been used to demonstrate multiple expression of γ chain subclasses in IgG synthesising human lymphoblastoid cell lines, (Simmons et al., 1979). As previously mentioned, Kishimoto et al., (1978) claimed to induce concurrent IgG synthesis in IgM-synthesising human lymphoblastoid cell lines by co-culturing with T lymphocytes using a radioimmune assay to identify the two classes of immunoglobulin. The results of Kishimoto et al., (1978) could not be repeated in this project using the haemagglutination inhibition assay and there appears to have been no confirmation of any of the reports of dual or multiple heavy chain expression cited above nor structural analysis of the heavy chains involved. Structural analysis in the form of SDS PAGE of radiolabelled immunoprecipitates was used to differentiate between the concurrently expressed secreted IgG and cell surface IgM of a human lymphoblastoid cell line (Premkumar et al., 1975). However the synthesis of the two different heavy chains was not demonstrated in clones of the line in which they were initially detected.

Methodological errors stemming from the specificity of serological reagents, the polyclonal nature of cell lines and their possible contamination by other cultures or the accuracy of cloning techniques could explain the various observations of multiple heavy chain expression. However, the possibility exists that there are genuine cases of cells which synthesise more than a single heavy chain class. In dual heavy chain expression if both heavy chains were being transcribed from the same allelic set of heavy chain genes, that is from the same chromosome this would confound the current models of heavy chain switching by gene deletion (see Introduction, Genetics section) because cells clearly cannot go back to express a gene which has been deleted. Alternatively if two heavy chain genes were being synthesised by the same cell it could be as a result of the expression of both allelic sets of heavy chain genes. This, however, would represent a breakdown of allelic exclusion which is currently thought, from work on mouse myeloma cells, to be maintained by there being only one functionally rearranged immunoglobulin heavy chain gene per cell (see Introduction, Genetics section). The most probable combination of heavy chains simultaneously expressed by human lymphoid cell lines would be μ and δ as the majority of IgD bearing human lymphocytes are also positive for IgM (Rowe et al., 1973) and the presence of IgD is considered to be a marker for B lymphocytes in an intermediate phase of differentiation (Vitetta and Uhr 1977). A report has been made showing the detection of multiple immunoglobulin classes including IgD on the cell surface of human lymphoid cell lines by immunofluorescence (Van Boxel and Buell 1974). However, no other reports of IgD expression by human lymphoid cell lines have been made. Neither surface nor secretory IgD were assayed for in this project. In any case the transient simultaneous expression in <u>vivo</u> of IgM and IgD could be explained within the deletion model of heavy chain switching simply by the stability of immunoglobulin mRNA. In this case dual production could not persist through multiple cell divisions and therefore could not be present in continuously cultured cell lines. Alternatively if IgM and IgD synthesis could be maintained through multiple cell divisions, because of the specialised developmental nature of IgD expression the underlying mechanisms need not necessarily be related to later switches to the major classes of secreted immunoglobulin.

Apart from a single instance in the results of Van Boxel and Buell (1974) there have been no reported cases of dual light chain production in human lymphoid cell lines and none have been observed in this project. However, the production of λ chains by BR18 and κ chains by BR8T the tetraploid derivative of BR18 represents a situation similar to the synthesis of both light chain types by a single cell line. The normal expression of only one light chain type in B lymphocytes (genic exclusion) and allelic exclusion in light chains are thought to be controlled by mechanisms limiting the number of rearranged functional light chain genes to one per cell (see Introduction, Genetics section). However, production of both light chain types by a single cell line, which has been reported at the mRNA level in mouse myeloma cells (Storb et al., 1977), would suggest that other mechanisms in addition to those involving gene rearrangement control light chain expression. Furthermore, an order of gene rearrangement has been proposed in light chain genes such that λ genes are only rearranged following unsuccessful initial rearrangement in the κ alleles. The derivation of a κ synthesising human lymphoid line from a λ secreting parent, a situation which BR18 and BR18T would appear to represent, indicates that such a hierarchy may not be present in human light chain expression. Any inferences made from these two human lymphoid lines depend, of course, upon how similar BR18 and BR18T actually are.

The monoclonal pattern of immunoglobulin production observed in human lymphoid cell lines reflects that of normal blood lymphocytes; however the overall predominance of IgM synthesis does not. Steel et al., (1977) have argued that only IgM synthesising cell lines are derived from EB virus transformation of cord blood lymphocytes, an observation confirmed in this study (see Results section, Table 3) because there is selective transformation of those cells which are actively secreting immunoglobulin. This is in keeping with what is known about immunoglobulin synthesis in the human neonate where, although the proportions of IgM and IgG-bearing lymphocytes are similar to those of adults (Froland and Natvig 1972), IgM is the only class of circulating immunoglobulin synthesised by the infant's cells, as distinct from that transported across the placenta from the maternal plasma (Lawton et al., 1972). In contrast to Steel et al., (1977),

Rosen et al., (1977) view the EB virus as a polyclonal B cell stimulator which can infect and activate the majority of resting B lymphocytes to immunoglobulin secretion. However, using EB virus as a B lymphocyte activator in cord blood Bird and Britton (1979) observe exclusive IgM synthesis by the activated B lymphocytes and explain the lack of activation of IgG or IgA surface bearing lymphocytes in cord blood as representing a functional immaturity in this lymphocyte subset. Active immunoglobulin secretion is, of course, a definative marker of functional maturity in B lymphocytes.

Predominant, although not exclusive, secretion of IgM by "EB virus activated" B lymphocytes from healthy adult peripheral blood lymphocytes and peripheral blood lymphocytes from individuals with infectious mononucleosis (an in vivo EB virus infection) has also been demonstrated (Bird and Britton 1979). These observations are both regarded as normal responses by the authors and indeed the in vitro stimulation of human blood lymphocytes with polyclonal B lymphocyte activating substances usually results in the development of a majority of IgM secreting lymphocytes (Ringden et al., 1979). In comparison to B lymphocyte activation, IgM synthesis is relatively over-represented among the cell lines derived from healthy adults and under-represented among those lines from infectious mononucleosis patients (see Results, Table 3) which agrees with (and extends) the findings of Steel et al., (1977). This suggests that the populations of lymphocytes from which the transformed lines are derived are not the same in these two groups. Furthermore, as infectious mononucleosis is characterised by raised serum immunoglobulin of all classes the proportion of circulating immunoglobulin-secreting B lymphocytes is likely to differ from the norm. Thus the difference between the immunoglobulin secretion pattern of lines derived from infectious mononucleosis patients and those derived from healthy adults, (a phenomenon which is not observed among 'resting' B lymphocytes which have been activated in vitro), can be attributed to differences in the cells showing active immunoglobulin secretion in vivo.

The pattern of immunoglobulin secretion in human lymphoid cell lines derived from individuals with disorders of the lymphoreticular system closely resembles that of cell lines derived from the blood lymphocytes of healthy adults (see Results, Table 3). This suggests

that in the majority of cases the cell lines are derived not from the malignant cells characteristic of the disorder but from EB virus transformation of lymphocytes from the normal 'background' population. Exceptions, of course, are those lines derived from Burkitt's Lymphoma patients where the proliferating cells are also those carrying the EB virus genome. However, other exceptions are few as can be judged by the comparative rarity of EB virus nuclear antigen-negative B lymphoid cell lines.

The argument that EB virus acts upon those lymphocytes already secreting immunoglobulin (mature plasma cells are evidently insusceptible, Nilsson et al., 1971) defines that subpopulation of circulating lymphocytes as those which can be transformed. If there are no other restrictions on the transformable population this implies that the distribution of immunoglobulin classes secreted by human lymphoblastoid cell lines established from healthy peripheral blood lymphocytes reflects the relative frequences of the different immunoglobulin-secreting lymphocytes in healthy peripheral blood. Most of the published data on immunoglobulin secretion by cultured blood lymphocytes have been derived using mitogen stimulation. Recently, however, Freijd and Kunovi (1980) have reported the frequencies of the different spontaneous immunoglobulin-secreting cells from healthy adult human blood. These show a far higher proportion of IgM-secreting lymphocytes than IgG secretors which agrees with the immunoglobulin secretion patterns of normal human lymphoblastoid cell lines. Freijd and Kunori (1980) also report that the number of IgA secreting lymphocytes exceeds both IgG and IgM secretors. IgA secreting cell lines, however, are not as frequently established as IgM and IgG lines.

In order to define clearly the nature of those human lymphocytes which are susceptible to EB virus transformation it would be necessary to isolate and characterise all the subpopulations of circulating B lymphocytes but most importantly,those actively secreting immunoglobulin.

Any selection for the growth of cells producing a particular class of immunoglobulin during the establishment or culturing of lymphoblastoid cell lines or any instability of immunoglobulin production in culture would greatly influence arguments concerning normal B lymphocytes and drawing on immunoglobulin secretion by human

lymphoblastoid cell lines. However, the observation in this laboratory that subcultures taken from an early stage of establishment of a cell line secreting two different immunoglobulin molecules give rise to approximately equal numbers of clones secreting each of the parental immunoglobulins argues against selection in culture. In addition, as discussed below, immunoglobulin secretion appears to be a particularly stable feature of human lymphoblastoid cell lines and human lymphoid cell lines in general.

The level of immunoglobulin production

The haemagglutination inhibition test used to detect immunoglobulins produced by human lymphoid cell lines is also a semi-quantitative assay. Human type I collagen (Sykes and Solomon 1978) and chicken lense specific crystalins (de Pomevai and Clayton 1978) have been identified and assayed quantitatively using haemagglutination inhibition techniques similar to (and in the latter case derived from) that used in this project for immunoglobulins. Therefore, having discussed the nature of the immunoglobulins normally produced by human B lymphoid cell lines the level of immunoglobulin production can also be commented upon.

The figure of $\simeq$10µg/24hrs/10^6 cells given in the Results section for the production of secreted immunoglobulin from human lymphoblastoid cells appears higher than the previously reported rate of 1 - 4µg/24hrs/10^6 cells (Nilsson and Ponten 1975). However, the level of immunoglobulin detected by the haemagglutination inhibition assay increases by doubling amounts so that given the limits of accuracy of this technique, the values are in reasonably good agreement. Immunoglobulin secretion at this level represents an output greater than the 1.5µg/10^6cells/7 days of IgG (Mannrotti et al., 1980) or 2.9µg/10^6cells/7 days of IgM (Koopman and Schrohenloher, 1980) secreted by mitogen stimulated human blood lymphocytes and not much less than the 10 - 20µg/10^6cells/24hrs reported for the two human myeloma lines RPMI 8226 (Matsuoka et al., 1968) and U266BL (Nilsson 1971a). These figures, however are lower than the output reported for mouse myeloma cells in culture (Laskov and Scharff 1974) which approaches 100µg of immunoglobulin/10^6cells/24hrs. The level of immunoglobulin production in the human plasma cells may be lower than their murine equivalents as,of the two human cell lines

assayed RPMI 8226 secretes only light chains and U266BL synthesises IgE which is not one of the major classes of secreted immunoglobulin. These human myeloma cell lines were not quantitatively assayed in this project as neither of them secretes one of the major classes of immunoglobulin although their output could have been measured knowing the concentration of total immunoglobulin in the reference serum and estimating the proportion of immunoglobulin molecules having κ or λ chains. However the γ κ secreting cell line ARH 77 which was established from a multiple myeloma patient and has been described as a plasma cell line (Burk et al., 1978) was assayed for its level of secreted immunoglobulin. ARH 77 secreted consistently less immunoglobulin than some of the more rapidly secreting human lymphoblastoid cell lines and since the establishment of this line it has been found to carry the EB virus nuclear antigen indicating that it is in fact a lymphoblastoid cell line (Minowada, personal communication; confirmed in this laboratory also).

The proportion of total secreted protein which immunoglobulin represents in human lymphoblastoid cultures has been reported as varying from 3% - 10% though a single extreme value of 50% has been reported (Hutteroth et al., 1973). The sucrose gradient analysis of radiolabelled culture supernatants from IgM secreting cell lines also showed contributions of approximately 10% by 19S IgM to the total secreted protein in most instances. However cell lines where the 19S IgM accounted for 50% more of the secreted proteins appeared quite common. In fact when the secreted excess light chains are also taken into consideration the total proportion of secreted protein given over to immunoglobulin of one kind or another must exceed 70% in some lines. This is borne out by SDS PAGE analysis of unfractionated radiolabelled cell line supernatants together with sucrose gradient fractions (see Results, Fig. 14) where the polypeptides in the unfractionated sample which co-migrate with the μ chains and light chains are by far the most abundant components in that track. This remarkable level of immunoglobulin production may be explained in some part if these lines contain a larger proportion than usual of more differentiated cells. It has been noted that cultures of human lymphoblastoid cell lines contain a number of cells which appear

morphologically more differentiated than the normal lymphoblast cell type (de Harven 1967, Ben-Bassat et al., 1977). It is reasonable to assume that a more differentiated B lymphoid cell would synthesise more immunoglobulin so that the presence of a significant number of these cells could markedly increase the amount of immunoglobulin secreted by a cell line culture.

Not all of these cell lines consistently produce such a high proportion of immunoglobulin over a long period of time. This inconsistency may be related to culture conditions which have been shown to affect immunoglobulin production (Nilsson 1971a). Certainly it was noticed during the course of this project that in some lines immunoglobulin synthesis and protein synthesis in general was disturbed during changes in the batches of FCS used in the laboratory. Nevertheless, the high levels of immunoglobulin production shown by human lymphoblastoid cell lines in this project indicate that these lines synthesise more immunoglobulin than perhaps is generally thought to be the case. Although they are considered to be "second class" cells in terms of immunoglobulin synthesis, human lymphoblastoid cells can produce a significant amount of immunoglobulin and in a relatively pure form so that in the absence of a range of suitable human plasma cell lines they may provide a useful alternative for the formation of human/human hybrids in monoclonal antibody production.

Although the levels of immunoglobulin detected in the lysates of human B lymphoid cell cultures were not accurately quantified, it can be reported that samples from normal immunoglobulin-secreting cell lines showed complete or near complete inhibition of agglutination across the twelve wells of the microtitre plate. This would normally indicate an average immunoglobulin concentration for the three major classes assayed of $\simeq 5\mu g/ml$ at least. As the lysate was prepared using 5×10^6 cells/ml of lysis buffer, 5×10^6 cells must contain on average at least $5\mu g$ of immunoglobulin. This in turn gives a figure of $10^4 - 10^5$ molecules per cell which would include both intracellular and surface immunoglobulin. McConahey et al., (1971) have measured surface immunoglobulin on human lymphoblastoid cells at 2×10^4 molecules per cell and Litwin et al., (1974) have reported a figure of $1 - 5 \times 10^5$ molecules/cell for surface immunoglobulin alone. By comparison with the estimate of total cellular immunoglobulin from the lysates these figures

suggest that the greater portion of cellular immunoglobulin is present on the cell surface which is the expected situation in an actively immunoglobulin secreting B lymphocyte of intermediate differentiation, (Nilsson 1978).

The absence of variant or mutant immunoglobulin secreting human B lymphoid cells in cloning and mutagenesis experiments

In experiments involving the mutagen treatment and cloning of a human lymphoblastoid cell line no variants of immunoglobulin production were detected, yet a large number of mouse myeloma cell line clones which synthesise abnormal immunoglobulins or which have lost the capacity to synthesise immunoglobulin have been described arising spontaneously or after mutagen treatment. These variant or mutant mouse myeloma cells occur with a high frequency which has been estimated at 10^{-5}/cell/generation for spontaneously occurring structural mutants of immunoglobulin polypeptides (Adetugbo et al., 1977), at 1 - 2 x 10^{-3}/cell/generation for spontaneously occurring loss of synthesis or secretion of one or both polypeptides (Coffino et al., 1972) and as high as 6.5% of the surviving cells for loss of immunoglobulin synthesis or secretion after mutagen treatment (Scharff et al., 1975). In contrast to these rates, drug resistance in mouse myeloma cells has been estimated as arising with a frequency of 10^{-6} - 10^{-7}/cell/generation (Baumal et al., 1973) which is many orders of magnitude lower than the spontaneous instability of immunoglobulin production and approaches the 10^{-8}/cell/generation figure for spontaneous variation in other mammalian cell systems (Sharp et al., 1973, Chasin et al., 1974). This kind of evidence has led to the general consideration that immunoglobulin genes and their expression are abnormally labile, a phenomenon which, it has been implied, may have some bearing on somatic mutation theories on the generation of antibody diversity, (Adetugbo et al., 1977).

In this project the analysis of over 2 x 10^{3} untreated and mutagenised clones of human lymphoblastoid cell lines for the production of more than one immunoglobulin polypeptide, making a total analysis of over 4 x 10^{-3} clonal products of immunoglobulin genes, did not lead to the detection of any variation in immunoglobulin production. A

relatively small number of MNNG and EMS-treated clones of YAK_1 and F137 were screened for immunoglobulin production. The vast majority of untreated and mutagenised clones, however, were derived from the cell line RPMI 8866 which has been successfully used by other workers in mutagenesis experiments (Lever and Seegmiller 1976). "Melphalan" with which the cells were treated belongs to the phenylalanine mustard group of chemicals and is an established mutagen (McCann and Ames 1978). The treatment of mouse myeloma cells, using the same dose of Melphalan (6×10^{-7} g/ml) as the human lymphoblastoid cells were exposed to in this project, produced loss of immunoglobulin synthesis or secretion in 1.86% of the clones derived from the surviving cells (Scharff et al., 1975). Furthermore, Melphalan is used in the treatment of human multiple myeloma and following treatment with the drug patients have been reported to show an increase on the amount of light chain produced, changes in the structure of the myeloma protein or complete loss of its synthesis (Hobbs 1971). This suggests that Melphalan has an effect, in vivo on human immunoglobulin-synthesising cells.

Against this background of information the absence of any variant clones of human lymphoblastoid cells especially after treatment with Melphalan makes it tempting to conclude that the immunoglobulin genes and their expression in human lymphoblastoid cells are very stable and particularly so in comparison to the immunoglobulin genes of mouse myeloma cells. The stable nature of immunoglobulin synthesis in human lymphoblastoid cells is borne out by the lack of reports in the literature of loss of synthetic activity or changes in the immunoglobulins synthesised by these cell lines. However it is questionable if this result can be used in a comparative sense. First, because only a single mutagen and a single cell line were investigated in detail. Second, and more importantly, the cloning and mutagenesis experiments were carried out, perhaps naively, for the most part to generate variants and not to compare rates of variation so that a mouse myeloma cell line was not included in these experiments. Therefore, strictly speaking, as a mouse myeloma cell line was not treated and analysed in the same system with a human lymphoblastoid cell line a true comparison of the rates at which variants arise in the two cell

types cannot be made. Nevertheless, in addition to the findings in this project it has been reported that cloning experiments with human lymphoblastoid cell lines over a long period of time have failed to detect non-producing cells and that treatment of the human plasma cell line U266BL with Melphalan has failed to demonstrate any changes in immunoglobulin secretion in cloned sublines (Nilsson 1978). Such results support the notion that the immunoglobulin genes of human lymphoid cell lines are more stable than those of mouse myeloma cells. If this is the case it represents an important and fundamental difference between the two systems and suggests that the labile nature of the immunoglobulin genes in mouse myeloma cells is not a property of that class of genes but is related to the nature of the mouse myeloma cell. This, in turn, might indicate that the rearrangement, erroneous rearrangement and deletion of immunoglobulin genes may not have so prominant a role in immunoglobulin gene expression in normal B lymphocytes and plasma cells as has been implied from their frequent occurrence in mouse myeloma cells (see Introduction, Genetics section).

Excess light chain synthesis by human B lymphoid cell lines

The analysis of unfractionated biosynthetically radiolabelled culture supernatants by reducing and non-reducing SDS PAGE suggested that, in addition to intact immunoglobulin molecules, free light chains may also be present in the culture supernatants of human B lymphoid cell lines. This was confirmed by the serological and SDS PAGE analysis of radiolabelled culture supernatants, from IgM synthesising cell lines, fractionated by sucrose gradient velocity sedimentation which provided convincing evidence that human lymphoblastoid cells do synthesise and secrete free excess light chains.

Despite this evidence, the presence of light chains in excess could be explained by the cultures being mixtures of cells secreting intact molecules and other cells secreting only light chains. However, this biclonal explanation appears unlikely when excess light chains have been observed to varying degrees in all the normal immunoglobulin-synthesising cell lines examined, whereas human lymphoblastoid cell lines secreting only light chains have been comparatively rare in this project.

Furthermore, these lines have been in culture for some time, are monoclonal in other aspects of their phenotypes and one line SMI_1 which has been assayed in detail and secretes significant amounts of free light chain had been physically cloned from a single cell. A second alternative explanation to account for these results is that free light chains in the culture supernatant could be produced by the degradation of secreted IgM in such a way that the heavy chains are degraded far more rapidly than the light chains. This possibility could be experimentally tested, using the techniques which demonstrated the presence of excess light chains in this project, by incubating radiolabelled 19S IgM in non-radioactive culture supernatant then analysing this mixture for radiolabelled 19S IgM or its degraded products. However, although FCS probably contains proteolytic activity and one might expect some proteolytic enzymes to be liberated or secreted from dead or dying cells within cultures, it is unlikely that specific preferential degradation of heavy chains over light chains would occur in what would be such a broad spectrum proteolytic environment. Therefore, the most rational explanation of the presence of free light chains in culture supernatants of these cell lines is that, together with intact molecules, human lymphoblastoid cell lines secrete free excess light chains.

Excess light chains have been serologically identified in the culture supernatants of other human lymphoblastoid cell lines (Litwin et al., 1974) and of the human plasma cell line U266BL (Nilsson 1978a). Human myeloma tumours produce excess light chains (Zolla et al., 1970) and this observation has been frequently made in mouse myeloma tumours and in the in vitro cultured mouse myeloma cell lines, (Baumal and Scharff 1973a). One view of this phenomenon is that it represents an abnormal imbalance of heavy chain and light chain synthesis (Parkhouse 1977) symptomatic of the neoplastic nature of the myeloma cell. This view stems from the opinion that the most economical way for cells to assemble and produce immunoglobulins should be by synthesising equimolar amounts of heavy and light chains and from descriptions of instances of apparently balanced heavy and light chain synthesis (Askonas and Williamson 1967). On the other hand normal human serum contains free light chains (Berggard and Edelman 1963) and while the

synthesis of heavy chains and light chains can be balanced in mouse tissues, including myeloma cells, this is comparatively rare; and much more commonly there is some excess light chain produced (Baumal and Scharff 1973). These observations, together with the consistent finding of excess light chain synthesis in immature human B lymphocyte neoplasms (Gordon et al., 1978) suggest that this is a pattern of immunoglobulin synthesis related to normal cells. The demonstration in this project of excess light chain synthesis in non-malignant human B lymphoblastoid cells derived from normal B lymphocytes is consistent with the view that excess light chain synthesis is related to a normal pattern of immunoglobulin production.

If an excess of light chains is a normal feature of immunoglobulin synthesis the question then arises of its role in this process. A plausible explanation is that an excess of light chains is maintained in order that light chains combine immediately with newly synthesised heavy chains to prevent the formation of large, relatively insoluble, heavy chain complexes which would be crippling, if not lethal, to the cell. This argument gains some support from observations which are compatible with the idea that synthesis of predominantly or only heavy chains is toxic to the cell. These observations come from studies of mouse myeloma cells and suggest that loss of heavy chain expression is a frequent event which precedes loss of light chain expression (Coffino and Scharff 1971). The rarity of variant mouse myeloma cell line clones synthesising heavy chains only in comparison to those synthesising only light chains (Scharff et al., 1975) and the preferential loss of heavy chain synthesis in mouse hybridoma lines containing different numbers of heavy and light chain genes (Kohler 1980) also indicate that the production of free light chains could be lethal to the cell.

Several of the human B lymphoid cell lines whose patterns of immunoglobulin synthesis have been described in this project could be used to clarify further excess light chain synthesis and its role in controlling immunoglobulin biosynthesis. For example, a comparison of the rates of incorporation of a radioactive precursor into the secreted heavy chains and light chains of a cell line secreting little or no excess light chain e.g. BAY_5 (see Results, Fig. 14) with a cell line

secreting a significant amount of excess light chain e.g. CON_1 (see Results, Fig. 14) would demonstrate whether the secreted light chains represent the only source of excess light chains or whether there is an intracellular pool of excess light chains. The four human B lymphoid cell lines described in this project as synthesising only heavy chains are obviously also of interest in this area. If the role of excess light chain synthesis is to prevent the formation of heavy chain complexes then heavy chains synthesised in a low concentration of light chains or, as in these cell lines, in the absence of light chains should be abnormal or present as intracellular complexes larger than dimers. Thus an analysis of the state of the heavy chains synthesised by human B lymphoid cell lines in the absence of light chains could provide evidence consistent with or contradictory to the idea of excess light chains preventing the formation of heavy chain complexes. In those mouse myeloma cell lines where heavy chains are the only immunoglobulin polypeptides expressed they appear always to be defective heavy chains (Conan et al., 1974, Wilde and Milstein 1980). and the same is true of the variant human heavy chain disease proteins which are synthesised in the absence of light chains (Buxbaum et al., 1978). However, the human B lymphoid cells described in this project which synthesise only heavy chains are not plasma cells (as these other examples are) and may represent stages of B lymphocyte differentiation even earlier than the lymphoblasts, when immunoglobulin synthesis is not so rapid and normal heavy chains can be safely synthesised.

Apparent variations in light chain molecular weight.

The mobility of human immunoglobulin light chains analysed by SDS PAGE in this project has not been constant. However in several cases the differences in migration appeared to be consistent and to be related to light chain type in that κ chains migrated more rapidly than λ chains. Similar observations have been described for light chains from normal human serum (Virella and Coelho 1974), and in the analysis of light chains from the surface immunoglobulin of human lymphoblastoid cells (Singer and Williamson 1980). The migration of polypeptides in SDS preparations is determined by their molecular weight (Shapiro 1967) and yet human κ and λ chains have identical molecular weights (Edelman and Gall 1969). In this case differential

binding of SDS seems likely to account for the phenomenon. Indeed, the amount of SDS bound by a polypeptide can depend upon its nature as well as its size (Nelson 1971) and so the differences in the amino acid composition of the constant regions of κ and λ chains must give rise to different affinities for SDS and thus to the type specific mobilities observed. There appear to be no specific studies of normal light chain mobilities in other species, but comparative analyses of the immunoglobulins secreted by mouse hybridomas show type specific mobility in murine light chains (Wilde and Milstein 1980, Kohler and Shulman 1980). The difference in mobility here, however, is the reverse of that found in human light chains, that is murine λ chains migrate more rapidly in SDS PAGE than κ chains. This observation may initially seem strange when greater amino acid sequence homology exists within light chain types between species than between types within species (Nisonoff et al., 1975). However, there is no reason to expect that those sequences which have been conserved during evolution are those which are important in determining the affinity of a polypeptide for SDS.

Not all the light chains analysed by SDS PAGE in this project showed variations in mobility which could be accounted for by type specific variation. The κ chains secreted by Jijoye migrate far more slowly than normal κ or λ chains and have an apparent molecular weight of ≈30,000 (see Results, Fig. 21). The same analysis showed that anti-light chain precipitable components of the same apparent molecular weight were synthesised by KAT_1, a κ chain secretor, and RPMI 8226 a λ chain secretor in addition to components of a normal light chain size. Purified human IgM obtained from the American Red Cross also had an apparently large κ light chain (see Results, Fig. 11). Previous reports of abnormal light chains either secreted by mouse myeloma cells (Birstein et al., 1977) or present as human myeloma proteins (Franklin et al., 1979) have been of polypeptides showing a much reduced molecular weight and have been classified as structural mutants containing deletions. Out-sized light chains on the other hand cannot be so readily explained. The presence of carbohydrate would increase the total molecular weight of these polypeptides which then as glycoproteins would show anomalous binding of SDS (Grefrath and Reynolds 1974) and glycosylation has been discussed as a possible mechanism to explain these

observations, (see Results, The analysis of secreted chains from the presumptive variant immunoglobulin synthesising cell lines by immunoprecipitation). There are, however, other possible explanations involving additions of amino acid residues to the polypeptides which, if less probable than the addition of carbohydrate are of more interest and potential significance. These explanations would be least likely to account for the large light chain in the purified serum IgM sample as the presence of additional peptides would be likely to disrupt the initial hydrogen bonding of light and heavy chains and so prevent subsequent disulphide bond formation and assembly of an intact IgM molecule. In contrast to this, Jijoye, KAT_1 and RPMI 8226 do not secrete complete immunoglobulin molecules therefore, there would appear to be no such restrictions on the structure of their secreted light chains whose synthesis as well as structure can be further studied. Mouse myeloma light chains have additional N-terminal amino acids in their "precursor" form. These are the "signal peptides", which determine the membrane bound synthesis and eventual secretion of light chains and which are subsequently cleaved from the light chains during or immediately after their entry to the endoplasmic reticulum (see Introduction, Biosynthesis of immunoglobulins). Similar N-terminal signals have not yet been demonstrated in the biosynthesis of human light chains but it is reasonable to assume that a similar mechanism applies. Although the signal is required for secretion into the endoplasmic reticulum it has not been demonstrated that the cleavage of the signal from the polypeptide is an essential part of the process. In at least one protein, ovalbumin, the signal is not cleaved during secretion (Lingappa et al., 1979). Therefore these large light chains could be normal peptides which have been secreted with their signals intact. In this case Jijoye would represent a variant of the process by which proteins are secreted across membranes lacking the membrane endopeptidase activity which is part of that mechanism. KAT_1 and RPMI 8226 would possess inefficient forms of that activity and so they would produce a mixture of processed and unprocessed light chains to give the observed two anti-light chain precipitable components. As yet, little is known about the membrane bound apparatus of the endoplasmic reticulum which recognises and cleaves secretory signals and so variants of this type would be valuable in studying these processes.

A second explanation for the abnormally large light chains, which also involves an addition to the polypeptide content of normal light chains, is the mechanism by which it is considered the immunoglobulins have evolved, that is, duplication of the domain pseudosubunits. However, it would be unlikely that KAT_1 and RPMI 8226 could express both a semi-duplicated and normal light chain simultaneously. This leaves only Jijoye to fit this explanation and even here the apparent increase in light chain molecular weight might not be enough to encompass an extra domain. Nevertheless, the discovery of split genes and the direct relationship between immunoglobulin domains and exons (see Introduction, The Genetics of Immunoglobulins) make the immunoglobulins an excellent model for the predicted generation of novel proteins by changes at the exon/domain level (Gilbert 1978). Infact the transfer and exchange of domains between immunoglobulin genes appears to have been a major part of their evolution (Miyata et al., 1980). Therefore, any examples of such events may help to understand the mechanisms involved at the molecular level in the evolution of immunoglobulins and proteins in general.

The nature of the presumptive variant immunoglobulin synthesising cell lines and the significance of their patterns of immunoglobulin synthesis and expression

Those cell lines analysed during the course of this project which have not shown the normal pattern of synthesis, surface expression and secretion of a single heavy chain class and a single light chain type (see Results, Table 14) have been referred to as "presumptive variants". As this description suggests, one explanation of their patterns of immunoglobulin synthesis and expression is that these cell lines are variants or mutants blocked at different stages of immunoglobulin synthesis and secretion. EB_3, Jijoye and RPMI 8226 fit this description well as they synthesise and secrete only light chains so that, as mutants, they would be expected to have non-functional heavy chain genes which is a common occurrence within our current understanding of immunoglobulin genetics. In the other cell lines which secrete only light chains, EB_2, JIM_1, and KAT_1 the situation

becomes more complex as they also synthesise, but do not secrete, μ heavy chains. As mutants these lines would have a fault affecting heavy chain secretion. However, the heavy chains concerned behave normally regarding the signal hypothesis as they are expressed on the cell surface and to get there they must pass into the endoplasmic reticulum. Therefore, a previously unknown step in secretion has to be invoked at which these heavy chains are blocked. It may be relevant that mutations not affecting the signal peptides of mouse light chains have been reported to prevent their secretion (Mossman and Williamson 1980). On the other hand there are structurally distinct membrane bound and secretory μ chains which are transcribed differently from the same single gene (see Introduction, Biosynthesis of immunoglobulins, transcription of immunoglobulin genes). Thus a similar effect would be achieved if the mutation in these lines affected μ chain nuclear RNA processing such that the splicing pattern of the primary transcript could produce only mRNA coding for μ chains with plasma membrane binding C-termini and so no μ chains would be actively secreted. Of the other presumptive variant lines, none of which secrete immunoglobulins, Daudi synthesises μ heavy chains and κ light chains and expresses both on the cell surface. A description of a mutation event which could give rise to this phenotype would be similar to the argument already outlined for the restriction of synthesised μ chains to surface expression. In this case the light chains are also restricted to the cell surface and as there are no known cell surface membrane binding light chains specific structures, all light chains synthesised by Daudi must be bound to membrane specific heavy chains. The remaining non-secreting cell lines FAL_1, F89, F137 and Raji synthesise only μ heavy chains. Thus, if their phenotypes are mutant, they must have completely non-functional light chain genes. The μ chains of F137 and Raji are expressed on the cell surface, but, as previously mentioned, not secreted. Therefore, a second genetic lesion affecting primary transcript splicing, in a manner already described, must also be postulated in these lines. FAL_1 and F89 do not express their μ chains on the cell surface and the prevention of μ chain secretion across the membrane of the endoplasmic reticulum by, for example, a mutation in the region coding for the μ chain signal peptide would account for their lack of surface or secretory expression.

Therefore, it is possible within our current understanding of the events of immunoglobulin synthesis to describe these presumptive variant lines as mutants of normal immunoglobulin synthesis. As such they would provide useful information on further investigation about the stages of synthesis at which they are blocked and also perhaps outline previously unknown steps in immunoglobulin synthesis and secretion.

Alternatively, the patterns of immunoglobulin synthesis and expression shown by several of the presumptive variant cell lines may represent different stages of B lymphocyte differentiation. That is to say these cell lines may not be mutants of normal cells but different fixed stages of B cell ontogeny. This is an initially attractive hypothesis not least because the classical descriptions of mutant immunoglobulin synthesising cells come from the many studies of spontaneous and mutagen-induced variant clones of the plasma cell mouse myeloma lines (Milstein et al., 1975, Scharff et al., 1975) whereas most human lymphoid lines do not represent plasma cells but intermediately differentiated B lymphocytes and have far more stable immunoglobulin synthesising phenotypes, as has been previously discussed. Indeed, if those cell lines synthesising or secreting only a single type of immunoglobulin chain were mutants, their frequent occurrence - even including the hundreds of cell lines screened in this laboratory at one time or another - is incompatable with the lack of variant or mutant cells in mutagenesis and cloning experiments, unless there is extreme selection for variant cells during the establishment and culture of cell lines. Furthermore, the re-occurrence of the same relatively complex phenotype e.g. in EB_2, JIM_1 and KAT_1 and the repeated requirement for the same type of mutation affecting the splicing of the μ chain primary transcript make the mutation argument seem over-elaborate and clumsy. However, apart from the shortcomings of the mutation hypothesis what strengthens the B lymphocyte differentiation argument is the continuum of immunoglobulin synthesis that is apparent when the presumptive variant lines are looked at from the point of view of representing different stages of B lymphocyte ontogeny. This pathway is outlined in Table 15. According to this scheme, immunoglobulin synthesis begins during B lymphocyte differentiation with the synthesis of μ heavy chains which are not expressed on the

TABLE 15

SEVERAL OF THE PRESUMPTIVE VARIANT CELL LINES

REPRESENTING DIFFERENT STAGES OF B LYMPHOCYTE DIFFERENTIATION

DIFFERENTIATION ↓	Immunoglobulin expression			Representative cell lines
	Intracellular	Surface	Secretory	
	μ	-	-	FAL_1, F89
	μ	μ	-	F137, Raji
	μL	μL	-	Daudi
	μL	μL	L	EB_2, JIM_1, KAT_1
	μL	μL	μL	hundreds

L = Light chain

cell surface or secreted, as represented by immunoglobulin expression in FAL_1 and F89. The next step, shown by F137 and Raji, is the surface expression of the μ chains followed by the synthesis of light chains which are also expressed on the cell surface as in Daudi and presumably as intact surface IgM molecules. At this stage in B lymphocyte ontogeny the cell surface antibodies bind antigen and stimulate the lymphocyte to proliferate and produce more immunoglobulin. Thus as immunoglobulin synthesis accelerates light chain synthesis increases first and remains in excess to accommodate the subsequent increase in heavy chain synthesis which otherwise might be toxic to the cell, as previously discussed. These events are indicated by the cell lines EB_2, JIM_1 and KAT_1 which synthesise and express on the cell surface μ chains and light chains but secrete only light chains. As μ chain synthesis increases so B lymphocytes switch from the synthesis of predominantly surface bound μ chains to secretory μ chains giving rise to the pattern of immunoglobulin expression shown by the vast majority of human B lymphoblastoid cell lines.

There is evidence from other sources to support the claims of some of these cell lines as examples of early B lymphocyte differentiation. There have been several reports from studies of murine foetal liver hybridomas (Burrows et al., 1979), Abelson murine leukaemia virus transformed mouse bone marrow cells (Siden et al., 1979) and isolated murine and human pre- B cells (Levitt and Cooper 1980) that pre-B lymphocytes synthesise μ chains and no light chains which is the same immunoglobulin synthesis pattern shown by FAL_1 and F89. However, unlike F137 and Raji, none of the pre-B cells described in these reports express their μ chains on the cell surface and in one report (Levitt and Cooper 1980) the pre- B cell μ chains are freely secreted. Although F137 may put some μ chains into the culture medium it could not be described as freely secreting μ chains, as previous analysis has shown. The immunoglobulin phenotype of Daudi is well established as one which represents an intermediate stage of B lymphocyte differentiation (Nilsson 1978) and the shift in synthesis from membrane bound immunoglobulin to secreted immunoglobulin is also a well documented step in B lymphocyte ontogeny. Athough there is evidence both from this project and elsewhere that light chains are synthesised in excess of heavy chains there appear to be no other reports of

B lymphoid cells which, like, EB_2, JIM_1 and KAT_1 synthesise both heavy chains and light chains but secrete only light chains.

The hypothesis that these cell lines represent relatively undifferentiated B lymphocytes raises the point of their origins especially as it has been argued earlier that human B lymphoblastoid cell lines are normally derived from B lymphocytes actively secreting immunoglobulin. All the cell lines in question carry the EB virus nuclear antigen (Steel, personal communication). However the majority are derived from Burkitts Lymphoma which makes credible their establishment as earlier B cells than lymphoblasts. The exceptions are F89, F137, FAL_1, JIM_1 and KAT_1. Of these F89 and F137 were established from leukaemias which are also sources of clonal expansion of B lymphocytes in early stages of differentiation. FAL_1, JIM_1 and KAT_1 on the other hand, were established from a normal cord blood, normal adult blood and blood from an infectious mononucleosis patient respectively. Bearing in mind the large number of normal, immunoglobulin secreting B lymphoblastoid cell lines derived from these sources they do not seem likely origins for relatively undifferentiated B lymphoid cell lines. However support for the possibility that EB virus transformed lines representing early cells in B lymphocyte ontogeny can be established from a non-malignant source comes from a recent report by Fu et al., (1980) that pre- B cell-like lines can be established using EB virus from the bone marrow of X-linked agammaglobulinaemia patients.

The possibility exists then that here is a group of cell lines which represent fixed stages of human B lymphocyte differentiation in finer detail than ever before as regards the onset and development of immunoglobulin synthesis. If this is the case these cell lines show that μ chain synthesis precedes light chain synthesis in human B lymphocyte ontogeny. The significance of this asynchromous onset of immunoglobulin chain synthesis, as suggested by Burrows et al., (1979) from murine data, may be that by not expressing light chains immature B lymphocytes are free to expand the number of individual cells expressing a specific V_H gene without the external regulation that might accompany the presence of an IgM surface receptor. It is equally interesting that these results suggest that immature B lymphocytes express free μ chains on the cell surface as T lymphocyte receptors may resemble or have the conformational properties of V_H domains, Eichman and Rajewsky (1975).

These two pieces of information raise the attractive hypothesis that the surface μ chain V_H domains in immature B lymphocytes have a role in the lymphocyte interactions which modulate the humoral response.

Further structural studies of the immunoglobulin polypeptides and their relevant N-terminal and/or C-terminal additions synthesised by the above cell lines should give an insight into the mechanisms which underly the control of the various patterns of immunoglobulin synthesis and expression. In addition structural studies will also determine whether or not the immunoglobulin chains synthesised by these putative immature B lymphocyte lines are analogous to those found in the more mature normal B lymphoblastoid cell lines. If this is not the case the status of these lines as representing earlier stages in differentiation would be questionable. In addition to analysis of their immunoglobulin phenotypes the presumptive immature B lymphocyte cell lines represent a valuable source for the study at the DNA level of the structure and organisation of immunoglobulin genes either in early or in variant human B lymphocyte development.

ACKNOWLEDGEMENTS

Dr. R.M. Clayton of the Institute of Animal Genetics, University of Edinburgh and Dr. C.M. Steel of the Medical Research Council Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh supervised this work. I am grateful to several members, past and present, of the MRC Clinical and Population Cytogenetics Unit, in particular Dr. B.B. Cohen for his help and advice throughout this project.

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APPENDIX I

Human B lymphoid cell lines secreting a single heavy chain class and a single light chain type

Cell lines were established from blood lymphocytes unless otherwise indicated and with the exception of cord bloods all donors were adult unless otherwise stated. Donors, other than infectious mononucleosis patients were considered to have had no recent infection.

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
AIR_1	M	Bladder cancer	+	-	-	+	-
ALF_2	M	Bladder cancer	+	-	-	-	+
ALP_1	F	Normal	+	-	-	+	-
BUR_2	F	Infectious mononucleosis	+	-	-	+	-
DUN_1	M	Infectious mononucleosis	+	-	-	-	+
FLE_1	M	Infectious mononucleosis	+	-	-	-	+
HUT_1	M	Manic depressive	+	-	-	-	+
ICH_2	F	Manic depressive	+	-	-	-	+
MAR_1	M	Infectious mononucleosis	+	-	-	+	-
NAY_2	M	Waldenstroms Macroglobulinaemia	+	-	-	-	+

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
ODY_1	M	Waldenstroms Macroglobulinaemia	+	-	-	-	+
ROS_1	F	Normal	+	-	-	+	-
SHA_1	F	Acute lymphatic leukaemia	+	-	-	+	-
SIG_2	M	Achiasmate	+	-	-	+	-
TON_1	F	Infectious mononucleosis	+	-	-	+	-
ALF_3	M	Bladder cancer	-	+	-	+	-
ARH 77	F	Multiple myeloma	-	+	-	+	-
ART_1	F	Chronic myeloid leukaemia	-	+	-	-	+
BEC-11	F	Tonsilitis (established from tonsil lymphoid cells)	-	+	-	+	-
BRI-7	M	Normal adult	-	+	-	+	-
CAN_1	F	15/7 translocation	-	+	-	+	-
COL_1	M	Aortic incompetence	-	+	-	-	+
DEW_1	F	Infectious mononucleosis	-	+	-	+	-
EB_4	F	Burkitt lymphoma	-	+	-	-	+
GEF_1	M	Normal	-	+	-	+	-
HUC_1	M	Normal	-	+	-	+	-
ICH_1	F	Manic depressive	-	+	-	-	+

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
JOF	M	Normal	-	+	-	-	+
LOR_1	F	Infectious mononucleosis	-	+	-	-	+
LYN_2	F	XY	-	+	-	+	-
MAZ_1	F	Trisomy 21	-	+	-	+	-
MEK_1	F	Trisomy 21	-	+	-	+	-
MICH	F	Idiopathic thrombocytopaenia (established from spleen cells)	-	+	-	+	-
ORI_1	F	Normal	-	+	-	-	+
PAR_1	M	Infectious mononucleosis	-	+	-	-	+
POS_3	M	XX	-	+	-	-	+
PRA_1	M	Infectious mononucleosis	-	+	-	+	-
RIC_1	F	Thorotrast treated patient	-	+	-	+	-
RIL_1	F	Normal	-	+	-	-	+
ROS_2	F	Normal	-	+	-	-	+
RPMI 8866	F	Acute myelogenous leukaemia	-	+	-	+	-
TAB_2	F	Hurlers syndrome	-	+	-	-	+
TIL_1	M	XXXXY	-	+	-	+	-

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
WAL_1	F	Infectious mononucleosis	-	+	-	+	-
WIK_1	M	Infectious mononucleosis	-	+	-	+	-
ANA_1	M	Cord blood	-	-	+	-	+
ANA_2	M	Cord blood	-	-	+	-	+
ARD_1	F	$15pH^+$	-	-	+	+	-
ARO_1	F	Aortic incompetence	-	-	+	+	-
ARY_1	M	Manic depressive	-	-	+	-	+
BAK_1	M	XXY	-	-	+	-	+
BAX_1	F	Cord blood	-	-	+	-	+
BAY_1	F	Cord blood	-	-	+	+	-
BAY_2	F	Cord blood	-	-	+	-	+
BAY_3	F	Cord blood	-	-	+	+	-
BAY_4	F	Cord blood	-	-	+	+	-
BAY_5	F	Cord blood	-	-	+	+	-
BET_1	M	Cord blood	-	-	+	+	-
BET_5	M	Cord Blood	-	-	+	+	-

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
BET_7	M	Cord blood	-	-	+	+	-
BLA_1	M	Acute leukaemia	-	-	+	+	-
BLA_4	M	Acute leukaemia	-	-	+	+	-
BOB_1	M	Cord blood	-	-	+	-	+
BOK_1	F	Cord blood from known chromosomal mosaic	-	-	+	+	-
BON_1	F	Cord blood	-	-	+	+	-
BR18	F	Normal	-	-	+	-	+
BR18T	F	Tetraploid derivative of BR18	-	-	+	+	-
BUR_1	F	Infectious mononucleosis	-	-	+	-	+
CAR_1	F	Chronic myeloid leukaemia	-	-	+	+	-
CHU_1	M	Acquired agamaglobulinaemia	-	-	+	-	+
CLA_1	F	Cord blood	-	-	+	+	-
CLA_4	F	Cord blood	-	-	+	-	+
COA	F	Myelofibrosis	-	-	+	+	-
CON_1	F	Cord blood	-	-	+	+	-
CRO_1	M	Cord blood	-	-	+	+	-

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
EB_1	F	Burkitt lymphoma	-	-	+	+	-
GAL_2	M	Normal	-	-	+	+	-
GAL_3	M	Normal	-	-	+	+	-
GAL_4	M	Normal	-	-	+	+	-
G-S	F	Chronic lymphatic leukaemia	-	-	+	+	-
HIL_1	F	Cord blood	-	-	+	-	+
HOL_1	M	Cord blood	-	-	+	-	+
HUC_2	M	Normal	-	-	+	+	-
JED_1	M	Small Y	-	-	+	+	-
KEL_4	F	Cord blood	-	-	+	+	-
KIT_1	F	Manic depressive	-	-	+	-	+
LAX_1	M	XX	-	-	+	-	+
LID_1	F	Normal	-	-	+	+	-
MAC_1	F	Cord blood	-	-	+	+	-
MON_1	M	Cystic fibrosis (child)	-	-	+	-	+
MUN_1	F	Normal	-	-	+	+	-

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
NAMALVA	F	Burkitt lymphoma	-	-	+	-	+
NOR_1	M	Manic depressive	-	-	+	-	+
OCH_1	F	Bladder cancer	-	-	+	+	-
PAL_1	F	Cord blood	-	-	+	-	+
PRA_1	M	Infectious mononucleosis	-	-	+	+	-
POS_1	M	XX	-	-	+	+	-
RAF_1	M	Trisomy 21	-	-	+	-	+
RHE	M	2/10 translocation (child)	-	-	+	-	+
RIC_2	F	Normal	-	-	+	-	+
RIC_3	F	Normal	-	-	+	-	+
RIT_1	F	Manic depressive	-	-	+	+	-
RPMI 1788	M	Normal	-	-	+	-	+
RUS_1	M	Leukaemia (Acute myeloblastic ?)	-	-	+	+	-
SAR_2	F	Manic depressive	-	-	+	-	+
SEB_3	F+M	Pooled cord bloods	-	-	+	+	-
SEC_1	F+M	Pooled cord bloods	-	-	+	+	-

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
SEC_2	F+M	Pooled cord bloods	-	-	+	-	+
SEC_3	F+M	Pooled cord bloods	-	-	+	+	-
SEC_4	F+M	Pooled cord bloods	-	-	+	+	-
SEC_5	F+M	Pooled cord bloods	-	-	+	-	+
SEC_6	F+M	Pooled cord bloods	-	-	+	-	+
SEY_2	M	Normal	-	-	+	+	-
SIM_1	M	Aypogammaglobulinaemia	-	-	+	+	-
SOB_1	M	XX	-	-	+	+	-
SMI_1	F	Cord blood	-	-	+	+	-
SMI_2	F	Cord blood	-	-	+	+	-
SMI_4	F	Cord blood	-	-	+	-	+
SUT_1	M	Cord blood	-	-	+	-	+
TAF_1	M	Ataxia telangiactasia (child)	-	-	+	+	-
TAF_2	M	Ataxia telangiactasia (child)	-	-	+	+	-
TAF_3	M	Ataxia telangiactasia (child)	-	-	+	-	+
VEN_1	M	Acute lymphatic leukaemia	-	-	+	+	-

Cell line	Sex	Donor Condition	Pattern of immunoglobulin secretion				
			α	γ	μ	κ	λ
WAD_2	F	Normal	-	-	+	+	-
WAM_1	M	C-band heterozygote	-	-	+	-	+
WAR_1	M	Acute lymphatic leukaemia (child)	-	-	+	-	+
WEB_1	F	Cord blood	-	-	+	-	+
WEB_2	F	Cord blood	-	-	+	-	+
WES_1	M	Cord blood	-	-	+	-	+
WHE	M	Cord blood	-	-	+	-	+
WIG	M	Cord blood	-	-	+	-	+
YAK_1	M	Cord blood	-	-	+	-	+
XER_1	F	Xeroderma pigmentosim	-	-	+	+	-
XER_2	F	Xeroderma pigmentosim	-	-	+	+	-

APPENDIX II

(Reprinted from Nature, Vol. 270, No. 5639, pp. 729–731, December 22/29, 1977)

Possibility of EB virus preferentially transforming a subpopulation of human B lymphocytes

PERMANENT lymphoblastoid cell lines can be established by 'transformation' of human lymphocytes with EB virus[1,2]. The lines secrete immunoglobulin (Ig) *in vitro*[3,4] and represent, morphologically, an intermediate stage between the resting lymphocyte and the fully developed plasma cell[5,6]. It has been suggested that the EB virus stimulates inactive B lymphocytes to secrete immunoglobulin and that the virus is behaving, in this respect, like a polyclonal B cell mitogen. The evidence comes from observations that most (possibly all) human B lymphocytes carry surface receptors for EB virus[8], that an increase in Ig secretion is detectable within a few days of adding the virus to a culture of lymphocytes[7], and that in the early stages of growth, lymphoblastoid lines established in this way secrete multiple classes of Ig heavy and light chain[4,9,10]. It seems, however, that only a fraction of 1% of lymphocytes in an EB virus-infected culture respond either by Ig synthesis or by proliferation and furthermore that the two events occur simultaneously[7,11,12]. The possibility therefore remains that EB virus does not stimulate Ig synthesis *de novo* but selectively 'transforms' (that is, induces to proliferate) that minor population of B cells which has already begun to secrete Ig. This alternative is supported by our analysis of the major classes of Ig heavy and light chains secreted by lymphoblastoid cell lines (derived from peripheral blood lymphocytes) in relation to the age and clinical status of the donors.

The data are set out in Table 1. The most striking finding is that, of fifty unselected lines derived from cord bloods, forty-nine secrete only IgM. This is at variance with the expected outcome of polyclonal stimulation of resting B cells, since the ability to synthesise cytoplasmic Ig of differing heavy chain classes in response to a polyclonal B cell mitogen seems to be acquired earlier in ontogeny than the class and subclass segregation of cell surface immunoglobulin determinants that is already well established among the circulating lymphocytes of the human foetus many weeks before birth[13,14]. Although IgM-secreting cells have been shown to predominate among pokeweed-stimulated human cord lymphocytes, IgG secretors form a substantial proportion in most samples and, in some cases at least, IgA-producing cells are also readily found[15]. It therefore seems incompatable with this mode of action of EB virus that no γ or α heavy chains could be detected in the supernatants of any of our cord blood lines even when tested repeatedly during the early weeks following their establishment *in vitro* and even though polyclonal composition could still be demonstrated by the presence of both kappa and lambda light chains. Conversely, our findings are in keeping with the view that EB virus induces proliferation in those rare cells which are actively secreting immunoglobulin since, for the first few weeks after birth, the healthy human infant synthesises Ig exclusively of class M (ref. 16).

Adult blood lymphocytes stimulated with pokeweed mitogen typically contain populations of B cells secreting IgM, IgG and IgA respectively, in the approximate proportions of 2:2:1 (refs 15 and 17). By comparison, IgM secretors seem to be relatively over-represented among the monoclonal lines derived from healthy adults and under-represented among those from infectious mononucleosis patients (Table 1). The difference between the two groups is difficult to explain if the lines are derived from resting blood B cells, but since infectious mononucleosis is characterised by a substantial increase in serum Ig of all three major classes[18], the population of Ig-secreting cells in the circulation is likely to be markedly disturbed. If this is the population which gives rise to lymphoblastoid lines, a deviation from the normal distribution of G,M and A secretors would not be unexpected. It would also provide a rational explanation for the observation that lymphoblastoid lines can be established exceptionally easily from the blood of patients in the acute or early convalescent phase of infectious mononucleosis[19–21] and other virus infections[22,23], when the absolute number of Ig-secreting cells in the circulation is abnormally high.

Table 1 Patterns of Ig secretion by human lymphoblastoid cell lines

Source of blood Lymphocytes*	No. of cell lines (no. of different donors)	Ig secreted†						
		Heavy chain				Light chain		
		G	A	M	None‡	K	L	None
Cords	Total 50(25)							
	Monoclonal Ig secretors 39(20)	0	0	49	1	30	29	1
		0	0	39	—	20	19	—
Adults (no recent infection)§	Total 79(62)							
	Monoclonal Ig secretors 66(59)	31	10	49	0	54	40	0
		20	8	38‖	0	39	27	0
Adults (infectious mononucleosis)	Total 19(16)							
	Monoclonal Ig secretos 17(15)	9	5	7	0	14	7	0
		7	5	5‖	0	12	5	0

*All cord blood lines, all infectious mononucleosis-derived lines and all but two of the lines from other adult donors were established in our laboratory.

†Ig secretion was detected by a highly sensitive and reproducible haemagglutination–inhibition technique[4]. In most cases supernatants had been tested on several occasions.

‡One cord-derived line (FAL_1) has consistently failed to secrete any detectible Ig (ref. 4). Morphologically it is indistinguishable from other lymphoblastoid lines, and complete EB virus particles were seen in electron microscope preparations. The cells have surface Ig MK determinants (by indirect immunofluorescence) and react strongly with a rabbit antiserum specific for human B lymphoid cells.

§Although described as 'adults', three of these donors were below the age of 16 yr. The group comprised healthy individuals and those suffering from genetic (including cytogenetic) disorders or from malignant disease not affecting the lymphoreticular system.

¶Lines were considered monoclonal when only a single class of Ig heavy chain and single class of light chain could be detected in the supernatant.

‖The difference between these two groups in the proportion of monoclonal lines secreting M heavy chain is significant ($\chi^2 = 4.3$; $P < 0.05$). There are no significant differences between any group in the proportion of lines secreting K or L light chains.

Arguments based on the study of Ig secretion by monoclonal lymphoblastoid lines would, of course, be invalid if there was any suspicion of a selective advantage for the growth of cells producing Ig of a particular class, or if the commitment of a given cell and its progeny to secrete a particular class of heavy chain were unstable in the conditions of culture. On the first point, our experience has been that when multiple aliquots of 10–100 cells are taken, at an early stage of growth, from a line which is secreting comparable amounts of two immunoglobulin molecules (distinguished by heavy and/or light chain class), the subcultures ultimately give rise to approximately equal numbers of clones secreting each of the parental Ig species. Selection of the dominant clone from a newly established line therefore seems to be a random process with respect to class of Ig secreted. On the second point, in spite of one contradictory report[24], it seems that lymphoblastoid cell clones secreting more than one class of heavy and/or light chain are exceptionally rare[4,25], while stability of the commitment to secrete a particular class of Ig is one of the most striking features of human lymphoblastoid lines[26]. We have been unable to detect any change in the pattern of Ig synthesis among several hundred clones derived from lines exposed to a variety of physical and chemical mutagens, although a considerable number of other biochemical changes have been induced[27,28].

The distribution of monoclonal heavy and light chains ultimately produced by lines from a particular set of donors is therefore likely to be a valid index of the relative proportions of their blood lymphocytes secreting (or committed to secrete) each class of Ig and susceptible to EB virus 'transformation'.

It is important to an understanding of the mechanism of EB virus transformation to know whether the stage of differentiation of the host B lymphocyte is a critical factor. Our conclusion, that there is preferential, if not exclusive, transformation of those cells already secreting Ig *in vivo* (though mature plasma cells are evidently insusceptible[25]), seems to be more compatible with the available data than the proposition that EB virus acts as a polyclonal B cell mitogen. If the subpopulation of Ig-secreting blood lymphocytes can be isolated—for example, by velocity gradient sedimentation[29]—the question may be resolved.

We thank Mrs Marilyn Woodward, Mrs June Jenkins, Mr Gordon Martin and Mr Ross Maynard for assistance, and Mr J. Davies for advice on statistical analysis.

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Received 26 July; accepted 30 October 1977.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset