Immunological aspects of ageing in the laboratory mouse

Andrew C. Payne BSc. (Lond)

Thesis submitted for the degree of

Doctor of Philisophy

University of Edinburgh

1974

EX BIBL UNIV. EDINBURGEN

INDEX

SUMMARY

CHAPTER 1 - Introduction 1

1. The development of lymphoid tissues and immunological responsiveness 1

2. The antigen trapping capabilities of young mature lymphoid tissues 6

3. Cellular interactions in the immune response 9

4. The effect of age on the immune response 16

(a) Lymphoid organs in relation to age 18

(b) Cellular aspects of immune function related to age 22

(i) The affect of age on the formation of antibodies 22

(ii) The age related incidence of auto-antibodies 31

(iii) The age related incidence of auto-immune disease and diseases with auto-immune components 32

(iv) The effect of age on cell-mediated immunity 33

(v) The effect of age on the inductive and proliferative events in the immune response 34

Aims of the present study 39

CHAPTER 2 - Materials and Methods

Materials 40

Methods 41

Preparation of rabbit anti-mouse Ig serum 41

Injection of and sensitisation with antigen 42

Collection and preparation of serum samples 43

Preparation and injection of cell suspensions 43

Thymus grafting 44

Thymectomy 45

Irradiation 45

Assay techniques 46

CHAPTER 3, - Primary responses to sheep erythrocytes 70

1. The background response to sheep erythrocytes in CBAT6 male mice 70

2. The primary response to sheep erythrocytes in male CBAT6 mice 72

3. The primary response to sheep erythrocytes in CBAT6 mice aged 3 and 21 months and in CBA mice aged 18 and 22 months 79

4. The primary response to sheep erythrocytes in male CBAT6 mice aged 3, 7 and 16 months 80

5. The primary response to sheep erythrocytes in CBAT6 female mice compared to that in males 81

6. The primary serum antibody response in male CBA mice 82

7. The primary response to sheep erythrocytes in female C57Bl mice 83

8. The primary response to sheep erythrocytes in (CBA x C57Bl)F_1 male mice 85

9. The primary response to sheep erythrocytes in very old (CBA x C57Bl)F_1 female mice 87

Conclusions 90

CHAPTER 4 Secondary responses to sheep erythrocytes 91

1. The secondary response in 9 and 24 month old CBAT6 mice 91

2. The secondary response to sheep erythrocytes in CBAT6 mice assayed by a passive haemagglutination technique 92

3. The secondary response to sheep erythrocytes in (CBA x C57Bl)F_1 male mice 93

Conclusions 94

CHAPTER 5. - Effect of age on the proliferative response to oxazolone 95

1. The primary response to oxazolone in CBAT6T6 mice 95

2. The primary response to various doses of oxazolone in CBA male mice aged 3 and 21 months 97

Conclusions 99

CHAPTER 6 - Discussion 1 101

Conclusions 112

CHAPTER 7 - The repopulation of old mice with young cells and tissues 114

1. A preliminary study on the repopulation of old mice with foetal liver cells with or without a young thymus graft. 114

2. The repopulation of 22 month old CBAT6 female mice with young cells and tissues 115

Conclusions 117

CHAPTER 8 - The potential of old bone marrow as a scurce of T and B cells and the ability of the old thymus to support a proliferating T cell population. 118

1. The ability of old Bone marrow to produce mature functional B cells. 118

2. The ability of old bone marrow to produce mature T cells. 120

3. The long term ability of old bone marrow to produce functional T and B cells, when transferred to young irradiated recipients. 124

4. The ability of the old thymus to support a proliferat-
-ing T cell population. 126

Conclusions 130

CHAPTER 9 - Discussion 2 131

Section A - haemopoietic and lymphoid cell deficiences 131

Section B - structural deficiences in lymphoid organs 141

Section C - Generalised deficiences and changes 145

Conclusions 153

ACKNOWLEDGMENTS 155

APPENDIX 1 156

REFERENCES 158

FIGURE LEGENDS

Fig. 1 The direct plaque response in CBAT6 male mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 2 The indirect plaque response in CBAT6 male mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 3 The direct plaque response in CBAT6 and CBA male mice after the injection of 4×10^7 sheep erythrocytes.

Fig. The indirect plaque response in CBAT6 and CBA male mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 5 The direct plaque response in CBA male mice aged 3, 7 and 16 months after the injection of 4×10^7 sheep erythrocytes.

Fig. 6 The indirect plaque response in CBA male mice aged 3, 7 and 16 months after the injection of 4×10^7 sheep erythrocytes.

Fig. 7 The direct plaque response in 3 and 16 month old male and 3 and 18 month old female CBAT6 mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 8 The indirect plaque response in 3 and 16 month old male and 3 and 18 month old female CBAT6 mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 9 Serum haemagglutination titres in male CBA mice in response to sheep erythrocytes, before and after reduction and alkylation.

Fig. 10 Serum haemagglutination titres in male CBA mice after the addition of a rabbit anti-mouse Ig serum, following reduction and alkylation.

Fig. 11 The direct plaque response in female C57Bl mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 12 The indirect plaque response in female C57Bl mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 13 The direct plaque response in (CBA x C57Bl)F_1 male mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 14 The indirect plaque response in (CBA x C57Bl)F_1 male mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 15 The direct plaque response in (CBA x C57Bl)F_1 female mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 16 The indirect plaque response in (CBA x C57Bl)F_1 female mice after the injection of 4×10^7 sheep erythrocytes.

Fig. 17 The direct plaque response in 9 and 24 month old CBA male mice after a second injection of 4×10^7 sheep erythrocytes.

Fig. 18 The indirect plaque response in 9 and 24 month old CBA male mice after a second injection of 4×10^7 sheep erythrocytes.

Fig. 19 'developed' serum haemagglutination titres in CBAT6 male mice after a second injection of 4×10^7 sheep erythrocytes.

Fig. 20 The direct plaque response in 16, 20 and 24 month old male (CBA x C57Bl)F_1 mice after a second injection of 4×10^7 sheep erythrocytes.

Fig. 21 The indirect plaque response in 16, 20 and 24 month old male (CBA x C57Bl)F_1 mice after a second injection of 4×10^7 sheep erythrocytes.

Fig. 22 Cellular proliferation in the draining lymph nodes of male CBAT6T6 mice after sensitisation with 10mg oxazolone and measured by the incorporation of ^{125}IUdR

Fig. 23 Cellular proliferation in the contralateral lymph nodes of male CBAT6T6 mice after sensitisation with 10mg oxazolone measured by the incorporation of ^{125}IUdR

Fig. 24 Cellular proliferation in the draining lymph nodes of female CBAT6T6 mice after sensitisation with 10mg oxazolone measured by the incorporation of ^{125}IUdR.

Fig. 25 Cellular proliferation in the contralateral nodes of female CBAT6T6 mice after sensitisation with 10mg oxazolone measured by the incorporation of ^{125}IUdR.

Fig. 26 Background levels of incorporation of ^{125}IUdR in the regional draining and contralateral lymph nodes of 3 and 21 month old CBA male mice.

Fig. 27 Cellular response to various doses of oxazolone in the regional lymph nodes of 3 and 21 month old CBA male mice.

Fig. 28 The cellular response to various doses of oxazolone in the draining inguinal nodes of 3 and 21 month old CBA male mice

Fig. 29 The cellular response to various doses of oxazolone in the contralateral inguinal nodes of 3 and 21 month old CBA male mice.

Fig. 30 The proliferative response to various doses of oxazolone in the spleens of 3 and 21 month old CBA male mice

Fig. 31 The direct plaque response to sheep erythrocytes in 19 month old male CBAT6 mice irradiated and repopulat--ed with young cells and tissues.

Fig. 32 The indirect plaque response to sheep erythrocytes in 19 month old CBAT6 male mice irradiated and repopulat--ed with young cells and tissues.

Fig. 33 The direct plaque response to sheep erythrocytes in young female CBAT6 mice repopulated with young bone marrow cells and old female mice repopulated with young or old bone marrow cells.

Fig. 34 The indirect plaque response to sheep erythrocytes in young female CBAT6 mice repopulated with young bone marrow cells and old female mice repopulated with young or old bone marrow cells.

Fig. 35 Individual plaque responses used in the preparation of Fig. 34.

Fig. 36 The direct plaque response to sheep erythrocytes in CBAT6T6 mice irradiated and repopulated with young or old bone marrow cells.

Fig. 37 The indirect plaque response to sheep erythrocytes in CBAT6T6 mice irradiated and repopulated with young or old bone marrow cells.

Fig. 38 Cellular proliferation in CBA mice, irradiated and repopulated with young or old bone marrow cells 6 months previously, in response to oxazolone and measured by the incorporation of ^{125}IUdR in the regional lymph nodes.

Fig. 39 The indirect plaque response to 4 x 10^7 sheep erythro-cytes in thymectomised CBAT6T6 male mice repopulated one month previously with foetal liver cells and young or old, normal or irradiated thymus grafts.

SUMMARY

A brief account is given of the development and structure of the lymphoid organs and cells and is followed by a description of the cellular interactions which are thought to take place after antigen stimulation. A more detailed account is provided of the information presently available on the effect of age on the immune system.

The haemolytic plaque technique was used extensively and precise details are given both of the assay procedures and the various titrations performed to establish the characteristics of the developing serum used to identify indirect, 7S, antibody forming cells.

Initial studies on the response to sheep erythrocytes were carried out in CBA, C57Bl and (CBA x C57Bl)F_1 mice. Although species differences were apparent the general pattern of depression was concluded to be similar in all the species investigated and was reflected in delays, both in the onset of the response and the time, after injection, at which maximum responsiveness was attained. Deficiences were more apparent in the indirect, 7S, response, although in very old (CBA x C57Bl)F_1 mice the 19S response was also severely depressed. The results of serum haemagglutination titres broadly confirmed the results obtained with the plaque technique.

The cellular response to oxazolone was seen to decline gradually with age in CBA mice but did not involve a delay in the time at which maximum proliferative activity was observed.

There was some indication, both in the response to sheep

erythrocytes and oxazolone that female CBA mice are not affected as rapidly as males by advancing age.

The results of experiments on intact mice of various ages are discussed within the context of reports from other laborat--ories.

Repopulation studies with old mice showed that their immune responsiveness was not improved by supplying them with young stem cells and thymus grafts. Experiments in which young or old bone marrow cells were injected into young irradiated syngeneic recipients indicated that stem cells from old mice were capable of proliferating and maturing into functional T and B cells in a manner similar to that observed with young bone marrow stem cells.

The results of an experiment in which old thymus grafts and foetal liver cells were placed in young thymectomised, irradiated syngeneic recipients were inconclusive but did not exclude the possibility that the old thymus is capable of supporting a young lymphocyte population derived from the stem cell inocula.

The inadequacies seen in old mice were discussed in the context of other author's work. It was concluded that the deficiences were not a reflection of an inadequate stem cell pool but may be the result of microenvironmental changes in old mice which prevent the maturation and/or function of lymphoid cells. The nature of the environmental deficiences were discussed with reference to changes in the structure of the lymphoid organs and in factors such as hormonal patterns and skeletal structures.

CHAPTER 1

INTRODUCTION

The effect of age on the immune system has for some years been the subject of intensive research by several authors, some of whom have even suggested that the ageing process itself is a reflection of immunological disorders (BURCH, 1968; BURNET, 1959; WALFORD, 1962, 1969; CORNELIUS 1972). It is clear that changes do occur in the immune capabilities of the animals or persons concerned.

Before the data on the effects of age on the immune system are reviewed, some mention will be made of the development of lymphoid tissues, antibody synthesis and the cellular inter--actions which underlie immune responses. In this way a greater understanding of the young, mature, immune system will be gained and attention focused on the various processes which may become modified or suppressed with increasing age.

1. THE DEVELOPMENT OF LYMPHOID TISSUES AND IMMUNOLOGICAL RESPONSIVENESS.

The development of lymphoid tissues has been studied by many authors. In the mammal the tissues concerned are the thymus, lymph nodes, spleen, and the lymphoid tissues of the gut, including the tonsils, adenoids, Peyers patches and intestinal mucosa.

In the mouse the first lymphoid organ to gain its structural integrity is the thymus (AUERBACH 1960) and its development seems to be dependent on the close proximity of the

mesenchymal tissue to the thymic epithelial anlage (AUERBACH, 1964). It is generally agreed that the thymus is a 'primary' lymphoid organ and in the foetus and newborn animal dissem--inates lymphoid cells to the secondary lymphoid tissues, such as the spleen and lymph nodes, where their immunological function can be exercised (RUTH, 1960; AUERBACH, 1964). It has, however, become increasingly clear in recent years that the precursors of the thymocytes do not originate in the thymus, but migrate there, their earliest detected source being the foetal yolk sac (OWEN and RITTER, 1969; OWEN and RAFF, 1970).

The development of secondary lymphoid organs and tissues depends on the degree of antigenic stimulation to which they are subjected. In germ-free animals there is very little activity in the peripheral tissues (THORBECKE, 1959; MIYAKAWA, 1959). Similar conclusions, on the effect of antigenic stimulation, have been arrived at in work on the developing sheep foetus (SILVERSTEIN et al, 1963).

The passage of cells from the thymus is not the only source of lymphocytes in the peripheral tissues. Cells which do not pass through the thymus have been seen to populate secondary lymphoid tissues (BARNES et al, 1967). Work on the mouse (MILLER, 1963), rat (ARNASON et al, 1962; JANKOVIC et al, 1962; WAKSMAN et al, 1962) and lamb (COLE and MORRIS, 1971) showed that very early thymectomy, whilst preventing the passage of cells via the thymus, did not completely stop the development of the secondary lymphoid tissues, or their ability to respond to certain antigens. Work in the mouse includes observations

that follicles and medullary red pulp cords of neonatally thymectomised animals develop normally, in contrast to the thymus-dependent areas (PARROTT and De SOUSA, 1966) which develop very little. These animals show very pronounced activity in their thymus-independent areas and may have heightened levels of circulating antibody to certain test antigens (HUMPHREY et al, 1964).

The capacity of the foetus to produce antibodies naturally follows the development of the lymphoid tissues. Most early work was performed on the neonatal animal because of the delicate operative procedures involved in sensitising and collecting serum from the foetus. It has been demonstrated that the first antibodies formed in the early post-natal period of children were of the 19S macroglobulin variety (FINK et al, 1962).

Work on the ability of the foetal animal to respond to antigen has, for the most part, been restricted to the lamb (SILVERSTEIN et al, 1963, 1964). The studies showed that there was an apparent stepwise maturation of immunological capability in the foetus. Early in development the spectrum of antigens to which the foetus made a response was very narrow. As the foetus matured so the range of antigens to which responses were made increased. The steplike maturation was reported to show no very obvious pattern and it was concluded that this may reflect the maturation of essentially non-immunological processes. The immunological potential against a large number of antigens may exist in appropriate cells at a very early stage

in development, while the capability to process antigen may still be underdeveloped (LEVINE and BENACERRAF, 1964).

The ability to trap and process antigen would seem to be fundamental to the development of immune responsiveness. Early studies on antigen localisation were made by SABIN (1939), when the process of phagocytosis was seen to occur in tissues of the lymphoid organs. This initial work, together with that perform--ed on adult tissues (NOSSAL et al, 1964a) led to extensive work on the neonatal rat (WILLIAMS, 1966; WILLIAMS and NOSSAL, 1966).

At birth the secondary lymphoid organs of the rat and mouse are very small; the spleen is essentially alymphoid and the lymph nodes are little more than condensations of fibrous and reticular tissues. The newborn rat appears able to clear bacteria and inert particles from the circulation, much of the phagocytosed material being found in the developing lymph nodes (WILLIAMS and NOSSAL, 1966). At this stage there is no division into red and white pulp in the spleen, nor any demarcation into cortex and medulla in the lymph nodes. Between 8 and 16 days after birth, the lymph nodes (popliteal and aortic first) develop a cortex and medulla (WILLIAMS and NOSSAL, 1966). Higher levels of stimulation with antigen were found to acceler--ate this process of differentiation, a conclusion also reached by SILVERSTEIN et al (1970) in the foetal lamb and also suggested by the rapid development of the mature structure of lymphoid tissues in germ-free animals which were brought into conventional conditions and stimulated with antigen (THORBECKE, 1959).

The primary event in the ontogeny of an efficient antigen trapping system appears to be the development of a system of dendritic cells in the cortex of lymph nodes capable of localising antigen around them. During the first 6 weeks of life in the rat there was a gradual accumulation in the cortical region of the lymph nodes which led to the development of recognisable primary follicles. At this time there was no evidence of germinal centre formation, which required a much greater period of antigen stimulation. In the spleen, follicular areas matured from a continuous rim of diffuse tissue in the marginal zone. The maturation of the adult medulla in the lymph nodes, with the associated ability of macrophages to localise antigen, also takes place over this time (WILLIAMS and NOSSAL, 1966)

Not only initial antigen localisation improved over the first few weeks of life, but also the ability to retain antigen for considerable periods of time. It was concluded (NOSSAL and WILLIAMS, 1966; NOSSAL and ADA, 1971) that optimal development of an antibody forming clone required more than one contact with antigen, and poor antigen retention would hinder this process. WILLIAMS (1966) reported that antibody production could be stimulated in rats over the first 2 weeks of life by 3 injections of polymerised flagellin, but that a single injection, of no matter how high a dose, failed to cause any significant antibody production. The earlier observations by FINK and his colleagues (1962) may be included in the observations that the change from 19S to 7S antibody production is closely associated with the development of primary lymphoid follicles in both lymph

nodes and the spleen (WILLIAMS, 1966).

The development of the mature structural state, rather than simply the maturation of lymphocytes capable of interacting with antigen, becomes important in the light of previous work by Nossal (1959) in which it was found that mature lymphocytes transferred to very young recipients failed to mount a full immune response, as they did when placed in adult recipients.

2. THE ANTIGEN TRAPPING CAPABILITIES OF YOUNG MATURE LYMPHOID TISSUES.

It is difficult, in a short account, to generalise on the structure of lymphoid organs or on their ability to trap and retain antigen. Spleens and lymph nodes differ somewhat in their organisation. Different antigens may be handled in different ways. Not all species behave in exactly the same way. However, as long as these points are kept in mind, it should not prevent an attempt to summarise the features of young, mature, lymphoid organs and the events that have been observed following antigen stimulation.

Most studies have involved the injection of radiolabelled antigen and autoradiographic techniques. In the rat localisation in the lymph nodes has been studied as early as 3 minutes after antigen injection (NOSSAL et al, 1968a, 1968b). Antigen was seen to localise, initially, within medullary macrophages. More recent studies using electron microscope autoradiographs (NOSSAL et al, 1968a, 1968b) have confirmed the initial localisation in the medulla and also produced evidence that the uptake of antigen occurs by pinocytosis and possibly

by direct penetration of the plasma membrane.

In vitro studies by Unanue and Askonas (1968) and Unanue and Cerottini (1970) showed that up to 20 percent of haemocyanin taken up by peritoneal macrophages appeared to remain immunogenic and could lead to antibody production when the appropriate cells were introduced into the system. Although it is possible that not all antigens would be treated in the same way and also that *in vitro* experiments may not accurately reflect events *in vivo*, Nossal and Ada (1971) have suggested that the presence of undena--tured antigen on the surface of macrophages could play an important role in stimulating lymphocytes which come into contact with it.

Localisation in the diffuse cortex of lymph nodes did not occur at any time (NOSSAL et al, 1966), but was seen to occur in the more dense follicular areas and germinal centres (KAPLAN et al, 1950; MELLORS and BRYOSKO, 1962; WHITE et al, 1967; WHITE 1963; NOSSAL et al, 1964a, 1964b). The studies by White (1963; WHITE et al 1967) and more recent work by Nossal and his colleagues (1968a, 1968b) showed that whereas in the lymph node medulla antigen was taken up intracellularly, in the follicular areas it remained attached extracellularly to long, rather complex, dendritic processes.

Antigen localisation in the spleen differs from that in the lymph nodes. Although the pattern of localisation in the follicles of the white pulp is essentially the same as that in the follicles of the lymph nodes, there is virtually no local--isation in the splenic red pulp, even 24 hours after antigen

after antigen injection (NOSSAL et al, 1966). Some labelling of the inner marginal zone occurred with flagella antigen, but not with flagellin or polymerised flagellin (NOSSAL et al, 1966).

Since antibody formation is known to occur mostly in the lymph node medulla and the splenic red pulp, it is important to correlate localisation patterns with areas of antibody production and in particular to see whether antigen localises within plasma cells. Most studies have failed to demonstrate the presence of antigen within plasma cells (McDEVITT et al, 1966; De PETRIS and KARLSBAD, 1965) and these are confirmed by more detailed studies using the electron microscope (NOSSAL et al, 1968a, 1968b). It has been concluded that those studies which have claimed to show antigen within plasma cells did so because of the excessive amounts of antigen used and consequent false labelling of certain areas and cells (NOSSAL and ADA, 1971). Since the spleen has not been seen to localise antigen in the red pulp, and yet appears to produce antibody at that site (McDEVITT et al, 1966), it is possible that if macrophage processed antigen is important to the induction of antibody formation, then spatial proximity of macrophage localised antigen to the devloping clones of antibody producing cells may not be an essential prerequisite. However, at this time this conclusion cannot be upheld with any certainty since there is no reason to suppose that antibody-forming cell precursors cannot migrate to the red pulp from some other source.

A great deal of interest has been shown in the role of follicular localisation, particularly in relation to germinal centres (HANNA et al, 1968, 1971 ; NOSSAL and ADA, 1971). It is clear that the antigen-retaining dendritic cells of both primary

and secondary follicles are very complex, with a highly convoluted plasma membrane which has the ability to retain antigen in an undenatured form for long periods of time. There is therefore ample oportunity for lymphocytes which migrate through the follicular areas to come into close contact with antigen.

There is considerable evidence that the presence of large amounts of extracellularly-bound antigen in follicular areas of either the spleen or lymph nodes is due to the presence of antibody, possibly naturally occuring opsonins (WHITE, 1963; NOSSAL et al, 1965b; WILLIAMS 1966; McDEVITT et al, 1966; HUMPHREY and FRANK, 1967; NOSSAL and ADA, 1971).

The precise role of germinal centres, whether formed de novo or pre-existing and showing increased proliferative activity as a result of antigen stimulation, is not known. Considerable evidence suggests, however, that the cellular events which occur within germinal centres are closely concerned with the formation of 7S, IgG antibody (NOSSAL et al, 1964; WILLIAMS, 1966; WILLIAMS and NOSSAL, 1966) and more specificly with the formation of IgG antibody-forming cells and their pre--cursors (COTTIER et al, 1967; HANNA et al, 1966; NOSSAL et al 1964; THORBECKE and HURLIMANN, 1965; WAKEFIELD and THORBECKE, 1968a, 1968b).

3. CELLULAR INTERACTIONS IN THE IMMUNE RESPONSE.

The way in which the lymphoid system reacts to antigen, with the production of humoral antibody or a population of sensitised lymphocytes, is by no means precisely formulated.

However, some of the mechanisms which take place after antigenic stimulation have, in recent years, been established with some certainty.

It should be stressed that most of the work on cellular interactions and the conclusions drawn from these experimental studies have been restricted to a very small number of mammalian species. Most recent work, for example that on subpopulations of lymphocytes and their possible role, has been performed in the mouse or on mouse cells. It should not be automatically assumed that what is true for one species is characteristic of all mammals.

The presence of more than one type of lymphocyte population has already been hinted at in the section on the development of the lymphoid system. Work in the mouse has shown that in these animals, two basic populations occur. One is derived from the bone marrow and passes directly to the peripheral tissues and has been called the B cell population (ROITT et al 1969). The other is characterised by a period of residence in the thymus before passing to the peripheral tissues and has been called the T cell population (ROITT et al, 1969). Identification of these two populations has been assisted in a number of ways. They may be distinguished by their electrophoretic mobility (WIOLAND et al 1972; NORDLING et al, 1972), or their ability to be distinguished serologically, by the presence of specific surface antigenic markers (BOYSE et al, 1968; REIF and ALLEN, 1964; RAFF, 1969; SCHLESINGER and YRON, 1970). B cells are readily recognised by surface immunoglobulin staining (RABELLINO et al, 1971) and by

the presence of Fc receptors detected by the uptake of agregated gamma globulin (DICKLER and KUNKEL, 1972). A recent study made on human lymphocytes has attempted a clear identification of 'T' and 'B' cells by external appearance (POLLIACK et al, 1973). A more straightforward study of internal differences, based on ultrastructural studies, has also been attempted (MATTER et al, 1972).

Co-operation between T and B cells is an established feature of many immune responses (CLAMAN and CHAPERON, 1969; MILLER and MITCHELL, 1969; TAYLOR, 1969; SCHWARTZ et al, 1970; MILLER et al, 1971). This fact was very clearly demonstrated in experiments where mixtures of T and B cells, transferred to irradiated syngeneic recipients, produced more antibody in response to antigen than was predicted from the performance of either cell type alone (CLAMAN and CHAPERON, 1969). It has been shown, however, that the production of antibody is a function of the B cell alone (MILLER and MITCHELL, 1969; NOSSAL et al, 1969).

Although it is possible that the underlying mechanism of all immune responses is basically similar, there is a pronounced difference at the effector level. In one instance, the production of antibody is fundamental to the effectiveness of the response. On the other hand, there are immune responses in which antibody may be formed specifically, but has no clearly understood function. The latter reactions involve the formation of a population of sensitised lymphocytes in delayed hypersensitivity reactions, graft rejection and graft versus host responses. These responses have been shown to be the function of T cells

(DAVIES et al, 1969). Early evidence in support of this conclusion was based on experiments in the chicken in which the B cell population is derived from a distinct organ, the bursa of Fabricius. For example, delayed hypersensitivity reactions occur with normal vigour in chickens that have been bursectom--ised (JANKOVIC et al, 1963). Those reactions in which circu--lating antibody production is important, are dependent on the presence of B cells and are severely depressed by neonatal bursectomy (JANKOVIC et al, 1966). The failure of cellular reactions to occur in mammals after neonatal thymectomy provides indirect evidence of the importance of T cells in animals other than birds. More direct evidence of the role of the T cell, in the mammal, was provided by DAVIES et al (1969) using chromosome marked lymphocyte populations.

It has been found that not all reactions involving the production of humoral antibody imply co-operation between T and B cells. The response to some antigens (large molecules of repeating units) appears to be independent of the presence of T cells (HOWARD et al, 1971a, 1971b). Indeed it is becoming increasingly clear that some T cells, far from enhancing immune responses, may actually have a suppressive effect in some (BAKER et al, 1970; GERSHON and KONDO, 1971; GERSHON et al, 1972; GORCZINSKI et al, 1972; KERBEL and EDINGER, 1972). It is possible that whilst some T cells do initiate co-operative events others do not and are suppressive in nature. There is some evidence that two populations of T cell occur in the mouse (BACH et al, 1971; CANTOR and ASOFSKY, 1972).

The possibility of there being two distinct populations of B cell has also been suggested (GERSHON and KONDO, 1970) and more recently it has been proposed that they may be distinguished by their dependence on T cell co-operation (PLAYFAIR and PURVES 1971a, 1971b).

No mention so far has been made of the macrophage and its involvment in the events which lead up to antibody formation. The activity of macrophages has been extensively reviewed (SCHWARTZ et al, 1970). The conclusions drawn were that macroph--ages participate in immune responses in several different ways. One of the most important may be in antigen handling. The handling and consequent processing may be important to intrinsically weak antigens such as bovine serum albumin in the mouse (MITCHISON, 1969). Macrophages may have another important function. By ingesting, storing and eventually releasing antigen macrophages compete with lymphocytes for it. In this way macrophages may control the amount of antigen available to immunocompetent cells and ultimately the amount of antibody produced. For example, when peritoneal exudate cells were cultured with sheep erythrocytes and then transferred to syngeneic recipients, the immunogenicity of the erythrocytes was inversely proportional to the number of phagocytosed cells (PERKINS and MAKINODAN, 1965).

Within the last two years extensive <u>in vitro</u> experiments have been attempted to explain the events of cellular co-operat--ion and bring together much of the earlier <u>in vivo</u> work within a single framework (FELDMANN and NOSSAL, 1972).

Initial studies gave direct evidence of some form of collaborative factor between T and B cells separated by a cell-impermeable membrane. Cellular co-operation took place with no direct contact between T and B cells (FELDMANN and BASTEN, 1972a, 1972b). In the system used, only the T cells which were 'educated' or 'activated' (i.e. those cultured *in vivo* in the presence of antigen) to the particular macromolecule or carrier antigen used *in vitro* were effective as helpers.

Evidence that the macrophage was required for T - B collaboration was obtained by inhibiting macrophage activity (FELDMANN and PALMER, 1971; SHORTMAN and PALMER, 1971). This was seen to affect those responses which were T cell dependent, but the response to a T cell independent antigen, DNP-flagella, was unaffected (FELDMANN, 1972a). The requirement for macrophages in most responses studied suggested to the investigators that the collaborative factor produced by the T cells acted not on the B cell directly but via the macrophage. The same report (FELDMANN, 1972a) also proposed that the specific factor liberated by the T cell was an IgM-like molecule. There is some evidence that the Ig molecule present on the surface of T cells is not the same as that found on the B cell (CONE et al, 1973).

The collaborative events which occur between T and B cells and macrophages have been visualised as follows. Activated T cells release their IgM-like molecules, probably already complexed to antigen, This complex binds to the surface of macrophages where it forms a concentrated depot of antigenic determinants. B cells are immunised upon contact with these

determinants (FELDMANN and NOSSAL, 1972).

Such a proposal is attractive because the last event, namely the immunisation of the B cell, could involve the same pattern of interaction between antigenic determinants and receptor molecules as that which occurs in the thymus independent responses to such substances as DNP-flagella (FELDMANN and NOSSAL, 1972).

The studies so far carried out favour the interpretation that T - B collaboration takes place in germinal centres (FELDMANN and NOSSAL, 1972). Evidence in support of this conclusion is taken from work on congenitally thymusless 'nude' mice. Several reports have revealed that responses in these animals to T cell dependent antigens is severely depressed (WORTIS, 1971; MITCHELL et al, 1972; PRITCHARD and MICKLEM, 1972). MITCHELL's work, which included a detailed histological study, showed that 'nude' mice possess primary lymphoid follicles and can localise antigen in them, yet the antibody response was primarily 19S in nature. It was suggested that T cell dependent responses and consequent 7S antibody production is dependent on germinal centre formation.

FELDMANN's work, using <u>in vitro</u> systems, may not answer all the queries regarding cellular co-operation but does provide an explanation for a great variety of enhanced and suppressed events which may alter the normal pattern of immune responsive--ness. For example, T cell suppression may be explained on the basis of over-production of collaborative factor by the T cells which would saturate the macrophages and then attach to the B

directly, leading to antibody-mediated tolerance (FELDMANN and NOSSAL, 1972). Other explanations regarding experimental tolerance induction (FELDMANN, 1972b) and antigenic competition (SCHRADER and FELDMANN, 1972) have also been put forward.

4. THE EFFECT OF AGE ON THE IMMUNE RESPONSE

It is not the purpose of this thesis to support any particular theory of ageing. However, as has already been mentioned, several authors have expressed the opinion that ageing is fundamentally concerned with immunological phenomena (BURNET, 1959; WALFORD, 1962, 1969; BURCH, 1968; CORNELIUS, 1972; BURSTEIN et al, 1973). Several other theories have been advanced. Among the most prominant are those that focus on RNA and the possible failure of protein synthesis pathways (ORGEL, 1963; HARRISON and HOLLIDAY, 1967).

The immune theory of ageing, as proposed by BURNET (1959) and supported at length in the 'Immunologic theory of ageing' (WALFORD, 1969), suggests that the reticulo-endothelial system undergoes antigenic diversification with age, as a result of somatic mutation, and that the mutant cells come to recognise the body as foreign and react against it, proliferating in the process. Lymphocytes suffering gene loss at a strong histo--compatability locus might well die as a result of 'allergic suicide'. Cells mutated at 'weak' loci might well proliferate yielding clonal proliferation of the mitotic error (JARVIC 1965).

The concept of gene loss in lymphoid cells, which undergo clonal proliferation in the presence of excess host antigen, represents the form in which the immune theory of ageing is most easily understood. Other events can, however, be visualised

A failure in the homoeostatic mechanisms controlling tolerance for self antigens might have similar effects without requiring actual genetic diversification or somatic mutation (WALFORD, 1969). Alternatively, as a result of increasing age and somatic mutation the lymphoid system may lose some of its functional acuity: its consequent failure to destroy aberrant cells may permit wider antigenic diversification in the soma and threaten the orderly functioning of entire systems of cells (HUEMER, 1967).

The possibilities for antigenic diversification of the body's cells are in some sense proportional to the degree of complexity within the body: the more complex it is, the greater the chance for divergence. Mutation of DNA coding for several sites on a biochemically active protein might occur without actually affecting the functioning of the molecule (CHILDS and KALOUSTIA, 1968). In such cases the molecule might well be recognised as foreign by the body. WALFORD (1969) envisaged a similar process occurring in the lymphoid cells where large numbers of mutations might occur without loss of vitality.

The experimental evidence for the effects of age on the immune system falls largely into two groups. Firstly, there is information on the size, cell content and morphology of the lymphoid organs. Secondly, there is more direct evidence, involving for example, variations in the quantity and quality of antibody produced, transplantation immunity and autoimmune phenomena.

(a) LYMPHOID ORGANS IN RELATION TO AGE.

Much of the work related to this field has not involved systematic research but rather chance observations, for example, during post mortem examination. This is true of the data on organ weights. Within the last 15 to 20 years some of the earlier work has been repreated and extended under more clearly defined conditions.

PEPPER (1961), working with A strain mice, ranging in age from 5 to 440 days, recorded thymus and lymph node weights as a function of age. Considered in relation to body weight, the thymus attained a maximum value at 15 days of age and thereafter declined in an exponential manner. Body weight increased with age, levelled off and only declined in extreme old age. METCALF et al (1967) found that the weight of the thymus in 2 month old C57Bl mice averaged 44mg and in 20 month old mice was only 16mg. Similar findings related to thymus and body weights have been reported by several authors (MAKINODAN and PETERSON, 1966a; THUNG, 1966; WALFORD, 1967).

The structure of the thymus has been analysed in humans with respect to age (BOYD, 1932). Coupled with the decrease in size and weight was a relatively greater decrease in the size of the cortical and medullary tissues and a relative accumulation of connective tissue and fat. Similar findings have been reported by WALFORD (1967). However, it has been stressed (MILLER, 1962) that although these changes are apparent in the thymus, the functional ability of the organ does not appear to show the same rapid involution.

The rather limited data on lymph nodes includes a report by PEPPER (1961) who showed that after their initial growth and development in young A strain mice, the lymph nodes changed very little in weight with increasing age.

Data on spleens show that after their initial increase in size and weight, the organs continue to grow at a slow rate and decline only when the animal is very old. Typical of these results were those obtained by WALFORD (1969), where the average spleen weight in female CBA mice 1 month after birth was about 80mg and in 109 week old mice was about 140mg. A similar trend was observed in males.

Although structural changes in the peripheral tissues do not appear as early as those in the thymus, several reports have shown that they may be very pronounced. Germinal centres were reported to decline in number in C57Bl mice (METCALF et al, 1967). Similar findings were reported by LEGGE and AUSTIN (1968), who also demonstrated that the changes in the lymph nodes included an age-dependent loss of the detailed structure; the differentiated areas of the cortex and medulla became rather diffuse in nature. HANNA et al (1971a) confirmed these findings in the spleen and also reported on the age-dependent rise in the incidence of reticulum cell sarcoma in this organ. The report, in this last case, was based on observations in (C57Bl x C3H)F_1 mice.

Details of changes in the Peyers patches include a report by ANDREW and ANDREW (1957), which showed that the number of lymphocytes per unit area in the gut of young C57Bl mice was

227, compared to about 109 per unit area in mice over 573 days of age.

The above information, on the state of the lymphoid organs in conventionally bred mice, contrasts with that from animals reared in germ-free conditions. In germ-free pigs the thymus has been reported to remain fully developed even into old age, whilst the peripheral tissues, not having experienced antigenic stimulation, remained poorly developed (GORDON, 1959). Similar results were obtained by other authors (MIYAKAWA, 1959; THORBECKE, 1959), in guinea-pigs and a variety of laboratory animals. These differences were lost and the animals conformed to the conventional condition very rapidly, once they were placed in an antigenic environment (MIYAKAWA, 1959).

As will be discussed later, the effects of hormones on the lymphoid organs may be of considerable importance in gaining a greater understanding of the mechanisms involved in the 'ageing' of the immune system.

The thymus especially has been shown to be very sensitive to changes in hormonal patterns. In A strain mice, it declined to 20 percent of its normal size during pregnancy and lactation (PEPPER, 1961). After the young were weaned the weight of the thymus increased, with some over compensation, before dropping to its pre-pregnant state. It has been demonstrated experimentally that both oestrogens and androgens can induce a marked involution of the thymus (WEAVER, 1955).

PEPPER (1961) also showed that both the thymus and the lymph nodes were sensitive to the action of corticosteroids.

Their effects on the thymus have been claimed to be the most important factor opposing thymus lymphopoiesis (ISHIDATE and METCALF, 1963; SANTISTEBAN, 1960). Adrenalectomy has been found to increase the weight of the thymus and the spleen in CBA mice of both sexes and all ages (MILLER and OSOBA, 1967). The increase in weight of the thymus was shown to be associated with mitotic activity of the thymic lymphocyte population.

Stress conditions such as restraint, overcrowding, fighting and infection, presumed to be reflected in an increase in adrenocortical activity, were found to cause atrophy of the thymus (DOUGHERTY et al, 1964). The decrease in cellular activity associated with corticosteroid action was shown to be a result of lymphocytolysis rather than any massive export of cells into the extrathymic lymphatics (ISHIDATE and METCALF, 1963). Recent work on the effects of corticosteroids have demonstrated that both T and B cells are sensitive to their action during certain stages of development (COHEN and CLAMAN 1971).

Clearly the effects of hormonal changes on the lymphatic system are very complex but they may underlie the anatomical changes shown to occur with increasing age in the lymphatic tissues, with pronounced effects on the functional ability of the lymphocyte populations.

(b) CELLULAR ASPECTS OF IMMUNE FUNCTION RELATED TO AGE

(i) The effect of age on the formation of antibodies.

Quantitative variation with age in titres of so-called naturally occuring isoantibodies have been reported by a number of authors. Both anti-A and anti-B antibodies have been reported to exhibit a decrease in titre with age (THOMSEN and KETTEL, 1929; GINGOLD et al, 1958; LEWI, 1968). A similar decrease in the titre of 'naturally' occuring haemolysins to sheep erythrocytes and rabbit erythrocytes have been reported in humans (FRIEDBERGER et al, 1929; PAUL and BUNNEL, 1932). Antibodies to poliomyelitis virus, measles and asian influenza have all been reported to decrease with age (SCHWICK and BECKER, 1969).

Included with those experiments which involved the injection of antigen are those of GOULET and KAUFMANN (1964/5), who studied the responses of 3 and 23 month old rats to injections of bovine serum albumin. The younger rats responded sooner and to a higher titre. On continued stimulation the older rats narrowed, but did not close, the gap. Experiments with strain 13 guinea-pigs aged 2, 6 and 24 to 36 months , showed that the response to 6.0mg bovine gamma globulin was significantly lower in the older group compared to both of the two younger groups (BAER and BOWSER, 1963).

The most extensive work of recent years on the cellular basis of humoral activity in relation to age is that of Makinodan and his colleagues. Their initial studies (ALBRIGHT and MAKINODAN, 1966) were carried out on (C3H x 101)F_1 mice,

ranging in age from 1 to 126 weeks. They injected a fixed number of spleen cells from donor animals of various ages into heavily irradiated, young, syngeneic recipients. Under these conditions only the injected donor cells were able to respond and form antibody against an erythrocytic antigen. The antibody levels in the serum of the antigen injected, recipient mice were measured by a haemagglutination technique. A linear relationship was found to exist between the antibody level and the number of spleen cells injected from any one source. The response of the donor cells was assayed on day 6 after the injection of antigen with the donor cells. It was maximal in mice injected with cells from 40 week old donors and thereafter declined gradually with age, so that 126 week old donor cells produced one seventh to one fourth the titre of antibody produced by the younger cells. However, the ability of the intact animal to produce antibody was reported to decrease more rapidly than did the number of immunocompetent cells as assayed by the transfer system. This was used as preliminary evidence that environmental change in the old mouse inhibited the function of the reduced number of immunocompetent cells (ALBRIGHT and MAKINODAN, 1966).

The data on the primary antibody forming capacity of fixed numbers of spleen cells from donor mice of different ages were confirmed in (C57Bl x C3H)F_1 mice, although the maximum response in these animals was found in 20 week old donors (MAKINODAN and PETERSON, 1966a). A later study included information on splenic size and responsiveness. Spleens of old mice were often enlarged. In 108 week old mice the spleens, whether enlarged or

not, were all about equivalent in primary antibody forming capacity. However, in 120 week old mice the potential of enlarged spleens was as low as 6 percent of that of normal sized spleens from animals of the same age (MAKINODAN and PETERSON, 1966b). This fact has more recently been associated with reticulum cells sarcoma (CHINO et al, 1971; HANNA et al, 1971a; MAKINODAN et al 1971c).

The above work was based on haemagglutination titres. The results were extended by using the haemolytic plaque technique (JERNE et al 1963) for the identification of antibody producing cells (mainly 19S). The work was based on the number of plaque-forming cells in the spleens of CBA mice 5 days after the injection of sheep erythrocytes. Maximal responses were found in spleens of 7 month old mice. By 24 months there was an 80 percent drop in the number of plaque forming cells and by 36 months a 94 percent drop (WIGZELL and STJERNSWARD, 1966).

A drop in the maximal response of 18 month old C57Bl and (C57Bl x AKR)F_1 mice, to sheep erythrocytes, has also been reported (METCALF et al, 1967). However, the same paper pointed out that depressed responses seen in the above two strains, were not a feature of similarly aged C3H mice which responded as well as young animals. A later report (KISHIMOTO et al, 1969) confirmed the depressed response in the C57Bl mice to sheep erythrocytes but went on to report that the response to bacterial alpha amylase showed no reduction in C57Bl mice up to 19 months of age.

LEGGE and AUSTIN (1968) also showed that the primary response to another protein antigen, this time polymerised

flagellin, was equivalent in young and old C57Bl mice when measured by a bacterial immobilisation technique.

Both WIGZELL and STJERNSWARD (1966) and MAKINODAN and PETERSON (1966b) showed that the greatest reduction in the antibody type, produced as a result of antigen injection, was in the 7S classes, as measured by 2-mercaptoethanol resistance in serum antibodies. RAM (1967) went so far as to state that the antibody response of old animals was characteristically of the 19S macroglobulin variety. The results of experiments using the haemolytic plaque assay, which showed a reduction in the ability to produce 19S antibody forming cells (WIGZELL and STJERNSWARD, 1966; METCALF et al, 1967; KISHIMOTO et al, 1969) were extended by the additional use of the indirect plaque technique for the identification of 7S antibody producing cells by MAKINODAN and his colleagues (1971a).

It should be pointed out that reference to direct plaque forming cells refers to those which produce mainly 19S antibodies and indirect plaque forming cells refers to those which produce mainly 7S antibodies. There is a full explanation of these terms and the modified plaque technique in the methods section.

MAKINODAN et al (1971a) used 3 and 30 month old (C57Bl x C3H)F_1 mice of both sexes. Maximum numbers of direct plaque forming cells (DPFC) and indirect plaque forming cells (IPFC) were obtained on days 4 and 6 in young mice and on days 6 and 12 in old mice respectively. Mice were grouped according to the conditions in which they had been raised, i.e. in 'clean' or 'dirty' animal farms. Background responses showed

considerable variation and there was no significant difference between ages or between environmental conditions with the exception of the old mice raised in the 'clean' farms, which had more DPFC and IPFC in their spleens than old mice from the 'dirty' farms. Following antigenic stimulation slightly, but significantly, higher DPFC and IPFC responses were detected in young mice from the dirty farms compared to those raised in the 'clean' farms. This effect was not seen in the old mice. Both the DPFC and IPFC responses of older mice were significantly lower than those of the younger animals. The DPFC responding capacity of old mice from 'clean' and 'dirty' farms decreased 17-fold and 52-fold respectively and the IPFC responding capacity decreased 8-fold and 30-fold respectively. The approximately 2-fold difference in the relative decrease with advancing age between 19S, DPFC and 7S, IPFC was not significant. However, it is interesting that these results show a tendancy for a greater decline in the capacity to produce 19S rather than 7S antibodies (confirmed by haemagglutination titres), when all other information talks of a greater decrease in 7S classes.

This general pattern of a reduced immune capability in old mice was confused by the occurence, in old animals, of reticulum cell sarcoma, with variable involvement of the spleen.

Information on the avidity of antibodies produced in response to antigenic stimulation includes a report by BAUMGARTNER (1934) who measured the avidity of antibody in rabbits following the injection of Bacillus enteritidis and

sheep erythrocytes. The avidity was measured by flocculation and haemolysis tests. For both antigens the avidity of the antibody produced was seen to decrease in rabbits over 2 years old.

Although work on the secondary response of old animals is not, perhaps, as extensive as that on primary responses there have been reports that it too decreases with age. OBERHOFFER et al,(1957) reported that persons over 60 years of age showed a decreased ability to produce anti-A antibody, following a second injection of group specific substance, than did persons 30 to 40 years of age. However, LEWI (1968) reported that there was no difference in the ability of old persons, compared to young, to produce antibodies to either group A or group B substances after a second injection.

The secondary antibody forming capacity of (C57Bl x C3H)F_1 mice has been examined by means of a system of cell transfers similar to those used to assay the primary response (MAKINODAN and PETERSON, 1966b). The assay involved priming the cells in donor mice of various ages to an erythrocytic antigen. Spleen cells from the primed mice were transferred, at intervals after injection, together with the challenge dose of antigen, into irradiated, young, syngeneic recipients. The results, although higher in titre, followed very closely the results obtained for the primary response: a decline in antibody forming capacity with age.

Using the intact animal GOULET and KAUFMANN (1964/5) showed that significant differences occurred between old and young

rats after repeated injections of bovine serum albumin. The primary response to polymerised flagellin was found to be similar in young (3 to 4 months) and old (18 to 27) C57Bl mice, but in the secondary response the younger animals showed a higher antibody titre (LEGGE and AUSTIN, 1968).

MAKINODAN et al (1971b) used the same plaque technique as that used for the primary response for the identification of DPFC and IPFC after secondary stimulation with sheep erythrocytes in (C57Bl x C3H)F_1 mice of both sexes, of 3 and 30 months of age and raised in 'clean' or 'dirty' animal farms. Mice assayed for plaque numbers one month after priming, but before secondary injection, showed that old mice, whether raised in 'clean' or 'dirty' farms, were not significantly above unstimulated background controls with respect to either DPFC or IPFC numbers. In young mice, although DPFC numbers were approximately back to unstimulated levels, IPFC numbers were considerably above background. The greatest number of IPFC in young mice, at this time, were in those that had been raised in 'dirty' conditions; they had approximately ten times as many as young mice raised in 'clean' conditions.

Preliminary studies on the response to a second injection of antigen showed that in young mice and old mice maximum numbers of both DPFC and IPFC were obtained on days 4 and 5 respectively. Differences related to sex for both DPFC and IPFC were small, as were age related differences in DPFC numbers. The greatest increase in the secondary response was reflected in the number of indirect plaques. Young mice mounted a strong

secondary response, about 6 times higher than in old mice. In both 'clean' and 'dirty' mice the relative increase in the indirect plaque response (i.e. the number of IPFC in the secondary response compared to the number in the primary response in any one age group) was greatest in the old mice, with a relative increase of about 15-fold, compared to an increase of about 4-fold in the young mice.

This last report (MAKINODAN et al, 1971b) and the one by LEGGE and AUSTIN (1968) commented on the importance of germinal centres. Since germinal centres had been shown to decrease in number in old mice LEGGE and AUSTIN concluded that germinal centre activity was important only to the secondary response. This conclusion was based on their observations that the primary response to polymerised flagellin was similar in young and old mice whilst the secondary response was significantly different. MAKINODAN and his colleagues, however, concluded that germinal centres were not important in the secondary response, since they had demonstrated a relatively greater increase in the number of IPFC in old mice compared to young in the secondary response to sheep erythrocytes. HANNA et al (1971b), in a series of experiments involving transfers of young and old spleen cells into young syngeneic recipients, concluded that 7S antibody formation, reflected both in IPFC numbers and serum antibody titres, was dependent on germinal centre activity.

Levels of antibodies directed against agents which are more or less endemic in nature, or to which part of the population may have had prior exposure, have not been reported

to show any clear-cut age-dependent rise or fall. One problem is that the investigator cannot be sure, especially in human populations, where most of the work has been done, whether he is measuring a primary or secondary response. Most observations appear to suggest that older people respond rather well to complex endemic agents, or at least not as poorly as might be expected from the results of experiments involving the injection of well defined antigens.

DAVENPORT et al (1953) measured antibody levels in a sample of the population to various types of influenza virus (types A and C and swine influenza). Sera were collected in 1952 during a non-epidemic period and 18 months after the last outbreak of type A virus in the area. For type A virus isolated since 1946, the titres in the 1953 population were maximal at age 12 and low or undetectable after 20 years of age. For type A virus isolated between 1933 and 1943, no antibody was detected in pooled sera from persons less than 11 years of age. For swine influenza, antibodies were just evident at age 29 and were maximal between 35 to 38 years of age. Substantial antibody levels were still found in all old sera tested. For type C virus, antibody was present in all old sera at levels similar to those of young children.

These data were interpreted as indicating that in early years the antibody spectrum is narrow, that it becomes broader in later life and that successive experiences with viruses of related, but varying, antigenicity result in a composite of antibodies orientated towards a larger number of antigens

common to influenza viruses, which confer a broader immunity with age. This immunity limits the infection even with more recent strains to which the older population may never have been previously exposed. This view was supported by a later study following the 1953 influenza epidemic in the United States (HENNESSY et al, 1955).

The presence of somewhat lower titres for specific antigens, accompanied by a broader, more protective immune state needs to be taken into account when considering responses to more complex natural antigens. Because of his background of exposure an older person may demonstrate better immunity, in certain cases, than younger persons. Nevertheless, as suggested by KOLODNY (1939), the increased resistance to infection sometimes noted clinically with age may not be attributable merely to past exposure to the same or related antigen(s). In a rat colony never previously exposed to Trypanosoma cruzi or to T. lewisi, he observed that the rats were as resistant to infection at 18 to 24 months as at 3 to 6 months of age. Both groups were far more resistant than young rats less than 75 days old. The resistance could not be passively transferred. Clearly non-specific factors also influence susceptibility to infection.

(ii) The age related incidence of auto-antibodies.

With the refinement in recent years of various serological techniques for assaying auto-antibodies, it has become clear that many such antibodies can be demonstrated in the serum of an animal or person showing no outward sign of auto-immune disease.

Whether these antibodies are actually deleterious to the host is less certain. It has been claimed that all individuals possess rheumatoid factors (MULLER-EBERHARD, 1961). Twenty percent of normal sera are thought to contain antibodies to buried determinants of gamma globulin (OSTERLAND et al, 1963).

It has also been claimed that auto-antibodies show increased incidence with age, a subject which has been extensively reviewed by WALFORD (1969). Some auto-antibodies may reflect a genuine immunological disorder. There is, for instance, little doubt that anti-DNA or anti-nuclear antibodies are of real pathogenic significance in the glomerulonephritis of the NZB-NZW mouse and of human systemic lupus erythematosus (STROM et al, 1968). Auto-antibodies include such substances as rheumatoid factor, anti-nuclear factor, antibodies directed against the thyroid gland and gastric parietal cells and anti-erythrocytic antibodies (WALFORD, 1969). For most factors, the incidence is higher in females in younger life but with advancing age, the male incidence tends to equal or even surpass that of the females.

(iii) <u>The age related incidence of auto-immune diseases and diseases with auto-immune components</u>.

The increasing incidence of auto-antibodies with age has been interpreted by some as evidence that the ageing process itself is an auto-immune phenomenon (WALFORD, 1969). There is some evidence that certain pathologic conditions in aged individuals result from auto-immune phenomena. For example, it

was reported that auto-immune haemolytic anaemias can show an age-related increase in incidence (DAUSSET and COLOMBANI, 1959; DACIE, 1962). Rheumatoid arthritis has been reported to be most common in persons between 50 to 60 years of age (ROBINSON, 1963). Polyarteritic lesions were not seen in Sherman strain rats before the seven hundredth day of life (WILENS and SPROUL, 1938). Hashimoto's thyroiditis, a disease with clear autoimmune affiliations, has been reported to be most common in persons over 50 years of age.

Other examples have been cited in support of an auto-immune theory of ageing. For a more complete survey the reader is referred to 'The immunologic theory of ageing' (WALFORD, 1969).

(iv) The effect of age on cell mediated immunity.

With few exceptions it is becoming clear that cell-mediated responses may also be severely affected by age. TELLER et al (1964), grafting across strong histocompatibility barriers, reported that there was a decrease in the ability to reject with age. On the other hand, KROHN found that when skin was grafted between inbred strains of mice differing at the H-2 histocompatibility locus, the allografts were rejected as vigorously in old as in young animals (KROHN, 1961, 1962).

SIMONSEN (1960) found that spleen cells from aged (3 years old) C3H mice were incapable of producing the characteris-tic splenomegaly in infant (C3H x DBA)F_1 hybrids that is produced by young adult C3H cells. Maximum resistance to transplants of 3-methylcholanthrene-induced sarcomas in CBA mice

was about 6 months of age. At 22 months there was a 100 percent take of the sarcoma (STJERNSWARD, 1966).

Experiments with strain 13 guinea-pigs of 2, 6 and 24 to 36 months of age showed that the contact skin sensitivity reaction to synthetic 3-Pentadecylcatechol, after a course of injections of the sensitising agent in Freunds complete adjuvant, was greater in the 2 month old animals than in either of the two older groups (BAER and BOWSER, 1963).

KISHIMOTO et al (1973) have reported that the ability of lymphoid cells from 15 to 19 month old C57Bl mice to induce a GVH reaction was reduced, compared to the ability of cells from 2 to 3 month old mice. A decline in the ability of human lymphoid cells to respond to PHA has been reported (PISCIOTTA, 1967), also the ability of humans to give delayed hypersensitivity reactions (WALDORF et al, 1967).

(v) <u>The effect of age on the inductive and proliferative events in the immune response</u>.

It is clear from the review above that changes do occur in the immunological capabilities of old animals. Although some evidence is available of decreased activity in cell-mediated immunity, the most convincing evidence of decreased activity has been presented for humoral responses. It is true that most of these responses have been to sheep erythrocytes and what is true for one antigen does not follow for all others. At the same time a number of more recent reports indicate that the depression is a rather general phenomenon and affects the

responses to a number of different antigens.

In recent years a number of investigations have been made in an attempt to find out which stage (or stages) of the immune response is affected by age-related changes.

The first studies were directed towards the ability of old mice to localise antigen. It has been demonstrated that antigen localisation, especially in germinal centres, is impaired in old mice (LEGGE and AUSTIN, 1968; HANNA et al, 1971a, 1971b). The use of radio-labelled polymerised flagellin showed that the total amount of antigen localised in old and young mouse spleens, 24 hours after injection, was approximately the same. But, whereas the young mice, with clearly defined white and red pulp and many primary and secondary follicles, showed a distinct pattern of localisation, old mice in which these areas were less clearly defined, localised antigen in a more diffuse pattern (LEGGE and AUSTIN, 1968) Localisation in young mice occurred in and around the follicles of the white pulp, whereas 7 out of 11 old mice showed no clear localisation at all. However, if the old mice were pre-immunised, either passively or by a previous injection of antigen, follicular localisation increased. This study was used as evidence that naturally occurring opsonic factors, in this case directed towards the flagellin, were absent or decreased in quantity and that their absence prevented cortical localisation occurring as it did in young mice (LEGGE and AUSTIN, 1968).

HANNA et al (1971a) made a study in (C57Bl x C3H)F_1 mice 13 weeks, 18 months and 30 months old. In young animals, one

hour after the injection of radio-labelled human gamma globulin, label was found in the spleen, occurring in greatest quantity in the marginal zone of the splenic lymphatic nodules and in active germinal centres. Between days 3 and 14 label was progressively lost from these areas. In 18 month old mice, both the concentration of label per follicle and the total number of follicles was markedly reduced. The formation of new germinal centres in response to antigenic stimulation in these older animals was rare. Of 24 mice 30 months old which showed no obvious sign of malignancy, only 3 showed any tendancy to form germinal centres. The results of antigen clearance studies showed that both groups of old mice exhibited a pronounced inability to clear antigen, either from the blood stream or from the spleen.

The rate of appearence of reticulum cell sarcoma in the spleen has been studied by HANNA et al (1971a) and at great length by CHINO et al (1971). HANNA reported that the growths, in their initial stages, were confined to the splenic tissues, but on further development sometimes invaded extra-splenic areas. The first alteration in splenic lymphatic nodules associated with the sarcoma was the proliferation of large cells in the thymus-dependent areas. Next, there was an accumulation of reticular cells in the thymus dependent areas, which upon further development invaded the rest of the cortical tissue and the red pulp.

The report by CHINO and his colleagues (1971) accompanied the reports on the primary and secondary haemolytic plaque responses in old mice (MAKINODAN et al, 1971a, 1971b). The study

compared the pathology of old mice from 'clean' and 'dirty' animal farms. Housing conditions were found to have no dramatic influence on the pathology of old mice. The males had a slightly longer life expectancy. Females from the 'dirty' farms which were less than 1000 days old seemed to have a slightly higher death rate from neoplasia, other than reticulum cell sarcoma, than did males from both farms or females from the 'clean' farms. In 80 percent of mice from both farms, death was associated with the development of tumour growth, 50 percent of which were reticulum cell sarcoma.

The antigen clearance study made by HANNA et al (1971a) suggests that macrophage activity may be impaired in old mice. Direct evidence of macrophage activity in old mice is limited. Aoki et al (1965) reported that clearance of colloidal carbon was more rapid in 12 week old random bred Swiss mice than in 35 to 78 week old animals. This difference was found to be even greater after zymosan treatment, which is said to increase phagocytic activity. A slight decrease in the phagocytic activity of ICR female mice has also been reported by Di CARLO et al (1963). This report only assayed mice up to 12 months of age, so that although the decrease in activity was not very great, it was detectable at an early age.

Other studies on the sub-populations of lymphocytes in aged mice have also been carried out. Several authors have concluded that the thymus derived T cell population does not decrease in size with age(PRICE and MAKINODAN, 1971a; HORI et al, 1973; KISHIMOTO et al 1973; MATHIES et al, 1973). This

conclusion was based on limiting dilution analyses (PRICE and MAKINODAN, 1972a) and the proportion of theta positive cells (RAFF, 1969) in the lymphoid tissues of old mice (HORI et al, 1973; KISHIMOTO et al, 1973; MATHIES et al, 1973). The report by Price and Makinodan suggested that not only was the T cell population of old mice equivalent to that in young animals but that it may be even larger in size.

The size of the bone marrow derived B cell population was thought by PRICE and MAKINODAN (1972a) to decrease slightly with age, but that this decrease could not account for the large deppression in the immune response of aged animals.

PRICE and MAKINODAN (1972a, 1972b) also concluded that the efficiency of antigen handling was impaired since the total amount of antigen required to evoke a response in old mice was ten times that required in young mice. It was also concluded that environmental factors had an immediate effect on the capacity of mature lymphocytes to respond to antigen.

Aims of the present study.

The purpose of the work reported here is two-fold. Firstly to study as fully as possible the effect of age on humoral and cell-mediated immune responses in intact mice. Extensive use will be made of the modified haemolytic plaque technique for the identification of both 7S and 19S antibody producing cells.

The second part is planned to continue studies initiated in other laboratories on the possibility of improving the immune responsiveness of old mice by providing them with young cells and tissues. Possible inadequacies in the lymphocyte populations of old mice will also be investigated by studying the ability of old bone marrow inocula to provide young irradiated recipients with both T and B cells and to compare their potential with that of lymphocytes derived from young bone marrow.

CHAPTER 2

MATERIALS AND METHODS

MICE

CBA/H (CBA) and CBA/HT6T6 (CBAT6T6); stock originally derived from Harwell (1966) and maintained by strict brother-sister matings.

CBA/HT6 (CBAT6), derived from matings between CBA/H and CBA/HT6T6 mice and maintained in the same manner.

C57Bl/10.ScSn (C57Bl); stock originally obtained from Searle Laboratories (1969) and maintained by strict brother-sister matings.

(CBA/H x C57Bl/10.ScSn)F_1 mice produced from matings set up in this laboratory.

Stock mice were housed in large polypropylene cages (North Kent Plastics Ltd.) in groups of 20. Experimental mice were housed in similar cages but at lower density.

All mice were maintained in conventional conditions with free access to food and water

ANTIGENS

Sheep erythrocytes (SRBC) were received as a 20 percent suspension in Alsevers solution from Tissue Culture Services Slough, England. All the blood obtained was taken from the same sheep.

4-ethoxymethylene-2-phenyl oxazolone (oxazolone) obtained from B.D.H. chemicals, Poole, England.

MEDIA - see appendix 1

AGAROSE

Two varieties - A37 and A45, obtained from L'Industrie Biologique Francaise, Gennevillievs, France.

THE PREPARATION OF RABBIT ANTI-MOUSE Ig SERUM

The antisera directed against mouse immunoglobulins and used in both the plaque technique and in haemagglutination assays, was prepared in this laboratory by Mrs. M.J.Riddaway. The method used was as follows.

Serum was collected from mice during their peak response to killed B. pertussis organisms (WELLCOME). The antiserum was used to coat killed B. pertussis with mouse immunoglobulins. The complex was washed 4 times by centrifugation and then injected in Freunds complete adjuvant (Difco) into a number of subcutaneous sites in a rabbit. The rabbit was bled 5 weeks later and the serum obtained used as the source of rabbit anti-mouse Ig serum for the various techniques. Since there was very little purification of the serum it inevitably contained antibodies to mouse γM antibodies. The amount of activity against γM was limited since the original anti-pertussis serum was obtained during the peak response.

More recently antisera have been raised in this laboratory to all the individual mouse antibody classes Amongst the advantages of being able to study individual class responses is the fact that these antisera do not inhibit the direct (mainly γM) plaque response, making the technique more accurate.

INJECTION OF AND SENSITISATION WITH ANTIGEN

Injection of sheep erythrocytes (SRBC) the cells were washed twice in citrate saline and twice in Duttons balanced saline (MISHELL and DUTTON, 1967). The cells were counted and injected at a dose of 4×10^7 in 0.2ml Duttons balanced saline (DBS).

Sensitisation with oxazolone. The oxazolone was dissolved in absolute alcohol in a 60°C water bath. The dose to be used was calculated in 0.1ml of alcohol. The mice were anaesthetised with ether and shaved on their left flank. The oxazolone was administered onto the shaved area through a 0.1ml pipette. Considerable caution was taken in the handling of this material because of its properties as a skin sensitising agent and rubber gloves were worn throughout the handling procedure.

COLLECTION AND PREPARATION OF SERUM SAMPLES

Sera from experimental animals were obtained from the tail vein or the retro-orbital sinus of the mouse concerned. The samples were left to clot at room temperature, the clot was removed and the serum centrifuged to remove any remaining cells. Samples were stored at -20°C.

Guinea pig serum was obtained from animals of both sexes and of various ages. The guinea pigs were anaesthetised with nembutal (Veterinary supplies, London, England) with a dose of about 12.0mg (in 0.5ml), administered intraperitoneally. The animals were either bled to obtain as much whole blood as possible, or only about 20.0ml were removed and the animal allowed to recover

so that it could be used again. The blood was removed, in both cases, by cardiac puncture, dispensed into test tubes and allowed to clot at room temperature. The clots were removed and transferred to 4°C to extract additional serum. All the serum obtained was absorbed with sheep cells (1 part packed SRBC to 5 parts serum) for about 35 minutes at 4°C. The serum was centrifuged to remove any cells and stored at -60°C. For use in the haemolytic plaque technique, or for any cytotoxic assay the serum was diluted 1 part in 10 with DBS immediately before use. Hamster serum was obtained by cardiac puncture from Golden hamsters. Preparation, absorption and storage was as described for guinea pig serum. In the anti-theta cytotoxicity assay the serum was absorbed against mouse cells and used undiluted.

PREPARATION AND INJECTION OF CELL SUSPENSIONS

Foetal liver injections. Livers were obtained from foetal mice of 16 to 18 days gestation. This was ensured by placing males in with the females overnight and removing them the next morning. The livers were homogenised in Hanks solution with loose fitting teflon homogenisers in round bottomed centrifuge tubes. The cells were passed through a 200-mesh sieve and washed once by centrifugation. They were then counted and injected intravenously at a dose of 5×10^6 viable cells per mouse in 0.2ml Hanks solution. The absence of T cells, where required, was ensured by treating the cell suspension with anti-theta serum and complement (RAFF, 1969) prior to counting.

Bone marrow injections. Cells were obtained from the bones of the hind limbs and pelvic girdles of donor animals (femur, tibia and iliac crest). The cells were washed out of the bones with Hanks solution using a syringe and 25g needle. Usually a single cell suspension could be obtained simply by drawing the cells up into a syringe through a 25g needle. If the cells showed a tendancy to clump together they were washed once by centrifugation. The cells were counted and injected intravenously at a dose of 5×10^6 viable cells per mouse in 0.2ml Hanks solution. As the complete procedure took some time to complete it was important to observe simple sterile routine and to keep the cells, once isolated, on ice to ensure a reasonable percentage viability.

Thymus cell injections. Cells were obtained from 4 week old donor mice. The thymus lobes were gently homogenised and then washed 3 or 4 times by centrifugation in Hanks solution and injected intravenously at a dose of 50×10^6 viable cells per mouse.

When there was more than one cell population to inject into one mouse (e.g. bone marrow and thymus cells) the cells were mixed after preparation and washing and injected together in a volume not exceeding 0.4ml.

THYMUS GRAFTING

Thymus grafts were obtained from young (2 to 3 day old) and old (18 to 21 month old) donors. One lobe of a thymus was placed in the axillary region of the recipient mice, subcutaneously. The operation was carried out under nembutol

anaesthesia (0.6mg nembutal per gm. body weight) and the wound made for the introduction of the graft was sealed with an autoclip (Clay Adams N. Y.).

THYMECTOMY - was carried out on mice no more than 4 weeks old under nembutal anaesthesia. The procedure involved holding the mouse out ventral side uppermost with the head held over a small bolster. An incision was made in the mid line of the neck and a small tube, connected to a suction pump, introduced to suck out the thymus. The force of the suction pump could be controlled by means of a small hole in the tube which could be covered by a finger when the apparatus was in the correct position. The operation did not necessitate the cutting of any bone and the mouse suffered no ill effects. The wound made for the extraction of the thymus was sealed with an autoclip (Clay Adams N.Y.). The clips were removed within two weeks of the operation. All thymectomised mice were routinely inspected after an experiment to ensure that no thymus remains had been left.

IRRADIATION

All irradiations were carried out with a Westinghouse X-ray machine with the following constants: 15mA; 0.5mm Cu and 1.0mm Al filtration; source to target distance of 75cm and a dose rate of 66r/minute.

ASSAY TECHNIQUES

The haemolytic plaque technique for the identification of antibody producing cells.

The assay employed was based on that described by DRESSER and WORTIS (1967), an elaboration of the original technique described by JERNE et al (1963).

9cm disposable plastic petri dishes (Sterilin Ltd, Richmond, Surrey, England). were prepared in advance. Each dish had 5.0ml of a 1.2 percent solution of agarose in DBS poured into it. The solution was prepared in a household pressure cooker placed under 10lb/sq. in. pressure for 2 to 3 minutes The 5ml of agarose was dispensed whilst in a liquid state by means of a repeating syringe, spread evenly over the bottom of the petri dish and allowed to settle on a level surface at room temperature. The plates prepared in this way could be stored for up to two weeks if sealed in plastic bags and kept at 4°C.

On any one assay day sufficient plates were removed from the cold, numbered and placed at 37°C for about 30 minutes before pouring on the top layer.

The top layer consisted of a 0.6 percent agarose solution, again made up in DBS. The solution was brought to the boil on a heated magnetic stirrer and then passed through a sintered glass filter into a vacuum flask. This procedure removed any undissolved particles of agarose. The solution was covered, to prevent water loss and maintained at about 60°C until required. 2.0ml aliquots were then dispensed into Khan tubes placed in a water bath at 45°C. The developing serum (described below)

for the identification of developed, 7S plaque forming cells, was then added to the required tubes in a total volume of 0.1ml. For each cell suspension to be assayed for antibody forming cells two tubes without developing serum (for direct, mainly 19S plaque forming cells) and two tubes with developing serum (for indirect, mainly 7S, plaque forming cells) were prepared.

Sheep erythrocytes were washed twice in citrate saline and twice in DBS. They were then made up to a 20 percent suspension, based on packed cell volume.

Spleens and lymph nodes were excised, weighed and placed in 2.0ml DBS in large round bottomed centrifuge tubes. The tissues were gently homogenised with loose fitting teflon homogenisers. The cell suspensions were then passed through a 200-mesh sieve into graduated centrifuge tubes, washed once by centrifugation and resuspended carefully with a pasteur pipette in 1.0ml DBS. The suspensions were then made up to the desired concentration. The cells were kept on ice at all times after removal from the mouse except during centrifugation.

The plating operation involved taking a tube (containing 2.0ml top layer agarose, with or without developing serum) from the water bath and dispensing into it 0.1ml of the 20 percent sheep erythrocyte suspension and 0.2ml of the spleen, or lymph node suspension. The contents of the tube were mixed rapidly on a rotamixer and poured onto a prepared plate. The contents of the tube were spread over the bottom layer of agarose and the plate placed on a level surface to allow the top layer to set.

The plates were incubated for 2 hours at 37°C in a humid

atmosphere. They were then removed from the incubator and 1.0ml of a 1 in 10 dilution of sheep erythrocyte absorbed guinea pig serum was added to each plate as the complement source. The serum was spread evenly over the surface of the plates which were then replaced in the incubator for a further one hour. Control plates, without complement, were used at each assay to ensure that all the plaques formed were complement induced.

On removal from the incubator the plates were usually scored at once. When this was not possible the cells were fixed with a 2.0 percent solution of formaldehyde in DBS and stored at 4°C. Plates were never left for more than 48 hours before counting. Only plaque forming cells which produced plaques which were round and regular in outline were scored. When irregular plaques occurred their presence was not attributable to the presence of complement and were a result of air bubbles, bacterial lysis and small irregularities in the agarose. When the number of plaques exceeded about 400 per plate a mask, which revealed several random areas totalling 10 percent of the surface area of the plate, was used. Plaques were counted on a conductivity type colony counter (Scientifica Ltd) modified for dark ground illumination.

A37 and A45 AGAROSE - up to december 1969 the agarose supplied was of the type that set at 42° to 45°C. This meant keeping the agarose at about 45°C in the water bath before plating. After the above date a new variety was introduced by the manufacturers which remained fluid down to 37°C. This new variety was used in all experiments from the end of december 1969. Comparative

tests showed that lowering the temperature of the water bath to 40°C, with the hope of increasing cell viability, resulted in no significant increase in plaque numbers. This conclusion was confirmed by DRESSER (personal communication). In order not to introduce any major modification into the technique the temperature of the water bath was lowered only 1°C to 45°C. This allowed the investigator a little extra time in which to add the sheep erythrocytes and lymphocyte suspensions.

THE TITRATION OF DEVELOPMENT AND INHIBITION CONSTANTS - the plaque technique depends on antibody - mediated, complement dependent lysis. It has been argued by several authors that most of the direct plaques (i.e. those detected without developing serum) are formed by cells producing γM antibody. This was based on observations which showed a close correlation between the number of direct plaques in the spleen and the titre of γM antibody (haemolysins) in the serum (STERZL and MANDEL, 1964; ROWLEY and FITCH, 1965; MÖLLER and WIGZELL, 1965), and on the arguement that cells releasing non γM antibodies would probably not be detected as direct plaques, because of the relatively low haemolytic efficiency of 7S as compared to 19S antibody. For example it has been calculated that one molecule of rabbit 19S antibody when bound to an erythrocyte can fix complement and effect lysis, whereas several hundred or even thousands of 7S molecules would be required (HUMPHREY and DOURMASHKIN, 1965; BORSUS and RAPP, 1950). DRESSER and WORTIS,(1965) by incorporating a rabbit anti-mouse Ig serum into the top layer agarose, found that the number of plaque forming cells was greatly increased,

and this mostly in the latter stages of the response. It was argued that these plaques were due to the release of 7S antibodies and they were named developed or indirect plaque forming cells to distinguish them from the direct type. It appears, however, that some 7S molecules may effect direct lysis and that some 19S molecules may require a specific antiserum before they may be detected (PLOTZ et al, 1968).

It has been assumed, in this work, that the direct plaque response was attributable to 19S, γM antibody producing cells and the indirect response to 7S, γG antibody producing cells. From the work reported by WORTIS et al (1969) it appears unlikely that γA plaque forming cells were produced as a result of a sheep erythrocyte dose of 4×10^7. Cell doses of about 10^{10} sheep erythrocytes are necessary to cause the production of appreciable numbers of γA plaque forming cells.

The detection of 7S antibody forming cells depends on the incorporation of a rabbit anti-mouse Ig serum into the top layer agarose. The problems associated with this procedure are discussed by DRESSER and WORTIS (1967). The antiserum used in the work reported here inhibited the formation of direct plaques; in consequence it had to be used at a concentration which limited the inhibition to a tolerable, known, level. At this concentration the antiserum was less efficient than at a higher concentration in producing developed, indirect, plaque forming cells. The inhibiting and developing characteristics of the serum had, therefore, to be ascertained and described in terms of numerical constants. The calculation of the constants was

based on two assumptions. First, that on day 2 after immunisation with sheep erythrocytes the great majority of the plaque forming cells were of the direct 19S variety. Secondly, that direct plaque forming cells remained as susceptible to inhibition by the rabbit anti-mouse serum at all times after immunisation as they were on day 2.

Initially the constants were calculated by the method described by DRESSER and WORTIS (1967). A number of mice were injected with 4×10^7 sheep erythrocytes intraperitoneally (i.p.). On day 2 of the response the mice were killed and their spleens removed and pooled. The cells were used as a source of potential direct plaque forming cells in titrations against different concentrations of the developing serum which was incorporated in the top layer tubes in the normal way. Serial dilutions of the antiserum, in 0.1ml were made up prior to titration from a concentrated stock of the antiserum. The plating procedure was that already described but using the same spleen cell pool for all the lymphocyte additions, and preparing 4 plates for each dilution. A number of plates were also prepared without the developing serum to give a count of the potential number of direct plaque forming cells present in each aliquot of spleen cells.

The results of the titrations performed on day 2, to establish an inhibition constant for the serum, are shown in Tables A and B.

EX BIBL UNIV. EDINBURGEN

Results of two seperate inhibition constant (KI) titrations carried out on CBAT6 male mice.

TABLE A

Log_2 dilution of antiserum	PFC/plate (geom.mean)	regression analysis value
5	75	97
6	144	136
7	177	176
8	239	216
9	262	256
10	305	297
11	313	337
No serum	408	-

TABLE B

Log_2 dilution of antiserum	PFC/plate (geom.mean)	regression analysis value
5	60	77
6	126	123
7	178	171
8	228	218
9	279	265
10	306	312
11	346	357
No serum	421	-

PFC plaques per plate.

$$\text{Geometric mean} = \text{antilog}\left[\frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 \ldots + \text{Log}_{10}X_4}{n}\right]$$

Where X_1, X_2 etc. refer to numbers of duplicate plates and n = number of duplicate plates, in this case 4.

DRESSER and WORTIS (1967) calculated their constants from values obtained from graphs using the geometric means and fitting a line through them by eye. In this work it was considered to be more reliable to plot a regression analysis using all the individual plate counts. The regression analysis value was then used in all further calculations. The geometric mean is shown here for comparative purposes only.

The inhibition constants (KI) were calculated from the following equation:

$$\text{KI at } \text{Log}_2 \text{ dilution } x = \frac{\text{No. PFC at dilution } x}{\text{No. PFC on direct plates}}$$

The value for the plates without serum (direct plates) was the geometric mean of all the direct plates. From the data in Tables A and B it was then possible to calculate inhibition constants for the serum at Log_2 dilution 10.

(1) from Table A $\quad \frac{297}{408} = 0.728$

(2) from Table B $\quad \frac{312}{421} = 0.742$

As mentioned above the antiserum must be used at a concentration that limits the inhibition but at the same time provides reasonable development. Since the developing serum had to be used in a diluted form another titration had to carried out to determine a development constant (KD). This time the mice were assayed on day 8 or 9 of their response to sheep erythrocytes (4×10^7 SRBC i.p.), when there should be a large number of indirect (7S) plaque forming cells and rather few direct plaques. Serial dilutions of the antiserum were

prepared in the same way as for KI titrations. The results of a typical titration are shown in Table C.

TABLE C

Log_2 dilution of antiserum	PFC/plate (geom,mean)	regression analysis value
7	565	-
8	732	-
9	865	878
10	747	732
11	595	585
12	427	439
No serum	291	-

As in the KI titrations the geometric mean was used for the number of direct plaques; the regression analysis values were used for the plates with developing serum.

It was a common feature of all the antisera investigated that they inhibited plaque formation in high concentration. This is shown clearly in Table C for the serum used in the work presented here. The regression analysis was performed from dilution 9 to 12 inclusive.

The development constant (KD) was calculated from the following equation:

KD at Log_2 dilution x =

$$\frac{\text{Max. No. PFC obtained - (No. direct PFC x KI at that concn.)}}{\text{No. PFC at dilution x - (No. direct PFC x KI at dilution x)}}$$

The particular serum in question was used at Log_2 dilution 10. As in all calculations of KD values in the CBAT6 mouse, the KI titrations shown in Tables A and B were used. A particular KI value was calculated as the mean of the two values obtained from the two sets of values. Using the information contained in Tables A, B and C the KD equation can be represented as follows:

$$\text{KD at dilution 10} = \frac{878 - (291 \times 0.624)}{732 - (291 \times 0.735)} = 1.34$$

Because the developing serum was to be used to develop all the classes of 7S antibodies (i.e. G_1, G_{2a}, G_{2b}; and remembering that γA antibodies would probably not be formed) and since the appearance of these classes varies with time after injection (SWORTIS et al, 1969), it was decided to calculate KD values throughout the greater part of the response to 4×10^7 sheep erythrocytes, incase there was any variation.

The days chosen for the assays were 5, 8 and 11 after antigen injection. Duplicate groups, using different mice, but injected and assayed at the same time, were assayed on each of the days. The KI values used were those obtained from Tables A and B. All the KD values were calculated at Log_2 dilution 10.

TABLE D

Day 5 development constant titrations in CBAT6 male mice

	Log_2 dilution of antiserum	PFC/plate (geom.mean)	regression analysis value
(1)	9	334	331
	10	307	315
	11	306	299
	12	282	274
	No serum	265	-
(2)	9	312	300
	10	263	280
	11	257	260
	12	247	240
	Noserum		

$$KD = \frac{331 - (265 \times 0.624)}{315 - (265 \times 0.735)} = 1.37 \text{ --------- (1)}$$

$$KD = \frac{300 - (243 \times 0.624)}{280 - (243 \times 0.735)} = 1.46 \text{ --------- (2)}$$

TABLE E

Day 8 development constant titrations in CBAT6 male mice

	Log_2 dilution of antiserum	PFC/plate (geom.mean)	regression analysis value
(1)	9	743	791
	10	736	629
	11	396	466
	12	315	304
	No serum	227	-
(2)	9	597	613
	10	552	523
	11	424	433
	12	340	343
	No serum	183	-

$$KD = \frac{791 - (227 \times 0.624)}{629 - (183 \times 0.735)} = 1.40 \text{ --------- (1)}$$

$$KD = \frac{613 - (183 \times 0.624)}{523 - (183 \times 0.735)} = 1.29 \text{ --------- (2)}$$

TABLE F

Day 11 development constant titrations in CBAT6 male mice

	Log_2 dilution of antiserum	PFC/plate (geom.mean)	regression analysis value
(1)	9	351	354
	10	268	264
	11	176	175
	12	83	85
	Nosserum	42	-
(2)	9	377	389
	10	315	298
	11	210	207
	12	109	116
	No serum	53	-

$$KD = \frac{354 - (42 \times 0.624)}{264 - (42 \times 0.735)} = 1.40 \text{ --------- (1)}$$

$$KD = \frac{389 - (53 \times 0.624)}{298 - (53 \times 0.735)} = 1.36 \text{ --------- (2)}$$

An analysis of variance performed on all the KD values showed that the variance within days (i.e. day 5 or 8 or 11) was as great as that between days. This being so it was concluded that with this serum there was no change in the KD value at different stages of the response to sheep erythrocytes and the calculation of experimental results was based on a mean value of all the KD values available.

Thus at Log_2 dilution 10; KI = 0.735 and KD = 1.38

Although the method for calculating the KI and KD values was essentially that of DRESSER and WORTIS (1967), there were a number of points which suggested that the semi-log plot used in the regression analysis was incorrect. Duplicate plate counts are not normally distributed but conform to a Poisson distribution. The use of the geometric mean should normalise the data at any one dilution, but it is also true to say that the standard error of the mean increases with the magnitude of the mean value (DRESSER et al, 1967). In consequence a regression analysis cannot, strictly, be applied, when using arithmetic plaque counts. It should be pointed out that the use of a regression analysis served only as a means of line fitting, considered more accurate than the method used by Dresser and his colleagues, i.e. fitting the line by eye. To be strictly accurate the regression analysis should be made on log transformed data.

When these criticisms were appreciated most of the experimental work had been completed and the results and graphs calculated and drawn. It was, however, decided to recalculate

the KI and KD values using a log-log plot, to investigate any inaccuracy which may have resulted from the previous work. The two recalculated KI titrations are shown in Tables G and H

TABLE G

Recalculation of Table A Inhibition constant titration using log transformed data.

Log_2 dilution	regression nalysis value	antilog
5	2.0241	103
6	2.1082	128
7	2.2024	159
8	2.2961	198
9	2.3903	245
10	2.4844	305
11	2.5782	378
(No serum	-	408)

TABLE H

Recalculation of Table B inhibition constant titration using log transformed data.

Log_2 dilution	regression analysis value	antilog
5	1.9391	87
6	2.0554	114
7	2.1718	148
8	2.2882	194
9	2.4046	255
10	2.5209	331
11	2.6367	434
(No serum	-	421)

KI values based on the log-transformed regressions were calculated using the antilog values indicated in Tables G and H. The value for the direct plates is the geometric mean, as used in previous calculations. The recalculated constants at Log_2 dilutions 9 and 10 are shown below.

	KI at Log_2 dilution 9	KI at Log_2 dilution 10
Table G	0.602	0.750
Table H	0.610	0.770
Mean value	0.606	0.760

The KD titrations were also replotted and the results are shown in Tables I to K. The KD values were calculated using the KI values from Tables G and H.

An analysis of variance performed on the recalculated KD values showed that they were not significantly different from the values obtained from a semi-log plot but did have a higher mean value. Values for any one day showed as much variance as that which existed between days.

The KI values obtained from log-transformed data were, however, very similar to those already calculated and used in the preparation of experimental results. Since it is this constant that determines the presence, or absence, of indirect plaques, as far as recording results is concerned, the results already calculated were not altered. A greater appreciation of this reasoning will be obtained from the section dealing with the calculation of experimental results.

TABLE I

Recalculation of Table D development constant titration using log-transformed data. (Day 5).

	Log_2 dilution	regression analysis value	antilog
(1)	9	2.5195	331
	10	2.4977	315
	11	2.4760	299
	12	2.4543	285
(No serum		-	265)
(2)	9	2.4788	301
	10	2.4350	272
	11	2.3912	246
	12	2.3474	223
(No serum		-	243)

$$KD = \frac{331 - (265 \times 0.606)}{315 - (265 \times 0.760)} = 1.48 \text{ --------- (1)}$$

$$KD = \frac{301 - (243 \times 0.606)}{272 - (243 \times 0.760} = 1.73 \text{ --------- (2)}$$

TABLE J

Recalculation of Table E development constant titration using Log-transformed data. (Day 8).

	Log_2 dilution	regression analysis value	antilog
(1)	9	2.9231	838
	10	2.7802	603
	11	2.6373	434
	12	2.4944	312
(No serum		-	227
(2)	9	2.8035	636
	10	2.7072	510
	11	2.6109	408
	12	2.5246	335
(No serum		-	183

$$KD = \frac{838 - (227 \times 0.606)}{603 - (227 \times 0.760)} = 1.62 \text{ --------- (1)}$$

$$KD = \frac{636 - (183 \times 0.606)}{510 - (183 \times 0.760)} = 1.41 \text{ ---------(2)}$$

TABLE K

Recalculation of Table F development constant titration using log-transformed data. (Day 11)

	Log_2 dilution	regression analysis value	antilog
(1)	9	2.5937	392
	10	2.3974	250
	11	2.2011	159
	12	2.0048	101
(No serum		-	42
(2)	9	2.6281	425
	10	2.4486	281
	11	2.2691	186
	12	2.0896	123
(No serum		-	53

$$KD = \frac{392 - (42 \times 0.606)}{250 - (42 \times 0.760)} = 1.68 \text{ --------- (1)}$$

$$KD = \frac{425 - (53 \times 0.606)}{281 - (53 \times 0.760)} = 1.63 \text{ --------- (2)}$$

Exhaustive tests were carried out on mice of various ages and these showed that there were no significant differences between the KI and KD values obtained with young mice (i.e. the results shown above) compared to those obtained with older mice. This fact is not, perhaps, surprising since the only reason why a change should occur would be because of a change in the relative amounts of any one antibody class compared to another. Since there was no significant difference in the values between different days, there is no reason why they should do so with age.

The detailed information given above refers to work on male CBAT6 mice. Further titrations were carried out, as the need arose, with CBA and CBAT6T6 mice of both sexes and in female CBAT6 mice. The values obtained did not differ signif-icantly from those obtained with CBAT6 male mice.

The constants obtained fromC57Bl and (CBA x C57Bl)F_1 mice appeared to be slightly different from those obtained in CBA animals. The number of titrations performed was limited but on the basis of a consistantly high KD value in C57Bl mice (2.16 compared to 1.38 in CBA and 1.45 in the F_1 mice) it was decided to assign separate KI and KD values to these two strains. the KI values obtained were 0.71 in C57Bl mice and 0.728 in (CBA x C57Bl)F_1 mice. Both the KI and KD values refer to female C57Bl mice, but to both male and female F_1 mice.

There can be little doubt that one of the basic inaccuracies of the haemolytic plaque technique is in the establishment of the various constants to the developing serum.

Variations do occur and may give rise to apparent anomalies in results. For instance in Fig. 16 of the results reported here which shows the indirect plaque response in female (CBA x C57Bl)F_1 mice there are cases recorded of 7S plaque forming cells on day 2 of the response. As pointed out here the calculation of KI values is based on the assumption that all the plaques on day 2 are of the direct, 19S type. There can be little doubt that in most cases this is essentially true. Variations which do occur, however, will be obscured during KI titrations because the spleen cells used were pooled from a number of mice.

It is not to be supposed that the results presented here, or indeed any others based on the same technique, are grossly inaccurate. Small variations do occur and should be taken into account when analysing results.

THE CALCULATION AND PRESENTATION OF HAEMOLYTIC PLAQUE RESULTS - Duplicate plates were made for both direct and indirect plaque responses for each individual spleen cells suspension and the geometric mean calculated. An example might be as follows:

Direct PFC per plate. 500 and 540; geometric mean = 520
Indirect PFC per plate. 2600 and 2200; geom. mean = 2391

The corrected number of indirect PFC per plate would therefore be
(PFC/indirect plate - (PFC/direct plate x KI)) x KD
or: (2391 - (520 x 0.735)) x 1.38 = 2770

It will be appreciated that an indirect response will only be seen when the number of direct PFC x KI is less than the total number of plaque forming cells on the indirect plate. It is because of this fact that the KI value is so critical and why the value based on the semi-log plot was retained since it was very similar to that based on a log-log analysis. The KD value only increases the magnitude of the 7S response.

All the results are expressed as plaque forming cells per spleen and are usually shown in graph form. The vertical bars in all instances refers to the standard error of the mean. The mean values for a particular group and all statistical analysis was performed on log-transformed data, based on the total number of direct and indirect plaque forming cells per spleen from individual mice. In plotting these results it was decided, in many instances to omit either the upper or lower standard error bar. The reason was simply that its inclusion would have unnecessarily complicated already overcrowded material.

Passive haemagglutinations

Passive haemagglutinations were performed in'Linbro' type plastic trays with conical wells (Flow Laboratories, Irvine, Scotland). Mice were bled from the tail or retro-orbital sinus. Sera were always assayed in duplicate, e.g. in rows A and B. In row A a serum which had been reduced and alkylated and in row B a similar dilution of the untreated serum. In both cases the row of wells had 0.05ml phosphate buffered saline (PBS) containing 0.01% gelatine, added to them. 0.05ml of the serum

sample was added to the first well of a row and doubling dilutions made along the row. Finally 0.05ml of a 0.75% suspension of sheep erythrocytes was added to each well. The plates were incubated for 90 minutes at 37°C. The final pattern of sttlement was allowed to take place in the cold overnight. The agglutination patterns were read and recorded. The supernatant was removed from each well and the cells washed by resuspending them in 0.1ml PBS and allowing them to settle again. The supernatant was removed and the cells resuspended in 0.1ml medium. In the case of the reduced and alkylated serum row the medium contained a suitable concentration of rabbit anti-mouse Ig serum. The row that had contained untreated serum had PBS alone added to the wells. The addition of the antiserum allowed an estimation of the total 7S antibody response to sheep cells. Agglutination patterns were read after settlement. The technique therefore allowed an estimate of both 19S and 7S antibody titres.

Treatment of sera with reducing agent.

The reducing agent used was Clelands reagent - Dithiothreitol (DTT). 0.1ml of the antiserum to sheep cells was added to 0.4ml of Tris-HCl buffer, 0.05M and pH 7.3. 0.5ml of a 0.1M solution of DTT was added, giving a final concentration of 0.001M DTT. The serum was left for 30 minutes at room temperature and a slight excess of iodo-acetic-acid (IAA) was then added. This produced a treated serum sample one tenth of its original concentration. The untreated serum was diluted one in ten with PBS before assaying.

The oxazolone / IUdR assay.

The proliferative response of lymphoid cells to sensitisation with oxazolone was measured by the incorporation of ^{125}IUdR (labelled 5-iodo-2-deoxyuridine) as described by PRITCHARD AND MICKLEM, 1972)

. Mice received a sensitising dose of oxazolone and on the assay day were given 5 x 10^{-8} moles fluorodeoxyuridine (FUdR) in 0.2ml sterile distilled water. This was followed after one hour by an injection of 1 μCi ^{125}IUdR which had been adjusted to a specific activity of 5 μCi/gm. The mice were killed 2 hours later and the various organs to be studied removed. The organs were fixed in neutral formalin for 24 hours and were then washed in several changes of 70% alcohol until no further radioactivity was leached out (BRYANT and COLE, 1967). Individual nodes, or other organs were counted in a Packard Autogamma crystal scintillation spectrometer for 2 minutes. The results for individual organs were expressed as a fraction of the number of injected counts x 10^{6}. The values for the axillary and brachial nodes for a given side of an individual mouse were pooled. The mean values and standard errors were calculated and statistical treatment made on log-transformed data.

CHAPTER 3

The primary response to sheep erythrocytes in CBA, CBAT6, C57Bl and (CBA x C57Bl)F_1 mice of various ages.

1. The background response to sheep erythrocytes in CBAT6 males

The background number of plaque forming cells to sheep erythrocytes was assayed in male mice aged 3, 6, 9, 12, 15, 18 and 21 months. The spleens were made up in 1.0ml DBS and the whole suspension plated over 5 petri dishes in 0.2ml aliquots. The results are shown in Table 1.

TABLE 1

The background number of PFC to sheep erythrocytes in CBAT6 males

Age in months	Log_{10} PFC /spleen *	Age in months	Log_{10} PFC /spleen *
3	1.5563	15	1.7324
	2.2175		2.3802
	2.0000		2.1673
6	2.1847	18	1.9243
	2.4150		1.7993
	2.2041		1.8573
9	2.3766	21	2.2788
	1.9541		2.0221
	1.6532		1.9031
12	2.1004		
	1.6021		
	1.9792		

* all plaques detected were of the direct type - no developed plaque forming cells were detected in any of the mice assayed.

Addition of developing serum to any of the plates in the normal way, prior to incubation, would have necessitated the use of the inhibition constant. As a result no developing serum was added to the plates prior to scoring for direct plaque forming cells. When all the plates had been scored developing serum was added on top of the agarose and the plates replaced in the incubator for a further one hour. No further plaques were revealed and it was concluded that there were no background indirect antibody forming cells. The procedure of adding developing serum after scoring for direct plaques, or after the direct plaques had been shown up after the addition of complement, was not used as a routine procedure.

Experiments were carried out to compare the efficiency of early and late addition of developing serum. The results showed that the standard early addition revealed more plaque forming cells than adding the serum late. In all background determinations where the number of plaque forming cells per spleen was low, it was important to assay as accurately as possible for direct plaques. This involved using the whole spleen suspension on plates without developing serum. Late addition of developing serum to the plates, although not as efficient as the normal procedure, was considered sufficient to conclude that there were no background indirect plaque forming cells in any of the mice assayed, both with CBAT6 mice and in all other strains assayed.

An analysis of variance performed on all the results in Table 1 showed that the variance within age groupings was as

great as that between ages. This was taken to indicate that there was no significant change in the background number of plaque forming cells to sheep erythrocytes with age. The data was pooled and the mean and 95% confidence limits used in all future reference to the CBAT6 mouse.

The use of large numbers of mice for background studies in CBA and CBAT6T6 mice was inpracticable, since insufficient mice were available at any one time. However, mice of various ages and both sexes were assayed when they became available and were included as background controls in experiments of specific responses to sheep erythrocytes. At no time did any of the background data obtained in this way fall outside of the 95% confidence limits of the data obtained for male CBAT6 mice. Since the CBAT6 data was gained in a single assay the mean and 95% confidence limits were used in all reference to the CBA varieties used in the experiments which follow.

2. The primary response to sheep erythrocytes in male CBAT6 mice.

The first study of a specific response to sheep erythrocytes was performed on male mice aged 3, 9, 16 and 21 months. The mice were all injected with 4×10^7 SRBC i.p. and their spleens assayed for plaque forming cells at intervals up to 11 days after injection.

The full results of this experiment are shown in Tables 2 to 5 as an example of the data made available during these studies. The results are also shown in graph form in Fig. 1 and 2.

TABLE 2

The primary response to sheep erythrocytes in 3 month old male CBAT6 mice.

Day	Body wt. (g)	Spleen wt. (mg)	Total cells /spleen x 10^6	Log_{10} No. direct PFC /spleen	Log_{10} No. dev. PFC /spleen
2	25.2	98	121.6	3.4048	none
	26.7	93	160.9	3.4260	none
	25.9	145	181.4	3.9122	none
3	21.2	78	133.8	3.9117	none
	25.5	84	152.4	3.2014	none
	20.0	85	137.4	3.9782	none
4	21.5	95	120.0	4.6530	3.0792
	27.6	83	77.8	4.0120	2.8388
5	24.8	92	123.6	3.7738	4.8434
	22.5	95	126.6	5.4051	3.8602
	25.1	88	133.2	5.1363	4.3629
7	26.5	68	116.0	3.9031	4.8561
	31.6	89	166.0	3.9294	4.7872
	30.3	94	156.0	4.1931	4.8253
8	29.0	108	NOT	3.8156	plates
	29.5	70	DONE	3.8162	not dev.
	26.5	71		3.0569	
11	24.7	71	NOT	3.2136	4.5601
	25.3	112	DONE	3.4044	4.1820
	22.4	73		2.6892	4.3193

Day = day after injection.

TABLE 3

The primary response to sheep erythrocytes in 9 month old male CBAT6 mice.

Day	Body wt. (g)	Spleen wt. (mg)	Total cells /spleen x 10^6	Log_{10} No. direct PFC /spleen	Log_{10} No. dev. PFC /spleen
2	33.0	105	175.2	2.6355	none
	33.2	87	180.0	2.5416	none
	29.3	78	158.0	2.5750	none
4	34.1	105	60.6	4.7604	none
	30.3	92	65.4	4.6489	none
	31.0	81	40.6	4.6946	none
7	27.6	69	80.8	3.8943	3.7559
	19.2	93	100.4	4.1139	3.9243
	29.5	211	204.8	3.9138	3.3979
8	33.9	121	151.0	4.2148	3.9494
	24.3	147	229.0	4.0107	3.9085
	29.5	99	93.0	4.1055	4.0969
11	29.7	145	180.0	3.5746	4.2375
	29.7	122	163.8	3.1761	4.4832
	31.1	123	122.4	3.5419	4.6303

Day = day after injection.

TABLE 4

The primary response to sheep erythrocytes in 16 months old male CBAT6 mice.

Day	Body wt. (g)	Spleen wt. (mg)	Total cells /spleen x 10^6	Log_{10} No. direct PFC /spleen	Log_{10} No. dev. PFC /spleen
2	30.5	125	193.6	2.6946	none
	32.3	116	224.8	2.6232	none
	34.9	87	134.4	1.9823	none
4	26.8	167	103.8	5.0060	none
	34.1	140	132.0	4.5263	none
	26.5	133	102.0	4.9004	none
7	27.1	132	175.2	4.4346	4.1061
	36.5	156	174.4	4.2148	3.6920
	29.3	125	184.8	4.1139	4.1386
8	26.2	202	308.0	4.5593	3.9320
	30.5	219	349.0	4.4843	4.4082
	28.2	135	235.0	3.9165	3.2788
11	28.4	128	258.0	3.3404	3.9180
	31.8	145	124.4	3.4900	3.3222
	32.6	261	316.5	3.5635	3.5989
	27.1	128	130.0	3.2765	3.5302

Day = day after injection.

TABLE 5

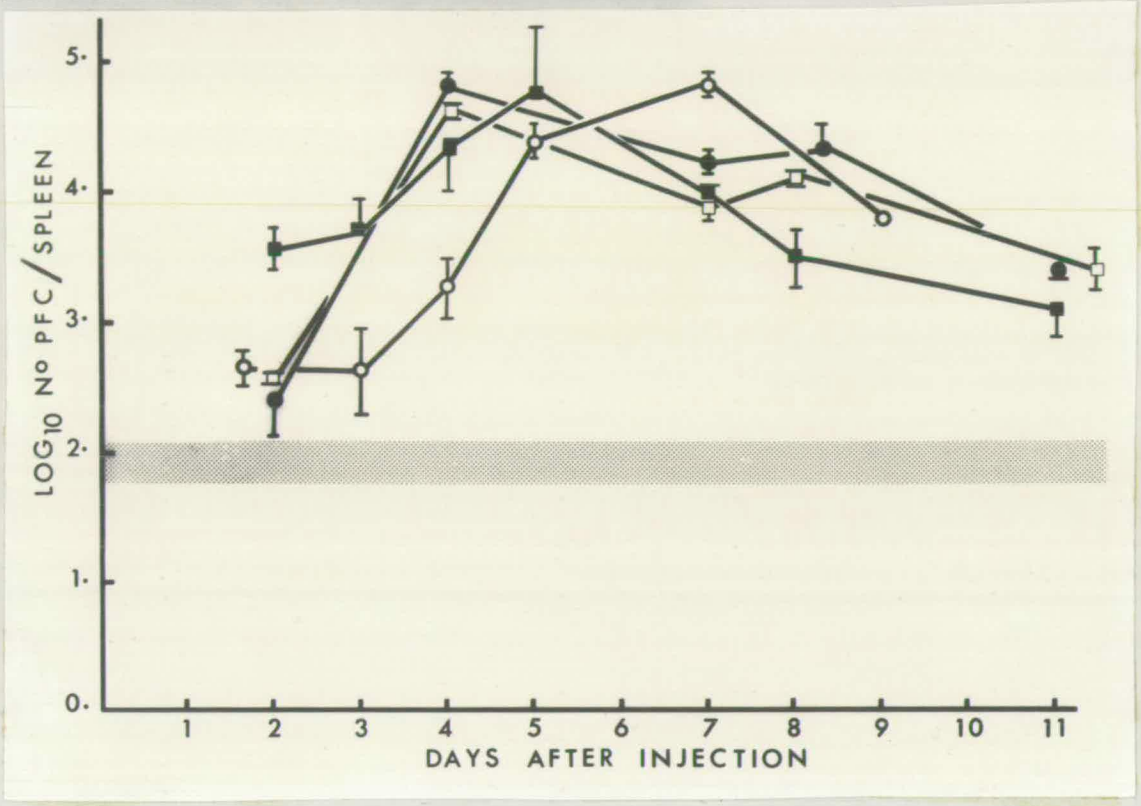
The primary response to sheep erythrocytes in 21 month old male CBAT6 mice.

Day	Body wt. (g)	Spleen wt. (mg)	Total cells /spleen x 10^6	Log_{10} No. direct PFC /spleen	Log_{10} No. dev. PFC /spleen
2	25.2	98	121.6	2.7634	none
	26.7	93	160.9	2.6212	none
3	28.4	121	160.4	3.3128	2.0828
	25.0	92	138.0	2.0828	2.5150
	27.1	91	156.0	2.5051	2.2930
4	27.7	135	176.8	3.0338	3.5506
	29.6	119	136.4	3.4757	3.0033
	26.8	136	146.3	3.2601	3.2813
5	25.9	90	NOT	4.5871	4.3133
	28.0	148	DONE	4.3096	4.0086
	27.3	131		4.4513	4.1603
7	23.8	98	159.0	4.9503	3.1703
	22.9	94	101.4	4.7604	2.9031
9	28.8	119	187.0	3.7818	2.9164
	26.8	150	240.0	3.7226	3.4808
	19.9	131	187.0	3.7818	2.9164

Day = day after injection.

FIG. 1

The direct plaque response in CBAT6 male mice after the injection of 4 x 10^7 sheep erythrocytes.



■ 3 months old; □ 9 months old; ● 16 months old; ○ 21 months old.
Shaded area represents 95% confidence limits of background data.

TABLE 6

Results of Student t tests performed on data from Fig. 1

Day after injection	test between ages	d.o.f.	t	P
2	3 and 21	4	4.84	<0.01
4	3 and 21	3	3.11	<0.05
7	3 and 21	3	6.39	<0.01
7	9 and 21	3	7.47	<0.01
7	16 and 21	3	4.48	<0.01

d.o.f. - degrees of freedom; t - value of t; P - probability.

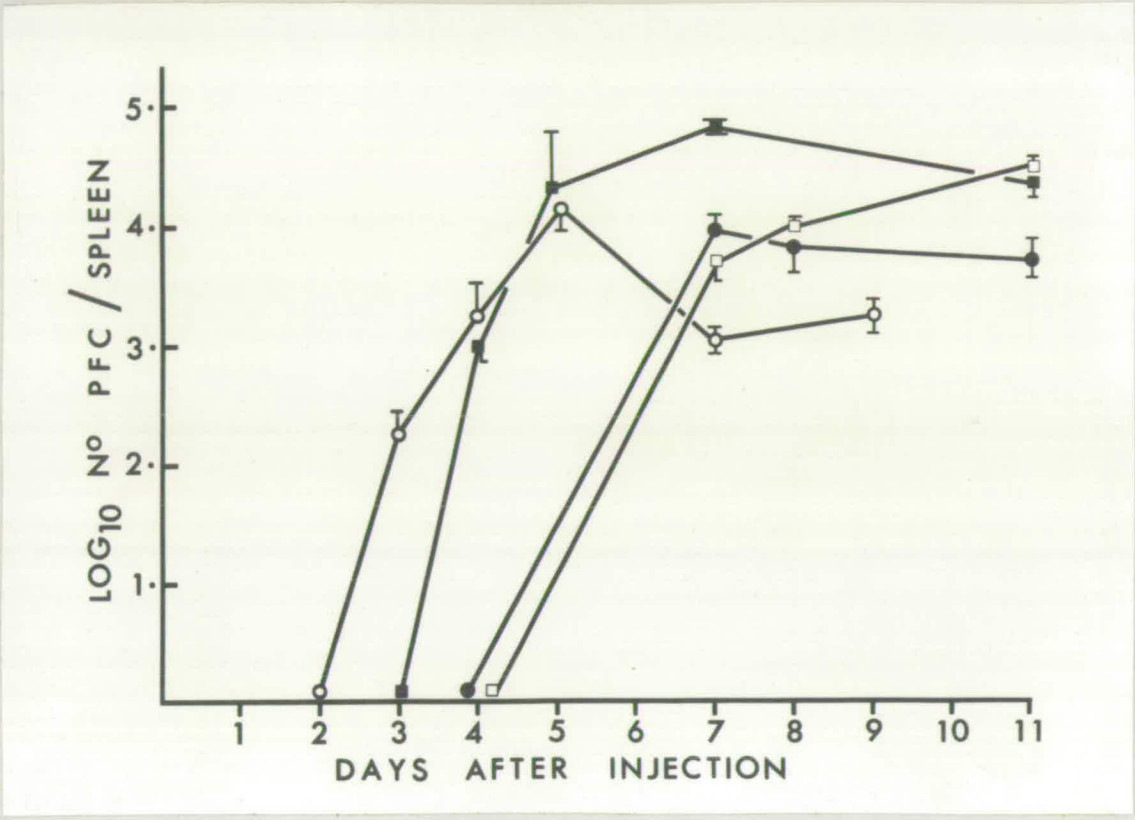
The results showed that the young mature mice 3 months of age produced a rapid rise, first in γM plaque forming cells (Fig. 1) then in γG plaques (Fig. 2). The peak of the γM response in these mice was observed on day 5 with about 65×10^3 PFC per spleen. The time of the peak γG response was not clear from the days assayed. The largest number of indirect plaques was seen on day 7 with about 85×10^3 PFC per spleen, but the shape of the graph suggests that the number on day 6 may have been greater (Fig. 2). The γM response declined in a linear manner from day 5 on and on day 11 there were only about 2×10^3 PFC per spleen. The γG response, on the other hand, was maintained at a higher level for a longer period of time with about 25×10^3 PFC per spleen remaining on day 11.

The γM response in all of the other groups appeared to be delayed (Fig. 1) since plaque counts were only marginally above background on day 3. With the exception of this initial delay the responses in the 9 and 16 month old mice was rather similar to that seen in the 3 month old animals. Analyses of variance failed to show any significant differences between these 3 groups on either day 5 or day 7 (the only days where direct comparisons could be made).

In contrast the 21 month old mice showed a more marked delay in their γM response, with a peak between days 5 and 7. With the exception of day 7, however, when the 21 month old mice were significantly higher in their response compared to the other groups assayed, no significant differences could be

FIG. 2

The indirect plaque response in CBAT6 male mice after the injection of 4 x 10^7 sheep erythrocytes.



■ 3 months old; □ 9 months old; ● 16 months old; ○ 21 months old.

TABLE 7

Results of Student t tests performed on data from Fig. 2

Day after injection	tests between ages	d.o.f.	t	P
7	3 and 9	4	7.22	< 0.02
7	3 and 16	4	5.82	< 0.02
7	3 and 21	3	22.16	< 0.001

d.o.f. - degrees of freedom; t - value of t; P - Probability.

found between any of the groups in their γM responses. The higher response seen in the 21 month old group may have been a reflection of the delay in the initiation of the response.

The γG response in the 9 and 16 month old mice was delayed for at least 24 hours compared to the 3 month old group. The 9 month old mice although still depressed in their response on day 7, compared to the 3 month old mice, appeared to make up their lack of activity in the later stages. On day 11 the response was equivalent in the 3 and 9 month old mice. Since this was the last day assayed it is possible that the 9 month old group may have improved their response on subsequent days.

Up to day 8 after injection the 16 month old mice were equivalent to the 9 month old mice. After this time the response in the 16 month old mice declined slowly but progressively.

The γG response in the 21 month old mice did not correspond to the trend seen in the other ages. In fact the initiation of the γG response appeared to be fastest in this group, with a clear developed response on day 3. The pattern of events in these old mice was further complicated by the slightly, though not significantly higher response on day 9 compared to day 7.

Data on body weights, spleen weights and total lymphocyte counts are shown in Tables 2 to 5. There appeared to be a tendancy for spleen weight to increase with age. This trend was substantiated in subsequent experiments but there were many individual exceptions. No clear relationship was discernable between the weight or cellularity of the spleen

and the number of plaque forming cells produced.

Since the response in the 21 month old group appeared not to follow the trend of the other ages assayed in this experiment a further experiment was designed to investigate the response in a number of old groups of mice.

3. The primary response to sheep erythrocytes in CBAT6 mice aged 3 and 21 months and in CBA mice aged 18 and 22 months.

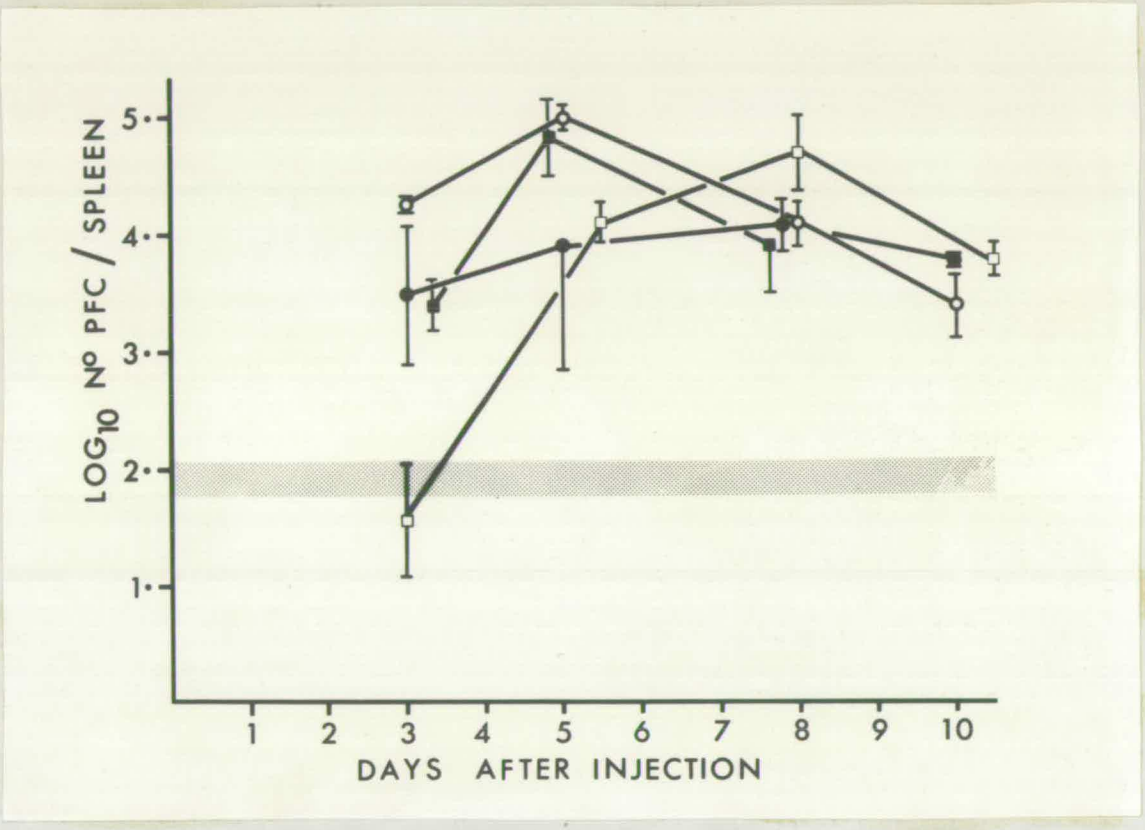
The results of this experiment with male mice are shown in Fig. 3 and 4 and relate to 3 and 21 month old CBAT6 and 18 month old CBA mice assayed at the same time. The data includes the results of a group of 22 month old CBA mice assayed on another occasion. All points on the graph show the mean and standard error of the mean of 3 mice and were responses made to 4×10^7 sheep erythrocytes injected intraperitoneally.

The γM response (Fig. 3) showed that there was a delay in the initiation of the response made by the older mice compared to the response made by the 3 month group. This trend was not however seen in the separately assayed 22 month old group. The γG responses were, however, consistently depressed in all the older groups, (Fig. 4).

It was concluded that both CBAT6 and CBA male mice exhibit a marked inability to respond to antigen in old age. The greatest depression was in the γG response; the γM response, although subject to delay, was not greatly affected. It would seem that the γG response seen in the 21 month old mice previously assayed (Fig. 2) was atypical of that age.

FIG. 3

Direct plaque response in CBAT6 and CBA male mice after the injection of 4 x 10^7 sheep erythrocytes.



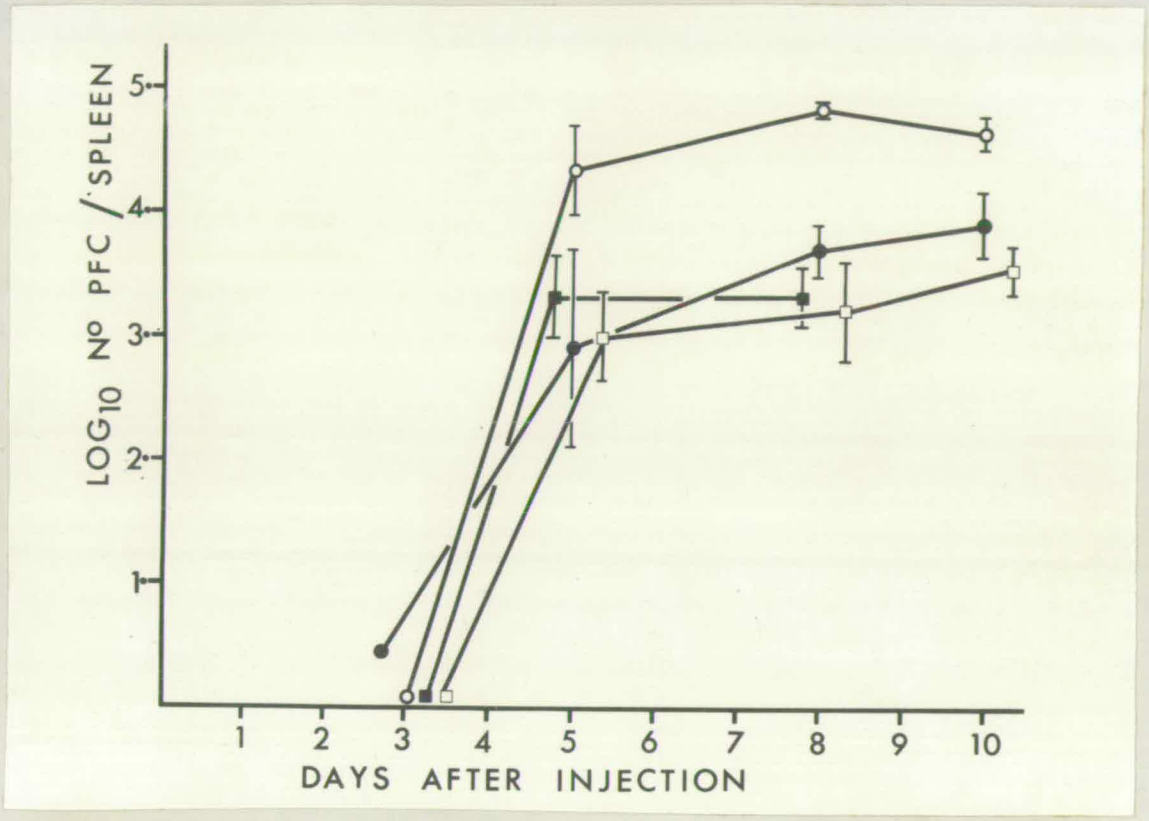
○ 3 month old CBAT6; ● 18 month old CBA

□ 21 month old CBA ■ 22 month old CBAT6

Background 95 percent confidence limits

FIG. 4

The indirect plaque response in CBAT6 and CBA male mice after the injection of 4×10^7 sheep erythrocytes.



○ 3 months old CBAT6; ● 18 months old CBA;

□ 21 months old CBA; ■ 22 months old CBAT6

TABLE 8

Results of Student t tests performed on data from Fig. 4

Day after injection	test between ages	d.o.f.	t	P
5	3 and 18	4	4.50	<0.02
8	3 and 18	4	4.36	<0.02
10	3 and 18	4	2.47	<0.05
5	3 and 21	4	3.50	<0.05
10	3 and 21	4	4.30	<0.02

d.o.f. - degrees of freedom; t - value of t; P - probability.

4. <u>The primary response to sheep erythrocytes in male CBAT6 mice aged 3, 7 and 16 months</u>.

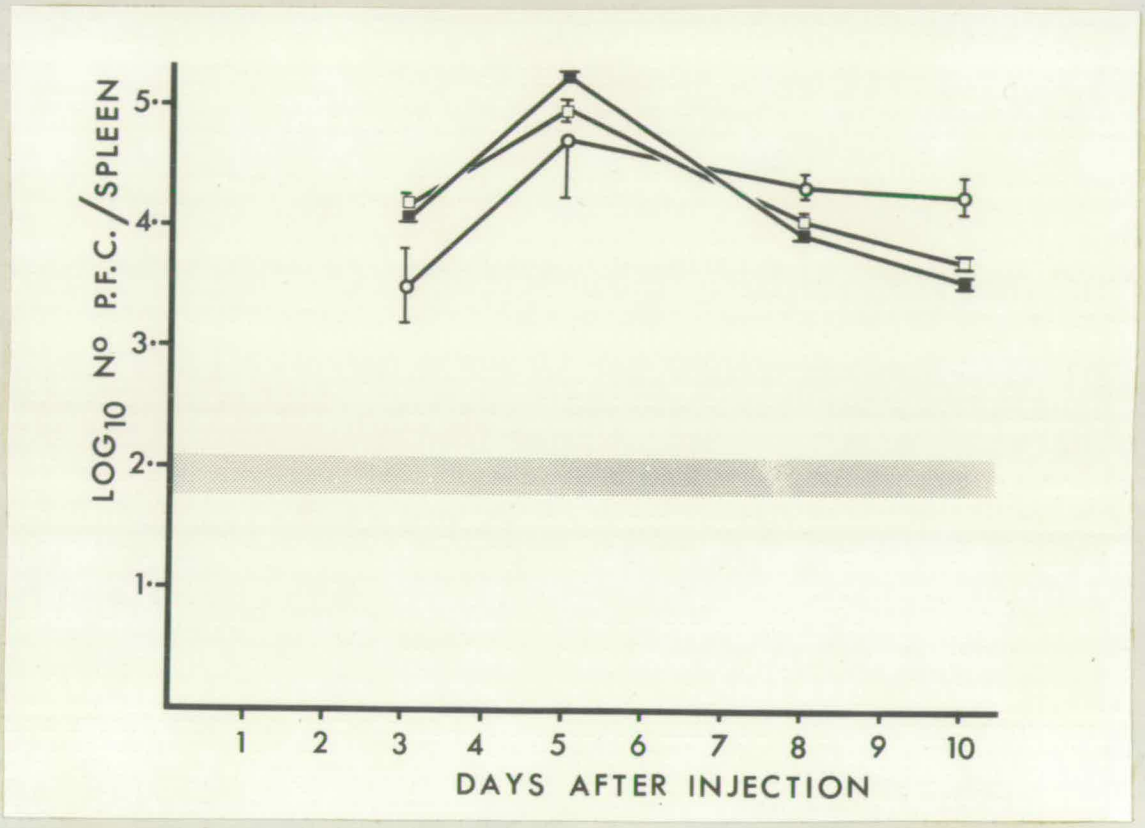
In view of reports from other research workers (WIGZELL and STJERNSWARD, 1966) that the response in CBA mice to sheep erythrocytes was maximal in animals 7 months of age, a further experiment was carried out using CBAT6 mice aged 3, 7 and 16 months. The results are shown in Fig. 5 and 6.

The γM response was similar in all the groups assayed; no one group was significantly different from another on any of the days assayed. There was a slight tendancy for the response of the 16 month old mice to be maintained at a higher level compared to the younger mice but this was not significant.

The γG response (Fig. 6) was similar in the 3 and 7 month old mice; no significant differences were established between these two groups on any of the days assayed. The 16 month old mice exhibited some delay in their γG response and peaked at a lower level compared to the other two groups.

FIG. 5

The direct plaque response in male CBA mice aged 3, 7 and 16 months after the injection of 4×10^7 sheep erythrocytes.

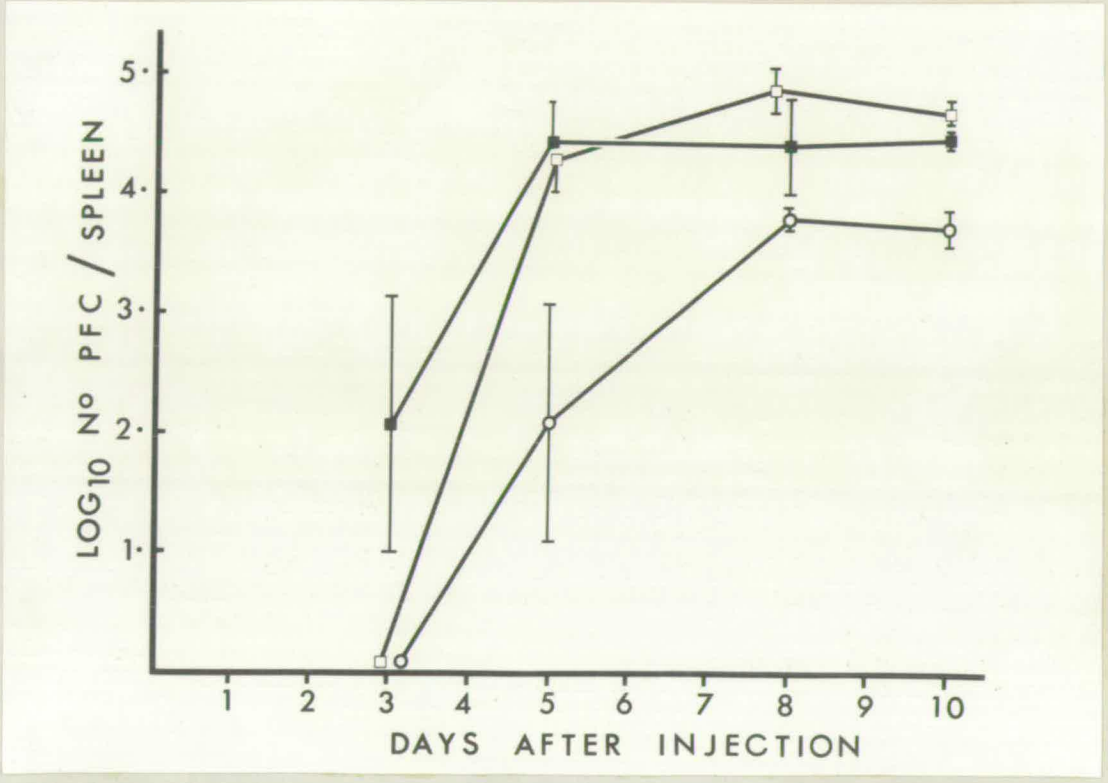


□ 3 months old; ■ 7 months old; ○ 16 months old.

Shaded area represents 95% confidence limits of background data

FIG. 6

The indirect plaque response in male CBA mice aged 3, 7 and 16 months after the injection of 4 x 10^7 sheep erythrocytes.



□ 3 months old; ■ 7 months old; ○ 16 months old.

TABLE 9

Results of Student t tests performed on data from Fig. 6

Day after injection	test between ages	d.o.f.	t	P
10	3 and 16	4	2.78	< 0.05
5	7 and 16	4	2.15	< 0.05
10	7 and 16	4	2.21	< 0.05

d.o.f. - degrees of freedom; t - value of t; P - probability.

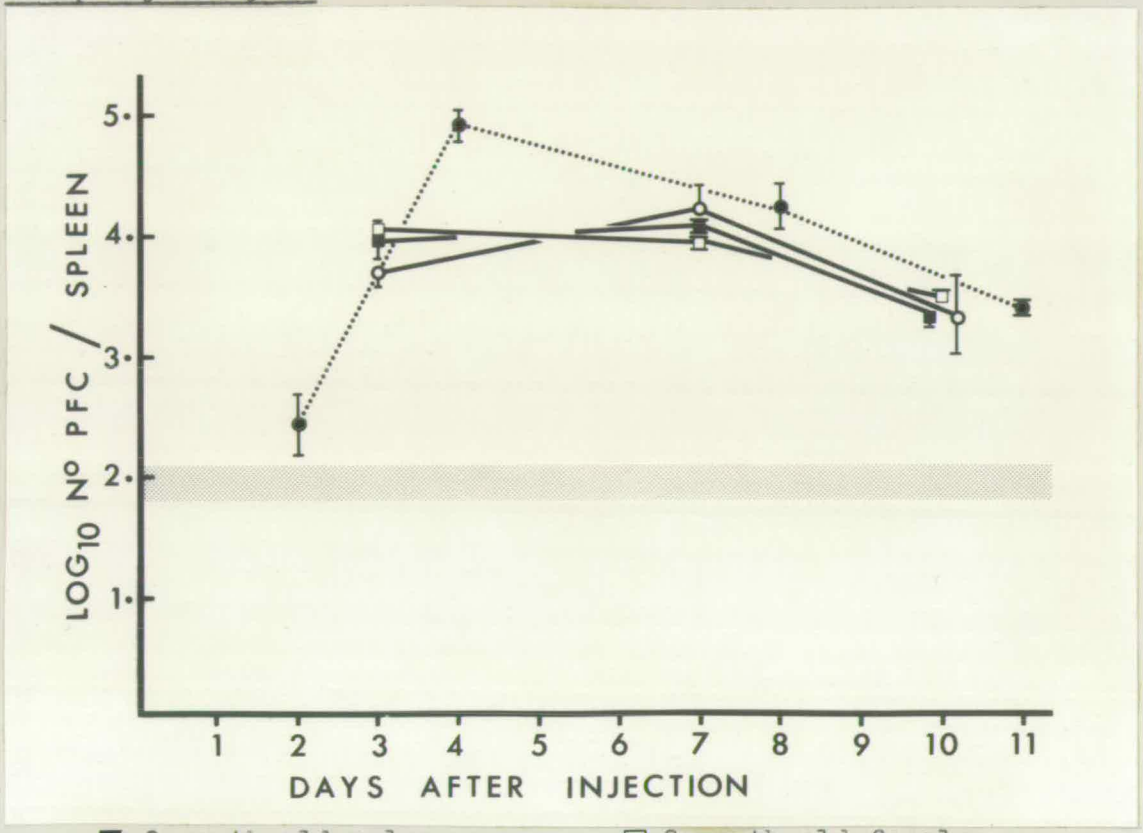
5. The primary response to sheep erythrocytes in CBAT6 female mice compared to that in males.

The ability of female mice to mount an immune response to sheep erythrocytes was compared to that in males. 3 month old male and female and 18 month old female mice were assayed on days 3, 7 and 10 after injection. The results are shown in Fig. 7 and 8 and include, for comparative purposes, a group of 16 month old male mice assayed at a different time. All points refer to the mean and standard error of the mean of 3 mice.

Both the γM (Fig. 7) and γG responses (Fig. 8) in the 3 month old groups and the 18 month old females were very similar and analyses of variance performed on the data showed that there was no significant difference between them. In comparison to previous results the γG responses were initiated rather earlier. The results when compared to the 16 month old males and previous experiments, suggest that female mice may not show the same rapid decline in the ability to produce γG antibody forming cells with age as do males.

FIG. 7

The direct plaque response in 3 and 16 month old male and 3 and 18 month old female CBAT6 mice after the injection of 4×10^7 sheep erythrocytes.



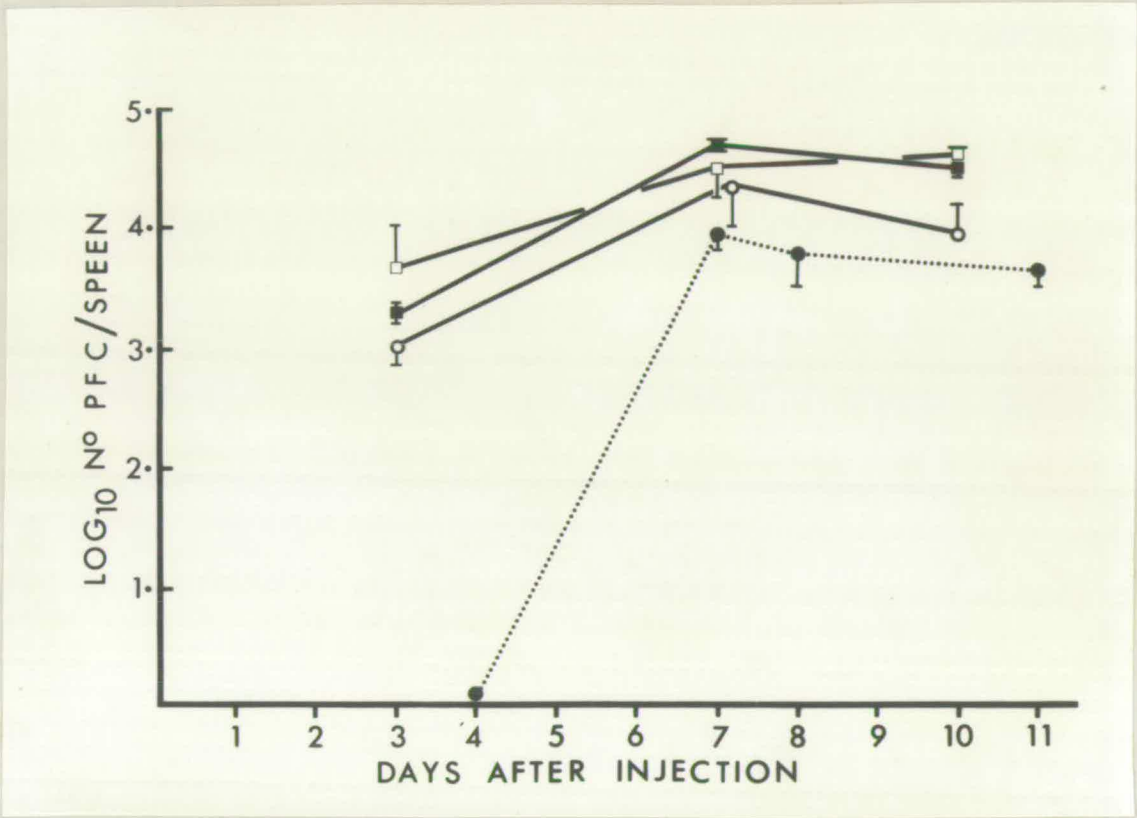
■ 3 month old male; □ 3 month old female;

●16 month old male; ○18 month old female.

Shaded area represents 95% confidence limits of background data.

FIG. 8

The indirect plaque response in 3 and 16 month old male and 3 and 18 month old female CBAT6 mice after the injection of 4×10^7 sheep erythrocytes.



■ 3 month old male; □ 3 month old female;

●16 month old male; ○ 18 month old female.

6. The primary serum antibody response in male CBA mice.

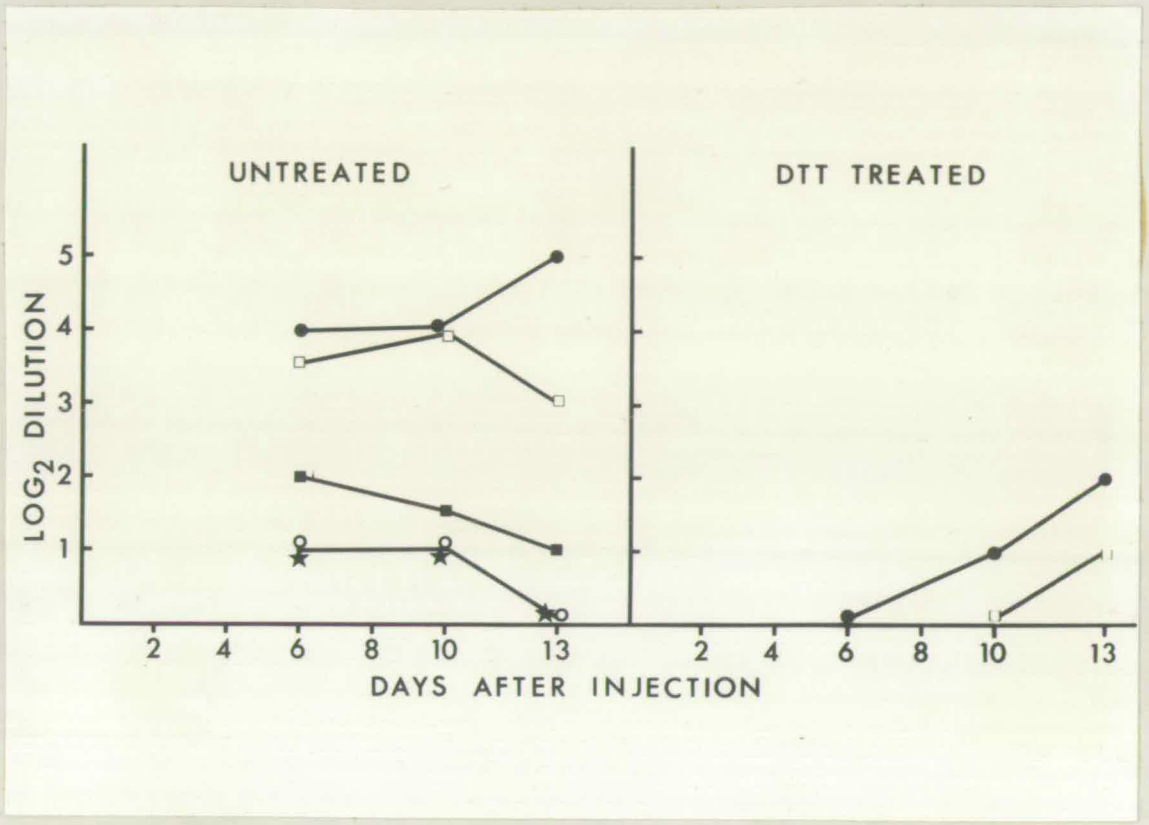
In this assay mice of 3, 14, 18, 24 and 1 month of age were bled at intervals during their response to a single injection of 4×10^7 sheep erythrocytes. The haemagglutination titres are shown in Fig. 9 and 10 and are the result of bleeding 6 mice from each group. It was not found possible to bleed mice repeatedly so as to provide individual samples large enough to perform a reduction and alkylation. As a result the sera from the 6 mice were pooled for each age group on any one day.

The results of DTT treatment (reduction and alkylation) are shown in Fig. 9. The ability of older mice to produce circulating 7S antibodies was markedly reduced. In the normal situation sera which had not been treated would contain both 19S and 7S antibodies. The 19S, γM type is far more efficient at causing haemagglutination. The DTT treated sera showed that only the 3 and 14 month old mice gave any response, indicating that these mice alone had sufficient 7S, γG antibodies to produce 'direct' haemagglutination in the absence of γM antibodies.

Fig. 10 shows the effect of adding a rabbit anti-mouse Ig serum to develop the response and agglutinate γG coated erythrocytes passively. The results showed that the amount of serum γG antibody formed in response to a single injection of erythrocytes declined with age. The 24 month old mice showed little or no ability to produce circulating γG antibody (Fig. 10). There was no detectable background response in any of the mice assayed. These data broadly confirm the plaque results shown earlier.

FIG. 9

Serum haemagglutination titres in male CBA mice, in response to sheep erythrocytes, before and after reduction and alkylation.

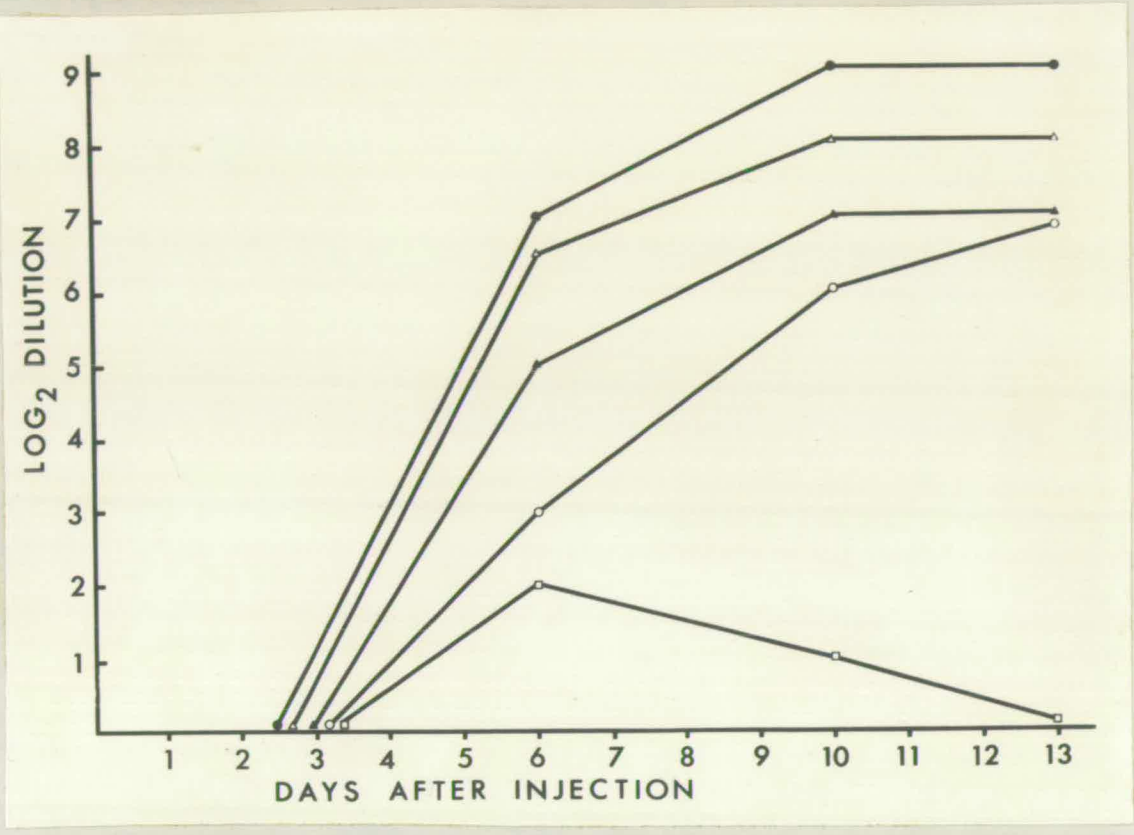


○ 1 month old; ● 3 months old; □ 14 months old;

■ 18 months old; ★ 24 months old.

FIG. 10

Serum haemagglutination titres in male CBA mice after the addition of a rabbit anti-mouse Ig serum, following reduction and alkylation.



○ 1 month old; ● 3 months old; △ 14 months old;

▲ 18 months old; □ 24 months old.

7. The primary response to sheep erythrocytes in female C57Bl mice.

As with the various CBA mice a number of individuals were used to ascertain the number of background plaques to sheep erythrocytes. Only a limited number of mice were available for the specific response to sheep erythrocytes and 2 mice from each age were assayed for background plaques. All the background plaque forming cells were of the direct type and no significant change occurred in their number with age. The data were pooled and the mean and 95% confidence limits calculated (Mean - 1.780; 95% C.L. - 2.135 and 1.445)

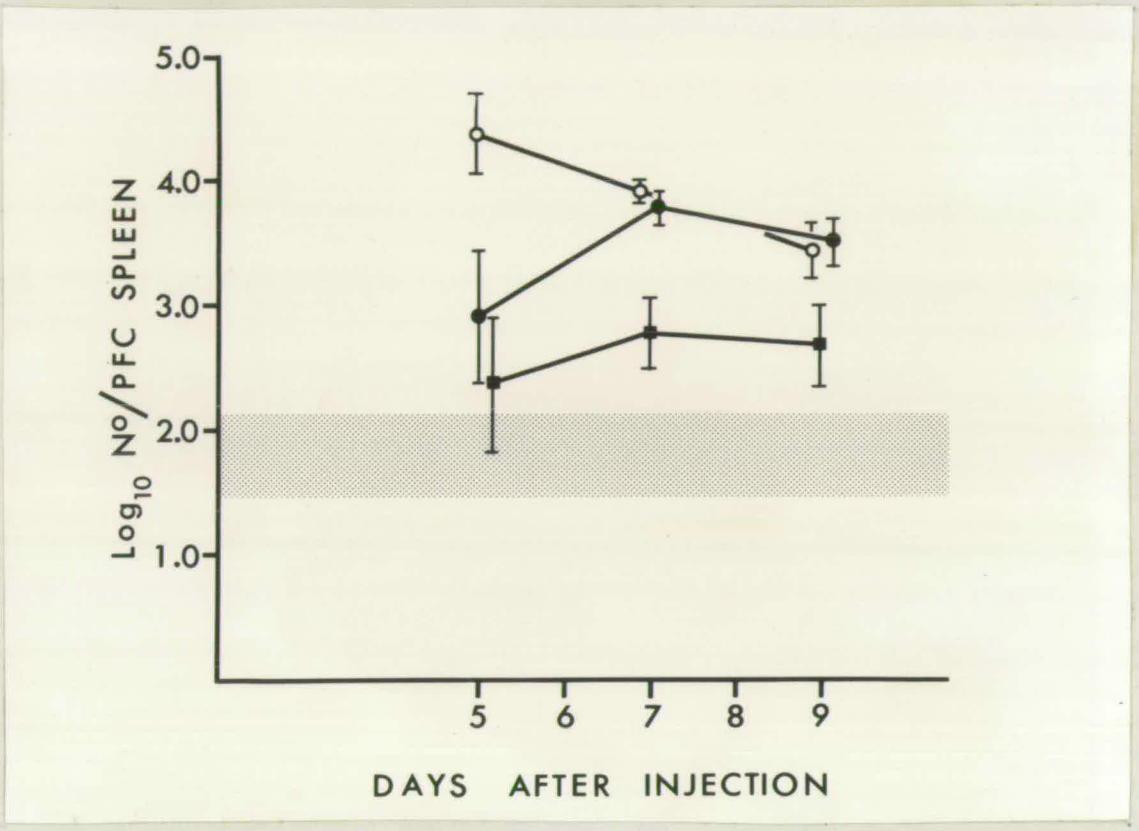
The response to 4×10^7 sheep erythrocytes was assayed on days 5, 7 and 9 after injection in 6, 12 and 24 month old mice. The results are shown in Fig. 11 and 12, each point represents the mean and standard error of the mean of 3 individuals.

The γM response (Fig. 11) showed, in contrast to the CBA mice, a rather rapid decline in the ability to respond with age. Dispite considerable variability in the results of individual mice the 24 month old animals were considerably lower in their response compared to the other two groups and barely exceeded the background 95% confidence limit.

The γG response appeared to remain rather constant in the 6 month old mice with approximately 10×10^3 PFC per spleen on each of the days assayed; considerably lower than the γG response of similarly aged CBA mice. The responses in the older mice were very variable. This clearly indicates a change in the older mice but makes statistical comparisons difficult.

FIG. 11

The direct plaque response in female C57Bl mice after the injection of 4 x 10^7 sheep erythrocytes.

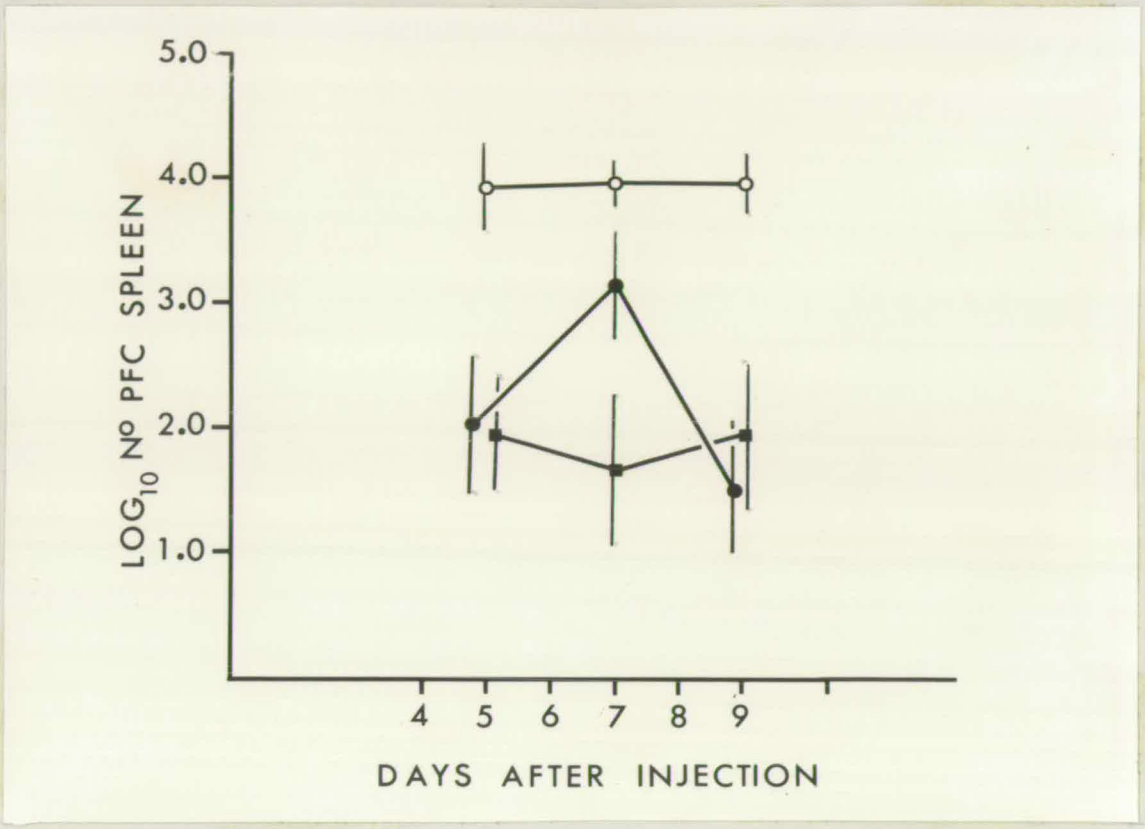


○ 6 months old; ● 12 months old; ■ 24 months old.

Shaded area represents 95% confidence limits of background data.

FIG. 12

The indirect plaque response in female C57Bl mice after the injection of 4×10^7 sheep erythrocytes.



○ 6 months old; ● 12 months old; ■ 24 months old.

The sera of the same mice were assayed for haemagglutin--ins. Individual variation was so great that no difference could be seen between any of the groups.

The general condition of the mice was poor compared to similarly ages CBA mice. This was not a feature peculiar to the sample used but was common to the stock as a whole. C57Bl mice exhibited a rapid loss of hair with age and were not as clean in their habits as CBA mice. Hardening of the skin was a common feature of 12 month old mice. A similar condition is not generally seen in CBA mice until about 20 months of age. However, the internal anatomy showed no obvious sign of malignancy and the spleens exhibited no great change in weight with age.

8. The primary response to sheep erythrocytes in (CBA x C57Bl)F_1 male mice.

The number of background plaque forming cells was calculated in a number of mice of different ages and was seen not to change significantly. The data were pooled and a mean value calculated (Mean - 1.939; 95% C.L. - 1.764 and 2.125).

Male mice aged 13, 17, 21 and 24 months were injected with 4×10^7 sheep erythrocytes and assayed on days 3, 5, 7 and 10. The results are shown in Fig. 13 and 14. Individual points refer to the mean response of 3 mice.

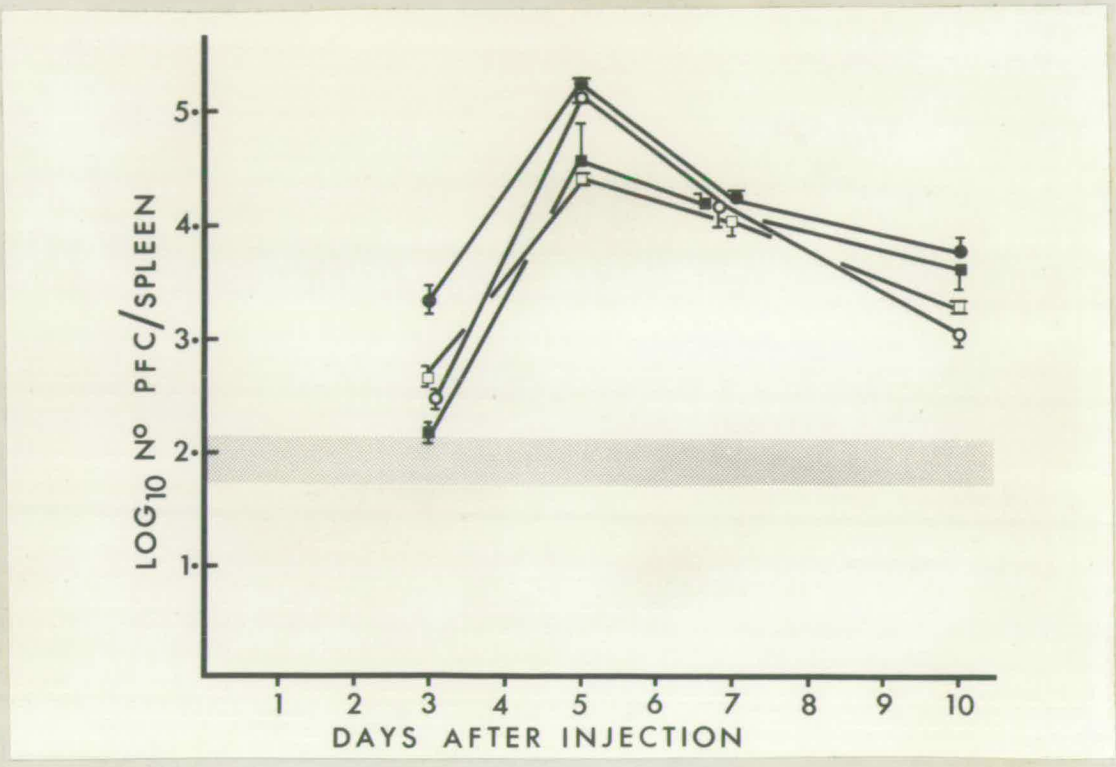
The γM response (Fig. 13)was rather more vigerous than that seen in CBA mice and the maximum response in the 13 and 17 month old groups exceeded 150×10^3 PFC per spleen on day 5. The higher response seen on day 3 in the 13 month old mice was significantly higher than the responses in the other three groups. The number of plaques produced by the 21 and 24 month old mice, on day 5, was lower than in the other groups but this was significant only in the case of the 24 month old mice.

The indirect, γG response was very rapid in the 13 month old mice. All the other groups exhibited a delay in their responses. In each of the three older groups only one mouse, of the three assayed on day 5, showed any indirect response and this is the reason for the large 'standard error' of the mean points at that time. The number of indirect plaque forming cells on later days in the response was not significantly different in any of the groups assayed.

The condition of the mice was good. The animals were

FIG. 13

The direct plaque response in male (CBA x C57Bl)F_1 mice after the injection of 4 x 10^7 sheep erythrocytes.



● 13 months old; ○ 17 months old; ■ 21 months old; □ 24 months old
Shaded area represents 95% confidence limits of background data.

TABLE 10

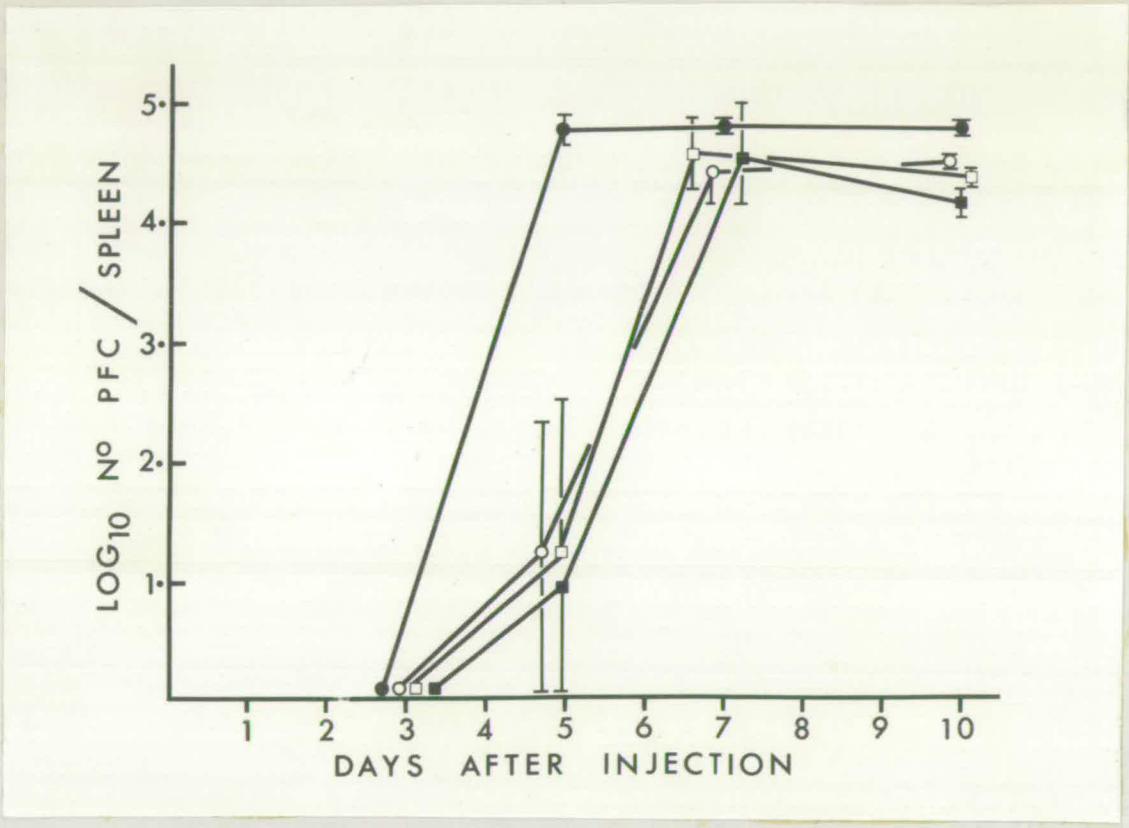
Results of Student t tests performed on data from Fig. 13

Day after injection	test between ages	d.o.f.	t	P
3	13 and 21	4	3.51	<0.05
5	17 and 21	4	2.18	<0.05
5	13 and 24	4	9.57	<0.01

d.o.f. - degrees of freedom; t - value of t; P - probability.

FIG. 14

The indirect plaque response in male (CBA x C57Bl)F_1 mice following the injection of 4 x 10^7 sheep erythrocytes.



● 13 months old; ○ 17 months old; □ 21 months old;

■ 24 months old.

clean and healthy and were quite active, even at 21 months of age. There were no signs of any gross malignancy in the internal anatomy. The body and spleen weights were rather higher on average than those seen in CBA or C57Bl mice but showed very little trend towards an increase in size with age.

9. The primary response to sheep erythrocytes in very old (CBA x C57Bl)F_1 female mice.

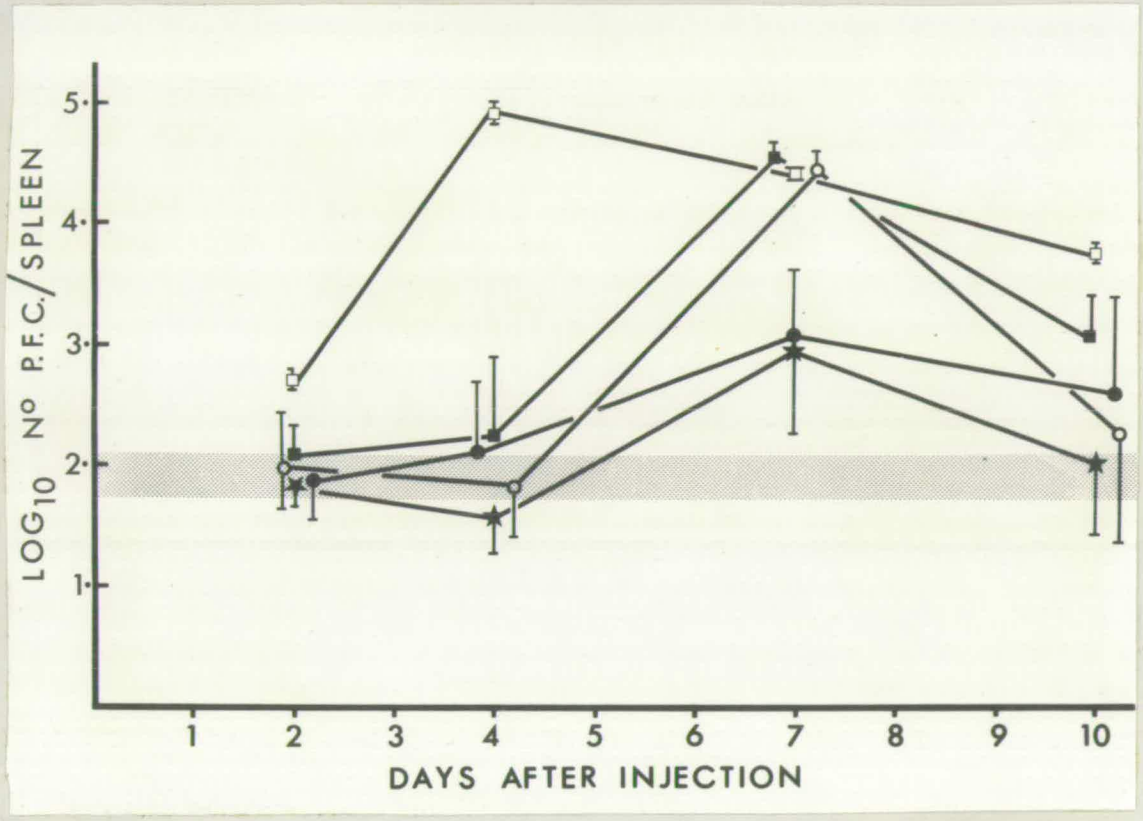
In view of the rather vigorous response obtained in male mice up to the age of 24 months (Fig. 13 and 14) a further experiment was planned in very old animals. Female mice aged 3, 30, 34, 37 and 40 months were assayed on days 2, 4, 7 and 10 after injection with sheep erythrocytes. The background number of plaque forming cells was assayed in a number of mice from each of the experimental groups and was found not to change significantly with age and fell within the 95% confidence limits established in the previous experiment. The results of the response to sheep erythrocytes are shown in Fig. 15 and 16.

The direct γM response (Fig. 15) in the 3 month old mice was very similar to that seen in the 13 month old males (Fig. 13). The response in the other, old, groups was considerably delayed. None of the older mice (30 to 40 months) showed a response significantly above background on day 2 and very few did on day 4. Mice in the 30 and 34 month old groups initiated their responses on or shortly after day 4 and reached a maximum level on or around day 7. The responses in the two oldest groups showed very little activity, even on day 7, when they were significantly lower than all the other groups.

The large 'standard errors' seen in Fig. 15 and 16 were largely attributable to the failure of one of the three mice assayed to give any detectable response. In such cases the data were not normally distributed and analyses of variance were not practicable. This problem is most clearly seen in the

FIG. 15

The direct plaque response in (CBA x C57Bl)F_1 female mice after the injection of 4 x 10^7 sheep erythrocytes.



□ 3 months old; ○ 34 months old; ✱ 40 months old;

■ 30 months old; ● 37 months old.

Shading represents 95% confidence limits of background data.

TABLE 11

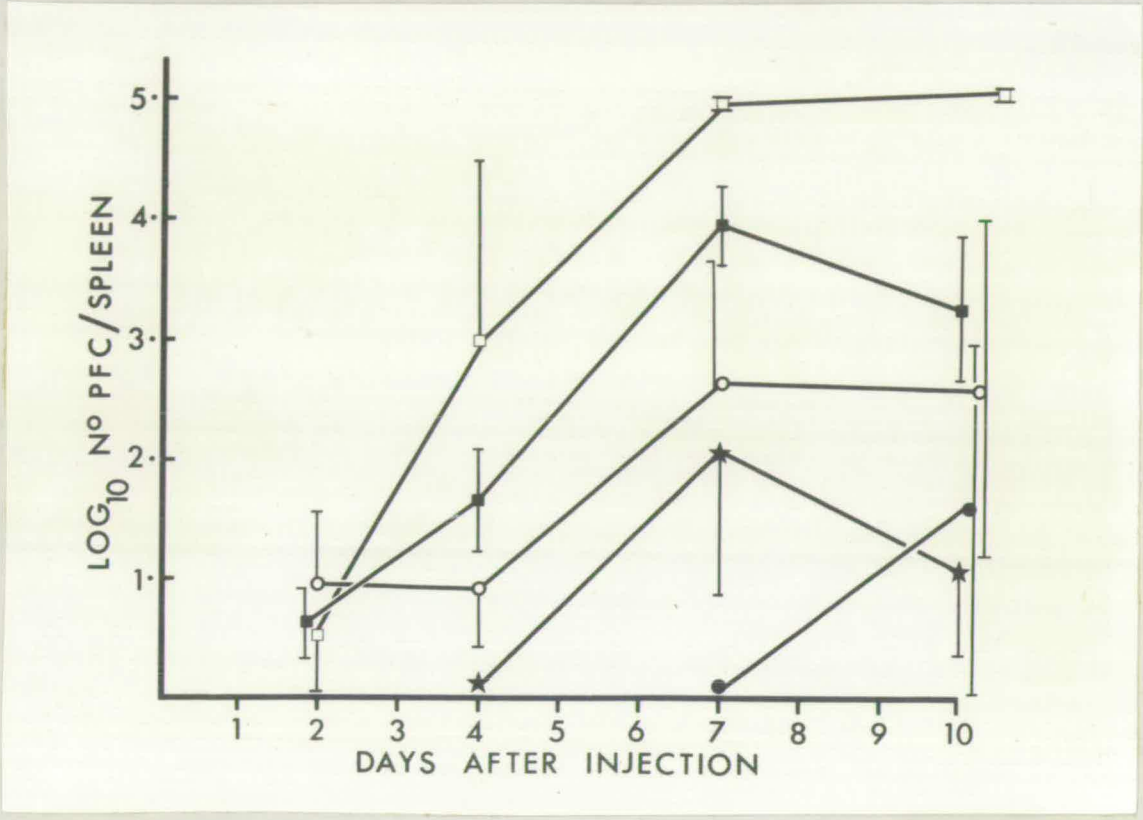
Results of Student t tests performed on data from Fig. 15

Day after injection	test between ages	d.o.f.	t	P
4	3 and 30	4	6.45	<0.01
10	3 and 34	4	2.88	<0.05
10	3 and 40	4	3.20	<0.05

d.o.f. - degrees of freedom; t - value of t; P - probability.

FIG. 16

The indirect plaque response in (CBA x C57Bl)F_1 female mice after the injection of 4 x 10^7 sheep erythrocytes.



□ 3 months old; ■ 34 months old; ★ 40 months old;

○ 30 months old; ● 37 months old.

TABLE 12

Results of Student t tests performed on data from Fig. 16

Day after injection	test between ages	d.o.f.	t	P
7	3 and 30	4	2.86	<0.05
10	3 and 30	4	2.87	<0.05
10	3 and 37	4	3.77	<0.02
10	3 and 40	4	5.55	<0.01

d.o.f. - degrees of freedom; t - value of t; P - probability.

indirect γG responses (Fig. 16). Significant differences could only be established between the 3 month old mice and the other ages but not between any of the four older groups. It was clear, however, that the ability of the old mice to mount a response was severely affected.

Some of the 40 month old mice showed some signs of malignancy in their spleens (probably reticulum cell sarcoma) and in every case this severely affected their ability to respond, even compared to other mice in the same group. The results of these mice were not, however, removed from the data as they were considered to represent a genuine trend in the pathology of the old mouse.

It was clear from this experiment that both the γM and the γG responses were affected by age to an almost equivalent extent. The fact that the γM responses were more uniform in nature than the γG responses, together with the observation that many more mice made no indirect plaque response than was true for the γM response indicate that the 7S, γG response was still affected to a greater extent.

Conclusions

The studies on the primary response to sheep erythrocytes support a number of other reports (WIGZELL and STJERNSWARD, 1966, METCALF et al, 1967; KISHIMOTO et al, 1969; MAKINODAN et al 1971a), which have revealed a marked age-dependent depression in the ability to produce antibody forming cells and circulating antibody. Despite differences in detail between the different strains used it would appear that the depression exhibited certain common features. The 7S, γG response was affected earlier and with greater severity. The time at which both the γM and γG responses were initiated was later in old mice and this was reflected in the time at which peak activity was seen. Very old mice displayed a more widespread depression in their responses with the γM response affected almost as severely as the γG response.

The possibility of a sex difference remains a possibility but requires more dta before any firm conclusion can be made.

CHAPTER 4

The secondary response to sheep erythrocytes in CBAT6 and (CBA x C57Bl)F_1 male mice.

1. The secondary response in 9 and 24 month old CBAT6 mice

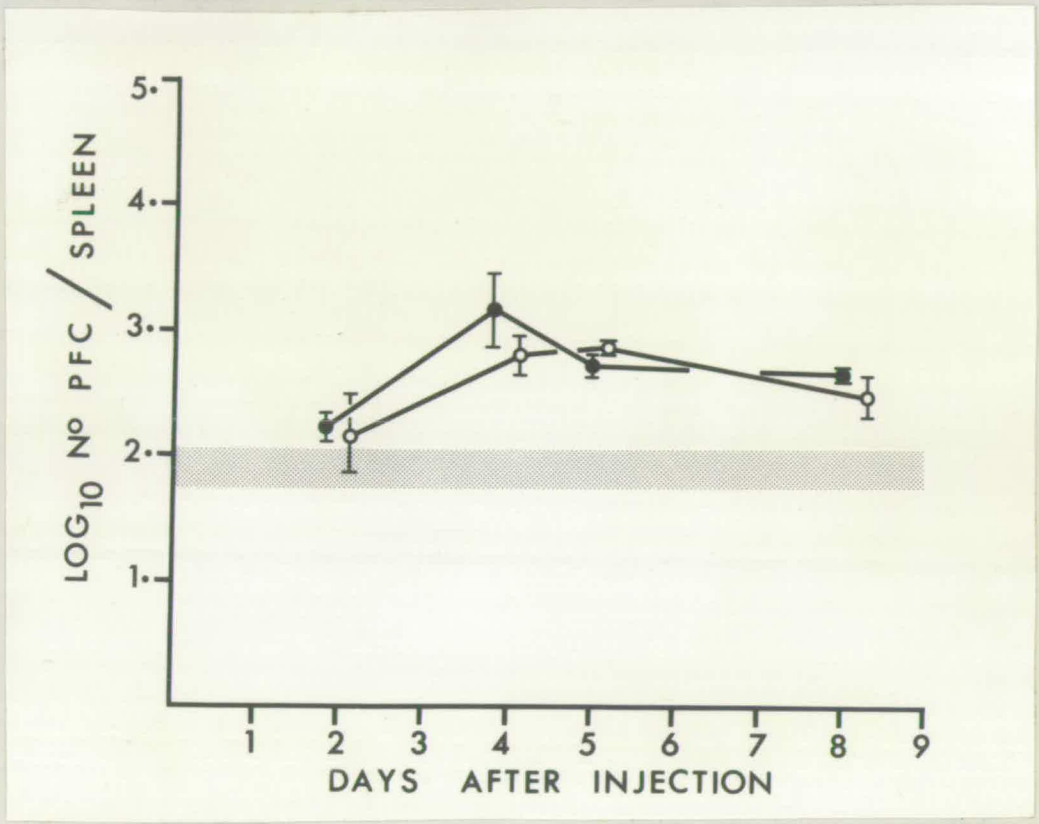
All the mice were primed with 4 x 10^7 sheep erythrocytes and given their second injections of the same dose 30 days later. The secondary response was assayed on days 2, 4, 5 and 8 after the second injection. The results are shown in Fig. 17 and 18. Each point represents the mean of responses in 3 mice.

No significant differences were found between the two groups, in either their γM or γG responses, on any of the days assayed.

The γG response was much lower than had been anticipated, especially in the 9 month old group. The general phenomenon of a reduced γM response and rapid γG response was seen in both groups, but it seems likely that the results as a whole did not reflect the true potential of the mice concerned. Two possibilities may account for this. Either the developing serum was defective, or the serum antibody levels from the primary response were still sufficiently high to inhibit the full exprssion of a secondary response. The second possibility has some foundation since the haemagglutination results of sera collected from mice before and after a second injection of sheep erythrocytes revealed considerable quantities of antibody still present, especially in the younger animals (Fig. 19).

FIG. 17

The direct plaque response in 9 and 24 month old CBA male mice after a second injection of 4×10^7 sheep erythrocytes.

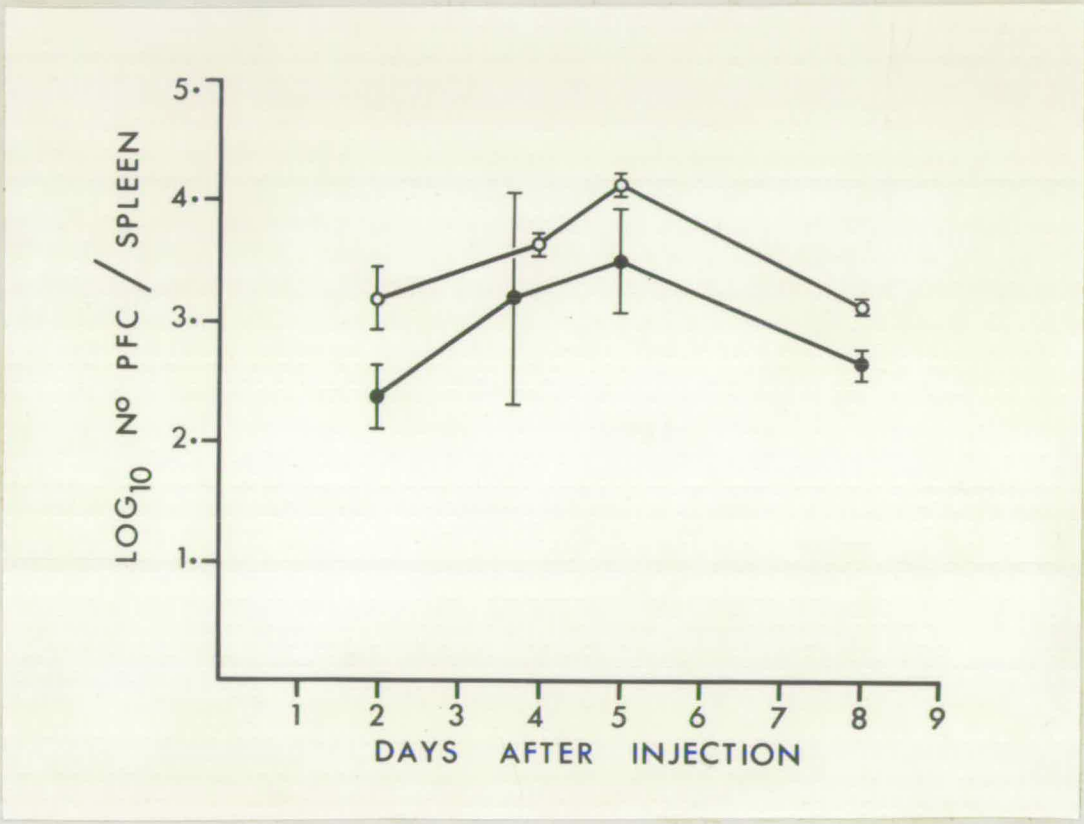


○ 9 months old; ● 24 months old.

Shaded area represents 95% confidence limits of background data.

FIG. 18

The indirect plaque response in 9 and 24 month old CBA mice after a second injection of 4×10^7 sheep erythrocytes.



○ 9 months old; ● 24 months old.

2. The secondary response to sheep erythrocytes in CBAT6 mice assayed by a passive haemagglutination technique.

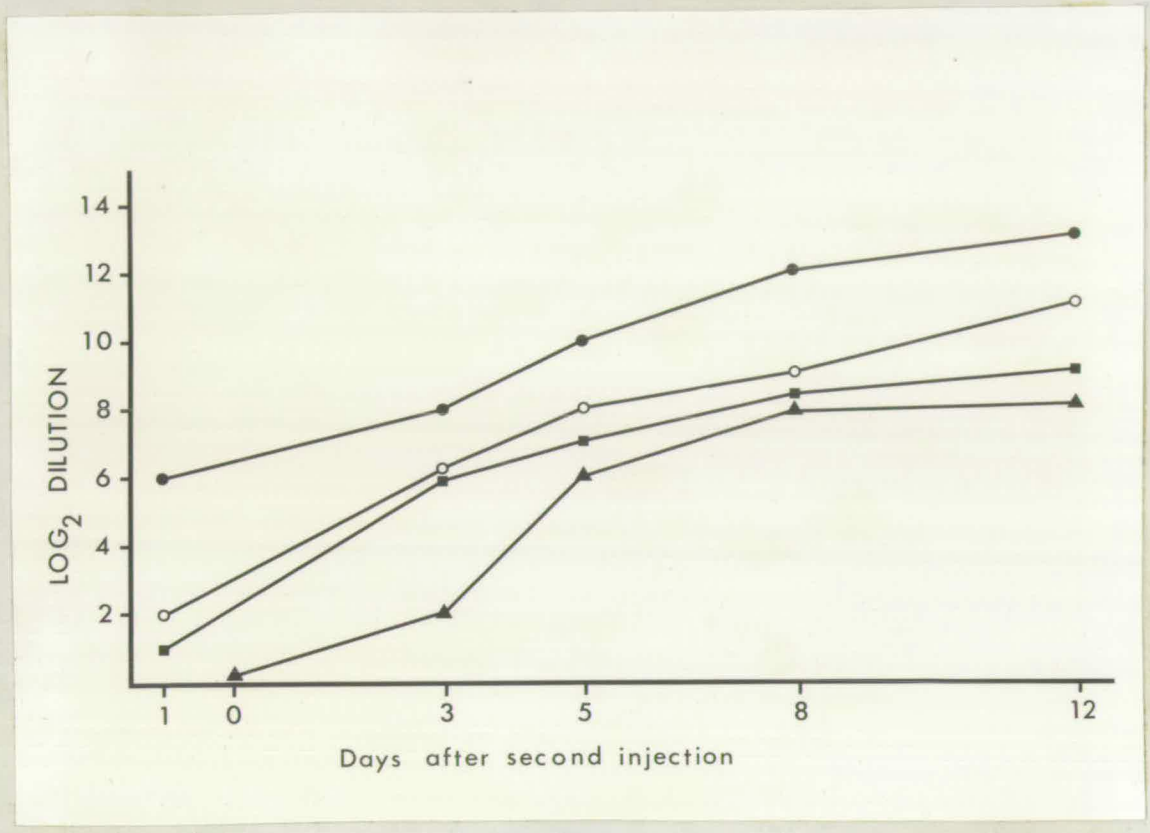
CBAT6 mice aged 3, 14, 18 and 24 months were injected with 4×10^7 sheep erythrocytes on two occasions separated by a period of 30 days. Each point represents one pooled sample from 5 mice. Sera were reduced and alkylated and a rabbit anti-mouse serum added to reveal more fully the γG response.

The results are shown in Fig. 19.

The titres of antibodies in the blood one day before the second injection was administered, showed that there was a considerable quantity of circulating antibody present, especially in the 3 month old mice. After the second injection there was a slow but steady increase in the titre of γG antibodies in all of the ages investigated to levels which exceeded those seen in the primary response (Fig. 10). The age dependent depression of serum antibody production seen in the primary response was reflected in the results seen after a second injection.

FIG. 19

'Developed' serum haemagglutination titres in CBAT6 male mice after a second injection of 4×10^7 sheep erythrocytes.



● 3 months old; ○ 14 months old; ■ 18 months old; ▲ 24 months old.

3. The secondary response to sheep erythrocytes in (CBA x C57Bl)F1 male mice.

Mice aged 16, 20 and 24 months were given two injections of 4×10^7 sheep erythrocytes separated by a period of 28 days. Their spleens were assayed for plaque forming cells on days 2, 5 and 7 after the second injection. The results are shown in Fig. 20 and 21. Each point refers to the mean value of 3 mice.

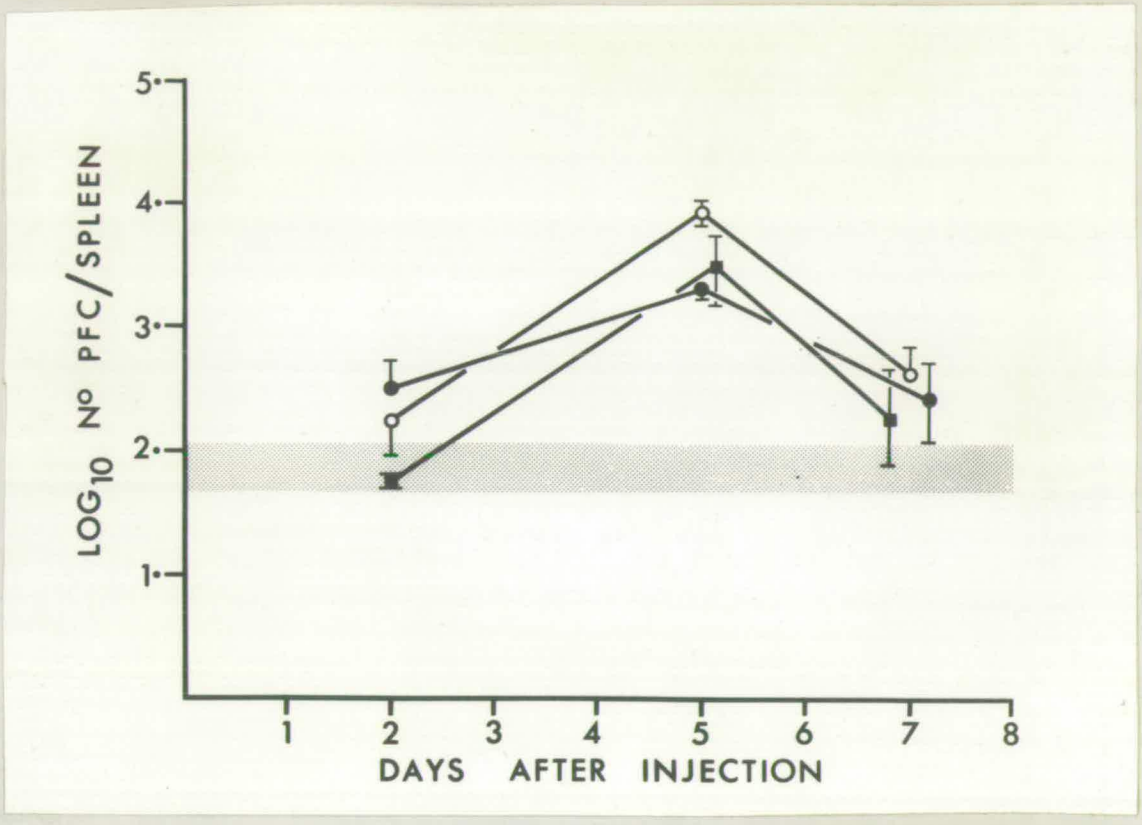
The direct γM response (Fig. 20) appeared to be initiated with approximately the same vigour as in the secondary response of CBA mice (Fig. 17) but was continued to a higher maximum level. The only significant difference occurred on day 5 when the 20 month old group were lower in their response compared to the 16 month old animals. However, this was not true of the 24 month old mice, which with the exception of one of the three mice assayed, responded rather well on this day compared to the 16 month old group.

The γG response (Fig. 21) was lower than in the primary response of similarly aged animals (Fig. 14) but higher than the secondary γM response (Fig. 20). No significant differences were found between any of the groups on any of the days assayed.

The remarks made on the CBAT6 secondary response apply equally to the results of this assay. The responses obtained were very low and although there were a limited number of differences between the groups assayed they cannot be treated very seriously as representing the true potential of the mice concerned.

FIG. 20

The direct plaque response in 16, 20 and 24 month old male (CBA x C57Bl)F_1 mice after a second injection of 4 x 10^7 sheep erythrocytes.

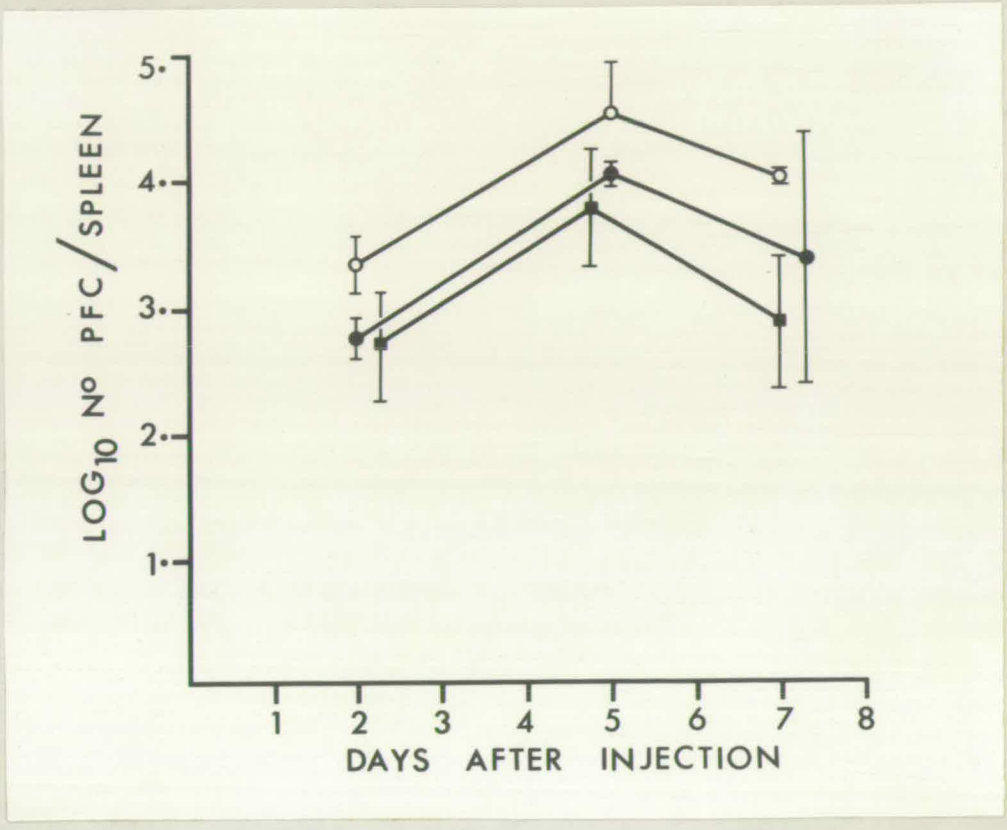


○ 16 months old; ● 20 months old; ■ 24 months old.

Shaded area represents 95% confidence limits of background data

FIG. 21

The indirect plaque response in 16, 20 and 24 month old male (CBA x C57Bl)F_1 mice after a second injection of 4 x 10^7 sheep erythrocytes.



○ 16 months old; ● 20 months old; ■ 24 months old.

Conclusions

There can be no doubt that full secondary responses were not assayed by the plaque technique in either the CBAT6 or the (CBA x C57Bl)F_1 mice. Although it is possible that serum antibody levels, as a result of primary stimulation, may have prevented a full secondary response it is clear that a reasonable age separation was achieved in the serum haemagglutination titres, using the same injection regime. In both the plaque experiments 3 mice from each group assayed were used for background studies. In every mouse assayed the only plaque forming cells present were of the direct type and the numbers fell within the 95% confidence limts data for unstimulated mice. This fact together with the very low responses achieved in the secondary plaque assay would suggest that there was a fault in the developing serum. However, the same batch of serum was used effectively in the haemagglutination technique.

It is concluded that the serum antibody levels reflect more accurately the effect of age on the secondary response since they conform to the general pattern reported by MAKINODAN and his colleagues (1971b) who studied the secondary response using a plaque technique similar to the one used here.

CHAPTER 5

The effect of age on the proliferative response to oxazolone

1. The primary response to oxazolone in CBAT6T6 mice

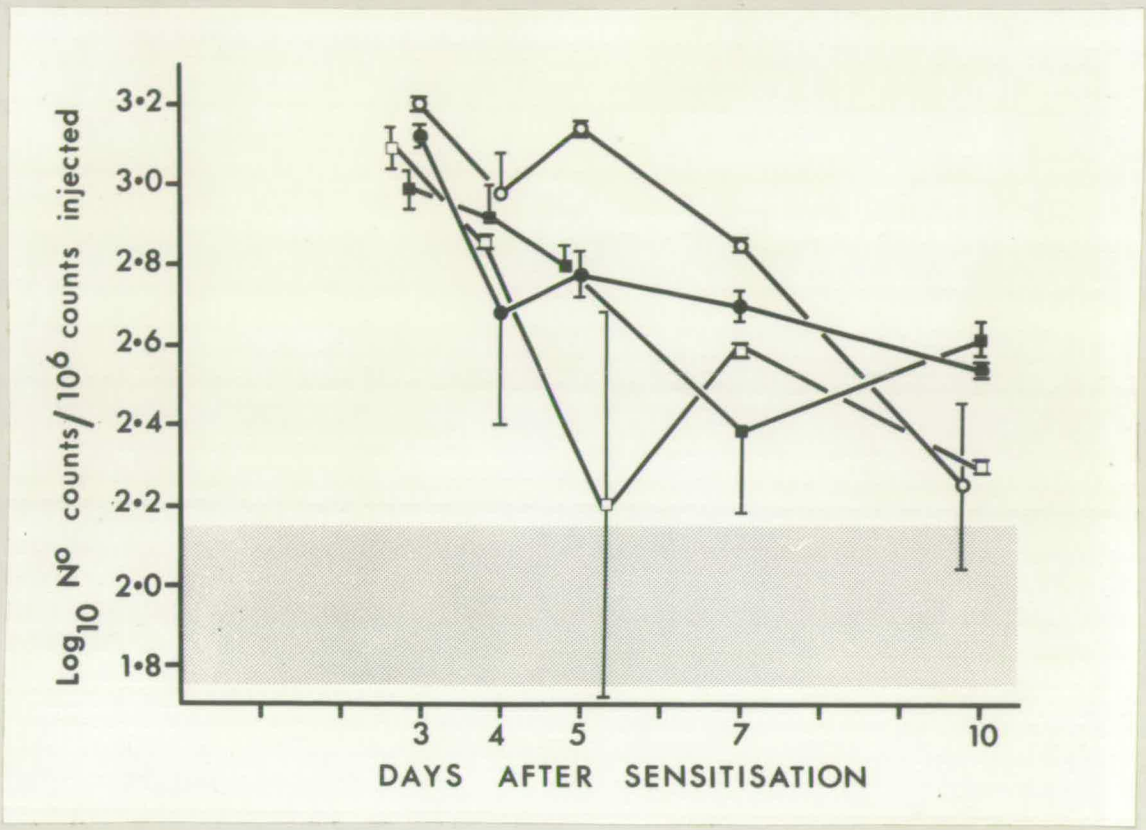
Male mice aged 3, 12, 17 and 19 months and females aged 3, 17, 21 and 24 months were sensitised with 10.0mg oxazolone and the resultant proliferation in the regional lymph nodes measured by the incorporation of ^{125}IUdR into DNA. Background levels of incorporation were measured in 3 mice from each group after the administration of alcohol alone. The specific response to oxazolone was measured in 3 mice from each group on days 3, 4, 5, 7 and 10 with the exception of the female mice aged 3 and 17 months which were not assayed on days 5 and 7 respectively.

The incorporation of ^{125}IUdR was measured in the axillary and brachial lymph nodes on both the draining and contralateral sides of the mice. The number of incorporated counts in the brachial and axillary nodes from any one side of an individual mouse were pooled and the results expressed first as arithmetic counts per 10^6 counts injected and then log-transformed. Mean values and statistical analyses were calculated on log-transformed data.

Initial work showed that there was no significant difference between the background levels of incorporation in draining and contralateral nodes in any one group or between the data from any one age group compared to another. The male background data were pooled and a mean value with 95% confidence

FIG. 22

Cellular proliferation in the draining lymph nodes of male CBAT6T6 mice after sensitisation with 10mg oxazolone, measured by the incorporation of ^{125}IUdR.



○ 3 months old; ● 12 months old; □ 17 months old; ■ 19 months old. Shaded area represents 95% confidence limits of background data.

TABLE 13

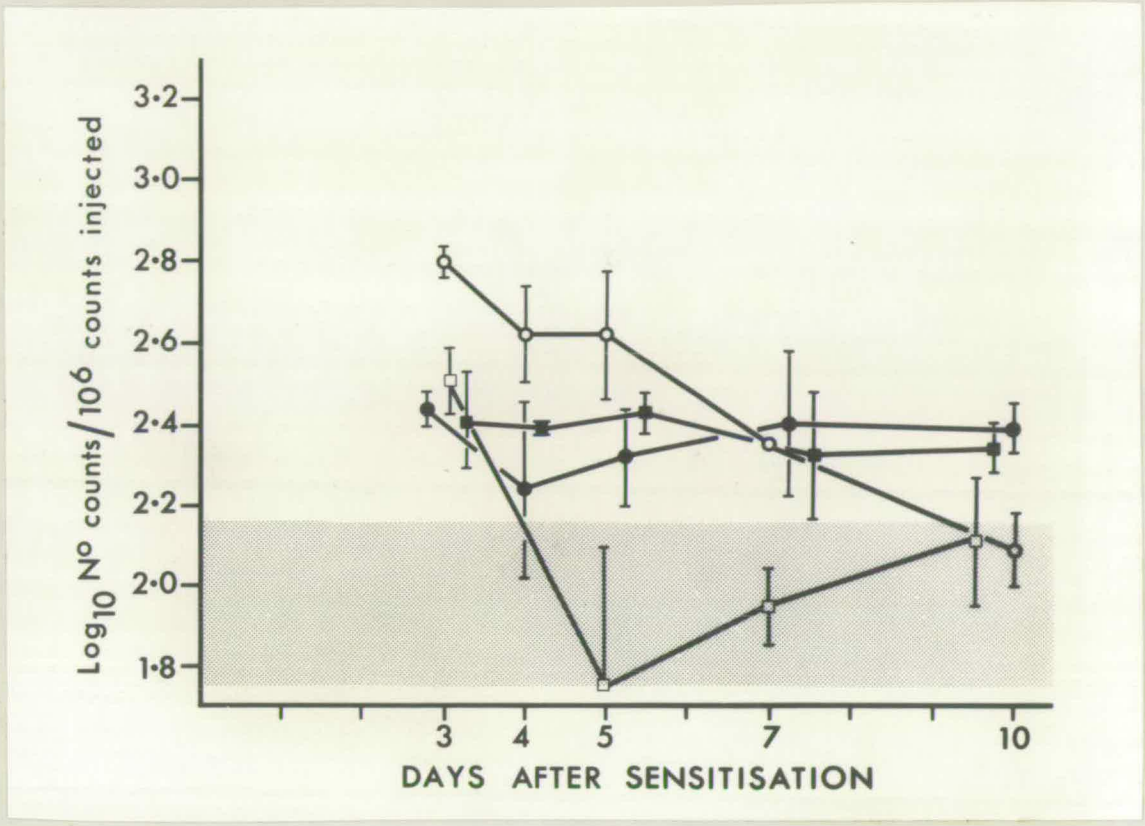
Results of Student t tests performed on data from Fig. 22

Day after sensitisation	test between ages	d.o.f.	t	P
3	3 and 19	4	4.01	< 0.02
5	3 and 12	4	6.03	< 0.01
5	3 and 19	4	5.92	< 0.01

d.o.f. - degrees of freedom; t - value of t; P - probability.

FIG. 23

Cellular proliferation in the contralateral lymph nodes of male CBAT6T6 mice after sensitisation with 10mg oxazolone, measured by the incorporation of ^{125}IUdR.



○ 3 months old; ● 12 months old; □ 17 months old; ■ 19 months old.

Shaded area represents 95% confidence limits of background data.

limits (mean = 1.9087; C.L. = 2.1653 and 1.7521). The background data in the female mice was not significantly different from that in the males but was less variable (mean = 1.9189; C.L.= 1.9759 and 1.8619).

The specific response to oxazolone in the draining nodes of male mice is shown in Fig. 22. The general response profile shows that the highest levels of incorporation were on day 3 in all ages. The early response was slightly lower in the old mice and, in comparison with the day 3 and day 5 data from 3 month old mice, appeared to decline rather rapidly after peak activity.

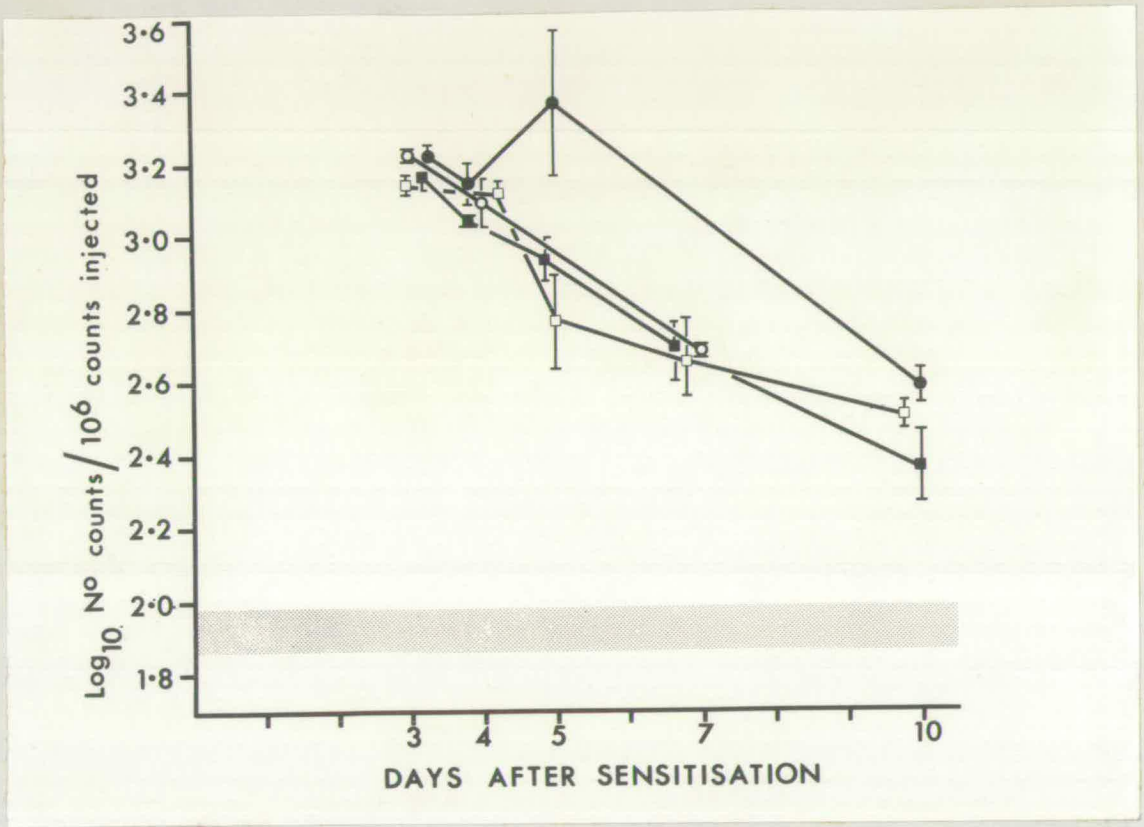
The response in the contralateral nodes (Fig. 23) was lower than in the draining nodes and the early response of the older mice was more clearly depressed compared that in the 3 month old animals.

The response in the females is shown in Fig. 24 and 25. With the exception of a very high but rather variable day 5 response in the 3 month old mice (Fig. 24) the data from the draining nodes showed little variation with age on any of the days assayed. Responses were substantially above background on all days assayed in contrast to the males where some mice failed to respond atall.

The responses in the contralateral nodes of female mice (Fig. 25) showed the same general trend of maximum incorporation on day 3 and decline over the period up to day 10. The response in all ages was lower than that in the draining nodes, but in general was higher than that in the contralateral nodes of male mice (Fig. 23).

FIG. 24

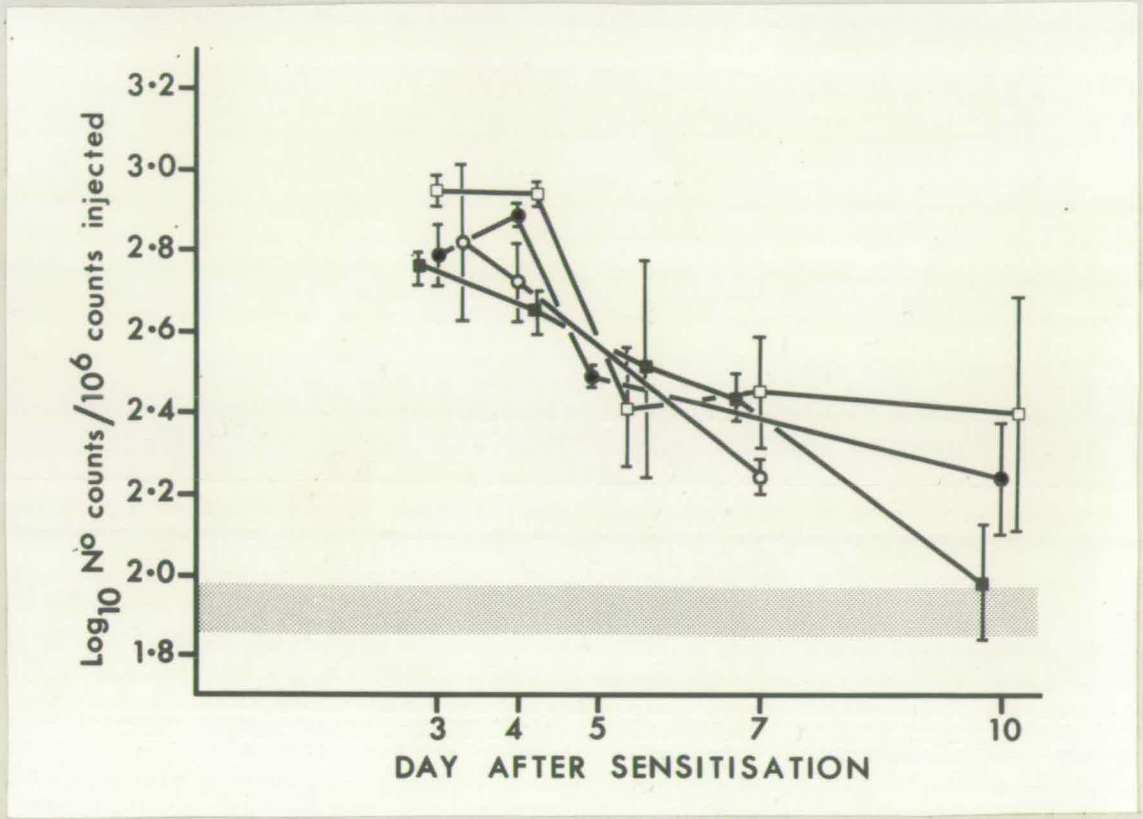
Cellular proliferation in the draining lymph nodes of female CBAT6T6 mice after sensitisation with 10mg oxazolone measured by the incorporation of ^{125}IUdR.



○ 3 months old; ● 17 months old; □ 21 months old; ■ 24 months.

FIG. 25

Cellular proliferation in the contralateral lymph nodes of female CBAT6T6 mice after sensitisation with 10mg oxazolone, measured by the incorporation of ^{125}IUdR.



○ 3 months old; ● 17 months old; □ 21 months old; ■ 24 months old.

Shaded area represents 95% confidence limits of background data.

2. The primary response to various doses of oxazolone in CBA male mice aged 3 and 21 months.

Control mice for measuring background levels of ^{125}IUdR incorporation were given alcohol alone. The experimental groups were sensitised with either 10.0mg, 1.0mg, 0.1mg or 0.01mg oxazolone in the normal way. The amount of incorporation was measured in the draining axillary and brachial nodes, the contralateral axillary and brachial nodes, the draining and contralateral inguinal nodes and the spleens of all animals. The axillary and brachial nodes from any one side were pooled and counted together. Since the peak level of incorporation in young mature mice occurs on day 3 after sensitisation (PRITCHARD and MICKLEM, 1972) and appears not to be affected by age (Fig. 22 and 23) all the mice, including those for background studies, were assayed on day 3 after sensitisation. Individual points represent the mean value of the results from 5 mice. Results were expressed as incorporated counts per 10^6 counts injected and log-transformed.

The background responses in the draining and contralateral axillary and brachial lymph nodes are shown in Fig. 26. No significant differences could be established between the two groups and the data were pooled. The mean value with 95% confidence limits is indicated in Fig. 27.

The specific responses to oxazolone in the axillary and brachial lymph nodes are shown in Fig. 27. The draining nodes showed a considerably greater level of incorporation than the contralateral nodes and also a very significant difference

FIG. 26

Background levels of ^{125}IUdR incorporation in the regional draining and contralateral lymph nodes of 3 and 21 month old CBA male mice.

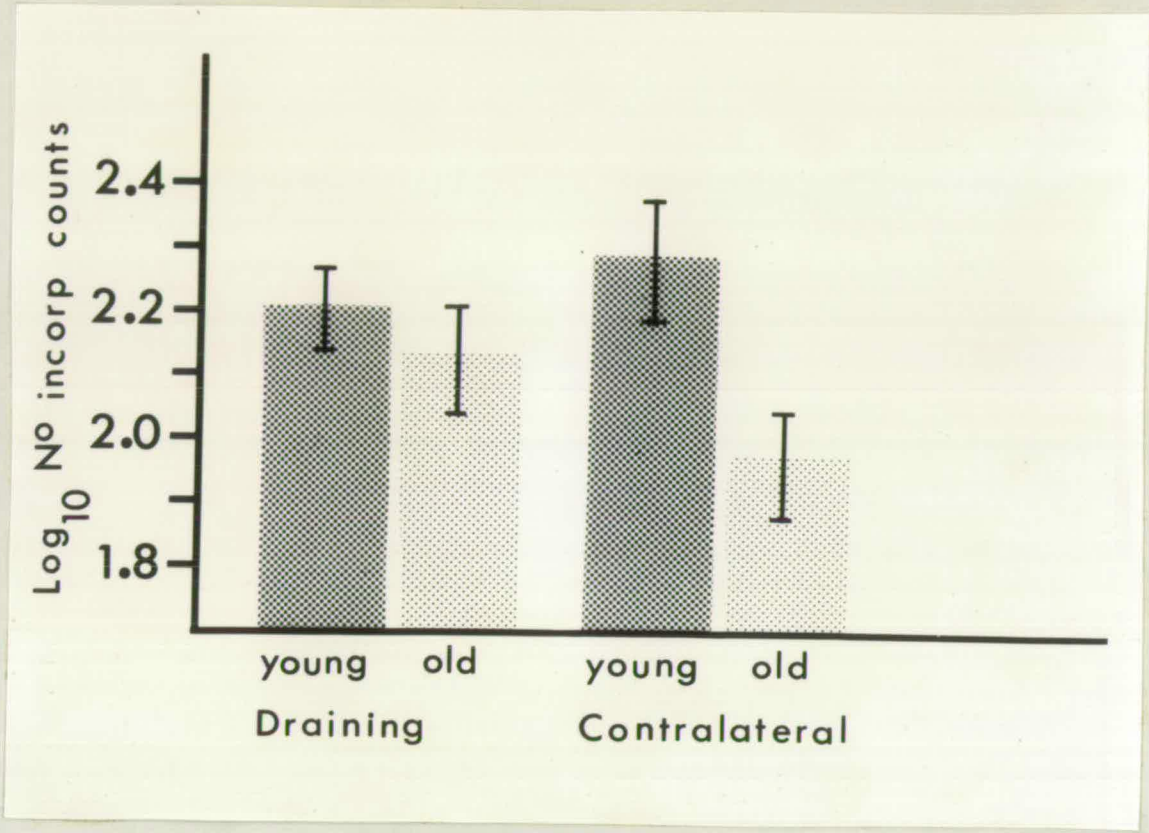
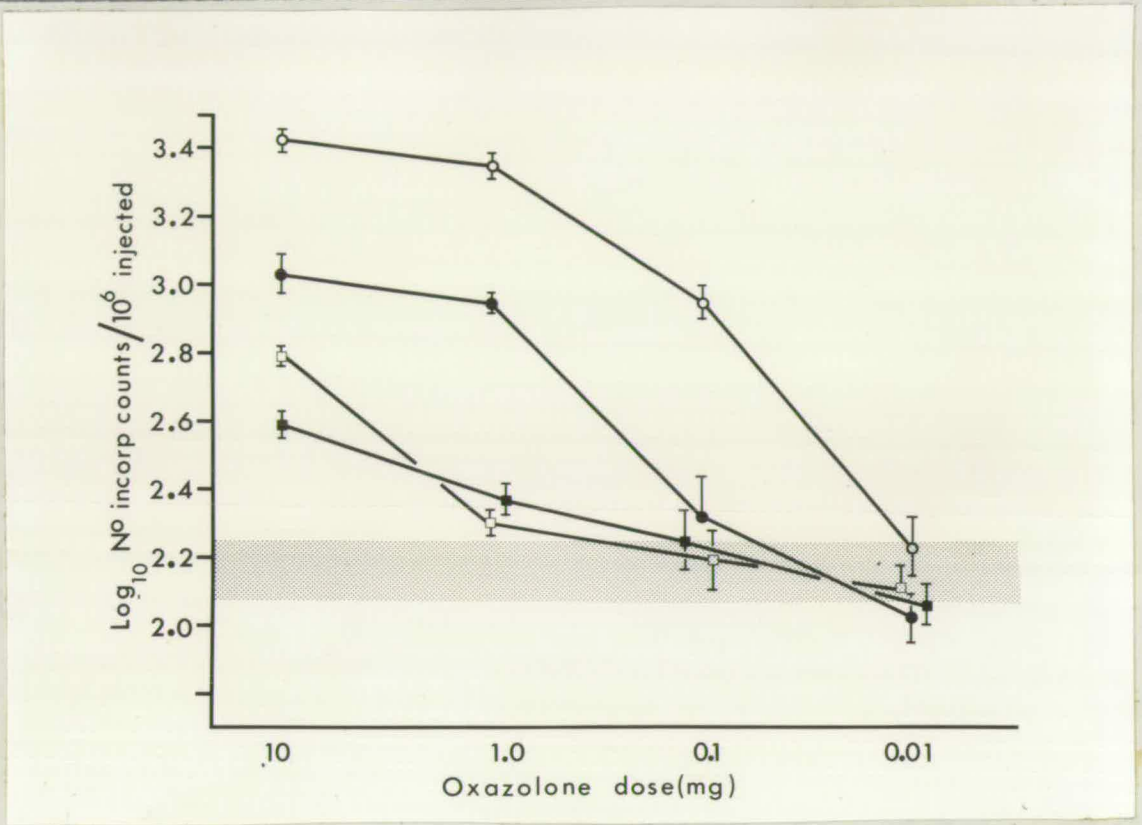


FIG. 27

The cellular response to various doses of oxazolone in the regional lymph nodes of 3 and 21 month old CBA male mice.



○ 3 months old - draining; □ 3 months old - contralateral.

● 21 months old - draining; ■ 21 months old - contralateral.

Shaded area represents 95% confidence limits of background data.

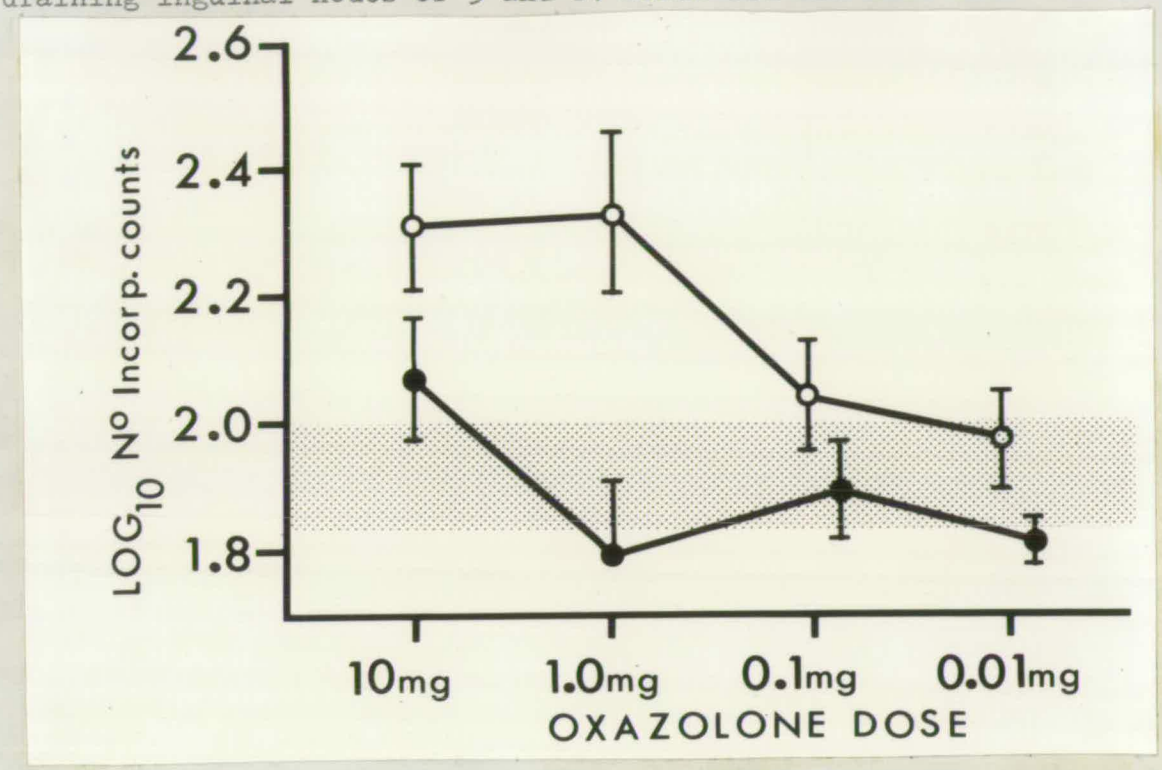
Statistical comparison (Student t-test) of the number of incorporated counts in 3 and 21 month old mice

	Oxazolone dose			
	10.0mg	1.0mg	0.1mg	0.01mg
Draining nodes	< 0.001	< 0.001	< 0.01	N.S.
Contralateral nodes	< 0.01	N.S.	N.S.	N.S.

N?S. - Not significant.

FIG. 28

The cellular response to various doses of oxazolone in the draining inguinal nodes of 3 and 21 month old CBA male mice.

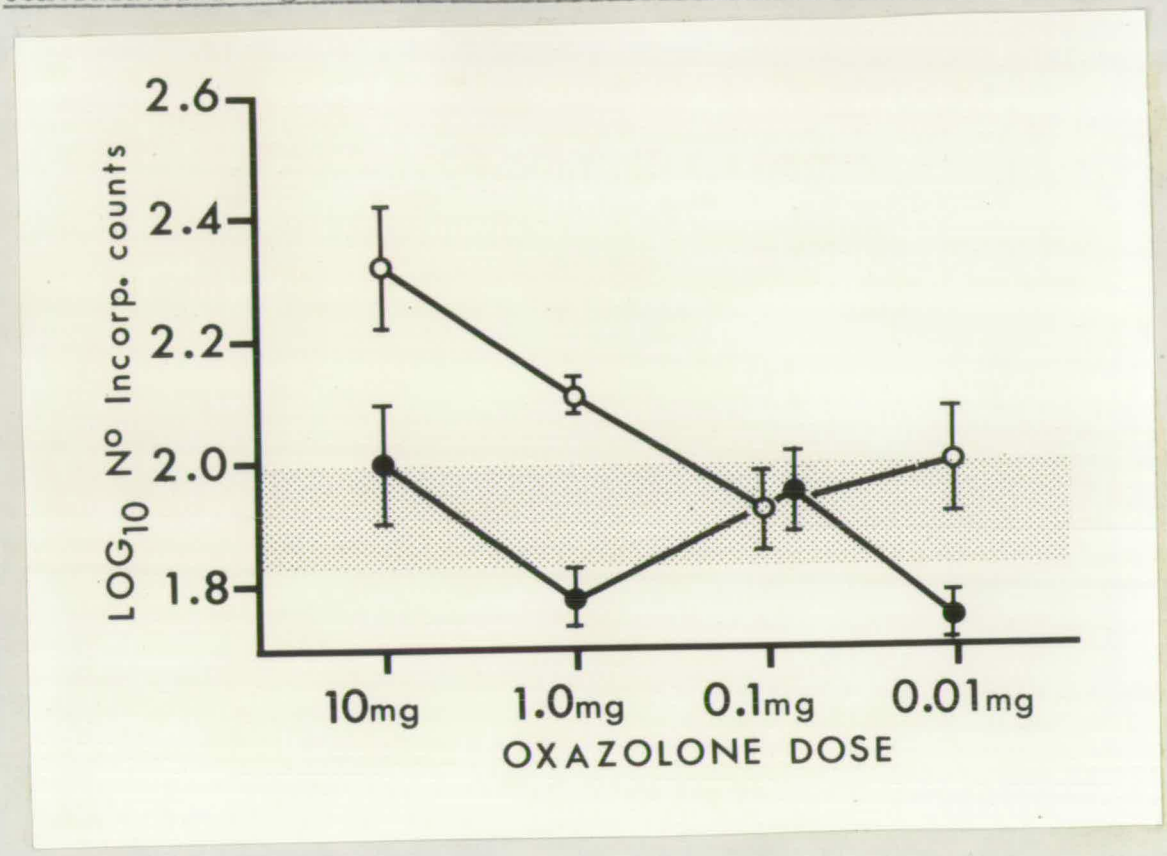


○ 3 months old; ● 21 months old.

Shaded area represents 95% confidence limits of background data

FIG. 29

The cellular response to various doses of oxazolone in the contralateral inguinal nodes of 3 and 21 month old CBA male mice.



○ 3 months old; ● 21 months old.

Shaded area represents 95% confidence limits of background data

between old and young groups. The highest responses were to 10.0mg, although in both old and young mice the response to 1.0mg was not significantly different to that obtained with the higher dose. In the young mice a significant response was made to 0.1mg oxazolone but the response to 0.01mg was not significantly above background. In the old mice the response to neither 0.1mg nor 0.01mg was significantly above background.

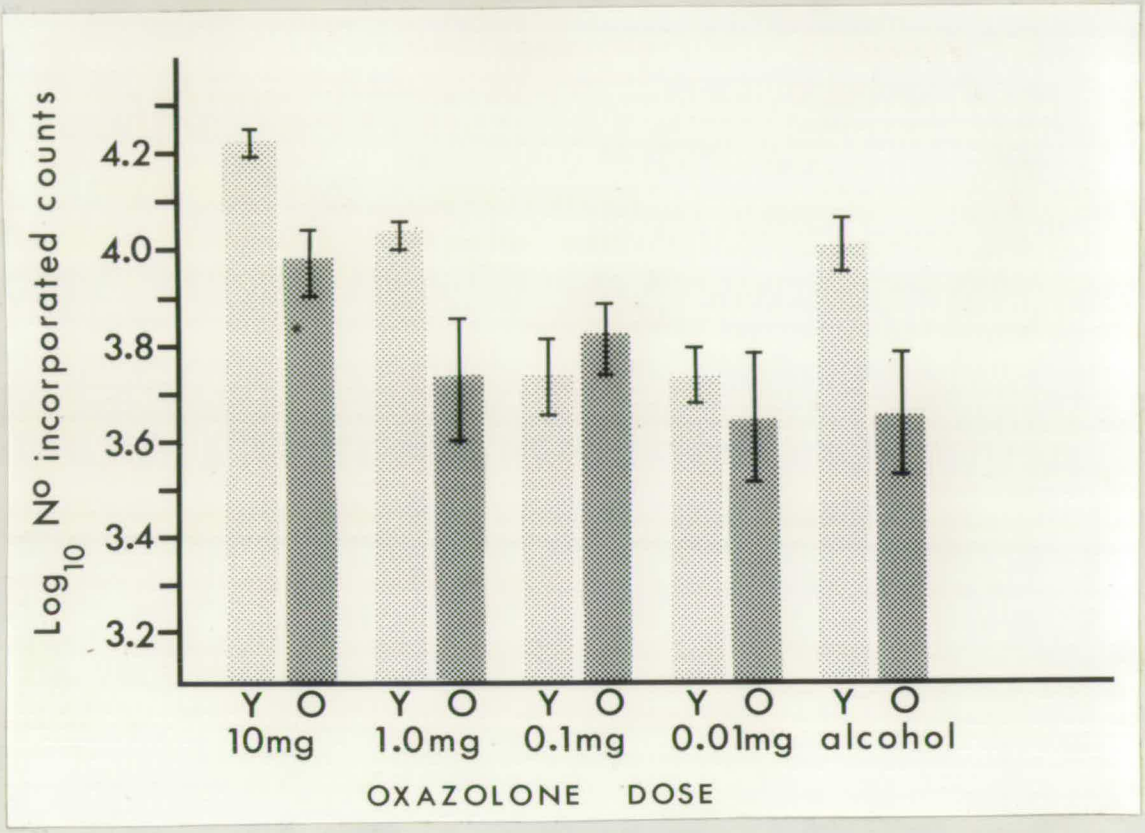
The level of incorporation in the young contralateral nodes was significantly higher than that in the old mice ($P < 0.01$) when the sensitising dose was 10.0mg. At all other doses there was no significant difference between the old and young or between these data and background.

The response in the inguinal nodes is shown in Fig. 28 and 29. The background responses were not significantly different in the young compared to the old mice. The data were used to calculate a mean value with 95% confidence limits which is shown in Fig. 28 and 29.

Even with high doses of oxazolone the responses in the inguinal nodes were somewhat variable. Statistical analysis failed to reveal any significant differences between either young and old or between draining and contralateral data. It does appear, however, that the responses in the young mice were higher than those in the old animals. The similarity between the draining and contralateral responses in the young mice to both 10.0mg and similarly with the old mice suggests that the data represent no more than a difference in background activity. Since the background data were obtained on the same

FIG. 30

The proliferative response to various doses of oxazolone in the spleens of 3 and 21 month old CBA male mice.



Y young (3 month old) spleens; O old(21 months) spleens.

day as the data for specific responses using the same operative techniques and materials, there can be little doubt that the mean and 95% confidence limits obtained reflected accurately the activity in the two groups. When compared to background data the young mice made significant responses to both 10.0mg and 1.0mg in the draining nodes and to 10.0mg in the contralateral nodes. None of the data from the old mice were significantly above background. The low levels of incorporation after sensitisation, especially in the old mice, remain difficult to explain but may reflect the movement of cells out of the area and their recruitment elsewhere.

The responses in the spleens are shown in Fig. 30. The only significant difference between old and young in a specific response was in that to 10.0mg oxazolone ($P < 0.01$). The data are confused by the rather high levels of incorporation in unsensitised control mice; in the young mice the background incorporation was significantly higher than that in the response to 0.01mg oxazolone ($P < 0.02$). The data suggest that the number of cells available to proliferate in response to oxazolone is limited and as in the inguinal nodes may reflect the movement of cells out of the spleen to other areas or indeed a failure to recruit cells from elsewhere.

Conclusions

The data available on responses to oxazolone make it clear that old mice do not respond as effectively as young animals. The depression in old mice does not appear to occur

as early as the depression in the response to sheep erythrocytes but is, nevertheless, significant at 19 months of age. Inadequacies in the response to oxazolone in old mice may reflect changes in both the lymphoid organs, where structural changes may inhibit proilferative activity, and in the T cell population. The data also provide information on the most effective dose of oxazolone for stimulating a praticular area.

The possibility that female mice do not exhibit the effect of age as rapidly as males is also suggested by the results of the first experiment where male and female mice were assayed at the same time. It was unfortunate that older male mice were not available but there can be little doubt that old female mice up to 24 months of age respond rather well. This fact is perhaps most clearly seen in the data relating to the contralateral nodes (Fig.22 and 24) which show that females of 24 months made a response considerably above background and well in excess of that achieved by all but the youngest male mice.

CHAPTER 6

DISCUSSION 1

There can be little doubt that the activity and potential of the immune system of most mammals decreases gradually from its peak in adolescence. From the information presented here, involving responses in intact animals, together with that reported from other laboratories it would appear that the depression reflects inadequacies in both the immunocompetent cell populations and in the old 'microenvironment'.

Of the work performed in intact mice of various ages the most comprehensive has involved responses to sheep erythrocytes.

The haemolytic plaque technique used in ageing studies has without exception been that based on the original report by JERNE et al (1963), with modifications in the case of the work presented here and that of Makinodan's group (MAKINODAN et al, 1971a, 1971b) for the identification of indirect 7S antibody forming cells. As described in Chapter 2 the use of a rabbit anti-mouse serum, or any serum directed against mouse immunoglobulin, involves extensive investigation to determine its characteristics. Only when these tests have been done can the investigator be reasonably sure of his ability to identify relatively small numbers of indirect plaques. It is unfortunate that details of the developing serum used and an assurance that the relevant tests have been carried out are, as a rule, not reported.

Since most studies made by research workers have

involved just one mouse strain at any one time and taking into account that individual authors may have utilised slight deviations from the assay stated to be in use, comparisons of one set of results with another are difficult. The situation is further hindered by the fact that in every instance different laboratories have used different doses of sheep erythrocytes for their immunising injections. However, it does appear that the technique, as utilised by MAKINODAN et al (1971a, 1971b) was some ten times more sensitive, based on maximum plaque numbers, than that in this laboratory or in others where the technique has been extensively investigated to achieve the most favourable conditions (PLOTZ et al, 1968; WORTIS et al, 1969). No reasons were given for the increased sensitivity achieved, which cannot be accounted for simply on the basis of a strain difference. As far as can be judged from the available information the sensitivity achieved by other laboratories was comprable to that seen in this work.

Considerable emphasis has been placed, in the work presented here, on assaying a number of days throughout the greater part of the response. This policy is also true of responses to oxazolone and of the experiments to be seen in the following chapters. Since the number of mice available for individual studies was often severely limited, this has resulted in the procedure of assaying 3 mice from each group on any one day. Usually the results of young animals assayed in this way exhibit considerable uniformity but it is true to say that variation within some of the older groups was often quite

extensive (Fig. 1, 15, 16, 22 and 23). No doubt more significant differences could have been achieved if more mice had been assayed on fewer days. However, it is clear that the assay of a number of days provides the investigators with information which gives them a greater understanding of the immunological activities of the animals concerned. For instance delays in the response may be appreciated and this allows a more accurate assessment of the time and magnitude of the peak response. Amongst the plaque results are those of WIGZELL and STJERNSWARD (1966) who assayed CBA mice (the sex was unspecified) up to 36 months of age by the direct plaque technique, on day 4 of the response to sheep erythrocytes only. The investigators concluded on the basis of one day's results that maximum responsiveness was not achieved until 7 months of age and then declined rather rapidly with increasing age. The results of the first experiment with CBAT6 male mice (Fig. 1 and 3) indicate that considerable delays may be expected in the response of older mice. Similar conclusions have been made by MAKINODAN et al (1971a) and are evident from the results of experiments in C57Bl female mice (Fig. 11) and (CBA x C57Bl)F_1 mice (Fig. 15). WIGZELL and STJERNSWARD (1966) may have severely underestimated the ability of older animals to respond to the antigen, since they probably assayed too early. Since the 7S antibody response appears to be more severely affected by age and is accompanied by even more severe delays the advantage of assaying several days becomes more apparent.

The pattern of depression in the immune response to sheep erythrocytes as a result of increasing age appears to differ from one strain to another. However, there are a number of features which are common to most if not all of the results reported from different laboratories.

In CBA mice, on the basis of the results reported here together with those of WIGZELL and STJERNSWARD (1966) it would appear that maximum responsiveness is achieved at about 3 months of age and is maintained at this level until about 7 months. It should be noted that the experiments from this laboratory have involved CBA/H and single and double T6 chromosome marked populations. The mass of work undertaken leads to the conclusion that the performance of these three types is equivalent. The 19S, γM response assessed by serum haemagglutinin and plaque responses in the spleen, does not show the same rapid deterioration with age as does the 7S, γG response. The first sign of a depressed 7S response was seen in 9 month old male mice (Fig. 2) at a time when the 19S response was almost identical to that seen in 3 month old mice assayed at the same time (Fig. 1). This conclusion is confirmed by serum haemagglutinin responses (Fig. 9) and by the results of 2-mercaptoethanol resistance reported by WIGZELL and STJERNSWARD (1966). There can be little doubt, however, that the 19S response is severely affected in very old mice (WIGZELL and STJERNSWARD, 1966).

The pattern in C57Bl mice appears to involve a more rapid deterioration. The results of both spleen PFC content (Fig. 11 and 12) showed that both the 19S and 7S response was severely

depressed even in 12 month old animals. Similar conclusions may be drawn from the results of METCALF et al (1967) and KISHIMOTO et al (1969) who utilised the direct plaque technique alone. Despite the depression of the 19S response at a relatively early stage in the life of the mice the results of serum haemagglutinations (METCALF et al1966) and the plaque results shown here (Fig. 11 and 12) show a more rapid deterioration of the 7S compared to the 19S response.

Whereas in CBA male mice a severely depressed response to sheep erythrocytes is a common feature of mice aged about 20 months, in (CBA x C57Bl)F_1 mice of a similar age and sex the response was not greatly altered from that in much younger animals (Fig. 13 and 14). However, the 7S response was delayed in the older mice (up to 24 months of age). In very old female mice (Fig. 15 and 16) both the 19S and the 7S responses were severely depressed. There can be little doubt that the 7S response was more severely affected with delays of at least 5 days in its initiation compared to that in young mature mice (Fig. 16). Severe depressions in the responses of 35 month old (C57Bl x C3H)F_1 mice have been reported by MAKINODAN et al (1971a).

Although some of the most convincing evidence for ageing effects have been established using sheep erythrocytes as the antigen, there have been a number of other reports, some of them very recent, which have examined the effect of age on humoral and cell mediated responses to several different antigens. The great majority of these reports support the general conclusion that the immune potential of old animals

is impaired. Thus the response to bovine serum albumin is depressed in aged rats (GOULET and KAUFMANN, 1964/5). The response to bovine gamma globulin is affected by age in guinea pigs (BAER and BOWSER, 1963). Both the above antigens and also sheep erythrocytes are dependent on the co-operation of T cells if a full response is to occur. Depressions in the response to any one of them may reflect inadequacies in either the T cells or the antibody producing B cells, or indeed in both populations. More recently a number of attempts have been made to investigate cellular responses, so as to gain a greater understanding of the activities of the T cell population alone.

The response to oxazolone, which has been shown to reflect T cell proliferation in the early maximal stages (DAVIES et al, 1969) is significantly depressed in old CBA mice (22,23 and 26 to 29). Although the proliferative activity of the T cells in intact animals may be inhibited to some extent by the disruption of the lymphoid tissues in old animals, there can be little doubt that the size or potential of the T cell population is impaired. This fact must be concluded from the experiments carried out <u>in vitro</u> with variously aged spleen cells. The response to PHA declines with age in (C57Bl x C3H)F_1 mice and Balb/c mice (HORI et al, 1973) and also in (C57Bl x 129)F_1 animals (MATHIES et al, 1973). The response to pokeweed antigen has also been reported to decline in (C57Bl x 129)F_1 mice (MATHIES et al, 1973). The response to pokeweed antigen has been shown to reflect mostly T cell activity but with some involvement of the B cell population (JANOSSY and GREAVES, 1972).

The response to PHA, has, like the response to oxazolone in its early stages, been reported to reflect T cell activity alone (DUKOR and DIETRICH, 1967). Since it has been demonstrated on a number of occasions that the size of the theta positive population remains essentially unaltered with age (HORI et al, 1973; KISHIMOTO et al, 1973; MATHIES et al, 1973) the depressed cellular responses outlined above would indicate that a substantial proportion of the T cell population has lost its ability to respond to antigen.

To the authors knowledge no attempt has been made to investigate the response in intact old mice to a thymus independent antigen. PRICE and MAKINODAN (1972a) have claimed, on the basis of antigen dose response analyses with polysaccharide and isolated spleen cells, to have demonstrated that the number of B cells reactive to the antigen in old spleen cells is fewer than that in young spleens. It is unfortunate that the plaque assay employed for this investigation was not used by the authors to investigate the response in intact old mice.

The great majority of assays performed on intact animals have shown depressed responses in old animals. There are, however, a number of reports which have claimed that the response to a certain antigen or the response made by a particular strain of mouse remains unaltered with age.

LEGGE and AUSTIN (1968) assayed the response to polymerised flagellin in young and old C57Bl mice. The primary response to this antigen was claimed to be equivalent in the young and old mice. The secondary response, on the other hand,

was significantly lower in the old group. The primary response was assayed over the first 3 weeks after injection and towards the end of this period there was some indication that the response in the old mice was failing compared to that in the young animals. Since the primary response was terminated by a second injection of antigen at this stage the extent of the difference was not assayed. It may be that the bacterial immobilisation technique was not sensitive enough to establish a clear difference between the two groups at other stages in the primary response.

In another report METCALF et al (1967) claimed that the response to sheep erythrocytes in 19 month old C3H mice was equivalent to that seen in 2 month old mice and therefore indicated that the C3H strain was not affected by age in its response to sheep erythrocytes. Since the same paper also described depressions in both C57Bl and (C57Bl x C3H)F_1 mice it would seem unlikely that the assay procedures were inadequate. More important are the ages of the animals used. The young 'mature' mice may have been too young at 2 months. Several different reports in a number of strains of mice have demonstrated that maximum responsiveness has not been achieved by 2 months of age (HORI et al, 1973; MAKINODAN and PETERSON, 1966a, 1966b; WIGZELL and STJERNSWARD, 1966). It is possible that the immunological capabilities of 2 month old C3H mice are rather limited. In the same way the 19 month old mice may have been too young. The age at which the immune response becomes significantly depressed varies from strain to strain.

In C57Bl mice it appears to be relatively early whilst in (CBA x C57Bl)F_1 there is very little sign of a depression in either the direct or indirect plaque response in mice up to 24 months of age. It may be that C3H mice respond rather well at 19 months, but this does not mean that their response to sheep erythrocytes is completely unaffected by age.

KISHIMOTO et al (1969) have claimed that the response to bacterial alpha amylase remained unaltered in C57Bl mice up to 19 months of age. At this age the response to sheep erythrocytes was severely depressed (Fig. 11 and 12; KISHIMOTO et al, 1969). The results obtained with bacterial alpha amylase are not easily accounted for. The fact that the response was assayed by a haemagglutination technique by coupling the antigen to sheep erythrocytes may be significant since there was no attempt to investigate differences in 19S compared to 7S antibodies. Any haemagglutination technique, without the use of an anti-Ig serum to 'develop' the response, would be rather insensitive to changes in 7S antibodies. It may be that the response of the old mice was sufficiently improved by the injection of the antigen in Freunds adjuvant that no differences between their performance and that of young mice were appreciated using a basic haemagglutination technique.

As yet no mention has been made about the influence of sex on ageing studies. In experiments reported here there were indications that female mice do not show the same rapid deterioration in their response as do males, (Fig. 7, 8, 22 to 25). It is interesting that no real attempt has been made

to compare males and females in ageing experiments. Several authors have stated that both sexes were employed but gave no details to indicate that particular experiments were matched for sex, or as seems more probable, that groups were mixed. It is not sufficient to show that the response in very old male and female mice is similarly depressed (MAKINODAN et al, 1971a) and conclude that the two sexes exhibit a similar rate of decline in their ability to respond to antigen.

In most animals that have been studied the male sex is the shorter lived (COMFORT, 1956). This is true in species as dissimilar as fish (BIDDER, 1932), Drosophila (ALPOTOV and PEARL, 1929) and Man (COMFORT, 1956). It has been suggested that the homogametic sex is the longer lived. Thus in all mammals the female should live longer but in birds, reptiles and Lepidoptera, in which the female is the heterogametic sex, the male should be the longer lived. The evidence available would not appear to support such a hypothesis (COMFORT, 1956; LANDAUER and LANDAUER, 1931; RAU and RAU, 1914). If it should transpire, by further studies, that a sex difference does occur in mice, as suggested by the experiments with CBA mice reported here, it may be that hormonal patterns are important in the depression seen in old animals.

Further observations related to the experiments described here include those on animal and spleen weights and cellular content. The data available in this regard are fairly numerous and have been reviewed (chapter 1). With the exception of a very small number of the oldest (CBA x C57Bl)F_1 female mice

with enlarged spleens, none of the animals assayed showed any sign of gross abnormailities, such as reticulum cell sarcoma. Any changes that occurred in the wieght or cellularity of spleens or lymph nodes (with the exception of the female mice mentioned above) with age were not reflected in changes in background levels of PFC in the spleen or in the background proliferative activity in the lymph nodes prior to sensitisation with oxazolone.

The great majority of responses assayed in laboratory animals of various ages have been to a single injection of antigen. Two attempts were made to assay secondary responses to sheep erythrocytes, one in CBAT6 mice and the other in (CBA x C57Bl)F_1 male mice. In addition to the criticism that has already been made of these results, which showed very low responses and no significant difference between old and young mice, might be added the further point that the (CBA x C57Bl)F_1 mice were too young since the primary response in similarly aged animals was almost normal. There can be little reason to doubt that MAKINODAN'S work on the secondary response both with isolated spleen cells (MAKINODAN and PETERSON, 1966b) and in intact animals (MAKINODAN et al, 1971b), reflects more accurately the effect of age on the secondary response. With the exception of the haemagglutination responses in CBAT6 mice (Fig.19) which showed a decline in the ability of old mice to mount a secondary response, the other results based on the plaque technique (Fig. 17, 18, 20 and 21) must be considered to be inaccurate.

Other attempts have been made to assay secondary responses and include the response to bovine serum albumin in rats (GOULET and KAUFMANN, 1964/5) and the response to polymerised flagellin in C57Bl mice (LEGGE and AUSTIN, 1968). Both demonstrated that the performance of old animals was lower than that of young adults.

The significance of these results and the details supplied by MAKINODAN et al (1971b) regarding the relative change in the number of direct and indirect plaque forming cells in young mice, together with changes in the structure of lymphoid organs will be more usefully discussed in the context of repopulation studies.

Conclusions.

At this point there seems to be no reason to doubt that there is a general tendancy for both the humoral and cell mediated immune response to become depressed in old age. The depression is gradual and involves delays in the time taken for humoral responses to become initiated. The 7S response is affected more rapidly and with greater severity compared to the 19S response. Cellular responses to oxazolone, PHA and Pokeweed mitogen do not appear to be affected as rapidly as the responses involving the production of circulating antibody. It is possible that the humoral responses studied to date, since they involve a complex series of co-operative events, may be more suscetible to the effects of age.

Dispite the lack of detailed studies on the B cell population alone, it would appear from the results of humoral

and cellular responses that both B and T cell activity becomes progressively impaired in old mice. No doubt structural changes which occur in the lymphoid organs reduce the potential of the available cells since the microenvironment may be unsuitable for lymphoid cell proliferation and inadequate to ensure rapid contact with antigen.

Differences with regard to sex may reflect a genuine phenomenon but, as yet, the evidence is inconclusive.

CHAPTER 7

The repopulation of old mice with young cells and tissues.

1. A preliminary study on the repopulation of old mice with foetal liver cells with or without a young thymus graft.

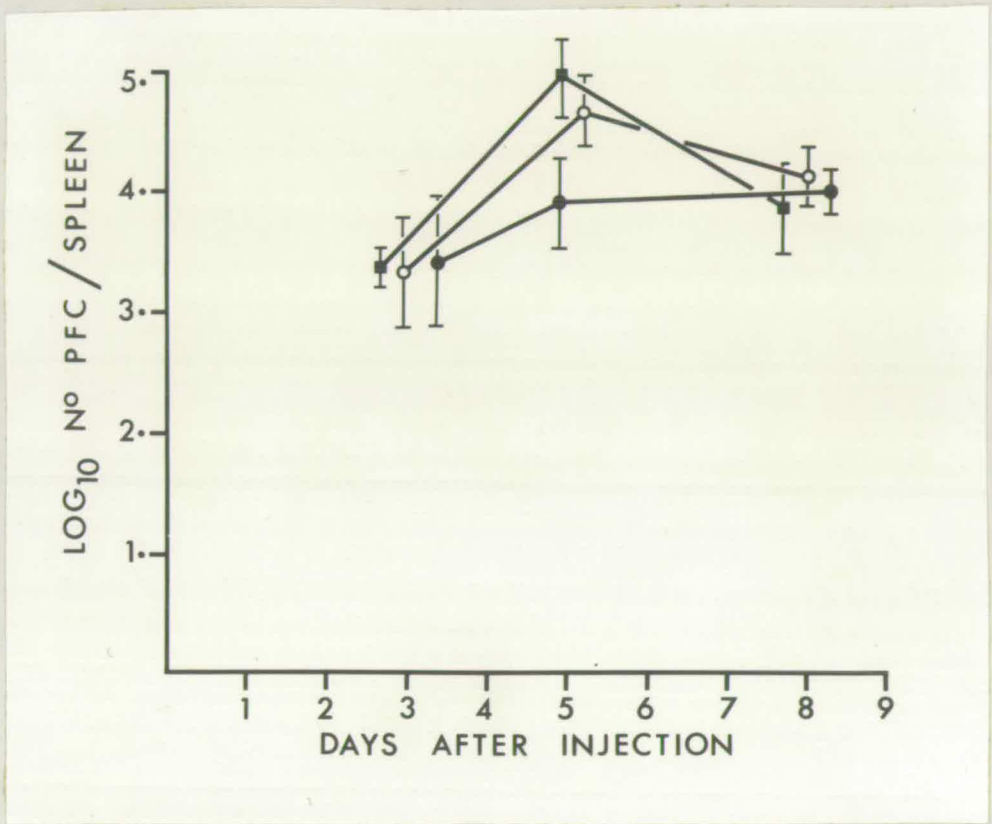
Previous experience with old mice had shown them to be very susceptible to infection after whole body lethal irradiation. In this study 19 month old male CBAT6 mice were given 900r to the hind body region only; the anterior half having been shielded with lead. The irradiated mice were repopulated with 5×10^6 foetal liver cells with or without a young thymus graft. The foetal liver cells were obtained from 18 day old CBAT6 foetuses, the thymus grafts from 2 to 3 day old male CBA mice. One lobe of the donor thymus was placed in the subcutaneous axillary region of the recipient mice.

3 months after repopulation, when all the mice were 22 months old, they were given a single injection of 4×10^7 sheep erythrocytes and assayed for plaque forming cells on days 3, 5 and 8. The results are shown in Fig. 30 and 31 and includes a group of unirradiated 22 month old CBAT6 mice assayed at the same time.

The responses made were rather erratic. On no day was one group significantly different from another, either in their 19S response (Fig. 29) or their 7S response (Fig. 31). There was no indication of an improvement in the old, part body irradiated, mice which had received young cells or young cells and a thymus graft. However, it might be argued that the irradiated

FIG. 31

The direct plaque response to sheep erythrocytes in 19 month old male CBAT6 mice irradiated and repopulated with young cells and tissues.



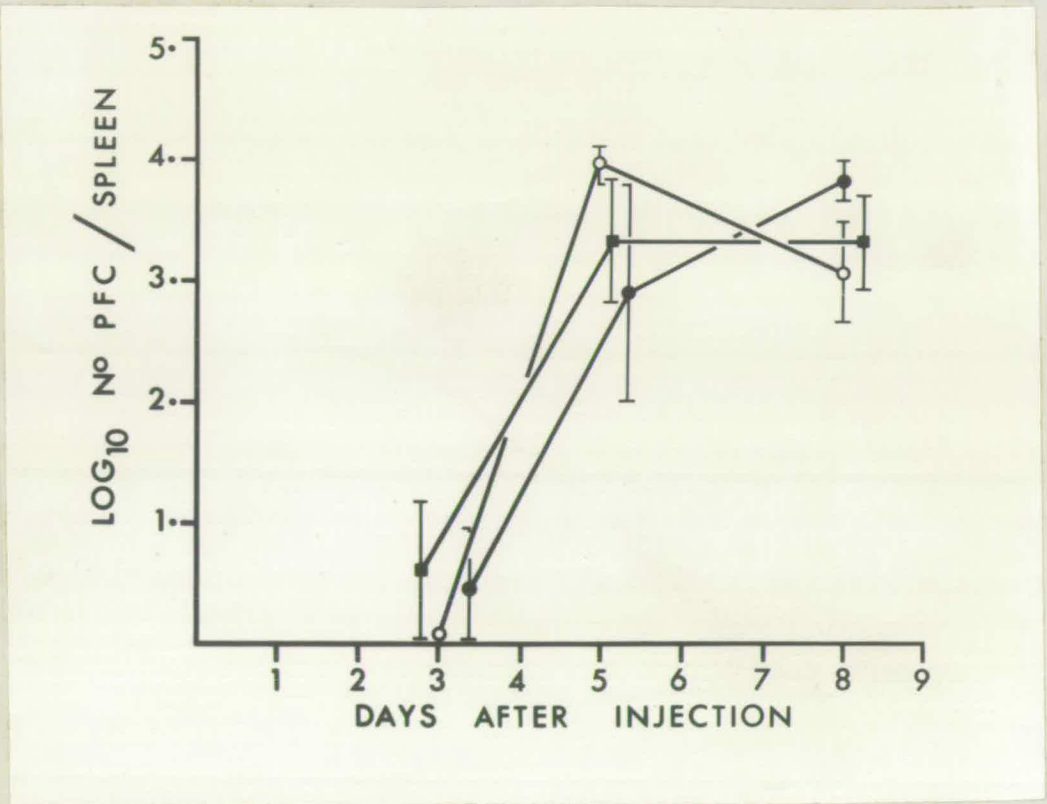
○ 5 x 10^6 foetal liver cells only

● 5 x 10^6 foetal liver cells and thymus graft

■ Untreated old mice.

FIG. 32

The indirect plaque response to sheep erythrocytes in 19 month old CBAT6 male mice irradiated and repopulated with young cells and tissues.



○ 5 x 10^6 foetal liver cells only.

● 5 x 10^6 foetal liver cells and thymus graft.

■ Untreated old mice.

and repopulated groups had responded rather well to compete with the normal old mice. As a result a further experiment was planned with greater attention paid to control groups.

2. The repopulation of 22 month old CBAT6 female mice with young cells and tissues.

The only mice available for this study were females. In view of the fact that 18 month old females had been shown to respond rather well to sheep erythrocytes 3 of the prospective experimental mice were compared to a similar number of 3 month old females in their day 7 response to sheep cells. The results showed that although the direct plaque response was almost normal the indirect response was significantly depressed in the old animals. On the basis of this preliminary study the female mice were considered to possess a sufficiently depressed response for use in the repopulation study. The experimental design for the repopulation of part body irradiated 3 and 22 month old mice is shown in Table 14.

TABLE 14

Experimental design for the transfer of young cells into old mice

Age of recipient mice	Treatment
3 months	900r, 5×10^6 3month B.M.
22 months	900r, 5×10^6 22 month B.M.
22 months	900r, 5×10^6 3 month B.M.
22 months	900r, 5×10^6 3 month B.M. and thymus graft

3 month B.M. = 3 month old bone marrow cells.

The bone marrow suspensions were obtained from 3 and 22 month old CBAT6T6 mice and the thymus grafts from 7 day old CBAT6T6 female mice. The bone marrow suspensions were treated with anti-theta serum and complement (RAFF, 1969) to remove the theta positive population and were injected within 5 hours of irradiation of the recipient mice. The thymus grafts were placed in the subcutaneous axillary region within 24 hours of irradiation.

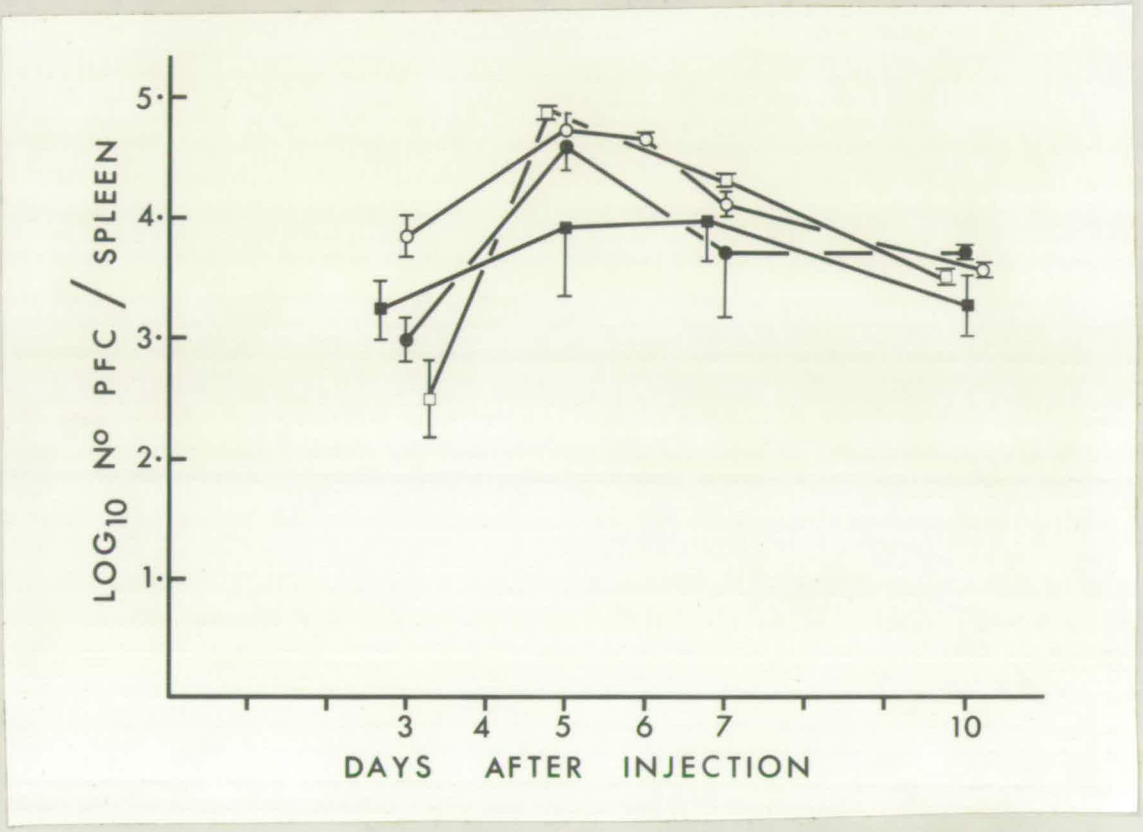
3 months after repopulation all the mice were injected with 4×10^7 sheep erythrocytes and the response assayed by the haemolytic plaque technique on days 3, 5, 7 and 10. The results are shown in Fig. 33 and 34. The individual points represent the mean value of 3 mice.

The direct plaque response (Fig. 33) was not significantly different in any one group compared to another on either day 5, 7 or 10. The apparent difference between the old and young mice injected with 3 month old bone marrow cells has doubtfull biological significance since the results of the other two groups fell mid-way between them. The apparent delay in the time of the maximum response in the thymus grafted group (Fig. 33) was due to one of the three mice producing a very poor response on day 5.

The indirect plaque response (Fig. 34) showed that the young mice responded well and compared favourably with similarly aged normal animals. No significance is attached to the higher response made by the thymus grafted group on day 3. The response in the young mice was clearly higher than any of the older groups. However, due to abnormally distributed data statistical

FIG. 33

The direct plaque response to sheep erythrocytes in young female CBAT6 mice repopulated with young bone marrow cells and old female CBAT6 mice repopulated with young or old bone marrow cells.



○ 3 month old recipient given 5 x 10^6 3 month old B.M.

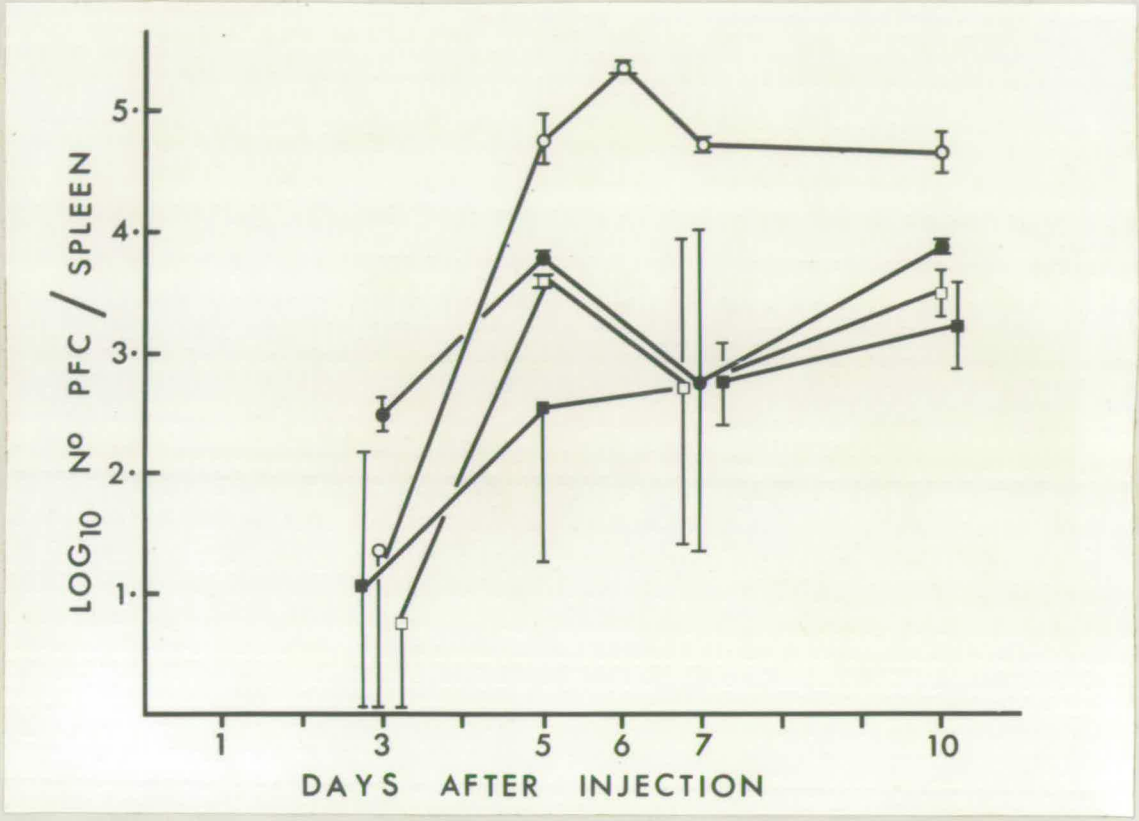
● 22 month old recipient given 5 x 10^6 22 month old B.M.

□ 22 month old recipient given 5 x 10^6 3 month old B.M.

■ 22 month old recipient given 5 x 10^6 3 month old B.M. and a thymus graft.

FIG. 34

The indirect plaque response to sheep erythrocytes in young female CBAT6 mice repopulated with young bone marrow cells and old female CBAT6 mice repopulated with young or old bone marrow cells.



○ 3 month old recipient given 5 x 10^6 3 month old B.M.

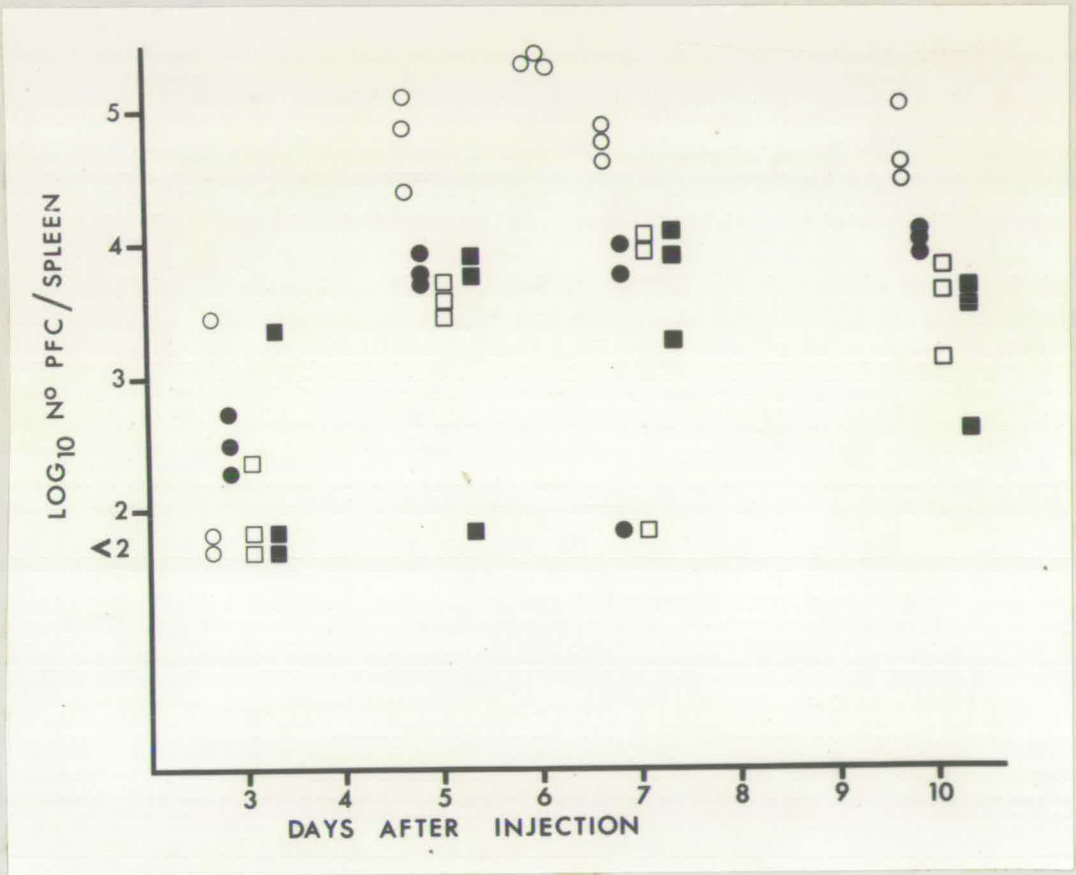
● 22 month old recipient given 5 x 10^6 22 month old B.M.

□ 22 month old recipient given 5 x 10^6 3 month old B.M.

■ 22 month old recipient given 5 x 10^6 3 month old B.M. and a thymus graft.

FIG. 35

Individual indirect plaque responses used in the preparation of Fig. 34



○ 3 month old recipient given 5 x 10^6 3 month old B.M.

● 22 month old recipient given 5 x 10^6 22 month old B.M.

□ 22 month old recipient given 5 x 10^6 3 month old B.M.

■ 22 month old recipient given 5 x 10^6 3 month old B.M. and a thymus graft

analysis was not always practicable. In every case where large 'standard errors' appear in the results, for instance in the old mice with young cells alone on day 7, it was due to one of the three mice assayed having very few indirect plaques. The data are shown as individual responses in Fig. 35. It is clear from this information that the response of old mice is not improved by the transfer of young cells or tissues.

Conclusions

There is no evidence that the immune responsiveness of old mice is simply a case of a lack of stem cells or the need for a young thymic environment.

CHAPTER 8

The potentiality of old bone marrow as a source of mature, functional T and B cells and the ability of the old thymus to support a proliferating T cell population.

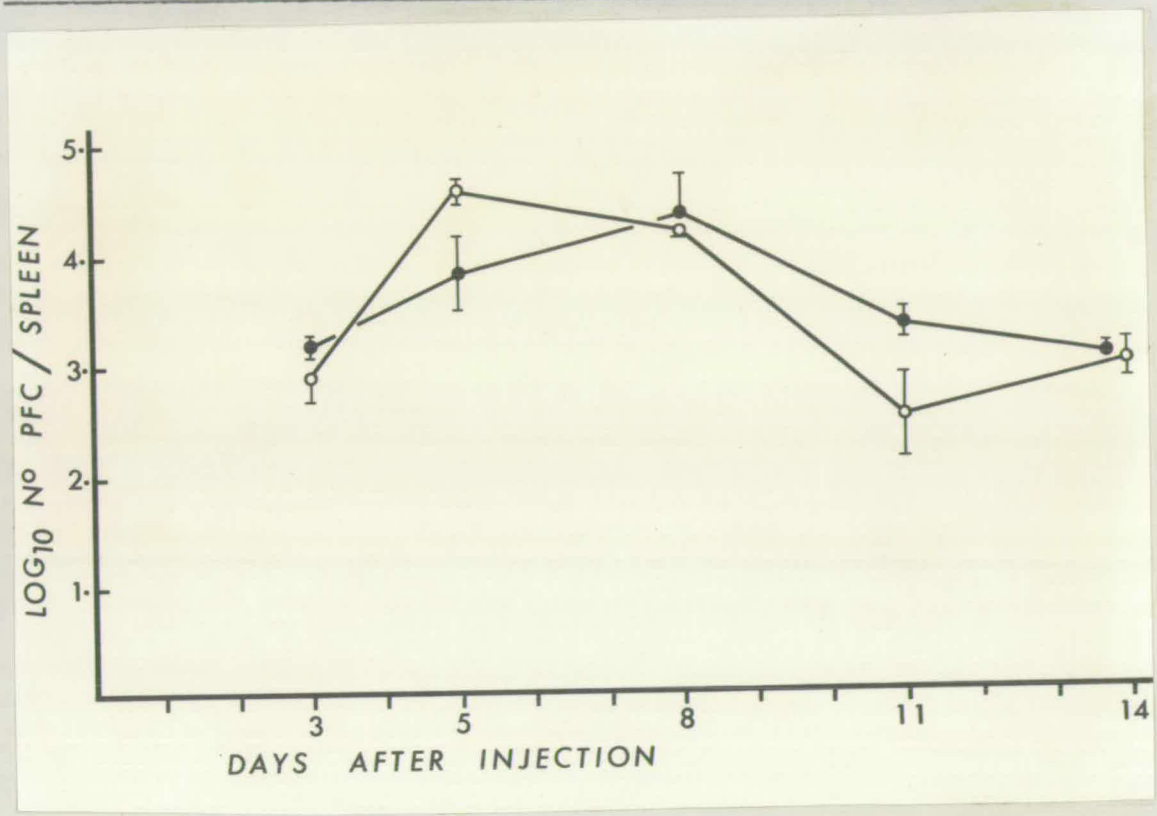
Both the work presented here and that reported by other authors (WIGZELL and STJERNSWARD, 1966; METCALF et al, 1967; KISHIMOTO et al 1969; MAKINODAN et al, 1971a) has demonstrated that the immune response to sheep erythrocytes is severely depressed in old mice. It has also been demonstrated that young cells transferred to old mice failed to raise their response. Experiments were therefore designed to investigate whether the progenitors of T and B cells were present in old bone marrow and if so whether they were capable of proliferation and normal function when placed in a suitable young environment. Investigations were also carried out on the ability of old thymus grafts to support a young proliferating T cell population when placed in young, thymectomised and irradiated mice.

1. The ability of old bone marrow to produce functional B cells

3 month old male CBAT6T6 mice were given 900r whole body irradiation and were repopulated within 5 hours with either 5×10^6 3 month old bone marrow cells and 50×10^6 4 week old thymus cells, or with 5×10^6 22 month old bone marrow cells and 50×10^6 4 week old thymus cells. Both the bone marrow and thymus cells were taken from CBA donors. 6 mice were used as radiation controls.

FIG. 36

The direct plaque response to sheep erythrocytes in CBAT6T6 mice irradiated and repopulated with young or old bone marrow cells.



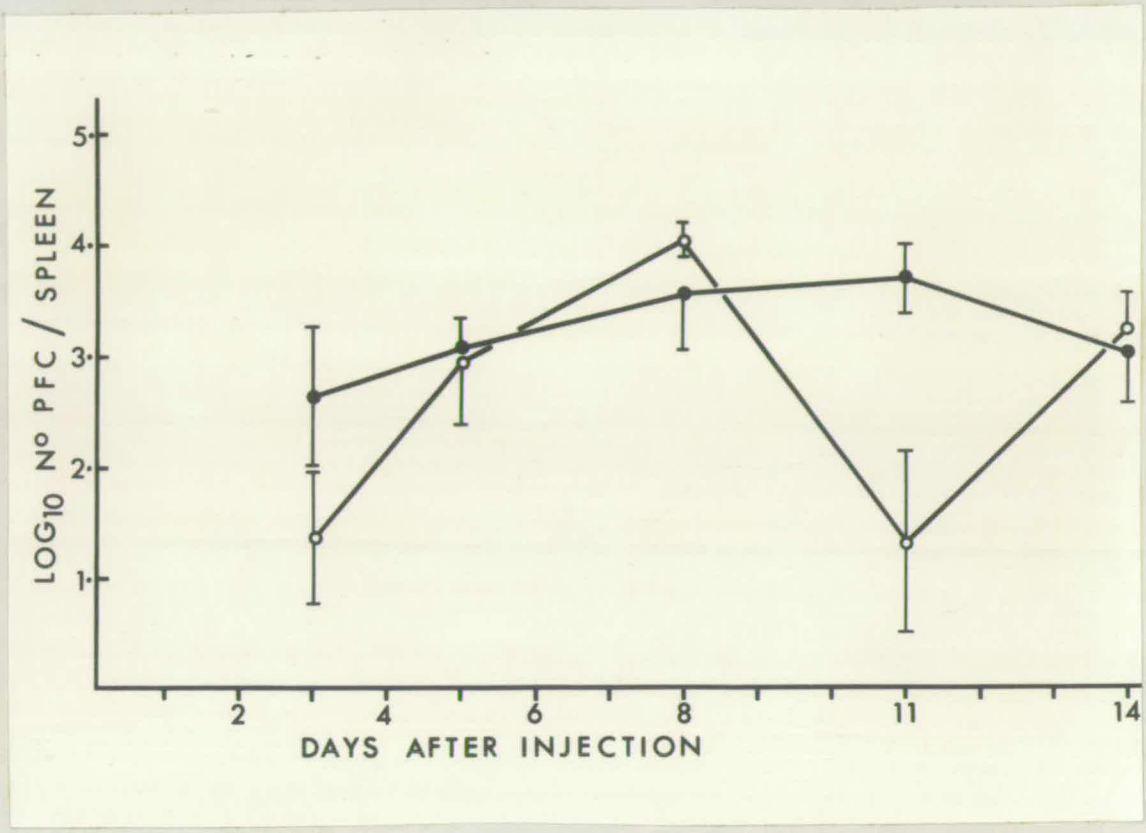
○ 3 month old recipient given 5×10^6 3 month old B.M.

● 3 month old recipient given 5×10^6 22 month old B.M.

Both groups received in addition 5×10^7 young thymus cells.

FIG. 37

The indirect plaque response to sheep erythrocytes in CBAT6T6 mice irradiated and repopulated with young or old bone marrow cells.



○ 3 month old recipient given 5×10^6 3 month old B.M.

● 3 month old recipient given 5×10^6 22 month old B.M.

Both groups received in addition 5×10^7 young thymus cells.

On day 17 after repopulation the mice were given a single injection of 4×10^7 sheep erythrocytes. The plaque forming cell responses were assayed on days 3, 5, 8, 11 and 14. The results are shown in Fig. 36 and 37. Individual points represent the mean of 3 mice.

The direct plaque response (Fig. 36) was considerably lower than in normal young mice; on no day were the plaque numbers in excess of 35×10^3 PFC per spleen. The response profiles in the two groups were rather similar with the exception of the apparently later peak in the old marrow injected group. This was due to one of the three mice assayed on day 5 having only 450 PFC in its spleen compared to the other two which had in excess of 25×10^3 PFC per spleen. Significant differences could not be established between the two groups on any of the days assayed.

The indirect plaque response (Fig. 37) reflected the low direct plaque response with maximum counts of less than 10×10^3 PFC per spleen. With the exception of the day 11 data there were no significant differences between the two groups. The peak response in both groups would appear to have occurred between day 8 and day 11. The drop in the number of indirect PFC in the group given young cells on day 11 is not easily explained since it represented the number of plaques present in the spleens of two of the three animals assayed.

Control mice given no cells after irradiation exhibited a rapid deterioration in their condition and died several days before the other mice were assayed.

2. The ability of old bone marrow to produce mature T cells

The purpose of this study was to compare the ability of a fixed number of old and young bone marrow cells to produce mature, functional T cells when placed in a young environment. The proliferative activity of the T cells produced from the bone marrow inocula were assayed by the oxazolone /IUdR technique.

Young, 3 month old CBA mice were given 900r whole body irradiation and injected with either 5×10^3 anti-theta treated (RAFF, 1969) 3 month old bone marrow cells or 5×10^6 anti-theta treated 24 month old bone marrow cells. Both the recipient mice and the bone marrow cells were male CBA. The response to 10.0mg oxazolone was measured in both groups on 3 occasions after grafting; at 1, 6 and 10 months.

(a) 1 month assay - By one month the mice should have had sufficient time to recover from the effects of irradiation but the activity in the mice as a result of oxazolone sensitisation would probably reflect activity of residual T cells.

2 mice from each group were used as controls and were given alcohol alone. All the mice were assayed on day 3 after sensitisation. The results are shown in Table 15. The mean values were calculated from the response of the three mice.

The background levels of incorporation, assayed in mice that had received alcohol alone, were not significantly different in the two groups, neither were the specific responses to oxazolone. Both the background data and the results of specific responses to oxazolone were considerably lower than in normal

TABLE 15

The response to oxazolone in mice irradiated and repopulated with young or old bone marrow cells one month previously.

Source of cells for repopulation	sensitising agent	Log_{10} counts in draining nodes/10^6 counts injected
3m B.M.	alcohol	1.4671 ± 0.2364
3m B.M.	oxazolone	2.4194 ± 0.1486
24m B.M.	alcohol	1.6914 ± 0.0100
24m B.M.	oxazolone	2,3661 ± 0.1248

3m B.M.= 3 month old bone marrow cells; counts shown as the mean and standard error of the mean.

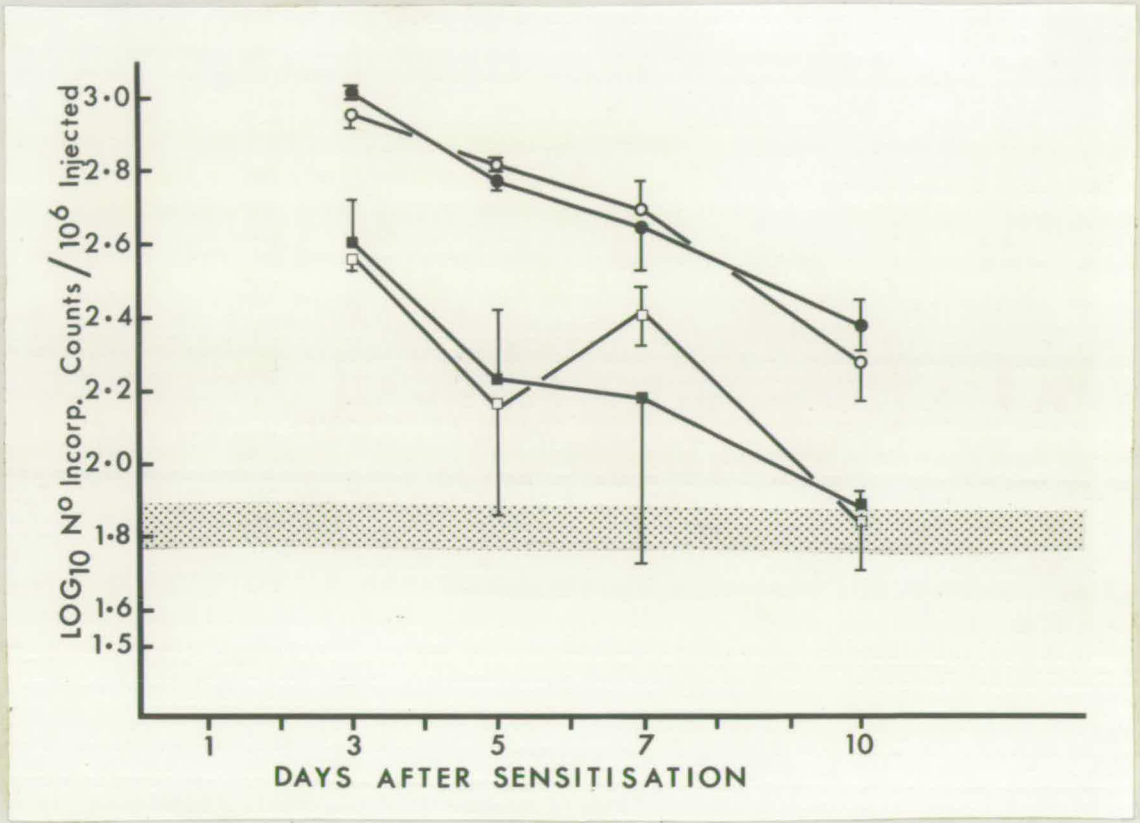
young or old animals (Fig. 22). The results were taken to indicate that the number of functional T cells remaining after irradiation was equivalent in the two groups.

(b) 6 month assay - the response to 10.0mg oxazolone was measured in the regional (draining and contralateral) lymph nodes of 3 mice from each group on days 3, 5, 7 and 10 after sensitisation. Three mice from each group were used as background controls and received alcohol alone. The results are shown in Fig. 38. Individual points represent the mean value of three mice.

The background data showed there to be no significant difference between the two groups. All the background data were pooled to calculate a mean value. The 95% confidence limits are shown in Fig. 38.

FIG. 38

Cellular proliferation in CBA mice irradiated and repopulated with young or old bone marrow cells 6 months previously in response to oxazolone and measured by the incorporation of ^{125}IUdR in the regional lymph nodes.



● Draining nodes - young marrow recipients

○ Draining nodes - old marrow recipients

■ Contralateral nodes - young marrow recipients

□ Contralateral nodes - old marrow recipients

Shaded area represents 95% confidence limits of background data.

The activity in the draining (pooled axillary and brachial) nodes was clearly very similar in the two groups. No significant differences were found between the two groups on any of the days assayed. The highest levels of incorporation were seen on day 3, declined over the rest of the days assayed but was still significantly higher than background on day 10.

The activity in the contralateral nodes was more erratic and lower than that in the draining nodes but on none of the days assayed was one group significantly different from another. The response in the contralateral nodes had declined to background levels by day 10.

(c) 10 month assay - The response to 10.0mg oxazolone was assayed on day 3 and 7 after sensitisation. Background levels of incorporation were assayed in 2 mice from each group. Specific responses to oxazolone were assayed in 4 mice from each group on day 3 and 6 mice from each group on day 7. The results are shown in Fig 16. No significant differences were found between the two groups either in background activity or in their specific responses to oxazolone with the single exception of the day 3 response in the contralateral nodes ($P < 0.02$). Expressed arithmetically the counts on this day were 320 in the young marrow recipients and 190 in the old. Taken collectively the results indicate that the number of T cells available for the specific response and their proliferative activity as a result of sensitisation, was equivalent in the two groups. Compared to the 6 month response

the data at 10 months were rather low. This was due to the low activity of the ^{125}IUdR used; more than the usual quantity of 'cold' IUdR had to be added to provide the normal sensitising dose since there was a limited amount of the radio-labelled material available.

TABLE 16

Cellular proliferation in CBA mice irradiated and repopulated with young or old bone marrow cells 10 months previously.

Group	Log_{10} No. incorp. counts/10^6 counts injected			
	DAY 3		DAY 7	
	Draining LN	Contra. LN	Draining LN	Contra. LN
Young B.M. Alcohol	1.6165 ±0.0360	1.6090 ±0.1615	-	-
Young B.M. Oxazolone	2.7561 ±0.0223	2.4973 ±0.0331	2.1886 ±0.0547	1.7165 ±0.0447
Old B.M. Alcohol	1.6409 ±0.1220	1.7481 ±0.0042	-	-
Old B.M. Oxazolone	2.7064 ±0.0360	2.2821 ±0.0469	2.2699 ±0.0574	1.8151 ±0.0842

Incorporated counts shown as the mean and standard error of the mean. Incorp = incorporated; Contra. = contralateral; LN = lymph node; B.M. = bone marrow cells.

3. The long-term ability of old bone marrow to produce functional T and B cells when transferred to young irradiated recipients

3 month old male CBAT6 mice were given 900r whole body irradiation. They were repopulated with either 5×10^6 3 month old anti-theta treated bone marrow cells or 5×10^6 26 month old bone marrow cells, also treated with anti-theta serum and complement before injection. The bone marrow inocula were obtained, in both cases, from CBA male mice. After an interval of 12 months the mice were used in anti-theta cytotoxicity assays and a response to sheep erythrocytes.

The anti-theta assays were performed on cells from the spleens, before antigen injection and on days 2 and 7 after the injection of 4×10^7 sheep erythrocytes. The same mice were used to determine the number of direct and indirect plaque forming cells in the spleens. The results are shown in Table 17.

No significant differences were found between the two groups. The proportion of theta positive cells in the spleens of old marrow recipients was very similar to that seen in in young marrow recipients. The total cellularity of the spleens was rather variable but the estimated proportion of T cells (71 to 86%) did not appear to differ between the two groups. The results of the plaque assay also showed that there was no significant difference between the potential of old compared to young bone marrow cells. The numbers of plaque forming cells seen in the spleens was approximately that which would be expected in 15 month old mice.

TABLE 17

Cellular composition and PFC content of the spleen of male CBA mice immunised with sheep erythrocytes 12 months after lethal total-body irradiation and repopulation with bone marrow from young or old syngeneic donors.

Age of marrow donor (months)	Days after immunisation	Number of spleen cells ($\times 10^{-6}$)		Log_{10} PFC/spleen (mean S.E.)	
		Theta-positive	Theta-negative	Direct	Indirect
3	0	28.2 ± 5.6	95.2 ± 1.7	1.81 ± 0.13	None
26	0	27.7 ± 8.2	91.4 ± 27.9	1.65 ± 0.05	None
3	2	27.3 ± 3.3	74.2 ± 9.1	2.23 ± 0.09	None
26	2	31.0 ± 2.9	97.3 ± 17.9	1.87 ± 0.17	None
3	7	42.0 ± 3.3	73.3 ± 1.9	3.97 ± 0.16	4.21 ± 0.13
26	7	44.9 ± 2.7	86.1 ± 4.3	4.11 ± 0.65	4.06 ± 0.61

4. The ability of the old thymus to support a proliferating T cell population.

The experiment involved placing the old thymus into young recipients and comparing its ability to support a T cell population with that of a similarly treated young thymus graft.

CBAT6T6 mice were thymectomised at 4 weeks of age. 2 months later they were lethally irradiated (900r) and divided into 5 groups. The experimental plan for their repopulation is shown in Table 18

TABLE 18

Repopulation schedule for TX male mice irradiated at 3 months of age.

Group	Source of cells and tissues for repopulation
1	5×10^6 F.L. + young thymus graft
2	5×10^6 F.L. + old thymus graft
3	5×10^6 F.L. + young irradiated thymus graft
4	5×10^6 F.L. + Old irradiated thymus graft
5	5×10^6 F.L. ONLY

TX = thymectomised; F.L. = foetal liver cells.

The foetal liver cells were obtained from 17 day old CBAT6T6 foetuses and were injected within 4 hours of irradiation. The young thymus grafts were taken from 2 to 5 day old newborn CBAT6T6 male mice, the old grafts from 17 to 18 month old CBAT6T6 male mice. Older donors could not be used as a source of thymuses because of the difficulty experienced in excising the organ precisely.

Unirradiated thymus grafts were placed in the subcutaneous axillary region within 7 hours of irradiation of the recipient mice. The thymus grafts which were to be irradiated were first excised from the donor mice and then placed on ice in medium 199. They were then given 1100r and placed in the subcutaneous axillary region of the recipient mice within 3 hours of their removal from donor animals. Since 50 mice were used in each group it proved impossible to complete all the operative procedures on one day and the irradiated thymus grafts were therefore transferred 24 hours after irradiation of the recipient mice.

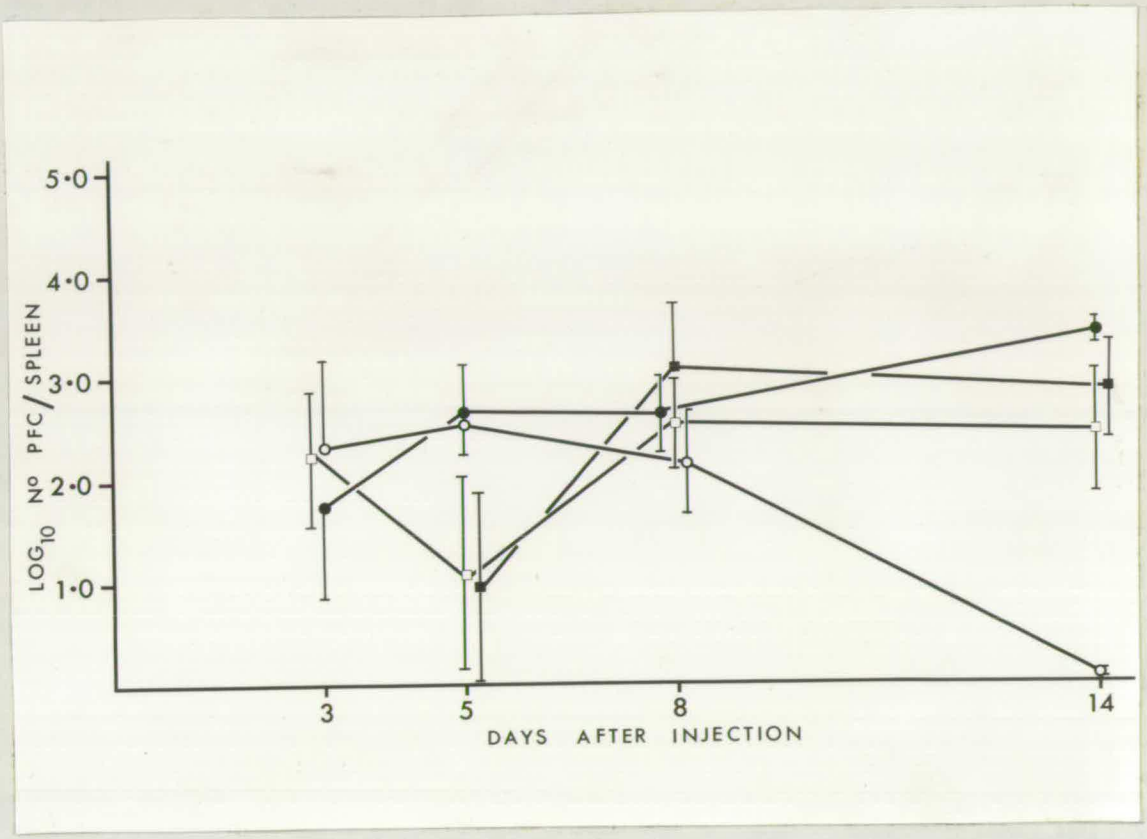
Despite very careful cleaning and feeding arrangements many of the mice died. The greatest number of deaths occurred in those groups that had recieved irradiated grafts. 4 weeks after irradiation and repopulation the number of mice remaining in the various groups was as follows: 30 from group 1, 25 from group 2, 21 from group 3, 13 in group 4 and 25 from group 5.

The surviving mice were to be used in plaque assays at 4 weeks and 3 months after irradiation. With this plan in mind the first response to sheep erythrocytes was performed on mice from all the groups except number 4 (those that had received irradiated grafts from old donors), since it was argued that they might be better employed in the later assay.

Mice in the other 4 groups were given 4×10^7 sheep erythrocytes and assayed on days 3, 5, 8 and 14 after injection. Due to a shortage of mice the group with young

FIG. 39

The indirect plaque response to 4 x 10^7 sheep erythrocytes in thymectomised CBAT6T6 male mice repopulated one month previously with foetal liver cells and young or old, normal or irradiated, thymus grafts.



○ Foetal liver cells only

□ Foetal liver cells and a young thymus graft

■ Foetal liver cells and old thymus graft

● Foetal liver cells and young irradiated thymus graft

irradiated grafts were assayed on days 5, 8 and 14 only. Generally 3 mice from each group were assayed on each day, exceptions were 2 mice only in the old thymus group on days 8 and 14 and 2 mice in the young irradiated group on day 14.

The results are shown only for the indirect response since the response assayed by the direct plaque technique showed no significant difference between the groups and very little variation with time (about 5×10^3 PFC per spleen in all groups on all of the days assayed).

The indirect plaque response (Fig. 39) reflected the rather low direct plaque numbers. No significant differences could be established between any of the groups on any day with the exception of the foetal liver cells only group on day 14 where none of the three mice assayed produced any 7S plaques at all. Clearly the significance of this result must await further research but the possibility is raised that the group that had foetal liver cells only produced no indirect plaque forming cells on day 14 because they lacked a thymus. Since the thymus grafted groups were equivalent in their responses on day 14 it might be concluded that old thymus grafts can act as a source of thymus derived lymphocytes in a manner equivalent to that seen in young thymus grafted mice.

The second plaque response planned for 3 months had to be abandoned because of further deaths in the remaining mice. The remaining mice (6 each from groups 1 and 2 and 3 in group 3) were assayed in their response to oxazolone on day 3 after sensitisation. Specific responses were assayed in 4 of the 6

mice in groups 1 and 2 (young and old unirradiated thymus grafted groups) and in all 3 of the young irradiated thymus group. The remaining 2 mice from each of groups 1 and 2 were used as background controls. The results are shown in Table 19.

TABLE 19

The primary response to oxazolone in thymectomised, irradiated mice repopulated with foetal liver cells and an old or young thymus graft.

Group	sensitising agent	Log_{10} No. incorp. counts/10^6 counts injected (mean S.E.)
1	alcohol	2.0240 ± 0.0316
1	oxazolone	2.6240 ± 0.1076
2	alcohol	1.9191 ± 0.1063
2	oxazolone	2.5033 ± 0.1383
3	oxazolone	2.6108 ± 0.1571

An analysis of variance showed that there was no significant difference between the 3 groups, either in their specific response to oxazolone or in background data where it was available. In both groups 1 and 2 the specific response was significantly higher than background($P < 0.02$). It is unfortunate that the experiment lacked groups 4 and 5 since no firm conclusions can be made about the ability of old irradiated thymus grafts. Nevertheless the possibility that the old thymus can support a proliferating T cell population is not ruled out by the results available.

Conclusions

The experiments described in this chapter indicate that old bone marrow retains its ability to produce functional T and B cells in a manner equivalent to that in young marrow, provided the cells are placed in a young environment. Since it has not proved possible to improve the response of old mice with young stem cells it is concluded that microenvironmental conditions in the old mouse prevent the proliferation both of the native bone marrow cells and young marrow stem cells when they are transferred to the old mouse environment. The possibility has been raised that the old thymus may not lose its ability to support a proliferating cell population.

CHAPTER 9

DISCUSSION 2

The purpose of this discussion is to relate the depression seen in the immune response of old animals and in particular the mouse, to studies which have investigated possible deficiences both in the lymphoid cell populations and to various microenvironmental changes which may, or do, occur with advancing age. Table 20 lists various mechanisms which may underlie immunological decline in senescence. Although Section A deals specifically with changes which may occur in the lymphoid cell populations and their precursors and Section B and C may be broadly entered under the heading of microenvironmental changes, it is not intended that either one of the sections should be thought of in isolation from another. Clearly, any changes which occur, for instance, in the structure of the lymphoid tissues may be reflected in inadequacies in the lymphoid cells which mature and proliferate within them.

SECTION A - haemopoietic and lymphoid cell deficiences.

Several authors have made studies on the size of the pluripotent stem cell population in relation to age (ALBRIGHT and MAKINODAN, 1966; PROUKAKIS and LINDOP, 1967; YUHAS and STORER, 1967; DAVIS et al, 1968; 1971; COGGLE and PROUKAKIS, 1970; CHEN, 1971). The report by CHEN (1971) concluded that although the proportion of spleen colony forming units (CFU) in the bone marrow of ageing (C57Bl x C3H)F_1 mice declined with

TABLE 20

Possible mechanisms underlying immunological decline in senescence

(A) HAEMOPOIETIC AND LYMPHOID CELL DEFICIENCES

1) Pluripotent stem cells
   (CFU, bone marrow, repopulating cells)
2) Bone marrow progenitors of thymus lymphocytes.
3) Bone marrow progenitors of B lymphocytes.
4) Mature T lymphocytes: (a) numerical
   (b) functional
5) Mature B lymphocytes: (a) numerical
   (b) functional
6) Bone marrow progenitors of macrophages.
7) Macrophages (a) numerical
   (b) change of distribution in body
   (c) functional

(B) STRUCTURAL DEFICIENCES IN LYMPHOID TISSUES

1) Thymic epithelium
2) Spleen, lymph nodes etc.
   (a) milieu for lymphocyte migration/ co-operation/ proliferation/ differentiation.

(C) GENERALIZED DEFICIENCES AND CHANGES

1) Hormonal regulation: (a) adrenocorticoids
   (b) sex hormones
   (c) somatotrophic hormone
   (d) thymic hormone
   (e) other
2) Other changes - e.g. availablility of nutrients, vascular changes etc.

(From NICKLEM et al, 1973)

progressively, the size of the CFU pool remained approximately the same, since the total cellularity increased. The conclusions of the report do not, however, neccessarily imply that the quality of the CFU pool remains unaltered. Several differences have been reported between CFU from different sources or recovered under different conditions. SIMINOVITCH et al (1964) reported that serial transplantation of CFU rendered them progressively less capable of self renewal. Reports from this laboratory, using CBA mice, have shown that CFU from foetal liver gave rise to larger more rapidly proliferating spleen colonies, judged by the incorporation of ^{125}IUdR into DNA, than did CFU derived from young adult marrow (MICKLEM, 1972). Further experiments with chromosome marked populations in irradiated mice supported the conclusion that the proliferatice potential of foetal liver CFU was greater than those derived from young adult marrow (MICKLEM et al, 1972). However, no comparable differences were found between populations derived from young and old adult donors.

Preliminary studies using a serial transfer technique (MICKLEM et al, 1973) suggested that old stem cells have a lower proliferative potential than do young ones, when the two are placed in a competition situation. However, more recent studies using the same technique have suggested that this may have been due to chance (OGDEN and MICKLEM unpublished).

It would appear, therefore, that any differences that do occur in either the size or the quality of the stem cell pools from old and young mice cannot be determined by the techniques presently employed.

Since there is no doubt that one stem cell (CFU) may differentiate into both T and B cells, as well as into other blood cells (BARNES et al, 1959; TRENTIN et al, 1967; WU et al, 1968; EDWARDS et al, 1970; NOWELL et al, 1970) any difference in the behaviour of CFU from old mice, or the haemopoietic populations derived from them, might be reflected in the populations of mature lymphocytes and in their responsiveness to antigens.

In a rather complex series of experiments PRICE and MAKINODAN (1972a) have concluded that the rate at which B cells, derived from progenitors in old bone marrow, produced functional antibody forming cells was significantly slower than B cells derived from progenitors in young bone marrow. Young or old bone marrow cells were injected into thymectomised, irradiated, syngeneic recipients. 8 days later spleen cells from the recipients were used as a source of young or old bone marrow derived B cells in a cell dose response analysis. Using a constant dose of spleen cells from normal old intact mice as a source of T cells, varying numbers of young or old marrow derived B cells were injected, together with the T cells and a maximally stimulating dose of sheep erythrocytes (10^9 cells) into thymectomised irradiated young syngeneic recipients.

Conclusions were based on 'maximum' direct plaque forming cell counts in the spleens. The authors claimed, on the basis of extrapolated take-off points, that the number of functional antibody forming cell precursors was the same at the start of the specific response. But on the basis of a less steep slope in

the dose response analysis in the old population they concluded that the rate of production of functional antibody forming cells in response to antigen stimulation was less rapid in the old compared to the young population. All of these conclusions have their basis on the slope of the dose response analysis calculated by regression analysis on very low, rather variable, plaque forming cell counts. A slight alteration in the slope of the line would suggest quite the opposite conclusion, i.e. that the number of functional antibody forming cell precursors at the start was lower in the old population, but that their proliferative potential was equivalent to the young.

However, on the basis of the information given there did appear to be a difference in the maximum cell dose response achieved by the B cells derived from the young bone marrow progenitors compared to the old. The young population reached a significantly higher plateau level than did the old. Since the responses were made in the presence of a constant number of similarly aged T cells the technique and assay employed by these investigators would seem to have been sensitive to inadequacies in the B cells derived from old bone marrow progenitors.

The results used in the dose response profiles were reported to be 'maximum' responses at any one particular cell dose. One might assume from this that a number of days post repopulation and immunisation were assayed so that the maximum responses were in fact recorded. If so, it is unfortunate that details of when this maximum response occurred and whether the

time was the same for young and old, were not reported.

The results of an experiment in which equal numbers of young and old bone marrow cells were injected with a large number of young thymus cells were shown in Fig. 36 and 37. In this case the irradiated and repopulated CBA mice were injected with sheep erythrocytes 17 days later and were assayed by the haemolytic plaque technique on several days after immunisation. The responses obtained were considerably higher than those obtained by PRICE and MAKINODAN (1972a) with an equivalent dose of marrow derived B cells, suggesting that the production of mature antibody forming cells had involved considerable proliferation of the progenitor cells in the inocula. Since the responses obtained in the two groups were not significantly different it was concluded that at the end of the 17 day pre-immunisation period, B cells, derived from old bone marrow, were capable of producing functional 19S and 7S antibody forming cells in equivalent numbers to those derived from young marrow inocula. Any possible differences in the number of stem cells injected was evidently compensated by differential proliferation and maturation within the experimental period.

The relative ability of old and young marrow to produce functional T cells was shown in Tables 16 and 17 and Fig.38. Lethally irradiated young mice were injected with equal numbers of young or old anti-theta treated (RAFF, 1969) bone marrow cells and assayed in their response to oxazolone 1, 6 or 10 months afterwards. No significant differences were found between the two groups on any of the assay occasions and

furthermore the responses obtained at 6 months after repopulation were very similar to those obtained in normal young animals (Fig. 22 and 23). The increase in mitotic activity, reflected in the incorporation of ^{125}IUdR into DNA in the lymph nodes 3 days after sensitisation with oxazolone is mainly, if not entirely, due to T lymphocytes (DAVIES et al, 1969). Mice which are congenitally deficient in T lymphocytes show no response to oxazolone (PRITCHARD et al, 1972). Furthermore in radiat--ion chimeras, injected bone marrow can only restore the response to oxazolone if the recipients have a thymus (PRITCHARD et al, 1973). In the ageing study it is presumed that the proliferation was due to the activity of T cells which had matured from theta-negative precursors in the injected bone marrow under the influence of the recipients thymus.

Further evidence that old marrow can produce functional T and B cells, in a manner equivalent to young marrow, was shown in Table 18. Young irradiated mice were repopulated with young or old anti-theta treated (RAFF, 1969) bone marrow. 12 months later the response of the recipients was assayed after the injection of sheep erythrocytes, a T cell dependent antigen. The responses of the two groups were equivalent and were at a level to be expected from normal 15 month old mice. In addition the relative proportions of theta-negative and theta-positive cells in the spleens were equivalent.

The conclusions from the experiments carried out in this laboratory is that a small fraction (5×10^6 cells) of the total bone marrow of 2 year old CBA male mice contains sufficient

progenitors to endow its recipient with both T and B cells approaching normal in both numbers and functional efficiency. This being so, it seems unlikely that the deficiency in old mice is a reflection of the number, proliferative potential, or ability to produce functional cells, of old bone marrow cells.

In spite of the conclusion that old marrow stem cells have the potential to produce mature, functional, lymphocytes, the performance of mature cells derived from old mice is impaired. The work by MAKINODAN and his colleagues (1964, 1966a, 1966b) in which spleen cells from various ages of mice were used as an indication of both primary and secondary antibody forming ability, when transferred into young irradiated, syngeneic, recipients, clearly indicated that there was a progressive decline in the functional ability of the cells used. Possibly both T and B cell function is impaired, a conclusion that could also be made of all the work that has involved haemolytic plaque responses or serum antibody estimations in normal intact mice of various ages (WIGZELL and STJERNSWARD, 1966; METCALF et al, 1967; KISHIMOTO et al, 1969; MAKINODAN et al, 1971a, 1971b; results of this thesis).

The results of experiments which have stimulated individual T or B cell populations of old mice indicate that both main cell lines are affected by ageing factors (HORI et al, 1973; MATHIES et al, 1973; PRICE and MAKINODAN, 1972a; Fig. 22, 23,27). Yet it appears that the overall size of the theta positive and theta negative populations remains essentially unaltered (KISHIMOTO et al, 1973; HORI et al, 1973;

MATHIES et al, 1973). The conclusions would seem to be that some micro-environmental influence causes alterations in at least some of the lymphocyte population. Such an alteration might involve, for instance, details of the plasma membrane configuration, as suggested by PRICE and MAKINODAN (1972a).

Whatever the cause it is clear that any inability on the part of old lymphocyte populations to respond to antigen cannot be restored by giving the animals young stem cells or a young thymus graft. Attempts were made to enhance the immune responsiveness of ageing mice by injecting them with haemopoietic stem cells from young syngeneic donors, with or without a neonatal thymus graft, after part body irradiation (Fig. 30 to 34). There was presumably no shortage of stem cells since 5×10^6 nucleated cells (approximately 5×10^3 stem cells) were injected. A more likely possibility seems to be that whatever micro-environmental conditions prevent the production of mature functional T and B cells in normal old mice, the same prevent the full expression of function in the young donor populations. The deficit does not appear to be a reflection of thymus environment since METCALF et al (1967) failed to improve the response of C57Bl mice by giving them multiple young thymus grafts, although this feature will be discussed at greater length below.

It has been claimed (PERKINS and MAKINODAN, 1971) that resistance to Salmonella typhimurum in old mice, which were normally highly susceptible, was greatly improved by transferring young cells primed to the organism into them. The beneficial

effect of this treatment, considered to reflect a partial immunological rejuvenation, was apparent up to 12 weeks after injection. It is clear that at the present time no long-lasting effect can be made to improve the responsiveness of old animals.

Although macrophages are undoubtedly important in many immune responses (SCHWARTZ et al, 1970), their precise functions are not yet fully understood. On the basis of phagocytic activity following the injection of inert particles (AOKI et al, 1965; Di CARLO et al, 1963) and the results of antigen clearance studies in old mice (HANNA et al, 1971a) all of which demonstrated decreased activity in old animals, it was concluded that macrophage activity in the old mouse is impaired.

No direct studies have been made on macrophage progenitors alone. However, experimental studies which have involved the repopulation of lethally irradiated young recipients with old or young syngeneic bone marrow cells, in addition to investigating the activity of T and B cell progenitors, would also involve macrophage maturation from their progenitor cells in the bone marrow inocula. Since no inadequacies were found in the performance of mice injected with old bone marrow, compared to the performance of mice injected with young marrow, over a considerable period of time after inoculation, it is concluded that macrophages are formed from progenitors in old and young marrow inocula in an equivalent manner.

In the intact old animal it may be that the same, or similar, environmental conditions which have been thought to

oppose T and B lymphocyte maturation also affect the maturation or functioning of macrophages. It is unknown whether depressed macrophage activity in the old mouse is due to a decrease in the overall size of the macrophage population, a decrease in the activity of individual cells, or a change in macrophage distribution within the body.

An attempt has been made (ZATZ et al, 1971) to investigate the localisation patterns of ageing Cr^{51} labelled lymphoid cells from CBA mice, when injected into syngeneic adult recipients. Most of the changes occurred over the first 3 months and were attributed to normal maturation processes. No great change was seen in the distribution of lymph node or thymus cells but there was an age dependent increase in the proportion of recirculating cells derived from spleen cell suspensions. The proportion of non-recirculating cells derived from spleens was also seen to increase gradually with the age of the donor. Unfortunately the study included mice only up to the age of 12 months. Although some attempt was made to relate these changes to the possibility that the number of newly generated, uncommitted cells decreases with age in the CBA mouse the data must be regarded as inconclusive. No doubt further studies of this nature, should they include groups of older mice, will prove valuable to a greater understanding of the behaviour of lymphoid cells in old mice.

In summary of this section, which more than any other has been the subject of the research carried out here, the following conclusions may be made. It would appear that

haemopoietic stem cells derived from old bone marrow exhibit no deficiences in their ability to produce functional lymphocytes when injected into a suitable (i.e. young) environment. It seems unlikely that the availability of lymphocyte or macrophage progenitors is a limiting factor in the immune responsiveness of old mice. Since there is mounting evidence that the overall size of the mature lymphocyte populations remain essentially unaltered in old mice, a proportion of the mature cells produced may be altered in such a way as to prevent them from participat--ing in responses to antigen. The micro-environmental conditions which prevent the maturation or functioning of lymphocytes in the old mouse would appear to suppress the activities of young stem cells when they are injected into old recipients.

SECTION B - Structural deficiences in Lymphoid Tissues.

Structural deficiences in lymphoid tissues may have profound effects on the immune capabilities of the animals concerned. MAKINODAN and his colleagues (ALBRIGHT and MAKINODAN, 1966; MAKINODAN and PETERSON, 1966b) pointed out that spleen cells from mice of various ages, exhibited a less rapid decline in responsiveness to sheep erythrocytes when transferred into young, irradiated, syngeneic recipients, than was evident from the performance of the intact mouse. Information of this kind provided some of the earliest awareness that environmental conditions. as well as lymphoid cell deficiences, were implicated in the depressed immune response of old mice.

ERRATUM

The first 4 lines of page 142 should read as follows:

Although there is some evidence to the contrary (CHAPERON et al, 1968; ANDERSON and DRESSER, 1972), the thymus as an organ has not been thought of as a site of antibody production

Although views to the contrary have been expressed (CHAPERON et al, 1968) the thymus, as an organ, has not been thought of as a site of antibody production, at least in the mouse. But as a source of T cells, it has been the centre of discussion regarding immunological deficiences in aged mice. Since 7S antibody production, which appears to have a greater dependence on T cell co-operation than 19S antibody (TAYLOR and WORTIS, 1968; AIRD, 1971; DRESSER, 1972; TORRIGIANI, 1972), becomes more severely depressed in old animals the involvement of the thymus in the ageing process, as it affects the immune system, still remains the subject of discussion by some authors. BURNET (1970) concluded that changes occurring in the thymus 'system' by which he presumably includes peripheral T cells in addition to the thymic environment, represents the organ system responsible for ageing in the vertebrate animal. In a discussion of WALFORD'S book, 'The immunologic theory of ageing' (WALFORD, 1969) Burnet attempted to involve the thymus in the gaps he considered to have been left in Walford's arguments. Burnet argued that Walford had omitted to discuss differences in longevity between species, the incidence of malignant diseases in relation to age and the importance of thymus atrophy and the Hayflick limit (HAYFLICK, 1965. - the conclusion that cells are capable of a limited number of mitotic divisions before serious structural abnormalities occur). In an attempt to involve the thymus in all of these aspects BURNET stated that : the ageing process in any species is genetically determined and programmed as a result of evolutionary processes; that the programme is

mediated, essentially, by an inbuilt metabolic clock, whose most direct manifestation is the Hayflick limit to cellular proliferation in the euploid state. He concluded that the organ most likely to exhaust its limit of cells was the thymus and that this would lead to inadequacies in immune surveillance and eventual death.

If it were the case that the old thymus was implicated to the extent envisaged by BURNET, its replacement by young thymic tissue should improve the performance of the animals concerned. This has, however, been shown not to be the case (METCALF et al, 1967). Unfortunately little attempt has been made to study the activity or potential of old thymic tissue when it is placed in a suitable environment. An attempt was made here (Fig. 39) but the results were inconclusive. However there was some indication that old thymus grafts can survive in a young mouse and supply functional T cells to the recipient. Unfortunately there is no evidence of a conclusive nature that the old thymus can support the maturation of T lymphocytes from progenitor cells derived from young mice.

Several reports have demonstrated that the structure of the spleen and lymph nodes is severely disrupted in old mice (LEGGE and AUSTIN, 1968; HANNA et al, 1971a, 1971b; CHINO et al, 1971). Many events which are affected by, or are dependent on, the highly complex structure of lymphoid organs may be modified in old mice. Studies on the ontogenic development of lymphoid structures have indicated a very close temporal relationship between the ability of a mouse to trap antigen and the formation

of specific antibody (WILLIAMS, 1966; WILLIAMS and NOSSAL, 1966). In young adult animals antigen localises in the follicles of the spleen and lymph nodes in a highly efficient manner shortly after injection (KAPLAN et al, 1950; NOSSAL et al, 1964; WHITE, 1963). In association with follicular antigen localisation is the wealth of information supporting the increased incidence of germinal centres and the close association of this event with the formation of 7S antibodies and memory cells (WILLIAMS, 1966; NOSSAL et al, 1964; WILLIAMS and NOSSAL, 1966; WHITE, 1963; COTTIER et al, 1967; HANNA et al, 1966, 1971b; THORBECK and HURLIMANN, 1965; WAKEFIELD and THORBECK, 1968a, 1968b).

It seems that localisation, at least of radio-labelled protein antigens, is severely depressed in old mice (LEGGE and AUSTIN, 1968; METACLF et al, 1967; HANNA et al, 1971a). The loss of the structural demarcation between cortex and medulla in the lymph nodes and between the red and white pulp of the spleen appears to take place at the same time as the loss of germinal centre activity (LEGGE and AUSTIN, 1968; CHINO et al, 1971; HANNA et al, 1971a, 1971b). Even when the inadequacies of the basic lymphocyte populations are taken into account it seems probable that these structural deficiences affect the immunological capabilities of the animals concerned.

LEGGE and AUSTIN (1968), on the basis of a deficient secondary but equivalent primary response in old mice, concluded that germinal centre activity was important only to the secondary response. MAKINODAN et al (1971a, 1971b) however,

on the basis of a relatively greater increase in indirect (7S) plaque forming cells in the secondary response compared to the primary in old mice compared to young, considered that germinal centre activity was important to the primary response but not the secondary. For reasons that have already been discussed the results reported by LEGGE and AUSTIN (1968) may not have reflected the complete potential of young mice and their conclusions may be based on insufficient information. The conclusion made by MAKINODAN disregards the possibility of increased antigen localisation and retention in the secondary response of old mice compared to the primary response, due to the presence of specific antibody in the lymphoid organs. It has been demonstrated by LEGGE and AUSTIN (1968) that prior immunisation or injection of passive antibody markedly improves the ability of lymphoid tissues in old mice to retain antigen.

The importance of the structural changes which occur in the lymphoid organs of old mice cannot at the present time be fully evaluated. That they contribute to the depression of immune responses in old mice can hardly be doubted. What remains uncertain is how the observed structural changes in old age affect the micro-environment for lymphocyte and macrophage differentiation and maturation.

SECTION 6 - Generalized deficiences and changes.

This section of Table 20 includes many factors which may prove to be of fundamental importance to immunological deficiences in old mice.

In recent years considerable interest has been shown in the effects of various hormones on immune responses. With their capacity to affect many metabolic processes it is not surprising that a number of hormones have been found to influence lymphoid tissues and cells, sometimes exercising their control in a very specific manner. The difficulties in determining hormone concentrations in intact animals creates severe obstacles in any determination of their effects on the ageing process. The problems associated with estimating or investigating the effect of hormones and their role in senescence were very ably summarised by COMFORT (1956). Although immunoassay of many hormones is now possible the problems envisaged by COMFORT must still prevent a fuller investigation. He stated that there appeared to be no single hormonal change which correlates with senescence, no single endocrine organ, of those that could be removed without fatal results, whose extirpation produced the syndrome of senescence in mammals and no hormone or combination of hormones which was known to produce more than a limited and apparently secondary reversal of senile changes. Furthermore, many hormones form part of a complex pattern involving inter-dependence of one hormone with several others. Disruption of any individual hormone may affect any number of others.

Despite these problems, which were specified some 20 years ago, the information presently available on hormonal influences in neonatal and young adult lymphoid cells and tissues may be worth consideration in a discussion of senescence.

Most attention has, it appears, been focussed on

corticosteroids and immune responses. Numerous studies have demonstrated that adrenocortical hormones affect lymphocytolysis (HECHTER and JOHNSTONE, 1949) and thymic involution (SANTISTEBAN and DOUGHERTY, 1954; SHEWELL and LONG, 1956) and are associated with cell death (CLAESSON and ROPKE, 1969). Inhibition of antibody formation has also been described (BJORNBOE et al, 1951; McMASTER and FRANZL, 1955).

Although corticosteroid effects on lymphoid tissues may be quite considerable, a recent review (CLAMAN, 1972) points out that these effects may not be seen with equal severity in all mammalian species. Some species have been described as corticosteroid sensitive and are, generally speaking , very susceptible to hormone treatment. This group includes such species as the mouse, rat and rabbit. Other species such as the guinea pig, monkeys and man are described as resistant and require a far greater concentration of hormone to achieve similar effects (SHEWELL and LONG, 1956).

CLAMAN, 1972) has attempted to summarise the available information on the effects of corticosteroids on individual tissues and cells populations. The cortex, but not the medulla, of the thymus has been described as sensitive (ISHIDATE and METCALF, 1963; WARNER, 1964). It has also been suggested that the development of T and B lymphocytes from their progenitors represents a sensitive phase in their development, whilst the mature antigen-sensitised T cell and the antibody producing B cell were described as resistant (COHEN and CLAMAN, 1971). It is this kind of conclusion, one based on sensitivity at

particular stages in the development of the lymphoid populations which may prove to be relevent to senescence since one of the basic conclusions of the repopulation studies described here has been that both T and B cells are inhibited in their maturation from precursors in the old mouse.

Another aspect of corticosteroid action has been suggested by AMBROSE (1970). The conclusion from this work, based on in vitro organ cultures, was that corticosteroids were essential for the induction of the immune response. It appeared that the elimination of cortisol from the culture medium prevented the induction of the response. The elimination of the hormone after the induction phases have been accomplished appeared to have no adverse effect. AMBROSE also investigated the role of several other hormones and concluded that hormones of low polarity, such as progesterone, testosterone and oestradiol, even when in very low concentration (as little as 1μM), prevented the induction of the immune response. A previous report from DAVIDSON et al (1963) had demonstrated that plasma and mitochondrial membranes of liver or kidney cells, bound steroids of low polarity more avidly than they did hormones of high polarity. AMBROSE (1970) suggested that low polarity corticosteroids may inhibit immune responses in a more or less non-specific manner, by coating and saturating various cellular membranes and blocking normal exchange through them.

Since the thymus exercises such an important role in the lymphoid system it is not surprising that many authors have attempted to demonstrate direct hormonal control, both during

its development and in the maintainance of a functional immune system. Considerable interest has been shown in the role of somatotropin (STH) and thyroxin. Most studies have been directed at the role of the anterior pituitary on thymic devlopment. Inhibition of STH production by the injection of anti-STH serum in mice results in rapid involution of the thymus and depletion of the thymus dependent areas in the spleen (PIERPAOLI and SORKIN, 1968). Beneficial,effects for both STH and thyroxin injection have been claimed in a number of studies. The injection of spleen cells and hormone, into newborn, histo-incompatible mice or rats, speeds up the course of runt disease compared to that seen with spleen cells alone (PIERPAOLI et al, 1970). The same paper also claimed that STH therapy induced competence in thymocytes from newborn donors in the absence of the recipients thymus. The suggestion has been made that senescence represents a protracted wasting disease whose cause is the effect of a depletion in the number of hormone dependent, multifunctional thymus-derived lymphocytes as a result of a failure in the endocrine system (PIERPAOLI, 1972). The inter-dependence of the lymphoid and endocrine systems was indicated by the observation that the lifespan of hormone deficient, hypo-pituitary dwarf mice could be extended equally well by either STH treatment or the injection of young lymph node cells from normal litter mates (FABRIS et al, 1972).

The possibility that the thymus itself functions as an endocrine organ has been suggested in a number of reports in recent years. A thymic activity factor, produced by medullary

epithelial cells of the thymus has been demonstrated in the serum of normal young adult mice (BACH and DARDENNE, 1972a, 1973a; BACH et al, 1973). It has also been claimed that partially purified, cell-free extracts of thymus tissue can induce the devlopment of T cell characteristics, in the form of the surface antigens TL and theta, on the membranes of bone marrow and foetal liver cells (KOMURO and BOYSE, 1973a, 1973b). At present it is not clear where the hormone exercises its function: it may be restricted to the thymic environment (BACH and DARDENNE, 1973a) or it may control the further maturation and maintenance of T cell characteristics in cells previously seeded from the thymus (BACH and DARDENNE, 1972b, 1973b).

Clearly any hormone which claims to have such extensive effects on the growth and maintenance of T cell characteristics in the intact mouse would be worthy of serious consideration in any discussion related to senescence. STRAUSSER and GOLDSTEIN (1972) claimed that a partially purified 'thymosin' extract, derived from the thymic tissues, when injected into old mice improved their performance in a response to sheep erythrocytes. The significance of this work awaits the use of a more purified extract to exclude endotoxin or other effects as a source of the observed enhancement. The serum concentration of a factor with thymosin-like characteristics has been said to decrease with age in mice (PFOCH, 1971). This report also stated that the frequency of residues in the golgi field of the thymic entodermal cells of newborn and 13 month old

Wistar rats, supposed to represent the site of production of thymic hormone, declines with age, and they become increasingly electron-dense.

The discussion of some of the effects that hormones may have on the immune response and the claims made concerning their role in the ageing process underlines the fragmentary nature of our knowledge. Very often there is an impression that too much is attributed to the effects of a single hormone. It is, nevertheless, possible that as the knowledge and technical skill of research workers increases fundamental ageing phenomena may be attributed to hormonal patterns. The results reported by PRICE and MAKINODAN,(1972b) in which both the lymphoid cells and the environment were defective demonstrated that a non-cellular factor, rather than the structural deficiences of the lymphoid organs, caused a depression in the response of young cells placed in cell-impermeable chambers within old mice. The authors concluded that it could not be determined whether the depression was due to a non-cellular factor which was detrimental to cell growth or to the absence of some factor which was required for the growth and maintenance of immunocompetent cells.

Any discussion which hopes to encompass all the factors that may have a part in the ageing process as it affects the immune system, will include many which are essentially non-immunological in nature. No attempt has been made to investigate their effects as they lie well outside the scope of the work presented here. However, it is important that factors of this nature are mentioned since any change which occurs in

other body systems with age may well affect the immune system. The vascular and skeletal systems have been the subject of extensive research because of the in the structural changes in two of their basic components, collagen and elastin. These two substances alone are employed in numerous sites throughout the body and any change in their molecular structure which results in a change in their properties may have an effect on the immune system.

Although it has been stated that molecular cross-linking of the collagen molecule may occur before the onset of more marked ageing effects (PIEZ. 1969) the general concensus of opinion would appear to support the observation that cross-linkage of the molecule, resulting in decreased elasticity, occurs gradually with age (HALL, 1972; DELBRIDGE and EVERITT, 1972; CANNON and DAVISON, 1973). In addition to the decrease in elasticity of collagen, which itself may have profound effects on the functioning of the lymphoid organs such as the spleen and lymph nodes, another aspect has been described by HALL (1972). He observed that as age progresses many connective tissues, especially the skin and walls of larger blood vessels appear to contain increasing amounts of a protein very similar to elastin, called pseudo-elastin, which is derived from the break-down products of collagen. In heavily cross linked collagen, found in old animals, the normal degradation of collagen to hydroxyproline, which is normally excreted in the urine, is not completed. The molecule collects in the various tissues after partial degradation to pseudo-elastin. There has also been a

report that the rate of wound healing in old rats was slower than in young animals (SUSSMAN, 1973).

Changes in any other structures or processes cannot at the present time be usefully discussed.

Conclusions

The inclusion, in the discussion, of non-immunological factors, even if only to encourage the reader not to ignore them completely, highlights the problems and complexities involved in elucidating age related changes as they affect the immune system. So much has been written on the effects of age, both within and outside the field of immunology, much of which no doubt affects the functioning of the immune system, that it may be many years before all the information presently available is brought together. Research workers seldom encompass within their knowledge details of the effects of senescence in other disciplines. It may be that this has lead to rather sweeping claims that a particular physiological system, for instance the lymphoid system, holds all the answers to the ageing process.

No attempt has been made to discuss in any detail the various immunological theories of ageing or to relate the various results of this thesis to them, since it is argued that insufficient information is presently available for it to worthwhile.

There may be a desire, on the part of certain people, to increase the lifespan of man by understanding and controlling

the mechanisms which underlie the phenomenon of senescence. The value of such an undertaking would certainly be questioned by many people and certainly has not been the motivation behind the work presented here. But there are aspects of the ageing process and old age, involving the increased incidence of immunological disorders, which if controlled might well improve the quality of life in elderly people. It is hoped that the work presented in this thesis will provide a framework on which to base future research which will increase our knowledge of immunological mechanisms both in the old and the young.

ACKNOWLEDGMENTS

I would like to offer my thanks: to Dr. David Dresser for his help and encouragement in my application to Edinburgh University for a postgraduate place; to the University authorities, both in the department of zoology and in the post-graduate committee, for allowing me to register despite my lack of financial support; to all the members of the immunobiology unit for their help and friendship especially Dr. Chris Inchley, Dr. Helen Briscoe, Dr. Sam Black, Jo Riddaway and David Ogden for their helpful discussions; to Helen Pritchard and Norman Anderson for all those occasions when night was fast approaching and the lab appeared to be littered with irradiated mice requiring their dose of life-giving cells, when their help with injections was greatly appreciated; to Norman for his help in irradiating mice and for counting theta treated cell suspensions; to David Ogden for his help in preparing bone marrow cell suspensions; to Dr. Bob Riddaway who, despite spending several hours explaining the intricacies ofcomputer programming, decided to do without my help in providing a programme for calculating plaque results; to Helen Lauder who must have wished she would never see another Khan tube that needed washing; to Mrs M. Philp ourlibrarian; to my wife Claire who has spent so many days and nights talking to herself, for her patience and encouragement. Most of all I would like to thank my supervisor Dr. Spedding Micklem for his constant help and encouragement and for his seemingly inexhaustible patience. It is due to him, more than any other person, that this work is to be found in its present form.

APPENDIX 1

MEDIA

Dulbecco's Phosphate Buffered Saline (PBS)

Solution A:

NaCl	-	8.09g
KCl	-	0.29g
KH_2PO_4	-	0.29g
Na_2HPO_4	-	1.159g

Made up to 80.0ml with distilled water (x 10 conc)

Solution B:

NaCl	-	0.19g

Made up to 100ml with distilled water.

Solution C:

$MgCl_2.6H_2O$ - 0.19g

Made up to 100ml with distilled water.

To prepare 1L take 80.0ml A, 1.0ml B and 1.0ml C and add 900ml distilled water.

Citrate Saline

Use 20.0ml of the concentrated PBS solution A, add 38.0g sodium citrate and make up to 1L with distilled water.

Duttons Buffered Saline

Solution A:

Dextrose	-	10.0g
KH_2PO_4	-	0.6g
$Na_2HPO_4.12H_2O$	-	4.78g

20.0ml 20% phenol red solution

Made up to 1L with dist. H_2O

Solution B:

$CaCl_2.2H_2O$	-	1.86g
KCl	-	4.00g
NaCl	-	80.00g
$MgCl_2.6H_2O$	-	2.00g
$MgSO_4.7H_2O$	-	2.00g

Made up to 1L with dist. H_2O.

Duttons Buffered Saline cont.

To make 1L medium take 100ml of soln A and 100ml soln. B add 800ml distilled water.

Medium 199

Obtained ready for use from Biocult Ltd. Glasgow, Scotland

A synthetic medium based on Hanks salts.

REFERENCES

ADA. G.L., NOSSAL. G.J.V. and AUSTIN. C.M. (1964) Antigens in immunity. V. The ability of cells in lymphoid follicles to recognise foreignness. Aus. J. Biol. Med. Sci. 42, 331.

AIRD. J. (1971) Thymus dependence of the immune response: response to haptenic determinant NIP in mice. Immunol. 20, 617.

ALBRIGHT. J.F. and MAKINODAN. T. (1966) Growth and senescence of antibody forming cells. J. Cell. Physiol. 67 suppl.1,185.

ALPATOV. W.W. and PEARL. R. (1929) Experimental studies on the duration of life. XII. Influence of temperature during the larval period of adult life on the duration of life in the imago of Drosophila melanogaster. Am. Nat. 63, 37.

AMBROSE. C.T. (1970) The essential role of corticosteroids in the induction of the immune response in vitro. In, Hormones and the immune response. Ciba foundn. study group No. 36 Churchill, London, p. 100.

ANDERSON. H.R. and DRESSER. D.W. (1972) The long term distribut--ion of antibody forming cells. Eur. J. Immunol. 2, 410.

ANDREW. W. and ANDREW. N.V. (1957) An age involution in the small intestine of the mouse with a description of the fundamental process of lymphoepithelial metamorphosis in intestinal mucosa. J. Gerontol. 12, 136.

AOKI. T., TELLER. M.N. and RUBITAILLE. M.L. (1965) Ageing and carcinogenesis. II. Effect of age on phagocytic activity of reticulo endothelial system and on tumour growth. J. Nat. Canc. Inst. 34, 255.

ARNASON. B.G., JANKOVIC. B.D., WAKSMAN. B.H. and WENNERSTEN. C. (1962) Role of the thymus in immune reactions of rats. II. Suppressive effect of thymectomy at birth on delayed (cellular) hypersensitivity and the circulating small lymphocyte. J. Exp. Med. 116, 177.

AUERBACH. R. (1960) Morphogenetic interactions in the devlopment of the mouse thymus gland. Dev. Biol. 2, 271.

AUERBACH. R. (1961) Genetic control of thymus lymphoid differ--entiation. Proc. Nat. Acad. Sci. 47, 1175.

AUERBACH. R. (1960b) Experimental analysis of the origin of cell types in the development of the mouse thymus. Dev. Biol. 3, 336.

AUERBACH. R. Developmental studies of mouse thymus and spleen Nat. Cancer Inst. Monogr. 11,23.

BACH. J.F. and DARDENNE. M. (1972a) Thymic dependency of rosette forming cells. Evidence for circulating thymic hormone. Transplant. Proc. 4, 345.

BACH. J.F. and DARDENNE. M. (1972b) Antigen recognition by T lymphocytes II. Similar effects of azothioprine, ALS and anti-theta serum on rosette forming lymphocytes in normal and neonatally thymectomised mice. Cell. Immunol. 3, 11.

BACH. J.F. and DARDENNE. M. (1973a) Studies on thymus products II demonstration and characterization of a circulating thymic hormone. Immunol. 25, 353

BACH. J.F. and DARDENNE. M. (1973b) Antigen recognition by T lymphocytes III. Evidence for two populations of thymus dependent rostte forming cells.; Cell. Immunol. 6, 394.

BACH. J.F., DARDENNE. M., DAVIES. A.J.S. (1971) Early effect of thymectomy. Nature N.B. (Lond) 231, 110.

BAER. H. and BOWSER. R.T. (1963) Antibody production and development of skin sensitivity in guinea pigs of various ages. Science. 140, 1211.

BAKER. P.J., BARTH. R.F., STASHAK. P.W. and AMSBAUGH. D.F.(1970) Enhancement of antibody response to type 3 pneumococcal polysaccharide in mice treated with antilymphocyte serum.; J. Immunol. 104, 1313.

BAKER. P.J., STASHAK. P.W., AMSBAUGH. D.F., PRESCOTT. B. and BARTH. R.F. (1970) Evidence for the existance of two functionally distinct types of cells which regulate the antibody response to type III pneumococcal polysaccharide J. Immunol. 105, 1581.

BALL. W.D. and AUERBACH. R. (1960) In vitro formation of lymphocytes from embryonic thymus.; Exp. Cell Res. 20, 245.

BARNES. D.W.H., BRECKON. G., FORD. C.E., MICKLEM. H.S. and OGDEN. D.A. (1967) Fate of lymphoid cells injected into lethally irradiated mice: further experiments. In 'The lymphocyte in immunology and haemopoiesis (Ed. J.M.Yoffey) p.207. Edward Arnold, London.

BARNES. D.W.H., FORD. C.E., GRAY. S.M. and LOUTIT. J.F. (1959) Spontaneous and induced changes in cell populations in heavily irradiated mice.; Progr. Nucl. Energy. SerVI 2, 1.

BASTEN. A. (1971) Specific inactivation of thymus derived (T) and non thymus derived (B) lymphocytes by ^{125}I labelled antigen. Nature N.B. (Lond). 231, 104.

BASTEN. A., MILLER. J.F.A.P., WARNER. N.L. and PYE. J. (1971) Specific inactivation of thymus derived (T) and non-thymus derived (B) lymphocytes by ^{125}I labelled antigen; Nature N.B. (Lond) 231, 104.

BAUMGARTNER. L. (1934) Age and antibody production. 1. Quantitative changes in antisera associated with age; J. Immunol.; 27, 407.

BENACERRAF. B., GREEN. I. and PAUL. W.E. (1967) The immune response of guinea pigs to Hapten-Poly-L-Lysine conjugates as an example of the genetic control of the recognition of antigenicity.; Cold. Spr. Harb. Symp. Qu. Biol. 32, 569.

BIDDER. G.P. (1932) Senescence; Brit. Med. J. ii, 5831.

BJORNBOE. M., FISCHAL. E.A., and STOARK. H.C. (1951) The effect of cortisone and adrenocorticotrophic hormone on the concentration of circulating antibody. J. Exp. Med. 93, 37

BOYD. E. (1932) The weight of the thymus gland in health and disease; Am. J. Dis. Child.; 43, 1162.

BOYSE. E.A., OLD. L.J., and STOCKART. E. (1968) An approach to the mapping of antigens on the cell surface.; Proc. Nat. Acad. Sci.; 60, 886.

BRYANT. B.J. and COLE. L.J. (1967) Evidence for pluripotentiality of marrow stem cells: Modifications of tissue distribution of *in vivo* ^{125}IUdR labelled transplanted marrow. In 'The lymphocyte in immunology and haemopoiesis (Ed. J.M.Yoffey) p. 170; Edward Arnold, London.

BURCH. P.R.J. (1968) Growth, disease and ageing. Oliver and Boyd (Edin).

BURNET. F.M. (1959) Autoimmune disease II. Pathology of the immune response. Brit. Med. J.; 2, 720.

BURNET. F.M. (1970) An immunological approach to ageing Lancet ii/7668, 358.

BURSTEIN. R., FRANKEL. S.,and SOULE. S.D. (1973) Ageing of the placenta: Autoimmune theory of senescence. Am. J. Obstet. Gynecol.;116, 271.

CANNON. D.J. and DAVISON. P.F. (1973) Cross linking and ageing in rat tendon collagen. Exp. Gerontol. 8, 51.

CANTOR. H. and ASOFSKY. R. (1972) Synergy among lymphoid cells mediating the graft versus host response III. Evidence for interaction between 2 types of thymus derived cells J. Exp. Med.; 135, 764.

CHAPERON. E.A., SELNER. J.C., and CLAMAN. H.N. (1968) Migration of antibody forming cells and antigen sensitive precursors between spleen, thymus and bone marrow. Immunol.; 14, 55.

CLAESSON. M.H. and ROPKE. C. (1969) Quantitative studies on cortisol induced decay of lymphoid cells in the thymo-lymphatic system. Acta. Pathol. Microbiol. (Scand.);76,376

CLAMAN. H.N. (1972) Corticosteroids and lymphoid cells. New. Eng. J. Med.; 287, 388.

CHEN. M.G. (1971) Age related changes in haemopoietic stem cell populations of a long lived mouse strain. J. Cell. Physiol. 78, 225.

CHINO. F.,MAKINODAN. T., LEVER. W.E. and PETERSON. W.J. (1971) The immune system of mice reared in clean and in dirty conventional laboratory farms. 1. Life expectancy and pathology of mice with long life spans. J. Gerontol.; 26, 497

CLAMAN. H.N. and CHAPERON. E.A. (1969) Immunologic complementation between thymus and marrow cells - a model for the two cell theory of immunocompetence. Transplant. Rev. 1, 92

COGGLE. J.E. (1970) The effect of age on the bone marrow cellularity of the mouse. Gerontologia.; 16, 25.

COHEN. J.J. and CLAMAN. H.N. (1971) Thymus - marrow immunocompetence and hydrocortisone resistant cells and processes in haemolytic antibody response of mice J. Exp. Med.; 133, 1026.

COLE. C.J. and MORRIS. B. (1971) The effect of thymectomy in the sheep. II. The response to antigen. Aus. J. Exp. Biol. Med. Sci.

CONE. R.E., FELDMAN. M., MARCHALONIS. J.J., and NOSSAL. G.J.V. (1973) Cytophilic properties of surface immunoglobulin of thymus derived lymphocytes. Immunol.; 26, 49

COMFORT. A. (1956) The biology of senescence. Routledge and Kegan Paul, London.

COTTIER. H., KEISER. G., ODARTCHENKO. N. , HESS. M. and STONER. R. (1967) In germinal centres in immune responses. Ed. H. Cottier, N. Odartchenko, R. Schindler and C. Conglon. Springer-Verlag. N.Y. p.270

CORNELIUS. E. (1972) Increased incidence of lymphomas in thymectomised mice - evidence for an immunological theory of ageing. Experientia,;28, 459.

DACIE. J.V. (1962) The haemolytic anaemias - congenital and acquired Part II. The autoimmune haemolytic anaemias Grune and Stratton. N.Y.

DAUSSET. J. and COLOMBANI. J. (1959) The serology and the prognosis of 128 cases of autoimmune haemolytic anaemia Blood.; 14, 1280

DAVENPORT. F.M., HENNESSY. A.V. and FRANCIS. T. (1953) Epidemiologic significance of age distribution of antibody to antigenic variants of influenza viruses.; J. Exp. Med. 98, 641.

DAVIES. A.J.S., CARTER. R.L., LEUCHARS. E. and WALLIS. V. (1969) The morphology of immune reactions in normal, thymectomised and reconstituted mice II. The response to oxazolone. Immunol.; 17, 111.

DAVIS. M.L., UPTON. A.C., COSGROVE. G.E. and SATTERFIELD. L.C. (1968) Effect of age of recipient mice on growth and differentiation of transplanted bone marrow cells. Proc. Soc. Exp. Biol. Med.; 128, 1149.

DAVIS. M.L., UPTON. A.C. and SATTERFIELD. L.C. (1971) Growth and senescence of the bone marrow stem cell pool in RFM/Un mice. Proc. Soc. Exp. Biol. Med.; 137, 1452.

DELDRIDGE. L. and EVERITT. A.V. (1972) The effect of hypophysectomy and age on the stabilization of labile cross links in collagen. Exp. Gerontol.; 7, 413.

De PETRIS. S. and KARLSBAD. G. (1965) Localisation of antibodies by electron microscopy in developing antibody producing cells. J. Cell Biol.; 26, 759.

Di CARLO. F.G., HAYNES. L.J. and PHILLIPS. G.E. (1963) Effect of Mycobacterium phlei upon reticulo-endothelial system of mice of different ages. Proc. Soc. Exp. Biol. Med. 112, 651.

DICKLER. H.B. and KUNKEL. H.G. (1972) Interaction of aggregated gamma globulin with B lymphocytes. J. Exp. Med.; 136, 191.

DOUGHERTY. J.F., BERLINER. M.L., SCHREBELI. G.L. et al (1964) Hormonal control of lymphatic structure and function. Ann. N.Y. Acad. Sci.; 113, 825.

DRESSER. D.W. (1972) The role of T cells and adjuvant in the immune response of mice to foreign erythrocytes. Eur. J. Immunol.; 2, 50.

DRESSER. D.W. and WORTIS. H.H., (1965) Use of an antiglobulin serum to detect cells producing antibody of low haemolytic efficiency. Nature (Lond); 208, 859.

DRESSER. D.W. and WORTIS. H.H. (1967) Localised haemolysis in gel. 'Handbook of Experimental Immunology' (Ed. D.M.Weir) p.1054. Blackwell Scientific Publications. Oxford.

DUKOR. P., and DIETRICH. F.M. (1967) Chemical suppression of immune responses in thymectomised mice. Int. Arch. Allergy 32, 131.

EDWARDS. G.E., MILLER. R.G. and PHILIPS. R.A. (1970) Differentiation of rosette forming cells from myeloid stem cells J. Immunol.; 105, 719.

FABRIS. N., PIERPAOLI. W. and SORKIN. E. (1971) Hormones and the immunological capacity IV. Restorative effects of developmental hormones or of lymphocytes on the immune deficiency syndrome of the dwarf mouse. Clin. Exp. Immunol. 9, 227.

FABRIS. N., PIERPAOLI. W. and SORKIN. E. (1972) Lymphocytes, Hormones and ageing. Nature;(Lond); 240, 557.

FELDMANN.M. (1972a) Induction of immunity and tolerance *in vitro* by Hapten protein conjugates. The relationship between the degree of hapten conjugation and the immunogenicity of dinitrophenylated polymerized flagellin; J. Exp. Med; 135, 735.

FELDMANN.M. (1972b) Induction of immunity and tolerance to hapten protein conjugates *in vitro* III. Hapten inhibition of antigen binding in immunity and tolerance.; J. Exp. Med; 136, 532.

FELDMANN.M. and BASTEN. A. (1972a) Specific collaboration between T and B lymphocytes across a cell impermeable membrane *in vitro*.; Nature N.B.(Lond); 237, 13.

FELDMANN.M. and BASTEN. A. (1972b) Cell interactions in the immune response *in vitro*. III Specific collaboration across a cell impermeable membrane.; J. Exp. Med.; 136, 49.

FELDMANN.M. and NOSSAL. G.J.V. (1972) Tolerance, enhancement and the regulation of interactions between T and B cells and macrophages.; Transplantation Rev.; 13, 3.

FELDMANN.M. and PALMER. J. (1971) The requirement for macrophages in the secondary immune response to antigens of small and large size.; Immunol. 21, 685.

FELDMANN.M., WAGNER. H., BAKER. A. and HOLMES. M. (1972) Humoral and cell mediated responses *in vitro* of spleen cells from mice with thymic aplasia (Nude mice).; Aus. J. Exp. Biol. Med. Sci.; 50, 651.

FINK. C.W., MILLER. W.F., DORWARD. B. and LOSPALLUTO. J. (1962) The formation of macroglobulins antibodies. II Studies on neonatal infants and older children.; J. Clin. Invest.; 41, 1422.

FRANZL. R.E. (1962) Immunogenic subcellular particles obtained from spleens of antigen injected mice. Nature (Lond); 195, 457.

FRIEDBERGER. E., BOCK. G. and FURSTENHEIM. A. (1929) Zur normalantikorperkurve des menschen durch die verschiedenen lebensalter und ihre beduntung fur die erklarung der hautteste (Schick, Dick).; Z. Immunitatsforsch; 64, 294. English transpation of summary.

GERSHON. R.K., COHEN. P., HENCIN. R., and LIEBHABER. S.A. (1972) Suppressor T cells.; J. Immunol. 108, 586.

GERSHON. R.K. and KONDO. K. (1970) Cell interactions in the induction of tolerance: the role of thymic lymphocytes.; Immunol.; 18, 723.

GERSHON. R.K. and KONDO. K. (1971) Infectious immunological tolerance.; Immunol. 21, 903.

GERSHON. R.K. and PAUL. W.E. (1971) Effect of thymus derived lymphocytes on the amount and affinity of anti-hapten antibody.; J. Immunol.; 106, 872.

GINGOLD. N., CAMPEANO. S. and PODHORSCHI. A. (1958) L'aspect hematologique de la viellesse. V. Facteurs de groupe sangvin et problemes d'immunite.; Sanguin; 29, 327

GOLDSTEIN. S. The biology of ageing.;N. Eng. J. Med.;285, 1120, (1971).

GORCZYNSKI. R.M., MILLER. R.G. and PHILLIPS. R.A. (1972) Initiation of antibody production to sheep erythrocytes *in vitro*. Replacement of the requirement for T cells with a cell free factor isolated from cultures of lymphoid cells.; J. Immunol.; 108, 547.

GORDON. H.A. (1959) Morphological andphysiological characterization of germfree life.; Ann. N.Y. Acad. Sci.; 78, 208.

GOWANS. J.L. and McGREGOR. D.D. (1965) The immunological activities of lymphocytes.; Prog. Allergy; 9, 1.

GOULLET. P., and KAUFMANN. H. (1964/5) Etudes sur la response immunitaire selon L'âge chez le rat.; Gerontologia.; 10, 76.

HALL. D.A. (1972) Theories of ageing - extracellular aspects.; Proc. Roy. Soc. Med.; 65, 671.

HANNA. M.G.Jr. (1965) Germinal centre changes and plasma cell reaction during the primary immune response.; Int. Arch. Allergy.; 26, 230.

HANNA. M.G.Jr., FRANCIS. M.W. and PETERS. L.C. (1968) Localisation of ^{125}I labelled antigen in germinal centres of mouse spleen. Effects of competitive injection of specific or non-cross reacting antigen.; Immunol.; 15, 75.

HANNA. M.G.Jr., NETTESHEIM. P., OGDEN. L.,and MAKINODAN. T. (1967) Reduced immune potential of aged mice. Significance of morphologic changes in lymphatic tissue.; Proc. Soc. Exp. Biol. Med.; 125, 882.

HANNA. M.G.Jr., NETTESHEIM. P. and PETERS. L.C. (1971b) Evidence of functional microenvironments in lymphatic tissue response to antigen.; Nature N.B. (Lond) 232, 204.

HANNA. M.G.Jr., NETTESHEIM. P. and SNODGRASS. M.J. (1971a) Decreasing immune competence and development of reticulum cell sarcoma in lymphatic tissues of aged mice. J. Nat. Canc. Inst.; 46, 809.

HANNA. M.G.Jr., SWARTZENDRUBER. D.C. and CONGDON. C.C. (1966) Morphologic changes in spleen lymphatic tissues during antibody production.; Exp. Mol. Path.; Supp. 3, 75.

HARRISON. B.J. and HALLIDAY. R. (1967) Senescence and the fidelity of protein synthesis in Drosophila.; Nature (Lond) 213, 990.

HARRISON. D.E. (1972) Normal function of transplanted mouse erythrocyte precursors for 21 months beyond donor life spans.; Nature N.B. (lond); 237, 220.

HAYFLICK. L. (1965) The limited in vitro life time of human diploid cell strains.; Exp. Cell. Res.; 37, 614.

HENNESSY. A.V., DAVENPORT. F.M. and FRANCIS. T. (1955) Studies of antibodies to strains of influenza virus in persons of different ages in sera collected in post-epidemic period; J. Immunol.; 75, 401.

HORI. Y., PERKINS. E. and HALSALL. M.N. (1973) Decline in phytohemagglutinin responsiveness of spleen cells from ageing mice.; Proc. Soc. Exp. Biol. Med (N.Y.); 144, 48.

HOWARD. J.G., CHRISTIE. G.H., COURTENAY. B.M., LEUCHARS. E. and DAVIES. A.J.S. (1971a) Studies on immunological paralysis VI Thymic independence of tolerance and immunity to type III pneumococcal polysaccharide.; Cell. Immunol.; 2, 614

HOWARD. J.G., CHRISTIE. G.H., and COURTNAY. B.M. (1971b) Studies on Immunological paralysis IV. The relative contributions of continuous antibody production and central inhibition to paralysis with type III pneumococcal plysaccharide.; Proc. Roy. Soc. (Lond)B; 178, 417

HUEMER. R.P. (1967) Autoimmune meahanisms in normal and pathological physiology. Am. Acad. Opthal. Otolaryng. Trans.; 71, 46.

HUMPHREY. J.H. and DOURMASHKIN. R.R. (1965) Electron microscope studies of immune cell lysis.; Complement (Ed. G.E.W. Wolstenholme and J. Knight) p. 175, Churchill, London.

HUMPHREY. J.H. and FRANK. M.M. (1967) The localisation of non-microbiol antigens in the draining lymph nodes of normal, tolerant and primed animals. Immunol.; 13, 87.

HUMPHREY. J.H., PARROTT. D.M.V., and EAST. J. (1964) Studies on globulin and antibody production in mice thymectomised at birth.; Immunol., 7, 419.

ISHIDATE. M. and METCALF. D. (1963) The pattern of lymphopoiesis in the mouse and thymus after cortisone administration or adrenalectomy.; Aus. J. Exp. Biol. Med. Sci.; 41, 637.

JANKOVIC. B.D. and ISAKOVIC. K. (1966) Antibody production in bursectomised chickens given repeated injections of antigen.; Nature (Lond); 211, 202.

JANKOVIC. B.D., ISAVENSKI. M., MILOSEVIC. D. and POPESKOVIC. L. (1963) Nature (Lond); 198, 298.

JANKOVIC. B.D., WAKSMAN. B.H. and ARNASON. B.G. (1962) Role of the thymus in immune reactions of rats. I. Theimmunologic response to bovine serum albumin (antibody formation, Arthus reactivity and delayed hypersensitivity) in rats thymectomised or splenectomised at various times after birth.; J. Exp. Med.; 116, 159.

JANOSSY. G. and GREAVES. M.F. (1972) Lymphocyte activation. II Discriminating stimulation of lymphocyte subpopulations

by phytomitogens and heterologous antilymphocyte serum. Clin. Exp. Immunol.; 10, 525.

JARVIC. L.F. (1965) Chromosomal changes and ageing. In, contributions to the psychology of ageing (Ed. R. Kastenbaum) Springer, N.Y. p.87.

JERNE. N.K., NORDIN. A.E. and HENRY. C. (1963) The agar plaque technique for recognising antibody forming cells,in 'Cell bound antibodies' p. 109. Wistar Institute press.

KAPLAN. M.H., COONS. A.H. and DEANE. H.W. (1950) Localisation of antigen in tissue cells III. Cellular distribution of pneumococcal polysaccharides types II and III in the mouse; J. Exp. Med.; 91, 15.

KERBEL. R.S. and EIDINGER. D. (1972) Enhanced immune responsiveness to a thymus independent antigen early after adult thymectomy: evidence for short lived inhibitory thymus-derived cells.; Eur. J. Immunol.; 2, 114.

KISHIMOTO. S., TSUYUGOCHI. I. and YAMAMURA. Y. (1969) Immune responses in aged mice.; Clin. Exp. Immunol.; 5, 525.

KISHIMOTO. S., SHOZO. S. and YAMAMURA. Y. (1973) Immune response in aged mice. Change in cell mediated immunity with ageing Transplantation; 15, 455.

KOLODNY. M. (1939) Studies of age resistance against Trypanosoma infections. I. The resistance of rats of different ages to infection with Trypanosoma cruzi; Am. J. Hyg.; 29, C13.

KROHN. P.L. (1961) The vigour of the homograft reaction in old mice.; Gerontologia; 5, 182.

KROHN. P.L. (1962) Review lectures on senescence II. Heterochronic transplantations in the study of ageing. Proc. Roy. Soc 157,(B) 128.

KOMURO. K. and BOYSE. E.A. (1973a) *In vitro* demonstration of thymic hormone in the mouse by conversion of precursor cells into lymphocytes. Lancet; 1, 740.

KOMURO. K. and BOYSE. E.A. (1973b) Induction of T lymphocytes from precursor cells *in vitro* by a product of the thymus. J. Exp. Med.; 138, 479.

LANDAUER. W. and LANDAUER. A.B. (1931) Chick mortality and sex ratios in the domestic fowl. Am. Naturalist. 65, 492.

LEGGE. J.S. and AUSTIN. C.M. (1968) Antigen localisation and the immune response as a function of age. Aus. J. Exp. Biol. Med. Sci.; 46, 361.

LEVINE. B.B. and BENACERRAF. B. (1964) Studies on antigenicity. The relationship between *in vivo* and *in vitro* enzymatic degradability of Hapten-polylysine conjugates and their antigenicity in guinea pigs. J. Exp. Med.; 120, 955.

LEWI. S. (1968) Studies on the ABO system isoantibodies and the effects of stimulation by blood group substances. Gerontologia; 14, 160.

LIND. P.E. (1970) Influence of neonatal thymectomy on antibody production in the Lewis rat. Int. Arch. Allergy.; 37, 258.

MAKINODAN. T., CHINO. F., LEVER. W.E. and BREWEN. B.S. (1971a) The immune system of mice reared in clean and dirty conventional animal farms II. Primary antibody forming activity of young and old mice with long life-spans. J. Gerontology; 26, 508.

MAKINODAN. T., CHINO. F., LEVER. W.E. and BREWEN. B.S. (1971b) The immune systems of mice reared in clean and in dirty conventional animal farms III. Ability of old mice to be

sensitised to undergo a secondary antibody response. J. Gerontology.; 26, 515.

MAKINODAN. T., PERKINS. E.H., and CHEN. M.G. (1971c) Immunologic activity of the aged. Adv. Gerontol. Res.; 3, 171.

MAKINODAN .T. and PETERSON. W.J. (1964) Growth and senescence of the primary antibody potential of the spleen. J. Immunol; 93, 886.

MAKINODAN. T. and PETERSON. W.J. (1966a) The primary antibody forming capacity of spleen cells as a function of age. Dev. Biol.; 14, 96.

MAKINODAN. T. and PETERSON. W.J. (1966b) The secondary antibody forming capacity of spleen cells as a function of age - its significance to senescence. Dev. Biol.; 14, 112.

MATHIES. M., LIPPS. L., SMITH. G.S. and WALFORD. R.L. (1973) Age related decline in response to phytohaemagglutinin and pokeweed mitogen by spleen cells from hamsters and a long-lived mouse strain. J. Gerontology.; 28, 425.

MATTER. A., LISOWSKA-BERNSTEIN. B., RYSER. S.E., LAMELIA. J.P. and VASSALI. P. (1972) Mouse thymus independent and thymus dependent lymphoid cells. II Ultrastructural studies. J. Exp. Med.; 136, 1008.

McDEVITT. H.O., ASKONAS. B.A. and HUMPHREY. J.H. (1966) The localisation of antigen in relation to specific antibody produced cells. I. Use of synthetic Polypeptide ((T-G)-A-L) labelled with ^{125}I.; Immunol.; 11, 337

McMASTER. P.D. and FRANZL. R.E. (1955) The effects of adrenocortical steroids upon antibody production. Metab.; 10, 990

MELLORS. R.C. and BRZOSKO. W.J. (1962) Studies in molecular pathology I. Localisation and pathogenic role of heterologous immune complexes. J. Exp. Med.; 115, 891.

METCALF. D., MOULDS. R. and PIKE. B. (1967) Influence of the spleen and thymus on immune responses in ageing mice. Clin. Exp. Immunol.; 2, 109.

METCALF. D. and STEVENS. S. (1972) Influence of age and antigenic stimulation on granulocyte and macrophage progenitor cells in the mouse spleen. Cell Tissue Kinet.; 5, 433.

MICKLEM. H.S. (1972) Cell proliferation in haemopoietic spleen colonies of mice. Difference between colonies derived from injected adult bone marrow and foetal liver cells. Cell. Tissue Kinet.; 5, 159.

MICKLEM. H.S., FORD. C.E., EVANS. E.P., OGDEN. D.A. and PAPWORTH. D.S. (1972) Competitive in vivo proliferation of foetal and adult haemopoietic cells in lethally irradiated mice. J. Cell. Physiol.; 79, 293.

MICKLEM. H.S., OGDEN. D.A. and PAYNE. A.C. (1973). Ageing, haemopoietic stem cells and immunity. In haemopoietic stem cells. Ciba Foundation Symposium No. 13 (new series) p. 285. Assoc. Sci. Publishers. Amsterdam.

MILLER. J.F.A.P. (1962) Immunological significance of the thymus of the adult animal. Nature (Lond); 195, 1318.

MILLER. J.F.A.P. (1962) The role of the thymus in transplantat--ion immunity. Ann. N.Y. Acad. Sci.; 99, 340.

MILLER. J.F.A.P. (1962b) Effect of neonatal thymectomy on the immunological responsiveness of the mouse. Proc. Roy. Soc. (B), 156, 415.

MILLER. J.F.A.P. (1963) Immunity and the thymus. Lancet (i), 43.

MILLER. J.F.A.P., SPRENT. J., BASTEN. A., WARNER. N.L., BREITNER. J.C., ROWLAND. G., HAMILTON. J., SILVER. H. and MARTIN. W.S. (1971) Cell to cell interaction in the immune response VII. Requirement for differentiation of thymus derived cells. J. Exp. Med.; 134, 1266.

MILLER. J.F.A.P., OSOBA. D. and DUKOR. P.(1964) A humoral thymus mechanism responsible for immunologic maturation. Ann. N.Y. Acad. Sci.; 124, 95.

MISHELL. R.I. and DUTTON. R.W. (1967) Immunisation of dissociated spleen cells cultures from normal mice. J. Exp. Med.; 126, 423.

MITCHELL. J., HOLMES. M.C., PYE. J. and NOSSAL. G.J.V. (1972) Antigens in immunity XVIII. Antigen localisation in congenitally athymic 'nude' mice. Aus. J. Exp. Biol. Med. Sci. 50, 637.

MITCHISON. N.A. (1969) The immunogenic capacity of antigen taken up by peritoneal exudate cells. Immunol.; 16, 1.

MIYAKAWA. M. (1959) The lymphatic system of germ-free guinea pigs. Proc. Natl. Acad. Sci. (U.S.); 78, 221

MOLLER. G. and WIGZELL. H. (1965) Antibody synthesis at the cellular level. Antibody induced suppression of 19S and 7S antibody response. J. Exp. Med.; 121, 969.

MOORE. M.A. and OWEN. J.J. (1966) Experimental studies on the development of the bursa of Fabricius. Dev. Biol.; 14, 40.

MOORE. M.A. and OWEN. J.J. (1967) Experimental studies on the development of the thymus. J. Exp. Med.; 126, 715.

MULLER EBERHARD. H.J. (1961) Serum factors which interact with aggregated gamma globulin. Proc. 10th. Int. Congr. Rheum. Diseases.; 2, 791.

NORDLING. S., ANDERSON. L.C. and HAYRY. P. (1972) separation of T and B lymphocytes by preparative cell electrophoresis.

NOSSAL. G.J.V. (1959) Antibody production by single cells III. Histology of antibody production. Brit. J. Exp. Path. 40, 301.

NOSSAL. G.J.V., ABBOT. A. and MITCHELL. J. (1968a) Antigens in immunity XIV. Electron microscope radioautographic studies of antigen capture in the lymph node medulla. J. Exp. Med.; 127, 263.

NOSSAL. G.J.V., ABBOT. A. and MITCHELL. J. (1968b) Antigens in immunity XV. Ultrastructural features of antigen capture in primary and secondary lymphoid follicles. J. Exp. Med.; 127, 277.

NOSSAL. G.J.V., ADA. G.L. and AUSTIN. C.M. (1964a) Antigens in immunity II. Immunogenic properties of flagella, polymerised flagellin and flagella in the primary response. Aus. J. Exp. Biol. Med. Sci.; 42, 283.

NOSSAL. G.J.V., ADA. G.L. and AUSTIN. C.M. (1964b) Antigens in immunity IV. Cellular localisation of ^{125}I and ^{131}I labelled flagella in lymph nodes. Aus. J. Exp. Biol. Med. Sci.; 42, 311.

NOSSAL. G.J.V., ADA. G.L. and AUSTIN. C.M. (1965) Antigens in imunity VIII. Localisation of ^{125}I labelled antigens in the secondary response. Immunol.; 9, 349.

NOSSAL. G.J.V. and ADA. G.L. (1971) Antigen, lymphoid cells and the immune response. Acad. Press Inc. N.Y. p. 107.

NOSSAL. G.J.V., AUSTIN. C.M., PYE. J. and ABBOT. A. Antigens in immunity XII. Antigen trapping in the spleen.; Int. Arch. Allergy.; 29, 368. (1966).

NOSSAL. G.J.V., CUNNINGHAM. A., MITCHELL. G. and MILLER. J.F.A.P. (1969) Cell to cell interaction in the immune response III Chromosomal marker analysis of single antibody forming cells in reconstituted, irradiated and thymectomised mice. J. Exp. Med.; 128, 839.

NOWELL. P.C., HIRSCH. B.E., FOX. D.H. and WILSON. D.B. (19) Evidence for the existence of multipotential lymph-haemopoietic stem cells in the adult rat. J. Cell. Physiol. 75, 151.

OBERHOFFER. G., PROKOP. O. and SCHUBERTH. G. (1957) Untersuchungen zu der frage lebensalter und immunis--ierungseffekt. Z. Immunitatsforsch.; 114, 10. English translation of summary.

ORGELL. L.E. (1963) The maintainance of the accuracy of protein synthesis and its relevence to ageing. P.N.A.S.; 49, 517.

OSTERLAND. C.K., HARBOE. M. and KUNKEL. H.C. (1963) Anti-gamma-globulin factors in human sera revealed by enzymatic splitting of anti-Rh antibodies. Vox. Sang.; 8, 133.

OWEN. J.J.T. and RAFF. M.C. (1970) Studies on the differentiation of thymus derived lymphocytes. J. Exp. Med.; 132, 1216.

OWEN. J.J.T. and RITTER. M. (1969) Tissue interaction in the development of thymus lymphocytes. J. Exp. Med.; 129, 431.

PANTELOURIS. E.M. (1972) Thymic involution and ageing. An hypothesis. Exp. Gerontol.; 7, 73.

PARROTT. D.M.V. and DeSOUSA. M.A.B. (1966) Changes in thymus-dependent areas of lymph nodes after immunologic stimulat--ion. Nature (Lond); 212, 1316.

PAUL. J.R. and BUNNELL. W.W. (1932) The presence of heterophile antibodies in infectious mononucleosis. Am. J. Med. Sci.; 183, 20.

PEPPER. F.J. (1961) The effect of age, pregnancy and lactation on the thymus gland and lymph nodes of the mouse. J. Endocrinol.; 22, 335.

PERKINS. E.H. and MAKINODAN. T. (1965) The suppressive role of mouse peritoneal phagocytes in agglutinin responses. J. Immunol.; 94, 765.

PERKINS. E.H. and MAKINODAN. T. (1972) Nature of humoral immunologic deficiences of the aged. Ist Rocky Mt. Symp. on Ageing.

PFOCH. M. (1971) Comparative electron microscopic investigations of the entodermal reticulum cells in the thymus of newborn and aged Wistar rats. Anat. Inst. Univ. Kiel.; 114, 271. English translation of summary.

PIERPAOLI. W. (1972) A thymus dependent function of the adrenal cortex and its relation to immunity. Experientia.; 28, 851.

PIEPAOLI. W., FABRIS. N., and SORKIN. E. (1970) Developmental hormones and immunological maturation. In'Hormones and the immune response'; Ciba foundn. study group No. 36 (Ed. G.E.W.Wolstenholme and J. Knight) P. 126. Churchill, London.

PIERPAOLI. W. and SORKIN. E. (1968) Hormones and immunological capacity. 1. Effect of heterologous anti-growth (ASTH) antiserum on thymus and peripheral lymphatic tissues in mice. Induction of wasting syndrome. J. Immunol.; 101 1036.

PIERPAOLI. W. and SORKIN. E. (1972) Hormones, thymus and lymphocyte functions. Experientia.; 28, 1385.

PIEZ. K.A. (1969) Chemistry of collagen and elastin and biosynthesis of covalent cross-links. Thule Int. Symp. 'Ageing of connective tissues and skeletal tissues' (Ed. A. Engel and T. Larsonn) Stockholm.

PISCIOTTA. A.V., WESTRING. D.W., DePREY. C. et al (1967) Mitogenic effect of phytohaemagglutinin at different ages Nature (Lond); 215, 193.

PLAYFAIR. J.H. and PURVES. E.C. (1971a) Antibody formation by bone marrow cells in irradiated mice 1. Thymus dependent and thymus independent responses to sheep erythrocytes. Immunol.; 21, 113.

PLAYFAIR. J.H. and PURVES. E.C. (1971b) Separate thymus dependent and thymus independent antibody forming cell precursors. Nature N.B. (Lond); 231, 149.

PLOTZ. P.H., TALAL. N. and ASOFSKY. R. (1968) Assignment of direct and facilitated haemolytic plaques in mice to specific immunoglobulin classes. J. Immunol.; 100, 744.

POLLIACK. A., LAMPEN. N., CLARKSON. B.D. and DeHAVEN. E. (1973) Identification of human T and B lymphocytes by scanning electron microscopy. J. Exp. Med.; 138, 607.

PRICE . G.B. and MAKINODAN. T. (1972a) Immunologic deficiencies in senescence. I. Characterisation of intrinsic deficiencies J. Immunol.; 108, 403.

PRICE. G.B. and MAKINODAN. T. (1972b) Immunologic deficiencies in senescence. II. Characterisation of extrinsic deficien--cies. J. Immunol.; 108, 413.

PRITCHARD. H. and MICKLEM. H.S. (1972) Immune responses in congenitally thymusless mice. 1. Absence of response to oxazolone. Clin. Exp. Immunol.; 10, 151.

PRITCHARD. H., RIDDAWAY. M.J. and MICKLEM. H.S. (1973) Immune responses in congenitally thymusless mice. II Qualitative studies of serum immunoglobulins. The antibody response to sheep erythrocytes and th e effect of thymus allografting. Clin. Exp. Immunol.; 13, 125

PROUKAKIS. C. and LINDOP. P.J. (1967) Age dependence of radiation sensitivity of haemopoietic cells in the mouse. Nature (Lond); 215, 655.

RABELLINO. E., COHEN. S., GREY. H.M. and UNANUE. R.E. (1971) Immunoglobulins on the surface of lymphocytes. 1. Distribution and quantitation. J. Exp. Med.; 133, 156.

RAFF. M.C. (1969) Theta isoantigen as a marker of thymus derived lymphocytes in mice. Nature (Lond); 224, 378.

RAFF. M.C. (1971) Surface antigenic markers for distinguishing T and B lymphocytes in mice. Tranplant. Rev.; 6, 52.

RAM. J.S. (1967) Ageing and immunological phenomena - a review. J. Gerontol.; 22, 92.

RAU. P. and RAU. N. (1914) Longevity in saturnid moths and its relation to the function of reproduction. Trans. Acad. Sci. St Louis.; 23; 1.

REIF. A.E. and ALLEN. J.M.V. (1964) The AKR thymic antigen and its distribution in leukemias and nervous tissues. J. Exp. Med.; 120, 413.

ROBINSON. W.D. (1963) Rheumatoid arthritis. Cited in Walford 1969.

ROITT. I.M., GREAVES. M.F., TORRIGIANI. C., BROSTOFF. J. and

PLAYFAIR. J.H. (1969) The cellular basis of immunological responses. Lancet (ii), 367.

ROWLEY. D.A. and FITCH. F.W. (1965) The mechanism of tolerance produced in rats to sheep erythrocytes. II. Plaque forming cells and antibody response to single and multiple injections of antigen beginning at birth. J. Exp. Med. 121, 683.

RUTH. R.F. (1960) Ontogeny of the blood cells. Fed. Proc.; 19, 579.

SABIN. F.R. (1939) Cellular reaction to a dye protein with a concept of the mechanism of antibody formation. J. Exp. Med.; 70, 67.

SANTISTEBAN. G.A. (1960) The growth and involution of lymphatic tissue and its inter-relationships to ageing and to the growth of the adrenal glands and sex organs in CBA mice Anat. Rec.; 136, 117.

SANTISTEBAN. G.A., AND DOUGHERTY. T.F. (1954) Comparison of the influences of adrenocortical hormones on the growth and involution of lymphatic organs. Endocrinology.; 54, 130.

SCHEID. M., BOYSE. E.A., CARWELL. L. and OLD. J. (1972) Serologically demonstrable alloantigens of mouse epidermal cells. J. Exp. Med.; 135, 938.

SCHLESINGER. M. and YRON. I. (1970) Serologic demonstration of a thymus dependent population in lymph nodes. J. Immunol.; 104, 798.

SCHRADER. J.W. (1973) Regulation of the immune response by IgM antibody: paradoxical suppression of the *in vitro* immune response to sheep erthrocytes by passive IgM. Aus. J. Exp. Biol. Med. Sci.; 51, 333.

SCHRADER. J.W. and FELDMAN. M. (1972) The mechanism of antigenic competition. 1. The macrophage as a site of a reversible block of T - B lymphocyte collaboration. Eur. J. Immunol.; 3, 711.

SCHWARTZ. R.S., RYDER. R.J.W. andGOTTLIEB. A.A. (1970) Macrophages and antibody synthesis . Prog. Allergy.; 14, 81

SCHWICK. H.G. and BECKER. W. (1969) Humoral antibodies in older humans. In Current problems in immunology.(Ed. Westphal. O., Beck. H.E. and Grundman. E).p. 253. Springer Verlag, Berlin.

SHEWELL. J. and LONG. D.A. (1956) A species difference with regard to the effect of cortisone on body weight, gamma globulin and circulating antitoxin levels. J. Hyg.; 54, 452.

SHORTMAN. K. and PALMER. J. (1971) The requirement for macrophages in the immune response in vitro, Cell. Immunol.; 2, 399.

SILVERSTEIN. A.M. (1964) Ontogeny of the immune response; Science; 144, 1423.

SILVERSTEIN. A.M., PRENDERGAST. R.A. and KRAMER. K.L. (1964) Foetal response to antigenic stimulus IV. Rejection of skin homografts by the foetal lamb.

SILVERSTEIN. A.M., PRENDERGAST. R.A. and PARSHALL. C.J.Jr. (1970) Cellular kinetics of the antibody response by the foetal resus monkey. J. Immunol.; 104, 269.

SILVERSTEIN. A.M., THORBECKE. G.J., KRANER. K.L. and LUKES. R.J. (1963) Foetal responses to antigenic stimulus III Gamma globulin production in normal and stimulated foetal lambs. J. Immunol.; 91, 384.

SIMMONOVITCH. L., McCULLOCH. E.A. and TILL. J.E. (1964) Decline in colony forming ability of marrow cells subjected to serial transplantation into irradiated mice J. Cell Comp. Physiol.; 64, 23.

SIMONSEN. M. (1960) On the aquisition of tolerance by adult cells. Ann. N.Y. Acad. Sci.; 87, 382.

STERZL J. and MANDEL. L. (1964) Estimation of the inductive phase of antibody formation by plaque techniques. Folia Microbiol (Praha); 9, 173.

STERZL. J. and RIHA. (1965) Detection of cells producing 7S antibodies by the plaque technique. Nature (Lond); 208,858.

STERZL. J. and SILVERSTEIN. A.M. (1966) Developmental aspects of immunity. Adv. Immunol.; 6, 19.

STJERNSWARD. J. (1966) Age dependent tumour-host barrier and effect of carcinogen-induced immunodepression on rejection of isografted methylcholanthrene - induced sarcomas.; J. Nat. Canc. Inst.; 37, 505.

STROM. T.B., LEVIN. B.S., DOURMANN. G.J. and POLLAK. V.E.(1968) The effects of antithymocyte globulin in NZB x NZW F_1 mice Proc. Soc. Exp. Biol. Med.; 129, 261.

SUSSMAN. M.D. (1973) Ageing of connective tissue: physiological properties of healing wounds in young and old rats. Am. J. Physiol.; 224, 1167.

TAYLOR. R.B. (1969) Cellular co-operation in the antibody responses of mice to two serum albumins: specific function of thymus cells. Transplant. Rev.; 1, 114.

TAYLOR. R.B., WORTIS. H.H. and DRESSER. D.W. (1967) In 'The lymphocyte in immunology and haemopoiesis' Ed. J.M.Yoffey, Edward Arnold, London.

TAYLOR. R.B., and WORTIS. H.H. (1968) Thymus dependence of antibody response. Variation with dose of antigen and class of antibody.; Nature (Lond); 220, 927.

TELLER. M.N., CURLETT. W. and KUBISEK. M.L. (1964) Ageing and carcinogenesis. 1. Immunity to tumour and skin grafts. J. Nat. Canc. Inst.; 33, 649.

THOMSEN. O. and KETTEL. K. (1929) Z. Immunitatsforsch.; 63, 67. English translation of summary.

THUNG. P.J. (1966) Note on the thymic epithelium in senile mice Exp. Gerontol.; 3, 19.

THORBECKE. G.J. (1959) Some histological and functional aspects of lymphoid tissue in germ free animals. 1. Morphological studies.; Ann. N.Y. Acad. Sci.; 78, 237.

THORBECKE. G.J. and HURLIMANN (1965) Les nodules lymphoides secondaires. Nouv. Rev. Franc. Haemat.; 5, 385.

TORRIGIANI. G. (1972) Qualitative estimation of antibody in the immunoglobulin classes of the mouse.II. Thymic dependence of the different classes. J. Immunol.; 108, 161.

TRENTIN. J.J., WOLF. N., CHENG. V., FAHLBERG. W., WEISS. D., and BONHAGE. R. (1967) Antibody production by mice repopulated with limited numbers of clones of lymphoid cell precursors. J. Immunol.; 97, 1326.

UNANUE. E.R. and ASKONAS. B.A. (1968) The immune response of mice to antigen in macrophages. Immunol.; 15, 287.

UNANUE. E.R. and CEROTTINI. J.C. (1970) The immunogenicity of antigen bound to plasma membrane of macrophages. J. Exp. Med.; 131, 711.

WAKEFIELD. J.D. and THORBECKE. G.J. (1968a) Relationship of germinal centres in lymphoid tissues to immunological memory. 1. Evidence for the formation of small lymphocytes upon transfer of primed splenic white pulp to syngeneic mice. J. Exp. Med.; 128, 153.

(1968b) II. the detection of primed cells and their progeny proliferation upon cell transfer to lethally irradiated syngeneic mice. J. Exp. Med. ; 128, 171.

WAKSMAN. B.H. and ARNASON. B.G., and JANKOVIC. B.D. (1962) Role of the thymus in immune reactions in rats. III. Changes in the lymphoid organs of thymectomised rats. J. Exp. Med.; 116, 187.

WALDORF. D.S., WILLIAMS. R.F. and DECKER. J.L. (1968) Impaired delayed hypersensitivity in an ageing population. Association with anti-nuclear reactivity and rheumatoid factors.; J. Am. Med. Assocn.; 203, 831.

WALFORD. R.L. (1962) Autoimmunity and ageing. J. Gerontol.; 17 , 281.

WALFORD. R.L. (1967) The general immunology of ageing. Adv. Gerontol. Res.; 2, 159.

WALFORD. R.L. (1969) The immunologic theory of ageing. Munksgaard, Copenhagen.

WARNER. N.L. (1964) The immunological role of different lymphoid organs in the chicken. II The immunolgical competence

of thymic cell suspensions. Aus. J. Exp. Biol. Med. Sci.; 42, 401.

WEAVER. J.A. (1955) Changes induced in the thymus and lymph nodes of the rat by the administration of cortisone and sex hormones and by other procedures. J. Path. Bact.; 69, 133.

WHITE. R.G. (1963)Functional recognition of immunologically competent cells by means of the fluorescent antibody technique. In, The immunologically competent cell Ciba Foundtn. Study Group. No. 16. p. 6.Boston.

WHITE. R.G., FRENCH. I., STARK. J.M., (1967) Germinal centre formation and antigen localisation in malpighian bodies of chicken spleen. In, germinal centres in immune responses. (Ed. H. Cottier, N. Odartchenko, Schindler and Congdon)

WIGZELL. H. and STJERNSWARD. J. (1966) Age dependent rise and fall of immunological activity in the CBA mouse. J. Nat. Canc. Inst.; 37, 573.

WILENS. S.L. and SPROUL. E.E. (1938) Spontaneous cardiovascular disease in the rat. II Lesions of the vascular system.; Am. J. Pathol.; 14, 201.

WILLIAMS. G.M. (1966) ontogeny of the immune response II. Correlation between development of afferent and efferent limbs. J. Exp. Med.; 124 , 57.

WILLIAMS. G.M. and NOSSAL. G.J.V. (1966) Ontogeny of the immune response 1. The development of the follicular trapping mechanism.; J. Exp. Med.; 124, 47.

WIOLAND. M., SABOLOVIC. D. and BRG. D. (1972) Electrophoretic mobilities of T and B cells. Nature N.B. (Lond); 237, 274.

WORTIS. H.H. (1971) Immunological responses of 'nude'mice Clin. Exp. Immunol.; 8, 305.

WORTIS. H.H., TAYLOR. R.B. and DRESSER. D.W. (1966) Antibody production studied by means of the localised haemolysis in gel (LHG) assay. 1. Splenic response of CBA mice to sheep erythrocytes. Immunol.; 11, 603.

WORTIS. H.H., TAYLOR. R.B. and DRESSER. D.W. (1967) Antibody production studied by means of the localised haemolysis in gel (LHG) assay. II assay procedure. Immunol.; 14, 69.

YUHAS. J.M. and STORER. J.B. (1967) The effect of age on two models of radiation death and on haemopoietic cell survival in the mouse. Radiat. Res.; 32, 596.

ZATZ.M , MELLORS.R and LANCE. E.M. (1971) Changes in lymphoid populations of ageing CBA and NZB mice. Clin. Exp. Immunol.; 8, 491.