AGEING AND THE HUMORAL IMMUNE RESPONSE IN MICE

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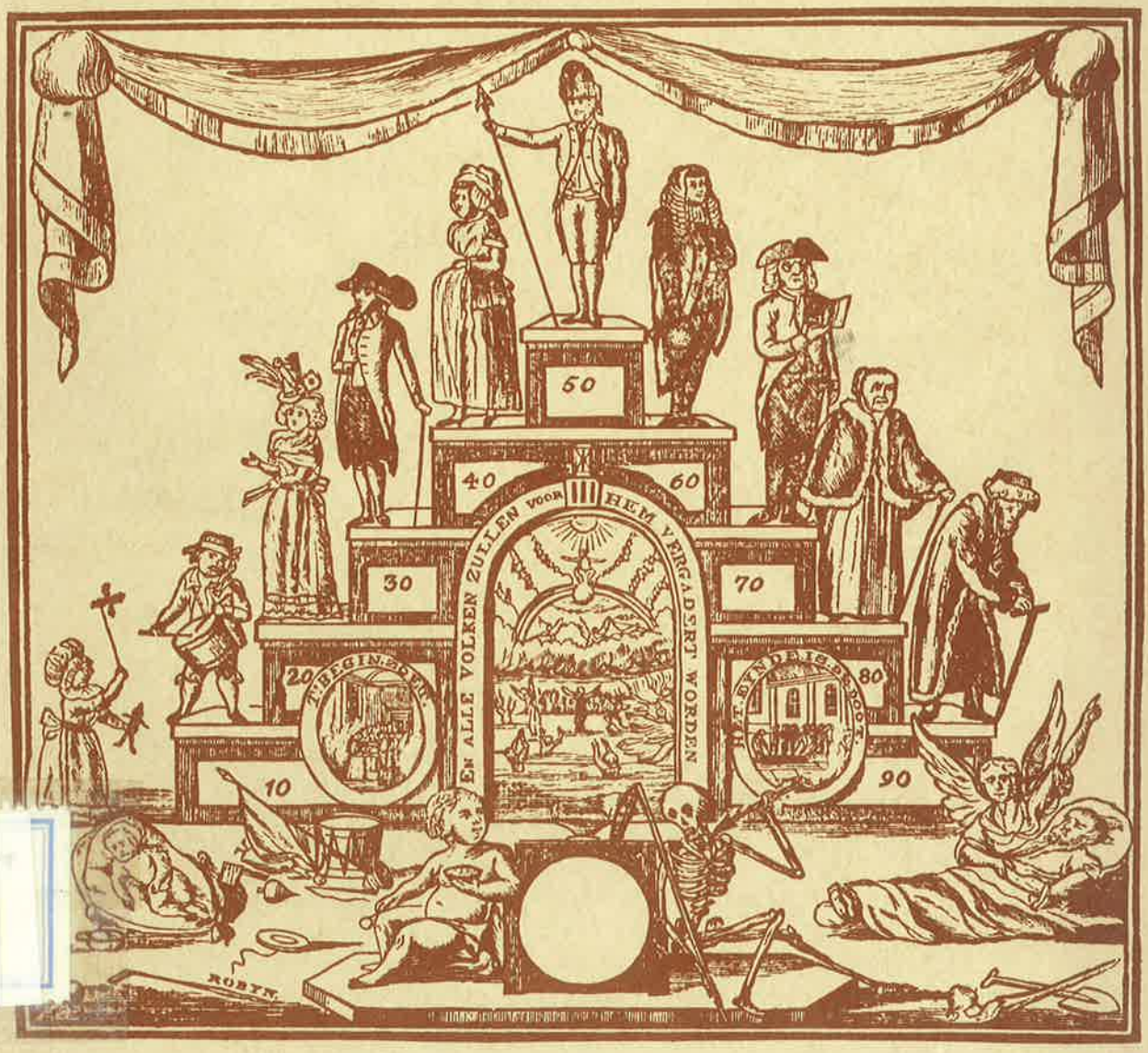

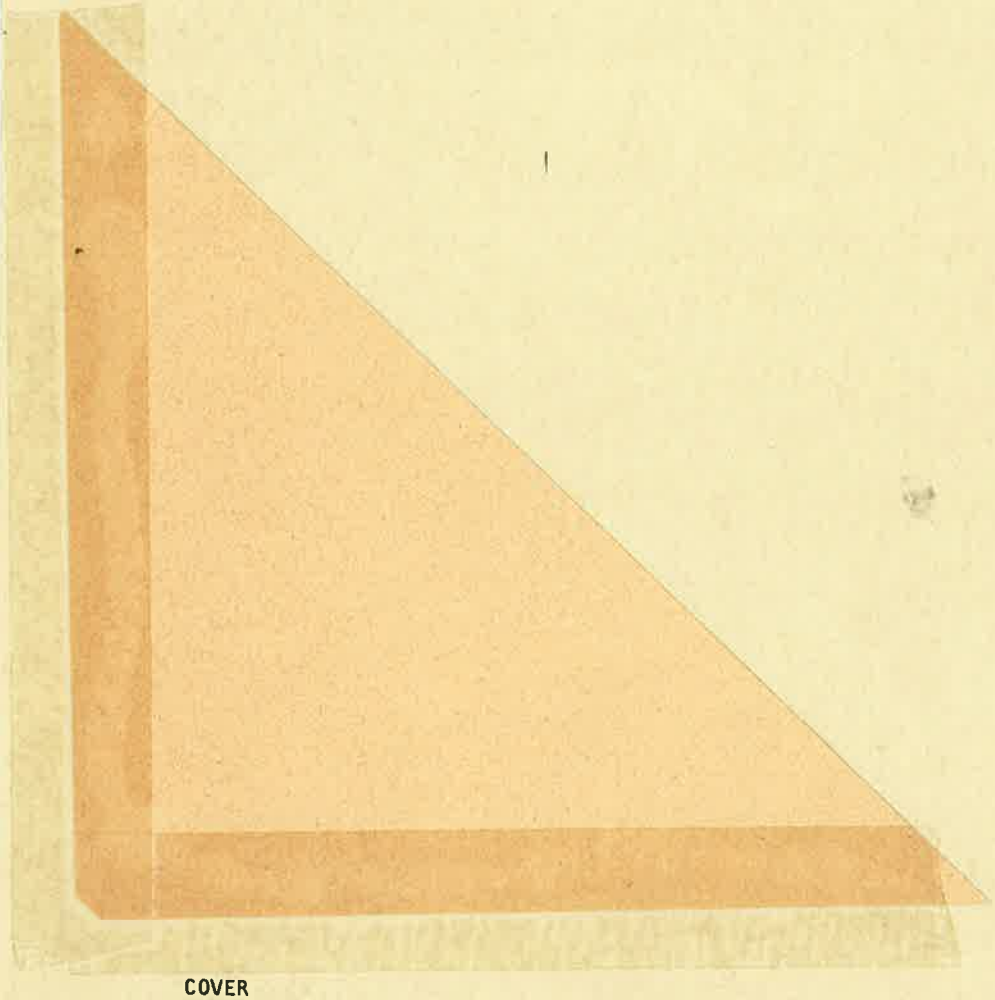

COVER

THE STAIRS OF AGE

A catchpennyprint from a wood-cut signed by Robyn abt. 1820.
Collection Rijksprentenkabinet, Amsterdam.

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CONTENTS

ABBREVIATIONS

BSS	balanced salt solution
C-Ig cells	cells containing cytoplasmic immunoglobulins
ConA	concanavalin A
FCS	foetal calf serum
i.p.	intraperitoneal
i.v.	intravenous
LPS	*E. coli* lipopolysaccharide
2-ME	2-mercaptoethanol
MRBC	mouse red blood cells
NLM	normal litter mates (of nu/nu mice)
PBS	phosphate buffered saline
PFC	plaque forming cell
PHA	phytohaemagglutinin
RRBC	rabbit red blood cells
SD	standard deviation
SF	serum factor
SRBC	sheep red blood cells
SIII	pneumococcal polysaccharide type III
TE	thymic epithelium
TES	thymic epithelial culture supernatant
TF	thymic factor
THF	thymic humoral factor
TL	thymic lymphocytes
TT	total thymus

"B" mice	thymectomized, lethally irradiated and bone marrow reconstituted mice
BC3F1	(C57BL/Cum x C3H/AnfCum)F1
B6CF1	(C57BL/6 x BALB/c)F1
B6C3F1	(C57BL/6 x C3H/An)F1
B6D2F1	(C57BL/6 x DBA/2)F1
CBF1	(C57BL/6J x BALB/cJ)F1
NZB/W	(NZB x NZW)F1

CHAPTER I

INTRODUCTION

A. IMMUNOLOGICAL ASPECTS OF AGEING

The interest of man in the mysteries of ageing has found expression in numerous words and pictures. However, the first attempts to methodically analyze the process of ageing date from the beginning of the twentieth century From that time, multiple theories have been proposed to explain the ageing process. It was not before 1960 that immunology became an integral part of the discipline of gerontology. This interest coincided with the then rapidly developing knowledge of the immune system. The pioneers in that borderland area of gerontology and immunology were Takashi Makinodan and Roy L. Walford. The studies of Walford have resulted in his Immune Theory of Ageing (Walford, 1969). In his opinion, "the key to the etiology of ageing may lie within the discipline of immunology and particularly that part having to do with self recognition" (Quotation - Walford, 1974).

The following are reasons why the role of the immune system in the ageing process has received increasing attention in recent years.

1) Since the immune system is interconnected with most other systems in the body, any alteration in the immune system would affect other systems. Therefore, a great majority of the "diseases of ageing" are thought to be connected either directly or indirectly with abnormalities of the immune system. Delay, reversal, or pre-prevention of the decline in normal immune functions may delay the onset and/or lessen the severity of "diseases of ageing".
2) The thymus, which shows such a striking alteration during ageing, forms a part of the immune system.
3) The current knowledge of the immune system is very comprehensive.
4) The system is greatly amenable to cellular and molecular analysis, which makes it an ideal system to determine alterations during ageing. A variety of in vivo and in vitro methods allows these studies.
5) The possibility of manipulation of the immune system makes it an attractive target for experimental studies.
6) Functional age-related changes in the immune system may present opportunities for fundamental studies in immunology itself (e.g., suppressor T cells, homogeneous immunoglobulins).

The following sections (C-H) will broadly cover the present knowledge of the relationship between the immune system and the ageing process. Section B gives a general introduction to various aspects of the humoral immune response.

B. IMMUNOBIOLOGY OF THE HUMORAL IMMUNE SYSTEM

1. Introduction

Two distinct types of specific immune reactions can be elicited when foreign material enters the body and is recognized as such by the immune system. The first type of reaction, the humoral immune response, is characterized by the synthesis and release of antibodies in the blood and other body fluids. These antibodies act by direct combination with the antigen. In the other type of reaction, the cell-mediated immune response, lymphocytes are the effector cells. This response is sometimes accompanied by the production of locally acting soluble mediators (lymphokines).

Antibody forming cells arise from the bone marrow-derived lymphocytes or B cells In birds, B lymphocytes differentiate from primitive progenitors in the Bursa of Fabricius. This structure is thought to provide a unique inductive stimulus, as the thymus in both birds and mammals is thought to provide a stimulus for the differentiation of thymus-derived or T lymphocytes. A bursal equivalent has not, however, been identified in mammals. Two proposed sites for the origin of B cells are the bone marrow and the gut-associated lymphoid tissue. After the finding of Miller (1961) that neonatal thymectomy impaired the antibody responses to some antigens, extensive evidence has been obtained that, in most cases, T cells are required for B cells to produce an optimal antibody response. Both cell types recognize the antigen but only the B lymphocytes give rise to antibody forming cells. T cells are considered to be helper cells (Claman, Chaperon and Triplett, 1966, and Mitchell and Miller, 1968). These conclusions were drawn from in vivo cell transfer studies in irradiated recipients. After the introduction of methods which permit antibody formation under in vitro conditions, it was demonstrated that a third cell type participates in humoral immune reactions. Accessory cells, such as macrophages, dendritic cells and granulocytes are also involved in the response (Mosier and Coppleson, 1968, and Unanue, 1972). This cell type, which is thought to facilitate the immune response in a

nonspecific manner, is relatively radioresistant and adheres to glass or plastic.

2. Cellular cooperation

The precise mechanisms underlying the interactions between the three cell types described above have not yet been conclusively defined. The development of antisera against B and T cells, which are raised against immunoglobulins identified on the B cells and Thy-1 antigens (formerly called theta) on the T cells, and cell separation techniques using these antisera have offered the possibility to prepare enriched T and B cell populations and to study their interactions. On the basis of the results obtained in those studies, the following models for the cooperation between T cells, B cells and accessory cells have been proposed.

(a) Specific cooperation

The carrier determinants of the antigen are bound by the T cells, so that the haptenic determinants are presented to the hapten-specific B cells. In this way, the T cells concentrate the antigens and an antigen bridge is formed between T and B cells (Rajewsky et al., 1969, and Mitchison, 1971). An alternative model eliminates the need for direct T-B cell bridging. In vitro experiments of Feldmann and Basten (1972) provided evidence that cell cooperation is mediated by soluble specific factors derived from T cells, since specific cell cooperation still took place when T cells and B cells were separated from one another by a cell impermeable membrane. The specific soluble T cell factor attaches cytophilically to the surface of macrophages. The macrophages then present the antigen to the B cells (Feldmann, 1972b). In the opinion of this author, the factor is a T cell-derived IgM like molecule (IgT). Taussig (1974) described another type of specific soluble mediator, a T cell replacing factor. This factor is produced in vitro by primed T cells in the presence of antigen to which the T cells are educated in vivo. It is active in vitro as well as in vivo and it is thought to be a product of the immune response (I) region of the major histocompatibility complex.

(b) Nonspecific cooperation

Evidence for a T-dependent, nonspecific cooperation comes from several studies. Hartmann (1971) and Waldmann, Munro and Hunter (1973) have shown that the response of B cells to SRBC can be increased by the addition of T spleen cells educated to a second non-cross-reacting

antigen to the B cell cultures. Since cell-free supernatants of T cells activated by non-cross-reacting antigens can facilitate the response of B cells to SRBC, this nonspecific cooperation appeared to be mediated by soluble factors (Waldmann and Munro, 1973). Another example of nonspecific cooperation is the activation of B lymphocyte function by the so-called allogeneic effect. Experiments of Katz et al. (1971) revealed that transfer of allogeneic cells which evokes a graft verus host (GvH) reaction obviates the need for helper T cells, provided that the B cells form part of the target cells of the GvH reaction. This effect could be reproduced in vitro and this study resulted in the detection of a factor, the "allogeneic effect factor" (AEF), which can replace helper T cells (Armerding and Katz, 1974b). Like the antigen specific T cell replacing factor of Taussig (1974), this nonspecific factor also appeared to be a product of the I region of the histocompatibility complex in the mouse. The involvement of the I region of the H-2 complex in the immune response has been further substantiated by the demonstration that the T-B cell interaction seems to depend on the compatibility of the interacting cells at the I region and that I region products play an essential role (reviewed by Katz and Armerding, 1976, and Munro and Bright, 1976).

3. Thymus-dependent and thymus-independent responses

Not all antigens require the participation of T cells in order to induce an optimal antibody response. Antigens with a very large number of identical repeating determinants such as polyvinyl pyrrolidone, polysaccharides and polymerized flagellin can bypass the T cell system and stimulate B cells directly. The requirement for T cells in antibody production appears to depend on the way in which the antigenic determinants are presented rather than on the specificity of the antigenic determinant concerned (Feldmann and Basten, 1971, and Feldmann, 1972a). An important observation was that most thymus-independent antigens trigger only an IgM response. From other studies it was also concluded that B cells committed to IgG synthesis require a greater degree of interaction with helper T cells for triggering than do B cells committed to IgM synthesis (Taylor and Wortis, 1968; Wortis, Dresser and Anderson, 1969, and Van Muiswinkel and Van Soest, 1975).

Coutinho et al. (1974) have demonstrated that thymus-independent antigens have more or less B cell mitogenic properties and are able to induce a polyclonal IgM antibody response in the absence of antigens. Therefore, the difference in thymus-dependence between IgM and IgG

synthesis may be explained by a difference in the capacity of the respective B cells to respond to a signal of a mitogen receptor. In this concept, IgG producing cells would need a second "T cell-derived" signal to be activated.

4. Suppressor T cells

So far, the positive side of the interaction between T and B cells has been described. However, over the past few years, a body of evidence has accumulated which indicates that negative interactions may be of equal importance in immunoregulation as are the helper T cells. In particular, T cells have been shown to suppress the immune response in a number of diverse situations (reviewed by Gershon, 1974). Both specific and nonspecific T-dependent suppressor activities have been described. Soluble mediators such as the antigen-specific suppressive T cell factor of Takemori and Tada (1975) are thought to play a role. A further characterization of this factor revealed that it is again a product of the I region of the H-2 complex (Taniguchi, Hayakawa and Tada, 1976). The central question in the understanding of suppressor cell activity is: "Are suppressor cells a distinct population from those cells which exert a helper activity or are T cells to be considered as regulatory cells which can either amplify or suppress the immune response depending on a number of factors such as antigen dose?" This question could be resolved with the use of antisera developed against cell surface antigens, termed Ly, present on thymocytes and peripheral T lymphocytes (Cantor and Boyse, 1977). Studies of Jandinksi et al. (1976) and Cantor, Shen and Boyse (1976) have revealed that suppression is a response of a specialized T cell subclass which expresses the Ly-23 phenotype, whereas the Ly-1 cells represent the helper cells.

5. Heterogeneity and affinity

A major characteristic of the immune system is its diversity. An extremely large immunoglobulin repertoire as a result of all possible combinations among the constant and the variable parts of the immunoglobulin molecules exists within an individual. This number more or less reflects the number of B lymphocyte clones, since an antibody forming cell produces only antibodies with a single specificity. Immunoglobulins with this specificity are present on the cell surface of B cells and act as receptors for the antigenic determinants. The same rule may apply to the T cells, although the precise nature of the antigen receptor on T cells remains controversial.

Diversity or heterogeneity is also found among the antibodies produced after antigenic stimulation. The antibodies within such a heterogeneous population differ in their affinity for the antigenic determinant. Davie and Paul (1973) have shown that the affinity of the antibodies increases during the course of the immune response due to the selective proliferation of precursor cells which produce the high affinity antibodies. Studies of Gershon and Paul (1971) and Anderson, Dresser and Wortis (1974) have demonstrated that the generation of high affinity antibodies is T cell-dependent. Indications that the thymus indeed plays an important role in the heterogeneity of immunoglobulins are obtained from the studies of Van Muiswinkel, Radl and Van der Wal (1976). Thymectomized mice which were irradiated and reconstituted with bone marrow more frequently showed homogeneous immunoglobulins in their sera as compared to nonthymectomized controls.

From the present knowledge of the humoral immune system as outlined hitherto, it can be concluded that, although the B cells give rise to the ultimate humoral products, the immunoglobulins, the thymus and the thymus-derived cells are of extreme importance in generating and regulating an optimally functioning humoral immune system.

C. AGEING AND HAEMOPOIESIS

A review on the influence of ageing on the immune system should include haemopoiesis and its changes during ageing. The stem cell of the haemopoietic system is a pluripotent cell which gives rise to both new stem cells and differentiated cells of the erythrocytic, myelocytic, lymphocytic and megakaryocytic lines. Any change in the behaviour of the pluripotent stem cells or the haemopoietic populations derived from them might be reflected in the lymphocyte population and ultimately in its immune responsiveness.

Age-related changes in the relative number of stem cells in the bone marrow, which contains 90% of the total stem cell population of adult mice (Chen, 1971), were investigated by counting the number of colony-forming units (CFU) in the spleen of lethally irradiated young recipient mice after injection of bone marrow cells from either young or old donors. It was found that the relative number of CFU in the bone marrow remains constant (Harrison, 1975, and Tyan, 1976, 1977) or decreases with age in mice (Davis, Upton and Satterfield, 1971; Chen, 1971; Silini and Andreozzi, 1974, and Coggle et al., 1975). Since the total number of nucleated cells in the bone marrow increases during

ageing (Chen, 1971; Coggle and Proukakis, 1970, and Silini and Andreozzi, 1974) the total number of stem cells in the bone marrow increases or remains constant thoughout the life-span.

It is known that stem cells subjected to in vivo serial cell transfer gradually lose their ability to replicate with increasing passage, independent of the time interval between passages (Siminovitch, McCulloch and Till, 1964; Cudkowicz et al., 1964, and Lajtha and Schofield, 1971). The fact that comparable numbers of CFU are found in the bone marrow of young and old mice indicates that the capacity of the stem cell to replicate in situ is not affected by age.

However, a study of Micklem et al. (1973) suggested that differences still do exist between the stem cells of young and old mice. They reported that stem cells from old donors have a poorer proliferative capacity than those from young donors, although this difference is less easy to demonstrate than that between foetal and young adult stem cells (Micklem et al., 1972). A recent study of Albright and Makinodan (1976) confirmed and extended the above observation. The authors investigated in detail the behaviour of stem cells in old (22-26 months) mice of three long-lived strains (BC3F1, B6D2F1 and C3H) in which comparable results were obtained. The changes that stem cells undergo with age are reflected in a reduction in the rate at which stem cells proliferate in young recipient mice shortly after transfer and a decrease in the number of cells present in the largest colonies at the end of the growth phase. Stem cells from old mice remain characteristically old, even after they were allowed to replicate in the bone marrow of young recipients. On the other hand, the spleen colony growth of young stem cells could be reduced by allowing them to replicate in old recipients. These results suggest that factors both intrinsic and extrinsic to the stem cells are responsible for the decline in their clonal expansion rate with age.

The question remains as to whether, in practice, any deficit in the pluripotent stem cell compartment is likely to be a limiting factor in the immune capacity of old mice. Therefore, the capacity of young and old bone marrow to reconstitute immune response in lethally irradiated young recipients was compared. The results revealed that recipients of old bone marrow produced antibody-forming cells as effectively as did recipients of young bone marrow (Micklem et al., 1973; Farrar, Loughman and Nordin, 1974; Harrison and Doubleday, 1975, and Harrison, Astle and Doubleday, 1977). Moreover, no difference in the thymus-dependent cellular response to oxazolone was detected between the two groups (Micklem et al., 1973). It should be noted, however, that the bone marrow from old mice contains a significantly

greater number of differentiated B cells than marrow from young mice (Farrar, Loughman and Nordin, 1974, and Haaijman, Schuit and Hijmans, 1977) which may be responsible as well for the observed similarity of the transplanted young and old bone marrow.

Finally, Toya and Davis (1973) have demonstrated that stem cells remain truly pluripotent during ageing, since no change in the differentiation capacity of mouse femoral stem cells of various ages was found.

On the basis of the observations documented so far, the conclusion may be drawn that differences do exist in the behaviour of young and old stem cells (i.e., the clonal expansion rate) but these changes cannot totally account for the age-related decline in the immune capacity.

D. EFFECT OF AGEING ON THE HUMORAL IMMUNE SYSTEM

1. Functional capacity

Initial studies on the effect of ageing on immunologic capacities concerned humoral immune functions. For instance, the level of natural serum antibodies in humans of several age groups was determined by measuring haemagglutinating and haemolysing antibody titres against blood group antigen B (Thomsen and Kettel, 1929), against sheep red blood cells (SRBC) (Friedberger, Bock and Fürstenheim, 1929) and against a number of bacteria and viruses (Rowley, Buchanan and Mackay, 1968, and Schwick and Becker, 1969). The results show a gradual decrease in the various titres to these antigens with advancing age. Natural antibody is the term used for antibody detectable in serum without prior immunization with the corresponding antigen. Assuming that the stimulatory factors responsible for the occurrence of natural antibodies are in fact antigens constantly present in the environment, the decrease in natural antibody titres reflects a diminished activity of humoral immune function in man. The pattern of the age-related decrease in natural antibody titres in man resembles the pattern found when age-related changes in antibody titres to SRBC were measured in mice following specific immunization (Makinodan, Perkins and Chen, 1971). The observation that the level of serum antibody decreased with age was also made in rats using serum albumin (Goullet and Kaufmann, 1964) and thyroglobulin (Schumacher and Premachandra, 1968) as antigen and in chickens (Wolfe et al., 1957) where bovine serum albumin served

as antigen. Whether the decrease in serum antibody levels could be attributed to a decrease in antibody forming cells has been investigated by Makinodan et al. (1971a) employing the PFC technique of Jerne and Nordin (1963). They reported that, in the spleen of 30-month-old BC3F1 mice, the number of cells producing antibodies of both IgM and IgG classes is only about 10% of that of young adult mice. Similar data for other strains of mice have been reported by Metcalf, Moulds and Pike (1966), Wigzell and Stjernswärd (1966), Kishimoto and Yamamura (1971), Finger, Emmerling and Bertz (1971), and Micklem, Ogden and Payne (1973). Studies were subsequently carried out to determine if the decline in the immune response was due to changes in the cellular milieu, changes in the cells of the immune system themselves or both. In these studies, a cell transfer assay in which known numbers of lymphoid cells from young and old donors were infused into syngeneic irradiated young and old recipients was employed. Antigenic stimulation was simultaneously given and the antibody forming capacity was determined. The results indicated that both the cells and the environment in the aged mice are defective. Ten per cent of the decline can be attributed to changes in the cellular milieu, while changes intrinsic to the old cells account for about 90% of the decline (Price and Makinodan, 1972a, b). The main cause of the decline in humoral immune capacity must therefore be sought for in the cells participating in an immune response and their interactions.

With regard to the cellular milieu in old animals, it has been established that lymphoid tissues undergo morphological changes with age (Andrew, 1952; Metcalf, Moulds and Pike, 1966, and Adler, Jones and Nariuchi, 1977). The principal change is seen in the thymus, where atrophy of the lymphoid tissue, chiefly in the cortex, occurs. Similar but less dramatic atrophic changes are observed in the spleen and lymph nodes of ageing mammals. In these organs, a disorganization of the follicles and a decrease in the number of active germinal centres have been noted.

2. Macrophages

Since macrophages participate in most humoral and cellular immune responses and confront antigens before lymphocytes do, a defect in these cells may result in a decrease in immune reactivity. Therefore, studies have been carried out to determine the effect of age on the function of phagocytic cells.

Macrophages are found in every tissue in the body and exhibit regional differences in their function. Even within a given site, macro-

phages show functional diversity (for a review, see Walker, 1976).

Properties of macrophages which have been studied in relation to ageing are the clearance of foreign particles from the blood and the capacity to promote a humoral immune response. Phagocytic cells which clear the blood stream of particles in vivo are primarily located in the liver (Kupffer cells) and in the spleen. A large proportion of the peritoneal macrophages, which are representative for a population of "free" macrophages, and splenic macrophages are able to process antigen and present it to lymphocytes in such a way that an immune response is initiated.

Age-related changes in blood clearance capacity were determined in ageing mice as well as in ageing rats. The results of Aoki, Teller and Robitaille (1965) and Jaroslow and Larrick (1973) for mice and of Bilder (1975) for rats indicate that the clearance rate is optimal in young animals; it later decreases, but remains constant in mature and old animals. Hanna, Nettesheim and Snodgrass (1971), however, found a progressive reduction in clearance capacity in 1.5 - 2.5-year-old mice.

Peritoneal macrophages from 24-38-month-old mice are as efficient as those from young adult mice in their capacity to both engulf and optimally digest opsonized SRBC in vitro (Perkins, 1971). The capacity of antigen-laden peritoneal macrophages from old mice to initiate primary and secondary antibody responses in vivo is comparable to that of young mice (Perkins and Makinodan, 1971). Likewise, Heidrick and Makinodan (1973) have demonstrated that the capacity of splenic accessory cells (among which are macrophages) to collaborate with T and B cells in the initiation of an antibody response in vitro is unaffected by age. So far, the majority of the data suggest that the age-related decrease in immune responsiveness does not appear to result from impaired phagocytic activity.

3. T and B cells

The remaining cells to be dealt with are the T and B cells. The question has to be resolved as to whether a depressed immune response during ageing can be explained by a quantitative rather than a qualitative defect in immunocompetent cells. The effect of age on the number of T cells (as judged by the presence of the Thy-1 antigen) in the lymphoid organs of mice has been studied by several investigators. Table I.1 summarizes literature data on changes in the number of T cells during ageing in the short-lived autoimmune susceptible NZB mice

TABLE I.1

EFFECT OF AGE ON RELATIVE AND ABSOLUTE NUMBERS OF Thy-1 POSITIVE CELLS IN MICE
(AS REPORTED IN THE LITERATURE)

strain	sex	ages studied in months	organ	changes in: relative number*	changes in: absolute number*	method**	references
NZB	♀	3 - 20***	spleen	↓	↓	cytotox	Stutman, 1972
			lymph node	↓			
			thymus	↓			
CBA/H	♀	3 - 20	spleen	=	↑		
			lymph node	↓/=			
			thymus	=			
NZB	♂ + ♀	4 - 14	spleen	=		cytotox	Waksman, Raff and East, 1972
			lymph node	=			
BC3F1****	?	3 - 34	spleen	↓		cytotox	Hori, Perkins and Halsall, 1973
BALB/c	?	3,22,27	spleen	=			
C57BL/6J	♀	2,17	spleen	=		cytotox	Kishimoto, Shigemoto and Yamamura, 1973
			lymph node	=			
(C57BL/6J x 129)F1	♂	2 - 30	spleen	=		cytotox	Gerbase-De Lima et al., 1974
C57BL/6	♂ + ♀	3 - 20	spleen		=	cytotox	Stutman, 1974
CBA/H	♂ + ♀	3 - 23	spleen		↑		
NZB	♂ + ♀	3 - 20	spleen		↓		
B6CF1****	♂ + ♀	3 - 30	spleen	↓		fluor	Brennan and Jaroslow, 1975
CBA/H	?	3,24	spleen	↓	=	fluor	Callard and Basten, 1977
BALB/c	?	2,19	spleen	=		fluor	Walters and Claman, 1975
NZB	♂ + ♀	3,10,19	spleen	↓	=	fluor	Hirano and Nordin, 1976

* ↓: decrease; ↑: increase; = : constant
** Cytotox : cytotoxicity test using anti Thy-1 sera
Fluor : immunofluorescence staining with anti Thy-1 sera
*** 3-20 indicates that various ages within these age limits were studied
****BC3F1 : (C57BL/Cum x C3H/AnfCum)F1; B6CF1 : (C57BL/6 x BALB/c)F1

and in 6 long-lived nonautoimmune strains. In most of the latter strains, the number of T cells remains constant or moderately increases with age. An exception is the B6CF1 mouse, where a decrease in Thy-1 positive cells as well as a reduction in the amount of Thy-1 antigen per individual lymphocyte was observed (Brennan and Jaroslow, 1975). Despite this discrepancy, which may be attributed to a strain-dependent difference, it can be concluded that, in most long-lived "nonautoimmune" strains of mice, no dramatic changes in the number of T cells occur during ageing. Table I.1 shows conflicting data on NZB mice, which cannot be easily explained. In contrast to Stutman (1972), Waksman, Raff and East (1972) and recently Hirano and Nordin (1976) did not find marked changes in the number of T cells in NZB mice.

Much less data are available on quantitative changes in the B cell compartment of mice during ageing. Stutman (1972) has shown that in both NZB and CBA/H mice either a constant level or a moderate increase with age occurs in the number of thymus-independent lymphocytes in the spleen, lymph nodes and bone marrow as judged by the presence of membrane immunoglobulins and antigen-antibody-complement receptors. Callard, Basten and Waters (1977) observed the same tendency during ageing for the spleen of their CBA mice. However, contradictory results have been reported with regard to bone marrow. Whereas Stutman (1972) did not find distinct changes in the B cell population in bone marrow, Farrar, Loughman and Nordin (1974) described a marked age-associated increase in lymphocytes bearing IgM on their membrane in the bone marrow of BC3F1 mice. This difference in the cellular constituents of old and young bone marrow has been confirmed by Haaijman (personal communication) for our colony of CBA mice. Preliminary data indicate that the number of Ig bearing lymphocytes in the bone marrow tends to increase during ageing.

From the aforementioned data, it can be concluded that no dramatic quantitative changes occur in the B and T cell compartments during ageing in long-lived mouse strains. It cannot be excluded, however, that changes in subpopulations of B and T cells do occur during ageing. At present, antisera against markers expressed on nearly all B and T cells are employed. Application of more defined antisera against, for instance, subpopulations of T cells which differ in Ly phenotypes will give a more definitive answer to this question. No data are available on age-related alterations in B and T cells in other species, except for man. This will be discussed later in this introduction.

Although the number of B cells does not change, ageing may affect their products, the immunoglobulins. Therefore, the level of immuno-

globulins in the serum has been compared in young and old mice. Serum IgG levels were determined in BC3F1 mice of several age groups by Quinn et al. (1973). Their results show a constant level of IgG in females and an increase in IgG in males during ageing. The catabolic half-life-time, however, changed with age. A decrease in catabolic half-life-time occurred between 1 and 8 months of age, followed by a more gradual decrease throughout the remainder of life. A recent study of Haaijman, Van den Berg and Brinkhof (1977) revealed that the levels of IgA, IgM and IgG2a and IgG3 in CBA mice remain fairly constant from the age of 6 months up to 2.5 years of age. The levels of IgG1 and IgG2b tend to increase. These results show that, with respect to the overall immunoglobulin production, the B immune system of old animals is as active as that of adult animals.

Summarizing this introduction on age-related changes in the humoral immune response, it can be concluded that the decreased antibody formation after immunization cannot be attributed to changes in the number of B and T cells nor to changes in the function of macrophages or a lower overall immunoglobulin production by B cells. As shown by Price and Makinodan (1972a, b), environmental changes are only partly responsible for the age-related decline in immune capacity. Therefore, the nature of the decline should be searched for in qualitative changes. The complexity of the humoral immune reactions as outlined in this introduction indicates that the qualitative changes responsible for the decline may be caused by multiple factors such as defects in the recruitment and proliferation of T and B cells, defects in antigen processing, changes in the capacity of B cells to interact with other cells and defects in the helper function of T cells or the increasingly dominant role of subpopulations of T cells which suppress the immune response. Literature data available with respect to these points will be discussed later in relation to the results obtained in the present study.

E. AGEING AND CELL-MEDIATED IMMUNE FUNCTIONS

Cell-mediated immunity is primarily dependent on T lymphocytes. The response is initiated by antigen recognition and subsequent activation of specifically reactive T lymphocytes. This type of response is important in host defense against many viruses, fungi and mycobacteria. Delayed-type hypersensitivity, graft rejection, graft versus host (GvH) reaction and antitumour immunity are also examples of cellular immunity.

In vivo studies comparing cell-mediated immune responses in young and ageing mice have been reported for contact sensitivity, graft versus host reaction and skin grafting. The results of these studies are presented in Table I.2. Variable results have been obtained with regard to the contact sensitivity and skin grafting experiments in the different strains. A reduced capacity to reject primary skin allografts was found in two-year-old B6CF1 mice, in contrast to other strains. It should be noted, however, that these studies did not include mice beyond the age of two years. Therefore, the only conclusion to be drawn is that the onset of a decrease, if any, in contact sensitivity and skin grafting differs for the different strains.

The *in vivo* graft versus host reactivity is reduced in all of the strains tested, except in C3Hf mice, where the GvH response remains constant during the life-span studied.

An *in vitro* method for measuring GvH activity was developed by Auerbach and Globerson (1966). As in the *in vivo* GvH procedure, splenomegaly can be detected *in vitro* by comparing the relative size of two matched spleen fragments: one fragment challenged by nonsyngeneic lymphocytes and the control fragment exposed to syngeneic lymphocytes. Such an *in vitro* approach offers the possibility to study whether the reduced GvH reactivity of old mice only results from a different homing pattern of old lymphocytes in the recipient mice. The results of Friedman, Keiser and Globerson (1974) showed that spleen cells from 32-33-month-old (C3H/eb x C57BL)F1 mice failed to produce a GvH response *in vitro*, indicating an intrinsic defect in the lymphoid cells.

The *in vitro* mixed lymphocyte reaction (MLR) corresponds to the recognition phase of the GvH response and the allograft rejection. The MLC reactivity of young and old mice has been compared by several groups (Table I.3). A significant decrease in MLR reactivity was reported for 2.5-3-year-old C57BL/6J and (C57BL/6J x 129)F1 mice. On the other hand, lymph node cells from 2.5-year-old CBF1 mice were as active as cells from adult mice in eliciting an MLR reaction. In the study of Meredith et al. (1975), it was demonstrated that lymph node cells from old mice of two strains lost the ability to synergize with syngeneic thymocytes in a MLR reaction. The results with regard to the MLR again illustrate that the onset of a decline may differ for the different strains. For instance, 35-month-old C57BL/6J mice showed a significantly depressed MLR reactivity, which was not yet observed at the age of 21 months (Konen, Smith and Walford, 1973).

The capacity to specifically generate immune lymphocytes capable of killing syngeneic or allogeneic tumour target cells *in vitro* (cell-mediated cytotoxicity) has been studied in young and old mice of

TABLE I.2

CHANGES IN IN VIVO CELLULAR IMMUNE FUNCTIONS DURING AGEING IN MICE
(AS REPORTED IN THE LITERATURE)

function	strain	sex	ages studied in months	organ	results*	references
contact sensitivity	BALB/c	?	3,18	-	=	Walters and Claman, 1975
	NZB/W	?	4,12	-	↓	
allograft rejection	CBA	?	3-24**	-	=	Krohn, 1962
	Swiss	♂ + ♀	2,14	-	♂ = ♀ ↓	Teller et al., 1964
	C3Hf	♂ + ♀	3-20	-	=	Stutman, Yunis and Good, 1972
	B6CF1***	o	5,24	-	↓	Menon, Jaroslow and Koesterer, 1974
graft versus host reaction	CBA	?	3-24	?	↓	Krohn, 1962
	C3Hf	♂ + ♀	3-20	spleen	=	Stutman, Yunis and Good, 1972
	C57BL/6J	♀	2,19	spleen lymph node thymus	↓ ↓ ↓	Kishimoto, Shigemoto and Yamamura, 1973
	NZB	♀	2,10	spleen lymph node	↓ ↓	Stutman, 1974
	C57BL/6	♀	2,23	spleen lymph node	↓ ↓	
	BALB/c	?	3,18	spleen lymph node	↓ ↓	Walters and Claman, 1975
	BALB/c	♂	3-24	spleen	↓	Perkins and Cacheiro, 1977

* ↓: decrease; = : constant
** 3-24 indicates that various ages within these age limits were studied
***B6CF1 : (C57BL/6 x BALB/c)F1

TABLE I.3

CHANGES IN IN VITRO CELLULAR IMMUNE FUNCTIONS DURING AGEING IN MICE
(AS REPORTED IN THE LITERATURE)

function	strain	sex	ages studied in months	organ	results*	references
mixed lymphocyte reaction	C57BL/6	?	3-12**	spleen	↓	Adler, Takiguchi and Smith, 1971
	A/J	?	3-12	spleen	=	
	CBA	?	3-12	spleen	↓	
	NZB	♂ + ♀	2,18,24	spleen	strong ↓	Rodey, Good and Yunis, 1971
	A/f	♂ + ♀	2,24	spleen	↓	
	C57BL/6J	♂	5,21,35	spleen	↓	Konen, Smith and Walford, 1973
	(C57BL/6J x 129)F1	♀	6,20,30	lymph node	↓	Meredith et al., 1975
	CBF1***	♀	6,20,30	lymph node	=	
	BALB/c	?	3,18	spleen	↑	Walters and Claman, 1975
	C57BL/6J	♀	3-29	lymph node	↓	Merhav and Gershon, 1977
cell - mediated cytotoxicity	C3Hf		1-23	lymph node	=	Stutman, 1972
	C57BL/6J		1-33	lymph node	=	
	CBA/H		1-33	lymph node	=	
	C57BL/6		3-18	spleen	↓	Menon, Jaroslow and Koesterer, 1974
	BC3F1***	?	3-32	spleen	↓	Goodman and Makinodan, 1975
	C57BL/6J	♀	2,20	spleen	=/↓	Shigemoto, Kishimoto and Yamamura, 1975
				lymph node	=	
	NZB	♂ + ♀	3,10,19	spleen	↓	Hirano and Nordin, 1976
	DBA/2	♂ + ♀	3,19	spleen	↓	

* ↓: decrease; ↑: increase; = : constant
** 3-12 indicates that various ages within these age limits were studied
***BC3F1 : (C57BL/Cum x C3H/AnfCum)F1; CBF1 : (C57BL/6J x BALB/cJ)F1

several strains (Table I.3). This reaction can be considered as the effector phase of cellular immune responses. A striking difference can be observed between spleen and lymph nodes of old mice. Whereas the cytolytic activity of spleen cells had already declined at the age of 18-20 months in C57BL/6, BC3F1, C57BL/6J, NZB and DBA/2 mice, lymph node cells of even older C3Hf, C57BL/6J and CBA/H showed the same activity as young adult animals. Since these results were obtained in separate studies, this possible difference in capacity between spleen and lymph node cells from old mice could also be due to strain differences or differences in techniques. Therefore, this observation has to be confirmed in a single study. Goodman and Makinodan (1975) demonstrated that the 4-fold reduction in the cytolytic capacity of cells from old mice to kill allogeneic target cells was due to a 2-fold decrease in relative numbers of immunocompetent precursor cell units. The other 2-fold decrease could be attributed to a decrease in the proliferative and transforming capacities of antigen-stimulated precursor cells of old mice.

It is generally accepted that the mitogens phytohaemagglutinin (PHA) and concanavalin A (ConA) selectively stimulate T cells to undergo blast formation and DNA synthesis (Greaves and Janossy, 1972). Therefore, PHA and ConA stimulation are commonly used as a measure of cell-mediated immunity. Nearly all investigators have reported that spleen cells showed a reduced capacity during ageing to respond to T cell mitogens as determined by incorporation of ^{14}C-or ^{3}H-thymidine (Table I.4), except for the spleen cells from CBA mice in the study of Rodey, Good and Yunis (1971). However, these mice were not older than 21 months, which is below the 50% survival time for this strain (see Chapter III). Nevertheless, some other long-lived strains of mice already showed a decline at this age. Meredith and Walford (1977) studied the age-related changes in the response to both PHA and ConA in 9 mouse strains congenic on A, C3H and C57BL backgrounds but with 3 different H-2 regional complexes substituted in any of the 3 backgrounds. An age-related decrease in responsiveness was noted in all strains (the data are not included in Table I.4). Congenic animals which respond similarly as young adults sometimes differ markedly when tested at a later period in life, indicating the influence of the H-2 complex upon mitogen responsiveness.

A decrease in T cell mitogen responsiveness during ageing was also found in hamsters (Mathies et al., 1973), dogs (Gerber and Brown, 1974) and rats (Kruisbeek, 1976). The studies of Hung, Perkins and Yang (1975a), Kruisbeek (1976), and Callard and Basten (1977) revealed that the depressed reactivity could not be attributed to a difference

between young and old animals in the requirements for mitogen dose and culture conditions. Therefore, a decrease in the number of mitogen responsive cells is thought to be responsible for the age-related defect in mitogen responsiveness.

This evaluation of data from the literature shows that, in old mice (2.5-3 years of age), most cell-mediated immune functions have declined in comparison with young mice. The only clear exception is the cytolytic activity of lymph node cells, which was shown to remain constant with age in separate studies using different strains. This should be substantiated in a single study using spleen and lymph node cells from one strain. The results obtained in middle-aged (1.5-2.5 years of age) mice show large variations, suggesting a difference in onset of the decline in various cell-mediated immune functions in different strains. Such a difference can even be found within one strain. Walters and Claman (1975) reported that 18-month-old BALB/c mice were as active as young mice with respect to contact sensitivity to dinitrofluorobenzene and MLR response, whereas the GvH reaction and T cell mitogen responsiveness were reduced.

The above data do not support the conclusion of Stutman (1974) as stated in his review on cell-mediated immunity and ageing that cell-mediated immunity is well-preserved in aged individuals of intermediate- and long-lived mice, in contrast to the decline in cellular immune functions in NZB mice. He suggests a correlation of such a decline with the autoimmune disease of NZB mice. The literature data clearly show decreased cellular immune responses in old mice, which may be related to a normal physiological ageing process rather than to autoimmune disease. However, the possibility that autoimmune disease may accelerate immune senescence in NZB mice cannot be excluded.

The nature of the age-related decline in cell-mediated immune functions has still to be elucidated. This decline cannot be attributed to a decrease in the number of Thy-1 positive cells during ageing, as was demonstrated at the same time in a few studies included in Tables I.2, I.3 and I.4 (see also Table I.1). However, the possibility that changes in subpopulations of T cells account for the defect cannot be excluded. Both recognition and proliferation are important components for the generation of an effective immune response. Especially the reduced MLR and T cell mitogen responses in old age suggest that the nature of the decline in cell-mediated immunity has to be sought for in changes in the recognition, proliferation and regulation processes.

TABLE I.4

CHANGES IN T CELL MITOGEN RESPONSIVENESS DURING AGEING IN MICE
(AS REPORTED IN THE LITERATURE)

strain	sex	ages studied in months	mitogen*	results**	references
A/f	♂ + ♀	2-24***	PHA	↓	Rodey, Good and Yunis, 1971
NZB	♂ + ♀	2-24	PHA	strong ↓	
CBA	♂ + ♀	2,21	PHA	=	
BC3F1****	?	3-36	PHA	↓	Hori, Perkins and Halsall, 1973
BALB/c	?	3-24	PHA	↓	
(C57BL/6J x 129)F1	♀	6,18,39	PHA	↓	Mathies et al., 1973
C58	?	3-16	PHA	↓	Lawton and Murphy, 1974
CBF1****	♂	6,18,30	PHA, ConA	↓	Meredith, Gerbase-De Lima and Walford, 1975
BALB/c	?	3,18	PHA, ConA	↓	Walters and Claman, 1975
BC3F1****	♀	4,30	PHA	↓	Hung, Perkins and Yang, 1975a + b
CBA/H	♂ + ♀	3,24	PHA	↓	Callard and Basten, 1977
C57BL/6J	♀	3-28	ConA	↓	Abraham, Tal and Gershon, 1977
BALB/c	♂	3,24	PHA	↓	Perkins and Cacheiro, 1977

* The T cell mitogen response of spleen cells was determined
** ↓ : decrease; = : constant
*** 2-24 indicates that various ages between these age limits were studied
****BC3F1: (C57BL/Cum x C3H/AnfCum)F1
CBF1 : (C57BL/6J x BALB/cJ)F1

F. ROLE OF THE THYMUS IN THE AGEING OF THE IMMUNE SYSTEM

The thymus is an intriguing subject for immuno-gerontological studies. It shows a marked involution early in life, although it is indispensable for the maintenance of an optimally functioning immune system. The thymus is responsible for the generation of thymus-dependent (T) lymphocytes. As described above, peripheral T lymphocytes are involved in cell-mediated immune functions and participate in most humoral immune responses. The factors necessary for the normal development and maintenance of T lymphocytes are an intact reticulo-epithelial framework in the thymus and a supply of cells of haemopoietic origin which are sensitive to the inductive action of the thymus (Stutman and Good, 1974). The precise mechanism by which the thymus exerts its inductive effect is unclear. One or more humoral factors, tentatively called thymic hormone(s), are considered to play a role in this process. Evidence is being obtained that the epithelial cells produce these factors. Secretory activity of thymic epithelial cells has been demonstrated using the electron microscope (Clark, 1968, and Meihuizen and Burek, personal communication), while the epithelial origin of a circultating thymic factor has been established (Dardenne et al., 1974b).

The principal change seen in the thymus with advancing age is atrophy of the lymphoid tissue, chiefly in the cortex, and an increase in macrophages and plasma cells (Hirokawa, 1977). This is accompanied by a decrease in volume and weight of the whole organ. In a study on age-related changes in the morphology of rat thymus, Burek (1978) showed that the onset and the morphological appearance of the atrophy was strain and sex dependent.

The capacity for generating functional T cells in young and old atrophic thymus glands has been compared. Thymus grafts of various ages were transplanted into thymectomized, lethally irradiated and bone marrow reconstituted mice and several immune parameters in the recipient mice were determined at various time intervals after the transplantation. These studies revealed that the generation of functional T cells was optimal when thymus grafts from one-week-old donors were used (Hirokawa and Sado, 1978) and declined gradually with the age of the graft (Yunis et al., 1972, and Hirokawa and Makinodan, 1975). It can be concluded, therefore, that the deficient generation of functional T cells by the aged thymus contributes to the age-related decline in T cell functions. To what extent ageing affects earlier and later stages of T cell differentiation and the functional capacity of the mature T cells remains to be elucidated. With regard

to the prethymic stage, the observations of Tyan (1976, 1977) suggested that the number of T cell progenitors in the bone marrow capable of repopulating the thymus of an irradiated host is reduced in older animals.

On the basis of the above observations, one should expect an accelerated decline in immunological functions after thymectomy. Neonatally thymectomized (nTx) animals indeed exhibit a severe impairment of cellular and humoral immune functions (reviewed by Hess, 1968). Studies in which the immune capacity of nTx mice older than 5-6 months is determined are lacking, since the mice succumb to wasting disease by that time. During these 5-6 months, immune responses of nTx mice remain decreased (Stutman, Yunis and Good, 1968, and Yunis et al., 1972). Since the wasting disease in rats is not as severe as that in mice (Hess, 1968), nTx rats could be observed for a longer period. A partial recovery of responsiveness to PHA was found in rats one year after neonatal thymectomy (Dabrowski and Dabrowska, 1972, and Dabrowski, 1974).

Adult thymectomy produces a delayed immunological defect in mice which can be detected as early as 5-7 months after the operation and increases progressively with age (Stutman and Good, 1974; Peterson et al., 1975; Perkins et al., 1975, and Pachciarz and Teague, 1976).

Some immune parameters, however, are decreased within a relatively short period of time after adult thymectomy. A decrease in numbers of spontaneous rosette-forming T cells has been observed in mice within 1-3 weeks after thymectomy (Bach, Dardenne and Davies, 1971). Similarly, serum levels of a humoral thymic factor decreased to undetectable levels within a few weeks after adult thymectomy in mice (Bach, Dardenne and Bach, 1973) and man (Astaldi et al., 1976). An early loss of circulating thymic factor is also observed during ageing. The serum level of this factor starts to decrease at the age of six months in various strains of mice (Bach, Dardenne and Salomon, 1973, and Dardenne et al., 1974a). The loss of such a thymic humoral factor may precede the decline in T cell functions later in life.

From the available data reported here and in the previous parts of the introduction it follows that the absence or dysfunction of the thymus has a large effect on immune reactions. Likewise, the observed dysfunction of the thymus during ageing should have consequences on the immune response of aged individuals. Therefore, the thymus has a central role in the search for the nature of the ageing of the immune system. Manipulations of the immune system in order to delay, reverse or prevent the functional decline should begin by improving the function of the thymus. A better insight into the mechanism by which the

thymus influences the maturation of T lymphocytes will help to achieve that goal.

G. AGEING AND IMMUNOPATHOLOGY

In the preceding parts of the introduction, it has been reported that a decline in both humoral and cellular immune functions occurs with advancing age producing a generalized state of immune deficiency. To which immunological aberrations and diseases of a possible immunological origin this state of immune deficiency may lead will be discussed here.

Little information is available on the relation between the impaired immune system in old age and the occurrence of infections. It is generally assumed that elderly people differ from young people in their susceptibility and response to certain infectious agents (Good and Yunis, 1974, and Hijmans and Hollander, 1977). For instance, gram-negative bacteremia is much more common in an elderly hospital population than in a young one and deaths from bronchopneumonia during an influenza epidemic were found predominantly in the age group over 60 (Gladstone and Recco, 1976). Unfortunately, appropriate experimental studies in animals are lacking. The following observations have been made in our own ageing animal colonies. Histological examination of untreated WAG/Rij rats which were allowed to complete their life-span revealed that the chance of developing inflammatory lesions increased with age. The involvement of the immune system was shown by the fact that old neonatally thymectomized rats had a higher prevalence of these lesions (Boorman and Zurcher, personal communication). On the other hand, evaluation of the mortality caused by an acute Sendai virus infection in ageing mouse colonies indicated that old mice (beyond the 50% survival value) were no more susceptible than were middle-aged mice (Zurcher et al., 1977). The mortality of ageing rats of various strains was also not increased during the period of the acute Sendai virus infection (Burek et al., 1977). It is impossible to draw any conclusions from these data on the role of a specific immune dysfunction in the occurrence of infectious diseases in aged humans and animals.

There is a striking age-related increase in the occurrence of autoantibodies, in both humans and mice (Walford, 1969). Manifestations of autoimmunity in mice include the development of antierythrocyte antibodies and antibodies to nuclear components. Some mouse

strains with a short or intermediate life-span, among which the NZB is the most notable, are extensively studied because of the appearance of autoantibodies at an early age. NZB mice develop autoantibodies against erythrocytes, nuclear components and thymocytes. The hybrid NZB/W is especially well-known for the early onset and high frequency of antinuclear antibodies (ANA) (for a review, see Talal and Steinberg, 1974). A high frequency of ANA later in life is reported for the A/J mouse and its sublines (Friou and Teague, 1964, and Teague, 1974). In addition to immunological factors, genetic and viral factors are considered to play a role in the development of autoantibodies in these strains. Evidence has been obtained that there is a relationship between the dysfunction of the immune system and the appearance of autoantibodies in NZB and NZB/W mice (Talal and Steinberg, 1974, and Stutman, 1974) and A/J mice (Teague et al., 1970). The influence of the thymus-dependent part of the immune system on the development of autoantibodies was clearly established by the finding that neonatal thymectomy accelerates autoantibody formation in NZB (Howie and Helyer, 1966), NZB/W (Steinberg, Law and Talal, 1970) and A/J mice (Teague et al., 1970). Recent studies in NZB and NZB/W mice have been focused on the defective immunologic regulation as a consequence of impaired thymus function. A loss of suppressor T cells is observed at an early age. This may account for the hyperresponsiveness to some antigens in very young mice, the impaired tolerance induction and maintenance and the loss of self tolerance leading to autoimmunity in older NZB mice (Talal, 1976). Additional evidence for an impaired thymus function is the disappearance of a circulating thymic factor at the age of 2-4 months in both NZB and NZB/W mice (Bach, Dardenne and Salomon, 1973).

An age-related increase in autoimmune manifestations is not limited to the well-known autoimmune susceptible strains with relatively short and medium life-spans but has also been reported for mice with long life-spans. Peterson and Makinodan (1972) have shown that the frequency of mice with circulating autoantibodies against erythrocytes increased with advancing age in BC3F1, C57BL/6J and SWR/J mice. Female BC3F1 mice tended to have a higher frequency than males. An increased frequency of antierythrocyte autoantibodies was also found in ageing C57BL/6 mice (Linder, Pasternack and Edgington, 1972). It must be noted, however, that these antierythrocyte autoantibodies cannot be detected by the direct and indirect Coombs' test. In the above-mentioned studies, the autoantibodies were demonstrated by indirect haemagglutination of enzyme-treated syngeneic red blood cells, suggesting that their activity is directed towards the cryptic HB auto-

antigen (Linder and Edgington, 1972). Autoantibodies of anti-X specificity capable of binding to erythrocytes in vivo as demonstrated in NZB mice were not found (Linder, Pasternack and Edgington, 1972).

Likewise, the occurrence of ANA has been reported for several long-lived mouse strains. Mice of various strains showed an age-related increase in ANA, but these antibodies were weaker and appeared less frequently than those observed with NZB and NZB/W mice (Siegel, Brown and Morton, 1972, and Abe et al., 1976).

The next question to be dealt with is the association of the appearance of autoantibodies with autoimmune diseases. NZB and NZB/W mice exhibit a broad spectrum of diseases and pathological lesions of autoimmune character including Coombs' positive haemolytic anaemia, tissue-lymphocytic and plasmacytic infiltration, immune complex vasculitis and glomerulonephritis (Howie and Helyer, 1968). Immunofluorescence and elution studies of the kidneys of NZB and NZB/W mice indicate that nuclear antigen and its antibody are concentrated in the glomerular basement membrane, implicating this particular immune complex in the pathogenesis of the nephritis (Dixon, Oldstone and Tonietti, 1971).

The occurrence of antierythrocyte autoantibodies in mice of the long-lived strains was not always accompanied by a severe haemolytic anaemia as found in the NZB mice. There was a slight correlation between the presence of autoantibodies in the blood plasma and the low haematocrit in old BC3F1 mice (Peterson and Makinodan, 1972). Linder, Pasternack and Edgington (1972) and Porter, Porter and Cox (1973) described the occurrence of immune complex glomerulonephritis in 8-12-month-old C57BL/6 mice. Immune complexes containing autologous soluble erythrocyte antigen and autoantibodies were detected in the eluates of the kidneys from these mice.

Immune complex glomerulonephritis has also been shown to increase with age in other strains of mice (Gude and Upton, 1960; Guttman and Kohn, 1960, and Peter, 1973), rats (Guttman and Kohn, 1960, and Couser and Stilmant, 1976) and mastomys (Van Noord et al., 1972). The nature of the antigens in these complexes has not always been established. In addition to autologous antigen-antibody complexes, renal depositions of viral antigen and antibody have been described (Hanna et al., 1972; Peter, 1973; Porter, Porter and Cox, 1973, and Van Pelt et al., 1976). The underlying immunological mechanism responsible for the deposition of immune complexes which can lead to tissue damage is not known. It is assumed that the ratio of antigen to antibody and the affinity of the antibody play a critical role in the occurrence of immune complex diseases (Cochrane and Koffler, 1973). In addition, the severity of

the glomerular lesions may be aggravated by an age-related impaired removal of the complexes by mesangial phagocytes (Couser and Stilmant, 1976).

A pathological lesion which is often linked to the immune system is amyloidosis. Spontaneous amyloidosis has been shown to increase with age in humans (Schwartz, 1970) as well as in mice (Dunn, 1967; Scheinberg et al., 1976, and Chapter III of this thesis). There is great variation in the occurrence, severity and distribution of amyloid deposits in the different inbred strains of mice (Dunn, 1967).

Various types of amyloid deposits differing in chemical composition exist, but a common feature of all amyloid proteins is their β - pleated sheet conformation and their resistance to proteolysis and solubilization. The accumulation of this indigestible material results in organ dysfunction (Glenner and Page, 1976). Three major types of amyloid fibrils can be recognized: a) amyloid of immunoglobulin origin consisting of light chain polypeptides or parts thereof; b) amyloid consisting of amyloid A protein; and c) amyloid of polypeptide-hormone origin. The first type is found in human patients with B cell dyscrasias, for example multiple myeloma. The second type occurs in human patients with chronic inflammatory processes such as rheumatoid arthritis. A serum component known as amyloid A protein is considered to be a precursor of this type of amyloid. Aged humans exhibit an increased level of this protein in their sera (Rosenthal and Franklin, 1975). The third type of amyloid is observed in amyloid deposits associated with endocrine tumours.

The occurrence of amyloidosis in patients with B cell dyscrasias and with chronic inflammatory processes are suggestive of links between the immune system and formation of amyloid. Persistent infections may give rise to amyloid formation as a consequence of excessive antigenic stimulation (Good and Yunis, 1974). Kellum et al. (1965) have shown that a combined treatment of thymectomy, appendectomy and sublethal irradiation induces profound amyloidosis in a large proportion of rabbits. Moreover, this study revealed that amyloidosis did not occur when treated animals were maintained under pathogen free conditions.

The nature of the relation between the immune system and the development of amyloid is unknown. In their review on amyloidosis, Glenner and Page (1976) propose the hypothesis that "the development of amyloid is due to altered or impaired immune reactions, altered either because of the presence of an abnormal immune reaction or of an abnormal antigenic stimulus that overloads the capacity of the host's immune system". Further studies are required to determine the precise

role of the immune system in amyloid formation. Subsequently, a direct correlation between decreased immune functions in old age and the occurrence of amyloidosis may be established.

Studies on the occurrence of neoplasia in laboratory animals have supported the conclusion that increased tumour incidence is associated with advancing age (Teller, 1972; Smith, Walford and Mickey, 1973; Gardner et al., 1973; Hollander, 1976, and Burek, 1978). Efforts have been made to correlate the decrease in immune function seen in old age with the age-related increase in neoplasia.

Teller and Eilbert (1967) measured changes in cytotoxic antibody production during ageing in one random bred and five inbred strains of mice with different incidences of spontaneous tumours. They could not find a correlation between a decline in this immune parameter and the incidence of spontaneous tumours in the different strains.

The immune status of ageing SJL/J mice, which develop a high frequency of spontaneous reticulum cell sarcoma (Dunn and Deringer, 1968) by one year of age, was investigated by several groups. According to Haran-Ghera et al. (1973), thymus-dependent as well as thymus-independent immune functions decreased as the mice grew older. No difference in the various immune functions was found between normal and tumour bearing mice. Seibert, Pollard and Nordin (1974), on the other hand, did find a difference between normal and tumour bearing SJL/J mice in the capacity for developing an antibody response. Mice with lymphoreticular tumours had low or no antibody formation and low levels of immunoglobulins. Opposite results were obtained by Owens and Bonavida (1976). They reported that the capacity to generate cellular and humoral cytotoxicity to a tumour allograft was not impaired in 2- to 12-month-old SJL/J mice regardless of their tumour status. They even found a hyperresponsiveness to alloantigens in 12-month-old mice, which may be attributed to a loss of immune regulation. A consistent finding in these mice is the presence of homogeneous immunoglobulins in the serum of tumour-bearing mice (Wanebo et al., 1966; Haran-Ghera et al., 1973, and Seibert, Pollard and Nordin, 1974).

The relation between immune capacity and the development of reticulum cell sarcoma was also studied in B6C3F1 mice (Hanna, Nettesheim and Snodgrass, 1971). A significant age-related decrease in antigen clearance in the serum and in antigen localization in the spleen occurred before tumour development.

The studies described here and the conflicting data reported for SJL/J mice are illustrative of the difficulty of ascertaining the relation between the decreased immune capacity in old age and the development of neoplasia. This conclusion is not so surprising, since

the immunological mechanisms by which spontaneous tumours are rejected or accepted are still largely unknown. The concept of immunological surveillance against neoplasia has been challenged by many investigators (see, for instance, Prehn, 1976). Moreover, the decline in immune functions is only one of many physiological changes which occur with advancing age and most likely several of such changes might be involved in tumour development.

Infections, autoimmune disorders, glomerulonephritis, amyloidosis and neoplasia are all diseases where incidences increase with advancing age and which are more or less associated with the immune system. However, the precise mechanisms by which the immune system is involved and the influence of factors outside the immune system are still largely unknown.

H. AGEING AND IMMUNE FUNCTIONS IN MAN

The effect of ageing on the immune capacities of man has been hardly discussed up to now. Therefore, this section of the Introduction will be devoted to studies on age-related changes in the immune system and immune responsiveness of man.

In section D.1, it was reported that the titre of natural antibodies gradually decreases during ageing. This decline is thought to reflect a reduced activity of the humoral immune system in man. In the only study where a primary humoral immune response to a given antigen was determined, the following results were obtained (Roberts-Thomson et al., 1974). After immunization with flagellin, the levels of total antibody were comparable in young adults and persons over 60 years of age. The IgG antibody formation to flagellin was also similar in young and old persons at the peak of the response, but afterwards a decline in IgG antibody titres in the serum of old individuals was observed. This result as well as the decrease in natural antibodies lead to the conclusion that there is an age-related failure to retain antibody levels.

On the basis of these findings, one should expect that serum immunoglobulin (Ig) levels change during ageing. However, many studies have indicated that the overall Ig levels in sera of ageing humans did not show a decrease. On the contrary, a gradual increase in the levels of IgG and IgA was found, while IgM remained at about normal levels (Hallgren et al., 1973; Buckley, Buckley and Dorsey, 1974; Radl et al., 1975, and Riesen et al., 1976). The Ig levels showed a marked in-

crease in variability during ageing. In addition, an imbalance in the κ/λ ratio and the occurrence of restricted heterogeneity of the Ig can frequently be seen in the sera of old humans; these findings suggest that the changes are not quantitative but rather qualitative, in other words, that the antibody repertoire is altered during ageing (Radl et al., 1975, and Riesen et al., 1976).

Various immune parameters have been used to study cellular immunity with human ageing. These studies include in vivo delayed hypersensitivity (DHS) reactivity and in vitro mitogen responses and mixed lymphocyte reactions. Expression of DHS type reactivity was assessed by summation of positive responses to ubiquitous antigens to which previous exposure could be expected. There was a progressive fall in number of positive reactions throughout life, especially after the age of 60 years (Toh et al., 1973; Roberts-Thomson et al., 1974, and Girard et al., 1977). Responsiveness was significantly lower in patients with immunopathological diseases (Toh et al., 1973). Age-related changes in a primary DHS response were determined by Girard et al. (1977) using dinitrochlorobenzene (DNCB) as antigen. The results demonstrate that the majority of persons over 65 years of age failed to become sensitized to DNCB.

The mitogen responsiveness of peripheral blood lymphocytes to ConA and PHA has been widely used to study cell-mediated immunity with advancing age. Nearly all studies revealed that the capacity to respond to T cell mitogens was markedly depressed in old age (Pisciotta et al., 1967; Hallgren et al., 1973; Roberts-Thomson' et al., 1974; Weksler and Hütteroth, 1974; Fixa, Komárková and Chmelař, 1975, and Girard et al., 1977). Ben-Zwi et al. (1977), however, are the only authors who found no difference in ConA and PHA responses between young adult and old individuals.

The mixed lymphocyte response of old persons was decreased when compared with the response of young persons (Weksler and Hütteroth, 1974).

The results obtained in the above studies justify the conclusion that changes in cell-mediated immunity in man are more striking than changes observed in humoral immunity with age.

Whether this decline in cellular immune responses was due to either quantitative or qualitative changes in the T cell compartment has been investigated by several groups. Human T cells are capable of forming nonimmune rosettes with sheep red blood cells. This property has been used to establish changes in the number of T cells in the peripheral blood, although it is uncertain whether the number of peripheral T cells accurately reflects the proportion of T cells in

the body. Unfortunately, the results of the various studies do not allow a definite conclusion. A significant decrease in the proportion and total number of T cells was reported by Smith, Evans and Steel (1974), Carosella, Mochanko and Braun (1974), Diaz-Jouanen, Williams and Strickland (1975), Girard et al. (1977), and Ben-Zwi et al. (1977), whereas Weksler and Hütteroth (1974), Cohnen et al. (1975), and Davey and Huntington (1977) found a constant number of T cells during ageing.

The data obtained in studies dealing with age-related changes in the number of B cells in the peripheral blood are not conflicting. The percentage and total number of B cells as judged by the presence of membrane-bound Ig remained the same during ageing (Weksler and Hütteroth, 1974; Gajl-Peczalska et al., 1974; Diaz-Jouanen, Williams and Strickland, 1975; Cohnen et al., 1975; Ben-Zwi et al., 1977, and Davey and Huntington, 1977).

Whether the immune deficiency in old age may lead to immunological aberrations and diseases of a possible immunological origin has been discussed in the previous section of the Introduction. As stated earlier, the complex interrelations between immune deficiencies and diseases such as infections, autoimmune phenomena, glomerulonephritis, amyloidosis and neoplasia have still to be elucidated.

In man, the prevalence of autoimmune phenomena with age has been extensively demonstrated (reviewed by Walford, 1969, and Mackay, 1972). More autoantibodies were found in females of all ages. An attempt was made to find a correlation between the occurrence of autoantibodies and various immune parameters. Studies of Hallgren et al. (1973) and Riesen et al. (1974) revealed that correlations could not be detected between serum Ig levels, titres of autoantibodies and T cell mitogen responses. On the other hand, Mackay (1972) found a significant correlation between autoantibodies and degenerative vascular diseases in a large population study in Australia.

In summary, the available data show that immunological functions do decline with advancing age in man. Changes in cellular immune functions are more prominent than changes in humoral immune functions, where qualitative rather than quantitative alterations have been observed. These results are suggestive for a failing thymus function with age in man, since cell-mediated immune responses are carried out by T cells and these cells have an important regulatory role in the maintenance of the Ig diversity.

I. OUTLINE OF THE PRESENT STUDY

The previous sections of the Introduction presented evidence for an age-related decline in humoral and cellular immune functions in both experimental animals and man on the basis of data from the literature.

In the present study, the effect of ageing on the humoral immune response of mice is investigated in detail in order to obtain more insight into the nature of the decline in immune functions with age. Therefore, various strains of mice which differ in their immunological ageing behaviour are compared. Attention will be paid to the role of the thymus and T cells in the ageing of the humoral immune system.

The following Chapter deals with the experimental procedures and techniques which have been used during this study. Emphasis is placed on a micro *in vitro* immune response system.

In Chapter III, the strains of mice which have been compared during this study are introduced. The survival patterns and age-related spontaneous lesions characteristic for each strain are described.

Chapter IV is devoted to age-related changes in the *in vivo* primary humoral immune response in the spleen of mice of the different strains. Antibody formation with or without T cell influence will be compared.

In addition to the study of the primary immune response in the spleen, the effect of ageing on a secondary immune response in both the spleen and bone marrow of one of the strains under investigation is described (Chapter V).

To eliminate *in vivo* environmental factors which may influence the immune system of aged mice, the capacity to form antibodies *in vitro* is investigated in young and ageing mice. The results are presented in Chapter VI. A comparison of the changes in the *in vivo* and *in vitro* antibody formation of ageing mice is included.

Chapter VII deals with literature data and our own attempts at the restoration of impaired immune responses in aged mice by thymus transplantation. A future approach to improve the failing immune functions in old age may be found in the administration of thymic humoral factors. These factors are thought to influence the maturation of precursor T cells to functional T cells. However, before application of thymic humoral factors in immuno-gerontological studies, more basic information on the nature and mode of action of the factors has to be accumulated. In this Chapter, various thymic factors either derived from thymic extracts or thymic epithelial cultures are introduced.

Their effect on an *in vitro* humoral immune response of spleen cells from athymic nu/nu mice is shown and discussed.

The final Chapter (VIII) contains a general discussion of the results obtained in the above studies.

CHAPTER II

MATERIALS AND METHODS

A. ANIMALS

The following strains of mice were used: CBA/BrARij, C57BL/KaLwRij, NZB/Lac and BALB/cAnCrlRij (for a list of abbreviations for symbolizing substrains, see Staats, 1976). The strains will hereafter be referred to as CBA, C57BL/Ka, NZB and BALB/c. The mice have been inbred in Rijswijk by brother-sister matings and are presently in their 68th (CBA), 34th (C57BL/Ka), 12th (NZB) and 26th (BALB/c) inbred generation.

The survival curves and the age-related pathology of the strains will be presented in Chapter III.

In addition, homozygous athymic nude (nu/nu) mice as well as their normal litter mates were used for a part of the study. The nu/nu mice were bred by intercross and backcross either to CBA (6th generation) or to BALB/c (4th generation).

The mice were born and reared under conventional conditions at the REP[1] Institutes TNO (Rijswijk, The Netherlands). Cohort groups of animals of the CBA, C57BL/Ka, NZB and BALB/c strains born in the same week were transferred to the animal quarters of the Institute for Experimental Gerontology. Cohort groups for experimental studies were separately reared from the cohort groups which were allowed to live out their life-spans and were used for longevity studies. All mice were housed under conventional conditions in polycarbonate Makrolon® cages with a bedding of sterilized wood shavings and fed a commercial pellet diet (AM II, Hope Farms, Woerden, The Netherlands). Water was provided ad libitum. Approximately 15 mice were kept per cage.

B. ANTIGENS AND IMMUNIZATION

Sheep red blood cells (SRBC) and lipopolysaccharide (LPS) from E. coli were used as thymus-dependent and thymus-independent antigens, respectively.

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[1] REP stands for the Radiobiological Institute, Institute for Experimental Gerontology and Primate Center.

Sheep blood was obtained from sheep from our own colony. During the course of the study, two sheep in succession were used as donors. They were selected because their red blood cells induced a high response after stimulation in in vitro cultures (see D) and a low number of background PFC in nonstimulated cultures.

The sheep blood was diluted with an equal volume of Alsever's solution. This stock suspension was kept at 4°C and renewed every 2-3 weeks. The concentration of erythrocytes in the Alsever's solution was counted in a haemocytometer. Before immunization, the desired number of red cells was washed three times (1300 g, 5 min) with sterile saline and resuspended to the appropriate concentration in sterile saline.

LPS from E. coli 055:B5 was obtained from Difco Laboratories (Cat. No. 3120-25, Detroit, Mi., U.S.A.). It was prepared according to the phenol-extraction method (Westphal, Lüderitz and Bister, 1952). For some experiments, it was necessary to decrease the toxicity of LPS in order to study the immune response of old NZB mice to it. Detoxification was performed with weak alkali as described by Britton (1969). Both LPS preparations were diluted in sterile saline to the desired concentration.

The antigens were injected in a volume of 0.5 ml into the tail veins.

C. IN VIVO RESPONSE TO SRBC AND LPS

1. Spleen and bone marrow cell suspensions

Mice were killed with aether at various times following immunization. Spleens and/or femurs were removed immediately after killing and placed into a balanced salt solution (BSS). This solution was prepared according to Mishell and Dutton (1967) and was supplemented with 5 per cent newborn calf serum (Flow Laboratories, Irvine, Scotland).

The spleen was cut into small pieces with a pair of scissors and the fragments were pressed through a sieve consisting of six layers of nylon gauze. During this procedure, the sieve was washed with BSS. Bone marrow cells were obtained by flushing the femurs with BSS. A bent needle was inserted into the distal end of the shaft and moved slightly up and down to break the small bone spicules. Bone fragments were removed by filtering the cells through a nylon sieve. The spleen and bone marrow cells were collected in calibrated glass tubes and

were washed once with BSS by centrifugation (150 g, 10 min). The cells obtained from single spleens and femurs were usually suspended in 5 ml and 2 ml BSS, respectively. All manipulations were carried out at $0^{o}C$ and the cell suspensions were kept in melting ice before use.

2. Assay for antibody forming cells

The plaque forming cell (PFC) assay was employed for the determination of the number of spleen or bone marrow cells which released antibodies after immunization. In the original PFC assay of Jerne and Nordin (1963), the plaques were developed in an agar layer. A modification of the PFC assay, in which plaque formation takes place in a liquid monolayer, was used during this study (Cunningham and Szenberg, 1968, and Zaalberg, Van der Meul and Van Twisk, 1968). The procedure was as follows.

Cells were taken from the spleen or bone marrow cell suspensions and diluted in BSS to an adequate concentration. This adequate concentration was based on the expected number of PFC per spleen or femur so that approximately 100 PFC would develop in the incubation chamber. To avoid a high density of cells in the incubation chamber, the maximum concentration was 4 x 10^{7} cells per ml. If less than 100 PFC per chamber were expected, more than the usual two chambers (see below) were prepared per spleen or femur in order to obtain a reliable number of PFC. To 0.15 ml of the diluted cell suspension, 0.15 ml of a 3% suspension of SRBC or coated SRBC (see C.3) and an appropriate amount of complement (see C.4) were added. The SRBC or coated SRBC were washed three times by centrifugation and resuspended in BSS. The incubation mixtures, which were kept in melting ice during preparation, were then warmed up in a $37^{o}C$ water bath to avoid the formation of air bubbles in the incubation chamber. Samples of each mixture were applied to two glass chambers (see C.5) with a calibrated pipette. The volume of the chambers was determined. The chambers were sealed with warm vaseline ($60^{o}C$, Vaselinum album, Brocacef, Maarssen, The Netherlands) and incubated at $37^{o}C$ for 90 minutes.

During the incubation, the concentration of viable nucleated cells in the original spleen and bone marrow cell suspensions was determined in order to allow calculation of the number of PFC per 10^{6} spleen or bone marrow cells in addition to the number of PFC per organ. The cells were counted in a haemocytometer and the viability was assessed by trypan blue exclusion (0.2% trypan blue in BSS).

Immediately after incubation, all chambers were read at 16x magnification using a dissecting microscope.

3. Coating of SRBC with LPS

For the determination of anti-LPS antibody forming cells, SRBC coated with LPS by the method of Andersson and Blomgren (1971) were used as targets in the PFC assay. LPS (1 $mg.ml^{-1}$ phosphate buffered saline, PBS) was boiled for 2 hours while the pH was kept at pH 8.0 by adding 0.1 M NaOH. To 1.5 ml of the boiled LPS solution, 0.5 ml of packed, three times washed SRBC were added. This mixture was incubated at 37°C for 45 minutes. Fresh batches of SRBC-LPS were prepared daily during the course of an anti-LPS response. The extent of coupling was routinely tested for by measuring the haemagglutination titre of a standard mouse anti-LPS serum with each batch of SRBC-LPS. Each spleen cell suspension was assayed with both control and coated SRBC and the number of anti-SRBC-PFC was subtracted from the number of anti-SRBC-LPS-PFC to give the number cells producing antibodies directed against LPS.

4. Complement

Normal guinea pig serum was used as the source of complement. Blood was collected from guinea pigs (Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands) by cardiac puncture and allowed to clot at room temperature for only one hour in order to preserve the complement activity. Serum was removed from the clotted blood after centrifugation at 4°C (1300 g, 20 min).

Naturally occurring antibodies in the guinea pig serum directed against SRBC and murine antigens were removed by absorption of the serum with SRBC and mouse red blood cells (MRBC). One volume of a mixture of packed SRBC and MRBC was added to two volumes of undiluted guinea pig serum. The absorption was carried out at 0°C for one hour with occasional stirring. Complement used for the anti-LPS PFC response was absorbed with SRBC coated with LPS. The absorbed complement was stored at -70°C in small volumes.

The amount of complement giving an optimum number of PFC was determined for each batch of complement. This quantity varied from 0.05-0.07 ml per incubation mixture for the PFC assay. One-half of the batches had an optimum at 0.06 ml. A representative experiment is shown in Fig. II.1A. The number of PFC found with the optimum amount of complement varied sometimes from batch to batch. Therefore, the same batch of complement was used within one series of experiments.

Later in this study, a commercially available, lyophilized complement was used (Grade B, Cat. no. 8-720 G, Flow Laboratories,

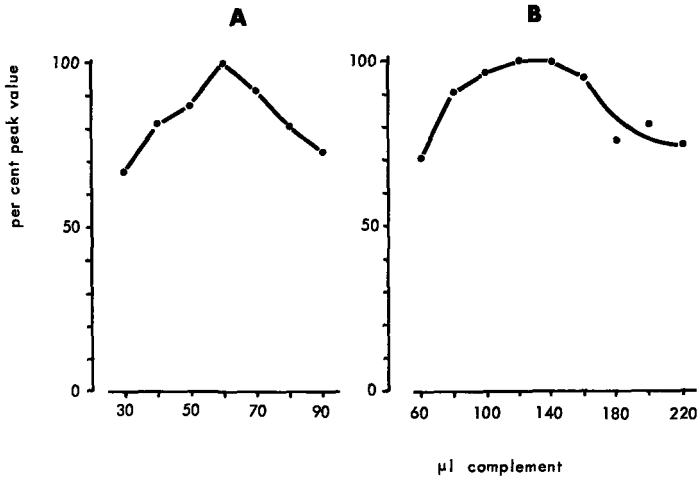

Figure II.1 Effect of the amount of fresh undiluted complement (A) and commercial 1/3 diluted complement (B) on the development of IgM-PFC.
Pooled spleen cells of 5 CBA mice 4 days after immunization with 4 x 10^8 SRBC were used.

Irvine, Scotland). The lyophilized material was redissolved in distilled water. The absorption procedure was also changed. The absorption with MRBC was replaced by absorption with agarose (BDH Chemicals, Poole, England). Cohen and Schlesinger (1970) have shown that absorption of complement with either agar or agarose effectively eliminates the antibodies directed against mouse antigens. This method was also effective in removing the antibodies to LPS (Zaalberg, personal communication). To minimize the loss of complement, the agarose was swollen in saline and spun down before use. The complement was diluted threefold in saline for the same reason. Anti-SRBC antibodies, if present, were eliminated by absorption with packed SRBC. The optimum amount of the diluted complement which had to be added to the mixture of spleen or bone marrow cells and SRBC was determined for each batch. A representative experiment is shown in Fig. II.1B. The addition of 0.10-0.16 ml diluted complement to the cells resulted in maximum numbers of PFC. The quantity chosen as optimal was 0.12 ml. Comparable dose-response curves were found for the various commercial complement batches used during this study.

5. Incubation chambers

The incubation chambers were prepared in the following way. Double-sided adhesive tape (Gudy, no. 5492, Neschen International, Bückeburg, Western Germany) was cut into 4 mm wide slips (Majoor, Van

't Veer and Zaalberg, 1975). The slips were laid along the long sides of clean 76 x 26 mm microscope slides. A glass cover slip (46 x 24 mm) was pressed firmly onto the tape. The content of these chambers was approximately 0.08 ml.

6. Indirect plaque formation

The PFC assay as described above is limited to the detection of cells forming antibodies of the IgM class (Dresser and Wortis, 1965) and is commonly called the direct PFC assay. Cells forming antibodies of another immunoglobulin class become visible in an indirect assay in which an antiserum directed against that class is added to the incubation mixture for the PFC test (Šterzl and Říha, 1965, and Dresser and Wortis, 1965).

During this study, the indirect assay was used to determine cells forming antibodies of the IgG class. An antiserum against mouse IgG was raised according to the method of Milgrom, Luszczymski and Dubiski (1956). The procedure consists of the immunization of rabbits with autologous erythrocytes coated with murine antibodies directed against these erythrocytes. The advantage of this method is that extensive purification of mouse Ig before immunization is avoided.

To obtain antibodies predominantly of the IgG class, mice were hyperimmunized with rabbit red blood cells (RRBC). The mice were primed intraperitoneally (i.p.) with 0.5 ml of a 5% suspension of RRBC. Four weeks later, 6 injections with the same dose (twice a week for 3 weeks, i.p.) were given. The mice were bled two weeks after the last immunization. The titre of the pooled sera was $1/2^{13}$, as measured by haemagglutination (see C.8).

Rabbit erythrocytes were incubated with a subagglutinating dilution of the mouse-anti-RRBC serum (one dilution past the end point of the titration) at 37°C for 1 h and then left overnight at 4°C. The coated erythrocytes were extensively washed with PBS to remove the unbound mouse serum proteins.

Six intravenous injections of the coated and washed erythrocytes (10 ml of a 5% suspension) were given twice a week for 3 weeks to the same rabbit from which the erythrocytes were originally obtained. Blood was collected from the rabbit one week after the last immunization. The haemagglutination titre of the rabbit-anti-mouse-Ig serum determined against the coated erythrocytes was $1/2^{14}$.

Immunoelectrophoresis using a serum pool of normal mice revealed that the antiserum reacted with both IgM and various IgG subclasses. To remove the anti-IgM, anti-light chain and anti-Fab activity, the antiserum was absorbed by two different immunoadsorbents. A column of

Fab fragments of mouse heterogeneous IgG coupled to Sepharose 4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden) was employed to remove anti-light-chain and anti-Fab activity. IgM-λ (MOPC-104E) isolated and polymerized by glutaraldehyde served as an immunoadsorbent for the anti-IgM antibodies (for technical details, see Bloemmen et al., 1976) After these absorptions, the antiserum was tested for specificity in an immunodiffusion technique against purified mouse paraproteins of all individual classes and subclasses. The paraproteins were isolated from sera of mice bearing plasma cell tumours (Bloemmen et al., 1976). A sensitive immunodiffusion technique performed in agar plates containing 3% polyethyleneglycol 6000 was used (Harrington, Fenton and Pert, 1971). The antiserum was shown to be specific for IgG, i.e., the γ1, γ2a, γ2b, and γ3 heavy chains. No activity against IgM, IgA and Fab was observed.

To determine the optimum dilution of the antiserum for the development of the greatest numbers of IgG-PFC, 0.01 ml aliquots of varying antiserum dilutions were added to the incubation mixtures for the PFC test. Ten-to-eighty-fold dilutions of the antiserum gave the highest numbers of PFC (Fig. II.2). A 20-fold dilution was routinely used in the indirect plaque test. Furthermore, it was demonstrated that

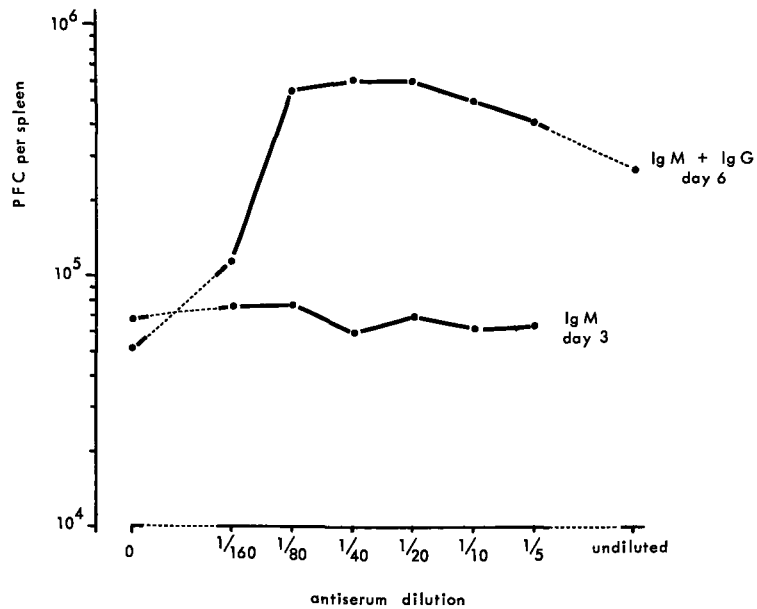

Figure II.2 Effect of rabbit anti-mouse IgG serum on the development of IgM- and IgG-PFC.
Aliquots of 10 ul antiserum of varying dilutions were added to the mixture of complement, spleen cells and SRBC. Pooled spleen cells of 5 CBA mice 3 and 6 days after immunization with 4 x 10^8 SRBC were used.

addition of the antiserum in this dilution did not inhibit IgM-plaque formation (Fig. II.2). This was tested on day 3 after immunization, since IgG antibody forming cells do not appear before the 4th day after immunization with SRBC (Benner et al., 1974).

The anti-mouse-IgG serum described here was used throughout the study. The antiserum distributed in small aliquots was stored at -70°C.

7. Calculations and statistics

The following formula was used to calculate the number of PFC per spleen or femur:

$$\text{PFC per spleen or femur} = \frac{A}{B} \times C \times \frac{DE}{F}$$

A: Total volume of the incubation mixture consisting of spleen or bone marrow cell suspension, complement and SRBC suspension (and anti-IgG serum in the indirect assay);
B: the summed volume of the chambers to which the mixture was applied;
C: number of PFC in these chambers;
D: original volume of the cell suspension obtained from one spleen or one femur (commonly 5 ml and 2 ml, respectively);
E: factor by which the original cell suspension was diluted;
F: volume of the cell suspension which was added to the incubation mixture.

From the number of PFC counted in one femur, the number of PFC present in the marrow of the whole animal can be estimated. The data of Chervenick et al. (1968) which showed that one femur contains 5.9 per cent of the total bone marrow cells in mice were used.

The number of IgG-PFC was calculated by substracting the number of IgM-PFC obtained in the direct assay from those obtained in the indirect assay (IgM + IgG-PFC) only if the number of PFC in both assays differed significantly. The significance was calculated as follows.

The number of PFC taken from one cell suspension and divided over separate incubation chambers can be expected to follow a Poisson distribution. The standard deviation (SD) of the distribution of p plaques was calculated by Jerne as the square root of $(p + 0.004\ p^2)$ in which formula an estimate for the variation due to technical inaccuracies is included (Jerne et al., 1974). The presence of IgG-PFC was considered to be significant when there was no overlap between the

upper limit (p + 2 SD) of the number of IgM-PFC in the direct assay and the lower limit (p - 2 SD) of the number of IgM + IgG PFC in the indirect assay.

The following statistical approach was used to compare the PFC response of experimental groups of mice. Since the PFC response in mice is not normally distributed and since a normal distribution is required for the application of most statistics, a transformation of the data is necessary. Gottlieb (1974) found that the log transformation provides a close approximation to normalize the statistical distribution of plaque-forming cells. Therefore, the number of PFC was logarithmically transformed and geometric mean, standard deviation and standard error were thus calculated. Significance of differences between experimental groups was assessed by either a two-tailed Student's t-test or by calculating the 95 per cent confidence limits. If the 95 per cent confidence limits of two means did not overlap, the means were considered to differ significantly.

8. Antibody titrations

Circulating antibodies were measured by a haemagglutination assay. Blood was collected from the retro-orbital sinus of the mice and allowed to clot at room temperature. Serum was separated by centrifugation and stored at -20°C. Serial twofold dilutions in PBS were made by an Automatic Diluter in U-shaped microtitre plates (both from Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va., USA). An equal volume (0.025 ml) of a 2 per cent SRBC or SRBC-LPS (C.3) suspension in PBS was added to the serum dilution. For the determination of 2-mercaptoethanol (2-ME) resistant antibody titres, 0.025 ml of 0.2M 2-ME, in addition to SRBC, was added to each serum dilution. The plates were incubated at 37°C for 1 h and overnight at 4°C. Titres of anti-SRBC sera were recorded as the reciprocal of the highest dilution of serum showing macroscopic agglutination. Agglutination of anti-LPS sera was examined under a microscope with a magnification of 25x to be able to discriminate between sham-agglutination and real agglutination.

D. IN VITRO RESPONSE TO SRBC

For the in vitro immunization of murine spleen cells with SRBC, two modifications of the culture system of Mishell and Dutton (1967)

were employed. The initial experiments were carried out in a macroculture system using petri dishes. A microculture system was developed later. The advantage of the latter system is its greater capacity per experiment and the economical use of mice, medium and calf serum. The culture conditions of the two systems will be described separately. A table at the end of this section summarizes the different culture conditions of the two systems.

1. Spleen cell suspension

The mice were sacrificed with aether. The spleens were removed under sterile conditions and collected in supplemented Ham's F12 medium (Ham, 1965; see D.2). Cell suspensions were prepared as described in part C.1, except that Ham's F12 medium was used instead of BSS. The cell suspensions were collected in calibrated tubes (2070, Falcon, Oxnard, USA) and washed once. After counting the number of cells in a haemocytometer and assessing the viability of the cells by trypan blue exclusion, the cell number was adjusted to the adequate cell concentration. The desired number of SRBC was washed three times (1300 g, 5 min) with the culture medium and resuspended in the appropriate volume. The suspensions were kept in melting ice before culturing.

2. Macroculture system

A modification of the original Mishell and Dutton (1967) system as described by Rossi and Zaalberg (1974) was used. The spleen cells were cultured in Ham's F12 medium (1F-100, Flow Laboratories, Irvine, Scotland) supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% foetal calf serum (see section D.4). The medium was buffered with 0.118% (w/v) sodium bicarbonate according to the publication of Ham (1965). The cell suspensions, containing spleen cells and SRBC in a total volume of 1 ml medium, were cultured in 35 x 10 mm plastic petri dishes (Greiner, Würtingen, West Germany). The dishes were placed in a box and incubated at 37°C in a humidified atmosphere of 5% CO_2, 7% O_2 and 88% N_2. The box was placed on a rocker platform (Bellco Glass Inc., Vineland, New Jersey, USA) which rocked at 3-4 cycles per minute. Twenty-four hours after the start of the culture, 2 ml of supplemented medium was added to the dishes. This modification made daily addition of a nutritional mixture unnecessary (Rossi and Zaalberg, 1974).

At the time of harvest, the cultured cells were resuspended by gentle agitation and collected. The remaining cells were harvested by washing the petri dishes with BSS supplemented with 5% newborn calf

serum and by scraping the dish with a plastic policeman. This procedure was done twice. All cells from one culture were collected in a calibrated glass tube. After centrifugation (150 g, 10 min), the cells were resuspended in supplemented BSS in an appropriate volume depending on the number of PFC to be expected. The number of PFC was determined as described in part C of this Chapter. Only IgM antibody producing cells were determined, since no IgG-PFC is to be detected in a primary *in vitro* response against SRBC (Pierce, 1969; Van Muiswinkel and Van Soest, 1976, and personal observation). The cell survival was assessed by trypan blue exclusion.

The optimum spleen cell concentration found by Mishell and Dutton (1967) was 1-2 x 10^7 spleen cells per culture. This high cell density is an absolute requirement for successful *in vitro* immunization (Schreier and Nordin, 1977). The concentrations compared by us were 10^7 and 2 x 10^7 spleen cells per culture. As shown in Fig. II.3, 2 x 10^7 spleen cells gave a better response than 10^7 cells and 2 x 10^7 cells were therefore routinely used in the macrocultures. In the same figure, the effect of antigen dose is presented. The number of PFC was

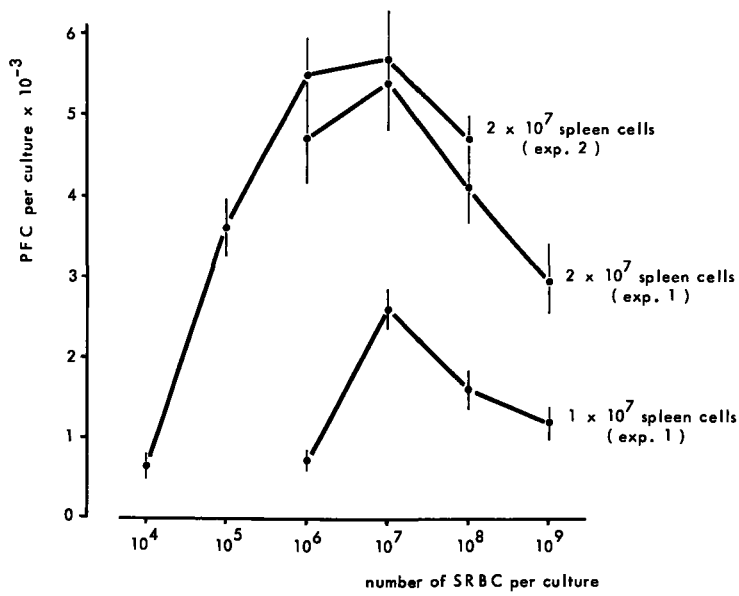

Figure II.3 Effect of the number of SRBC on the response of 10^7 and 2 x 10^7 spleen cells in the macroculture system.
Each point represents the mean PFC ± S.D. of three cultures after four days of culture. Pooled spleen cells from 5 CBA mice were used in both experiments. Numbers of PFC in the absence of SRBC: 65 ± 10 (exp. 1; 10^7 spleen cells), 117 ± 24 (exp. 1; 2 x 10^7 spleen cells), 322 ± 33 (exp. 2; 2 x 10^7 spleen cells). Cell survival varied from 20-30 %.

determined at day 4. When 2×10^7 spleen cells were cultured, a significant response was already observed with 10^5 SRBC. An optimum number of PFC was detected with 10^7 SRBC. When not stated otherwise, the cultures in the macrosystem contained this number of SRBC.

In most instances, the highest response was found on day 4. Occasionally, larger or similar numbers of PFC were obtained after 5 days of culture (see Chapter VI). Unless stated otherwise, results represent determinations after four days of culture.

3. Microculture system

For the microcultures flat-bottomed microtest II™ tissue culture plates (3040, Falcon, Oxnard, USA) were used. The spleen cells were cultured in a volume of 0.2 ml medium. The cells and SRBC were mixed and 0.2 ml aliquots were dispensed into the wells of the culture plate.

During the pilot experiments, it was observed that fewer PFC developed in the wells at the outside of the culture plate when compared to the wells in the centre. Therefore, the outermost wells were not used for culturing but filled with medium. The same box as described above was used for incubation. The cells in the microsystem could be cultured without rocking and extra nutrition (Armerding and Katz, 1974a; Pike, 1975, and personal observation during pilot experiments).

The optimum numbers of both spleen cells and SRBC in the microcultures were investigated. Since many experiments with spleen cells from nu/nu mice were done in this phase of the study (see Chapter VII), the culture conditions were assayed for both CBA and nu/nu mice. The data illustrated in Fig. II.4 show that 10^6 spleen cells per culture gave the best responses for both CBA and nu/nu mice. The optimum number of SRBC was 10^6 SRBC.

As was found with the macroculture system, optimum numbers of antibody-forming cells developed after 4 culture days, although similar or somewhat higher responses were occasionally observed on day 5. The response was routinely determined on day 4.

The supplemented Ham's F12 as used for the macrocultures appeared not to be sufficient in supporting the microcultures, since very low and variable responses were obtained during initial experiments. According to Pike (1975), the type of buffering system employed in microcultures is crucial. Therefore, the buffering system was modified by the addition of 20 mM HEPES. Since bicarbonate should also be added as a nutritional requirement, the effect of various concentrations of

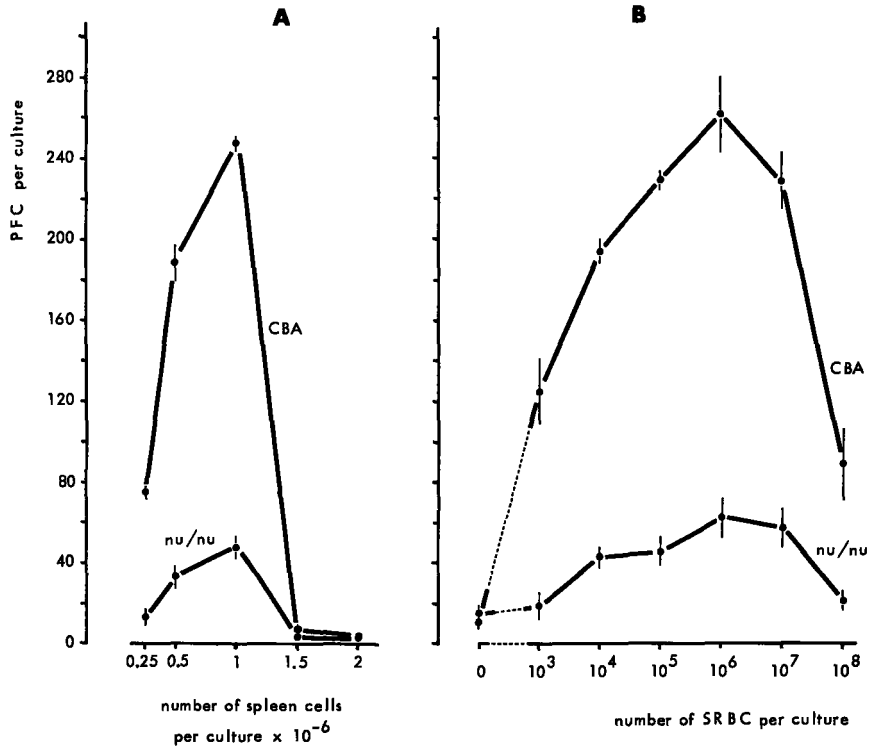

Figure II.4 Effect of the number of spleen cells (A) and the number of SRBC (B) on the in vitro anti-SRBC response of CBA and nu/nu mice in the microculture system.
Each point represents the mean PFC ± S.D. of quadruplicate cultures harvested on day 4. Pooled spleen cells from 3 CBA mice and 3 nu/nu mice were used.

$NaHCO_3$ as suggested by others (Pike, 1975; Manual Flow Laboratories, Irvine, Scotland) was investigated. A representative experiment in which spleen cells from CBA, nu/nu and C57BL/Ka mice were cultured with the various buffer combinations is shown in Table II.1. The induction of antibody forming cells was improved by the addition of 20 mM HEPES (HEPES buffer, pH 7.3, Gibco-Cult, Glasgow, Scotland). Optimum responses were obtained with 0.2% $NaHCO_3$ (w/v) in the culture medium in addition to HEPES. This buffer combination was therefore routinely used in subsequent experiments. The observed improvement in the response was not due to a better survival of the cultured cells, since comparable numbers of viable cells were recovered (Table II.1). It must be noted that cells from C57BL/Ka mice were less sensitive to changes in the buffer system than were those from CBA and nu/nu mice. Furthermore, cells from C57BL/Ka mice exhibited a higher in vitro re-

sponse than those from CBA mice, confirming previous data of Click, Benck and Alter (1972b). Since the anti-SRBC response is partially thymus-dependent (see Chapter IV, part B), the in vitro response of athymic nu/nu mice is lower than that of other mice under optimum culture conditions.

At the end of the culture period, the culture supernatant was removed by inverting the culture plate and gently shaking. Residual medium on top of the plate was immediately removed by tissue paper. The cultured cells were resuspended by adding BSS supplemented with 5% newborn calf serum and by shaking the plates on a microshaker (AM 69, Cooke Microtitre System, Dynatech Laboratories Inc., Alexandria, Va., USA). The volume of the added BSS was dependent on the expected number of PFC in the wells. When fewer than 200 PFC were expected to be obtained, the cells were resuspended in 0.07 ml BSS. Further addition of 0.06 ml complement (diluted 1/3, see C.4) and 0.07 ml of a 4% SRBC suspension completed the plaque revealing mixture. After thorough mixing on the microshaker, the suspensions were transferred to the incubation chambers as described in C.5. When more than 200 plaques were expected in the cultures, the cultured cells were resuspended in 0.05 ml supplemented BSS. From these starting suspensions, appropriate dilutions were made in the wells of a new microculture plate so that, after addition of complement and SRBC, approximately 100 plaques would develop per chamber.

For the determination of cell survival, separate cultures were included in the experiments.

The standard deviation within each set of replicate cultures varied from 5-20%. A larger variation was found when the culture plates were centrifuged and the supernatant removed by inverting the culture plates. Individual harvesting of the cultures by transferring the cell suspensions to tubes and washing the wells did not improve the reproducibility and was time consuming.

A summary of the differences in culture conditions between the macro- and microculture systems is presented in Table II.2.

4. Foetal calf serum and mercaptoethanol

For culturing lymphoid cells under physiological conditions, it would be preferable to use medium containing syngeneic or allogeneic serum. However, normal mouse serum did not support a primary in vitro immune response to SRBC (Mishell and Dutton, 1967); instead, it inhibited the response (Veit and Michael, 1972a, and Schreier and Nordin, 1977). The concentration of this serum inhibitor increased after im-

TABLE II.1

EFFECT OF HEPES AND VARIOUS AMOUNTS OF $NaHCO_3$ ON THE MICRO IN VITRO ANTI-SRBC RESPONSE OF CBA, nu/nu AND C57BL/Ka MICE

cultures set up with		CBA		nu/nu		C57BL/Ka	
per cent $NaHCO_3$ (w/v)	20 mM HEPES	PFC per culture	per cent cell survival	PFC per culture	per cent cell survival	PFC per culture	per cent cell survival
0.118	-	40 ± 9	36	11 ± 4	17	259 ± 30	27
0.085	+	57 ± 12	34	38 ± 8	26	369 ± 33	26
0.118	+	76 ± 6	37	41 ± 3	25	260 ± 31	30
0.200	+	218 ± 7	30	62 ± 5	24	464 ± 22	30
0.360	+	116 ± 18	32	52 ± 11	22	338 ± 25	31

Data represent the mean PFC ± S.D. of 5 replicate cultures determined after 4 days of culture. Pooled spleen cells from 4-5 mice of each strain (3 months of age) were used. Cultures contained 10^6 spleen cells and 10^6 SRBC.

Table II.2

DIFFERENCES IN THE CULTURE CONDITIONS BETWEEN THE MACRO- AND MICROSYSTEMS FOR IMMUNIZATION IN VITRO

culture conditions	macro	micro
culture vessel	petri dishes 35-10 mm	wells of a microtitre plate 6-10 mm
volume	1 ml	0.2 ml
number of spleen cells per culture	20×10^6	1×10^6
number SRBC per culture	10×10^6	1×10^6
rocking	yes	no
per cent $NaHCO_3$ (w/v)	0.118	0.200
HEPES (20 mM)	no	yes
extra feeding	2 ml after 24 h	no

munization (Veit and Michael, 1973). Therefore, foetal calf serum (FCS) is mostly used as the serum source for culturing mouse lymphocytes. It has been recognized that not all FCS batches support the in vitro humoral immune response equally well (Mishell et al., 1972, and Schreier and Nordin, 1977). Shiigi and Mishell (1975) suggested that serum samples which were temporarily contaminated during processing were strongly supportive. Samples which were likely never to have been contaminated were deficient. Schreier and Nordin (1977), however, found no correlation between endotoxin activity and the supportive activity of FCS. In their opinion, the difference between supportive and deficient FCS batches cannot be attributed to a single factor but is of a much more complex nature.

The FCS batches investigated by us are summarized in Table II.3. Serum B from Flow Laboratories (Irvine, Scotland, Cat. no. 4-055, Batch no. 413135 U) was selected and used throughout the time that the macroculture system was employed. The responsiveness of CBA spleen cells to SRBC was highest in the presence of this batch. When this batch was used up, it was replaced by serum E from Gibco Bio-Cult (Glasgow, Scotland, Cat. no. BCL-005, Batch no. U 654501). This gave a similar response to that obtained with serum B in microcultures. Table II.3 also shows a frequently observed phenomenon: the relative number of spontaneous PFC in unstimulated cultures compared to stimulated cultures was larger in the micro- than in the macrocultures. In the macrocultures, this "background" response never exceeded 10% of that obtained by stimulated cultures. The percentages for microcultures varied from 10-20%. The reason for this difference is not clear.

TABLE II.3

SUPPORTIVE EFFECT OF VARIOUS BATCHES OF FOETAL CALF SERUM (FCS) ON THE IN VITRO ANTI-SRBC RESPONSE OF CBA MICE

culture system	FCS batch	PFC per culture + SRBC	PFC per culture - SRBC
macro	A Flow	2569 ± 272	98 ± 14
"	B "	4536 ± 430	240 ± 31
"	C "	1601 ± 157	101 ± 9
"	D "	3302 ± 396	187 ± 23
micro	B Flow	230 ± 28	44 ± 3
"	E Gibco	223 ± 19	33 ± 5

Data represent the mean PFC ± S.D. of 4 replicate cultures determined after 4 days of culture. Pooled spleen cells from 5 mice (3 months of age) were used. Macrocultures contained 2 x 10^7 spleen cells and 10^7 SRBC. In the microcultures, 10^6 of both spleen cells and SRBC were used. The culture medium contained 10% FCS.

Many investigators add 2-mercaptoethanol (2-ME) in a concentration range of 2-5 x 10^{-5} M to the culture media used for lymphoid cells. The enhancing effect of 2-ME on the primary in vitro anti-SRBC response was first described by Click, Benck and Alter (1972a).

Our data concerning 2-ME are presented in Table II.4. Addition of 2-ME to the culture medium neither changed the anti-SRBC response under the culture conditions used by us nor did it influence the cell survival in the cultures. It was therefore decided that the presence of 2-ME was not required in the cultures.

TABLE II.4

EFFECT OF 2-MERCAPTOETHANOL (2-ME) ON THE IN VITRO ANTI-SRBC RESPONSE OF CBA AND nu/nu MICE

		CBA		nu/nu	
culture system	5 x 10^{-5} M 2-ME	PFC per culture	per cent cell survival	PFC per culture	per cent cell survival
macro	-	not done	not done	1256 ± 118	24
	+	not done	not done	1243 ± 131	26
micro	-	271 ± 32	24	71 ± 12	28
	+	263 ± 24	29	70 ± 4	32

Data represent the mean PFC ± S.D. of 4 replicate cultures determined after 4 days of culture. For additional legends, see Table II.3.

2-ME was shown to substitute for macrophages in the in vitro humoral immune response of macrophage-depleted spleen cells (Chen and Hirsch, 1972; Schreier and Nordin, 1977, and Opitz et al., 1977). In addition to this, Opitz et al. (1977) found that 2-ME can convert a

deficient FCS into a supportive one. Mishell et al. (1973) reported that 2-ME had no effect when an optimum FCS batch was used. Opitz et al. (1977) further analyzed the mode of action of 2-ME. The active component is not 2-ME itself but a serum factor which is activated by 2-ME. When this activated serum factor was isolated, it effectively replaced macrophages and FCS in the cultures. In view of the above observations, it is likely that the FCS batches used by us belong to the supportive ones, which cannot be further activated by 2-ME. Furthermore, it may be concluded that the number of macrophages was not limiting in our system.

CHAPTER III

SURVIVAL DATA AND AGE-RELATED PATHOLOGY OF CBA, C57BL/Ka AND NZB MICE

A. INTRODUCTION

The determination of the survival ages combined with data on the natural causes of death of the animals used is a prerequisite for any experimental study on ageing (Hollander, 1973). These baseline data are necessary to establish the 90%, 50%, 10% and maximum survival ages of the animals as well as to select the appropriate animal model for ageing studies (Hollander and Burek, in press). Furthermore, it allows a comparison of data for the same strain maintained at different institutes.

In this Chapter, information on the life-spans and the age-related disease patterns of the three mouse strains CBA, C57BL/Ka and NZB, used most extensively in this study, will be presented.

B. SURVIVAL DATA

Ageing cohorts, each of approximately 30 mice, of male and female CBA, C57BL/Ka and NZB mice were allowed to live out their natural life-spans under well controlled conventional conditions (Hollander, 1976). The ages of the mice varied from 4-12 weeks when they were transferred to the animal quarters of the Institute for Experimental Gerontology. During the past 5 years, such cohorts were established every 3 months. As mentioned in the previcus Chapter, animals for longevity studies were reared separately from the animals used for experiments.

The longevity data of the 3 strains were derived from ageing cohorts established in 1973 and 1974. The C57BL/Ka and NZB mice, which were necropsied and examined histopathologically, were taken from these cohorts but also included untreated control mice from long-term experiments carried out during the same period. With regard to the CBA mice, the histopathological data were collected from animals kept in the above-mentioned ageing cohorts and from untreated mice kept prior to 1973, which died spontaneously. The reason that mice not kept in specific ageing cohorts were also included in the histopathological study was that the loss due to autolysis left only a relatively small number of animals for histological examination. As can be concluded

from Tables III.1, 2 and 3, the survival ages of mice derived from the ageing cohorts were comparable to those of the series of mice examined histopathologically.

TABLE III.1

SURVIVAL DATA OF CBA, C57BL/Ka AND NZB MICE

strain	sex	number of cohorts*	percent survival age (months) 90	50	10	maximum survival (months)
CBA	male	5	15 ± 3** (11 - 17)	29 ± 2 (28 - 30)	33 ± 2 (30 - 35)	34 ± 2 (31 - 36)
	female	5	18 ± 3 (13 - 22)	29 ± 3 (25 - 31)	33 ± 1 (32 - 35)	36 ± 3 (34 - 40)
C57BL/Ka	male	7	16 ± 5 (8 - 21)	24 ± 2 (21 - 27)	29 ± 1 (28 - 30)	31 ± 2 (29 - 33)
	female	6	16 ± 3 (13 - 20)	22 ± 1 (21 - 23)	27 ± 1 (26 - 28)	29 ± 2 (26 - 30)
NZB	male	5	13 ± 1 (12 - 14)	19 ± 1 (18 - 20)	23 ± 1 (21 - 24)	25 ± 2 (23 - 28)
	female	5	9 ± 3 (5 - 12)	13 ± 2 (11 - 16)	17 ± 1 (15 - 18)	20 ± 2 (16 - 21)

* Each cohort consisted of approximately 30 mice.
**Mean ± standard deviation; range of the values between parentheses.

Aged CBA and C57BL/Ka mice used for the experiments reported in the following Chapters were born in 1973 and 1974. The experiments with NZB and BALB/c mice were carried out prior to this time.

The 90%, 50%, 10% and maximum survival ages are shown in Table III.1. Survival curves for each strain are illustrated in Figs. III.1, 2 and 3. Mortality occurring before 3 months of age is not included in the table and the figures, since some of the mice entered the colony at the age of 3 months. Survival curves are characterized by a post-weanling plateau corresponding to the young adult period, a downward bend, a period of rapid decline in the number of survivors and finally a bend to the X axis. According to Comfort (1964), the period of rapid decline can be considered as the onset of senescence for the population studied. In this study, the 50 per cent survival point is accepted as the onset of senescence, in accordance with the definition proposed by Walford (1976). A post-weanling plateau with few deaths (possibly caused by fighting) was observed in the survival curves of male and female CBA, C57BL/Ka and male NZB mice, although the length of this period differed for the different strains. Female NZB mice lacked such a plateau, since the mice began to die at an early age. A comparison of the survival ages in Table III.1 shows that CBA mice had a survival pattern superior (in 50%, tenth decile and maximum survival) to those of C57BL/Ka and NZB mice. The shortest life-span

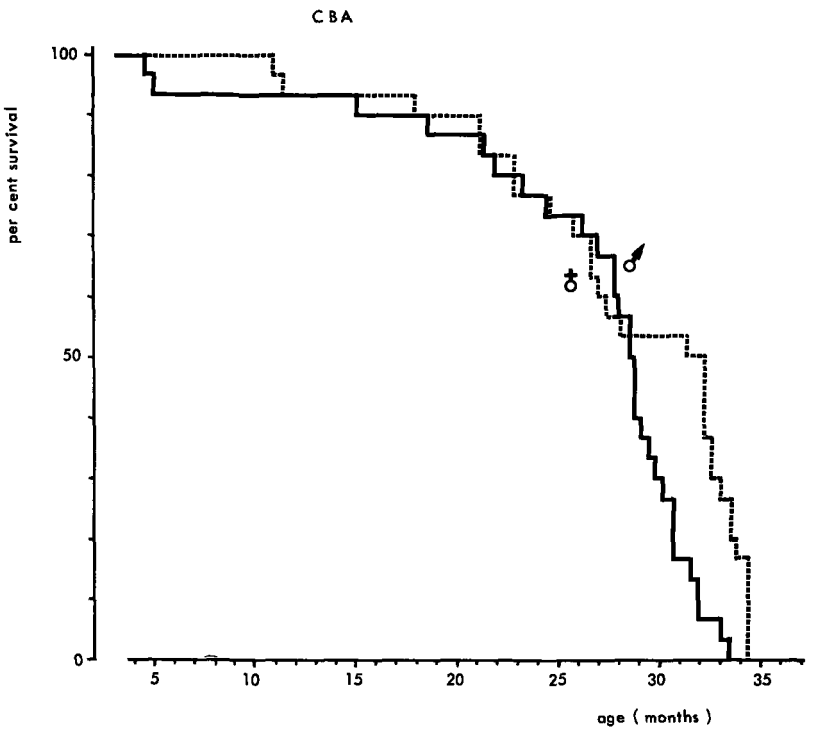

Figure III.1 Survival pattern of CBA mice.
Survival curves of 30 male and 30 female mice belonging to a single ageing cohort are shown.

was observed for female NZB mice. The NZB was the only strain with a difference in survival between male and female mice.

A comparison of the above mentioned data with those reported in the literature for CBA, C57BL and NZB reveals the following picture. Our male and female CBA mice live longer than the CBA/J mice used in the study of Smith, Walford and Mickey (1973). The difference at the 50% survival age is 3 and 7 months and at the maximum survival age 5 months and 9 months for male and female mice, respectively. In the study of Festing and Blackmore (1971), female CBA mice had a mean life-span of 27 months, while the mean life-span for males was only 16 months. The data recently given for substrains of C57BL mice (C57BL/10 and C57BL/6J) show that they have a longer life-span than our C57BL/Ka mice (Smith, Walford and Mickey, 1973; Goodrick, 1975, and Kunstýř and Leuenberger, 1975). Conflicting data are reported with regard to differences in longevity between male and female mice. Smith, Walford and

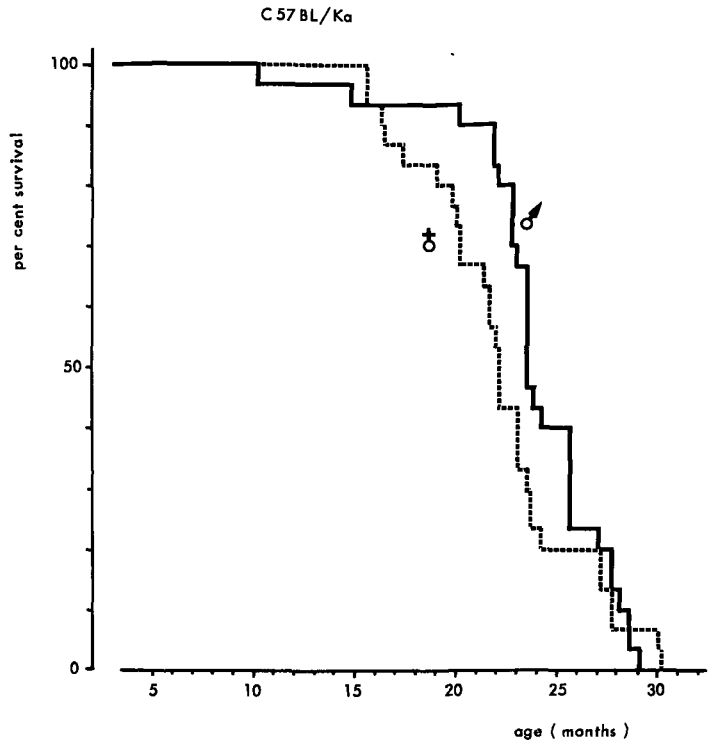

Figure III.2 Survival pattern of C57BL/Ka mice.
Survival curves of 30 male and 30 female mice belonging to a single ageing cohort are shown.

Mickey (1973) found that the longevity of males was generally significantly greater than that of the females of CBA/J, C57BL/10 and the 9 other inbred strains studied by them. The same was reported for C57BL/6J mice by Kunstýř and Leuenberger (1975). On the other hand, Goodrick (1975) did not find differences in longevity between male and female A/J, C57BL/6J and DBA/2J mice, which is in accordance with our findings for C57BL/Ka and CBA mice.

The survival data for NZB mice reported here are comparable to those recently given by Croft, Adams and Purves (1975) for males and Fernandes, Yunis and Good (1976) for females. In earlier studies, much shorter mean life-spans, varying from 8 to 15 months, were found (East, de Sousa and Parrott, 1965; Howie and Helyer, 1968, and Festing and Blackmore, 1971).

In the following Chapters, we will refer to the survival patterns of CBA, C57BL/Ka and NZB mice in terms of the survival age (i.e., per cent survival) as well as chronological age in months.

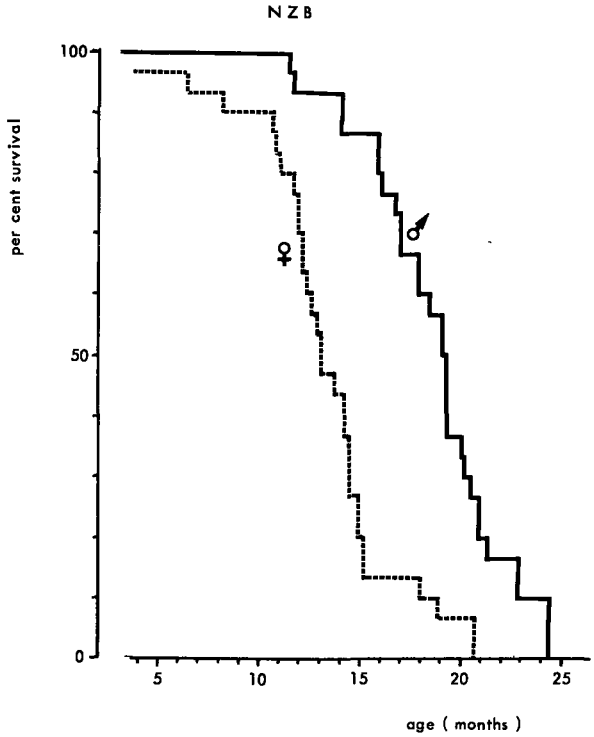

Figure III.3 Survival pattern of NZB mice.
Survival curves of 30 male and 30 female mice belonging to a single ageing cohort are shown.

C. NEOPLASTIC AND NONNEOPLASTIC LESIONS IN CBA, C57BL/Ka AND NZB MICE

When CBA, C57BL/Ka and NZB mice of the ageing cohorts and the other groups mentioned above were found moribund or dead, they were submitted to the same routine histopathological examination as has been established for rats (Burek, 1978). Autolytic animals were discarded. The neoplastic and nonneoplastic lesions found in these three strains are listed in Tables III.2 and 3, respectively. Some conditions commonly found in many mice of the 3 strains are not shown in the tables; these include mild chronic dermatitis secondary to acariasis, intestinal protozoan infestation (most frequently with Hexamita muris, and less commonly with Giardia muris and Entamoeba muris) and intestinal nematodiasis (pinworms). Glomerulonephritis was

a common finding in mice of all 3 strains, although the histological appearance and the severity varied among the strains. Other common histological changes, varying in extent and severity in individual mice as well as among mice of the various strains, were fibromyxomatous change of cardiac valves, focal adrenocortical atrophy with fibrosis and ceroid deposition in several organs, especially the ovary and adrenal glands. Finally, sparse to moderate infiltration by lymphocytes and plasma cells, without evidence of accompanying parenchymal lesions, was frequently found in many organs, including lungs (peribronchial and perivascular), liver (portal tracts), kidneys (pelvis and perivascular), salivary glands (periductal), urinary bladder (submucosal), mesentery and reproductive organs.

1. CBA

The most common tumours found in CBA mice were tubular adenomas and granulosa-theca cell tumours of the ovary, which occurred in 79% of the females. Hepatocellular neoplasms (type A and type B nodules according to Walker, Thorpe and Stevenson, 1973) were also common and occurred in about a third of the males and in 19% of the females. While occasionally the type B tumours were quite large, metastases were not found in this small series. Hepatocellular neoplasms are reported to be quite common in CBA and C3H mice (Andervont, 1950, and Smith, Walford and Mickey, 1973) and both genetic and dietary factors influence the incidence of these tumours (Heston, Vlahakis and Deringer, 1960).

The reticulum cell sarcoma was uncommon, and both types A and B according to the classification of Dunn (Dunn and Deringer, 1968) were found only sporadically.

The nonneoplastic lesions consisted of a variety of conditions including dystrophic calcinosis mainly in heart muscle, smooth muscle of gastrointestinal tract and in renal tubules, extreme testicular atrophy, adrenocortical atrophy, more severe than in the other strains, and an inclination to cyst formation in a variety of tissues and organs such as the thyroid, ovary, thymus and serosa of the gastrointestinal tract. Renal lesions were also common and varied in severity from mild to moderate. Amyloid deposits were found only in a few female mice (5%).

2. C57BL/Ka

The study of this series of C57BL/Ka mice was complicated by the

TABLE III.2

NEOPLASTIC LESIONS IN AGEING CBA, C57BL/Ka and NZB MICE

type of tumour	prevalence in males (per cent)			prevalence in females (per cent)		
	CBA	C57BL/Ka	NZB	CBA	C57BL/Ka	NZB
lymphoreticular and haemopoietic tissue						
reticulum cell sarcoma type A[1]	-[2]	4	10	5	18	7
reticulum cell sarcoma type B	-	28	8	5	23	2
other[3]	4	11	12	2	16	14
hepatocellular neoplasm type A[4]	23	3	2	12	-	-
hepatocellular neoplasm type B	11	3	-	7	-	-
thyroid follicular adenoma	-	9	6	-	2	-
sarcoma[5]	-	1	12	5	7	-
ovarian tumours[6]				79	-	5
testis: interstitial cell tumour	12	13	-			
lung: alveologenic carcinoma	11	3	-	7	-	2
uterus: adenocarcinoma				7	-	-
other tumours[7]	11	7	6	24	2	16
number examined	44	105	50	41	44	44
mean age (range) in months	28 (17-36)	23 (6-34)	17 (4-27)	28 (12-32)	20 (8-29)	14 (5-24)

Only those lesions which occurred with a prevalence of 5% or more in one of the 3 strains are included.

[1] according to the classification of Dunn (Dunn and Deringer, 1968).

[2] lesion not observed.

[3] lymphoblastic lymphoma, plasmacytoma, unclassifiable lymphomas, myeloid leukemias, and mast cell tumours.

[4] according to the classification of Walker, Thorpe and Stevenson (1973).

[5] fibrosarcoma, rhabdomyosarcoma, osteosarcoma, undifferentiated haemangiosarcoma, liposarcoma.

[6] tubular adenoma and granulosa-theca cell tumour.

[7] includes sporadic cases of benign and malignant neoplasms in a variety of tissues.

fact that an epizootic of Sendai virus infection occurred during a small part of the observation period. The data for the CBA mice presented above were from animals which died in the period before the Sendai virus infection. The acute viral pneumonia increased the mortality in the pre-weanling mice from 10-20% to 50-60%, while the mortality in mice younger than 16 months of age increased from 1 to 2%. After this age, mortality due to the Sendai virus infection alone was difficult to distinguish from mortality caused by age-related diseases. Although 20% of the male and female mice necropsied had histological signs of Sendai virus pneumonia, no effect on the survival curve was seen (Zurcher et al., 1977). It was noted that the mean age of the mice with a Sendai virus pneumonia was even somewhat higher than that of the whole population. Thus, the Sendai virus infection is either not an important cause of death in adult and ageing animals or the virus kills only those in the terminal stage of life.

The pathology of old age in this strain was dominated by a high prevalence of lymphoreticular tumours and amyloidosis. Deposition of amyloid was most frequent in the lamina propria of the terminal ileum. The next most frequent locations were coecum, spleen, liver, lung, thyroid and mesenteric lymph node. Mites, pinworms and Hexamita muris were regular findings. In sporadic cases, cryptosporidia or Entamoeba muris were found in the intestinal tract. Severe chronic inflammation caused by parasites was not frequent and could not explain the high prevalence of amyloidosis in this strain. Another nonneoplastic lesion which was especially common in female mice (36%) was periarteritis nodosa. Coronary and carotid arteries were often involved, leading to heart failure and cerebral infarction, respectively. The latter was often evident clinically as head tilt and circling to one side.

Most of the lymphoreticular tumours involved the mesenteric lymph node and fit the criteria of reticulum cell sarcoma type B of Dunn (Dunn and Deringer, 1968). In some cases, a mixed pattern of lymphosarcoma and reticulum cell sarcoma type B was found. In other cases, the tumour was difficult to differentiate from a malignant plasma cell tumour. Studies using immunofluorescence in human patients with so-called reticulum cell sarcomas have revealed that they are mainly lymphoid in nature. Preliminary results of electron microscopic studies on reticulum cell sarcoma type B of mice are also suggestive of a lymphoid origin (Meihuizen, personal communication).

The main localization of reticulum cell sarcoma type A in C57BL/Ka mice was the liver, which is in accordance with the findings of Dunn and Deringer (1968). At times, it was difficult to differentiate it from a Kupffer cell tumour. This, and the striking erythrophagocytosis

TABLE III.3

NONNEOPLASTIC LESIONS IN AGEING CBA, C57BL/Ka AND NZB MICE

diagnosis	prevalence in males (per cent)			prevalence in females (per cent)		
	CBA	C57BL/Ka	NZB	CBA	C57BL/Ka	NZB
amyloidosis	-*	83	12	5	73	-
periarteritis nodosa	2	16	2	-	36	-
fibrinoid vascular necrosis + hyalinization	-	-	12	-	2	27
thrombosis (mainly atrial)	2	4	42	2	-	32
ischaemic liver necrosis						
not due to torsion	-	1	26	-	4	33
due to torsion	16	-	-	-	-	-
focal myocardial necrosis + fibrosis	-	-	56	-	-	48
gastrointestinal tract ulceration	-	-	10	-	2	18
cystic endometrial hyperplasia				45	52	34
endometrial polyp				10	2	2
dystrophic calcinosis (cardiac + smooth muscle)	75	-	2	78	-	18
multifocal liver necrosis	16	3	16	17	14	16
serosal epithelial cysts	11	-	-	2	-	-
thyroid cyst	36	10	19	51	4	17
ovarian follicular cysts				50	34	2
hydronephrosis	2	6	4	-	9	14
"mesenteric disease"	7	10	-	-	18	-
purulent inflammation**	5	6	22	-	9	20
eosinophilic macrophage pneumonia	5	30	-	2	16	-
severe testicular atrophy	77	5	4			
spermatocele	-	7	-			
cystic sinuses of lymph nodes	-	-	8	-	-	9
number examined	44	105	50	41	44	44
mean age (range in months)	28 (17-36)	23 (6-34)	17 (4-27)	28 (12-32)	20 (8-29)	14 (5-24)

Only those lesions which occurred with a prevalence of 5% or more in one of the 3 strains are included.
* lesion not observed
**skin excluded

by the tumour cells, is more suggestive of a monocytic rather than a lymphoid origin of this tumour.

3. NZB

The majority of the lesions observed in this series of NZB mice tended to be either of a degenerative or an inflammatory nature. Nearly all mice examined had evidence of glomerulopathy characterized by thickened, hyalinized glomerular basement membranes (GBM) with an increased amount of mesangial matrix. These changes varied from uniform diffuse thickening of the GBM to severe, segmental hyalinization and sclerosis of glomerular tufts, often with prominent fibrinoid necrosis of a portion of a tuft. In some animals, the renal lesions were limited to the changes just described, but in most mice they were accompanied by tubular changes which varied from mild focal dilatation to severe widespread tubular dilatation accompanied by atrophy and collapse. Casts were prominent in these kidneys and these changes were often accompanied by interstitial fibrosis and chronic inflammation, resulting in a picture of "end-stage" renal disease.

Hyalinization and fibrinoid necrosis of small arteries occurred in 27% and 12% of females and males, respectively. Such arterial changes were found in a number of organs, but predominantly in the spleen, lymph nodes and gastrointestinal tract.

Localized or widespread vascular thrombosis was frequently seen, occurring in 32% and 42% of female and male mice, respectively. Large atrial or auricular thrombi were present in many of these mice.

Changes presumed to be secondary to the vascular and thrombotic lesions were found primarily in the liver and myocardium. These consisted of focal or multifocal ischaemic necrosis, with accompanying mild fibrosis and mixed inflammatory cell infiltration.

This mouse strain is also characterized by the occurrence of purulent inflammatory processes in a number of sites, but predominantly involving the genito-urinary tract. Seminal vesiculitis and prostatis, with and without accompanying cystitis and pyelonephritis, occurred in 22% of the males examined. In females, the vagina was the most common localization showing purulent inflammation in 20% of the cases.

Extensive extramedullary haemopoiesis in various tissues was also a typical finding in the NZB mice. This was the cause of the enlargement of the spleen often noted on gross examination. Particularly conspicuous was the prominent component of large, occasionally bizarre, megakaryocytes amongst the haemopoietic cells in many cases. In a few cases, this aspect was so pronounced that a diagnosis of

megakaryocytic myelosis was made. Marked haemosiderosis, primarily in the spleen and liver, was present in most of these mice.

The most frequent neoplasms observed in this series arose from the lymphoreticular and haemopoietic tissues. Reticulum cell sarcomas, both types A and B (Dunn and Deringer, 1968), were similar histologically to those found in the C57BL/Ka mice and together they were observed in 18% of males and 9% of female NZB mice. Six males and 6 females had other types of lymphoreticular and haemopoietic neoplasms, including lymphoblastic lymphoma (4 cases), unclassifiable lymphomas (5 cases), myeloid leukemia (2 cases) and mast cell tumour (1 case).

Other neoplasms were relatively rare in this series of mice, although sarcomas of several types (see Table III.2) were diagnosed in 6 males.

D. DISCUSSION

It is clear from the data presented above that the 3 strains used in this study differ considerably in their survival characteristics as well as in type and frequency of lesions observed. It will be difficult to determine whether the variety of lesions seen with age within a specific strain are interrelated and more specifically whether they have a bearing on the functional changes in the immune system with advancing age.

In general, the age-related pathology of NZB mice is characterized by immune complex glomerulonephritis as well as fibrinoid changes in glomeruli and small arteries and thrombosis and ischaemic necrosis in different tissues. The latter phenomena may be secondary to the presence of circulating immune complexes. The iron deposition in the tissues can be related to the Coombs' positive haemolytic anaemia known to be present in this strain (see Chapter I, part G). The age-related pathology of C57BL/Ka mice is dominated by the high prevalence of amyloidosis and of malignant lymphomas, while such lesions are almost absent in CBA mice. In the latter strain, lesions seem to have predominantly an endocrine basis.

Since our study is concerned with the immune system of ageing mice, attention was paid to the histological changes in the lymphoid organs of the 3 strains. The lymphoid tissues of the 3 strains differed in the frequency of lymphoreticular tumours causing disorganization of the lymphoid structures and presumably interfering with normal functioning. Apart from differences in involvement by lymphoreticular

tumours, the 3 strains also differed in the morphology of the lymphoid tissues that were not affected by neoplasia. Although such differences were not measured quantitatively, the observations made allow some conclusions. While an increased number of plasma cells was found in most of the aged mice, NZB mice often showed an excessive increase. Another characteristic of lymph nodes in this strain was the presence of cystic dilatation of sinuses. The structure of lymph nodes of aged C57BL/Ka mice frequently showed an increased size and a moderate lymphocytic depletion of the paracortical areas. Germinal centres were found infrequently in this strain, in contrast to the situation in CBA and NZB mice. CBA mice showed the least severe changes in lymphoid tissues with age as compared to the C57BL/Ka and NZB mice. Age-related atrophic changes in the thymus were common to all 3 strains. Specific differences in thymus morphology among the strains, with the exception of cyst formation in the CBA mice, were not noted on routine histological examination. Detailed quantitative morphological studies were not performed, however.

The fact that the CBA mice do not develop diseases that are clearly of an immunopathological nature makes this strain an appropriate animal model for the study of ageing, i.e., the study of an ageing pattern which is unencumbered by the influence of lesions of the immune system itself. Therefore, information obtained with the CBA mice may serve as a reference for the study of mouse strains such as C57BL/Ka and NZB which display specific immune dysfunctions and/or lesions of the immune system. The above are the reasons why a large portion of the studies covered in this thesis and other immunological investigations at the Institute for Experimental Gerontology (Haaijman, 1977) were initially conducted with the CBA mice.

In contrast to CBA mice, C57BL/Ka mice develop lesions which can be clearly related to the immune system. The high incidence of lymphoreticular tumours (about 50% of all C57BL/Ka mice died with a lymphoreticular tumour) is striking. In addition, old C57BL/Ka mice exhibit a high prevalence of amyloidosis. As outlined in part G of Chapter I, the development of amyloid is often linked to the immune system, although a direct relationship has not yet been proved. Another characteristic finding in ageing male and female C57BL/Ka mice is the high incidence of homogeneous immunoglobulins in the sera which closely resemble those found in idiopathic paraproteinaemias of man (Radl and Hollander, 1974, and Radl et al., 1978). At present, the possible relationship between the idiopathic paraproteinaemia, amyloid deposition and the high frequency of lymphoreticular neoplasia is not clear. The C57BL/Ka mouse may serve as a valuable animal model in

which to study this relationship. Such a study may also contribute to the understanding of the significance and origin of idiopathic paraproteinaemia in ageing man. C57BL/Ka mice were included in our study in order to compare changes in the humoral immune response of these mice with those of ageing CBA mice.

A few mouse strains, notably the NZB, NZW and NZB/W mice, serve as animal models for the study of autoimmune phenomena and related disorders during ageing. A short review of the current knowledge of these strains is presented in Chapter I, part G. The studies on the underlying immunological mechanisms in NZB mice have been focused mainly on the thymus-dependent part of the immune system. Therefore, we have limited our study with NZB mice to an investigation of an immunological function exerted by B cells, the antibody response to lipopolysaccharide of *E. coli*.

CHAPTER IV

EFFECT OF AGE ON THE IN VIVO PRIMARY ANTI-SRBC RESPONSE IN THE SPLEEN OF CBA AND C57BL/Ka MICE AND ON THE IN VIVO PRIMARY ANTI-LPS RESPONSE IN THE SPLEEN OF CBA, C57BL/Ka, NZB AND BALB/c MICE

A. INTRODUCTION

A general introduction to the decline in immunological competence with advancing age observed in man and experimental animals has been presented in Chapter I. This and the following Chapters will be focused on the effect of age on the humoral immune system in mice.

Two different approaches can be envisaged for the estimation of age-related changes in the humoral immune system of experimental animals. The first is to determine either the levels of immunoglobulins (Ig) in the serum or the number of Ig-containing cells in the lymphoid organs or both, irrespective of the antigens to which these Ig's are directed. The values obtained in this way may give an idea of ongoing responses to stimulation by environmental antigens. In the second approach, the specific response to a known antigenic challenge is assessed by determining antibody levels or the number of antibody forming cells. This response is superimposed on the ongoing responses to environmental antigens.

The second approach was selected for the present study in order to study the changes in the humoral immune response with advancing age. With regard to the first approach, this has been extensively covered by Haaijman, Schuit and Hijmans (1977), and Haaijman, Van den Berg and Brinkhof (1977). In addition to this, it seems relevant now to investigate the immediate response to an antigenic challenge, since this parameter actually provides insight into the functional capacity of the humoral immune system at a given age. For instance, if the antigenic challenge is derived from a pathogenic microorganism, the magnitude of a specific response in its early phase will determine whether an individual will be resistant or susceptible to this microorganism. It will be of great interest to establish whether there is any correlation between the data obtained by using the two approaches mentioned above. This is facilitated by the fact that the estimation of age-related changes in serum Ig levels and numbers of Ig containing cells has been carried out with the same strain of our mouse colony, i.e., the CBA (Haaijman, Schuit and Hijmans, 1977, and Haaijman, Van den Berg, and Brinkhof, 1977), that was used for the present study. Thus

a valid comparison between these two parameters for the humoral immune system can be made.

The initial goal of our study was to compare the capacity to mount a humoral immune response to certain antigenic stimuli in various mouse strains which differ in their age-related pathology. However, it is not feasible to study all strains in a given time period to the same extent. Therefore, it was decided to study one strain (the CBA) in more detail, since this strain appears to be free of overt age-related immunopathological disorders (see Chapter III). Data obtained in this strain will give insight into an immunological ageing process which may be regarded as normal, i.e., in which pathological immune lesions do not interfere with the ageing process. In addition to investigations with CBA mice, a comparison was made between some parameters of humoral immune function in strains with a different immunological ageing behaviour. For this purpose, C57BL/Ka and NZB mice were selected. The rationale for this selection and the ageing patterns characteristic for each strain have been presented in the previous Chapter.

The following functional parameters of the humoral immune system have been studied in young and ageing mice of the above-mentioned strains.

1. The primary immune response to the particulate thymus-dependent antigen sheep red blood cells (SRBC) was determined in the spleen of CBA and C57BL/Ka mice. The anti-SRBC response of NZB mice was not included, since this particular response has been the subject of many other studies. The findings of those studies will be included in the final discussion of this Chapter.
2. In order to compare a thymus-dependent humoral immune function with a thymus-independent function, the response to lipopolysaccharide (LPS) of E. coli was determined as a function of age in CBA, C57BL/Ka, BALB/c and NZB mice. All of these investigations were carried out after in vivo immunization with antigen.

The effect of ageing on the in vitro response of spleen cells to SRBC will be presented in Chapter VI.

B. SOME CHARACTERISTICS OF THE IN VIVO ANTI-SRBC RESPONSE

Many experimental antigens may be considered for an investigation of the functional capacity of the humoral immune system. The use of SRBC as a thymus-dependent antigen has the following advantages.

1. This antigen has been employed widely over the past years and this has resulted in a good documentation of the backgrounds and the kinetics of this reponse. This makes a direct application in immunogerontological studies feasible.
2. Due to the many antigenic determinants present on SRBC, a large part of the immunocompetent cell population is involved in this response; this results in a wide range of values in which age-related changes are more easily detected.
3. The use of an adjuvant can be omitted when SRBC are used, in contrast to, for instance, protein antigens where an adjuvant is a prerequisite to obtain an immune response in mice. The physiological changes induced by adjuvants are largely unknown, which complicates the interpretation of the data obtained in, for instance, young and aged animals.
4. The anti-SRBC response has already been applied to the study of the functional capacity of the humoral immune system in old age in mice, as will be discussed later. Nevertheless, for an initial investigation of strains from our own ageing mouse colonies, it was valuable to use this type of response in order to be able to compare and extend the data obtained with other mouse strains.

The appearance of antibody forming cells in the spleen of young CBA mice during a primary anti-SRBC response is presented in Fig. IV.1. The mice received an intravenous injection of an optimal dose of SRBC (4×10^8, see also Fig. IV.2). The intravenous route of antigen administration was used throughout this study. The response is characterized by a logarithmic increase in cells producing antibodies of the IgM immunoglobulin class. The production of IgM-PFC reaches a peak 4 days after immunization and declines rapidly thereafter. Cells forming antibodies of the IgG immunoglobulin class do not appear until 4 to 5 days after immunization. A peak is observed on day 6 followed by a decline on day 8. This pattern is essentially similar to those reported previously by others (see, for instance, Sell, Park, and Nordin, 1970, and Benner et al., 1974). Figs. IV.2a and 2b show the dose response curves of splenic IgM- and IgG-PFC formation to SRBC at doses ranging from 10^6 to 4×10^9 cells. As can be concluded from Fig. IV.1, the IgM and IgG responses show separate peak days. The number of IgM-PFC was, therefore, determined 4 days after immunization. For the IgG-PFC response, day 6 was chosen. However, the time intervals of 4 and 6 days required to reach optimum numbers of IgM and IgG-PFC, respectively, are valid only for doses higher than 4×10^7 SRBC, as was shown by Wortis, Taylor, and Dresser (1966). These authors reported that, for IgM-PFC formation, the time interval between immu-

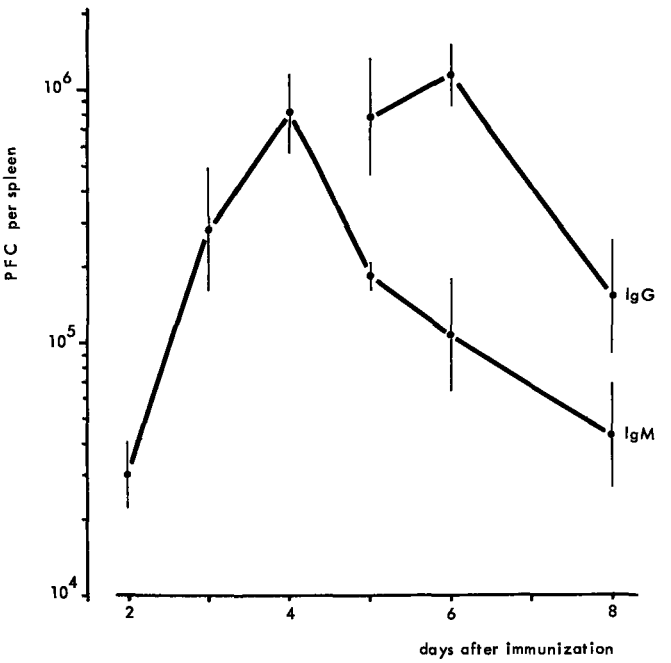

Figure IV.1 Time course of the primary IgM- and IgG-PFC response to SRBC in the spleen of 3-month-old CBA mice.
The mice were immunized with 4 x 10^8 SRBC. Each value represents the geometric mean of 5 mice. The 95 % confidence limits are indicated by bars. No IgG-PFC could be detected before day 5 in this experiment.

nization and peak becomes longer with decreasing amounts of SRBC (below 4 x 10^6), whereas, for IgG-PFC formation, this time interval becomes shorter with decreasing amounts of SRBC (below 4 x 10^7). With regard to the dose-response experiments recorded in Figs. IV.2a and 2b, the following conclusions can be drawn. At antigen doses below 10^8 SRBC, a ten-fold increase in SRBC resulted roughly in a 2-3-fold increase in IgM antibody forming cells. The IgM response reaches its maximum level at 2-4 x 10^8 SRBC. At higher doses, a decrease in PFC is found. When a dose of 4 x 10^9 SRBC is injected, the IgM response is significantly lower than the responses obtained with 2-4 x 10^8 SRBC. The IgG anti-SRBC response reaches a plateau at 4 x 10^8 SRBC. In contrast to the IgM response, no decline in PFC number is observed at higher doses.

Foreign red blood cells are considered to evoke a thymus-dependent immune response. The role of thymus-dependent lymphocytes (T cells) in the humoral immune response has been discussed in Chapter I, section C. The anti-SRBC response has been widely used to establish the func-

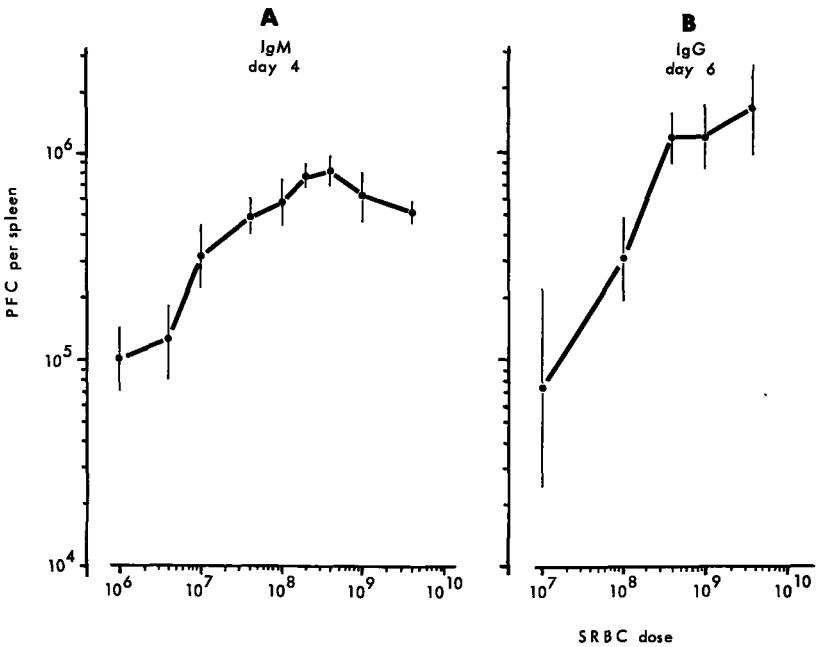

Figure IV.2 Effect of SRBC- dose on the primary IgM- and IgG-PFC response in the spleen of 3-month-old CBA mice.
The IgM (A) and IgG (B) responses were determined on day 4 and day 6, respectively. Each value represents the geometric mean of 5 mice. The 95 % confidence limits are indicated by bars.

tion of T cells as helper cells (Claman, Chaperon, and Triplett, 1966, and Mitchell and Miller, 1968) and as suppressor cells (Gershon, 1974, and Cantor, Shen, and Boyse, 1976). However, there are differences in the requirement of T cell help for the production of antibodies of the various Ig classes. This can be illustrated with the results of the following experiment with athymic nude (nu/nu) mice (Fig. IV.3). The ability to produce IgM-PFC in the spleen after a primary immunization with SRBC is significantly lower in nu/nu mice when compared to the response in normal litter mates (NLM) of nu/nu mice. However, no IgG-PFC response can be obtained. Six weeks after transplantation of a thymus obtained from sublethally irradiated newborn NLM, the IgM-PFC response is significantly enhanced in the grafted nu/nu mice. Moreover, the grafted mice are now capable of developing IgG-PFC. The newborn donor mice were sublethally irradiated with 300 rad of X-rays in order to remove the thymocytes and to leave intact thymic epithelial tissue (Trowell, 1961).

Histological examination of the transplanted thymuses revealed that the grafts were well-vascularized and had the characteristic

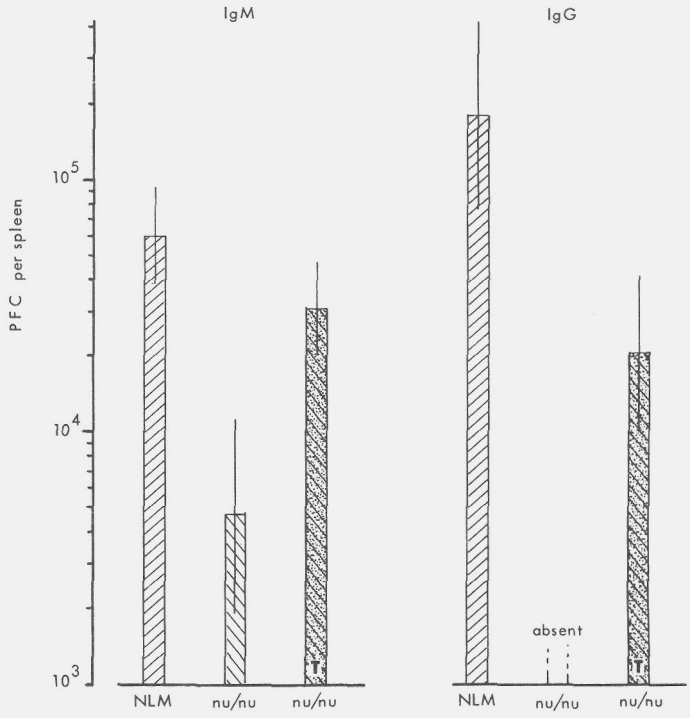

Figure IV.3 Effect of an irradiated thymus graft on the IgM- and IgG-PFC response to SRBC in the spleen of nu/nu mice.
Thymus grafts were derived from neonatal normal litter mates (NLM) of nu/nu mice and transplanted under the kidney capsule of 6-week-old nu/nu mice. The donor mice were irradiated with 300 rad of X-rays before removing the thymus. Six weeks after transplantation, the transplanted mice (indicated by a T in the column), age-matched NLM and nontreated nu/nu mice were immunized with 4 x 10^8 SRBC. Six days later, the IgM- and IgG-PFC responses were determined. Each figure represents the geometric mean of 5 mice. The vertical lines represent the 95 % confidence limits.

thymus architecture. These data indicate that the IgG-PFC response is totally thymus-dependent, whereas the IgM-PFC response is only partially thymus-dependent. In the presence of a thymus, the IgM response can be amplified, whereas the thymus is a prerequisite for the development of an IgG response. Similar results were obtained by Van Muiswinkel and Van Soest (1975) in experiments where B cells in the presence or absence of educated T cells were transferred to irradiated hosts and their ability to mount IgM and IgG antibodies to SRBC was determined. Comparative studies on serum Ig levels in thymus-deprived (nu/nu and thymectomyzed) mice and control mice also indicate that, in the absence of a thymus, the concentration of IgG, especially of IgG1, is more affected than the concentration of IgM (Luzzatti and

Jacobson, 1972; Pritchard, Riddaway, and Micklem, 1973, and Bankhurst, Lambert, and Miescher, 1975).

C. PRIMARY ANTI-SRBC RESPONSE IN AGEING CBA MICE

Some introductory remarks must be made before presenting the data obtained in ageing CBA mice. In the present study, only male CBA mice were used. We preferred to include investigations on other mouse strains in this study rather than determining whether male and female mice differ in immune responsiveness during ageing. A basis for such a comparison might have been morphological differences in the lymphoid organs between male and female CBA mice during ageing. Histological examination did not reveal marked differences as were observed, for instance, in WAG/Rij rats; in this strain, males show an earlier onset of thymus atrophy than do females (Burek, 1978).The mice were macroscopically examined for pathological lesions. Only spleens which appeared grossly normal were used. The very few mice with macroscopically visible lymphoid tissue disease (e.g., reticulum cell sarcoma; see Chapter III) were not included in the experimental groups. Mice with hepatocellular neoplasms (which were frequently found) were normally employed for the experiment. A comparison of age-matched mice with and without this tumour (data to be shown later) indicates that the presence of a hepatocellular neoplasm did not influence the response.

CBA mice of various age groups were compared as to their ability to mount a primary response to SRBC (Fig. IV.4). According to the survival pattern of male CBA mice as presented in Chapter III, 24-month-old CBA mice are beyond the 90% survival age. At 30 months of age, the CBA mice have reached the 50% survival age and are regarded to be senescent (Chapter III). The mice received an intravenous injection of 4×10^8 SRBC, since an optimal IgM- as well as IgG-PFC response was obtained in the spleen of 3-month-old CBA mice with this dose (Fig. IV.2). The number of IgM-PFC was determined 4 and 6 days after immunization. A comparison of the IgM response on day 4 in the various age groups reveals that 3- and 9-month-old mice developed about the same numbers of PFC (Fig. IV.4). When compared to the response in the 3-month-old mice, a significant decrease is observed from the age of 15 months onwards. The numbers of PFC in 24-month-old and 30-month-old mice were 4 and 10 times lower, respectively, than those obtained in the youngest groups. A comparable number of IgM antibody forming cells was found on day 6 in all age groups. It will be shown later that this effect cannot be attributed to a shift of the

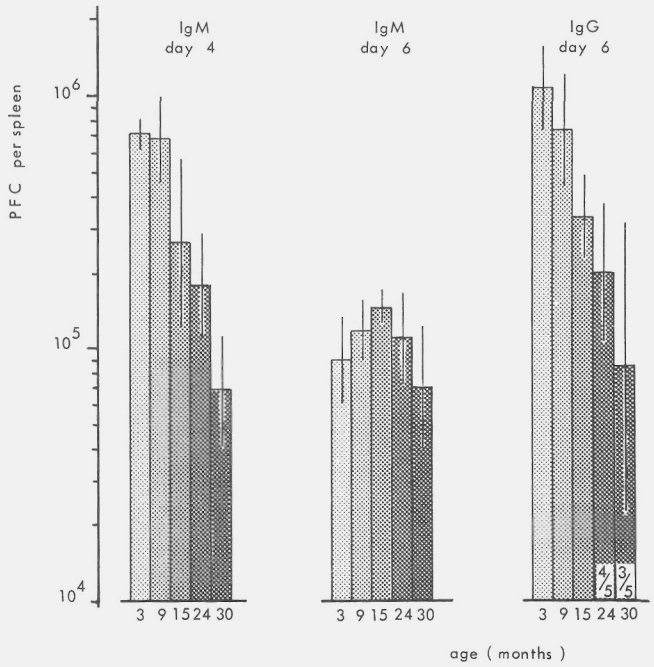

Figure IV.4 Effect of age on the IgM- and IgG-PFC response to SRBC in the spleen of CBA mice.
The mice were immunized with 4 x 10^8 SRBC. Each figure represents the geometric mean of 5 mice unless nonresponder mice were found, which is indicated by the fraction of responders over the total number of mice tested. In that case, only the mean and confidence limits of the responder mice were calculated. The 95 % confidence limits are indicated by vertical lines.

peak day of the IgM response in ageing mice, but results from a much slower decrease in the number of IgM-PFC in the phase of the response following the peak day.

With regard to the IgG-PFC response, this experiment indicates that again no significant difference existed between 3- and 9-month-old mice. The IgG responses of the 15, 24, and 30-month-old mice were about 3, 5, and 10 times lower than that obtained at the age of 3 months. There was no IgG-PFC response in one and two mice from the age groups of 24 months and 30 months, respectively. The above results were expressed as the total number of PFC per spleen. In addition, the number of PFC per 10^7 nucleated spleen cells was calculated. Although no significant alterations with age were observed in the total number of nucleated cells per spleen (Fig. IV.5), variations in either the total number of PFC per spleen or number of nucleated cells per individual animal might reveal a different picture. This was not the case, as is illustrated by the data presented in Fig. IV.6. The same

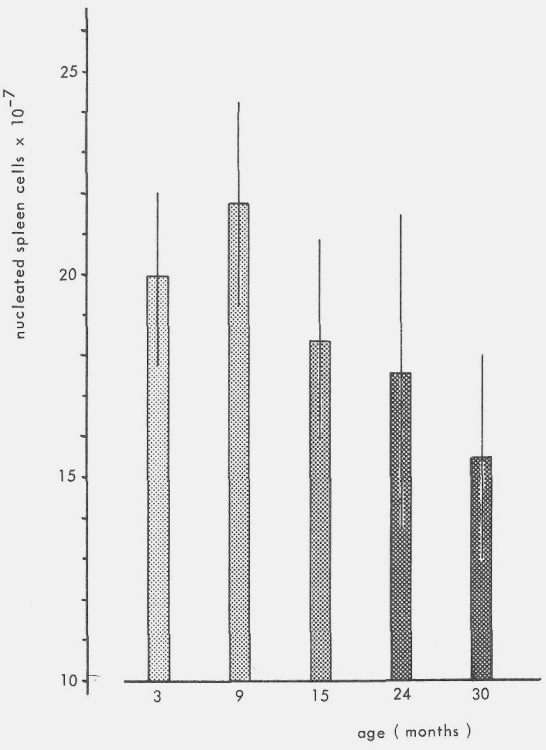

Figure IV.5 Number of nucleated cells in the spleen of CBA mice of various ages. Each figure represents the average of the indicated number of animals. The 95 % confidence limits are indicated by vertical lines.

pattern of age-related changes was observed when the PFC response was expressed per spleen or per 10^7 spleen cells.

Table IV.1 summarizes the results obtained in 4 separate experiments, including the experiment presented above. Since these data were collected over a period of 2 years, time-dependent variations in the CBA ageing cohorts can be detected. The mean PFC responses and the 95% confidence limits are given as percentages of the response of 3-month-old mice which is taken as 100%. In experiment 2, CBA mice of 6 months of age were included in order to have additional data on an age group near to or above the age-specific peak of the anti-SRBC response.

The following conclusions can be drawn from the data presented in Table IV.1:

1. At the age of 6 months, the day 4 IgM response is comparable to that of 3-month-old mice. Mice of 6 months develop about 50% more IgG-PFC than do 3-month-old mice, although the difference is not significant.

TABLE IV.1

INFLUENCE OF AGE ON THE PRIMARY IgM AND IgG ANTI-SRBC RESPONSE IN THE SPLEEN OF CBA MICE

antibody class and response day	exp. number	absolute number*	AGE IN MONTHS 3	6	9	15	22-24	28-30
IgM	1	502	100	-	63**	38	8	-
			149- 67***		97- 41	66- 22	17- 4	
day 4	2	677	100	116	114	-	28	-
			123- 81	133-102	128-102		36- 21	
	3****	713	100	-	95	37	25	9
			113- 88		140- 65	81- 17	40- 15	16- 6
	4	714	100	-	-	-	29	7
			118- 85				38- 22	28- 2
IgM	1	93	100	-	94	82	100	-
			124- 81		123- 71	138- 49	152- 66	
day 6	2	60	100	158	155	-	86	-
			115- 87	191-130	175-137		112- 65	
	3	90	100	-	130	160	122	77
			148- 68		171-100	186-138	183- 82	135- 44
	4	88	100	-	-	-	140	133
			121- 83				209- 93	165-107
IgG	1	1,151	100	-	83	29	4	-
			145- 69		164- 42	68- 12	16- 1	
day 6	2	870	100	152	127	-	10	-
			127- 79	198-117	155-105		14- 7	
	3	1,058	100		79	31	18 (4/5)*****	8 (3/5)*****
			148- 68	-	113- 41	45- 21	35- 10	29- 2
	4	960	100				12	9 (3/5)*****
			145- 69	-	-	-	51- 3	10- 8

The mice received an intravenous injection of 4×10^8 SRBC. Each age group in the different experiments consisted of 5-6 mice.

* Absolute number of PFC per spleen (geometric mean $\times 10^{-3}$) in 3-month-old mice
** Geometric mean given as a percentage of the mean number of PFC found in 3-month-old mice.
*** 95% confidence limits are expressed in percentages of the mean number of PFC in 3-month-old mice.
**** Experiment 3 corresponds with figure IV.4.
*****Number of responder mice over the total number tested.

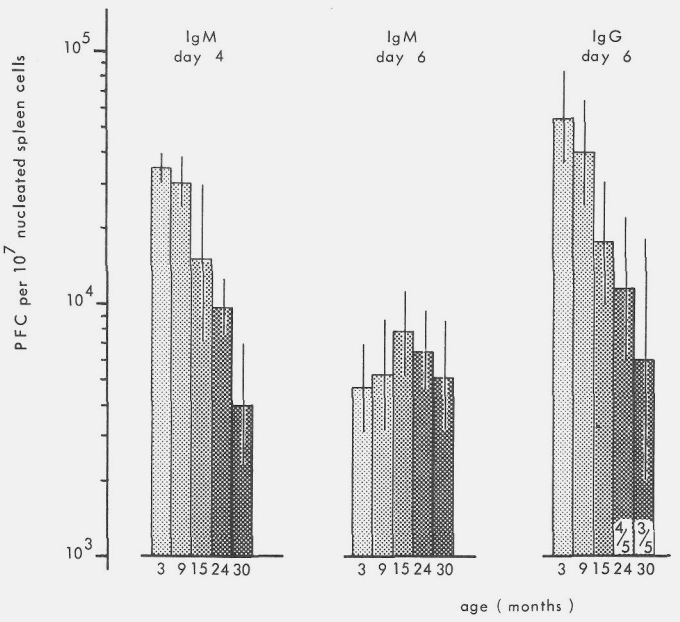

Figure IV.6 Effect of age on the IgM- and IgG-PFC response to SRBC in CBA mice expressed per 10^7 nucleated spleen cells.
See legend to figure IV.4.

2. With regard to the responses found in the older age groups recorded in Table IV.1, the same tendency of age-related changes as presented for experiment 3 (Fig. IV.4) can be observed, in spite of the occasionally large variations between experiments and the great variation within the age groups as reflected by the large 95 % confidence limits. Thus, the results obtained in the aged animals over an extended time period (2 years) indicate that the magnitude of the age-related decline was not markedly changed in the different ageing cohorts used.
3. It should be emphasized that 4 out of 10 mice in the oldest age group (28-30 months) failed to elicit any IgG-PFC response, indicating that the number of nonresponders with respect to IgG antibody formation increases with advancing age.

The above experiments were designed to establish differences in the anti-SRBC response between young and old CBA mice under conditions which have shown to be optimum for 3-month-old mice. However, there is the possibility that old mice require other doses or time intervals to attain optimal IgM and IgG responses.

To investigate this, a group of 22-month-old mice was used, because sufficient animals of older age groups were not available for such a study and a marked decline in the IgM and IgG responses to 4 x 10^8 SRBC had already been observed at this age. Since low doses of antigen were used in the following experiment, it was necessary to establish the number of background PFC to SRBC in the spleen of unstimulated mice of various age groups in order to determine above which number of PFC mice can be considered to have responded to the SRBC injection. The number of background anti-SRBC-PFC in 3, 22-24 and 28-30-month-old mice is presented in Table IV.2. A moderate, but not significant, increase with age can be observed in the background level of PFC. No IgG-PFC were detectable in the spleen of these mice. On the basis of these data, mice which develop more than 1500 PFC per spleen can, therefore, be considered as responders.

TABLE IV.2

NUMBER OF BACKGROUND ANTI-SRBC PFC
IN THE SPLEEN OF CBA MICE OF VARIOUS AGE GROUPS

age in months	PFC per spleen exp. 1	exp. 2
3	230* 523-101	195 640- 95
22-24	592 1149-305	224 547- 92
28-30	200 729- 55	449 1494-167

*Figures represent the geometric mean and 95% confidence limits of 5 mice.

The IgM and IgG antibody-forming cells appearing in the spleen 4 to 6 days after injection were determined by using a dose range of 10^6 to 10^9 SRBC. Doses of 10^8 and 10^9 SRBC were also employed to determine responses at day 7. From the results presented in Figs. IV.7 and 8, the following points can be noted:

1. The average numbers of IgM and IgG-PFC cells never exceeded those obtained in the age group of 3 months at all doses and days of observation, although the differences were not always significant.
2. With regard to the peak day of the IgM response, it was found that no significant shift occurred, neither when the dosage of SRBC was varied nor when the two age groups were compared. Only in 22-month-old mice was a (not significantly) higher response obtained

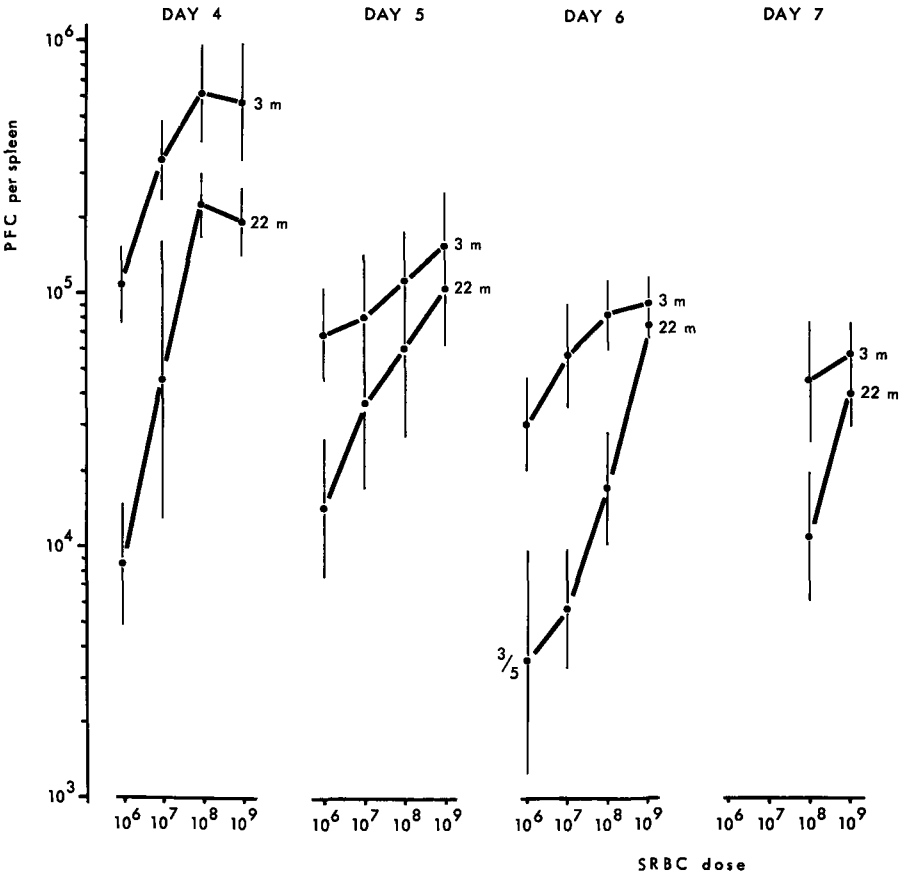

Figure IV.7 Effect of SRBC dose and time after immunization on the IgM-PFC response in the spleen of 3- and 22-month-old CBA mice.
Each value represents the geometric mean of 5 mice unless nonresponder mice were found, which is indicated by the fraction of responders over the total number of mice tested. In that case, only the mean and confidence limits of the responder mice were calculated. The 95 % confidence limits are indicated by bars.

on day 5 than on day 4 after immunization with 10^6 SRBC.

As demonstrated in Table IV.3, the ratio of the response at day 4 over that obtained at day 5 is dependent upon the SRBC dosage. At each dosage of SRBC, the response declined much slower in 22-month-old mice.

3. Young as well as 22-month-old mice reached a plateau level of the IgM response at a dosage of 10^8 SRBC. As the dose of antigen was decreased, the difference in the response between the two age groups became greater. This was observed on both the peak day of the response and the days thereafter. Two out of 5 mice immunized

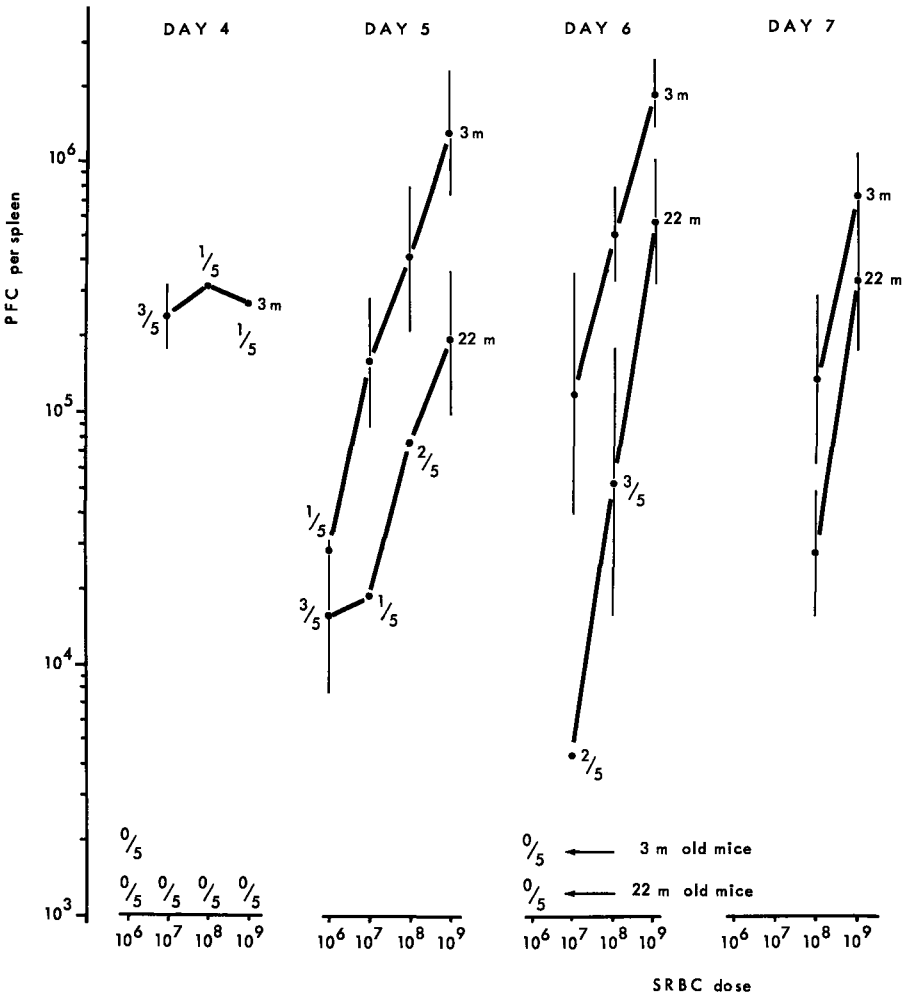

Figure IV.8 Effect of SRBC dose and time after immunization on the IgG-PFC response in the spleen of CBA mice.
See legend to figure IV.7. No confidence limits were calculated when less than 3 mice developed a significant PFC response.

with 10^6 SRBC developed numbers of PFC on day 6 within the range of PFC found in unstimulated mice. They were, therefore, considered as nonresponders.

4. As with the IgM response, the IgG response showed greater differences between the two age groups as the dose of antigen was decreased. In addition, a considerable number of 22-month-old mice displayed no IgG response at low doses of SRBC. Four days after immunization, only a portion of the young animals give an IgG response. No marked changes in the peak day of the IgG response

TABLE IV.3

COMPARISON OF THE DAY 4 AND DAY 5 IgM-PFC RESPONSES IN THE SPLEEN OF 3- AND 22-MONTH-OLD CBA MICE AFTER IMMUNIZATION WITH DIFFERENT DOSES OF SRBC

SRBC dose*	3 months day 4 PFC x 10^{-3}	3 months day 5 PFC x 10^{-3}	3 months ratio day 4/5	22 months day 4 PFC x 10^{-3}	22 months day 5 PFC x 10^{-3}	22 months ratio day 4/5
10^6	109**	69	1.6 n.s.***	9	14	0.6 n.s
10^7	338	81	4.2	46	36	1.3 n.s
10^8	617	110	5.6	223	60	3.7
$4x10^8$	1,004	188	5.3	84	83	1.0 n.s
10^9	565	156	3.6	190	105	1.8

* Data from the experiment presented in Fig. IV.7 were used for this table, except for the response obtained with 4 x 10^8 SRBC. These results were obtained in a separate experiment.
** Figures represent geometric mean of 5 mice.
***The difference between the day 4 and day 5 responses was not significant.

were evident.

The following conclusions can be drawn from the data presented above:

1. Under none of the conditions studied here (i.e., different days, varying antigen doses) did the 22-month-old mice reach the level of responsiveness observed in young mice.
2. At suboptimal doses of SRBC, the differences in the response between the 3- and 22-month-old mice became greater; in other words, when antigen becomes the limiting factor, the older mice have a smaller capacity to respond. This applies to the IgM as well as to the IgG response.

It has already been noted that a common tumour occurring in about a third of CBA mice from the age of 19 months on is the hepatocellular neoplasm. In the above experiments, the data from mice with or without macroscopically visible hepatocellular neoplasms were taken together, since it appeared that the results for the tumour-bearing and tumour-free mice fell in the same range. An example in which the IgM-PFC responses of both groups are shown separately is presented in Table IV.4. The results indicate that the responses obtained in mice with hepatocellular neoplasms were comparable to those of the mice without this tumour.

D. PRIMARY ANTI-SRBC RESPONSE IN AGEING C57BL/Ka MICE

The appearance of homogeneous immunoglobulins (Radl and Hollander, 1974) and of lymphoid tissue diseases (Chapter III) are characteristic

TABLE IV.4

COMPARISON OF THE ANTI-SRBC RESPONSE IN AGED CBA MICE WITH OR WITHOUT HEPATOCELLULAR NEOPLASMS

age in months	PFC per spleen * IgM day 4 without	IgM day 4 with	IgG day 6 without	IgG day 6 with
22-24	18**	28	6	0
	21	36	7	9
	23	38	10	
	25		11	11
	28		19	20
	30		21	25
	34			
	39			
30	4	12	0	6
	9		0	15
	11		6	
	12			

* The mice were immunized with 4 x 10^8 SRBC.
**Figures represent individual values expressed in percentages of the mean number of PFC of 3-month-old control mice included in the same experiment. The data correspond with exp. 2 and exp. 3 from Table IV.1.

features of the immune system of ageing C57BL/Ka mice. Therefore, this strain was selected for a first comparison with the CBA mice with regard to the age-related changes in the anti-SRBC response.

Similar remarks as were made for the experiments with old CBA mice apply for the present experiments with old C57BL/Ka mice. The mice were macroscopically examined. Animals with visible lymphoid tissue disease and animals with spleens which appeared grossly abnormal were excluded from the experiments. Only male mice were used.

The anti-SRBC response was studied in 3 different age groups (3, 18, and 24 months) employing varying doses of SRBC. C57BL/Ka mice of 18 and 24 months of age have reached approximately the 90% and 50% survival age, respectively (see Chapter III, Table III.1). The IgM and IgG responses were determined on days 4, 5, and 6. The number of background PFC towards SRBC in unstimulated mice of various age groups are given in Table IV.5. It can be concluded that mice of 3 and 18 months have responded to the SRBC injection when they produce more than 1,000 PFC per spleen, this number being the upper confidence limit of the background value. The background level of anti-SRBC PFC was increased in the 24-month-old mice. Therefore, mice of this age group which develop more than 5,000 PFC per spleen can be regarded as having responded to the injected SRBC. The data concerning the immunized mice are presented in Fig. IV.9. The following points can be noted:

1. If the maximum IgM response of the various age groups are compared - irrespective of the day on which this response is attained - a

TABLE IV.5

NUMBER OF BACKGROUND ANTI-SRBC PFC IN THE SPLEEN

OF C57BL/Ka MICE OF VARIOUS AGE GROUPS

age in months	PFC per spleen exp. 1	exp. 2
3	171* 621-47	133 253- 70
18	202 691-59	324 963-109
24	622 85-4,555	-

*Figures represent the geometric mean and 95% confidence limits of 5 mice.

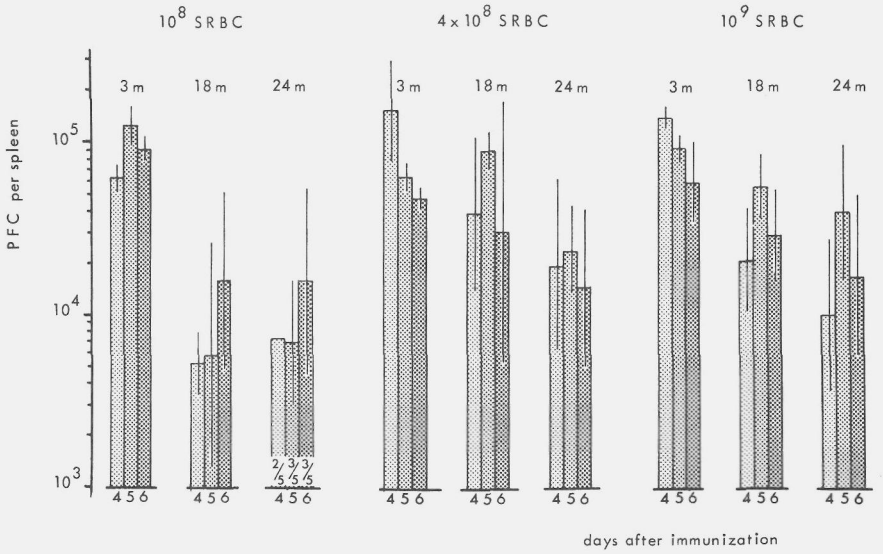

Figure IV.9 Effect of age on the IgM-PFC response to SRBC in the spleen of C57BL/Ka mice.
The mice were immunized with 10^9, 4 x 10^8 or 10^8 SRBC. Each figure represents the geometric mean of 4 to 5 mice unless nonresponder mice were found, which is indicated by the fraction of responders over the total number of mice tested. In that case, only the mean and confidence limits of the responder mice were calculated. The 95 % confidence limits are indicated by vertical lines. No confidence limits were calculated when less than 3 mice developed a significant PFC response.

decline can be noted at a dosage of 10^9 SRBC and this is even more pronounced when 10^8 SRBC were administered With the latter antigen dosage a number of the 24-month-old mice showed no response at all since their number of antibody forming cells was within the range of background PFC. After immunization with 4 x 10^8 SRBC, the response was decreased significantly only in 24-month-old mice

2 Another feature of the IgM antibody formation in C57BL/Ka was that the peak day had shifted to day 5 or even to day 6 (10^8 SRBC) in the older age groups. Only in the youngest age group did the response peak on day 4, except for the lowest SRBC dosage used

3 The data also show a remarkable variation in the IgM response obtained in the older mice. The 18- and 24-month-old mice display a wide variety in the number of antibody forming cells.

With regard to the IgG response, none of the C57BL/Ka mice studied developed antibody forming cells of this immunoglobulin class on day 4. On day 5, only in a minority of mice belonging to the youngest age group was a poor IgG response observed. Since significant numbers of IgG antibody forming cells appeared on day 6, only the data for the IgG response on day 6 in C57BL/Ka mice are listed (Table IV.6). Only two of the youngest mice gave a poor IgG response at the lowest dosage (10^8 SRBC). The response of these mice was increased after immunization with 4 x 10^8 and 10^9 SRBC. At these antigen doses, 8 out of 10 mice of 18 months and only one 24-month-old mouse showed the development of IgG-PFC. The average responses of the 18-month-old mice were lower than those of the 3-month-old mice, although the differences were not significant. The large 95% confidence limits and the high response observed in one 24-month-old mouse indicate that the number of PFC generated in the aged mice sometimes fell within the range of PFC found in the youngest age group.

When the data obtained in the C57BL/Ka mice are compared with those obtained in the CBA mice (part C of this Chapter). the following differences are noteworthy.

1. Generally, both the IgM and especially the IgG responses are much lower in all age groups of the C57BL/Ka mice.
2. The response profiles are different for both strains tested. A shift in the peak day of the IgM response was noted in the C57BL/Ka mice. In addition. the decline in the response after the peak day is less pronounced in the C57BL/Ka than in the CBA mice (compare Figs. IV.4, 7 and 9).
3. In contrast to the CBA, aged C57BL/Ka mice exhibit hardly any IgG response, even when a high dose (10^9) of SRBC is employed.

TABLE IV.6

IgG-PFC RESPONSE IN C57BL/Ka MICE OF VARIOUS AGE GROUPS 6 DAYS AFTER IMMUNIZATION WITH DIFFERENT DOSES OF SRBC

	10^8 SRBC		4×10^8 SRBC		10^9 SRBC	
age in months	fraction responder mice/ total number tested	PFC x 10^{-3} per spleen	fraction responder mice/ total number tested	PFC x 10^{-3} per spleen	fraction responder mice/ total number tested	PFC x 10^{-3} per spleen
3	2/5	17* 8	4/5	100** 139 - 71	5/5	66 168 - 26
18	0/5	no response	4/5	40 127 - 13	4/5	16 96 - 3
24	0/5	no response	0/5	no response	1/5	127

* Individual values
**Geometric mean of the responder mice and 95% confidence limits.

E. DISCUSSION OF THE SECTIONS C AND D

In the previous sections, the capacity of ageing male CBA and C57BL/Ka mice to generate antibody forming cells to SRBC was investigated and compared. Attention has been paid to the early phase of this response in the spleen, since that approach gives information on alterations with age in the immediate reaction to an antigenic stimulus. Looking at the data presented so far, the following should be emphasized. If the response of the various age groups had been determined on a single day, at a single dose or for only a single antibody class, a real picture of the differences between young and ageing mice would not have been obtained. The existing differences might even have been masked. As stated above, the limited availability of old mice, however, did not allow complete kinetic studies and this complicated the choice of the experimental design for the reported ageing studies. Despite these limitations, we have obtained data on the changes in the early kinetics of an anti-SRBC response with age which permitted the conclusions as outlined in sections C and D.

The loss with age of the capacity to generate antibody forming cells to SRBC during a primary response can be attributed to many factors. Changes in the splenic environment of ageing mice may interfere with antigen processing, recruitment of immunocompetent cells from outside the spleen and cooperation between B and T cells. In vitro studies could reveal to what extent environmental factors contribute to the decline in antibody formation. One of the following Chapters (VI) will deal with age-related changes in the capacity to generate antibody forming cells in vitro. Since the antibody production to SRBC is thymus-dependent, both B and T cells can be responsible for the reduced response. This defect can be either quantitative, due to a depletion of progenitor cells of both or one cell type(s), or qualitative, i.e., involving defects in differentiation and proliferation as well as in the interaction between B and T cells. The existence of the so-called thymus-independent antigens makes it possible to study alterations in the functional capacity of B cells with advancing age. This is the subject of the following section (F) of this Chapter.

With increasing antigen dose, the difference in responsiveness between young and old mice was diminished. Therefore, the question arises as to whether such differences can still be observed when a higher level of antigenic stimulation is used, e.g., after repeated immunization. In Chapter V, an investigation of the secondary antibody formation in the spleen and bone marrow of ageing CBA mice will, therefore, be presented.

F. PRIMARY ANTI-LPS RESPONSE IN AGEING CBA, C57BL/Ka, BALB/c AND NZB MICE

1. Introduction

The existence of antigens which do not require T cells for the induction of a splenic PFC response offers the possibility to study exclusively the functional capacity of B cells in the antibody forming process. In this way, the contribution of B cells to the age-related decline in humoral immune reactivity can be investigated. The characteristics of the so-called thymus-independent antigens which activate B cells without T cell help have already been discussed in Chapter I, section B.3. From the well-known thymus-independent antigens such as lipopolysaccharide (LPS) from E. coli, Pneumococcal polysaccharide (S III), polyvinylpyrrolidone (PVP) or polymerized flagellin, LPS was selected for the following reason. Evidence has been accumulated over the past few years that not all antigens mentioned above can be considered as completely thymus-independent, in the sense that T cells have an influence on the magnitude of the response to some of these antigens. It was found that after T cell depletion by either treatment with antilymphocyte serum (ALS) or by adult thymectomy, the response to S III and PVP is markedly enhanced (Baker et al., 1970; Kerbel and Eidinger, 1972; Rotter and Trainin, 1974, and Lake and Reed, 1976). These results are explained by the existence of suppressor T cells which regulate immune responses even to thymus-independent antigens. However, no evidence has yet been presented that suppressor T cells do play a regulatory role in the antibody response to LPS. This response is not affected by treatment with ALS (Veit and Michael, 1972b, and Barth, Singla, and Ahlers, 1973).

Because of the complete absence of T cell influence on the response to LPS, LPS was considered to be the appropriate antigen for our study. The thymus-independence of the immune response to LPS is well established for the mouse. Thus, an equally strong anti-LPS response was found in thymectomized, irradiated and bone marrow-repopulated mice, whether thymocytes were added or not (Möller and Michael, 1971, and Andersson and Blomgren, 1971). Similarly, athymic nu/nu mice responded as well as normal litter mates to LPS (Reed, Manning, and Rudbach, 1973).

Another characteristic of the anti-LPS response is that the antibodies produced are restricted to the IgM class. IgG-PFC were never encountered during an anti-LPS response (DiPauli, 1972; Benner and Van Oudenaren, 1976; personal observation).

The anti-LPS response was used to assess the age-related changes in a thymus-independent humoral immune function in the CBA, C57BL/Ka NZB mice and BALB/c mice.

2. CBA

Before measuring the response of ageing male CBA mice towards LPS, the number of anti-LPS PFC in unstimulated mice was determined. Background levels of anti-LPS PFC in 3- and 22-24-month-old CBA mice are presented in Table IV.7. The background value was increased in the oldest age group. Therefore, mice older than 2 years were regarded as responders when they produced more than 2,000 PFC per spleen, this number being the upper value of the 95% confidence limits.

TABLE IV.7

NUMBER OF BACKGROUND ANTI-LPS PFC IN THE SPLEEN OF 3- AND 22-24-MONTH-OLD CBA MICE

age in months	PFC per spleen
3	202* 371-110
22-24	651 1,974-215

*Figures represent the geometric mean and 95% confidence limits of 5 mice.

The primary anti-LPS response was determined in three different age groups (3, 24 and 30 months) of CBA mice. It is known that in contrast to the anti-SRBC response, the PFC activity after immunization with LPS reaches its peak on day 4 independent of the antigen dose used (Benner and Van Oudenaren, 1976). Therefore, this test day was initially used to compare the response in the different age groups following stimulation with doses of LPS ranging from 0.001 µg to 10 µg (Fig. IV.10). The following remarks can be made:

1. Three- and 24-month-old mice reached a plateau level of anti-LPS PFC activity at a similar dose (0.1 µg), whereas the 30-month-old mice exhibited a lower response as compared to the plateau values with that dose. In other words, the dose required for an optimum level of responsiveness is 10-100-fold higher for the age group of 30 months than for the other two groups.
2. After immunization with 10 and 1 µg LPS, the average number of PFC tended to be lower in the age group of 30 months than in the other two groups, although the differences were not significant.

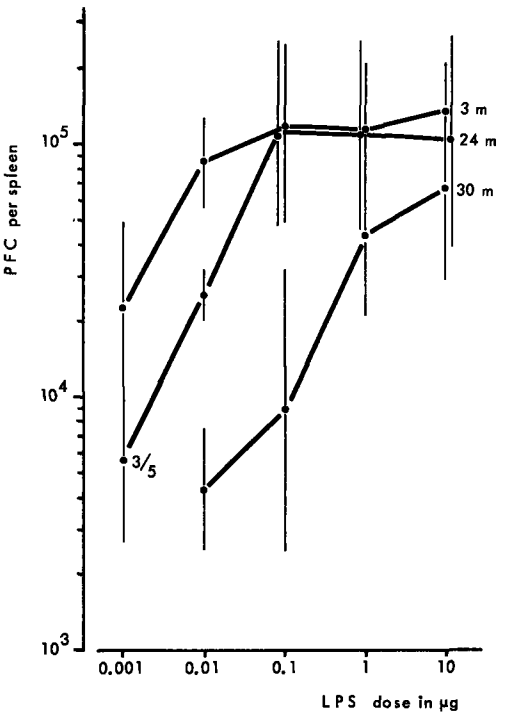

Figure IV.10 Effect of LPS dose on the PFC response in the spleen of 3-, 24- and 30-month-old CBA mice.
The response was determined 4 days after immunization. Each value represents the geometric mean of 5 mice unless nonresponder mice were found, which is indicated by the fraction of responders over the total number of mice tested. In that case, only the mean and confidence limits of the responder mice were calculated. The 95% confidence limits are indicated by bars.

3. As with the thymus-dependent anti-SRBC response, the response to LPS also revealed greater differences among the various age groups when suboptimal doses of antigen were employed.

A summary of three separate experiments, including the experiment shown in Fig. IV.10, is presented in Table IV.8. To provide a means of comparison among the experiments, the table gives the geometric means of the PFC responses and the 95% confidence limits as percentages of the response of the 3-month-old age group. The experiments were carried out at time intervals of approximately six months in order to investigate whether comparable results could be obtained with aged mice born and reared in different time periods. In experiments 2 and 3, two doses of LPS were used: 1 µg, a dose which was shown to give optimal responses in 3- and 24-month-old mice and 0.01 µg representing

TABLE IV.8

ANTI-LPS RESPONSE IN THE SPLEEN OF CBA MICE OF VARIOUS AGE-GROUPS

exp.	LPS dose in µg	3 months PFC x 10^{-3} per spleen	3 months per cent	24 months PFC x 10^{-3} per spleen	24 months per cent	30 months PFC x 10^{-3} per spleen	30 months per cent	36 months PFC x 10^{-3} per spleen	36 months per cent
1*	1	115**	100	114	99***	44	38	-	
		208	181	277	241	92	80		
		63	55	47	41	21	18		
2	1	129	100	109	85	-	-	-	-
		183	142	182	141				
		91	70	66	50				
3	1	91	100	82	90	55	60	13	14
		166	182	162	179	233	257	37	41
		50	55	41	45	13	14	5	5
1*	0.01	86	100	26	30	4	5	-	-
		129	151	32	37	8	9		
		57	66	20	24	2	3		
2	0.01	80	100	17	21	-	-	-	-
		136	171	36	45				
		46	58	8	10				
3	0.01	43	100	9	22	2****	7	-	-
		68	160	22	51	3	8		
		27	62	4	10	4	10		

* Experiment 1 corresponds with Fig. IV.10.
** Geometric mean and 95% confidence limits of 4-5 mice 4 days after immunization.
*** Geometric mean and 95% confidence limits are expressed in percentages of the mean number of PFC obtained in 3-month-old mice.
****Only 2 out of 5 mice responded to LPS. Mean and individual values of these two mice are presented.

a suboptimal dose (see Fig. IV.10). An additional age group consisting of 5 mice of the age of 3 years challenged with 1 µg LPS was included in experiment 3. These mice exhibited PFC activity to LPS but, in contrast to the 24- and 30-month-old mice, the response differed significantly from that obtained in 3-month-old mice. The remaining PFC responses found in experiments 2 and 3 were comparable to the results obtained in experiment 1; i.e., no significant differences were observed in the numbers of PFC until the age of 30 months following immunization with 1 µg LPS, whereas, with the lower dose of 0.01 µg, the response was decreased with advancing age. An additional finding in experiment 3 was that only 2 out of 5 mice of the 30-month-old age group reacted to the dose of 0.01 µg LPS.

In the above experiments, the PFC activity was estimated on day 4. An experiment in which the response of 3- and 23-month-old CBA mice was determined 4 and 5 days after immunization with 1 and 0.1 µg LPS is presented in Fig. IV.11. The results are not suggestive of a shift in the peak day of the anti-LPS response in older mice.

With regard to the variation in the numbers of PFC, the occasionally large 95% confidence limits reflect that there is a great variability among the individual mice in the anti-LPS response, which was independent of the dose or age group.

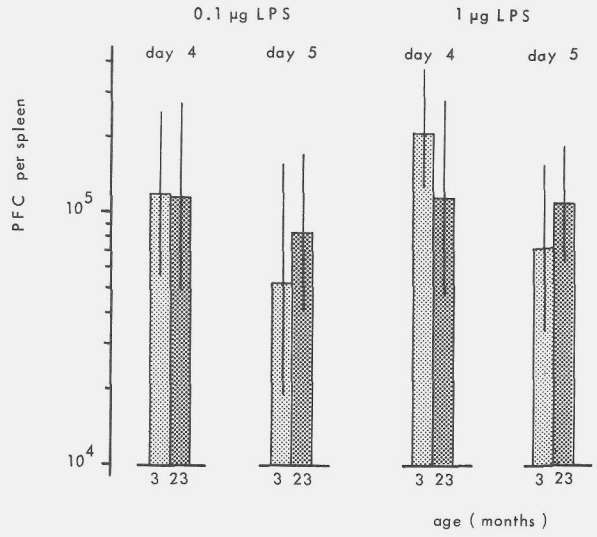

Figure IV.11 PFC response to LPS in the spleen of 3- and 23-month-old CBA mice determined 4 and 5 days after immunization with 0.1 or 1 µg LPS. Each figure represents the geometric mean of 5 mice. The 95 % confidence limits are indicated by vertical lines.

Before discussing the above data in more detail, we will first report on the data obtained with the antibody formation to LPS in C57BL/Ka and NZB mice.

3. C57BL/Ka

The antibody formation to LPS in ageing male C57BL/Ka mice was only partially studied, since mice older than 18 months were no longer available at that time. In two experiments, the age groups of 3 and 18 months were compared as to their capacity to mount a response to LPS four days after immunization. Since the numbers of PFC found in each experiment fell within the same range, the results of the two experiments are taken together and shown in Fig. IV.12. It can be observed that C57BL/Ka mice of 18 months of age, which is around the 90% survival age, exhibited the same level of responsiveness to 10 µg LPS as did 3-month-old C57BL/Ka mice. After immunization with 1 µg LPS, the PFC reactivity of 18-month-old C57BL/Ka mice was even significantly higher. All mice studied did respond to 10 and 1 µg LPS, since the numbers of antibody forming cells greatly exceeded the background values of anti-LPS PFC in unstimulated C57BL/Ka mice (Table IV.9).

A first comparison with the anti-LPS response obtained in CBA mice at the same doses of antigen shows that, also in C57BL/Ka mice, a de-

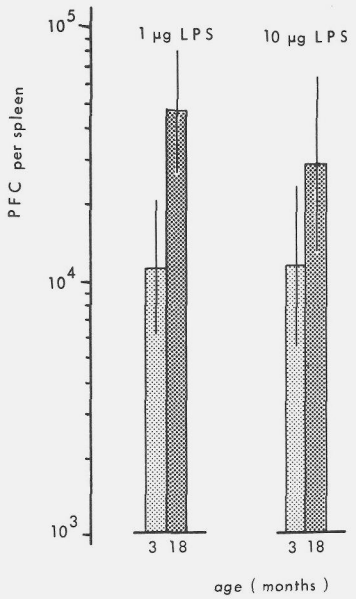

Figure IV.12 PFC response to LPS in the spleen of 3- and 18-month-old C57BL/Ka mice immunized with 1 or 10 µg LPS.
The response was determined 4 days after immunization. Each figure represents the geometric mean of 9-10 mice studied in two separate experiments. Vertical lines represent the 95 % confidence limits.

cline in the PFC activity to LPS cannot be noted until the 90% survival age.

4. NZB and BALB/c

The functional study of the thymus-independent part of the humoral immune system of NZB mice was a continuation of an extensive investigation of Elkerbout and Hijmans (1974a, b) concerning a thymus-dependent humoral immune function in this mouse strain. Their main finding was that ageing NZB mice exhibited a prolonged response to SRBC in comparison to other mouse strains. Their subsequent experiments revealed that this prolonged elevation of the response was possibly due to anti-SRBC antibodies with a low avidity. These results will be further discussed in relation with data from the literature on NZB mice at the end of this Chapter.

The rationale to initiate a study concerning a thymus-independent immune function in NZB mice was that so far much more attention has been paid to changes in the functional capacities of the T cell system, which were shown to decline with advancing age in this strain. It was, therefore, generally accepted that this decline mainly attributes

TABLE IV.9

NUMBER OF BACKGROUND ANTI-LPS IN THE SPLEEN OF 3- AND 18-MONTH-OLD C57BL/Ka MICE

age in months	PFC per spleen
3	172* 362-82
18	146 350-60

*Figures represent the geometric mean and 95% confidence limits of 5 mice.

to the autoimmune phenomena characteristic of this strain. In order to complete the immunological pattern of ageing NZB mice, it was necessary to investigate the contribution of the B cell system to the observed deviations from the normal immune reactions in these animals. For this purpose, the anti-LPS response was employed.

At the time these experiments were performed, the mouse strains for the ageing colonies of the Institute for Experimental Gerontology were not yet selected. In order to have a reference mouse strain to which the anti-LPS response of NZB mice could be compared, the histocompatible BALB/c mice were chosen. The 10- and 19-21-month-old BALB/c mice used consisted of retired female breeders.

In view of the findings of Elkerbout and Hijmans (1974a) that ageing NZB mice exhibited a prolonged anti-SRBC response, our experiments were designed to investigate a long time course of the splenic anti-LPS response. Such experiments required a large number of mice which were not available at that moment, at least not a large group consisting of mice of the same sex. It was decided, therefore, to combine male and female mice in this series of experiments. However, after the establishment of the ageing cohorts, it was shown that male and female NZB mice differ with regard to their survival pattern (see Chapter III, Table III.1). Thus, from the gerontological point of view, it was not justified to combine male and female NZB mice of the same age within one experimental group. Therefore, in the figures presented hereafter, the data will be expressed differently from those already published (Blankwater, Levert, and Hijmans, 1975), in the sense that male and female mice will be separately indicated. It will be shown, however, that male and female NZB mice of the same age behave in a comparable fashion with regard to their anti-LPS response.

In pilot experiments performed with NZB and BALB/c mice of various age groups, it was observed that even small quantities (1-10 µg) of LPS were highly toxic to 10-13-month-old NZB mice. Approximately half of the mice of this age died within 24-48 hours after administration of LPS. NZB mice of the ages of 2-4 months and BALB/c mice of all age groups studied were not sensitive to the given doses of LPS.

It is known that LPS derived from the cell wall of Gram-negative bacteria (also referred to as endotoxin) can cause a multitude of physiological changes (for a collection of reviews, see supplement J. Infect. Dis. 128, July 1973). However, the dose of LPS which is sufficient for inducing an immune response is normally not toxic for mice (Agarwal, Parant, and Parant, 1972). The most likely explanation for the effect of LPS in 10-13-month-old NZB mice can be found in the observations of Chedid (1973). He noted that mice became hyperreactive to LPS after either immunosuppression or immunostimulation, which suggests that susceptibility to LPS may be enhanced by disturbances in the immune system. Accordingly, the immunological disorders of NZB mice may account for the high sensitivity of this strain to LPS. It must be pointed out here that, after immunization with 10 µg LPS, neither the CBA nor the C57BL/Ka mice up till the age of 30 months and 18 months, respectively, nor young nu/nu mice exhibited the same sensitivity as NZB mice.

In order to still be able to study the response of NZB mice to LPS without selecting mice that were not susceptible to the effect of it, the toxicity of LPS had to be reduced. This was accomplished by treating the LPS with weak alkali according to the method of Britton (1969) (see Chapter II). This preparation was no longer toxic for NZB mice of the age of 10 months.

To establish the thymus-independence of detoxified LPS, the antibody response of nu/nu mice and their normal litter mates (NLM) to LPS and detoxified LPS was compared. Table IV.10 shows that the number of anti-LPS PFC in these two groups of mice did not differ significantly. This indicates that neither the response to LPS nor the response to detoxified LPS requires the participation of T cells. Untreated LPS gave a higher response than did detoxified LPS in nu/nu and NLM mice. For comparison, the response of young BALB/c and NZB mice to either LPS preparation is included in Table IV.10. A similar difference in immunogenicity as found in nu/nu and NLM was observed in 2-month-old NZB mice. The response to 5 µg detoxified LPS was approximately 3-fold lower than the response to an equal amount of untreated LPS. BALB/c mice, on the contrary, reacted as strongly to LPS in its normal or alkaline treated state.

TABLE IV.10

COMPARISON OF THE RESPONSE TO LPS AND DETOXIFIED LPS IN nu/nu, NLM, BALB/c AND NZB MICE

strain	age in weeks	PFC per spleen x 10^{-3}* normal LPS	detoxified LPS
nu/nu	8	102** (166 - 63)	30 (56 - 16)
NLM***	8	120 (167 - 86)	24 (48 - 12)
BALB/c	12	190 (304 - 118)	163 (188 - 141)
NZB	8	42 (62 - 28)	12 (27 - 5)

* Response was determined 4 days after immunization with 5 µg normal or detoxified LPS.
** Figures represent the geometric mean and 95% confidence limits of 4-5 mice.
***Normal litter mates of nu/nu mice.

It has been reported that the antigenic determinants of LPS were basically unchanged by alkaline hydrolysis (Britton, 1969). However, those properties associated with the lipid A region of LPS such as mitogenic, polyclonal and adjuvant activities were no longer present (Andersson et al., 1973, and Skidmore et al., 1975). Therefore, an explanation for the strain dependent difference in the response to LPS and detoxified LPS may be found in the lack of lipid A activity after detoxification, which may influence the level of responsiveness, at least in some strains.

On the basis of a limited number of pilot experiments in young NZB and BALB/c mice, a dose of 5 µg detoxified LPS was chosen for immunization. Time courses of splenic anti-LPS PFC responses were determined in various age groups in order to compare them with the data concerning the thymus-dependent anti-SRBC response in these strains as presented by Elkerbout and Hijmans (1974a). A group of 2-month-old mice was included, since NZB mice are known for their hyperresponsiveness at a relatively young age (Evans, Williamson and Irvine, 1968). Young adult animals are represented by the age group of 4 months. The oldest age group of NZB mice consisted of mice aged 10 months. Female NZB mice of that age have already reached or passed the 90% survival age whereas male NZB mice of 10 months are 2-4 months younger than when they reach that particular point in the survival curve (see Chapter III, Fig. III.3). Since BALB/c mice show a different survival curve (the 90% and 50% survival ages for female BALB/c mice are 18 and 24 months, respectively), an extra age group of

19-21-month-old BALB/c mice was included in this series of experiments.

The results of the anti-LPS response in the various groups are presented in Fig. IV.13 and Fig. IV.14 for BALB/c and NZB mice, respectively. Following immunization, the BALB/c mice showed an exponential increase in antibody forming cells with a peak response on day 4 or 5. A tendency to a shift in the peak from day 4 to day 5 can be observed with advancing age. When the maximal responses of the various age groups of BALB/c mice were compared, the response of the age group of 2 months was significantly lower than those of the 10- and 19-21-month-old mice. The other three age groups did not differ significantly from each other.

With regard to the NZB mice, the following points are noteworthy (Fig. IV.14).

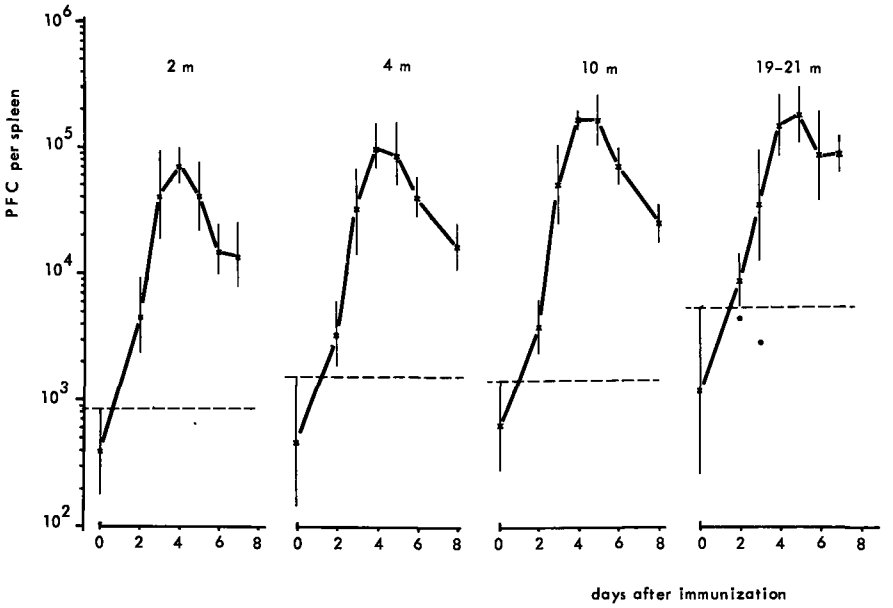

Figure IV.13 PFC response to detoxified LPS in the spleen of BALB/c mice of various ages.
The response was determined on various days after immunization with 5 µg detoxified LPS. The geometric means of 5 mice are indicated by crosses. Bars represent the 95 % confidence limits. Male and female mice were used for the age groups of 2 and 4 months, while the mice of 10 and 19-21 months were females. Day 0 values represents the number of background PFC obtained in unstimulated mice. The upper limit of the 95 % confidence limits of background PFC is indicated by a dashed line. Immunized mice which develop PFC below this upper limit are considered as nonresponders (e.g. two mice (•) of 19-21 months). In those cases, only the mean and confidence limits of the responder mice were calculated.

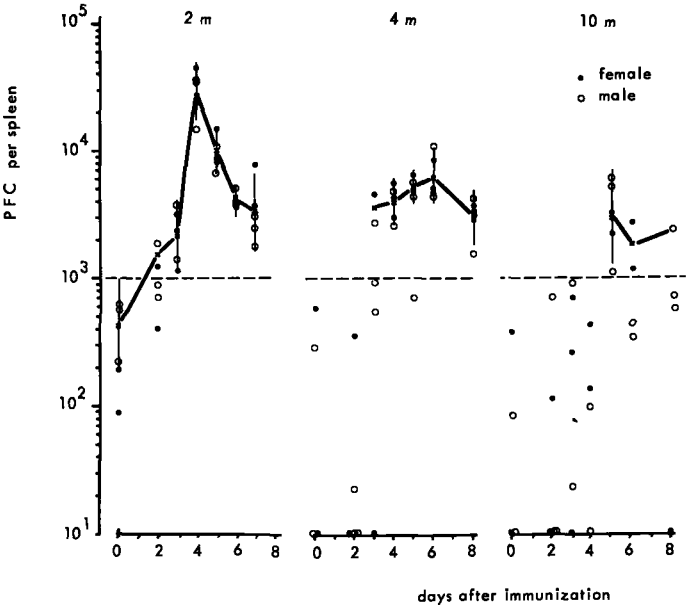

Figure IV.14 PFC response to detoxified LPS in the spleen of NZB mice of various ages.
The response was determined on various days after immunization with 5 µg detoxified LPS. The geometric mean of the responder mice are indicated by crosses. Bars represent the 95 % confidence limits. Individual values of male and female NZB mice are presented. Day 0 values represent the number of background PFC obtained in unstimulated mice. The upper limit of the 95 % confidence limits of background PFC is indicated by a dashed line. Immunized mice which develop PFC numbers below this upper limit are considered as nonresponders.

1. In general, the PFC values of male and female NZB mice of all age groups fell within the same range.
2. Two-month-old NZB mice exhibited a regular time course of the response with a peak on day 4 comparable to the time course of BALB/c mice of the same age, although the level of responsiveness was lower in NZB mice.
3. Very poor responses were obtained in 4- and 10-month-old mice, as was indicated by the large numbers of nonresponder mice in both age groups. Nonresponder mice are considered to be those animals which respond with the same PFC values as found in unstimulated mice of the same age. Among 4-month-old mice, nonresponders were observed on day 2 (5/5), day 3 (3/5) and day 5 (1/5). These nonresponders and the fact that the peak day is delayed (day 6 instead of day 4 as found in the 2-month-old mice) possibly reflect a slow start of the maturation and proliferation of the immune competent cells after antigenic stimulation in this age

group.

4. In the 10-month-old NZB mice, the 5th day of the time curve was the only day that all mice responded, while only 2 out of 4 mice (day 6) and one out of 4 mice (day 8) gave a response on the subsequent days.
5. The spleen weight and the number of nucleated cells in the spleen of 10-month-old NZB mice were increased in comparison with those of 2- and 4-month-old NZB mice (Fig. IV.15). A correlation between the spleen weight or number of nucleated cells per spleen and the anti-LPS PFC activity, however, was not observed.

Additional information was acquired by assaying the serum of NZB and BALB/c mice of the different age groups for antibody titres to LPS (Fig. IV.16). The antibody levels of BALB/c mice were always higher than those of NZB mice. The antibody titres of NZB mice decreased with

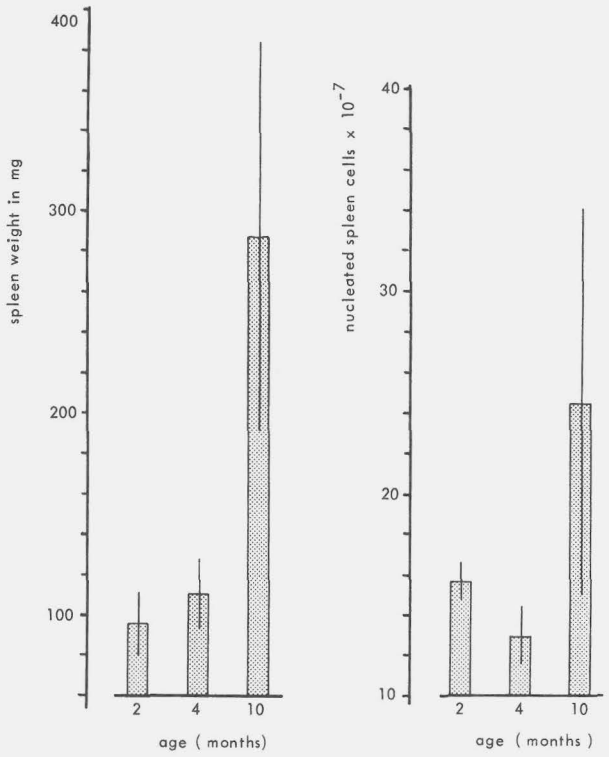

Figure IV.15 Spleen weight and number of nucleated cells in the spleen of NZB mice of various ages.
Each figure represents the average value of 9-10 animals. The 95 % confidence limits are indicated by vertical lines.

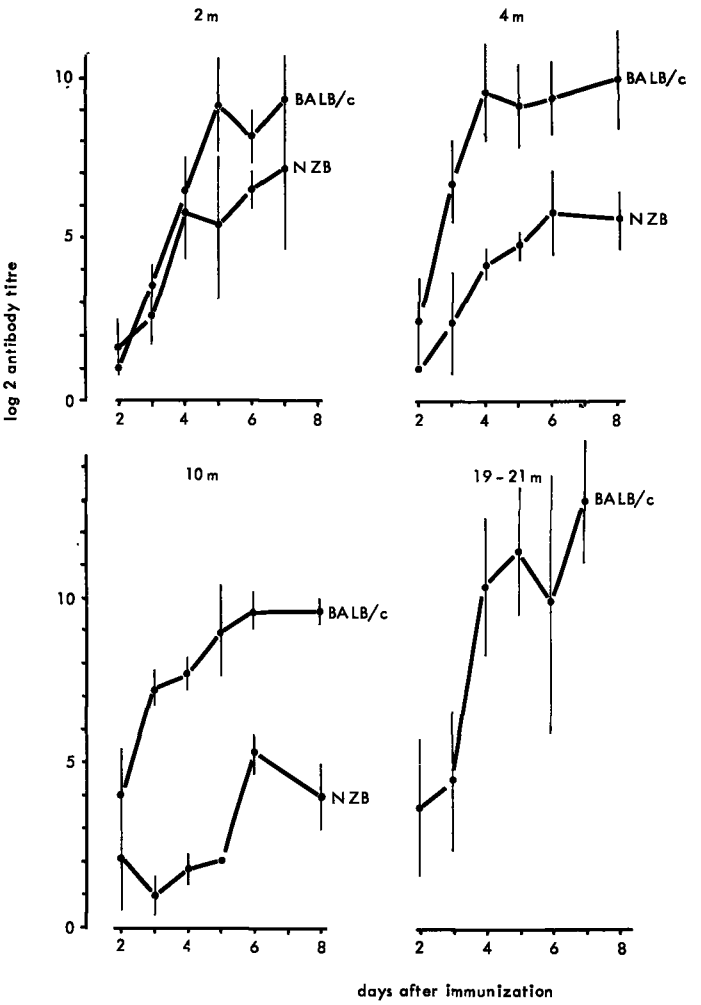

Figure IV.16 Comparison of the antibody titres to detoxified LPS in serum of NZB and BALB/c mice of various ages.
The response was determined on various days after immunization with 5 µg detoxified LPS. Each value represents the mean of 4-5 mice. Standard deviations are indicated by bars.

age. A low level of anti-LPS antibodies was detected in the sera of 10-month-old NZB mice. This does not seem to be in accordance with the absence of anti-LPS PFC activity in the spleen of a large number of mice of this age group. However, it is known that the bone marrow also contributes to the production of anti-LPS antibodies, since the bone marrow contains antibody forming cells to LPS (but not to SRBC) during

a primary response (Benner and Van Oudenaren, 1976).

The above results clearly demonstrate a progressive loss of cells forming antibodies to LPS with advancing age. Since this antibody response is exclusively dependent upon B cells (see F.1), this loss reflects a defective B cell function in NZB mice.

G. DISCUSSION

In this Chapter, age-related alterations in the humoral immune potential of different mouse strains were investigated and compared. For that purpose, the capacity to develop an in vivo primary antibody response after antigenic stimulation was studied by determining the number of antibody forming cells in the spleen. The investigations presented here were mainly focused on the early phase of a primary response, i.e., around the peak day.

With respect to the thymus-dependent anti-SRBC response, the results revealed that the maximum level of responsiveness as found in young adult mice could no longer be reached by either CBA or C57BL/Ka mice from approximately the 90% survival age onwards, which is about 3/5 of the expected life-span. After this age, the differences with the young adult mice became even more pronounced. The decline in responsiveness was found for the IgM as well as for the IgG response. Among aged mice of both strains - but most evident in the C57BL/Ka mice - mice were observed which failed to show any IgG response to SRBC. In contrast to the C57BL/Ka mice, old CBA mice did not exhibit clear-cut shifts in the appearance of peak responses in comparison with 3-month-old CBA mice. In C57BL/Ka mice, the profiles of the time course changed with age.

It was found in ageing mice of both strains that the immune reactivity to low doses of antigen was severely affected. This was more apparent for the IgG, as illustrated by the occurrence of nonresponders, than for the IgM response. Also with the latter response the difference between young and old mice became larger at lower antigen doses. This observation confirms previous data reported by Price and Makinodan (1972a), who made a dose response analysis for the IgM anti-SRBC antibody formation in young and old BC3F1 mice. They showed that about 10 times as much antigen is required to maximally stimulate IgM production in old mice; this is in accordance with our findings on the anti-LPS response in CBA mice (Fig. IV.10). Although such a conclusion cannot be firmly drawn from our data on the anti-SRBC response, Fig.

IV.7 suggests that young CBA mice reached their maximum response at a lower antigen dose than did 22-month-old CBA mice. Our data extend those of Price and Makinodan (1972a) by including the IgG response and different test days in the dose response analysis.

It should be noted, that compared with the CBA mice, 3-month-old C57BL/Ka mice generally developed lower numbers of IgM-PFC and were poor responders with regard to IgG antibody formation to SRBC. Silver and Winn (1973) observed the same paucity of IgG-PFC activity in young C57BL/6J mice during a primary response to SRBC; i.e., the values found for IgG never surpassed those found for IgM antibody formation, in contrast to other strains. Another interesting finding of Silver and Winn (1973) was that C57BL/6J mice were not capable of developing extra IgG-PFC activity after a second or multiple exposure to SRBC.

A survey of the results reported in the literature concerning the primary anti-SRBC response as estimated by either antibody titration or plaque formation (mainly IgM) reveals that all strains studied showed a decline in reactivity with advancing age (Wigzell and Stjernswärd, 1966; Kishimoto, Tsuyuguchi, and Yamamura, 1969; Metcalf, Moulds, and Pike, 1966; Finger, Emmerling, and Bertz, 1971, and Micklem, Ogden, and Payne, 1973). However, it should be emphasized that only antigen doses giving optimal immune responses were used, while hardly any attention was paid to possible changes in the time course of the response in old mice of these strains. The onset of the decline can differ dependent upon the strain used. However, the data are very difficult to compare, since the exact survival ages of the various strains are seldom indicated in the above reports (see also Walford, 1976).

Our observations on the changes in the primary response to SRBC with age can best be compared with those of Makinodan and co-workers. Their initial studies concerned the assessment of the primary and secondary antibody forming potential by transferring spleen cells of varying age groups together with foreign red blood cells to irradiated hosts and comparing their serum titre levels (Makinodan and Peterson, 1962, 1964, 1966a, 1966b). The PFC technique was employed in later studies of this group. The strain used by them was the long-lived BC3F1 hybrid. Detailed information concerning the survival pattern and age-related pathology of this strain is given by Chino et al. (1971). A remarkable difference with our CBA and C57BL/Ka mice was the very late appearance of an IgG peak response (day 12) in old BC3F1 mice even at the high dose of SRBC (10^9) used by them (Makinodan et al., 1971a). The shift in the peak of IgM antibody forming cells in the spleen of old BC3F1 mice (from day 4 in young mice to day 6 in old mice) resembles the response pattern found for old C57BL/Ka mice, but

not that for the old CBA mice nor for old (C3H x C57BL/6J)F1 used in a study of Friedman and Globerson (1978).

From the studies of Makinodan et al. (1971a), Micklem, Ogden, and Payne (1973), Goidl, Innes and Weksler (1976), and Friedman and Globerson (1978), it appeared that the IgG response was more affected by ageing than was the IgM response. Although the level of IgG antibody formation measured by them was low, nonresponder mice as observed by us were never encountered. Unfortunately, it cannot be concluded from the above-mentioned literature data whether significant numbers of IgG-PFC were always obtained, since the authors do not show the number of IgM-PFC on the days for which they report an IgG-PFC response. This may explain the discrepancy between their data and our own. As outlined in part C of Chapter II, a statistical analysis is necessary to ascertain significant IgG plaque formation.

The rapid decline after the IgM peak, as was found for 3-month-old CBA and C57BL/Ka mice at high antigen doses, disappeared in aged mice, suggesting changes in the regulatory mechanism(s) responsible for this decline. Although the processes occurring after a response has reached its maximum level are largely unknown, the following mechanisms can be envisaged. Based on previous work of Nossal et al. (1964) and recent work of Kolb and Bosma (1977), there is evidence that clones of antibody forming cells can switch from IgM to IgG production while retaining the same antigen specificity. This may also explain the sudden appearance of IgG-PFC 4 to 5 days after immunization. In older mice, this IgM-IgG switch may occur to a lesser extent, leaving relatively a larger number of cells which continue to produce IgM. In addition, a regulatory role has been ascribed to antibodies acting in a feedback inhibition either alone (Bystryn, Schenkein and Uhr, 1971) or as antigen-antibody complexes (Diener and Feldmann, 1972) and, recently, to anti-antibodies (anti-idiotypes, Jerne, 1974, and Cosenza, 1976) at certain stages of an immune response. Similarly, regulatory or suppressor T cells are considered to play a role in the ongoing response to a given antigen. Any change with age in one of the possible regulatory mechanisms will result in different response patterns for young and old mice. It is tempting to speculate as to whether the same phenomena (i.e., lack of antibody or complex mediated feedback inhibition and/or defect in regulatory interactions between helper and suppressor cells) also have a bearing on the appearance of homogeneous immunoglobulins with ageing, as found, for instance, in C57BL/Ka mice. This question can also be asked in relation to the increased frequency of autoimmune phenomena during ageing as will be shown in the following discussion on NZB mice.

As pointed out earlier, the determination of the <u>in vivo</u> anti-SRBC

response in ageing NZB mice was not included in the present study. From the extensive literature data, the following picture emerges. NZB mice show an early maturation of the anti-SRBC response, since neonatal mice of this strain gave a very high response when compared with neonatal mice of other strains (Evans, Williamson, and Irvine, 1968, and Playfair, 1968). Young adult NZB mice still showed an elevated antibody response in comparison with other strains (Baum, 1969; Morton and Siegel, 1969, and Cerottini, Lambert, and Dixon, 1969). This state of hyperresponsiveness waned in older NZB mice, which gradually developed a lower anti-SRBC response, not only in comparison with young adult NZB mice (Roder, Bell, and Singhal, 1975) but also in comparison with other strains (Morton and Siegel, 1969). On the other hand, Elkerbout and Hijmans (1974a) did not find peak values of 9-month-old NZB mice to be different from those in various other strains, but they noted a continuous high IgM and IgG-PFC activity to SRBC throughout 30 days after immunization. Their subsequent study revealed that old NZB mice produced IgG antibodies of a low avidity which may cause a disturbed feedback mechanism of the immune response and result in prolonged antibody formation (Elkerbout and Hijmans, 1974b). This was in accordance with the data of Petty and Steward (1972, 1977), who found that the relative affinity of antiprotein antibodies produced in NZB mice was lower than in other strains tested. Another explanation for the prolonged response in NZB mice - which does not necessarily exclude the above possibility - is the loss of suppressor T cells in ageing NZB mice. An accelerated loss of suppressor T cell function has been suggested for a variety of immunological functions in both NZB and NZB/W mice and has been associated with the autoimmune phenomena in these strains (see the reviews of Steinberg et al., 1975, and Talal, 1976). Recently, a study of Klassen, Krakauer and Steinberg (1977) suggested that naturally occurring thymocytotoxic antibodies caused this selective loss of suppressor cells in NZB and NZB/W mice. Although the data presented for NZB and NZB/W mice, especially by the groups of Talal and Steinberg, can be explained by a defect in suppressor T cell activity, it is appropriate to refer at this point to Gershon and Metzler (1977) who argued for a more cautious interpretation of the experimental data obtained in NZB and NZB/W mice. In their opinion, it is more likely to suggest that NZB mice have a defect in regulatory interactions between their different T cell subpopulations rather than having an intrinsic defect in suppressor T cells only. Support for this notion comes from a study of Roder, Bell, and Singhal (1977) who found increased suppressor activity instead of a loss of this activity in aged NZB/W mice, using a different assay system.

The role of suppressor cells during ageing has not only been studied in autoimmune susceptible strains but also in strains of mice free of overt autoimmune phenomena. Goidl, Innes and Weksler (1976) found a suppressive effect in spleen cells of old mice, which increased with the age of the donor. Suppressor activity was demonstrated by transferring a mixture of young and (pooled) old spleen cells to an irradiated host and measuring a primary immune response to an hapten-carrier complex. Whether T cells were responsible for this suppressive effect was not explored in the above experiments. Results obtained by Segre and Segre (1976a) are only partially in accord with the above observations. By studying the effect of immunized and nonimmunized old spleen cells on the secondary response of young spleen cells in diffusion chambers, these authors found that only immunized spleen cells from old animals exhibited suppression. Anti-Thy-1 serum abolished the suppressive effect. Further studies of Segre and Segre (1977) in which, instead of pooled old spleen cells, spleen cells of individual old mice were used, showed individual differences with respect to suppressor T cell activity, although a majority of the old mice still displayed suppression. Similar results concerning individual variation in suppressor activity of old spleen cells were obtained by Makinodan et al. (1976) by using mixtures of young and old spleen cells in an in vitro anti-SRBC response. They again found suppressive activity in spleen cells from nonimmunized old mice (about 65%) which resembles the results of Goidl, Innes and Weksler (1976) as described above.

In summary, these first studies concerning suppressor activity in spleen cells of nonautoimmune mice suggested an increase rather than a decrease in suppressor activity during ageing. Conflicting results were recently obtained on age-related changes in suppressor T cell activity in humans. In these studies, the property of concanavalin A (ConA) activated lymphocytes to suppress mitogen responses of normal lymphocytes was used. Hallgren and Yunis (1977) found a decrease in suppressor activity with age in human peripheral blood lymphocytes. Antel, Weinrich and Arnason (1978), on the other hand, described an increased activity of suppressor cells induced by ConA.

From our results obtained by measuring the anti-SRBC response in ageing CBA and C57BL/Ka mice, no conclusion can be drawn with respect to alterations in suppressor activity. The data can be explained by a reduction in helper T cell activity as well as by an increase in suppressor cell activity or by alterations in both populations, while a functional decline in the B cells can play an additional role. However, the difference found in response patterns between young and old CBA mice and especially young and old C57BL/Ka mice still indicate

that changes occur in the interactions between B cells and subpopulations of T cells with advancing age. The complexity of the antibody formation to thymus-dependent antigens makes it difficult to discover which cell population is totally or partially responsible for the age-related decline.

As outlined in part E of this Chapter, the anti-LPS response was, therefore, used to study a thymus-independent immune response in comparison with the thymus-dependent anti-SRBC response. The results indicate that, after immunization with optimal doses of LPS, the response of CBA mice up to the age of 24 months and of C57BL/Ka mice up to the age of 18 months is still comparable to the response in 3-month-old CBA and C57BL/Ka mice, respectively. In other words, both ageing CBA and C57BL/Ka mice maintained a B cell population capable of responding to LPS after optimal antigenic stimulation. It has to be emphasized that the antibody formation to optimal doses of SRBC was already markedly reduced in comparison with young animals at the above-mentioned ages (24 months for CBA and 18 months for C57BL/Ka). The data indicate that, in these strains under optimal antigenic stimulation, a thymus-dependent humoral immune function declines earlier than a humoral immune function which is only dependent upon B cells. Nevertheless, suboptimal doses of LPS induced a significantly lower response in 24- and 30-month-old CBA mice than in 3-month-old CBA mice (C57BL/Ka mice were not studied to this extent). It can be concluded, therefore, that old CBA mice are less capable of responding to a low antigen dose in the early phase of a primary immune response independent of the involvement of T cells.

Before discussing the anti-LPS response in NZB mice (which clearly exhibited a decline during ageing), the data reported in the literature with respect to thymus-independent humoral immune responses in ageing mice of nonautoimmune susceptible strains will be compared with our results. It has been reported by Gerbase-DeLima et al. (1974) that, in (C57BL/6J x 129)F1 mice, the anti-LPS response appeared to decline to a lesser degree and later than thymus-dependent humoral immune functions. This is in accordance with our data for CBA and C57BL/Ka mice. Opposite results were obtained by Zharhary, Segev and Gershon (1977) who reported that the IgM response of aged C57BL/6J mice to TNP coupled to a thymus-dependent carrier (KLH) was not impaired during ageing in contrast to the response to TNP-LPS which was decreased. Friedman and Globerson (1978), on the other hand, found the response to SRBC and to the thymus-independent antigen polyvinyl pyrrolidone to be decreased to the same extent in aged (C3H x C57BL/6J)F1 mice.

In another study, various mouse strains were compared as to their capacity to mount a thymus-independent response during ageing (Smith, 1976). It was found that both BALB/c and C3H mice retained the capacity to mount a high level of PFC response to pneumococcal polysaccharide type III (S III) at various ages throughout 2 years of life. BALB/c mice were also capable of producing high PFC responses to LPS during the same time period. The only strain in this comparative study which exhibited a decline in both the anti-S III and the anti-LPS responses was the SJL/J mouse. As stated earlier, the anti-S III but not the anti-LPS response is under the control of regulatory T cells. Therefore, Smith (1976) investigated the effect of antilymphocyte serum (ALS) on the anti-S III response in aged BALB/c and SJL/J mice. Using this approach for the assessment of suppressor T cell activity, he found a decline in this activity in BALB/c mice and an increase in SJL/J mice. Age-related changes in the anti-S III response were also studied by Callard, Basten, and Waters (1977) in CBA/H mice. They observed a significantly lower response to S III in 22-24-month-old mice after immunization with an optimal dose of S III. Treatment with anti-Thy-1 serum did not alter the response, indicating that suppressor T cells did not play a role in the defect and the decreased response must, therefore, be attributed to B cells.

In summary, the above data point to strain differences in the ageing behaviour of a thymus-independent humoral immune response. Nevertheless, in comparison with thymus-dependent humoral immune responses, the functional capacity of B cells seems to decline less rapidly with age in most strains studied.

With respect to the anti-LPS response, both the SJL/J mice as reported by Smith (1976) and the NZB mice as reported in this Chapter exhibited a decreasing capacity to respond to this antigen with advancing age. This was already apparent in NZB mice at 4 months of age. In both studies, the response in BALB/c mice was determined for comparison. The results agree very well, in the sense that BALB/c mice retained a high level of responsiveness up to 20 months of age. The progressive decline in the anti-LPS response in NZB mice is indicative of a defect in the B lymphocyte population of NZB mice. This defect can be attributed to a decrease in the size of the stem cell pool for B cells or a decline in the functional capacity of existing B cells. There are no indications, however, for a reduced number of stem cells in NZB mice. Although abnormalities in haemopoietic stem cell differentiation in NZB mice have been observed (Warner and Moore, 1971), an elevated rather than a reduced number of stem cells was found, as manifested by a large number of endogeneous colony forming units.

Other studies also point to a loss of B cell function in NZB mice with age. Staples, Steinberg and Talal (1970) reported that B cells of NZB mice are less easily made tolerant to bovine gamma globulin than are those of normal mice, even in the presence of competent thymus-derived cells. De Jesus, Holborow, and Brown (1972) showed that ageing NZB mice gradually lost the ability to localize aggregated human gamma globulin complexes in germinal centres, a function chiefly attributed to B cells. Furthermore, our results are in accord with those of Purves and Playfair (1973) who also found a hyporeactivity of 3-6-month-old NZB mice to two other thymus-independent antigens S III and bacterial levan. On the contrary, Barthold, Kysela, and Steinberg (1974) reported an increase in antibody formation to S III in ageing NZB mice, which they suggest to be due to a loss of suppressor T cell function. The use of S III as a thymus-independent antigen and, in combination with ALS, as a method to detect suppressor T cells has frequently led to conflicting results (Warr, Ghaffar, and James, 1975, and Baker and Prescott, 1975). Since Stutman (1972) clearly demonstrated that the number of B lymphocytes did not change with age in NZB mice, the conclusion can be reached that the reduction in B cell function in NZB mice is due to a qualitative rather than a quantitative defect in the B lymphocytes themselves.

In addition to the extensive literature data on impaired T cell functions in NZB mice as manifested by a decrease in cell-mediated immune functions and by a possible defect in the balance of helper and suppressor T cell function (for a review, see Talal, 1976), a loss of B cell function has to be considered in NZB mice. Therefore, in addition to a frequently suggested dominant role for T cells in the appearance of autoimmunity in NZB mice, aberrations at the level of B lymphocytes also have to be taken into account. A causal relationship of deficient T cell functions to the development of antierythrocyte autoantibodies has not been established so far. Recently, DeHeer and Edgington (1977) were the first to present evidence for a primary genetically determined immunoregulatory defect which is manifested at the level of the B lymphocyte of the NZB mice. Their conclusion was derived from experiments involving the transfer of NZB bone marrow to irradiated hosts and from studying the autoantibody production to red blood cells.

In summary, the data reported in this Chapter lead to the conclusion that ageing mice gradually lose the capacity to reach the same level of responsiveness as observed in young adult mice after a primary antigenic stimulation. Thymus-dependent responses generally show an earlier onset of the decline than thymus-independent respon-

ses, at least when optimal doses of antigen are used. At a lower level of antigenic stimulation, the difference in responsiveness with young adult mice is more pronounced, independent of the involvement of helper T cells.

This decreased capacity to mount a primary immune response makes ageing mice likely to be more susceptible to pathogens which they encounter for the first time. This is supported by the observation that an age-related decrease in resistance was observed in mice to infection with Salmonella typhimurium (Perkins, Makinodan, and Seibert, 1972), Toxoplasma gondii and Listeria monocytogenes (Gardner and Remington, 1977).

It has to be pointed out again that this immunological decay occurs in spite of the fact that the number of B and T cells in most strains studied does not undergo dramatic changes with age (see Chapter I, section D.8). In addition, the available literature data show no indications for age-related alterations in the functional capacity of macrophages, which are involved in humoral immune reactions. Therefore, the observed decline in immunocompetence must be attributed to qualitative rather than quantitative changes in the lymphocyte populations which are responsible for the generation of a humoral immune response.

As stated in the introduction to this Chapter, the number of cells containing cytoplasmic immunoglobulins (C-Ig cells) was determined as a function of age in various lymphoid organs during the life-span of CBA mice from the same ageing cohorts as used for the present study (Haaijman, Schuit, and Hijmans, 1977). The number of C-Ig cells in the spleen reached a maximum at around 6 weeks. Thereafter the number decreased gradually and became stable between 0.5 and 2 years of age. Thus, the observed decline in immunocompetence as observed in our study was not accompanied by a decrease in the number of C-Ig cells in the spleen of ageing CBA mice. Likewise, the levels of serum immunoglobulins in CBA mice do not show a decrease between 0.5 and 2 years of age; on the contrary, an increase in the level of IgG1 and IgG2b was observed (Haaijman, Van den Berg and Brinkhof, 1977). The parameters studied by Haaijman and co-workers are a reflection of all ongoing humoral immune responses. Primary responses, especially in aged individuals, will form only a small part of all current reactions, since fewer new environmental antigens are encountered with advancing age. Therefore, an explanation for the apparent discrepancy may be that secondary and later immune reactions are less altered during ageing when compared with a primary immune response, resulting in constant numbers of C-Ig cells and levels of serum immunoglobulins.

Whether the experimental data will support this notion will be discussed in the following Chapter which deals with secondary responses in ageing mice.

CHAPTER V

EFFECT OF AGE ON THE SECONDARY RESPONSE TO SRBC IN THE SPLEEN AND BONE MARROW OF CBA MICE

A. INTRODUCTION

It has been postulated in the previous Chapter that the capacity to evoke a secondary response may be less susceptible to alterations with age than is the primary response. This hypothesis was based on the finding that the number of immunoglobulin containing (C-Ig) cells in the spleen did not show an age-related decrease (Haaijman, Schuit, and Hijmans, 1977) and that this level of C-Ig cells was a reflection of secondary and later responses rather than primary immune responses in ageing mice. Indeed, several reports indicate that the difference between the responses of young and old mice was less after secondary antigenic stimulation than after primary stimulation (Makinodan et al., 1971b, and Haaijman, 1977). Finger et al. (1972) even found that the secondary response to SRBC was similar in young and 20-month-old NMRI mice. In these studies, the antibody forming capacity was determined by measuring either the number of antibody forming cells in the spleen or the level of antibodies in the serum.

An extensive study of Benner (1975) emphasized the important role of the bone marrow in the generation of a secondary humoral immune response against SRBC. During a primary response, the spleen is the major site of antibody production but hardly any activity can be detected in the bone marrow (Benner et al., 1974). The authors showed that, during the early phase of a secondary response, both the spleen and the bone marrow contain a large proportion of antibody forming cells and that the bone marrow takes over the role as the major antibody producing organ in a later stage. Therefore, the determination of antibody formation in the bone marrow has to be included in experiments concerning secondary immune responses.

Another reason to pay attention to the bone marrow, especially in ageing mice, stems from the following observation. In addition to the events in the ageing spleen mentioned above, Haaijman, Schuit, and Hijmans (1977) noted the following changes in the other lymphoid organs: from the age of 5 weeks up to 1 year of age, male CBA mice showed an increase in the number of C-Ig cells in the bone marrow; no change occurred after that age. In a later experiment, the number of C-Ig cells in the bone marrow was also found to increase gradually

after 1 year of age (Haaijman and Hijmans, 1978). The number of C-Ig cells in the mesenteric lymph nodes and Peyer's patches gradually decreased with age. When the relative contribution of the various lymphoid organs to the total number of C-Ig cells at different ages was calculated, the following pattern emerged: the contribution of the spleen diminished steadily with age, that of the bone marrow increased. It was suggested that this alteration in the major site of Ig synthesis might be due to a gradual shift in the individual animal from primary type responses to a pattern of secondary type responses which occur in the bone marrow. The constant or increasing value found for the number of C-Ig cells in the bone marrow may suggest that the immunological capacity of the bone marrow is not diminished during ageing. The following experiments were aimed at testing this hypothesis by determining the PFC response after a secondary antigenic stimulation in the bone marrow as well as the spleen of ageing male CBA mice.

B. RESULTS

Six days after a single immunization with 4 x 10^8 SRBC, the presence of PFC in the bone marrow above the normal background level (varying from 0 to 1,000 PFC) could not be detected in either 3- or 24-month-old CBA mice. These findings confirm the earlier observation of Benner et al. (1974) concerning the lack of antibody formation in the bone marrow during the early phase of a primary response to SRBC. In addition, it shows that this pattern was not altered during ageing.

According to the data of Benner (1975), a primary dose of 10^7 SRBC and a booster dose of 4 x 10^8 SRBC gave optimal numbers of PFC in the bone marrow at an interval of 3 months between priming and the booster injection in (C57BL/Rij x CBA/Rij)F1 mice. Using these experimental conditions, 6-month-old CBA mice developed a PFC response in the spleen and bone marrow comparable to that found in (C57Bl/Rij x CBA/ Rij)F1 mice with respect to the magnitude of the reaction and the response pattern. Therefore, this schedule was applied to investigate the capacity of aged CBA mice to elicit antibody forming cells in the bone marrow and spleen during the early phase of a secondary response.

Cells obtained from the femoral bone marrow were used for the estimation of the PFC response in the bone marrow. It was shown by Benner and Van Oudenaren (1975) that the antibody forming potential of the femoral compartment is representative for the total bone marrow in

adult mice. Hence, the data of Chervenick et al. (1968), who demonstrated that one femur contained 5.9% of the total bone marrow, were used to calculate the number of PFC of the total bone marrow from the number of PFC obtained in one femur. This calculation may also be valid for ageing mice, since no age-related change in the distribution of ^{59}Fe in the skeletons of ageing mice was found (Tyan, 1977). Histological examination of tissue sections of femurs from CBA mice of various ages, which was included in the study presented in Chapter III, also did not reveal striking alterations with age in the morphology of the bone marrow. However, quantitative information on age-related changes in the distribution of the various cell types in the bone marrow is lacking.

The PFC responses of 6-, 18- and 24-month-old CBA mice (Fig. V.1) and of a few mice of 30-32-months of age (Table V.1) were determined in the spleen and bone marrow on 4 and 6 days after the booster injec-

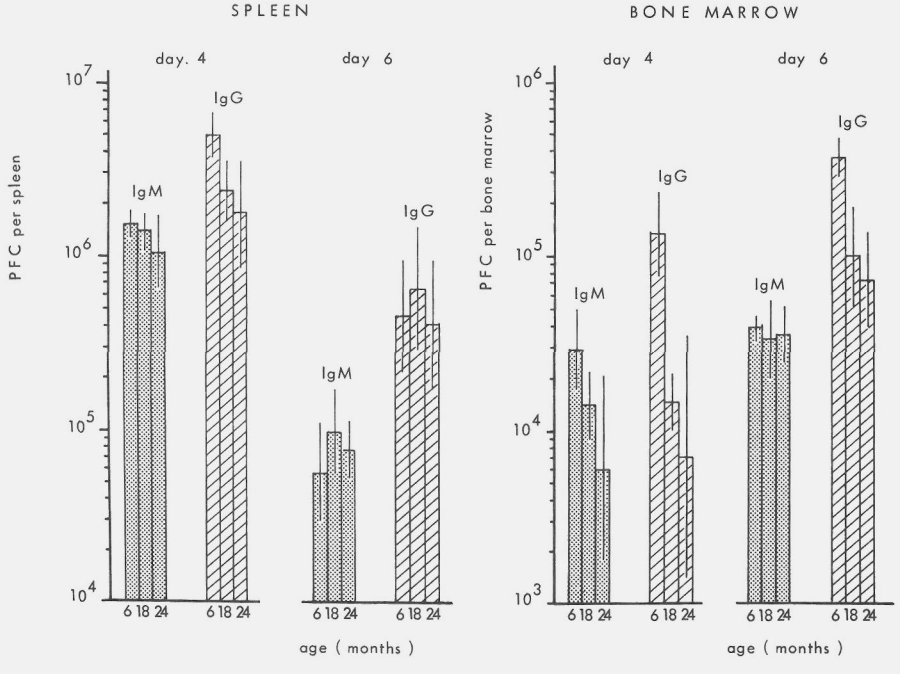

Figure V.1 Secondary PFC response to SRBC in the spleen and bone marrow of 6-, 18- and 24-month-old CBA mice.
The mice were primed with 10^7 SRBC. A second intravenous injection of 4×10^8 SRBC was given three months later. Each figure represents the geometric mean of 5-10 mice. The 95% confidence limits are indicated by vertical lines. The number of PFC in the bone marrow of 3- and 24-month-old mice which were either unstimulated or received only the booster injection of 4×10^8 SRBC did not exceed 10^3 PFC. Note the different ordinates for spleen and bone marrow.

TABLE V.1

SECONDARY PFC RESPONSE TO SRBC IN THE SPLEEN AND BONE MARROW OF 30-32-MONTH-OLD CBA MICE

		IgM-PFC*			
		spleen		bone marrow	
mouse number	response day	total x 10^{-3}	per 10^7 cells x 10^{-3}	total x 10^{-3}	per 10^7 cells
1	4	36.0 (2)**	5.8 (7)	7.1 (25)	205 (15)
2		203.8 (14)	15.2 (19)	4.7 (16)	135 (10)
3		245.1 (16)	19.5 (25)	6.5 (22)	210 (15)
4	6	24.3 (43)	1.5 (36)	8.9 (23)	298 (17)
5		41.2 (73)	4.5 (106)	21.4 (55)	592 (34)
6		18.6 (33)	1.3 (30)	15.5 (40)	462 (27)

The mice were intravenously primed with 10^7 SRBC. A second intraveneous injection of 4 x 10^8 was given 3 months later.

* No IgG-PFC were detected.
**Values in parentheses indicate the number of PFC expressed as a percentage of the mean number of PFC obtained in 6-month-old CBA mice (see Fig. V.1 and 2).

tion. Days 4 and 6 were chosen, since optimal responses in the spleen and bone marrow, respectively, occurred on these days (Benner and Van Oudenaren, 1975). The following points can be noted:

1. With regard to the spleen, the highest number of antibody forming cells was found on day 4 in all age groups studied.
2. The secondary IgM response in the spleen was not diminished up to the age of 24 months. The number of IgG-PFC on the peak day (day 4) showed about a 2-fold and 3-fold decrease in 18- and 24-month-old mice, respectively, when compared to that of 6-month-old mice. These differences were significant, as can be judged from the 95% confidence limits indicated in the figure. Six days after the booster injection, the splenic IgG-PFC response was comparable in the three age groups.
3. In the bone marrow of 6-month-old mice, an increase in IgG-PFC was found on day 6 in comparison to day 4, whereas the number of IgM-PFC was comparable on both days. The 18- and 24-month-old mice developed more antibody forming cells of both the IgM and IgG classes in the bone marrow on day 6.
4. A significant age-related decrease in the bone marrow was observed only in the production of IgG antibody-forming cells on both response days. Mice of the age groups of 18- and 24-months exhibited a 2 and 5 times lower IgM response, respectively, than did 6-month-old mice on day 4, although the differences were not significant. Six days after the second immunization, the IgM response in the three age groups was comparable.

5. No IgG-PFC response was detected in neither the spleen nor bone marrow of 30-32-month-old mice. The IgM responses found in the spleen and bone marrow of this age group varied from 2 to 73% and 16 to 55%, respectively, of the response exhibited by the mice of 6 months of age on the corresponding test days.

In the preceding Chapter, it was shown that the number of nucleated cells in the spleen of CBA mice does not change remarkably with age. Consequently, the differences in the primary anti-SRBC responses in the spleen among the various age groups revealed a comparable pattern when expressed per whole spleen or per 10^7 nucleated spleen cells. The same tendency was observed for the secondary anti-SRBC response in the spleen. Fig. V.2 shows that the overall pattern of the secondary PFC response in the spleen of the various age groups expressed per 10^7 nucleated spleen cells does not differ from the results presented above for the whole spleen.

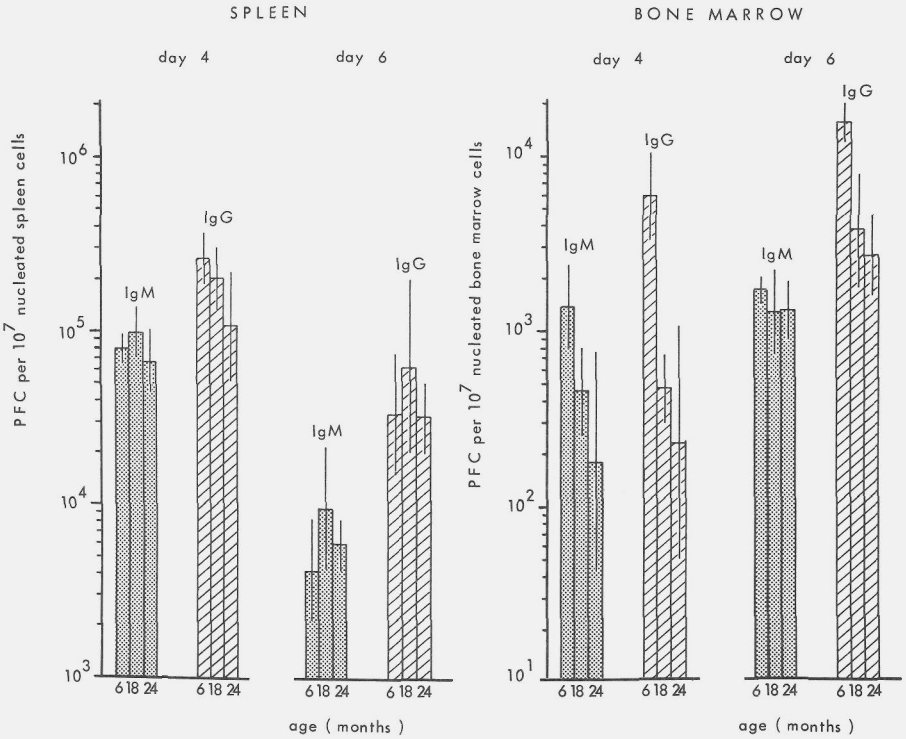

Figure V.2 Secondary PFC response to SRBC in the spleen and bone marrow of 6-, 18- and 24-month-old CBA mice expressed per 10^7 nucleated cells. See legend to figure V.1.

For the bone marrow, it had to be established whether the decreased responsiveness was accompanied by changes in the number of nucleated cells during ageing. It was found that, unlike the spleen, the total number of nucleated cells in the femur as representative for the bone marrow increased with age (Fig. V.3). Calculations of the PFC response in the bone marrow based on a fixed number of cells resulted therefore in a larger difference among the various age groups than was observed with the results expressed for the total bone marrow (Fig. V.1). Nevertheless, despite an increased cellularity in the bone marrow, the capacity of this organ to develop antibody forming cells in the early phase of a secondary response to SRBC was diminished with age.

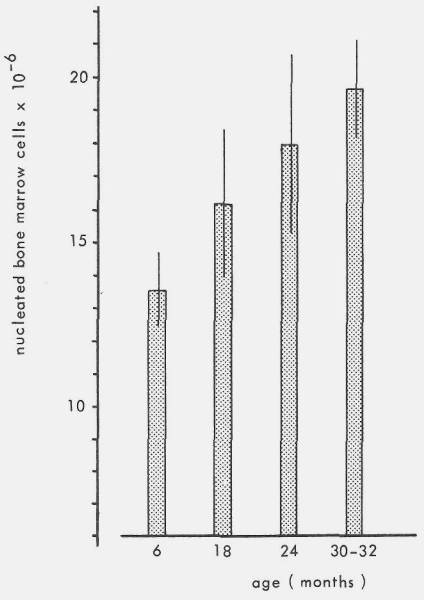

Figure V.3 Number of nucleated cells in the femur of 6-, 18-, 24- and 30-32-month-old CBA mice.
Each figure represents the average value of 5-10 mice. The 95% confidence limits are indicated by vertical lines.

Additional information concerning the secondary anti-SRBC response in ageing CBA mice was obtained by the determination of the serum haemagglutinin titres to SRBC. A distinction was made between 2-mercaptoethanol (2-ME)-sensitive and -resistant antibody titres. The results are presented in Fig. V.4. The total level of anti-SRBC antibodies in the serum was decreased with age. After treatment with 2-ME, the differences among the various age groups were increased, indicating that the production of 2-ME resistant antibodies which are con-

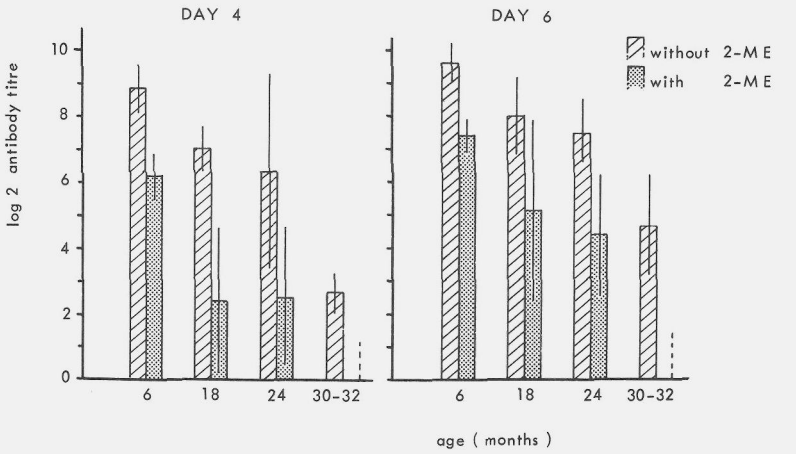

Figure V.4 Antibody titres in the serum of 6-, 18-, 24- and 30-32-month-old CBA mice during the secondary response to SRBC.
Serum was obtained from the same mice as used for the PFC responses depicted in figure V.1 and table V.1. Each figure represents the average antibody titre ± standard deviation. 2-ME resistant antibodies were not detected in the serum of 30-32-month-old mice.

sidered to belong to the IgG class was affected more than the production of IgM antibodies. 2-ME-resistant antibodies could not be detected in mice of the age group of 30-32 months, which is in accord with the results obtained with the PFC technique.

C. DISCUSSION

The results presented in this Chapter reveal a differential effect of ageing on the secondary anti-SRBC response in the spleen and bone marrow of CBA mice. Whereas the spleen maintained the same level of responsiveness up to the age of 24 months, the capacity of the bone marrow to develop antibody forming cells on a secondary antigenic stimulation was already decreased at that age. In 30-32-month-old mice, the production of IgM antibody forming cells was diminished in both the bone marrow and spleen, while there was no IgG response. The decreased responsiveness in the bone marrow might be attributed to a deficient migration of memory cells from the other lymphoid organs to the bone marrow and/or to a diminished capacity to generate antibody forming cells *in situ* from the memory cells present in the bone marrow.

The difference in the onset of the decline between IgM and IgG antibody formation in the bone marrow suggests a different mechanism for the development of either category of antibody forming cells. It

is known that the primary IgM response to SRBC is less thymus-dependent than is the IgG response (Chapter IV, section B). Whether the same phenomenon accounts for the difference between secondary IgM and IgG antibody responses in the bone marrow during ageing remains to be established.

In comparison with the effect of age on a primary response to SRBC in the spleen of CBA mice (Chapter IV), the secondary response in the spleen is much less affected by ageing, as can be concluded from the later onset of the decline. A comparison of the pattern of decline in a primary and secondary response in the spleen showed that the IgG antibody formation to SRBC was more affected in both types of responses.

Our results with regard to the spleen confirm the observations obtained in comparable studies (as referred to in section A) in which different mouse strains or antigens were used. Experiments of Segre and Segre (1976b) also revealed that spleen cells of old mice exhibited a lower *in vitro* secondary response to DNP-SRBC one month after an *in vivo* antigenic challenge. A comparison with a primary response was not included in their study. Furthermore, Krogsrud and Perkins (1977) demonstrated that the capacity of carrier-primed T cells to initiate a secondary response in hapten-primed (young) B cells decreased with advancing age.

The absence of PFC above a background level in the bone marrow of 24-month-old mice 6 days after a single injection with SRBC suggests that the bone marrow does not compensate for the already decreasing capacity of the spleen to respond to SRBC at that age. It must be noted, however, that the contribution of the bone marrow of ageing mice to antibody formation during later phases of a primary response has not been thoroughly investigated.

The above studies point to a decreased responsiveness in old age in the bone marrow and in the spleen on a secondary antigenic stimulation, but this decline is not reflected in the number of C-Ig cells in the spleen or in the bone marrow (Haaijman, Schuit, and Hijmans, 1977, and Haaijman and Hijmans, 1978). The size of this pool of C-Ig cells is a result of all types of reactions (primary, secondary and later) at various stages of their respective time courses. The different results obtained by using these two approaches for the estimation of the activity of the humoral immune system indicate that a decline in the early phase of a specific response (which will form only a minor part of all ongoing reactions) will not be recognized when an overall picture of these responses is determined. These findings demonstrate that functional studies such as

the estimation of the responsiveness to a specific antigen should be included to obtain complete insight into the changes in the humoral immune system during ageing.

CHAPTER VI

EFFECT OF AGE ON THE IN VITRO ANTI-SRBC RESPONSE OF SPLEEN CELLS FROM CBA AND C57BL/Ka MICE

A. INTRODUCTION

In the foregoing Chapters, the age-related changes in the capacity to generate antibody forming cells after antigenic stimulation were determined in the intact animal. The observed decrease with age in the competence of the humoral immune systems can, therefore, be attributed to deficiencies in the cells involved in a humoral immune response as well as to changes in the microenvironment. The latter changes may be respresented by an altered vasculature and/or architecture of the spleen during ageing and this may consequently affect antigen processing, recruitment of lymphocytes from outside the spleen and cooperation between B cells, T cells and macrophages. The major morphological changes in the spleen of aged mice are atrophy and disorganization of the follicles and a decrease in active germinal centres (Metcalf, Moulds, and Pike, 1966; Hanna et al., 1967, and Finger et al., 1972).

Price and Makinodan (1972b) demonstrated that the "old" environment indeed contributes to the age-related decrease in the humoral immune response. They found that adoptively transferred lymphocytes from young donors did not respond as well in old as in young irradiated hosts.

In order to investigate the age-related changes in the immunocompetent cells themselves, an approach had to be sought in which environmental factors were kept constant. One way to do so is by transferring young and old spleen cells to irradiated young recipients (Price and Makinodan, 1972a).

We chose the in vitro generation of antibody forming cells to investigate the immunocompetence of aged spleen cells isolated from their aged environment.

B. CBA

The initial experiments were designed to assess whether the time course and the required antigen dose for optimum stimulation differ for spleen cells from young and ageing male CBA mice. In Fig. VI.1, an

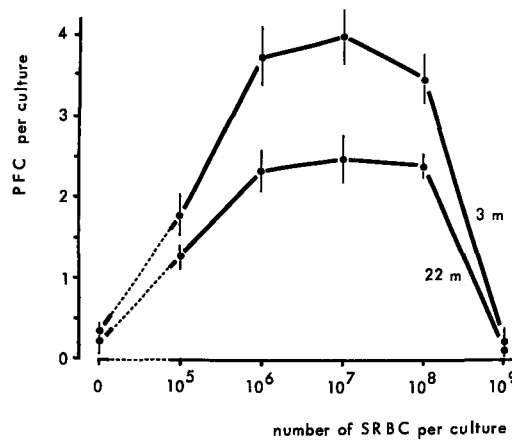

Figure VI.1 Effect of the number of SRBC on the in vitro PFC response of spleen cells of 3- and 22-month-old CBA mice.
Figures represent the mean number of PFC ± S.D. of 4 replicate cultures containing 2 x 10^7 spleen cells and the indicated number of SRBC (macroculture system). The culture time was 4 days. Pooled spleen cells of 6 mice were used in each age group.

experiment in which spleen cells from 3- and 22-month-old CBA mice were cultured in the presence of antigen doses ranging from 10^5 to 10^9 SRBC is shown. No difference in required antigen dose was detected, since optimum numbers of PFC were obtained in cultures containing 10^6 to 10^8 SRBC for spleen cells of both 3- and 22-month-old CBA mice. At these antigen doses, the response of the older mice was about 60% of that found in the cultures of young spleen cells. The difference was significant ($p < 0.001$) according to Student's t-test.

After 4 days of culture in the presence of 10^6, 10^7 and 10^8 SRBC, the number of recovered cells was 17 to 21% and 18 to 24% for the 3- and 22-month-old mice, respectively, indicating that the age of the spleen cell donor did not influence cell survival.

In order to investigate whether the time course of the in vitro anti-SRBC response was changed during ageing, the number of PFC was determined in various age groups of CBA mice on succeeding days of the culture period (Fig. VI.2a and 2b). Three days after initiation, only a low number of PFC was found. The response of the spleen cells of mice aged 18 and 23 months already exhibited a reduced response when compared to the response of the younger age groups. The peak day of the in vitro response was mainly found on day 4, with the exception of the response of spleen cells of 12-month-old mice which was (not significantly) higher on day 5 (Fig. VI.2a). In all age groups

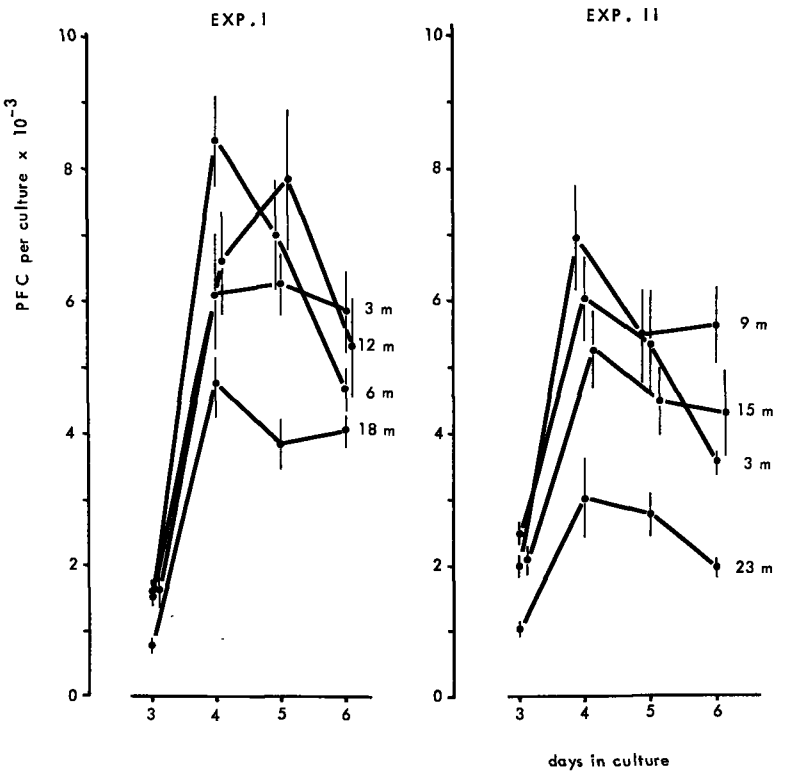

Figure VI.2 Time course of the in vitro anti-SRBC response of spleen cells of CBA mice of various ages.
Figures represent the mean number of PFC + S.D. of 5 replicate cultures containing 2 x 10^7 spleen cells and 10^7 SRBC (macroculture system). Pooled spleen cells of 6 mice were used in each age group. Two separate experiments are shown.

studied, there was no significant difference between the numbers of PFC obtained on day 4 and day 5. After 6 days, the response was either equal or lower in comparison with the response on day 5. The different patterns of the time courses shown here cannot be attributed to the ages of the spleen cells. They are due to inherent variations in this in vitro culture technique. The same variation was also obtained in separate (pilot) experiments in which spleen cells of 3-month-old mice were used to establish the optimal culture conditions. From the figures, it can be concluded that 18- and 23-month-old mice generally show a time course of the in vitro anti-SRBC response comparable to that of the younger mice. Up to the age of 15 months, no decrease in the in vitro generation of antibody forming cells was detected. The response of 6-month-old mice on day 4 (Fig. VI.2a) was even signi-

ficantly higher than the response of the 3-month-old mice in the same experiment. Significant reduced responses were found in the spleen cell cultures of 18- and 23-month-old mice, which were 78% and 50%, respectively, of the number of PFC in the spleen cells of 3-month-old mice in the same experiment.

The number of nucleated cells recovered at harvest did not markedly differ for the various age groups. In these experiments, the percentage of recovered cells found on day 3 was about 50% and was decreased to 20-30% after 6 days.

A summary of the results obtained in separate experiments in which spleen cells of various age groups of CBA mice were stimulated in vitro is presented in Fig. VI.3. To allow a comparison among the different experiments, the results are expressed as the percentages of the response obtained in spleen cells of 3-month-old mice. Since the data obtained in macro- and microcultures using spleen cell pools or cells from individual mice seemed to fall in the same range, the results were taken together and the averages were calculated. The results obtained within the various age groups exhibit a wide variety. When the average results of each group are compared, it can, nevertheless, be concluded that the capacity of spleen cells to develop PFC in vitro was decreased with advancing age. In spleen cell cultures of 22-24-month-old mice, the responses were lower and in one case equal to the response found in 3-month-old mice, whereas, in the cultures of the younger age groups (9, 15 and 18 months), more PFC were sometimes found than in the cultures of the 3-month-old age group. Spleen cells derived from mice older than 24 months displayed a markedly reduced response which was less than 5% of that found in 3-month-old mice.

It is difficult to compare the results of the anti-SRBC responses measured in vivo (Chapter IV) with the above data obtained in vitro, since there is such a large variation among the individual mice and among the different experiments. Nevertheless, a comparison of Table IV.1 where the in vivo data for CBA mice are summarized and Fig. VI.3 gives the following impression. A definite conclusion in terms of statistical significance cannot be drawn. It seems that the response of the spleen cells measured in the intact animal declines more rapidly with age than the response determined in isolated spleen cells. The average in vivo responses of 22-24-month-old mice (expressed as the percentage of the response of 3-month-old mice) varied from 8 to 29% (with confidence limits varying from 4 to 40%, Table IV.1), whereas, in the in vitro culture, this percentage varied from 14 to 100% with an average of 45% (Fig. VI.3). The above impression was confirmed in an experiment where CBA mice derived from the same cohorts were

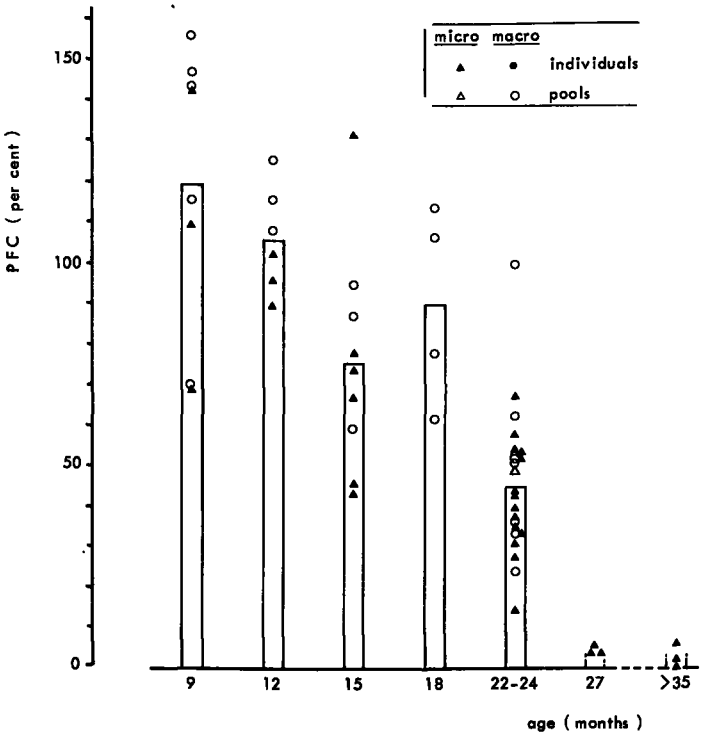

Figure VI.3 Summary of the in vitro anti-SRBC responses of spleen cells of CBA mice of various ages.
Figures represent the mean number of PFC in cultures of spleen cells from individual mice or spleen cell pools expressed as percentages of the mean number of PFC obtained in spleen cell cultures of 3-month-old mice in the same experiment. Results obtained in the macro- as well as in the microculture system after 4 culture days are given. The mean number of PFC ± S.D. in spleen cell cultures of 3-month-old mice from the separate experiments was 5,437 ± 2,142 (range of the values: 8,728 - 2,422; n = 8) for the macroculture system and 282 ± 80 (range of the values 444 - 204; n = 9) for the microculture system.

simultaneously studied in an *in vivo* and an *in vitro* anti-SRBC response (using the same batches of SRBC and complement). The *in vivo* response of the spleen cells of 22-month-old mice was decreased to 14% of that of 3-month-old mice, whereas age-matched mice of the same cohorts exhibited a 50% reduction in the *in vitro* anti-SRBC response (Table VI.1).

It must be noted that far fewer antibody forming cells are found *in vitro* than *in vivo*, indicating that the culture conditions are still not optimum to completely mimic the *in vivo* situation. Despite this shortcoming, this system has been proved to be a useful adjunct to the *in vivo* assay for the study of the humoral immune response

TABLE VI.1

COMPARISON OF THE *IN VIVO* AND *IN VITRO* ANTI-SRBC RESPONSE OF SPLEEN CELLS OF CBA MICE OF VARIOUS AGES

age in months	in vivo* IgM-PFC per $2x10^7$ spleen cells: geometric mean and 95% confidence limits	per cent	in vitro** IgM-PFC per culture: day 4 mean ± S.D.	per cent	day 5 mean ± S.D.	per cent
3	56,986 87,189 - 37,245	100 153 - 65	6,501 ± 642	100	5,359 ± 805	100
9	49,685 71,218 - 34,662	87 125 - 61	6,951 ± 775	115	5,478 ± 720	102
15	33,066 67,881 - 16,107	58 119 - 28	5,271 ± 592	87	4,500 ± 517	84
22	7,953 18,238 - 3,468	14 32 - 6	3,031 ± 589	50	2,780 ± 315	52

The mice used for the *in vivo* and *in vitro* assays were derived from the same cohorts.

* The *in vivo* PFC responses were determined 4 days after immunization with $4x10^8$ SRBC. Each group consisted of 5 mice. For comparison, the means and confidence limits are also expressed as percentages of the mean number of PFC obtained in 3-month-old mice.

**Pooled spleen cells from 5 mice ($2x10^7$ cells per dish) were cultured in the presence of 10^7 SRBC (macroculture system). The mean numbers of PFC ± standard deviation of 5 replicate cultures are given. The results are also expressed as percentages of the mean number of PFC obtained in spleen cell cultures of 3-month-old mice.

during the past years. Since the culture conditions are comparable for the spleen cells of the various age groups, the observed age-related decrease in responsiveness can be accounted for by a reduction in the number of immunocompetent cells in the spleen of aged mice and/or a decrease in their functional efficiency.

It is appropriate at this point to refer to a study of Haaijman and Schuit (personal communication) on age-related changes in the cellular composition of spleen cells of CBA mice. An immunofluorescence method was used to estimate the number of B and T cells in the spleen as judged by the presence of surface immunoglobulins and thymus lymphocyte antigens, respectively. The results show that no significant changes occur in the absolute number of B and T cells in the spleen of CBA mice with advancing age, confirming the data obtained by most authors for other mouse strains (see Chapter I, part D.3 and Table I.1). An experiment presented in Table VI.2 shows that even 35- and 38-month-old mice have comparable percentages of T cells, B cells and negative cells in their spleen as are found in 3-month-old mice. Nevertheless, the *in vitro* anti-SRBC response of these spleen cells was largely reduced. The loss in capacity to produce PFC *in vitro* cannot, therefore, be attributed to a decrease in the overall number of T and B cells.

TABLE VI.2

PERCENTAGES OF LYMPHOCYTES, T CELLS, B CELLS AND NEGATIVE CELLS IN THE SPLEEN OF CBA MICE AGED 3 MONTHS AND 35-38 MONTHS AND THE IN VITRO PFC RESPONSE TO SRBC

mouse number	age in months	total lymphocytes	T cells*	B cells*	negative cells: lymphoid	negative cells: non-lymphoid	PFC per culture**
1	3	85	26	47	12	15	253 ± 8
2	3	85	16	59	10	15	211 ± 16
3	3	87	22	60	5	13	148 ± 3
4	38	80	25	47	8	20	12 ± 2
5	35	83	30	48	5	17	0
6	35	86	13	69	4	14	3 ± 2

The cell numbers are expressed as percentages of the total number of mononucleated cells in the spleen.

* The number of T and B cells was estimated by an immunofluorescence method for surface markers (Bolhuis and Schuit, 1978) using a fluorescent rabbit antiserum directed against Fab fragments of mouse heterogeneous IgG and a rabbit anti-MTLA antiserum (mouse thymus lymphocyte antigen) (Nordic Immunological Laboratories, Tilburg, The Netherlands).

**Figures represent the average number of PFC ± S.D. of 5 replicate cultures containing 10^6 spleen cells and 10^6 SRBC (microculture system). The culture period was 4 days. The percentages of recovered cells were 23, 20 and 23% in the cultures of 3-month-old mice and 32, 25 and 25% in the cultures of 35-38-month-old mice.

C. C57BL/Ka

The age-related changes in the capacity to produce antibody forming cells in vitro was also investigated in male C57BL/Ka mice. Time courses of the response in spleen cell cultures of 3 different age groups after stimulation with 10^7 SRBC are presented in Fig. VI.4. Comparable profiles were found for the different age groups. On day 4, the number of PFC in the cultures of 15- and 24-month-old mice was significantly reduced to 63% and 47%, respectively, of that of the 3-month-old mice. A summary of responses obtained with individual mice and spleen cell pools of various age groups in separate experiments is shown in Fig. VI.5. Despite the wide variety in the level of responsiveness within each age group, the results indicate that the anti-SRBC response of the isolated spleen cells tends to decrease with advancing age. Generally, responses obtained in spleen cell cultures of 22-24-month-old mice are below that found at the age of 3 months. The four mice older than 26 months of age which were studied, showed responses which were less than 35% of that of the 3-month-old mice.

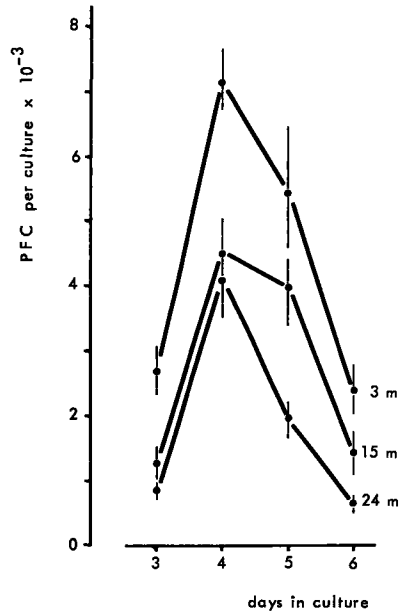

Figure VI.4 Time course of the in vitro anti-SRBC response of spleen cells of C57BL/Ka mice of various ages.
Figures represent the mean number of PFC ± S.D. of 5 replicate cultures containing 2 x 10^7 spleen cells and 10^7 SRBC (macroculture system). Pooled spleen cells of 6 mice were used in each age group.

D. DISCUSSION

The *in vitro* experiments presented in this Chapter were designed to establish whether the age-related decrease in anti-SRBC response found *in vivo* was solely due to environmental changes or whether intrinsic changes in the cell types were also involved. The *in vitro* results show that isolated spleen cells gradually lose the capacity to generate antibody forming cells upon antigenic stimulation with advancing age, although a large variation was found. In both CBA and C57BL/Ka mice, spleen cells from animals older than 22 months did not display the same level of responsiveness as did young mice, with the exception of a few mice which responded equally well. This indicates that, in addition to environmental factors (Price and Makinodan, 1972b), intrinsic changes in the cells which cooperate in a humoral immune response are responsible for the decrease in function. The time courses, dose dependence and survival of the cultured spleen cells were essentially comparable for the various age groups. Therefore, the reduced responses found in the aged mice cannot be explained by a

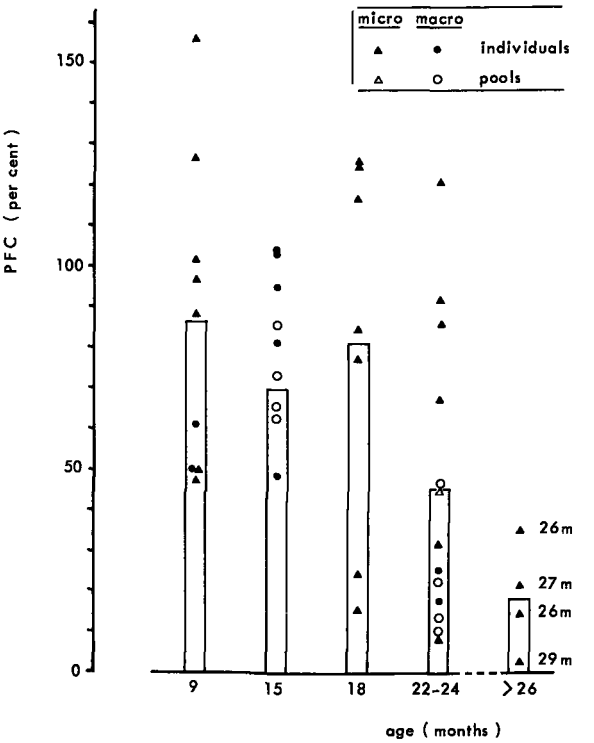

Figure VI.5 Summary of the in vitro anti-SRBC responses of spleen cells of C57BL/Ka mice of various ages.
Figures represent the mean number of PFC in cultures of spleen cells from individual mice or spleen cell pools expressed as percentages of the mean number of PFC obtained in spleen cell cultures of 3-month-old mice in the same experiment. Results obtained in the macro- as well as in the microculture system after 4 culture days are given. The mean number of PFC ± S.D. in spleen cell cultures of 3-month-old mice from the separate experiments was 6,265 ± 1,917 (range of the values: 9,744 - 3,973; n = 6) for the macroculture system and 401 ± 182 (range of the values: 851 - 230; n = 5) for the microculture system.

shift in the kinetics or changes in antigen requirement of the responding cells.

The diminished capacity of the spleen cells cannot be attributed to changes in the number of T and B cells. This pattern did not alter remarkably during the life-span of CBA mice from our ageing cohorts (Haaijman and Schuit, personal communication), which is in keeping with the data obtained in other strains (Table I.1). It must be realized, however, that the available data concern an overall picture of the B and T cell populations. Possible changes occurring with age in, for instance, subpopulations of T cells must await further

analysis with the aid of the anti-Ly sera (Cantor and Boyse, 1977). Data on the distribution of subpopulations of B and T cells in the spleen of ageing mice will give a more complete answer as to whether changes in those subpopulations attribute to the decrease in responsiveness. The present information on alterations in the function of suppressor T cells in the spleen during ageing is confusing (see discussion Chapter IV), so that the contribution of this specific cell population to the ageing process needs more investigation. With regard to the third cell type involved in humoral immune responses, the macrophages, the available data (summarized in Chapter I, part D.2) do not point to impaired phagocytic activity or to a reduced capacity to collaborate with T and B cells with advancing age. Therefore, the cause of the decrease in humoral responsiveness must be sought for in qualitative changes in the B and T cell populations. Their ability to cooperate, proliferate and differentiate may be affected by age, resulting in a loss of effector cells such as the antibody forming cells and helper T cells.

A few other studies deal with the effect of age on the capacity of isolated spleen cells to generate antibody forming cells. Heidrick and Makinodan (1973) demonstrated that the defect in the in vitro anti-SRBC response of 28-30-month-old BC3F1 mice was associated with the nonadherent cell population. The same mouse strain was used to investigate the in vitro responsiveness of mixtures of young and individual old spleen cells (Makinodan et al., 1976). The response of pure old spleen cells was always lower than that of young spleen cells. In mixtures, the spleen cells of old mice exhibited either a suppressive effect, a stimulatory effect or no effect upon the young spleen cells. These results indicate that different processes account for the decrease with age in the individual mice. It has been reported by Farrar, Loughman and Nordin (1974) and Kishimoto, Takahama and Mizumachi (1976) that, even in the presence of an optimal source of helper T cells (young T cells activated in the presence of antigen), B cells of aged mice have a decreased capacity to generate PFC in vitro. Segre and Segre (1976b) studied the secondary response of young and old spleen cells in diffusion chambers implanted in recipient mice. The old spleen cells exhibited a reduced response, whereas no difference could be detected in the kinetics of the response of young and old cells.

As described in part B of this Chapter, a comparison of the in vivo and in vitro results suggests that the anti-SRBC response measured in the intact animal declines more rapidly with age than the response determined in spleen cell cultures stimulated with SRBC.

Another difference is that the antigen dose required for maximum antibody formation in vivo is changed with age (Chapter IV), whereas young and old spleen cells stimulated in vitro display a comparable dose dependence. It is known that the ageing environment contributes to the age-related decline (Price and Makinodan, 1972b). The main reason for this difference is, therefore, that the exclusion of environmental factors in vitro diminishes the difference between, for instance, the age groups of 3 and 22-24 months (the largest groups studied by us). A possible way in which environmental factors influence a humoral immune response might be the following. During the first days of an immune response in vivo, an influx of circulating lymphocytes into the spleen is observed (Sprent, Miller and Mitchell, 1971, and Rowley et al., 1972). Among these recruited lymphocytes, there may be cells which are specifically equipped to respond to the antigen and enhance the ongoing response of the cells present in the spleen. In a study of Inchley et al. (1976) on age-related changes in the distribution of injected lymphocytes, it was found that mice older than 22 months of age display a decreased antigen-related localization of injected (young) cells in the lymph nodes draining the site of antigen injection. Although the spleen was not investigated in this respect, it is tempting to speculate that one of the reasons for the larger differences observed in vivo is a decreased recruitment of lymphocytes after antigenic stimulation. The spleen cells put in culture represent the cells present in the spleen of unstimulated animals, which may lead to a smaller difference between young and, for instance, 22-24-month-old mice.

In conclusion, the loss of immune responsiveness found in ageing mice can be partly attributed to changes in the immunocompetent cells themselves. In the intact animal, environmental factors play an additional role. A possible effect of ageing on the recruitment of lymphocytes during an immune response is discussed. Ageing does affect the magnitude of the response elicited in vitro but has no effect on the kinetics and dose requirement of the responding cells. The nature of the changes underlying the decline has still to be clarified. The large variation in our study and in the results reported by others (Makinodan et al., 1976, and Segre and Segre, 1977) point to the fact that the processes leading to the decrease in responsiveness may be different in individual mice. Future application of the in vitro immune response system for the clarification of the different changes occurring with advancing age will include the manipulation of cells after cell separation. In view of the individual variation, micromethods will have to be developed for the separation of cells from individual mice.

CHAPTER VII

THYMUS TRANSPLANTATION AND THYMIC HUMORAL FACTORS

A. INTRODUCTION

In spite of the fact that the underlying mechanism responsible for the loss of immune reactivity with age is not yet fully understood. various attempts to correct the state of immunodeficiency in old age have already been made (for a recent review, see Walford, Meredith and Cheney, 1977). These experimental approaches consisted of nutritional manipulation, reconstitution with cells or transplants and administration of humoral factors. Because of the important role of the thymus in the ageing process of the immune system, it could be expected that some of the experimental designs for immunological reconstitution were focused on the thymus. Experiments in which thymus grafts were transplanted into aged animals have given inconclusive results so far. Our experience and the data from the literature with regard to thymus transplantation will be presented in part B of this Chapter.

During the last decade indications have been obtained that humoral factors produced by the thymus, tentatively called thymic hormones, are involved in the differentiation of precursor T cells into mature T lymphocytes. Therefore, administration of these factors may be envisaged as an alternative method for the restoration of thymus-dependent functions in aged animals. Furthermore, studies on these thymic humoral factors in young and old animals may help to elucidate the precise nature of the loss of thymus function with age. The crucical question in this respect is whether the decreasing T cell functions stem from a decrease in the number of progenitor cells and/or to a failure of maturation of progenitor cells to immune reactive cells as a result of thymus involution accompanied by a diminished production of thymic humoral factors. The availability of well defined thymic factors and assays for T cell functions will help to resolve this question.

The above considerations indicate why investigations on thymic humoral factors are valuable for immunogerontology and explain why a study on these factors is included in the present thesis.

The various thymic factors which exhibit biological activities are derived from three different sources, viz., thymus extracts, serum and supernatant of thymic epithelial cultures. The main preparations

derived from calf thymus extracts are thymosin (Goldstein et al., 1972) thymic humoral factor, designated as THF (Trainin and Small, 1970) and thymopietin (Goldstein, 1974). Two circulating factors have been described: the thymic factor (TF) of Bach and Dardenne (1973) which is present in pig and mouse serum and the thymus dependent serum factor (SF) of Astaldi et al. (1976) found in human serum. Recently, Kruisbeek, Kröse and Zijlstra (1977) demonstrated the presence of active factors in rat thymic epithelial culture supernatant (TES).

The biological effects of the various factors are established in quite a variety of immune assays. These assays include the in vitro induction of T cell markers, in vitro tests for thymus-dependent immune functions such as T cell mitogen responsiveness, mixed lymphocyte reactivity and graft versus host response and a few in vivo experiments in which animals were treated with thymic factors followed by an in vivo or in vitro test for T cell function. Detailed information on the above-mentioned activities can be found in extensive reviews by Trainin (1974), Bach and Carnaud (1976) and in the proceedings of a conference and a workshop on thymic hormones edited by Friedman (1975) and by Van Bekkum (1975).

The present studies on thymic factors proceed along two lines of investigation. Firstly, the purification and chemical characterization of the various factors are being investigated or already completed. The amino acid sequence of thymopoietin (Schlesinger and Goldstein, 1975), serum thymic factor (Bach et al., 1977) and of a purified component of thymosin fraction 5 (Goldstein et al., 1977) has been reported. Secondly, further elucidation of the role of different factors in the several steps leading to mature T cells, including the characterization of the target cell, and the determination of the activities of each preparation in various assays for T cell function are being studied. In addition, preliminary clinical trials using thymosin (Goldstein et al., 1975; Wara et al., 1975) and THF (Zaizov et al., 1977) have been made.

A few studies deal with changes in the level of thymic factor in serum with ageing, on the one hand, and the effect of these factors on immune functions of aged animals on the other. Dardenne et al. (1974a) reported a decrease in the circulating thymic factor as assessed by the rosette inhibition assay starting at the age of six months in various strains of mice. In a preliminary study of Goldstein et al. (1974) in which serum levels of thymosin were determined in a radioimmunoassay, it was found that the concentration of thymosin in human serum was decreased in normal individuals after the age of 35

years. Twomey et al. (1977) used the bioassay developed for thymopoietin (induction of Thy-1.2 antigen on null lymphocytes from nu/nu mice) to measure activity in serum or plasma and to relate the observed activity with that of known amounts of thymopoietin. In this way, the authors demonstrated a decrease in "thymopoietin like" activity in plasma of 50-55-year-old humans. Likewise, Astaldi et al. (1977) observed that the level of serum factors, as measured by the stimulation of cyclic AMP in thymocytes, progressively decreased after the age of 30 years in humans. Although different assays were used in the above studies, a comparable conclusion implying that the level of circulating thymic factor(s) tend to decrease with advancing age was reached.

Literature data on the effect of thymic factors on immunological activities of aged animals are scanty. Friedman, Keiser and Globerson (1974) reported that spleen cells derived from 33-month-old mice exhibited a decreased in vitro graft versus host reactivity. They further showed that addition of THF to the cultures enhanced the response elicited by cells from the aged donors, whereas it did not lead to any change in reactivity of the cells from young animals. Bach (1977) observed that spleen cells of ageing mice gradually lose the capacity to kill target cells as assessed in an assay for cell mediated lympholysis (CML). Treatment of aged mice with thymic factor, however, depressed rather than improved the CML activity in these mice. In contrast, the decreased CML activity of adult thymectomized mice could be enhanced by in vivo administration of thymic factor.

The effect of thymic hormone preparations was also studied in the autoimmune susceptible NZB and NZB/W mice. Gershwin et al. (1974) showed that a short term in vivo treatment with thymosin enhanced several T cell-dependent immune functions in 7-month-old NZB/W mice. An abnormality of thymocytes of 8-week-old NZB mice was detected by measuring the DNA synthetic response to transplantation antigens (Dauphinee and Talal, 1973). This abnormal DNA proliferation pattern could be corrected to a normal pattern comparable to that of younger NZB mice and control strains after thymosin injections (Dauphinee et al., 1974). Furthermore, using a system of antigen-induced depression of DNA synthesis in the spleen as a measure of suppressor T cell activity (Zatz and Goldstein, 1973), a loss of suppressor cells was found in 2-month-old NZB mice (Dauphinee and Talal, 1975). Administration of thymosin could enhance this activity (Dauphinee and Talal, 1975). Despite these indications of a positive effect of thymosin on various immune functions in NZB mice, subsequent studies of the group of Gershwin, however, did not provide further evidence that thymosin

could have a beneficial role in the treatment of the autoimmune disease of NZB and NZB/W mice (Gershwin et al., 1976). In a long-term study in which several schedules for thymosin administration were used, no effect of thymosin on survival, autoantibodies and mitogen responsiveness of NZB and NZB/W mice was found.

Bach and Niaudet (1976) reported on a regulatory influence of their thymic factor on antibody production to polyvinylpyrrolidone (PVP) in NZB mice. Aged NZB mice showed an exaggerated production of anti-PVP antibodies, which was attributed to a loss of suppressor T cells. In vivo administration of Bach's thymic factor reduced the antibody response to PVP to normal levels in NZB mice.

In conclusion, the above data provide evidence for a loss of circulating thymic humoral factors with age. Less convincing data have been obtained so far with regard to the restorative effect of thymic humoral factors on the decreased immune functions in aged and autoimmune susceptible mice. This is not so surprising, since, in our opinion, clear-cut results on the restorative effect of thymic humoral factors in animal models for T cell deficiency such as nu/nu mice and thymectomized mice are also lacking.

Therefore, before treating aged animals with thymic humoral factors and selecting a specific preparation for this purpose, it is still necessary to gather more information on the nature and mode of action of these factors.

The aim of our initial studies on this subject was to compare the activity of two known preparations derived from thymus extracts, thymosin and THF, in an assay which would also be appropriate to monitor further purification. The in vitro anti-SRBC response of spleen cells from nu/nu mice proved to be a valuable assay. In addition, an attempt was made to establish the cellular origin of the active factor(s) (i.e., are the active factors derived from the epithelial or lymphoid part of the thymus?). The results are presented in part C of this Chapter.

During the course of this study, Kruisbeek and co-workers of our Institute established the presence of active material in the supernatant of rat thymic epithelial cultures which was designated as TES (Kruisbeek, Kröse and Zijlstra, 1977). Since this supernatant proved to be a more attractive starting material for the isolation of thymic factors than thymic extracts, the activity of TES was investigated in many immune assays, among which was the in vitro production of cells forming antibodies to SRBC (Kruisbeek et al., 1978). The effect of TES in this assay will be shown in part D. In part E, the results obtained with the preparations derived from thymic extracts and with TES will be compared and the prospects for future use will be discussed.

B. EFFECT OF THYMUS TRANSPLANTATION ON THE ANTI-SRBC RESPONSE OF AGED CBA MICE

The data obtained in the present study using CBA and C57BL/Ka mice and those presented in the literature as discussed earlier (see Chapter IV) point to the fact that humoral immune responses which are completely or partially T cell dependent decline more rapidly with age than totally thymus-independent responses. Therefore, an impaired function of the thymus and, as a consequence, of T cells appears to be a factor limiting the humoral immune response during ageing in some mouse strains. Therefore, attempts have been made to correct the impaired responses in aged mice by means of thymus transplantation (Metcalf, Moulds and Pike, 1966; Hirokawa, Albright and Makinodan, 1976, and Micklem, Ogden and Payne, 1973).

It was shown that a thymus graft obtained from an irradiated neonatal mouse and transplanted under the kidney capsule improved the IgM- and induced the IgG-PFC response to SRBC in athymic nu/nu mice (Chapter IV, part B; Blankwater and Lina, 1974). The same method was used to investigate the effect of a thymus graft on the anti-SRBC response of aged mice. In the experiment shown in Fig. VII.1, male CBA mice of 20 months of age received a thymus graft under the kidney capsule. The thymus grafts were derived from neonatal CBA mice which were sublethally irradiated with 300 rad of X-rays in order to remove the thymocytes and to leave intact thymic epithelial tissue (Trowell, 1961). Control mice were sham-operated. After 3 months, the antibody formation of the spleen to SRBC was assessed and compared with the response in 3-month-old mice and the sham-operated mice. In this particular experiment (Fig. VII.1), thymus grafting resulted in a significant increase in both IgM and IgG antibody formation as determined on day 4 and day 6, respectively. The response of the grafted mice was still below that found in the 3-month-old mice. Histological examination revealed that all thymus grafts were well-vascularized and showed the characteristic architecture of a thymus.

Subsequent experiments were carried out in an attempt to reproduce the above effect. Unfortunately, no significant differences were detected between the transplanted and control mice, although a good take of the thymus grafts in the majority of the treated mice was confirmed by histological examination. Mice of the same age as those in the experiment shown in Fig. VII.1 as well as older ones (26 months of age) were used. In the latter case, the absence of an effect may be attributed to the age of the treated mice. The possibility exists that, beyond a certain age, thymus transplantation is too late. In the

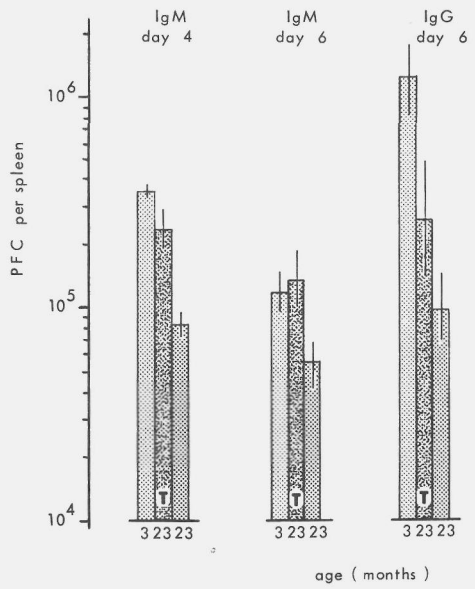

Figure VII.1 Effect of thymus transplantation on the anti-SRBC response of aged CBA mice.
IgM and IgG-PFC were measured in the spleen of 3- and 23-month-old mice with (indicated by a T in the column) or without a thymus graft. The grafts, obtained from neonatal mice irradiated with 300 rad of X-rays were transplanted under the kidney capsule 3 months before intravenous immunization with 4 x 10^8 SRBC. Each figure represents the geometric mean of 6 mice. The 95 % confidence limits are indicated by vertical lines.

other experiments, the number of PFC obtained in the treated and control groups showed a large individual variation and overlapped each other. It must be stated that, in the time period elapsing between the first and the later experiments, the late effects of a Sendai virus infection followed by a Mycoplasma pulmonis infection in our animal quarters became evident. This resulted in purulent bronchopneumonia and shortening of the life-span of the mice. It was, therefore, decided to terminate these experiments and to wait until healthy aged mice are available from new established ageing cohorts consisting of mice derived from breeding colonies under specific pathogen free conditions.

The literature data are more or less a reflection of our own experience: positive as well as negative effects of thymus grafts on the immune response of aged mice have been reported. The earliest publication on this subject is that of Metcalf, Moulds and Pike (1966). They found that twelve one-day-old thymus glands grafted in 14- to 15-month-old C57BL/Ka mice did not improve the haemagglutination titre or the PFC response to SRBC 1 to 3 months after grafting. Likewise,

Micklem, Ogden and Payne (1973) reported that thymus grafts from neonatal mice, whether in combination with bone marrow from 3-month-old mice or not, failed to improve the anti-SRBC response of 22-month-old CBA mice 3 months after transplantation. On the other hand, Hirokawa, Albright and Makinodan (1976) described a successful attempt to restore various immune functions in old mice by thymus transplantation. Four and a half months after grafting of a neonatal thymus under the kidney capsule, the anti-SRBC and PHA responses were elevated in 19.5-month-old B6D2F1 mice. The responses were increased further by combined bone marrow cells and thymus grafting. However, at 3 months after grafting, only the combined treatment consisting of bone marrow and thymus grafts improved the anti-SRBC response of 29-month-old BC3F1 mice; a thymus transplantation alone had no effect.

Further studies are required to unequivocally demonstrate the beneficial role of thymus transplantation for the manipulation of the immune system in old age.

C. EFFECT OF THYMIC FACTORS PREPARED FROM THYMUS EXTRACTS ON THE IN VITRO ANTI-SRBC RESPONSE OF SPLEEN CELLS FROM nu/nu MICE

1. Introduction

As outlined in part A of this Chapter, attention was paid in this study to humoral factors derived from the thymus for a possible future use in immunogerontology.

Initially, a simple and reproducible assay was sought to compare different preparations and to monitor purification procedures. Armerding and Katz (1975) demonstrated that spleen cells from normal and nu/nu mice exhibited an increased immune response to various antigens when cultured in the presence of a thymosin-like preparation. Especially the fact that a clear-cut effect of thymosin on the response of nu/nu mice was obtained prompted us to compare the activity of another thymic hormone preparation, THF, with thymosin and to test various control preparations.

Thymosin (fraction 5) and THF were prepared from calf thymus glands according to the procedures of Hooper et al. (1975) and Trainin and Small (1970). The same procedures were employed to prepare control substances from calf spleen and lymph nodes. Livers from WAG/ Rij rats were used to obtain a thymosin-like preparation from a nonlymphoid organ. To remove the blood, the livers were perfused with PBS at 37°C before starting the isolation procedure. (For further details, see Blankwater et al., 1978).

2. Effect of thymosin and THF

Spleen cells from nu/nu mice showed a reduced capacity to respond to SRBC *in vitro* as compared to the response of their normal litter mates, CBA/Rij and C57BL/Ka mice (Fig. VII.2). Initial experiments confirmed the observations of Armerding and Katz (1975) that addition of thymosin to spleen cell cultures of nu/nu mice resulted in an increased number of antibody-forming cells. Figure VII.3 shows two representative experiments in which varying quantities of thymosin prepared from total calf thymus and a corresponding spleen extract were added to spleen cell cultures. The factors were present during the entire culture period. For comparison, the results of both a macro- and a microculture assay in which the same batches of thymosin and spleen extract were used are presented. The results obtained in both culture systems were comparable: a bell-shaped dose-response profile and an increase in the antibody formation by a factor of 1.5.

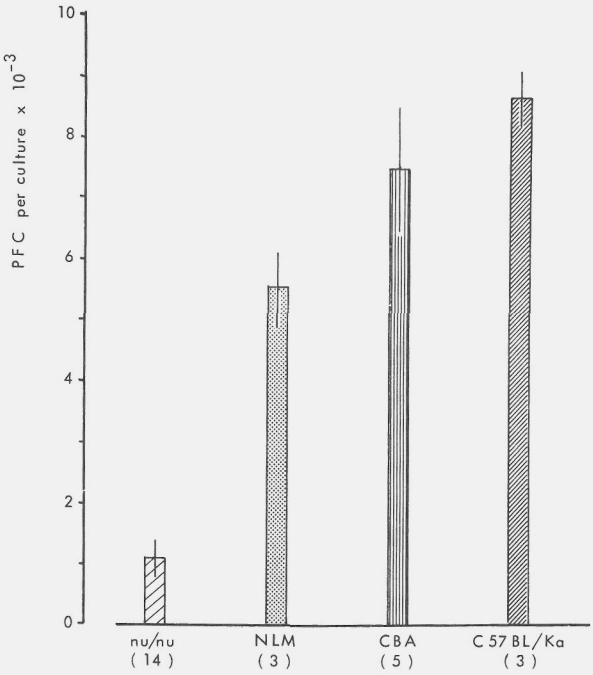

Figure VII.2 Comparison of the *in vitro* anti-SRBC response of spleen cells from nu/nu and normal litter mates (NLM) of nu/nu, CBA/Rij and C57BL/Ka mice. Figures represent the mean values of triplicate cultures (on day 4) of the indicated number of experiments (in parentheses) performed with the macroculture system in the same period. Standard deviations are indicated by vertical lines.

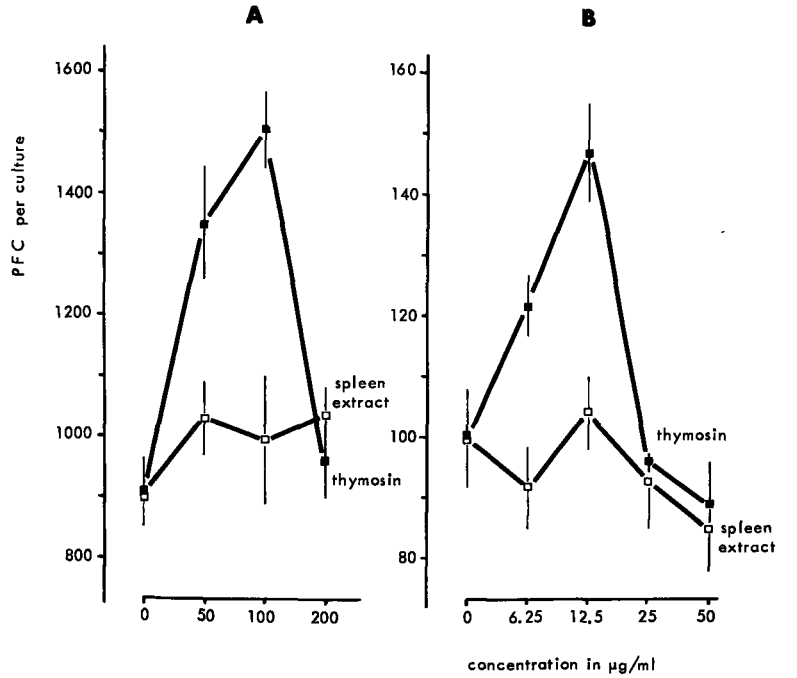

Figure VII.3 The effect of thymosin and its corresponding spleen extract on the in vitro anti-SRBC response of nu/nu mice as measured in (A) a macro- and (B) a microculture system.
The same batches of thymosin and spleen extract were used in both experiments. Each point represents the average PFC of either four (A) or five (B) replicate cultures. Standard deviations are indicated by bars. The preparations were present throughout the culture period of 4 days. Number of PFC in cultures without SRBC = 390 ± 71 (A) and 43 ± 3 (B).

The optimal doses were 100 and 12.5 µg/ml for the macro- and microcultures, respectively. The spleen extract did not influence the response.

The effect of another type of thymic hormone preparation, THF, and its corresponding spleen extract was studied in the same way. Figure VII.4 illustrates that THF was also active. Although the THF batches differed in the macro- and microassays shown here, the response was increased in both assays by a factor of 2. One of the three spleen extracts corresponding with THF was active, but the concentrations at which a positive reaction occurred were higher than needed with thymus-derived THF (Fig. VII.4a).

The above experiments showed a clear effect of thymosin and THF on the in vitro production of antibody-forming cells in spleen cell cultures from nu/nu mice and the near absence of activity in most of the spleen extracts. These data were extended by studying more batches

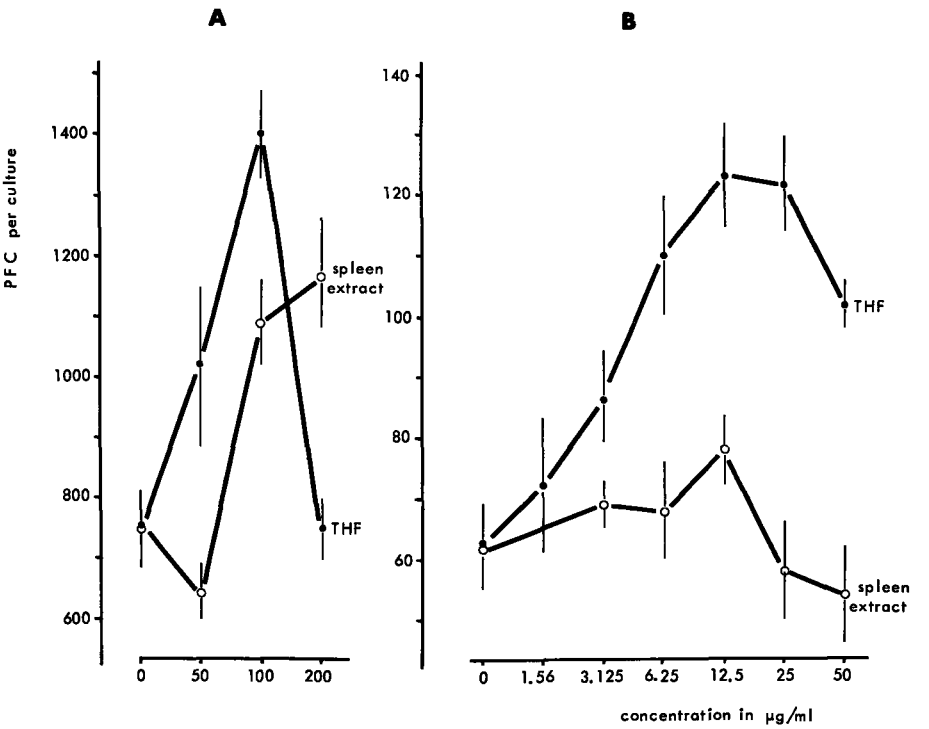

Figure VII.4 The effect of THF and its corresponding spleen extract on the in vitro anti-SRBC response of nu/nu mice as measured in (A) a macro- and (B) a microculture system.
Different batches of THF and spleen extract were used in experiments A and B. Each point represents the average PFC of either three (A) or four (B) replicate cultures. Standard deviations are indicated by bars. The preparations were present throughout the culture period of 4 days. Number of PFC in cultures without SRBC = 124 ± 16 (A) and 34 ± 2 (B).

of thymosin and THF and other control preparations derived from lymphoid and nonlymphoid organs. A survey of the results obtained in separate experiments is presented in Table VII.1. A dose-response curve was made for each preparation. To provide a means of comparison between the preparations, the table gives the concentration at which maximum activity was obtained or the highest concentration tested when no activity was observed. Unlike the THF batches, the various thymosin batches gave consistent results. Some of the THF batches were completely negative. With the exception of one spleen extract corresponding to THF as mentioned above, all control preparations obtained from spleen, lymph node, or liver resulted in either a slight enhancement (lymph node, thymosin type) or no enhancement.

To determine whether the preparations influence cell survival, extra cultures were included in the experiments. No difference was

TABLE VII.1

EFFECT OF THYMOSIN, THF, AND CONTROL PREPARATIONS ON THE IN VITRO ANTI-SRBC RESPONSE OF SPLEEN CELLS FROM nu/nu MICE

type of preparations	source	number of batches		concentration[a] (in µg/ml)		relative PFC response[b]	
		A[c]	B	A	B	A	B
Thymosin	Thymus	1	4	100	6.25-12.5-25	1.6 (1.4-2.1)	1.8 (1.4-2.7)
	Spleen	1	2	400	50	1.1 (1.0-1.2)	1.2 (1.0-1.3)
	Lymph node	1	-	400	-	1.3 (1.3-1.4)	-
	Liver[d]	1	-	400	-	1.0 (0.9-1.1)	-
THF	Thymus	1	2[e]	50-100	6.25-12.5-25	1.9 (1.8-2.0)	2.1 (1.5-3.0)
	Spleen	1	2	200	25-50	1.6 (1.4-1.8)	0.9 (0.8-1.3)
	Lymph node	-	1	-	50	-	0.8 (0.8-0.9)

[a] Concentration at peak response or highest concentration tested when no enhancement was observed.

[b] Relative response = response at peak/response in the absence of preparation. Values represent the mean and range of at least three experiments per batch. Three (A) or four (B) replicate cultures were performed in each experiment for each concentration tested. Number of PFC in the absence of preparations = 600-1400 (A) and 40-100 (B) PFC/culture.

[c] The experiments under column A were performed with the macroculture system and those under column B with the microculture system.

[d] Liver was obtained from WAG/Rij rats. Calves were used for the other preparations.

[e] Two out of six THF batches were positive.

found in the number of viable cells between cultures with and without the preparations. The cell survival in the macro- and microcultures was comparable and was approximately 20%.

3. Effect of thymosin and THF prepared from thymus epithelium and thymus lymphocytes

The above results indicate that thymosin and THF prepared from calf thymus are capable of improving the in vitro anti-SRBC response of spleen cells from nu/nu mice, whereas most of the control substances fail to enhance the response. Therefore, this assay was employed in a first attempt to establish the cellular origin of the active factor(s). Since the thymus consists of an epithelial and a lymphoid compartment, humoral factors derived from thymus tissue enriched in thymus epithelium (TE) were prepared separately from factors derived from thymus lymphocytes (TL). To obtain starting material enriched in epithelium, the thymus was depleted of lymphocytes by exposing the calves to total body irradiation from a ^{137}Cs source (dose rate, 100 rad/hr; focus object distance, 1.25 m) two days before extraction of

TABLE VII.2

EFFECT OF VARIOUS THYMIC FACTORS AND CONTROL PREPARATIONS ON THE IN VITRO ANTI-SRBC RESPONSE OF SPLEEN CELLS FROM NU/NU MICE

	thymosin-type			THF-type		
source*	number of batches	concentration** (in µg/ml)	relative PFC response***	number of batches	concentration** (in ug/ml)	relative PFC response***
Total thymus (TT)	4	6.25-12.5-25	1.8 (1.4-2.7)	2****	6.25-12.5-25	2.1 (1.5- 3)
Thymus epithelium (TE)	7	3.13-6.25-12.5	2.4 (1.4-4.7)	2****	0.78-1.56-3.13	3.0 (1.6-4.5)
Thymus lymphocytes (TL)	3	6.25-12.5	1.3 (1.1-1.4)	1	12.5-25	1.0 (0.8-1.3)
Spleen	4	25-50	1.6 (1.3-1.9)	3	25-50	1.0 (0.8-1.2)
Lymph node	4	25-50	1.8 (1.7-1.9)	3	6.25	1.0 (0.7-1.2)

* Thymus epithelium, spleen and lymph node were obtained from calves irradiated with 600 rad of whole-body irradiation.
** Concentration at peak response or highest concentration tested when no enhancement was observed.
*** Relative response = response at peak/response in the absence of preparation. Values represent the mean and range of at least three experiments per batch. Four replicate cultures per experimental point. Number of PFC in the absence of preparations = 40-100 PFC/culture.
****Two out of six TT-THF and two out of three TE-THF batches were positive.

the thymus (Van Bekkum, De Vries and Klouwen, 1965). Histological examination revealed that the thymus is largely devoid of thymocytes by that time and consists mainly of epithelial cells, reticuloepithelial cells, and macrophages. TE-thymosin and -THF were prepared from the irradiated thymus tissue. The effect of these TE preparations was compared with thymosin and THF made from total thymus (TT) and thymus lymphocytes (TL). Calf thymocytes were obtained by squeezing the thymus through nylon gauze. The thymocytes were washed once with phosphate buffered saline before starting the isolation procedure. The suspension of thymocytes was not contaminated by epithelial cells, as was demonstrated by histological examination. Control preparations were made from the spleens and lymph nodes of the irradiated calves. Table VII.2 gives a survey of the results obtained with various batches of each preparation in separate experiments. Representative experiments demonstrating dose-response profiles of TE-thymosin and THF in comparison with their corresponding TT preparations are shown in Fig. VII.5. The following points are noteworthy. The average enhancement of the response caused by both THF and thymosin-like TE preparations was greater than that induced by the TT preparations. Although the optimal dose of the various batches differed, the TE factors, especially the THF-type preparations, generally exhibited activity at lower concentrations as compared with the TT preparations. Both TL-thymosin and TL-THF showed marginal activity. Surprisingly, some activity was found in thymosin-type control preparations derived from irradiated spleens and, in particular, from irradiated lymph nodes (Table VII.2). It should be noted that these responses were obtained at higher concentrations than in the case of TE-thymosin. In contrast, THF-type preparations derived from spleens and lymph nodes of the irradiated calves had no detectable effect. However, the interpretation of the latter observation is uncertain, since inactive TT- and TE-THF batches have been occasionally obtained. One out of three TE-THF batches was ineffective, whereas all seven TE-thymosin batches showed activity. The reason for the lack of activity in some THF preparations is obscure. Although it cannot be concluded from the above data that the activity of TE-thymosin is exclusively derived from the epithelial part of the thymus, the results are still suggestive of active epithelial factor(s) because of the near absence of activity in the TL preparations. Both TT-thymosin and TT-THF show enhancing activity which cannot, therefore, be derived from thymus lymphocytes.

The effect of the preparations on the background anti-SRBC PFC was determined in order to establish whether the preparations possess B-cell mitogenic properties and are therefore capable of inducing a

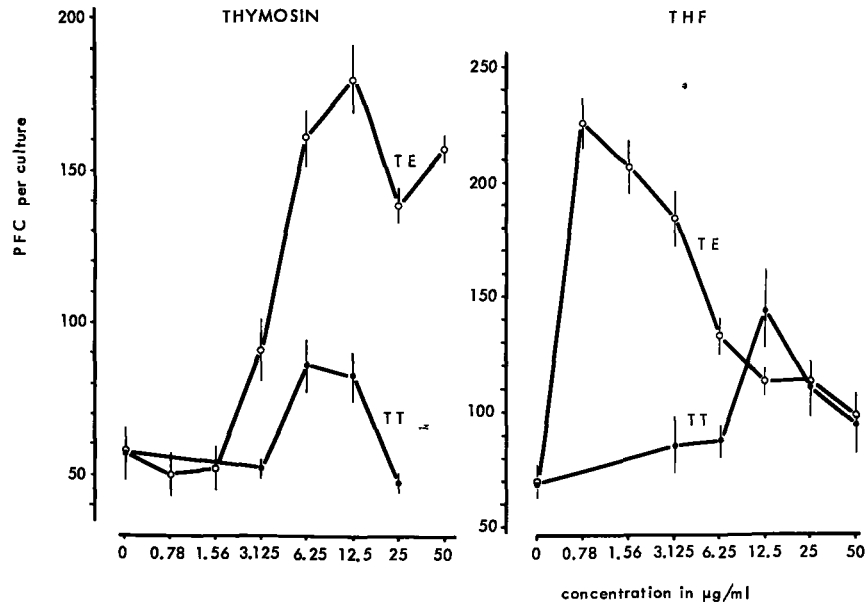

Figure VII.5 Comparison of the effect of thymosin and THF prepared from thymus epithelium (TE) and total thymus (TT) on the in vitro anti-SRBC response of nu/nu mice.
Each point represents the average PFC of four replicate microcultures. Standard deviations are indicated by bars. The preparations were present throughout the culture period of 4 days. Number of PFC in cultures without SRBC = 25 ± 5 (A) and 38 ± 6 (B).

polyclonal antibody synthesis (Coutinho and Möller, 1974). Control experiments in which various preparations were added to spleen cell cultures in the absence of the antigen SRBC demonstrated that these preparations did not influence the number of background anti-SRBC PFC (Table VII.3). These results suggest that the enhancement of the in vitro anti-SRBC response by the active preparations cannot be attributed to a B-cell mitogenic property of these preparations.

4. Comparison of the effect of thymosin and E. coli lipopolysaccharide

During the time-consuming purification procedures necessary to obtain thymic extracts such as thymosin or THF, contamination of the extracts with endotoxin due to bacterial proliferation is difficult to avoid. Endotoxin is known to affect the immune response and it has often been suggested that it mimics the activity of thymic extracts. Enhancement and suppression of the immune response by endotoxin both in vivo and in vitro have been described (Mishell et al., 1973;

TABLE VII.3

EFFECT OF VARIOUS THYMIC FACTORS AND CONTROL PREPARATIONS ON THE "BACKGROUND" ANTI-SRBC RESPONSE OF SPLEEN CELLS FROM nu/nu MICE

type of preparations	source	exp. number*	concentration (in µg/ml)	number of PFC per culture**	
				without	with preparations
Thymosin	Total thymus (TT)	1	100, 400	140	126, 107
		2	100	423	451
		3	100	399	287
		4	3.13-50***	35	37, 38, 42, 41, 40
	Thymus epithelium (TE)****	5	1.56-50	42	40, 42, 49, 42, 40, 44
	Lymph node****	6	1.56-50	37	43, 39, 43, 39, 30, 35
THF	Total thymus (TT)	4	3.13-50	35	40, 27, 37, 39, 26
		6	3.13-50	37	35, 34, 33, 34, 32
		7	3.13-50	27	27, 22, 20, 22, 33
	Thymus epithelium****	5	1.56-50	42	44, 41, 36, 38, 32, 35

* Expt 1-3, macroculture system; Expt 4-7, microculture system.
** Spleen cells were cultured in the absence of SRBC as antigen.
*** Concentration range tested (twofold dilutions).
****TE and lymph node extract were obtained from calves exposed to 600 rad of whole-body irradiation.

Armerding and Katz, 1974a, and Hoffmann et al., 1975). Since we use an in vitro immune response as an immune assay for monitoring the biological activity of thymic extracts, it was necessary to establish in what manner endotoxin influences the in vitro immune response under our experimental conditions. Therefore, lipopolysacchardide (LPS) of E. coli 055:B5 was added in varying amounts to cultures of spleen cells from athymic nude mice in the presence and the absence of sheep red blood cells (SRBC) as antigen. LPS was used because it is the main component of bacterial endotoxin. For comparison, cultures with the optimal amount (100 µg) of calf thymosin which was shown to enhance the in vitro antibody formation of spleen cells from nude mice were added to the experiments. As shown in table VII.4, LPS suppressed the in vitro immune response to SRBC. In the absence of antigen, the

TABLE VII.4

COMPARISON OF THE EFFECT OF LPS AND THYMOSIN ON THE IN VITRO ANTI-SRBC RESPONSE OF SPLEEN CELLS FROM nu/nu MICE

addition	Anti-SRBC PFC/culture* exp. 1 +SRBC	exp. 1 -SRBC	exp. 2 +SRBC	exp. 2 -SRBC
none	1680	423	1794	399
5 µg LPS	907	736	1291	-
25 µg LPS	760	845	1158	-
100 µg LPS	838	749	947	732
200 µg LPS	687	703	864	791
100 µg thymosin	3539	451	2594	287

*The macroculture system was used. Each value represents the mean number of PFC of at least three cultures.

opposite was observed. Increasing amounts of LPS increased the number of background plaque forming cells (PFC) towards SRBC. On the other hand, the antibody response was increased by thymosin in the presence of SRBC, whereas no increase in the number of background PFC was observed. The results clearly demonstrate that thymosin and LPS have a different effect on the in vitro immune response of spleen cells from nude mice. Therefore, the possibility that the enhancing activity of thymosin is due to contamination by endotoxin can be excluded.

5. Discussion of part C

A variety of in vitro immune assays has been employed in attempts to identify and assay thymic humoral factors and to monitor their isolation and purification. The aim of the present study was to investigate whether the in vitro anti-SRBC response using spleen cells from athymic nu/nu mice could be an appropriate test system for thymic hormone preparations. In particular, the use of an animal which lacks a thymus makes this assay attractive. Therefore, the observation of Armerding and Katz (1975) that a thymosin-like preparation enhanced the in vitro immune response of nu/nu mice has been extended by studying in addition to thymosin the effect of another thymic hormone preparation (THF and appropriate control substances). The results show activities for these well-known thymic hormone preparations, whereas most of the control preparations show none. Comparable results were obtained in the macro- and microculture systems. For future studies, the latter will be preferred because of its greater capacity. It is important to note that the extent to which thymosin and THF increase the response was comparable. At concentrations higher than the optimum concentrations, thymosin and THF exhibit suppressive effects. Whether this is due to the presence of inhibitors in the preparations or to a biological phenomenon such as the induction of suppressor cells remains to be investigated.

Our subsequent investigations concerned the cellular origin of the factor(s) active in the in vitro anti-SRBC response of nu/nu mice. It is well known that the thymus consists of a lymphoid and an epithelial compartment; the latter is considered to produce the thymic hormone(s) (Pyke and Gelfand, 1974, and Dardenne et al., 1974b). Therefore, the isolation procedures for THF and thymosin were used to prepare factors from thymus lymphocytes (TL) and from thymus tissue enriched in thymus epithelium (TE) by irradiation of the calves before extraction of the thymus (Van Bekkum, De Vries and Klouwen, 1965). Their activity in the in vitro antibody formation was assessed and compared with the

activity of factors derived from total thymus (TT). A comparison of the results obtained with the various preparations showed the TE preparations to be much more active than the TT preparations in terms of enhancement of the response and dose-response relations. Very little activity was found in the TL preparations. The high activity of the TE preparations cannot, however, be attributed exclusively to an enrichment in epithelium-derived factors, since addition of thymosin-like preparations from spleen and lymph nodes from irradiated calves also resulted in an increase in the anti-SRBC response. One could speculate whether a macrophage-derived factor contaminates the thymosin-type preparations obtained from the thymus, spleen, and lymph nodes of the irradiated calves, since the lymphoid organs remaining after irradiation are infiltrated by macrophages. Macrophage-derived factor(s), especially factor(s) released by culturing stimulated macrophages, have indeed been shown to improve the in vitro immune response of both normal and nu/nu mice (Schrader, 1973; Wood and Gaul, 1974, and Calderon et al., 1975). However, for the following reasons, it is unlikely that this type of macrophage-derived factor(s) is present in our thymosin preparations. Various macrophage factors which exhibited lympho-stimulatory activities had sizes ranging between 15,000 and 21,000 daltons (Calderon et al., 1975; Wood et al., 1976, and Gery and Handschumacher, 1974), but the thymosin preparation was passed through Amicon UM-10 ultrafilters which are impermeable to proteins with a molecular weight higher than 15,000 (Hooper et al., 1975). The macrophage factors were heat labile (Calderon et al., 1975, and Gery and Handschumacher, 1974), in contrast to thymosin which was heated to 80°C during its isolation.

Furthermore, macrophage-derived factors stimulate proliferation of thymocytes and spleen cells in the absence of lectins (Calderon et al., 1975, and Gery, Gershon and Waksman, 1972); neither TE-thymosin nor the corresponding control preparations had mitogenic properties (Betel and Kruisbeek, personal communication).

It would be of great interest to establish the origin of the active material derived from the irradiated spleens and lymph nodes. The first possibility is obviously that material different from thymus epithelium-derived factor(s) is present in the preparations obtained from the irradiated thymus, spleen, and lymph nodes. On the other hand, the possibility cannot be excluded that lymph nodes and spleens contain small numbers of epithelial cells with the same function as thymus epithelium. The relative proportion of these cells could have been increased as a result of the irradiation.

Apart from the results obtained with preparations from the irradi-

ated tissues, a comparison of the effect of the TT and TL preparations suggests that the thymus epithelium is the source of the active factor(s). TT preparations show activity in the in vitro anti-SRBC response which cannot be derived from thymocytes, since the TL preparations were nearly negative.

With regard to our test system, the target cell for the active thymic factor(s) in the spleen cell population of nu/nu mice still has to be identified. A direct effect on B cells by a B-cell mitogenic property of the active preparations could be excluded. Furthermore, Armerding and Katz (1975) demonstrated that thymosin failed to reconstitute in vitro anti-SRBC responses of spleen cells depleted of T lymphocytes by treatment with anti Thy-1 serum plus complement. They also showed that thymosin differs from a T-cell replacing factor such as the allogeneic effect factor which acts directly on B cells and exhibits a far superior enhancing activity. The present data suggest that thymosin and THF contain factor(s) which improve the anti-SRBC response of spleen cells from nu/nu mice by the induction of helper T cells from precursor cells. A supporting effect on a few already present helper T cells cannot, however, be excluded. It has been previously shown that nu/nu mice do possess precursor cells capable of being induced to express TL and Thy-1 surface markers after either in vitro treatment with thymosin (Komuro and Boyse, 1973) or in vivo treatment with thymopoietin (Scheid, Goldstein and Boyse, 1975). Furthermore, helper T cells develop in nu/nu mice given an irradiated thymus graft (Blankwater and Lina, 1974; Chapter IV, part B). This also suggests that nu/nu mice possess prethymic precursor cells. Likewise, responsiveness to concanavalin A and mixed lymphocyte reactivity were induced in a subpopulation of brain-associated T-antigen-positive cells in spleen and lymph nodes from nu/nu mice (Sato, Waksal and Herzenberg, 1976). Loor and Roelants (1974) suggested that such cells represent committed T lymphocyte precursors.

D. EFFECT OF THYMIC EPITHELIAL CULTURE SUPERNATANT (TES) ON THE IN VITRO ANTI-SRBC RESPONSE OF SPLEEN CELLS FROM nu/nu MICE AND "B" MICE

Since the epithelial compartment of the thymus is considered to be the source of thymic humoral factors, attempts have been made to establish cultures of thymic epithelial cells and to investigate the effect of cocultivation of lymphocytes with the cultured epithelial cells and with the supernatant. Mouse thymocytes incubated on thymic

epithelial monolayers displayed increased helper T cell activity as well as increased responsiveness to PHA and allogeneic cells (Mosier and Pierce, 1972). Likewise, spleen cells from thymus-deprived animals acquired the capacity to produce T lymphocyte reactions when incubated with cultured epithelial cells in vitro (Wekerle, Cohen and Feldman, 1973, and Waksal et al., 1975). Sato, Waksal and Herzenberg (1976) isolated brain-associated T antigen positive cells from nu/nu spleens with a fluorescence-activated cell sorter. When these cells which are thought to represent precursor T cells were cultured on thymic epithelial monolayers, they exhibited an enhanced responsiveness to ConA and allogeneic cells. Supernatants from human thymic epithelial cultures increased the capacity of human marrow cells to form rosettes with sheep erythrocytes (Pyke and Gelfand, 1974) as well as the sensitivity of spleen cells from adult thymectomized mice to anti-Thy-1 serum (Papiernik, Nabarra and Bach, 1975). In addition to these effects on the appearance of surface markers, it was recently demonstrated that a supernatant derived from rat thymic epithelial cultures (TES) enhanced the PHA and ConA responses of rat thymocytes (Kruisbeek, Kröse and Zijlstra, 1977). Since these cultures consist of epithelial cells only after a certain period of time, the supernatant provides a more homogeneous starting material for the isolation of thymic factors than do thymic extracts. In order to gain more insight into the biological activity of TES, its influence on a number of other thymus-dependent immune functions was investigated. Here, the effect of TES on the in vitro anti-SRBC response will be dealt with.

Thymic epithelial cultures were prepared from rat thymus glands as described by Kruisbeek, Kröse and Zijlstra (1977). The experiments reported here were performed with pools of supernatants harvested from cultures of 10 days and older, since from that time onwards the cultures consist mainly of epithelial cells as determined by electron microscopy (Kruisbeek, Kröse and Zijlstra, 1977).

When TES is added at the beginning of the culture period of an anti-SRBC response of spleen cells from nu/nu mice, a significant increase in responsiveness is observed. A representative experiment is shown in Fig. VII.6. Addition of kidney epithelial culture supernatant (Fig. VII.6) or supernatant from thymocytes or pancreas epithelial cultures (data not shown) did not influence the response. Peak of activity was usually observed at a dilution of 1/10 or 1/20. The factor by which the response was increased varied from 1.7 to 3.9 for the several batches tested. The survival of the spleen cells at the end of the culture period varied from 20-30% and was not affected by the presence of TES or control supernatant. TES exhibited no effect on

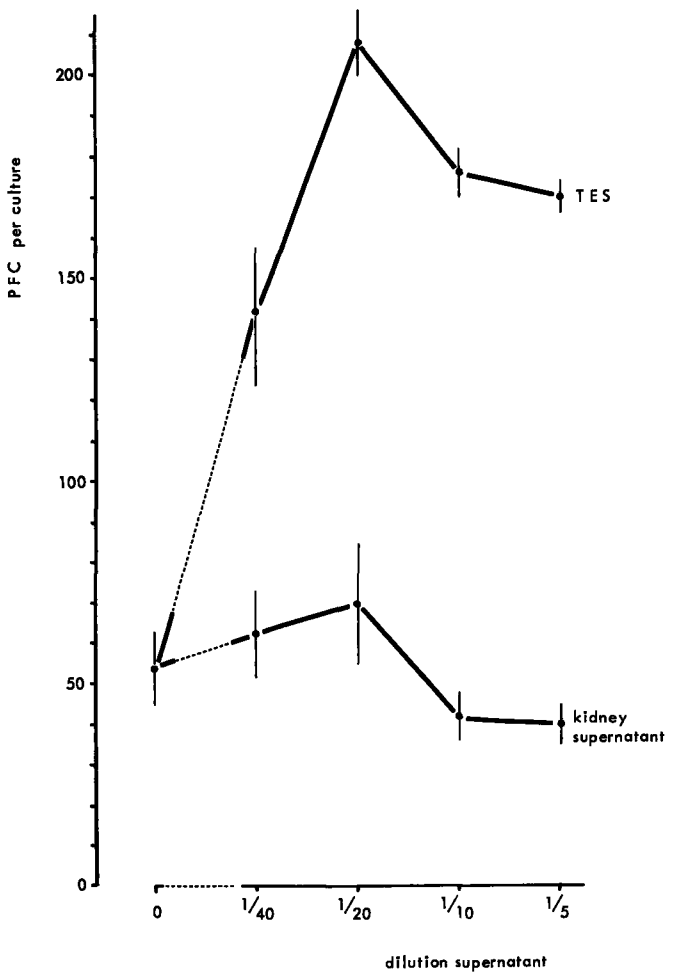

Figure VII.6 Effect of TES and kidney supernatant on the *in vitro* anti-SRBC response of spleen cells from nu/nu mice.
Each point represents the average number of PFC of four replicate microcultures. Bars indicate standard deviations. TES or kidney supernatant was present throughout the culture period of 4 days. The number of PFC in unstimulated cultures (without SRBC) was 16 ± 2 and was not affected by the presence of either supernatant.

the number of background PFC in unstimulated spleen cell cultures, indicating the absence of B cell mitogenic properties.

The increased response of nude spleen cells in the presence of TES could reflect a direct influence of TES on B lymphocyte precursors of antibody forming cells, on T lymphocyte precursors of T helper cells or both. Therefore, TES was also tested in cultures of spleen cells

from "B" mice in the presence or absence of thymocytes in order to discriminate between these possibilities. Fig. VII.7 shows that addition of TES to spleen cell cultures of "B" mice had no enhancing effect. This indicates that TES does not directly facilitate triggering of B cells. Addition of 10^5 thymocytes to spleen cell cultures of "B" mice in the absence of TES also did not result in a significant increase in the number of PFC. This finding confirms other reports (Mosier and Pierce, 1972, and Kontiainen and Feldmann, 1975) which showed that normal thymocytes exhibited poor helper cell function. However, when TES was added together with thymocytes to spleen cells

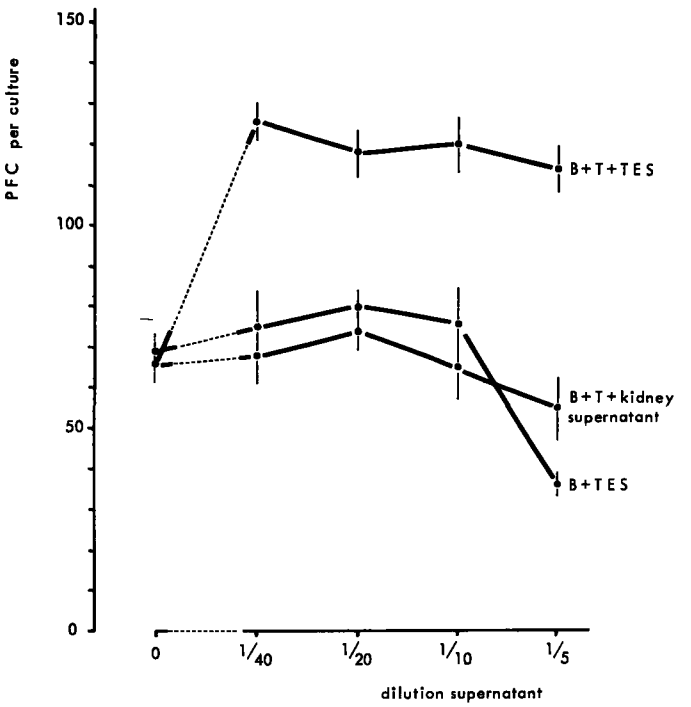

Figure VII.7 Effects of TES and kidney supernatant on the in vitro anti-SRBC response of spleen cells from "B" mice in the presence or absence of thymocytes. Spleen cells from B mice (10^6) were cultured with TES either alone (B) or in the presence of 10^5 thymocytes (B+T). "B" mice were female CBA mice thymectomized at the age of 6 weeks, lethally irradiated and reconstituted with 5×10^6 syngeneic bone marrow cells two weeks later. The effect of kidney supernatant on spleen cells from "B" mice cultured in the presence of 1×10^5 thymocytes is shown. TES or kidney supernatant was present throughout the culture period of 4 days. Each point represents the average number of PFC of four replicate cultures. Bars indicate standard deviations. The number of PFC in unstimulated cultures (without SRBC) was 23 ± 2 and 30 ± 2 for spleen cells from "B" mice alone and together with thymocytes, respectively, and was not affected by the presence of either supernatant.

of "B" mice cultured with SRBC, an increase in the number of PFC was observed. In the representative experiment shown here, 10^5 thymocytes were used. Similar results were obtained by adding 0.5 or 2 x 10^5 thymocytes plus TES to the B cell cultures.

These results indicate that TES influences the helper T cell function of thymocytes and has no effect on the generation of antibody forming cells in the absence of thymocytes. In view of these data, the possibility that the activity observed in nude spleen cell cultures can be attributed to a direct effect on B cells can also be excluded. This suggests that the in vitro anti-SRBC response of spleen cells from nude mice may have been increased due to TES by the induction of helper T cell function in precursor T cells. An enhancing effect on a few already present helper T cells cannot be excluded.

In addition to the previously reported effect of TES on T cell mitogen responsiveness (Kruisbeek, Kröse and Zijlstra, 1977) and the induction of helper T cell activity in thymocytes described here, it was further shown that TES enhances the responder capacity of thymocytes in a mixed lymphocyte reaction and increases cAMP levels in thymocytes (Kruisbeek et al., 1978).

E. DISCUSSION

Involution of the thymus with advancing age is probably accompanied by a decreased production of the putative thymic hormone which may result in the demonstrated decline in effector T cell functions. The observed decrease in the levels of various circulating thymic factors as described in the introduction lends some support to this speculation. An additional role of a decrease with age in T cell precursors which have to undergo the various differentiation steps of the T cell lineage cannot be excluded. Studies of Tyan (1976, 1977) suggest an age-related decrease in marrow T cell progenitors, since bone marrow from aged donors has a diminished capacity to repopulate the thymus of an irradiated (young) host. Assuming that prethymic precursor cells are still present in a reasonable quantity in the ageing immune system, one of the possibilities to correct or improve the thymus-dependent immune functions in old age may be found in the manipulation of the T cell system by thymus transplantation or administration of thymic humoral factors.

The data from the literature and our results concerning thymus grafting in aged mice have given inconclusive information so far. However, it cannot be denied that the few successful attempts which

have been reported should encourage further investigation in this direction.

Before thymic humoral factors can be applied in immunogerontological studies and ultimately in reconstitution experiments, more insight into the nature and mode of action of these factors is required. The experiments described in this Chapter represented a part of a combined project on thymic humoral factors of the Institute for Experimental Gerontology and the Radiobiological Institute TNO. It was found that thymosin and THF preparations derived from thymus extracts exhibited an enhancing effect on the in vitro anti-SRBC response of spleen cells from nu/nu mice. This finding is an extension of the already described activities of these preparations in other assays for T lymphocyte functions (see Van Bekkum, 1975, and Bach and Carnaud, 1976). However, in contrast to the literature data, clear-cut effects of these preparations on T cell mitogen- and mixed lymphocyte responses with mouse and rat lymphocytes were not obtained (Betel and Kruisbeek, personal communication). Furthermore, it was found that it was difficult to obtain active batches of one of the selected preparations, THF, in a reproducible way (see, for instance, Table VII.1). Finally, using the isolation procedures with thymic extracts as the starting material, it was not possible to definitively establish the origin of the active factors. The results suggest that the thymic epithelium may be the source of factors active in the anti-SRBC response, since preparations derived from thymus lymphocytes were only marginally effective. However, stimulatory material was also found in the control preparations derived of spleen and lymph nodes of the irradiated calves. This fact complicated the interpretation of the results.

Therefore, another method was sought to obtain thymic humoral factors (Kruisbeek, Kröse and Zijlstra, 1977). It was found that supernatant from rat thymic epithelial cultures (TES) contained material which showed clear-cut activities in more assays (Kruisbeek, Kröse and Zijlstra, 1977, and Kruisbeek et al., 1978) than was found with factors derived from thymic extracts in our hands. Moreover, the use of cultured epithelial cells as starting material circumvents the problem of whether the active factors are derived from the epithelial or lymphoid part of the thymus.

With regard to the effect of TES on the in vitro anti-SRBC response as described in this Chapter, evidence was provided that TES acts on precursors of helper T cell which reside in the thymocyte population. In addition to the other functional effects of TES such as the increase of the responsiveness of thymocytes to T cell mitogens and

allogeneic cells, it was also found that cAMP levels of mouse thymocytes were enhanced by TES (Kruisbeek et al., 1978). Since T cell differentiation is considered to be mediated by cAMP (Bach, Fournier and Bach, 1975, and Scheid et al., 1975), the combined data suggest that TES exerts its effect on T cell functions via differentiation of precursor T cells.

In conclusion, the results obtained with TES are more promising than those obtained with factors derived from thymic extracts. Therefore, our future investigations in this field will be focused on the factors present in the supernatant rather than on those present in thymic extracts.

The ultimate goal will be to reconstitute in vivo states of immunodeficiency, such as found in aged individuals, which are thought to be the consequence of failing thymus function. Therefore, animal models for T cell deficiencies such as nu/nu mice and thymectomized mice have to be used. As mentioned earlier, convincing results with regard to an in vivo reconstituting effect of the current thymic factors are lacking. Our preliminary studies in which nu/nu mice, adult thymectomized mice and B cell mice were treated with thymosin and THF according to various regimens as reported in the literature (Collins and Morrison, 1976; Morrison and Collins, 1976, and Ikehara, Hamashima and Masuda, 1975) have not given positive results. In the near future, it must be clarified which types of T cell deficiencies can be corrected by thymic factors alone and in which types contact with the thymic environment in addition to humoral factors is needed for reconstitution. These problems have to be resolved before a feasible application of thymic factors for the reconstitution of immunodeficient animals can be envisaged.

CHAPTER VIII

GENERAL DISCUSSION

In order to incorporate the experimental data described in the preceding Chapters into a broader context, it may be necessary to first summarize the major findings. Based on the studies with male CBA mice, the following conclusions concerning the age-related decline in the humoral immune response can be presented.

1. The primary response to SRBC in the spleen decreases earlier in life than does the response after secondary stimulation.
2. The generation of IgG-PFC is more affected by age than that of IgM-PFC.
3. The capacity to respond to the thymus-independent antigen LPS is preserved longer throughout life than that to respond to the thymus-dependent antigen SRBC.
4. There is an age-related loss of IgM-PFC and especially IgG-PFC in the bone marrow after a secondary stimulation with SRBC.
5. The responsiveness of spleen cells isolated from mice of various ages to SRBC generally declines later in life if the cells are stimulated *in vitro*.
6. The antigen dose required to induce maximum numbers of splenic PFC *in vivo* is higher in aged mice, while young and aged spleen cells display a comparable dose dependence *in vitro*.

The major findings indicate a greater influence of age on primary than on secondary responses and on thymus-dependent than on thymus-independent responses.

The mechanisms underlying the decline in humoral as well as cell-mediated immunity with advancing age are far from being defined. On the basis of our findings and the available literature data, one may present the following outline of the influence of ageing on the events leading to an immune reaction.

The first phase in an immune response concerns antigen processing and the presentation of antigen to the immunocompetent cells. Macrophages play an important role in this initial process. The *in vivo* and *in vitro* data on macrophages do not imply age-related changes in phagocytic activity or changes in the capacity to collaborate with the immunocompetent cells after antigen digestion (Chapter I, part D.2).

From the observation that young and old spleen cells stimulated *in vitro* display comparable dose-response patterns (Kishimoto, Takahama and Mizumachi, 1976; Segre and Segre, 1976b; and Chapter VI), it can

be concluded that the antigen sensitivity of the immunocompetent cells is not altered during ageing. However, the in vivo situation is different. Here, the maximum dose of antigen required for a maximum response was higher in aged mice (Price and Makinodan, 1972a; and Chapter IV). This difference in sensitivity to antigens indicates an influence of factors which do not play a role when the cells are stimulated in vitro and must, therefore, be microenvironmental. One may think of a decreased opportunity for lymphocytes to contact antigens due to changes in the architecture of the lymphoid organs or to amyloid depositions in certain conditions. It is not known whether the ageing environment influences only the antigen processing or the cellular interactions required for a response or both. Our finding that the reactivity of spleen cells stimulated in vitro under optimum conditions generally declines later during the life-span of the donor than in vivo also suggests an additional contribution of environmental factors to the loss of humoral immunity. Price and Makinodan (1972a, b) estimated that approximately 10% of the age-related decline can be attributed to changes in the cellular milieu.

For the triggering of both B and T cells, the antigens have to be recognized as non-self by these cells. Only one study deals with the binding and subsequent recognition of antigen by lymphocytes in relation to ageing. Callard, Basten and Waters (1977) demonstrated that the number of spleen cells which bind pneumococcal polysaccharide type III (S III, a thymus-independent antigen) and the intensity of binding is not changed with age. Previous investigations of this group showed that these cells are B cells and represent the precursors of cells producing antibodies to SIII. Despite the comparable antigen binding in young and old mice, old mice displayed a reduced response to SIII, indicating intrinsic changes in the B cells in the capacity of differentiating into antibody forming cells. No data are available as yet on changes in the number of B and T cells recognizing thymus-dependent antigens.

The finding of a decrease in the number of responding cells after antigen binding with SIII is consistent with the results of many other studies concerning the effect of age on the proliferation phase of a response after mitogenic or alloantigenic stimulation.

Abraham, Tal and Gershon (1977) and Merhav and Gershon (1977) reported that the age-related decline in the in vitro responses to ConA, LPS and allogeneic cells cannot be attributed to an altered generation time nor to a reduced capacity for repeated cell division. These findings support the suggestion of others (Kruisbeek, 1976, and Perkins and Cacheiro, 1977) that there are fewer responding cells

in aged animals as compared to young animals but that those cells which do respond display the same proliferative capacity as do cells of young animals. In addition, it has been demonstrated that the concentration of B and T cell mitogens and of alloantigens required for maximum proliferation is not changed during ageing (Mathies et al., 1973; Hung, Perkins and Yang, 1975b; Kruisbeek, 1976; Callard and Basten, 1977; Callard, Basten and Waters, 1977; Abraham, Tal and Gershon, 1977, and Merhav and Gershon, 1977). As discussed earlier, the dose of antigen required for optimum in vitro antibody production also remains the same during ageing.

The notion, that during ageing, fewer cells are capable of responding to a triggering signal finds support from the observation of Andersson, Coutinho and Melchers (1977) that the frequency of mitogen reactive B cells, as analysed in a limiting dilution system, decreases with age. A cytochemical study of human lymphocytes stimulated by PHA has revealed that the reduced response with age could be related to structural alterations in the chromatin resulting, at least in some of the T lymphocytes, in an irreversible blockage in the G1 phase of the cell cycle (Preumont, Van Gansen and Brachet, 1978). Arrest of cells in the G1 phase is one of the possible mechanisms proposed for cellular senescence (Gelfant and Smith, 1972).

The underlying causes for the age-related loss in responding cells may be numerous and have to be discussed in relation to the complex cell interactions which determine the ultimate responses measured in the experimental systems. From the summary at the beginning of this Chapter, one may conclude that the influence of age on the humoral immune system is particularly evident after a first encounter with antigen. A first explanation might be that the clonal diversity of the B and/or the T cell compartment becomes less during ageing. This would predict that the number of nonresponding animals should increase with age within an inbred strain. This hypothesis can be tested only with antigens carrying a limited number of determinants (e.g., synthetic oligopeptides). The fact that secondary responses are less affected than primary responses makes it necessary to restrict this hypothesis to only certain populations or subpopulations of lymphoid cells. It appears that the more the responses are dependent on helper T cells, the faster the age-related decline occurs. The latter conclusion follows from the observations that: (1) the thymus-independent anti-LPS response is less affected by age than the thymus-dependent anti-SRBC response; and (2) IgG antibody formation is more and earlier decreased than IgM antibody formation. As mentioned previously, the production of IgG antibodies is more dependent on helper T cells than

is the IgM response (Chapter IV, part B). Our findings and the literature data presented in Chapter IV may, therefore, indicate that helper T cells lose their functional capacity to help virgin B cells to proliferate and differentiate into antibody secreting cells. In addition, responses mediated by B cells only (such as the anti-LPS response) are also reduced during ageing but this seems to occur later in life in most strains studied (Chapter IV). From the observation that secondary responses are less affected by age (Chapter V), it may be tentatively concluded that T cells involved in the generation of memory cells are functionally intact for longer periods of time. It has been demonstrated that different T cell populations are responsible for the induction of antibody producing cells from virgin B cells and for the generation of B memory cells (Okumura et al., 1976). Again, a differential effect of ageing on the generation of IgM and IgG producing cells after secondary stimulation has been noted (Chapter V).

So far, the influence of T cells on the ageing immune system has been interpreted exclusively in terms of declining helper cell activity. An alternative explanation for the loss of humoral immunity may be found in changes occurring in the behaviour of suppressor T cells. As stated in Chapter IV, studies on suppressor cell activity in mice suggested an increase rather than a decrease in this activity during ageing. Suppressor T cells most probably act via helper T cells and not on the B cells directly (Herzenberg et al., 1976). The question as to what extent alterations in helper function and in suppressor function contribute to the declining immunocompetence with age cannot be answered. One may speculate that many phenomena characteristic for the ageing immune system result from changes in the balance between helper and suppressor activity. In this respect, the loss of heterogeneity within the immunoglobulin repertoire and the loss of self-tolerance with ageing have often been mentioned.

T cells play an important role in the expression of the heterogeneity within the B cell compartment, as evidenced by a restricted diversity of the immunoglobulins (Van Muiswinkel, Radl and Van der Wal, 1976) and a shift to production of low affinity antibodies (Gershon and Paul, 1971, and Anderson, Dresser and Wortis, 1974) following T cell depletion. A restriction of the affinity distribution with a selective loss of cells producing high affinity antibodies during ageing has been reported by Naor, Bonavida and Walford (1976), Kishimoto, Takahama and Mizumachi (1976), and by Goidl, Innes and Weksler (1976). Zharhary, Segev and Gershon (1977), however, observed comparable affinity patterns in young and old mice.

Another measure of the heterogeneity is the immunoglobulin spectrum in the serum. Homogeneous immunoglobulins and idiopathic paraproteins appear quite frequently in the serum of old humans and in mice, most notably in the C57BL/Ka strain (Radl et al., 1978). Presumably, this phenomenon indicates a specific loss of T cell regulation over B cell clones, since neonatally thymectomized C57BL/Ka mice display an earlier onset and a higher frequency of homogeneous immunoglobulins and idiopathic paraproteins (Radl, personal communication). Concerning the age-related changes in antibody production after antigenic stimulation as described in Chapter IV, the following can be noted for C57BL/Ka mice. The IgG-PFC response to SRBC was poor or even absent in aged C57BL/Ka mice, whereas the anti-LPS response was concomitantly unaltered. This finding is again suggestive of a disturbed function of T cells. In addition to idiopathic paraproteinaemia, a high prevalence of malignant lymphomas and amyloidosis is characteristic for the ageing C57BL/Ka mice. The relationship between these lesions and the severe changes in humoral immune function remains to be established.

The increased incidence of autoantibodies in old age may also point to a disregulation of certain B cell clones by T cells. Rheumatoid factors, antithyroglobulin and antinuclear antibodies are well-known in this respect. The age-related increase in autoantibodies was the basis of the "immunological theory of aeing" proposed by Walford (1969) in which the prime event in ageing was sought in increased autoaggression. It has been demonstrated that it is much easier to induce an immune response against modified self-antigens in old than in young mice of a long-lived mouse strain (Naor, Bonavida and Walford, 1976) as well as in those of the autoimmune susceptible NZB and NZB/W mouse strains (Naor et al., 1976). In our investigation on NZB mice, a rapid decline in the capacity to respond to LPS was found, which was in contrast to the other strains studied. Therefore, a loss of B cell function has to be taken into account in addition to the extensively reported impairment of T cell functions (Talal, 1976) for the formulation of hypotheses regarding the origin of the autoimmune disorders in this strain.

It may be concluded from the above discussion and from the extensive data on the loss of cell-mediated immune responses with age (Chapter I, part E) that foremost among the changes responsible for the decline and aberrations in immune functions with age are those in the T cell population. Therefore, the effect of age on T lymphocyte differentiation needs further study. The influence of the thymus on T cell differentiation was found to already decline drastically in the

preinvolution stage, since only 1- to 2-week-old thymus grafts were capable of completely restoring the immune potential of thymus deprived mice (Hirokawa and Sado, 1978). This finding means that the generation of thymocytes in mice is achieved during the early weeks in life. The reasons for the rapid decline afterwards have not yet been elucidated. With regard to later stages in life, an age-related decrease in bone marrow progenitors (Tyan, 1976, 1977) as well as in the level of putative thymic hormones (Dardenne et al., 1974a; Goldstein et al., 1974; Twomey et al., 1977, and Astaldi et al., 1977) has been reported. It must be noted again that these early and later changes in thymocyte differentiation do not result in an age-related decrease in the number of cells bearing Thy-1 antigen. However, within the T cell population, shifts may occur in the ratio between the various T cell subsets. In view of the prominent age-related changes in the activities mediated by T cells, it may be anticipated that an analysis of the T cells by the use of anti-Ly sera will reveal such a shift in T cell subsets with age.

Whether thymic humoral factors may be useful tools for the reconstitution of T cell functions in old age is still uncertain. A prerequisite for a beneficial effect of these factors is that the susceptible precursor cells from either the bone marrow or the thymus are still present in sufficient quantities during ageing. For reasons outlined in Chapter VII, it can be stated that factors present in the supernatant of thymic epithelial cultures (TES) rather than those in thymic extracts may be helpful to study in more detail the steps leading to T cell maturation not only in the early phase of life but also in various conditions of T cell deficiencies such as in old age.

Basic research is still needed for the understanding of the underlying mechanisms leading to the loss of homeostasis in the ageing immune system. Elucidation of these processes may eventually indicate ways in which the decline and aberrations in immune functions in old age can be lessened. The frequent occurrence of a number of diseases which are possibly related to a dysfunctioning immune system justifies this effort.

SUMMARY

The study presented in this thesis is concerned with changes in the humoral immune system as a function of age in different inbred mouse strains. Their capacity to develop humoral immune responses to experimentally given thymus-dependent and thymus-independent antigens under various conditions is compared. Furthermore, experiments employing thymus transplantation and thymic humoral factors which are directed at the restoration of the diminished T cell functions in old age are reported.

Immune functions decline with age. The increased incidence of some diseases in old age might be related to a dysfunctioning of the immune system. This possibility has prompted numerous investigations on the changes which occur in the ageing immune system. These studies are reviewed in Chapter I. Literature data on the effect of ageing on the haemopoietic stem cells, humoral and cell-mediated immune reactions and on the cells involved in these reactions are discussed. In addition, attention is paid to the thymus in relation to ageing as well as to phenomena and diseases which may be attributed to a dysfunctioning of the immune system in old age. The data discussed in Chapter I show a decline in immunological competence with age in both man and experimental animals, although the onset, magnitude and rate of decline seem to vary with the type of immune function, species or strain studied. The conclusion is reached that the decrease is not likely to be due to quantitative changes in haemopoietic stem cells or to changes in the overall numbers of T and B cells.

The materials and methods employed to determine the immune responsiveness of mice both in vivo and in vitro are described in Chapter II.

Chapter III is devoted to the survival patterns and age-related pathology of the following strains of mice: CBA, C57BL/Ka and NZB. The 50% and maximum survival ages were highest for male and female CBA mice, whereas the female NZB mice had the shortest life-span. The most frequently observed lesions in ageing NZB mice concerned autoimmune diseases and lesions due to immune complexes. Malignant lymphomas and amyloidosis were predominant in old C57BL/Ka mice. CBA mice showed the least severe changes in lymphoid tissues with age as compared to the other strains. On the other hand, a high prevalence of hepatocellular neoplasms and ovarian tumours was characteristic for this strain. The CBA mouse seemed to be an appropriate animal for the study of the ageing immune system which is unencumbered by the influence of lesions in the immune system itself.

The effect of age on the capacity to develop a primary immune response *in vivo* is discussed in Chapter IV. The immune responsiveness was determined by the number of antibody forming cells in the spleen. The results revealed that, with regard to the thymus-dependent immune response against sheep red blood cells (SRBC), the level of responsiveness, as found in young adult mice, can no longer be reached by either CBA or C57BL/Ka mice approximately from the age which corresponds with the 90% survival ages for these strains. The generation of cells forming antibodies of the IgG class (IgG-PFC) is more severely affected by age than is the IgM antibody formation. In particular, C57BL/Ka mice display a poor or absent IgG response during ageing. The development of IgG-PFC is known to be more dependent on helper T cells than that of IgM-PFC. The humoral immune response against lipopolysaccharide (LPS) from *E. coli*, for which helper T cells are not required, was also investigated as a function of age. In contrast to SRBC, an optimal dose of LPS elicits responses in CBA and C57BL/Ka comparable in magnitude to those of young adult mice up to the ages of 2 and 1.5 years, respectively. Thereafter, an age-related decrease was found in the number of splenic PFC which was less severe than the decrease observed with SRBC. This observation and the differential effect of ageing on IgM and IgG antibody formation in response to SRBC indicate that the more the immune reactions are dependent on helper T cells, the more rapid does the age-related decline occur. This conclusion applies to experiments in which mice were stimulated with optimal doses of the various antigens. With suboptimal doses of SRBC and LPS, the difference between young and old mice in the magnitude of the responses became even larger.

Much information is already available on thymus-dependent immune functions in NZB mice. Therefore, only the response to the thymus-independent LPS was studied in this strain. The capacity to respond to LPS was severely affected by age in NZB mice in contrast to the results obtained in CBA, C57BL/Ka and BALB/c mice. Therefore, in addition to the extensively reported impairment of T cell function, an age-related loss of B cell function was postulated for this autoimmune susceptible strain.

Chapter V deals with age-related changes in CBA mice in the secondary immune response against SRBC *in vivo*. The bone marrow, which is a major PFC forming organ during the secondary response, displayed a decreasing capacity to develop antibody forming cells with age, especially those producing IgG. It was found for splenic PFC that the secondary response decreases later in life than does the primary response to SRBC.

The capacity of isolated spleen cells from young and aged mice to respond to SRBC *in vitro* is discussed in Chapter VI. The response of spleen cells obtained from donors of different ages stimulated *in vitro* generally declines later in life than that of spleen cells stimulated *in vivo*. A further comparison of the results obtained *in vivo* and *in vitro* reveals a difference in sensitivity to antigens. Whereas the minimum dose of antigen required for a maximum *in vivo* response was higher in aged mice (Chapter IV), spleen cells from both young and aged mice display optimum *in vitro* responses at the same SRBC doses. These findings indicate the contribution of extrinsic (microenvironmental) factors to the decrease in immune reactivity with age.

Attempts to restore the decreased antibody formation to SRBC in aged mice by thymus transplantation are described in Chapter VII. No conclusion was reached as to whether thymus transplantation may prove to be a useful approach for the manipulation of the immune system in old age.

In addition to thymus transplantation, treatment with thymic humoral factors may be envisaged for the improvement or even restoration of thymus-dependent functions in aged individuals. Therefore, the activity of various thymic humoral factors is compared in Chapter VII. Thymosin, thymic humoral factor (THF, both derived from thymus homogenates) and thymus epithelial culture supernatant (TES) enhanced the *in vitro* anti-SRBC response of spleen cells from athymic nu/nu mice. In an attempt to determine whether the active factors present in thymosin and THF were derived from the epithelial or lymphoid part of the thymus, preparations made from the two compartments were compared for activity. Although the results suggested that the thymic epithelium was the source of the active factors, their origin could not be unequivocally established. Another approach to this problem was the use of TES, since the cultured cells producing the active factor(s) consist only of epithelial cells. Therefore, the activity of TES was investigated in more detail and it was demonstrated that TES acts on precursors of helper T cells residing in the thymocyte population.

In Chapter VIII, the results of the investigations are discussed in relation to recent findings as reported in the literature. It is concluded that the age-related decline in immune functions are particularly evident under conditions in which the immune system has to respond to an antigen for the first time and in which helper T cells are involved in the generation of a humoral immune response. In addition to intrinsic factors, extrinsic (microenvironmental) factors are also of importance, as evidenced by a different susceptibility of

spleen cells to antigens *in vivo* and *in vitro*.

Further elucidation of changes in the T cell population by analysing the possible disturbances in their maturation and in their activities as effector, helper and regulatory cells will be required to understand the loss and aberrations in immune functions with age.

SAMENVATTING

Het in dit proefschrift beschreven onderzoek is gewijd aan het effect van veroudering op de humorale immuunrespons. De veranderingen die tijdens veroudering optreden in het reactievermogen op experimenteel toegediende antigenen worden vergeleken in enkele ingeteelde muizenstammen. Daarnaast worden experimenten met thymus-transplantatie en humorale thymus factoren beschreven, met het oog op mogelijke toepassing in immuno-gerontologische onderzoek.

Het immunologisch reactievermogen neemt af met het vorderen van de leeftijd. Tegelijkertijd vindt een toeneming plaats van bepaalde ziekten die in mindere of meerdere mate het gevolg kunnen zijn van een slechter functioneren van het immunologisch apparaat. Dit gegeven heeft geleid tot velerlei onderzoek naar veranderingen die optreden in het immuunsysteem tijdens veroudering. Hoofdstuk I geeft een overzicht van de resultaten die uit deze studies verkregen zijn. Met name het effect van veroudering op de haemopoëtische stamcellen en op humorale en cellulaire immuunreacties en de daarbij betrokken celpopulaties wordt besproken. In dit hoofdstuk wordt verder aandacht geschonken aan de veranderingen in de thymus tijdens veroudering en aan verschijnselen en ziekten die mogelijk een gevolg zijn van een slechter functionerend immuunsysteem op oudere leeftijd. Uit de literatuurgegevens blijkt dat er zowel bij de mens als bij proefdieren verschillen zijn in het tijdstip waarop de verschillende immunologische functies achteruitgaan alsmede in de mate en de snelheid van dit proces. Het is verder duidelijk dat de achteruitgang niet toegeschreven kan worden aan veranderingen in het aantal stamcellen of in het totale aantal B en T cellen.

De technieken die gebruikt zijn om de immuunrespons van met antigeen gestimuleerde muizen of van geisoleerde miltcellen te bepalen zijn beschreven in Hoofdstuk II.

Hoofdstuk III is gewijd aan de overlevingsgegevens en verouderingspathologie van CBA, C57BL/Ka en NZB muizen. CBA muizen vertoonden de langste gemiddelde (50%) en maximale overlevingstijd. Vrouwelijke NZB muizen leefden het kortst. Auto-immuunziekten en immuuncomplex ziekten kwamen het meest voor bij NZB muizen. Oude C57BL/Ka muizen hadden een toenemende frequentie van maligne lymfomen en amyloidosis. In vergelijking met de andere twee stammen werden afwijkingen in de lymfoide organen het minst gevonden bij de CBA muizen. Hun verouderingspathologie werd daarentegen gekenmerkt door veelvuldig voorkomen van lever- en ovariumtumoren. De CBA muis lijkt daarom een geschikt diermodel voor het bestuderen van verouderingsverschijnselen in het

immuunsysteem, met name van die verschijnselen die niet een gevolg zijn van ziekten in het immuunsysteem zelf.

Hoofdstuk IV beschrijft de veranderingen bij toenemende leeftijd in het vermogen een primaire humorale immuunrespons te ontwikkelen. De sterkte van de respons werd bepaald aan de hand van het aantal cellen in de milt dat antilichamen maakt tegen het toegediende antigeen. Ongeveer vanaf de leeftijd die overeenkomt met de 90% overlevingstijd voor CBA en C57BL/Ka muizen, bereikten muizen van deze stammen niet meer hetzelfde reactievermogen als jong volwassen dieren. Dit geldt voor de reactie opgewekt door een optimale dosis van een thymus-afhankelijk antigeen, de rode bloedcellen van schapen (SRBC). Daarbij bleek ook dat het aantal cellen dat antilichamen van de IgG klasse maakte sterker daalde dan de cellen die IgM antilichamen produceerden. In oude C57BL/Ka muizen was de IgG productie erg laag of afwezig. Hierbij moet opgemerkt worden dat de invloed van helper T cellen groter is bij een IgG dan bij een IgM respons. Een respons waarbij helper T cellen helemaal geen rol spelen vindt plaats na stimulatie met het thymus-onafhankelijke antigeen lipopolysaccharide (LPS) van E. coli. In tegenstelling tot SRBC, wekte een optimale dosis LPS vergelijkbare responsen op in CBA en C57BL/Ka muizen tot op een leeftijd van respectievelijk 2 en 1,5 jaar. Na die leeftijd neemt ook de respons tegen LPS af, hoewel in mindere mate dan de respons tegen SRBC. Dit feit en het bovengenoemde verschil in daling tussen IgM en IgG antilichaamvorming wijzen erop dat, naarmate T cellen meer betrokken zijn bij een humorale immuunrespons, die respons eerder achteruitgaat tijdens veroudering. Daarnaast bleek dat bij lagere doses SRBC en LPS het verschil in respons tussen jonge en oude muizen steeds groter wordt.

Het onderzoek van NZB muizen beperkte zich tot het bepalen van leeftijdsafhankelijke veranderingen in de antilichaamvorming tegen LPS. Over de invloed van veroudering op thymus-afhankelijke functies in deze stam is al veel informatie beschikbaar. Het vermogen een antilichaamrespons tegen LPS te ontwikkelen nam sterk af met de leeftijd in NZB muizen, dit in tegenstelling tot de resultaten verkregen met CBA, C57BL/Ka en BALB/c muizen. Er moet daarom ook rekening gehouden worden met een verlies aan B cel functies in deze auto-immuungevoelige stam naast de veelvuldig beschreven defecten op T cel niveau.

Hoofdstuk V behandelt het effect van veroudering op de secundaire respons tegen SRBC in CBA muizen. Deze reactie speelt zich voor een belangrijk deel af in het beenmerg. Het aantal antilichaamvormende cellen en met name het aantal IgG vormende cellen daalde tijdens het

ouder worden. Ook het vermogen van de milt om een secundaire respons te ontwikkelen nam af, zij het op een latere leeftijd dan gevonden was voor de primaire respons tegen SRBC.

De capaciteit van geisoleerde miltcellen van jonge en oude muizen om na antigene stimulatie in vitro antilichaamvormende cellen te produceren wordt beschreven in Hoofdstuk VI. De resultaten suggereren dat de in vitro respons tegen SRBC op een latere leeftijd achteruitgaat dan de respons opgewekt in vivo. Een verdere vergelijking van de in vivo en in vitro verkregen resultaten toonde aan dat er een verschil is in de gevoeligheid voor antigeen. Oude muizen hadden in vivo een hogere dosis nodig dan jonge muizen om maximaal gestimuleerd te worden. In het in vitro systeem vertonen miltcellen van jonge en oude muizen een optimale productie van antilichaamvormende cellen bij vergelijkbare doses SRBC. Deze gegevens wijzen op een invloed van omgevingsfactoren (micromilieu) in de afname van de humorale immuunrespons tijdens veroudering.

Hoofdstuk VII beschrijft pogingen om door middel van thymustransplantatie de verminderde respons tegen SRBC in oude muizen te verbeteren. De tot nu toe verkregen resultaten geven nog geen antwoord op de vraag of thymustransplantatie een effectief middel is om het immuunsysteem op oude leeftijd te manipuleren.

Behalve het transplanteren van een thymus bestaat wellicht de mogelijkheid om door middel van humorale thymusfactoren thymus-afhankelijke functies op latere leeftijd te verbeteren. Om die reden zijn enkele preparaten geisoleerd uit thymusextracten (thymosine en thymus humorale factor, THF), en een supernatant van thymusepitheel kweken (TES) vergeleken. Alle preparaten waren in staat de in vitro respons tegen SRBC in kweken van miltcellen van thymusloze nu/nu muizen te verhogen. Om uit te zoeken of het epitheliale of lymfoïde gedeelte van de thymus, de actieve factoren in thymosine en THF produceert, werden van beide delen apart preparaten gemaakt en hun activiteit vergeleken. Het kon niet worden aangetoond dat het epitheel de bron is van de actieve factoren. Door supernatant (TES) te gebruiken van kweken die alleen uit thymusepitheelcellen bestaan kon dit probleem vermeden worden. Een nader onderzoek naar de activiteit van dit supernatant toonde aan dat TES de respons tegen SRBC verhoogde door inductie van helper T cellen in de thymocytenpopulatie.

In de algemene discussie (Hoofdstuk VII) worden bovengenoemde resultaten met elkaar in verband gebracht. Het blijkt dat het slechter functioneren van het humorale immuunsysteem tijdens veroudering vooral tot uiting komt bij de eerste confrontatie met een antigeen en wanneer bovendien voor de daaropvolgende reactie T cellen nodig zijn. Behalve

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In 1963 eindexamen Gymnasium-β aan het Lyceum "Sancta Maria" te Haarlem. Van 1963 tot 1964 werkzaam als laborante bij de Research Afdeling van Brocades N.V. te Haarlem.

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