A THESIS

entitled

IMMUNOLOGICAL STUDIES OF THE CONNECTIVE TISSUE DISEASES USING NEW ZEALAND MICE AS A LABORATORY MODEL

Submitted in part fulfilment of the requirements for the admittance to the degree of

Doctor of Philosophy

in

The University of Glasgow

by

KEITH WHALEY M.D.

University of Glasgow 1973

SUMMARY

Systemic lupus erythematosus (SLE) is primarily a disease affecting the connective tissues of the many organs of the body. It is associated with the production of a wide spectrum of autoantibodies, which, with one exception, have no defined pathogenetic role. The one exception is antibody to deoxyribonucleic acid (DNA), which complexes with DNA in the circulating blood, and the resultant complexes are deposited in the renal glomeruli, where they are responsible for the induction of glomerulonephritis. For many years research on SLE was hampered by lack of a suitable laboratory model. The discovery that New Zealand Black (NZB) and the F1 hybrid progeny (NZB x NZW F1; BWF_1) resulting from crossing NZB with New Zealand White (NZW) mice develop an autoimmune disease closely mimicking SLE, has since stimulated considerable research into the pathogenesis of the disease. Numerous immunological peculiarities have been shown to be present in these mice, yet no completely satisfactory explanation for their occurrence has been proposed. Phagocytosis and the processing of antigen by macrophages have been shown to be important events in the initiation of immune responses. In this thesis I describe studies

of the early events of the immune response in NZ mice in order to evaluate their importance, if any, in the production of the immunological peculiarities known to occur in these strains of mice.

An initial study of the development of the time of onset of autoimmune disease in our mouse colonies was undertaken in order to compare our mice with those previously reported from other centres. The results showed that although minor differences could be detected the mice in the main behaved very similarly to those previously reported.

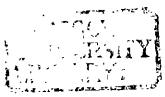
Studies of the in vivo phagocytosis of colloidal carbon by the fixed macrophages of the liver and spleen and the peritoneal macrophages were undertaken. Carbon was removed from the blood more slowly by NZB than other mice, but when correction was made for liver and spleen weight in relation to total body weight, phagocytosis was found to be normal. Peritoneal cellular responses to carbon, and its phagocytosis and subsequent tissue localisation, were normal. The phagocytosis and subsequent intracellular killing of Staphylococcus aureus by the peritoneal macrophages of NZ mice was measured and found to be normal in a supravital system. Serum factors did not appear to affect the rate of phagocytosis or killing of bacteria.

As NZB and BWF_1 mice have been shown to produce heightened immune responses to the soluble protein antigens, bovine gamma globulin (BGG) and bovine serum albumin (BSA), and have also been shown to be relatively resistant to the induction of both high and low zone tolerance to these antigens. it was decided to study the rates of non-immune elimination of these antigens. New Zealand mice were found to have faster rates of catabolism than control strains of mice. but a study of the antibody responses in these mice showed that there was no relationship between antigen elimination rate and the tit re or affinity of the antibody subsequently produced. However, it was found that the time of onset of immune elimination, indicating production of antibody, was earlier in those mice having rapid rates of antigen non-immune elimination. A new method of studying thyroxine secretion rates in mice (based on "the Occupancy Principle") was devised. and a study of endogenous thyroxine secretion rates was undertaken in order to investigate the possible role of thyroxine metabolism in relation to BGG elimination rates. No correlation was demonstrated in the strains of mice studied. Thus interstrain variations in non-immune antigen elimination probably result from differences in macrophage function, although the action of naturally occurring adjuvants cannot be excluded as adjuvants have also been shown

to accelerate non-immune antigen elimination.

Finally using E. coli DNA labelled with phosphorus-32 (^{32}P), a study of DNA catabolism in New Zealand mice was undertaken to investigate the possibility that abnormalities of DNA catabolism might be responsible for the production of DNA antibodies, which are known to occur spontaneously in these mice. NZB mice removed DNA more slowly from the blood than other strains of mice, but unlike the slow clearance of colloidal carbon, the slow rate of DNA clearance was not found to be due to the small size of liver and spleen.

It was found that phagocytosis rather than nuclease activity was responsible for the removal of DNA from the blood, as injected DNA was mainly localised in the liver and spleen, and its removal from the blood could be slowed by the administration of ethyl stearate, which is known to produce reticuloendothelial blockade. Apart from the relatively low rates of elimination, no peculiarities likely to be responsible for the proneness of NZ mice to produce antibodies to DNA could be demonstrated.



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Certain sections of the work described in this thesis have already been published or submitted for publication.

1. Immunological mechanisms in the pathogenesis of systemic lupus erythematosus. Scot. med. J. (1970), 15 : 261.

2. Catabolism of bovine gamma globulin in New Zealand mice. Rev. Eur. Et. Clin. Biol. (1972), 17 : 292.

3. Clearance of deoxyribonucleic acid from the blood in New Zealand mice. Rev. Eur. Et. Clin. Biol. (1972), 17 : 788.

4. In vitro studies of the phagocytosis of Staphylococcus aureus by the peritoneal macrophages of New Zealand mice. Immunology (in press).

5. In vivo studies of the phagocytosis of colloidal carbon by the fixed tissue and peritoneal macrophages of New Zealand mice. Scot. med. J. (in press).

6. A simple method for the measurement of thyroxine secretion rates in mice. Acta endocrinol. (in press).

7. Studies of thyroid function in the mouse: Interstrain differences in thyroxine secretion rates and organ thyroxine concentrations. Submitted to Acta endocrinol.

8. Antigen catabolism in New Zealand mice. I Catabolic rates of three antigens. Submitted to Immunology.

9. Antigen Catabolism in New Zealand mice. II Relationship to immune responses. Submitted to Immunology.

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LIST OF ABBREVIATIONS USED IN THIS THESIS

ABC 30	=	Serum antigen binding capacity = 30% of the quantity of antigen which can be bound by 1 ml. of serum (μg/ml).
ANF	=	Antinuclear factor.
BSA	=	Bovine serum albumin.
BGG	=	Bovine gamma globulin.
BWF_1 mice	=	NZB x NZW F1 hybrid mice.
DNA	=	Deoxyribonucleic acid.
HGG	=	Human gamma globulin.
LE factor	=	Lupus erythematosus factor.
NZ mice	=	New Zealand mice.
NZB mice	=	New Zealand Black mice.
NZW mice	=	New Zealand White mice.
PBS	=	Phosphate buffered saline.
PVP	=	Polyvinylpyrrolidone.
Radio T4	=	Radioactively labelled thyroxine.
SLE	=	Systemic lupus erythematosus.
T4	=	Thyroxine.
T4SR	=	Thyroxine secretion rate.

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CHAPTER 1.

INTRODUCTION

The concept of connective tissue as the seat of disease is of relatively recent acceptance, largely because of two factors: firstly, to the ubiquity of connective tissue and the absence of any apparent anatomical continuity or limit, and secondly to the emphasis placed by pathologists, notably Paul Ehrlich, to cellular pathology. According to Robb-Smith (1) the first significant contribution to the importance and function of connective tissue was made by Schade (2). Thus in his book "Physical Chemistry in Internal Medicine" published in 1923, Schade emphasised the fundamental alterations of the connective tissues in diseased states, and described changes in the elasticity of the walls of the affected vessels in aneurysms, haemorrhoids and varicose veins, as well as mucin deposition in myxoedema, and the changes occurring during wound healing. In 1928, Standendath (3) again emphasised the alterations in connective tissues in certain diseases. These conditions are, of course, very different from the concept of the connective tissue diseases as it is understood today.

There is no doubt that the modern trend of thinking from which the present-day concept of the connective tissue diseases evolved is from observations on the pathological changes in rheumatic fever (4-6). Thus Talajew (4) noted mucinous oedema in the connective tissues in rheumatic fever, and Klinge (5, 6) emphasised that the essential pathological change in this disease was "fibrinoid degeneration" in the sites of inflammation. Klinge pointed out that similar lesions were found in rabbits rendered hypersensitive to

foreign proteins and suggested that rheumatic fever was an allergic disease, and as he found "fibrinoid material" in the lesions of polyarteritis nodosa, dermatomyositis, malignant hypertension, nephritis, subacute bacterial endocarditis and thromboangiitis obliterans, he postulated that these diseases might also have an allergic pathogenesis. Jager (7) and Rössle (8) proposed that fibrinoid necrosis was the characteristic morphological change in diseases of allergic background and suggested that rheumatic fever, polyarteritis nodosa, and thromboangiitis obliterans should be grouped together as the "rheumatishe Formenkreis". Clark and Kaplan (9) described fibrinoid degeneration in the lesions of serum sickness in man, and Masugi and Ya (10) noted the similar changes in the vessels of a patient with progressive systemic sclerosis; they proposed an allergic pathogenesis for these conditions.

In 1941 Banks (11) published a paper entitled "Is there a common denominator in scleroderma, dermatomyositis, disseminated lupus erythematosus, the Libman-Sacks syndrome, and polyarteritis", in which he concluded that these diseases were related both clinically and pathologically and could be grouped together as diffuse vascular or mesenchymal diseases. In the following year Klemperer, Pollack and Baehr (12) published their short but historic paper entitled "Diffuse collagen disease, acute disseminated lupus erythematosus and diffuse scleroderma". These workers put forward the concept that the connective tissues could be the primary site of disease. The authors were careful however to point out

that although they regarded these "diffuse collagen diseases" as disorders of the connective tissues, this did not imply that they were necessarily aetiologically related, or were primarily diseases of any particular constituent of connective tissue, such as collagen. In their own words "..... to identify this system as the seat of certain diseases is by no means to identify these diseases with one another, or even to relate them. This would be an unjustifiable over-simplification".

This paper appears to have aroused little immediate interest, perhaps due to the Second World War, or to the fact that the concept was morphological rather that aetiological. However, the idea that polyarteritis nodosa might be due to tissue hypersensitivity received support from the experimental studies of Rich and his colleagues (13-15). These workers found lesions of polyarteritis nodosa in patients with serum sickness and sulphonamide reactions, and produced similar lesions experimentally by the injection of horse serum into previously sensitised rabbits. Rich (14) concluded that polyarteritis nodosa was an "allergic" disease, and suggested that rheumatic fever and disseminated lupus erythematosus were also due to anaphylactic hypersensitivity. In this way he reintroduced the concept of Klinge (6) of a common aetiological basis for the collagen disorders.

There is no doubt that the demonstration by Hench and his colleagues in 1948 (16) of the effectiveness of cortisone in rheumatoid arthritis, rheumatic fever and systemic lupus erythematosus popularised the concept of "the collagen diseases". In spite of the

TABLE 1

A LIST OF ILLNESSES INCLUDED UNDER THE HEADING "COLLAGEN, OR CONNECTIVE TISSUE DISEASES"

Cranial (temporal or giant cell) arteritis.
Polymyalgia rheumatica.
Thrombo-angiitis obliterans.
Thrombotic thrombocytopenic purpura.
Henoch-Schönlein purpura.
Weber-Christian disease (relapsing febrile non-suppurative panniculitis).
Erythema nodosum.
Acute glomerulonephritis (post streptococcal).
Rheumatic fever.
Malignant hypertension.
Ulcerative colitis.
Endocardial fibroelastosis.
Sjøgren's syndrome.
Serum sickness (hypersensitivity or allergic angiitis).
Loeffler's pneumonia.
Pemphigus.

These following diseases should also be added.

Takayashu's disease (young female arteritis).
Wegener's granulomatosis.
Goodpasture's syndrome.
Rheumatoid arthritis.
Systemic lupus erythematosus.
Progressive systemic sclerosis.
Dermatomyositis (polymyositis).
Polyarteritis nodosa.

warnings of Klemperer and his colleagues (12), the term "collagen disease" caught the imagination of the physician and an increasing number of diseases were included under the term, (Table 1), (1, 17, 18). These diseases were included either because of their presumed allergic and hypersensitivity aetiology, or because of the presence of "fibrinoid" in the pathological lesions. In 1950, Klemperer (19) ruefully wrote of the term collagen disease:

"By suggesting it we unwillingly became responsible for a deplorable usage of this term as a collective diagnosis for those puzzling maladies which we had tried to differentiate by rational pathogenetic investigation. The impatience of clinical investigators and a peculiar worship of diagnostic terms have led to an exaggerated popularity of the diagnosis of collagen disease. It is not a term applicable to diagnosis and certainly does not define the morbid process of the diseases grouped together".

The discovery of the rheumatoid (20) and LE factors (21) paved the way for the discovery of many other immunological abnormalities present in the connective tissue diseases. The demonstration of interconversion and transitional syndromes, overlapping of the immunological phenomena, and familial aggregation of the various syndromes and their associated immunological abnormalities have led to an inevitable tendency to consider the connective tissue diseases as forming a single nosological entity with several different manifestations. However, clinical, pathological and immunological similarities should not be considered as necessarily implying a

common aetiology. In the absence of any real understanding of the aetiology and pathogenesis of the connective tissue diseases it would seem correct to abandon the unitarian concept and to return to careful description and classification of clinico-pathological entities. It is however, reasonable to retain the concept of connective tissue diseases, provided it is used literally to mean a disease primarily affecting connective tissues, without aetiological implications. At this point it may be worthwhile indicating that, although immunological mechanisms are involved in the pathogenesis of these diseases, the presence of "fibrinoid material" per se is not itself evidence of an immunological process, as it is found in infarcts, thrombi and other lesions where its pathogenesis is clearly not immunological.

The major connective tissue diseases, often termed the "non-organ-specific autoimmune diseases" are rheumatoid arthritis, systemic lupus erythematosus, progressive systemic sclerosis and dermato-myositis. Of these diseases, two have assumed importance, rheumatoid arthritis and systemic lupus erythematosus, because intense study has provided considerable knowledge of their immuno-logical features. In this thesis, I have elected to study systemic lupus erythematosus as it is the only connective tissue disease with a suitable laboratory model.

Lupus erythematosus was first described by Hebra in 1845 (22) as a localised skin lesion but it was in 1851 that Cazenave (23) gave it the name because of its alleged association with cutaneous tuberculosis.

That lupus was more than simply a disease of the skin was proposed in 1872 by Kaposi (24), and it is from this time that the term disseminated lupus erythematosus dates. Osler wrote a series of essays between the years 1895 and 1903 (25-27), and in them he clearly delineated the clinical picture of the disease, stressing again the generalised nature of both symptoms and signs. He emphasised the presence of endocarditis, purpura, haemolytic anaemia, and the frequent occurrence of abdominal pain, and pointed out that the systemic manifestations of the disease could occur in the absence of the cutaneous lesions. Although some of his cases would not be accepted as proven examples of systemic lupus erythematosus today, Osler's observations are of fundamental clinical importance.

An atypical, non-bacterial, verrucous endocarditis (Libman Sacks endocarditis) in systemic lupus erythematosus was described by Libman and Sacks in 1924 (28), and at the same time they drew attention to the splenic lesions present in the disease. The typical "wire-loop" lesions and other renal changes present in lupus nephritis were described by Baehr and his colleagues in 1935 (29). It then remained for Klemperer et al (30) to describe the diffuse fibrinoid degeneration affecting the collagen connective tissues and to classify systemic lupus erythematosus a connective tissue disease (12).

The collections of haematoxylin-staining bodies, devoid of regular nuclei and cellular structure, were first observed by Gross in 1932 (31),

and Ginzler and Fox (32) described similar large haematoxylin-staining aggregates in the lymph nodes of patients with this disease. These are now called haematoxyphil bodies and were originally thought to consist of depolymerised DNA (33), although more recent evidence suggests that they consist of nucleo-protein (228).

The discovery by Hargraves of a specific phagocytic phenomenon, the "LE cell" in the bone marrow and peripheral blood of patients with systemic lupus erythematosus has been the single most important advance in both the diagnosis and understanding of the disease (21). The detection of this phenomenon has enabled not only the diagnosis of systemic lupus erythematosus to be broadened, and to be made with more certainty, but the discovery of the factor responsible for its production has opened a Pandora's box of immunological phenomena from which much of our present knowledge of the nature of systemic lupus erythematosus has stemmed. The LE cell factor has been shown to be circulating antibody to DNA-histone complex (34) and will react with cell nuclei of many different species. Using the immunofluorescence technique, antibody to DNA-histone is seen as a homogeneous pattern of nuclear staining (35). Three other patterns of nuclear staining may be seen, speckled (antibody to a saline-soluble component of the nucleus), nucleolar (antibody to nuclear RNA-associated protein, but not RNA), and membranous, also called shaggy or fibrillar (antibody to DNA) (35). The morphological patterns of nuclear fluorescence are not now thought to be as specific as previously supposed. The speckled and nucleolar patterns of antinuclear factor occur almost exclusively in Sjøgren's syndrome and progressive

systemic sclerosis (36, 37).

Apart from antinuclear antibodies, antibodies to ribosomes (38-40) and RNA (41, 42) have been described. In addition precipitating antibodies reacting with unidentified cytoplasmic constituents, rheumatoid factors and antibodies to erythrocytes, leucocytes and platelets may be present in the serum of patients (43). An increased prevalence of thyroid autoantibodies has been reported in SLE (44, 45).

Despite this abundance of autoantibodes in the sera patients with systemic lupus erythematosus, the large majority do not have any diagnostic significance. LE cells for instance may be found in the sera of 10% of patients with chronic discoid lupus erythematosus, although many of these patients have evidence of multisystem disease (46-48). Patients with rheumatoid arthritis (43, 49-53) and chronic active, or lupoid hepatitis (54-56), may also have positive LE cell tests in about 5-15% of cases. Rarely LE cells may be seen in other diseases such as Hodgkin's disease and aplastic anaemia (57). The only antibody which appears specific for systemic lupus erythematosus is antibody to native DNA. Apart from its specificity, antibody to native DNA is also different from the other autoantibodies occurring in systemic lupus erythematosus as it is the only antibody definitely known to have pathogenetic significance. Antibody to native DNA complexes with antigen, and the resulting antigen-antibody complexes, together with complement, are deposited in the renal glomeruli, where they induce acute glomerulonephritis. The antigen, immunoglobulin and complement

can be detected in the glomeruli by immunofluorescence (58, 59), and antibody to DNA can be eluted from the glomeruli (60-64), as well as DNA (64).

The explanation for the occurrence of autoimmune disease is not apparent, but the possibilities include immunological hyperresponsiveness (65, 66), loss of immunological tolerance to self antigens by the emergence of forbidden clones of antigen reactive cells (67), and a peculiar host response to some infective agent possibly due to an underlying genetic predisposition. Indeed recent work has shown the presence of increased titres of antibodies to certain viruses in the sera of patients with SLE (68, 69) and electron microscopic examination of the tissues of these patients has shown the presence of myxovirus-like particles (70-76) but their nature and significance have not yet been proven.

Stimulus to research on the connective tissue diseases was held up for many years because of a lack of a suitable animal model of the disease. However in 1959 Bielchowsky (77) described the occurrence of Coombs' positive haemolytic anaemia in New Zealand black (NZB) mice. These mice also develop glomerulonephritis and in 5% LE cells can be demonstrated (78, 79). When mated with New Zealand white (NZW) mice the resultant progeny (NZB x $NZWF_1$; or BWF_1 mice) fail to develop the haemolytic anaemia, but have a much higher incidence of LE cells (60-75%), and the glomerulonephritis is florid, especially in the females who die of uraemia under 1 year of age (80). As in human lupus, antibodies to DNA are common (81) and the nephritis has been shown to be due to the deposition of circulating DNA -anti DNA

complexes in the renal glomeruli (82, 83). On the basis of these facts, it is reasonable to accept that New Zealand mice provide a suitable laboratory model for the study of SLE. However, having accepted their suitability, it must be stressed that there are also dissimilarities between the diseases of New Zealand mice and human SLE. For example -

1) Human SLE is a disease which progresses by relapses and remissions whereas "murine lupus" progresses continuously.

2) Human SLE is a multisystem disease which may produce characteristic skin lesions, alopecia, arthritis, pericarditis, myocarditis, endocarditis, vasculitis, pleurisy with effusion, parenchymal lung disease, glomerulonephritis, haemolytic anaemia, leucopenia, thrombocytopenia, central nervous system disease, and peripheral neuropathy. New Zealand mice generally only develop haemolytic anaemia and glomerulonephritis, although lymphoid infiltrates can be seen in many organs on histological examination.

3) Malignant lymphoreticular neoplasms complicate human SLE uncommonly, whereas they are extremely common in murine lupus.

The aetiology of the autoimmune disease of NZB mice isstill unknown but three factors are of importance.

1. Genetic: This is the most obvious, the first investigated, and yet least understood. The homozygous NZB mouse develops autoantibody to erythrocytes detectable by a positive Coombs' test from 5 months of age, whereas when crossed with NZW mice, or any other strain of mouse, the introduction of different genetic material results in marked retardation of the appearance of Coombs' positivity. Several genetic studies, some of which were extremely elaborate, have been reported, and the conclusions of all were that the disease is not simply controlled by one gene (80, 84-91).

2. Viral: Electron microscopic studies have revealed that New Zealand mice carry C type murine leukaemia virus particles, which have been shown to be of the Gross strain (92-98). Although the virus is found in almost all strains of mice (99), only genetically susceptible hosts develop leukaemia/lymphoma. New Zealand mice are the only known strain to break tolerance to this virus, and after several months they produce antibodies to Gross antigen (100). Gross antigen-antibody immune complexes are then deposited in the renal glomeruli (101).

The role of the virus in the aetiology and pathogenesis of the autoimmune disease of NZB mice remains obscure, but the vaccination

of young mice with a Gross virus vaccine will prevent the onset of Coombs' conversion (100). Some authors claim that the administration of interferon inducing agents can delay the onset of autoimmune disease (102), whereas others have failed to confirm this (41, 103). Certainly a direct role of the virus in the production of autoimmune disease is doubtful, as the virus itself is ubiquitous, yet the autoimmune disease is unique. A report that cell free filtrates of tissue containing the virus transferred the disease (93), has not been confirmed despite extensive study (104).

3. Immunological: Numerous immunological peculiarities have been observed in NZ mice and they include -

1. Earlier maturation of the immune response to sheep red blood cells than in other inbred strains of mice (105, 106).
2. Excessive humoral antibody responses following injection of certain antigens including bovine gamma globulin (107, 108), human gamma globulin (108), bovine serum albumin (109, 110), polysaccharide (Vi) antigen (111) and sheep erythrocytes (110, 112-114). However antibody responses to pig erythrocytes, synthetic polypeptides (115) and keyhole limpet haemocyanin (110) are similar to those in other strains of mice.
3. Tolerance to bovine and human gamma globulins (107a, 107, 108) and bovine serum albumin (109) is difficult to induce, and neonatally induced tolerance fails to persist (108, 116).

4. Antibody responses to synthetic polynucleotides (polyinosinic-polycytidilic, and polyadenylic-polyuridylic acids) are excessive (41, 117) and these agents also accelerate the appearance of naturally occurring nucleic acid antibodies (41, 118).

5. With increasing age there is an accelerated reduction in thymic function, compared to other strains of mice, as shown by

   a) Tolerance to sheep erythrocytes induced by their concomitant administration with cyclophosphamide could be transferred to irradiated donors by thymus and bone marrow cells of C57BL/6 mice, and bone marrow cells of BWF_1 mice. However thymus cells from tolerant BWF_1 mice failed to induce tolerance in recipient mice (119).

   b) It is now well documented that the presence of the thymus is necessary for escape from a previously established state of tolerance to occur (120-123). Studies of BWF_1 mice have shown that thymus grafts from old mice given with bone marrow cells from young mice, failed to promote the escape of tolerance to bovine gamma globulin in irradiated recipients, whereas young thymus grafts given with young bone marrow cells enabled such escape to occur (108).

   c) Spleen cells from older mice are less efficient in the induction of graft-versus-host disease (124, 125).

   d) The response of spleen cells to the mitogenic agent, phyto-haemagglutinin is reduced in older mice (126, 127).

   e) Old mice are unable to reject Moloney sarcoma virus induced tumours (128).

(f) Immune responses to sheep erythrocytes are impaired in old mice (112, 129, 129a).

This host of immunological peculiarities may be explained by an imbalance between thymus and bone marrow cell populations. A deficiency of thymus cell precursors would account for the abnormalities of thymic function, and an excess of bone marrow stem cells would explain the generalised immunological hyper-responsiveness. Sophisticated cell transfer experiments have recently suggested that this in fact is the case (108, 130, 131).

Over the years, macrophages have been given roles of varying immunological importance. Metchnikoff in 1899 (132), reported that the phagocytic cells of the liver, (Kupffer cells), lymphoid tissue, spleen and peritoneum (macrophages), phagocytose particulate material, live cells and foreign proteins. In the 1920's and 1930's, it was thought that macrophages themselves not only phagocytosed antigen, but also synthesised antibody (229). Interest in their importance dwindled with the discovery that lymphocytes and plasma cells were involved in antibody responses. However, the controversial observations of Fishman and Adler in the early 1960's (148, 230, 231), showed that antibody to T4 phage was not produced by exposure of lymphocytes to antigen, whereas extracts of macrophages which had previously phagocytosed T4 phage were highly immunogenic. These experiments were important as they focussed attention upon the possible role of

antigen processing by macrophages as an essential step in immune recognition. Since then further experimental data have shown that this interpretation was not completely correct. Certainly lymphocytes do interact with macrophage bound antigens but sometimes this interaction may not be required, and may not need antigen processing. The role of the macrophage in immunogenesis is now well established but apart from this, macrophages are important in cell mediated immune reactions (232), and as a non-specific defence mechanism in host resistance to bacterial and viral infections (233).

The role of macrophages in the triggering of an immune response has been established using both in vivo and in vitro techniques.

Early workers were able to trace the tissue localisation of antigens either by trace labelling them, and studying the distribution of radioactivity in the tissues, or by preparing tissue extracts and assessing their immunogenicity (134, 135, 234, 255). These studies revealed that immunogenic material persisted in, and could be extracted from tissues many weeks or months after injection. The antigenic material was believed to be associated with RNA. Autoradiography and immunofluorescence have been employed as better methods for the detection of tissue bound antigens (236). Fluorescence is accurate, but not sufficiently sensitive to detect a small number of antigen molecules, hence the use of radioactively labelled antigens of high specific activity

has become the method of choice (138, 139). From the antigen tracing studies, two main sites of antigen trapping in lymph nodes were identified: the first was in the macrophages of the medulla and perifollicular zones of the lymph nodes, or the perifollicular or marginal zones of the spleen, and the second was associated with the lymphoid follicles. At the first site the antigen was taken up by cells morphologically similar to macrophages isolated from the bone marrow or the peritoneal cavity and was localised in the phagolysosomes. The distribution was similar in tolerant and normal animals (237, 238). The antigen was rapidly catabolised (144) so that after a few days, it was difficult to demonstrate. In the second site, the lymphoid follicle, the antigen appeared to be bound only to the surface of the dendritic macrophage, and this was markedly enhanced in immune animals (138, 239, 240). Humphrey and Franks (237) showed that follicular localisation of antigen did not occur in tolerant animals, whereas at the same time medullary macrophages were unaffected. In normal animals, follicular localisation did not occur until several days after antigen administration when antibody synthesis has started. A combination of autoradiography and immunofluorescence has shown that both antigen and immunoglobulin were present on the same sites in the follicles (241). Further evidence showed that the follicular localisation of antigen was associated with the presence of the Fc portion of the Ig molecule, and C3 was identified in the same area (244). This evidence supports the view that

antigen in the follicles is in the form of an immune complex. Antigen persists for long periods in the follicles, and whereas the extrafollicular antigen trapping mechanism is resistant to X-irradiation (239, 245), the follicular mechanism is highly sensitive (245-247). The mechanism whereby the antigen antibody complexes enter the follicle is unknown, but they could enter either as free complexes or already bound to cell membranes. White and his colleagures have presented evidence that the latter mechanism occurs in the chicken spleen (248), and Nossal (249) has provided evidence that a similar process occurs in the mammalian spleen. The association between the follicular and extrafollicular antigen trapping mechanisms and the immune response is unknown. However, as the extrafollicular macrophages handle antigen similarly to peritoneal macrophages (250, 251), and antigen bound to peritoneal macrophages is immunogenic, it has been assumed that antigen bound to the extrafollicular macrophages is available for immunogenesis for a certain time. Because antigen does not enter the follicles before immunity has occurred, and as it persists for long periods, it is not unreasonable to assume that it may be a mechanism for the maintenance of immunological memory, although this is purely hypothesis.

It has been known for many years that antigenic material persists in the tissues for relatively long periods of time after initial antigen exposure, and tissue extracts made days or weeks after injection provoked specific immune responses when injected

into a second animal. Later more sophisticated experiments confirmed these early observations. These experiments are as follows:

1) Mouse lymphocytes previously primed with human albumin made antibody when injected into immunologically incompetent mice which had previously received the same antigen (252). Using this same system it has been shown that immunogens from sheep erythrocytes, E. coli lipopolysaccharide and human albumin persisted for 14 days, 45 days and 17½ hours respectively (253-254).

2) The fact that antibody can block immune responses at the level of the antigenic stimulus, has also been used to study antigenic persistance. For instance Britton and Möller (255) were able to show the persistance of E. coli lipopolysaccharide in mice for up to 50 days after immunisation, and Graf and Uhr (256), by removing specific anti-albumin antibody by plasmophoresis after 2 to 3 weeks, found that antibody levels rose, probably as a result of persistance of antigenic material.

The site where immunogenic material persists has not yet been determined, but possibilities include the dendritic cells, extra-follicular macrophages and possibly other cells. Using the macrophage transfer system immunogens have been shown to persist in macrophages for at least 3 weeks (257), and Mitchison (258) has isolated cells containing antigen from mouse spleens for up to 7 months after antigen injection. The actual amount of

antigenic material which persists is extremely small, approximately 0.001% of the injected dose.

Although the above experiments leave no doubt that immunogenic material does persist in the tissues for prolonged periods of time, whether it serves any useful function is not at all clear. However there is the possibility that it may play a role in the induction and maintenance of immunological memory. There is evidence both for and against this hypothesis. Celada (259) showed, using human albumin as the antigen in the lymphocyte transfer system of Mitchison (258) (described earlier), that immunological memory declined with a half-life of 15 days during the first month and 100 days thereafter, whereas the immunogenicity of the same antigen in vivo had a half-life of $17\frac{1}{2}$ hours. Hence the memory response to albumin did not appear to depend upon the presence of retained antigen. However, optimal immunological memory to haemocyamin required the antigen to be present for at least 3 to 4 weeks, as judged by the administration of passive antibody (260). The role for antigen persistance in the production of immunological memory is thus not as yet established, although it may be important for some but not all antigens.

It is now accepted that there is an association between the degree of uptake of antigen by macrophages of the lymphoid organs and immunogenicity. For instance particulate antigens such as red cells and bacteria which are rapidly phagocytosed, provoke extremely good immune responses easily, whereas

soluble antigens such as proteins and polysaccharides are removed slowly from the blood and are poor immunogens. The best example of the relationship between the rate of uptake of antigen and immunogenicity comes from studies of protein antigens (generally albumin and gammaglobulin) which differ in their states of polymerisation (180, 261-265). Following injection into animals, polymers are rapidly taken up by macrophages, whereas the monomer is only slowly removed. Removal of polymer by ultracentrifugation (261, 262) or by biological filtration (180, 263) results in a significant reduction in immunogenicity. Furthermore, single or repeated injection of monomer may induce immunological tolerance (180, 261, 263, 264, 266). In contrast the polymeric forms of these proteins are highly immunogenic (267, 268). As a result of these observations, the "direct access" theory of tolerance was proposed by Dresser and Mitchison (266). This theory suggests that polymer which is rapidly taken up by macrophages becomes immunogenic, whereas monomer which is poorly phagocytosed may come directly into contact with lymphocytes and induce tolerance. Evidence in favour of this theory came from macrophage transfer experiments, in which antigen bound to macrophages is highly immunogenic in syngeneic un-immunised recipients. However it is possible that polymers could be removed rapidly from the circulation and be trapped in lymphoid tissue, not necessarily the reticuloendothelial system, and thus

act as the stimulus of an immune response as the antigen is available to reàct with lymphocytes.

Further evidence in favour of an important role for macrophages in the induction of the immune response came from experiments in which the function of the macrophage has been specifically depressed, either by the induction of reticulo-endothelial blockade (269-273) or the action of specific anti-macrophage antibody. Reticuloendothelial blockade is the term used when the reticuloendothelial system has been overloaded with a given colloid, and can be measured by estimating the rate of clearance of a test colloid from the circulation. Many of the studies on immune responses during reticuloendothelial blockade have suffered from the fact that blockade is never complete, its duration is variable, from five hours only for saccharated iron oxide (274) to 3 days for colloidal carbon, and blockade induced by some colloids is followed by a period of hyperphagocytosis. Thus, the results of many of the studies to date are of doubtful value and difficult to interpret. In fact, blockade has sometimes resulted in increased antibody formation (275-277). More carefully controlled studies have recently shown that the antibody response to sheep erythrocytes in mice was significantly impaired as a result of reticuloendothelial blockade by either colloid carbon or ethyl stearate (273, 278, 279). Carageenin, a high molecular weight polygalactose which is specifically cytotoxic for macrophages, when administered to unsensitised guinea pigs, led to depressed cell mediated immune responses (280).

Antimacrophage antibody which has been shown to depress the phagocytic activity of macrophages both in vivo (281) and in vitro (281-284), has been used to help evaluate the role of macrophages in immune responses, although with rather variable results. Using antimacrophage serum of unrecorded specificity, it was reported that its concomitant administration with antigen resulted in depression of the immune response (283, 285). One should be cautious in the evaluation of these results because apart from the unknown specificity of the antibody, normal serum has been shown to depress immune responses (286). Careful studies have concluded that antimacrophage antiserum lacks a consistent immunosuppressive effect (283, 286, 287). These findings do not however reduce the significance of the role of macrophages in the induction of the immune response, as it is possible that antimacrophage serum in the doses used did not reach the tissues in adequate concentration, or for an adequate period of time to block macrophage function. As a comparison, antilymphocyte serum produces its profound immunosuppressive effect because it opsonises circulating lymphocytes (288). Against this argument however is the observation that anti-macrophage serum does depress the phagocytic index, potentiates virus infections in which the degree of viraemia depends upon the ability of the macrophages to remove virus from the blood (281).

I have already mentioned the use of the macrophage transfer system in evaluating macrophage function. The system consists

of isolating macrophages containing antigen, washing them extensively to get rid of free antigen, and transplanting them into syngeneic hosts (257, 289-296), and thus the role of macrophage bound antigen alone may be studied. The antigens used in this system have included soluble proteins such as albumins, immunoglobulin, haemocyanin and lysozyme, or particulate antigens including E. coli, Shigellae and red cells. The use of radioactively labelled antigens has allowed the dose of antigen bound to the macrophages to be evaluated before transfer. Using this system it has been established that lymphocytes are necessary to interact with antigen and trigger an immune response, and that macrophages themselves do not synthesise antibody. The experiments on which these conclusions are based are the transfer of macrophages into irradiated (257, 291, 297, 298) or into immunologically tolerant recipients (294). Isolation of the macrophages in an impermeable diffusion chamber resulted in diminished immune responses, which suggests that an intimate association between macrophages and lymphocytes is required for optimal immune responses to occur (257, 297). The macrophages used in the transfer must be viable and injected into syngeneic recipients in order to obtain optimal antibody responses (257, 294). The importance of the transferred cells being macrophages is emphasised by the observation that antigen bound to other cells (e. g. fibroblasts) was poorly immunogenic (294). Primary immune responses, secondary immune responses, and

cell mediated immunity (299, 300) have all been induced by transferring antigen bound to live macrophages. The immune responses to known amounts of antigen given either in free or bound form have been compared; poorly phagocytosed antigens such as albumin are more immunogenic when given in the bound form (257, 294, 295), whereas well phagocytosed antigens such as erythrocytes or keyhole limpet haemocyanin, are more immunogenic when given in the free form (293, 297). These findings may be explained by assuming that the concentration of well phagocytosed antigens within the lymphoid tissues is not enhanced when they are bound to macrophages, whereas poorly phagocytosed antigens are more concentrated. Immune mice produce similar antibody responses whether the antigen is given in the free or bound form (294). This suggests that the small amounts of antibody present in primed mice serve to concentrate the antigen, or alternatively, immune mice with large numbers of immunocompetent lymphocytes are not so dependent upon antigen concentrating mechanisms.

The macrophage transfer system has also been used to study the interactions of macrophages with T and B lymphocytes, and their role in tolerance, the production of genetically determined high and low antibody responses in mice, and the immunosuppression of X-irradiation. That macrophages can react with both T and B cells, has been shown by the following series of experiments:

1) Animal injected with suitable macrophage bound antigens develop cell mediated immunity (299, 300). Presumably this represents macrophage-T cell cooperation.

2) Thymectomised mice did not produce antibody to haemocyanin even when the antigen was bound to macrophages. The macrophages from the thymectomised mice handled haemocyanin normally (301). Again this reflects macrophage-T cell cooperation.

3) Hapten-primed cells and carrier primed cells responded to hapten-carriers bound to spleen macrophages (302) or peritoneal macrophages (303). The carrier-primed lymphocytes are probably thymus derived whereas the hapten-primed cells are of bone marrow origin.

Macrophages from tolerant animals take up antigen normally (237) and such macrophage bound antigen has normal immunogenicity when injected into normal animals (294) or in an in vitro system (304). It is apparent that antigen processing by macrophages is a pathway for immunogenicity, and not tolerance induction. There is however competition between the tolerogenic form of an antigen and its immunogenic macrophage bound form. For instance Spitznagal and Allison (295) injected bo vine serum albumin in both free and bound form together into syngeneic mice. Macrophage bound antigen produced good immune responses which were markedly reduced by concomitant administration of free antigen. Certain inbred strains of mice exhibit genetically controlled immunological hyperresponsiveness or hyporesponsiveness to either haemocyanin

or the synthetic polypeptide poly L-(tyrosine, glutamic acid)-poly-DL-alanine-poly-L-lysine ((TG)-A--L). Macrophage bound antigens from hyper- and hyporesponders possessed the same immunogenicity (193, 305).

The importance of the macrophage in antigenic stimulation as shown by the previously outlined experiments has been re-emphasised during in vitro studies. In general two in vitro systems have been used, the antibody forming cell, or plaque forming cell assay to foreign erythrocytes, and the proliferative cell response to soluble antigens or allogeneic cells. The antibody forming cell response is measured in two similar systems, the Mishell-Dutton (306) or Marbrook (307) systems. Both systems employ spleen cells suspended in tissue culture medium, and the plaque forming cell (PFC) response is measured after 4 or 5 days. Three cell types have been identified as being required for the in vivo primary antierythrocyte response. Two of these cell types are lymphocytes, and correspond to T and B cells and the third cell, on the basis of the following observations, appears to be unlikely to be any other cell but a macrophage (308-320).

1) The cell adheres to glass or plastic surfaces
2) It has phagocytic properties
3) Morphologically it is macrophage-like
4) It is radioresistant
5) It does not synthesise antibody

6) It is affected by agents which induce reticuloendothelial blockade

7) It is affected by specific antimacrophage antiserum

The importance of the macrophage in in vitro antierythrocyte responses is generally accepted, yet a great controversy exists as to its mode of action. Three possible mechanisms exist; none of which are mutually exclusive:

1) An antigen handling role

2) A role in which it facilitates cell to cell interactions irrespective of antigen handling ability

3) Provision of essential nutrients or growth factors

Originally Mosier (308) suggested that erythrocytes had to be completely endocytosed before immune recognition was possible, but since then evidence has been presented that erythrocytes adherent to the macrophage surface are highly immunogenic (321). Recent, as yet unconfirmed, work showed that macrophage and erythrocyte free tissue culture supernatants were able to stimulate an antierythrocyte response by the non-adherent cells (lymphocytes) (318). It was thought that the macrophage secreted certain factors, which were able to release erythrocyte antigens, capable of provoking an immune response by lymphocytes. The immune response induced by these supernatants was however only 4% of that of macrophage bound erythrocytes.

That the macrophage favours cellular interactions has been deduced from morphological studies of the cell clusters present

in the Mishell-Dutton system after 24-48 hours. Clusters of cells are apparent, and a macrophage was necessary for non-adherent cells to form these clusters. Many of the non-adherent cells in the clusters were plaque forming cells (309, 312). Interruption of cluster formation, by interrupting the culture at 6 hours, followed by maintaining the culture in a stationary state rather than rocking, resulted in the formation of few clusters and few plaque forming cells (312).

Evidence that nutritional factors may be supplied by macrophages has been concluded from experiments in which adherent spleen cell tissue culture supernatants permitted non-adherent spleen cells to respond to antigen (314, 322). The factor has not yet been identified, but would possibly be an enzyme, an antibody-like molecule, or a macrophage modified culture medium factor (322).

The role of the macrophage in in vitro immune responses has recently been somewhat clarified by the studies of Shortman and Palmar (318). Their studies suggest that in vitro macrophages play an important role in antigen handling rather than nutrient role. Another interesting result of their experiments was that certain antigens e.g. polymerised flagellin did not require macrophage processing, whereas sheep erythrocytes did.

The other in vitro system studied, the lymphocyte proliferation system, is a system in which lymphocytes from immunised humans or animals are shown to proliferate an exposure to antigen by measuring the uptake of radioactive DNA precursors, or

counting the number of blast cells in a smear. In this system also, macrophages have been shown to be necessary for lymphocyte proliferation to occur (323-327) and by using purified preparations of both macrophages and lymphocytes, it has been shown that the antigen is able to provoke a good proliferative response when it is bound to the macrophages, whereas the response to the soluble antigen was poor (323, 325, 326, 328-330). Intimate macrophage lymphocyte contact was necessary, as separation of lymphocytes and macrophages by a millipore membrane markedly impaired the response (323-329). As found in the previously described macrophage transfer experiments, the macrophages in this system had to be viable (329) and macrophages from un-immunised and immunised animals were equally effective (329, 330). Culture fluid from macrophage cultures has been substituted successfully for macrophages in the lymphocyte proliferative response to allogeneic cells or soluble antigens (331), although earlier workers were not able to demonstrate this effect (329). This is probably because of technical differences between centres, and underlines the difficulty in distinguishing with confidence several possible effects in these in vitro systems.

After reviewing the evidence in favour of the role of the macrophage in antigenic stimulation it is pertinent to discuss the process of antigen handling by these cells, not only because of importance of the process in itself, but because it will provide

the reader with useful background information and insight into the experiments described in this thesis.

Phagocytosis consists of two stages; initially the antigen adheres to the macrophage cell membrane, and secondly it is endocytosed. Following endocytosis the majority of pinocytic or phagocytic vesicles fuse with the lysosomes after which the antigen is digested by the phagocytic lysosomes. From these processes two principle points emerge for discussion, the recognition of foreignness, and the site of the immunogenic portion of the ingested antigen.

In vivo and in vitro techniques have both been used in the study of antigen uptake by macrophages. In vivo methods include the clearance of labelled antigens from the blood, and the auto-radiographic localisation of antigen as described previously. In vitro methods study the uptake of antigen in tissue culture. The removal of labelled compounds from the blood, including colloidal suspensions of carbon (159a) saccharated iron oxide (332) chromium phosphate (333, 334) gold (335), and particulate antigens such as heat aggregated proteins (336) has been used to study the kinetics of phagocytosis. Indeed extensive studies of the clearance of colloidal suspensions from the blood (159a, 337-339) have shown that 80-90% of the injected material was removed by the liver and spleen, the former removing approximately two-thirds. The rate of removal of colloid was monoexponential at suitable doses and also dependent upon liver and spleen size.

Therefore using standard body weight, dependent doses of carbon, it was possible to arrive at a value termed the phagocytic index. The clearance of bacteria (340-342), viruses (343) and foreign red cells (344) was also studied. It was concluded on the basis of distribution of colloidal material in the tissues, the radioresistance of the process of clearance, and the increase in clearance after injection with M. tuberculosis (345), or mycobacterial products or endotoxin, that macrophages were the cells responsible for clearing the injected material, as they are radioresistant, and activated during delayed hypersensitivity reactions.

Although macrophages can take up a wide variety of colloidal substances, proteins, micro-organisms and red cells, the molecular basis of the process is not understood. Many antigens, e. g. certain encapsulated virulent bacteria require to be opsonised with antibody and complement before they can be phagocytosed (346-350), whereas other antigens for instance foreign red cells appear to be able to interact directly with the macrophage cell membrane. Macrophages are not able to take up foreign red cells unless the latter are effete (351, 352), and trypsin treatment of the macrophages prevented effete autologous erythrocytes from binding, which suggests that macrophages carry a receptor for this process (353).

In vivo and in vitro studies have shown that protein antigens can be taken up directly by macrophages (237, 268, 354). The degree of uptake of protein antigens by macrophages is related to the degree of polymerisation, monomeric albumin and immunoglobulin

being removed more slowly than polymeric forms (174, 268). In fact the clearance rates of foreign monomeric proteins is similar to the autologous molecules (242, 268). Thus it can be concluded that polymeric proteins are more easily attached to macrophage cell membranes than monomeric forms, and that macrophages have a poor capacity to discriminate between foreign and non-foreign substances. It is widely thought that any macrophage can take up any antigen under optimal conditions, but there is some evidence that selected macrophages preferred to take up foreign erythrocytes (355) although the state of maturity of the cells studied was not apparently ascertained.

Antigen complexed to antibody binds avidly to macrophages as a result of the antibody binding specifically to an immunoglobulin receptor on the macrophage cell membrane, such antibody is termed cytophilic (356). There is immunoglobulin class and subclass restriction of cytophilic antibody: in humans only IgG_1 and IgG_3 antibodies have cytophilic properties (357-359), whereas in mice IgG and IgM antibodies are cytophilic (360). Cytophilic antibody binds to the immunoglobulin receptor by the Fc portion of the molecule (358, 359, 361). The nature of the immunoglobulin receptor is unknown; trypsin treatment of macrophages destroys the ability to bind IgM coated red cells but not IgG coated red cells (360, 362, 363). Free sulphydril groups must form part of the receptor site, as iodacetamide treatment prevents the attachment of opsonised red cells. The mechanisms of

binding immunoglobulin, either free or in antigen-antibody complex are the subject of controversy. It was thought that the stronger binding of immunoglobulin as part of an immune complex was due to an allosteric change in the Fc portion of the molecule, but is now thought as being simply due to an increase in the number of available binding sites from each antibody molecule in the complex (344, 364, 365). Apart from the immunoglobulin receptor, macrophages also carry a complement receptor on their cell membrane (360, 366, 367). The receptor binds C_3 and complexes including only C_1, C_{14} or C_{142} are unable to become attached to the cell membrane (368). It is probable that macrophage bound C_3 is important in promoting phagocytosis in vivo. For example, whereas immunoglobulin blocked macrophage uptake of antigen-antibody complexes not containing complement, it did not affect the uptake of complexes which had fixed complement (366). In vivo studies of decomplemented mice showed that the ability to remove antibody coated E. coli, and rat erythrocytes from the circulation was impaired, but could be restored by incubating the complexes with complement prior to injection (368). It is probable that the macrophage immunoglobulin and complement receptors act in cooperation (366), as studies of human macrophages have shown that 100 molecules of C_3 lead to ingestion of IgG antigen-antibody complexes, whereas about 1000 are needed for IgM antigen-antibody complexes (as mentioned earlier IgM has no receptor

on human monocytes).

Therefore, although the basic molecular interactions involved in the uptake of antigenic and non-antigenic substances by macrophages is unknown, it can be concluded that it is not a process of recognition of foreignness such as described for lymphocytes (369-371) and if antigens do not reach the relevant lymphocytes, then immune recognition, and a subsequent immune response will not occur.

The small proportion of ingested antigen which is not digested becomes available for immune recognition. Attempts to identify the immunogenic moiety and its location have been made by several methods; these have included the use of live macrophages in the macrophage transfer system, disruption of cells and testing the fractions for immunogenicity, and a combination of tissue culture and the macrophage transfer system. This latter experimental technique has shown that only a small fraction of radioactively labelled haemocyanin which had been phagocytosed by macrophages remained undigested (147). This fraction was immunogenic, when injected into syngeneic hosts. A small fraction of this undigested antigen was retained on the plasma membrane, in native form, from which it can be eluted, and shown to react with antibody, or trigger an immune response (372-375). The amount of antigen retained on the plasma membrane varied with different antigens; for haemocyanin 1-2% of the antigen molecules were retained for prolonged periods, whereas for polymeric albumin

the amount was the same, but it dissociated faster. Moreover albumin was rapidly lost from the membrane (268). It is now thought that the antigen retained on the plasma membrane is the first part of the antigen to come into contact with the macrophage, and is localised on areas having little phagocytic activity (268, 354, 373).

Following endocytosis of labelled erythrocytes it was shown that small amounts of antigenic material were slowly released from the macrophage, and were capable of stimulating an immune response (297). Other experiments have demonstrated that immunogenic material could be extracted from intracellular vesicles for some time following endocytosis (136, 140-144, 376, 377), and ultracentrifugation studies have suggested that the antigen is bound to intracellular membranes (140, 378). Antigen bound to live macrophages was more immunogenic than antigen extracted from the cytoplasm (378).

The mechanism of release of intracellular antigen is unknown. Normally soluble antigens do not cross the membrane of the secondary lysosome, unless extensively catabolised (379). On cell death antigen could be released, but apparently the amount of antigen released is far in excess of that accountable for by cell death (297). Release of lysosomal enzymes following the ingestion of large particles occurs from both macrophages and polymorphs (380, 381), and presented ultrastructural evidence of channels communicating between the phagocytic vesicles of

polymorphs and the cell surface has been presented (381, 382). Exocytosis of antigen remains an unconfirmed possibility (383).

Garvey and Campbell (134) first postulated that BSA which persisted in the liver and spleen was possibly associated with nucleic acid. In the early 1960's it was reported that lymphocytes produced antibody to T4 phage in response to extracts of peritoneal macrophages which had previously been exposed to this antigen (148, 230, 231). The immunogenic fraction was found to be sensitive RNA-ase treatment, and extracting the RNA with phenol yielded antigenic material which sedimented as a low molecular weight RNA. These experiments suggested that antigen needed to be processed by macrophages in order to become immunogenic, yet close scrutiny of the results revealed low antibody titres, following immunisation with the RNA extract, and in many cases the results were not reproducible. Later, Askonas and Rhodes (149, 384) isolated RNA from mouse macrophages which had taken up ^{125}I-haemocyanin. About 0.01% of the antigen was bound to RNA, and on injection into mice, it was found to be highly immunogenic. Since then many studies have confirmed this finding (150, 385-389), but there is strong evidence that such fractions are of doubtful biological significance, and may possibly represent laboratory artifact (390, 391), resulting from cellular disruption which allows RNA and antigen to mix. RNA and antigen could complex as a result of in vivo cell death, and it has been reported that antigen-RNA complex

can be found in urine (392). Other evidence which militates against the biological significance of the antigen-RNA complex, is the ability of RNA from cells other than macrophages to form such complexes (393), and the lack of any clear relationship between the complexing of an antigen to RNA and its in vivo immunogenic capacity (390).

From the review of the role of the macrophage in antigenic stimulation it is obvious that this cell plays an important role in the removal of antigens, and although not capable of recognising foreignness, it presents the antigen to the lymphocytes in such a way that immune recognition is possible. The immunogenic moiety is probably undegraded antigen bound to the plasma membrane, or released from the intracellular vesicles.

Turning back to the immunological peculiarities of NZ mice, it is at once apparent that certain aspects of macrophage function may be of relevance. For instance, the greater the amount of bound antigen transferred in the macrophage transfer system, the better the immune response, and thus the immunological hyperresponsiveness of these mice might be due to rapid uptake of antigen by their macrophages. Following this same line of reasoning, the relative resistance of NZ mice to the induction of immunological tolerance might be due to an excellent antigen trapping mechanism which would not permit antigen to come into direct contact with lymphocytes and induce tolerance. The removal of potentially autoantigenic substances by macrophages

might also provide possible information as to why such substances do in some instances provoke immune responses.

For these reasons, I have elected to make the study macrophage function in NZ mice as the subject to this thesis.

CHAPTER 2.

DATA ON THE ESTABLISHMENT OF OUR NEW ZEALAND MOUSE COLONIES

INTRODUCTION

As New Zealand mice had not previously been studied in this department, it was considered important to gather data on the rate of Coombs' conversion, incidence of proteinuria and onset of nephritis, and the cumulative mortality in our mouse colonies. This would enable us to determine whether the mice in our colonies could be reasonably compared to those of other centres. In this chapter I present these data, and also the breeding statistics of our various mouse strains.

TABLE 2

BREEDING STATISTICS OF MICE STUDIED

Mouse Strain	Economic Breeding Life (months)	Average number of litters per pair	Average litter size	Pre-weaning death rate
NZB	9	5	6.1	1.3/6.1
NZW	9	5	4.4	2.8/4.4
BWF_1	9	8	7.5	0.4/7.5
BALB/c	15	8	5.9	0.7/5.2
CBA	15	8	8.9	0.8/8.9

NUMBER OF MICE OF EACH STRAIN STUDIED FOR THE TIME OF ONSET OF AUTOIMMUNE DISEASE

Mouse Strain	Number Studied	
	Males	Females
NZB	25	25
BWF_1	15	17
BALB/c	25	25
CBA	50	50
NZB x CBA F1	42	25
NZB x CBA F2	44	42

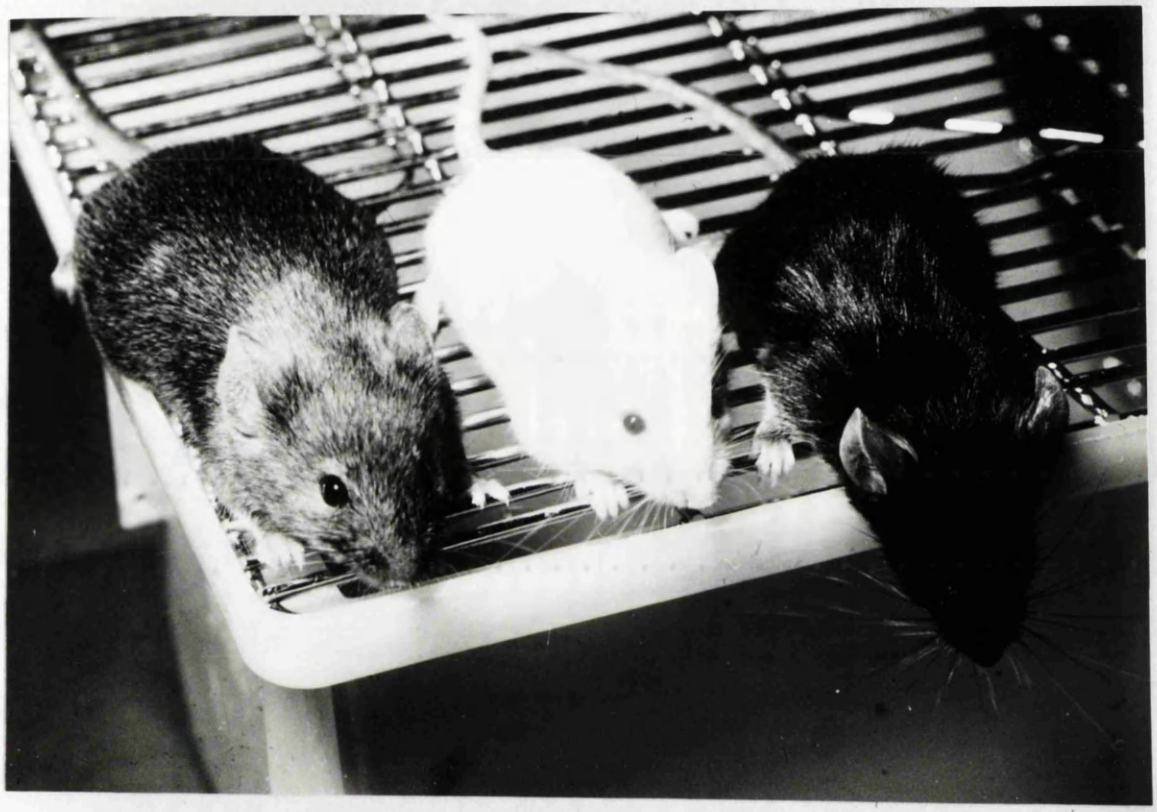


Fig. 1. The three New Zealand strains of mice, New Zealand Black (NZB), New Zealand White (NZW), and NZB x NZW F1 (BWF_1) hybrid mice.

MATERIALS AND METHODS.

The different mouse strains used in most studies are shown in Table 2. NZB mice were a gift from Dr. W.J. Irvine of the M.R.C. Endocrinology Unit in Edinburgh. The mice were derived from inbred New Zealand stock sent to Professor Woodruff's department. They were maintained as a strictly inbred colony by brother-sister mating, and the colony is now in its 6th generation. Inbred NZW mice were obtained from the M.R.C. Animal Centre, Carshalton, Surrey, England. BWF_1 hybrid mice were the result of mating either NZB males with NZW females or NZB females with NZW males. The three New Zealand mouse strains are shown in Fig. 1. CBA and BALB/c mice came from our own inbred colonies, and C3Hf mice were kindly supplied by Professor Jarret of the Glasgow Veterinary School. CBA x NZB F1 and F2 mice were included as extra control strains during studies of the time of onset of autoimmune disease. Table 3 shows the number of mice of each strain included in this study.

Direct Coombs' Tests

Mouse red cells were washed three times in warm saline ($37^{o}C$), and finally resuspended in an equal volume of saline. An equal volume of red cell suspension and a 1 in 10 dilution of Coombs' reagent were mixed on a slide and at the end of 5 minutes the presence or absence of agglutination was noted. The degree of agglutination was scored weak +, + or ++. Known positive and negative controls were used in all experiments.

The Coombs' reagent was an anti-mouse gamma globulin prepared by immunising rabbits with pooled mouse gamma globulin. Rabbits were initially immunised intramuscularly using gamma globulin in complete Freund's adjuvant, and boosted 4 weeks later with a single intravenous injection of antigen. The rabbits were exsanguinated by cardiac puncture 1 week following the booster injection. The antisera were tested by immunoelectrophoresis against normal mouse serum, and only lines of precipitation against the major mouse immunoglobulin classes were detected. Sera were then heat inactivated and absorbed against NZB, BALB/c and CBA erythrocytes sequentially. Only one serum was used throughout all the experiments.

Proteinuria

A single drop of urine was expressed by manual pressure to the lower abdomen, and the quantity of proteinuria estimated during Albustix (Ames Labs). Proteinuria was considered significant when 30 mg/100 ml or greater was present.

Tissue specimens for histological examination were taken at post-mortem examination of sick mice or from mice killed at selected ages. Animals found dead were not examined.

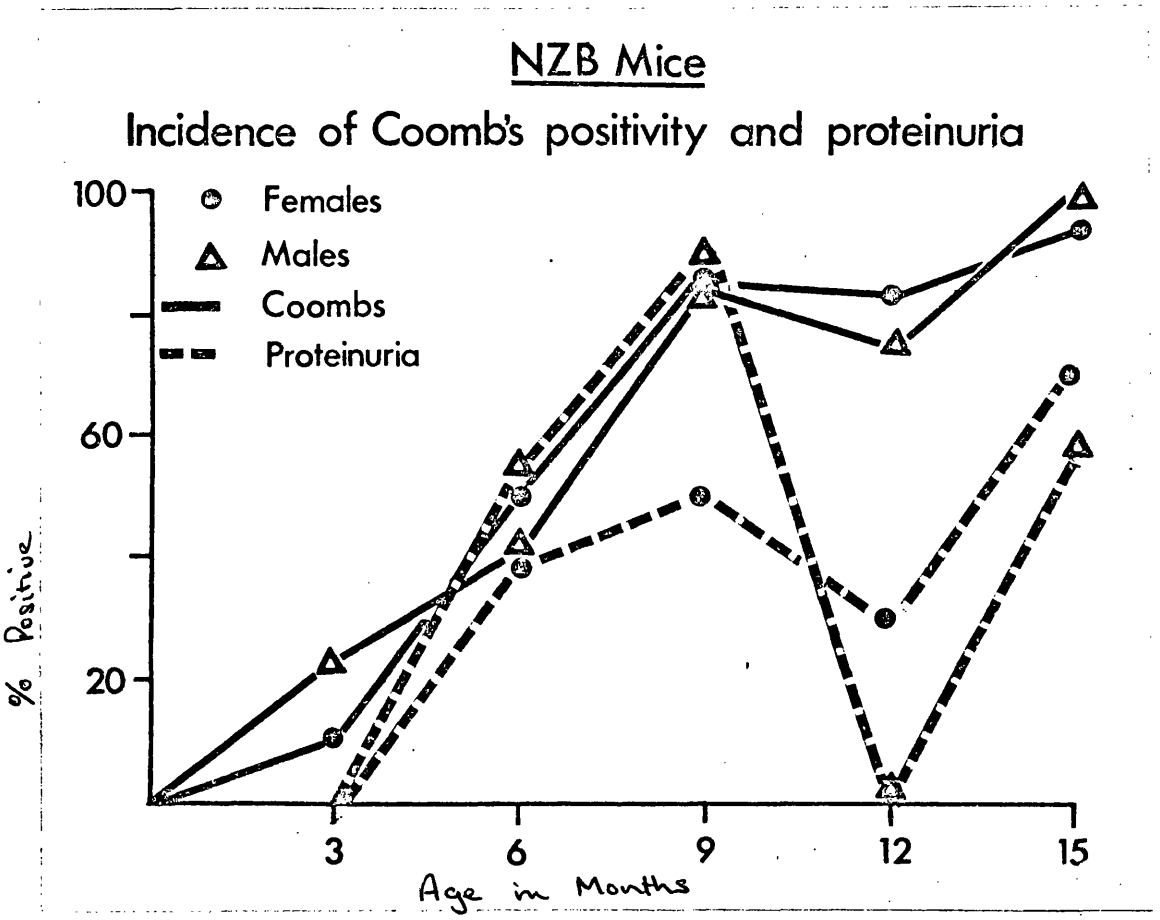


Fig. 2. Incidence of Coomb's conversion and proteinuria in NZB mice.

RESULTS

Breeding Statistics

Table 2 shows the breeding statistics of the five strains of mice bred in this department. Average litter sizes were highest in CBA mice (8.9), and lowest in NZW mice (4.4). NZB litter sizes averaged 6.1 mice. Pre-weaning mortality was highest in NZW mice, (2.8 deaths of 4.4 births) and lowest in BWF_1 mice (0.4 deaths of 7.5 births). NZB mice had a pre-weaning mortality of 21.3% (1.3 of 6.1 mice). Data on immediate post-weaning mortality are not available.

The average duration of the economic breeding life is longest for CBA and BALB/c mice, approximately 15 months, whereas breeding lives for NZB, NZW and BWF_1 mice were 9 months. The number of litters per pair of breeding mice parallels the duration of the economic breeding life, being 8 for CBA and BALB/c mice, and 5 for NZB and NZW mice. Eight litters BWF_1 were born in the short breeding lives of the parent mice.

Coombs' Conversion (Fig. 2)

At 3 months of age 22.5% of male and 10% of female NZB mice were Coombs' positive. The percentage with positive tests rose steeply from 3 months, until at 9 months 85% of both males and females had positive Coombs' tests. A slight fall in the percentage with positive Coombs' tests was found at 1 year, with a secondary rise at 15 months. The fall at 1 year is due to deaths among sick Coombs' positive animals.

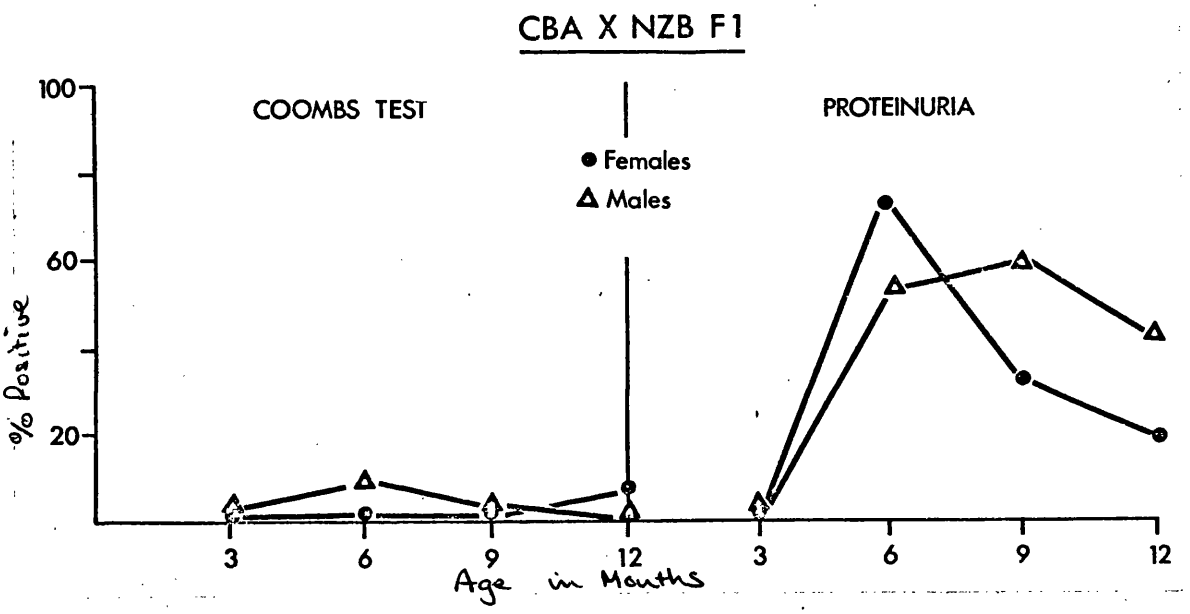


Fig. 3. Incidence of Coomb's conversion and proteinuria in CBA x NZB F1 hybrid mice.

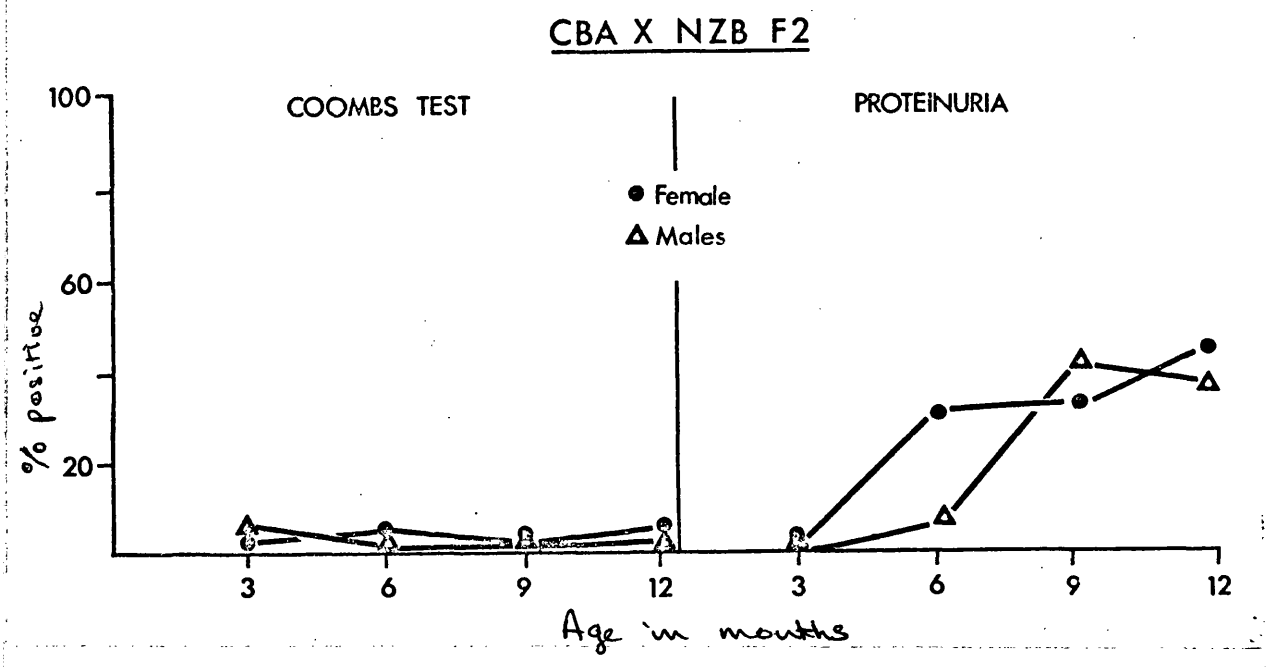


Fig. 4. Incidence of Coomb's conversion and proteinuria in CBA x NZB F2 mice.

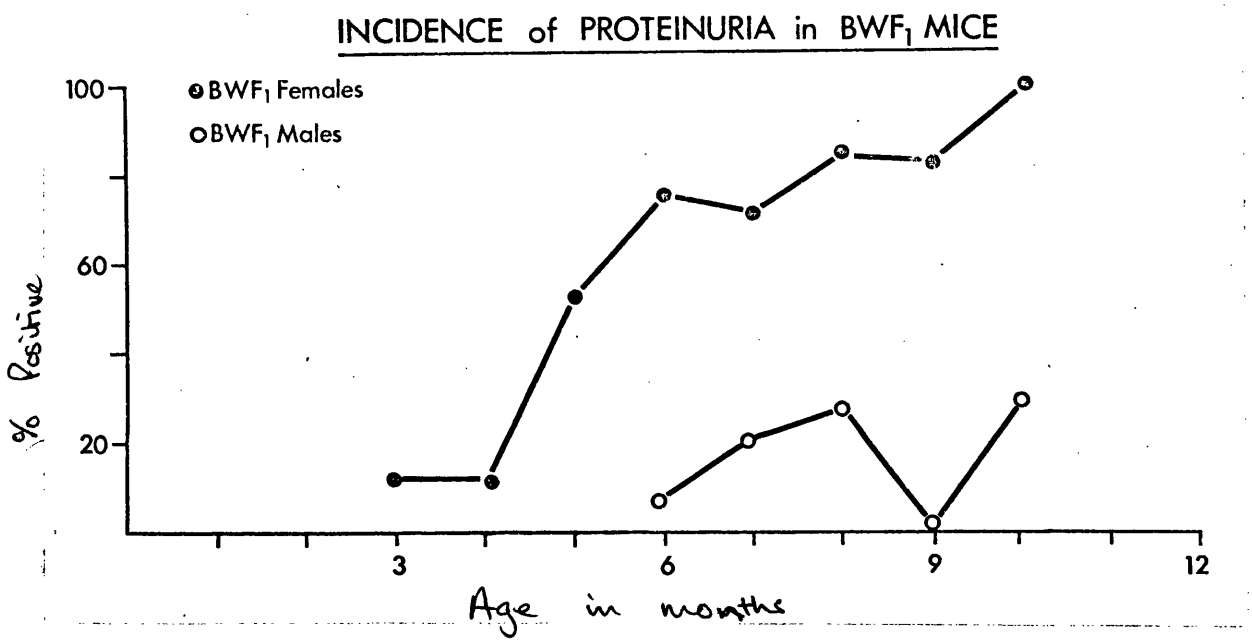
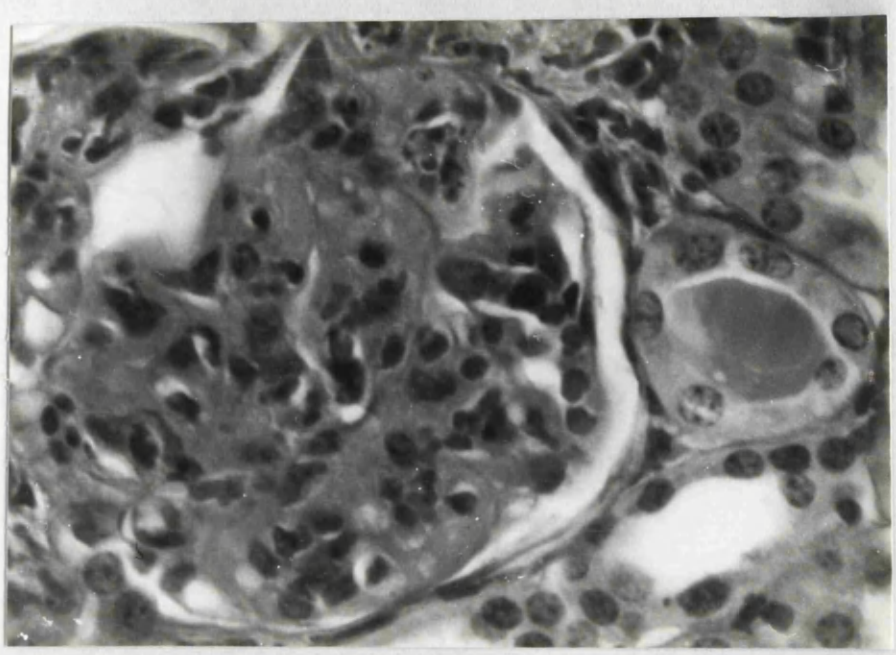


Fig. 5. Incidence of proteinuria in BWF_1 hybrid mice.

Fig. 6. Nine month old BWF_1 female mouse with massive ascites and oedema.
Healthy 4 month old female BW F1 on the left for comparison.



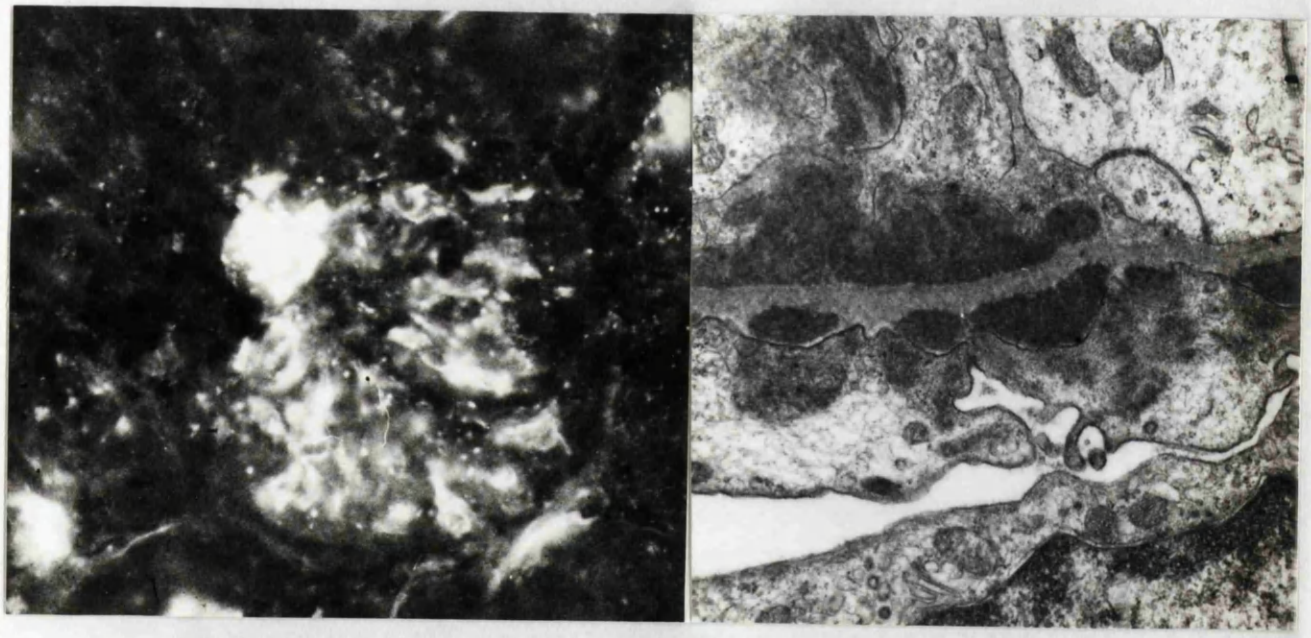


Fig. 7. Glomerulonephritis of BWF_1 female shown in Fig. 6.

(a) H. & E. x 400.

(b) Direct Immunofluorescence using fluorescein labelled antimouse immunoglobulin x 400.

(c) Electron Microscopy showing electron dense immune complexes on glomerular basement membrane x 12, 000.

In CBA x NZB F1 and F2 mice, only weak transiently positive Coombs' tests have been found up to 1 year; at no stage during this time did positive Coombs' tests occur in CBA and BALB/c mice.

Proteinuria

By 9 months of age 50% of NZB females (Fig. 2) were proteinuric. By 1 year this figure fell to 30%, but at 15 months 70% of these mice had albuminuria. A surprising feature of this NZB colony was a 90% incidence of proteinuria in NZB males at 9 months, with a marked fall to 0% in the incidence of proteinuria at 1 year. However by 15 months 50% had proteinuria. None of the male NZB mice exhibited over 100 mg% of albuminuria, whereas females showed increasing degrees of proteinuria with time. By 6 months 72% of females and 53% of males of the CBA x NZB F1 hybrid mice (Fig. 3) had developed proteinuria, but by 12 months the figures were 18% and 41% respectively. There was a progressive rise in the incidence of proteinuria to 44% in females and 37% for males of the CBA x NZB F2 hybrid mice by 12 months (Fig. 4). In neither of these two hybrid strains did proteinuria exceed 100 mg% at any stage.

Of the BWF_1 hybrid females (Fig. 5) 10% had mild proteinuria at 3 and 4 months followed by a sharp rise to 54% at 5 months, and by 10 months, 100% of animals were proteinuric. The proteinuria became heavy ($>$ 1000 mg%) pre-terminally, and was associated with massive ascites and subcutaneous oedema (Fig. 6), due to the rapidly developing renal lesion (Fig. 7). By 6 months only 7% of BWF_1 males had proteinuria, rising slowly to 26% at 8 months, followed by

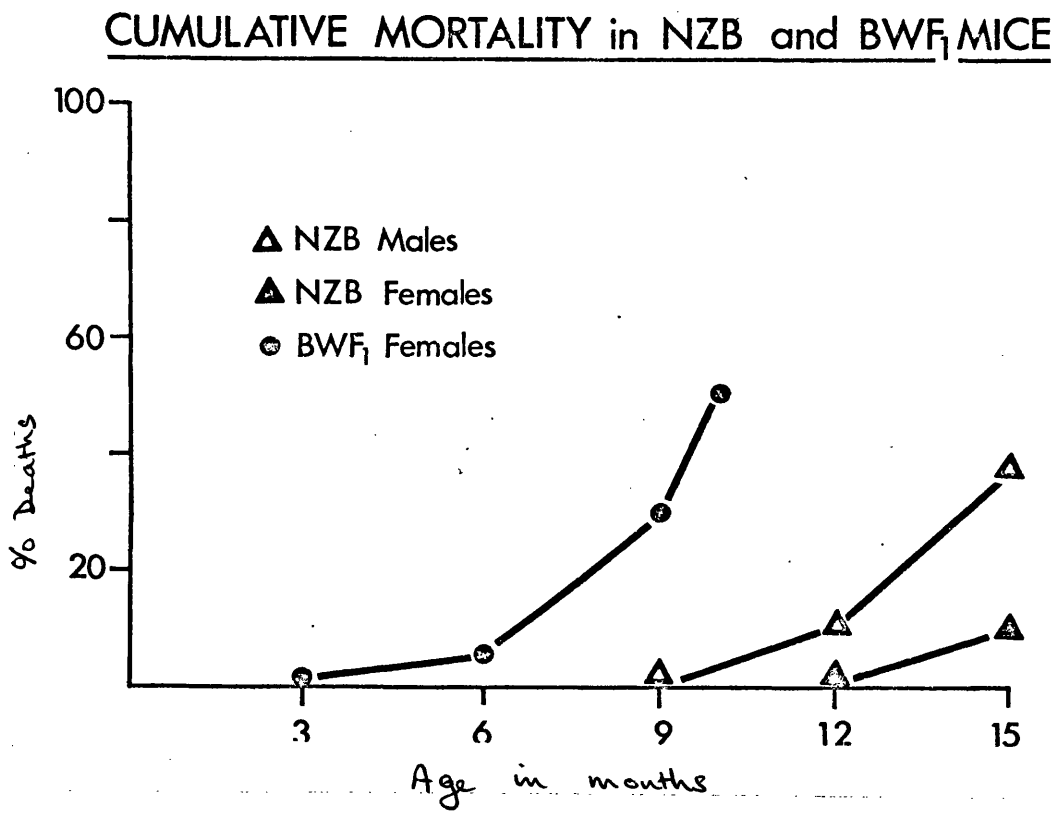


Fig. 8. Cumulative mortality in NZB and BWF_1 mice.

a sudden fall to 0% at 9 months. This fall could not be explained by mouse deaths, but could be due to the relatively small numbers of BWF_1 males examined. By 10 months 28% had proteinuria but in all cases it was less than 100 mg%. Whether the father or mother of a BWF_1 mouse was an NZB did not appear to affect the time of onset of proteinuria.

Significant proteinuria did not occur in CBA or BALB/c mice.

Cumulative Mortality (Fig. 8)

The highest death rate was found in BWF_1 female mice, 50% being dead by 10 months, whereas none of the males had died by this time. The increasing mortality closely parallels the increasing frequency and severity of proteinuria.

Deaths in the NZB females began to occur at 12 months by which time 11% had died, and by 15 months 38% were dead. This increasing death rate corresponds in time to the period of maximum haemolysis and increasing proteinuria (Fig. 2). Only 10% of NZB males however were dead at 15 months, when haemolysis was as severe as in females of the strain, but the severity of the proteinuria was much less. This possibly indicates that both haemolytic anaemia and uraemia contribute to the increased death rate of the females, as has been reported by other workers (78, 80, 153, 154).

No deaths occurred up to 1 year in CBA x NZB F1 and F2 hybrids, CBA or BALB/c mice.

Problems specific to the breeding of NZB mice

Like East and her colleagues (155). we have found that NZB males fight viciously, although rarely to the point of killing each other. However their fur becomes thin, alopoecia developing over the neck and back with ulceration and scabbing appearing later. Unlike the males, the females are fairly docile and retain a healthy appearance, even at 15 months, despite the presence of strongly positive Coombs' tests.

Infections of the eyes of NZB mice occurred frequently and did not appear to be related to bleeding from the retro-orbital venous plexus as it was not observed in the control strains. At the age of 2-3 months, a self-limiting bout of a diarrhoeal illness, affected the BWF_1 mice, but none of the other strains. No bacterial pathogens were isolated on culture, and no treatment required. Like Holmes and Burnet (153), we have found that the mice of the NZB strain are prone to the mite infestation, Myobia musculi, which was controlled by dusting with Gammexane dusting powder, or dipping in Tetmosol (156). No breeding problems occurred in any of the control strains studied.

DISCUSSION

The purpose of presenting these data on our New Zealand mouse colonies is to show that the animals used for the studies presented in this thesis are strictly comparable to those of other colonies. In this respect, the rate of Coombs' conversion and the onset of albuminuria are similar to those of other studies, although minor differences, especially the biphasic nature of proteinuria in NZB mice do exist (77-80, 153, 154, 157, 158). The cumulative mortality of the BWF_1 mice is similar to all other previous reports (79, 80, 158, 159) but that of the NZB mice is much closer to cumulative mortality of Holmes and Burnet, (153) and other authors (78, 79, 157) than to the data of East and her colleagues (155) who found that none of the mice in their colony lived for longer than 63 weeks.

A detailed study of the post-mortem and histological findings has not been presented here but it should be mentioned that the renal appearances are the same as those found in the previously mentioned studies (Fig. 7).

CHAPTER 3.

A COMPARATIVE STUDY OF THE PHAGOCYTOSIS OF COLLOIDAL CARBON BY THE FIXED TISSUE AND PERITONEAL MACROPHAGES OF NEW ZEALAND AND OTHER STRAINS OF MICE

INTRODUCTION

Phagocytosis of antigen is one of the earliest events occurring the immune response, and in this chapter I have attempted to compare phagocytic activity in NZB and BWF_1 mice with that in other strains of mice by following the peritoneal cellular response to and the subsequent localisation of carbon in the fixed tissue macrophages. The function of the fixed tissue macrophages has also been assessed by studying the clearance of colloidal carbon from the blood, following intravenous injection.

MATERIALS AND METHODS

Mice studied were from the inbred strains NZB, NZW, CBA, C3Hf, BALB/c and BWF_1 stock. Mice were aged 8-10 weeks at the onset of experiments.

Each mouse was given a single intraperitoneal injection of colloidal carbon as 0.1 ml. of a 1:4 saline dilution of Pelikan india ink (lot number C11/1431a, Gunther Wagner Ltd., Germany). Four male and four female mice of each strain were killed and examined as described below at 5, 30 and 60 minutes; 6, 15 and 24 hours; 3, 7 and 28 days, and 3 months post-injection. Mice were anaesthetised with ether, injected intraperitoneally with 10 units of preservative-free heparin in 2 ml. of phosphate buffered saline, and exsanguinated by axillary bleeding. After dissecting the skin carefully the parietal peritoneum was gripped gently with forceps, and a small hole made with pointed scissors. The peritoneal washings were aspirated as completely as possible using a siliconised Pasteur pipette. Peritoneal cell counts were performed in an improved Neubauer counting chamber (Hawksley). Smears of the peritoneal cells were prepared on a cyto-centrifuge (Shandon Ltd.), fixed in alcohol-ether and stained with May-Grunwald-Giemsa. Differential cell counts were performed on the smears, 300-400 cells usually being counted.

The omentum from each animal was removed and spread on a microscope slide, fixed in alcohol-ether, and stained with May-Grunwald-Giemsa. The number of focal lymphoid aggregates was counted macroscopically. The density of carbon in the lymphoid aggregates and in small aggregates of phagocytes in the omentum, together with

the character of the omental cellular infiltrate, were determined by microscopic examination.

Representative samples of liver, spleen, thoracic and mediastinal lymph nodes and thymus were fixed in 10% formol-saline, processed, and embedded in paraffin. Six micron sections were cut and stained with haematoxylin and eosin. The relative amounts of carbon in the various tissues was estimated microscopically and its distribution noted.

Intravenous Carbon Clearance Studies

Inbred NZB, CBA and BALB/c mice aged 8-10 weeks and 9-12 months were studied. Carbon clearances were performed using the method described by Biozzi, Benacerraf and Halpern (159a). Each mouse was injected intravenously into the lateral tail vein with 0.1 ml per 10 g. of body weight of a colloidal carbon suspension (16 mg/ml) (C11/1431a, Gunther, Wagner, Hanover, Germany) suspended in an aqueous solution of gelatin. The mice were bled from the retro-orbital venous plexus prior to and at 3, 6, 9, 12 and 15 minutes following the carbon injection. Twenty microlitre samples were collected in standard heparinised capillary tubes (Drummond Microcaps, Shandon Ltd), and lysed into 2 ml of 0.1% sodium carbonate solution. The concentration of carbon was determined using an EEL colorimeter. The colorimeter readings ($\log_{10}$) were plotted against time, and the phagocytic index (K) calculated using the following formula:-

$$K_{16} = \frac{\log_{10} C_1 - \log_{10} C_2}{T_2 - T_1}$$

Where K_{16} = the phagocytic index (using a carbon concentration of 16 mg/ml)

C_1 = colorimeter reading at time T_1

and C_2 = colorimeter reading at time T_2

Due to variation in the size of livers and spleens between animals, K is corrected for the ratio of liver and spleen weight to body weight. Thus the corrected phagocytic index (α)

$$= \frac{W}{wls} \times \sqrt[3]{K_8}$$

Where W = bodyweight

and wls = combined weight of liver and spleen

K_8 = the phagocytic index using a carbon concentration of 8 mg/ml.

This has been shown to be twice the value for K_{16}, and is converted to K_8 for the calculations as many of the early studies of phagocytosis in mice were performed using a carbon concentration of 8 mg/ml.

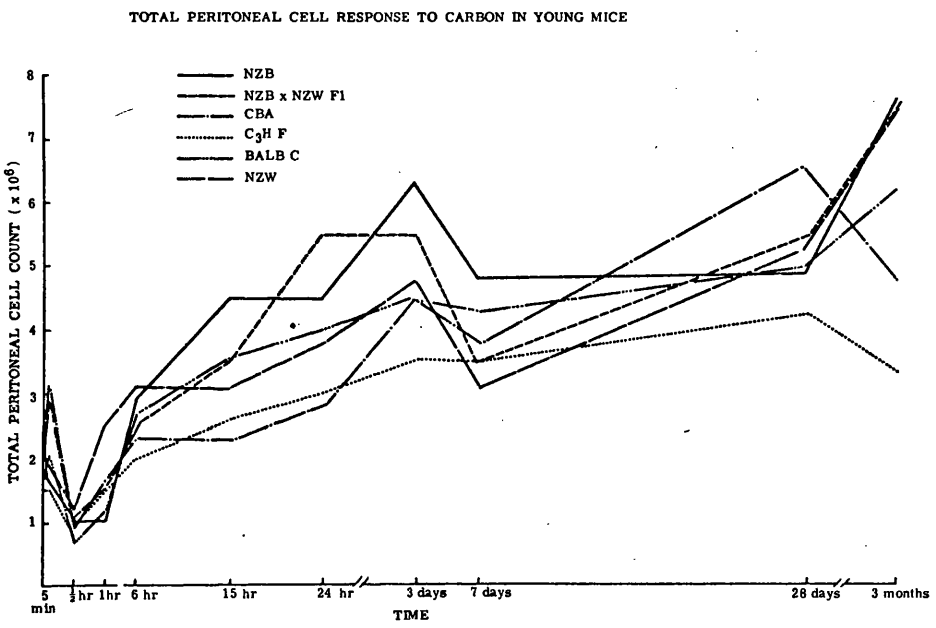


Fig. 9. Total peritoneal cellular response to colloidal carbon in 6 strains of mice.

RESULTS

No marked sex difference in the peritoneal cell counts was found and so the results in males and females of each strain have been considered together.

(1) Total peritoneal cell response (Fig. 9).

The mean control total peritoneal cell counts ranged between 1.5 and 2.5×10^6 cells/mouse in the six strains of mice studied, with wide overlap of individual counts between the strains. The highest mean counts were in NZB (2.5×10^6) and BWF_1 (2.0×10^6) mice.

The general pattern of total cell response following carbon injection was similar in all strains. There was an abrupt fall in the number of cells to minimum values at 30 minutes (range of means: 0.7 - 1.2×10^6) followed by a rise which reached a peak at 3 days (range of means: 3.5 - 6.3×10^6). At this point NZB (6.3×10^6) and BWF_1 (5.5×10^6) mice had the highest cell counts and BALB/c mice the lowest (3.5×10^6). Total cell counts then fell to a trough by day 7 (range of means: 3.1 - 4.8×10^6) and though NZB mice still had the highest mean cell count (4.8×10^6), the mean cell count in BWF_1 mice was appreciably lower (3.5×10^6) at this time. A progressive rise in total cell counts then occurred and persisted for up to 3 months, at which time the NZB, NZW and BWF_1 mice had higher counts (7.5 - 7.7×10^6) than the other three control strains of mice (3.3 - 6.2×10^6).

(2) Differential cell counts.

Due to the density of carbon in the peritoneal exudate, differential

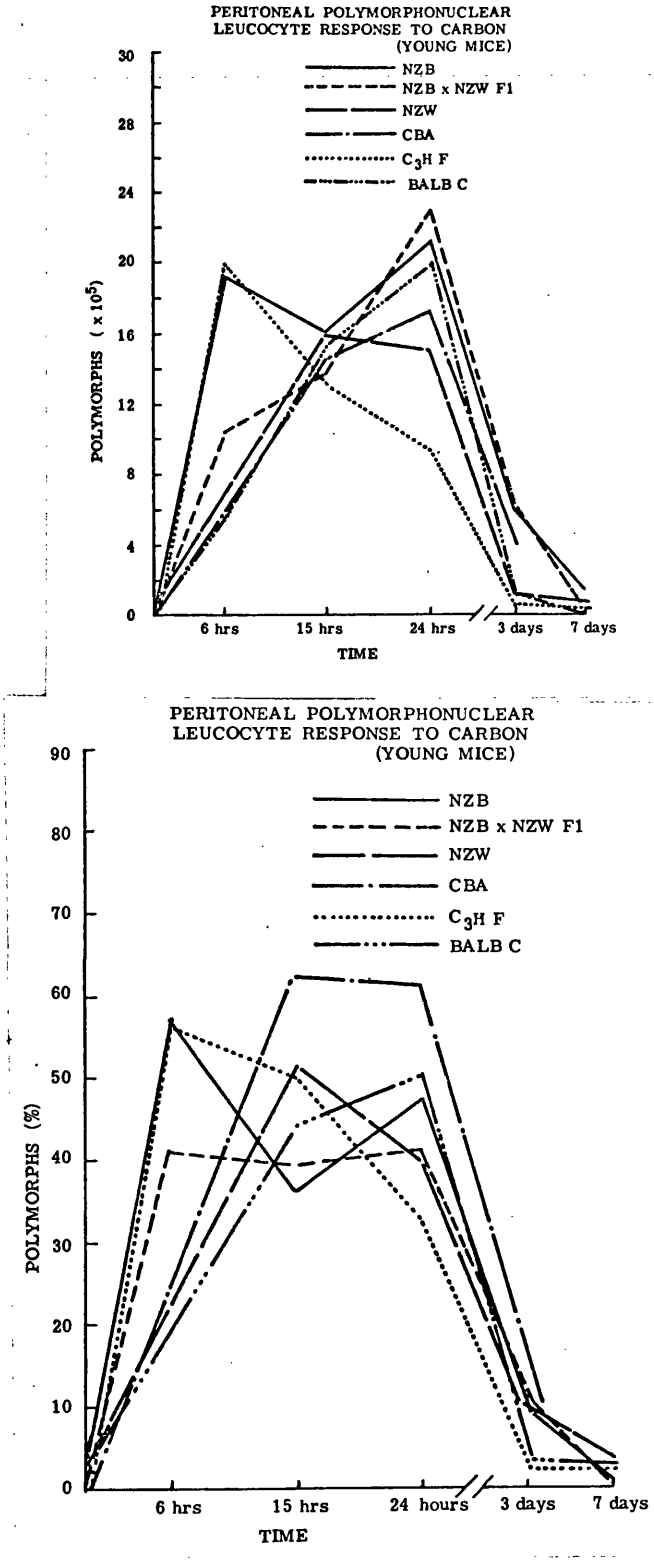
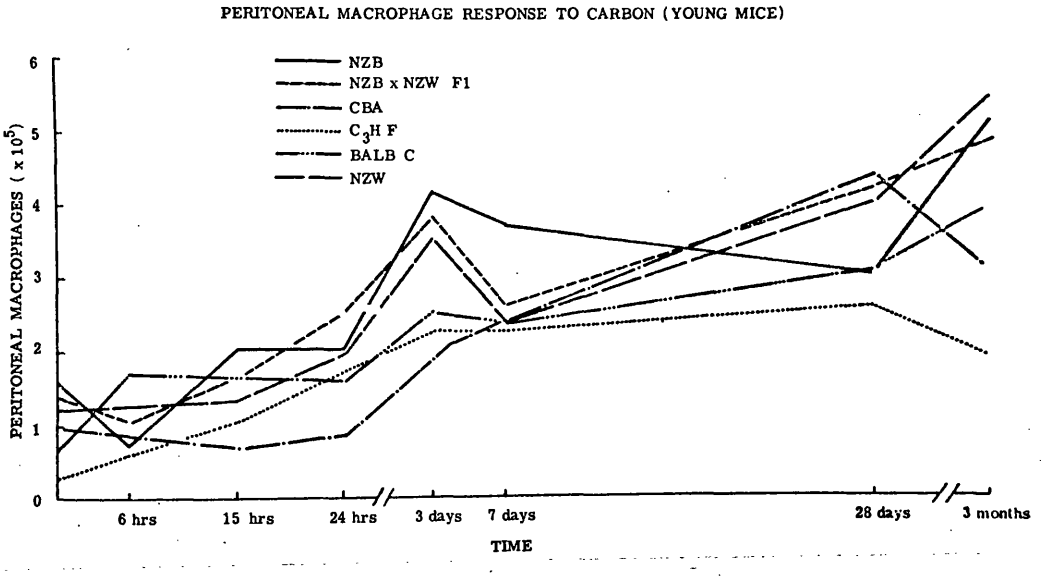


Fig. 10. Peritoneal polymorphonuclear leucocyte response to colloidal carbon in 6 strains of mice.

a) Total polymorph count.

b) Percentage of total peritoneal cell count.



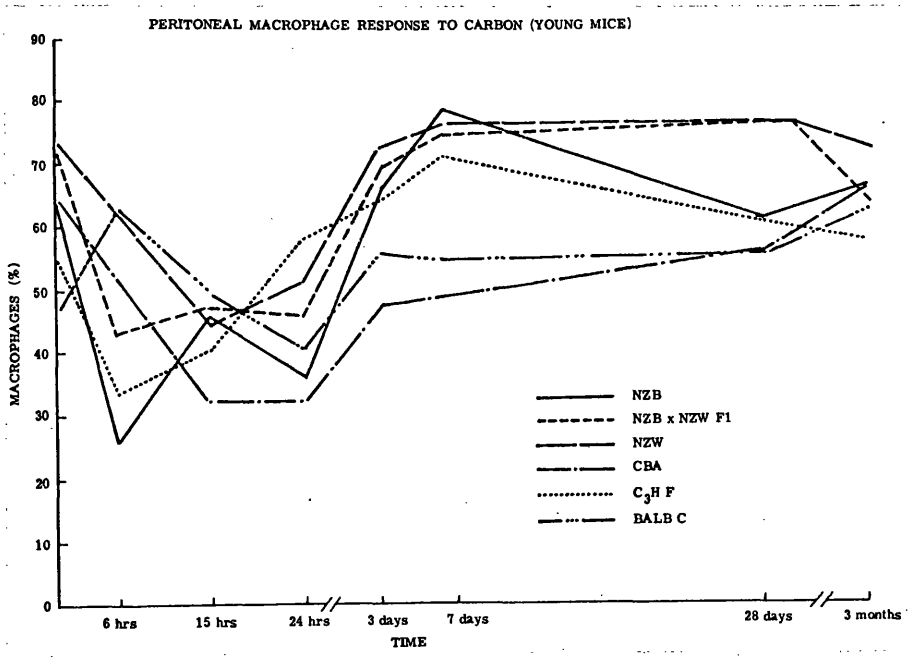


Fig. 11. Peritoneal macrophage response to colloidal carbon in 6 strains of mice.

a) Total macrophage count.

b) Percentage of total peritoneal cell count.

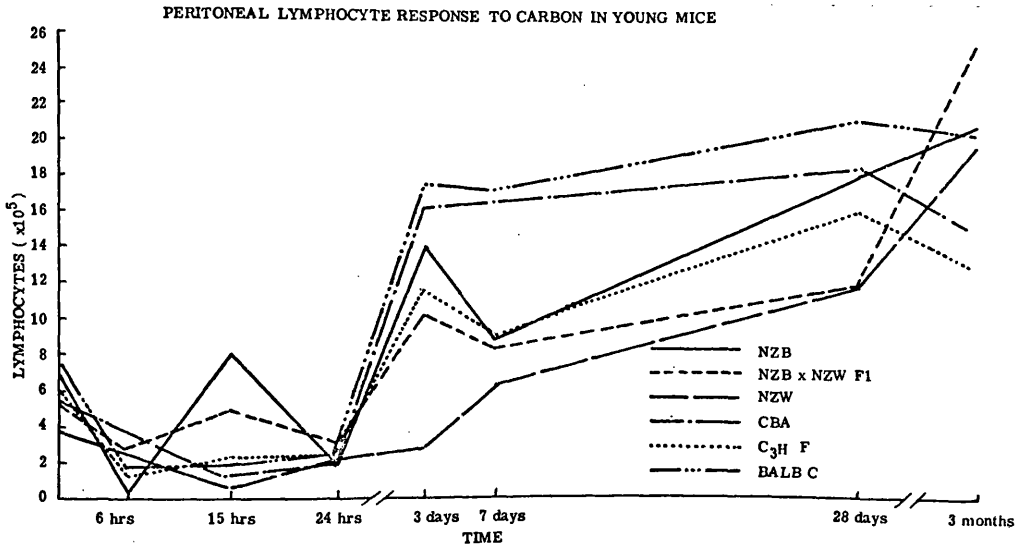
cell counts were not possible at 5, 30 and 60 minutes following injection. Due to unsatisfactory preparations, accurate differential cell counts could not be obtained at 6 hours in CBA and NZW mice, or at 7 days in CBA mice.

Polymorphonuclear leucocyte response

From Fig. 10 it can be seen that control uninjected mice had only very small numbers of polymorphs in the peritoneal exudate (mean counts $1.5 - 9.5 \times 10^4$) but following the injection of carbon, there was a rapid rise in both the absolute polymorph counts and their proportion of the total cell counts. Maximum polymorph responses occurred between 6 and 24 hours. By 3 days a marked fall in polymorph counts had occurred, and by 7 days they had returned to control levels. There were no marked interstrain differences in polymorph response.

Peritoneal macrophage response (Fig. 11).

The mean macrophage counts per mouse in control uninjected mice ranged from $0.33 - 1.6 \times 10^6$, the highest being in NZB (1.6×10^6) and BWF_1 (1.45×10^6), and the lowest in C3Hf mice (0.33×10^6). The macrophage counts did not change over the first 6-24 hours, although their proportion of the total peritoneal exudate cell counts fell. This period corresponds in time to the maximum polymorph response. After 24 hours there was a rise in macrophage counts, both absolute and in their proportion of total peritoneal cells. Though the absolute macrophage counts peaked at day 3, their proportion of total peritoneal cells continued to rise to day 7. Between 7 and 28 days, absolute



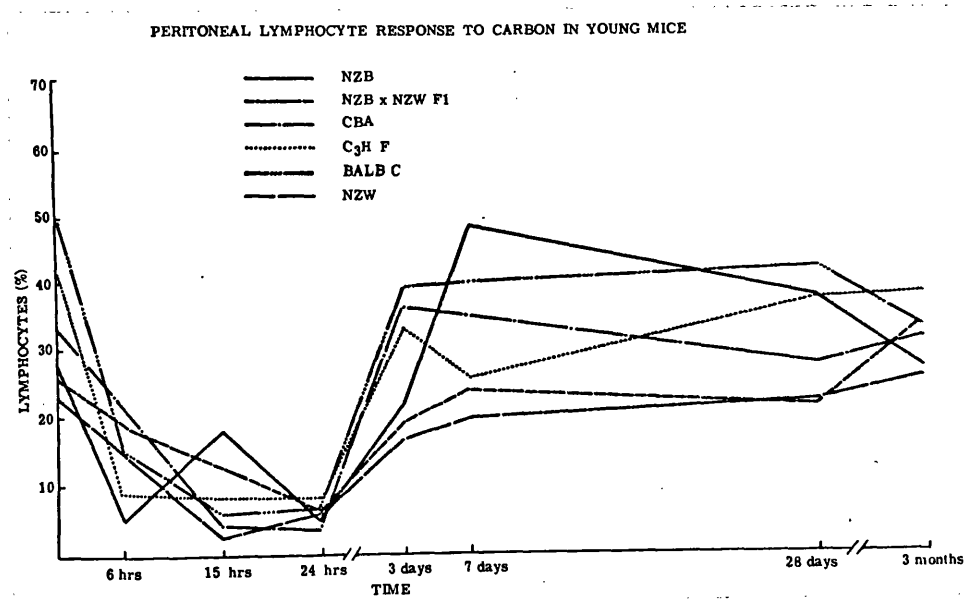
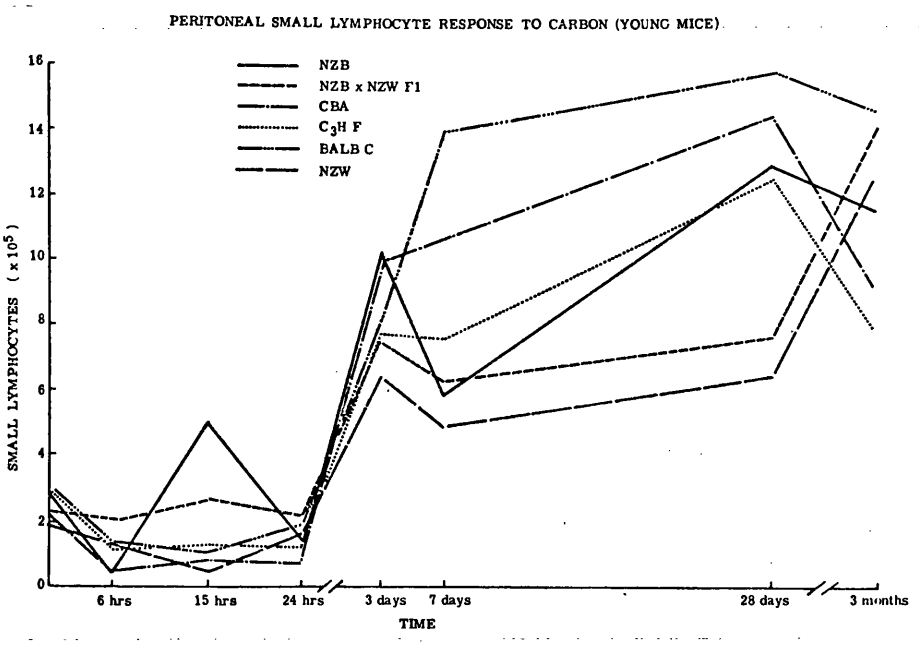


Fig. 12. Peritoneal lymphocyte response to colloidal carbon in 6 strains of mice.

a) Total lymphocyte count.

b) Percentage of total peritoneal cell count.



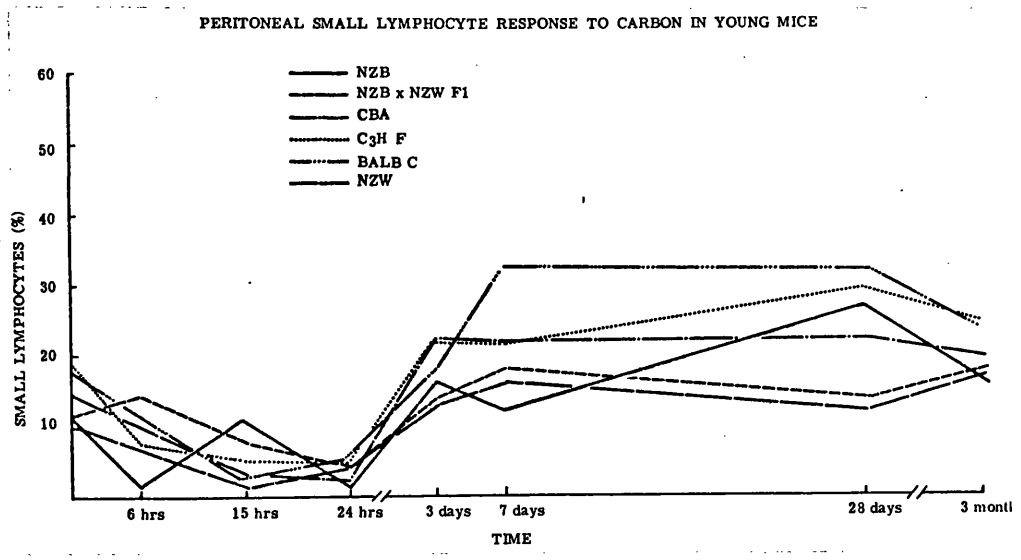
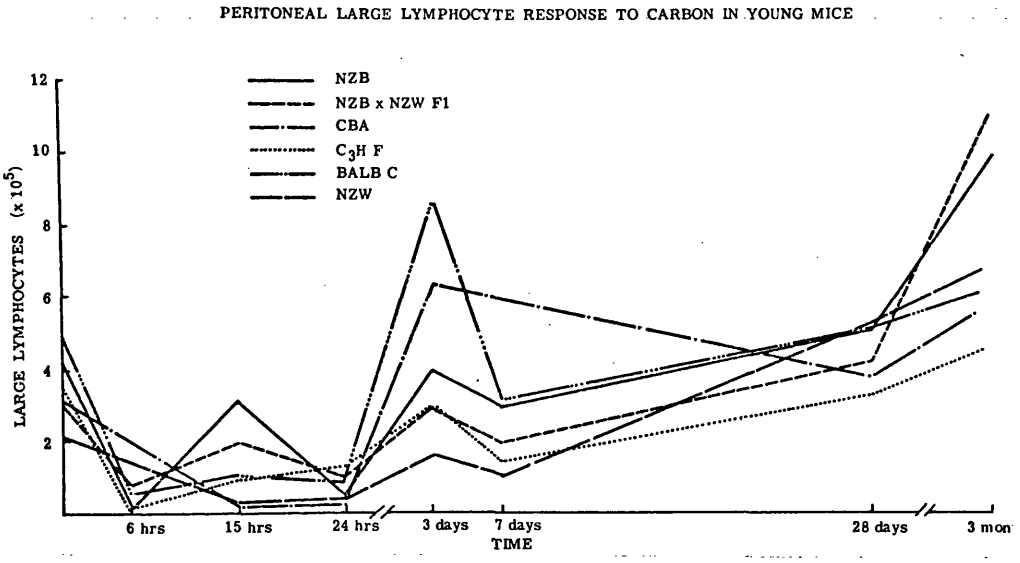


Fig. 13. Peritoneal small lymphocyte response to colloidal carbon in 6 strains of mice.

a) Total small lymphocyte count.

b) Percentage of total peritoneal cell count.



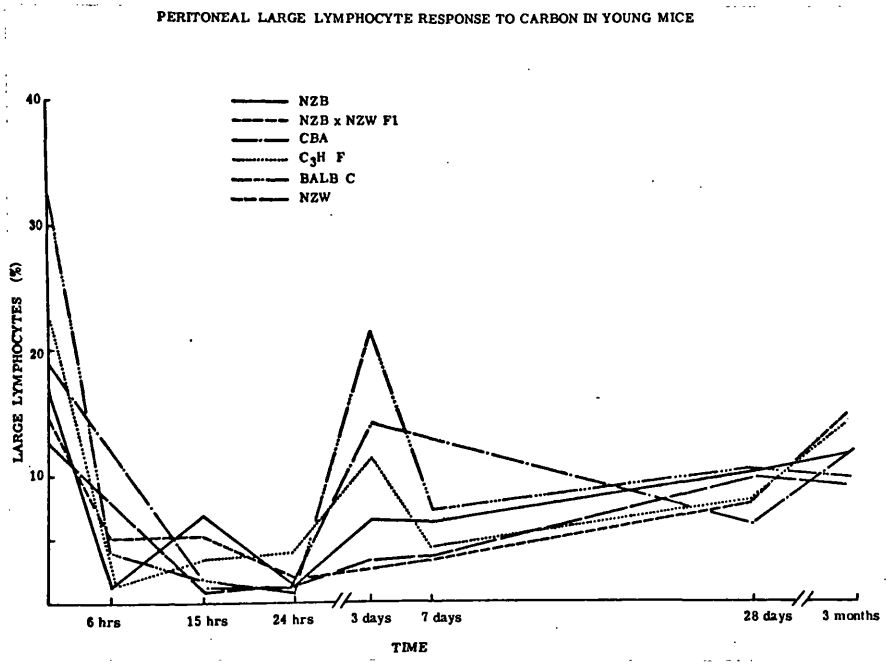


Fig. 14. Peritoneal large lymphocyte response to colloidal carbon in 6 strains of mice.

a) Total large lymphocyte count.

b) Percentage of total peritoneal cell count.

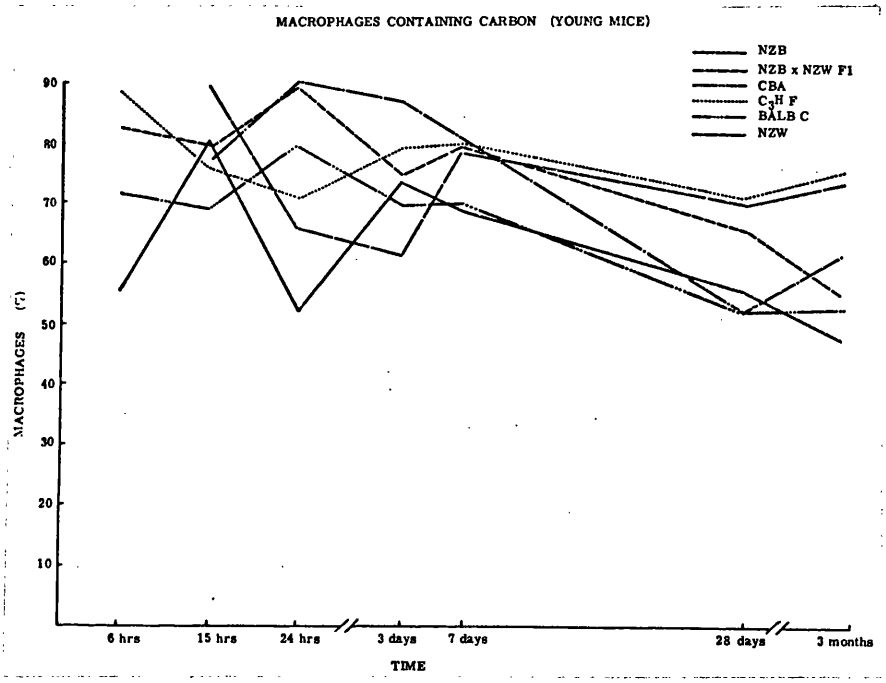


Fig. 15. Removal of carbon-laden macrophages from the peritoneal cavity. Carbon-laden macrophages expressed as a percentage of total peritoneal macrophages.

macrophage counts rose in all but NZB mice, and their proportion of total peritoneal cells rose in all but NZB and C3Hf mice. At 3 months, absolute macrophage counts remained at high levels, although a small decrease was evident in C3Hf and CBA mice.

Lymphocyte response (Fig. 12).

Control mean lymphocyte counts ranged from $3.8 - 7.7 \times 10^5$. Six hours following the injection of carbon, a marked fall in absolute lymphocyte counts had occurred in all strains and counts remained low for 24 hours except in NZB mice which showed a spike at 15 hours. A rise in absolute lymphocyte counts had occurred by day 3 in all but NZW mice, and apart from a minor trough at day 7, this rise was essentially maintained over 3 months by which time lymphocyte counts of NZW mice did not differ from those in the other strains. The patterns of responses of small lymphocytes (Fig. 13) and large lymphocytes (Fig. 14) were almost identical to that of the total lymphocyte response.

(3) Removal of carbon-laden macrophages from the peritoneal cavity (Fig. 15).

The percentage of macrophages containing carbon began to fall slowly after day 7 but had risen again slightly by 3 months at which time there was an increased proportion of less heavily laden cells; these have presumably phagocytosed carbon released from dead macrophages.

(4) Omental changes.

Aggregates of carbon were evident macroscopically on the peritoneal surfaces 6 hours after injection. Lymphoid aggregates were quite heavily laden with carbon at this time and microscopically there were also small aggregates of carbon within the omentum. From 6 hours and onwards, the proportion of different cell types in the omental cellular infiltrate paralleled those in the peritoneal cavity. At 3 months considerable carbon deposition was still present in lymphoid aggregates within the omentum. No strain differences were noted.

(5) Distribution of carbon within tissues.

Within 5 minutes of injection, traces of carbon were visible within the subcapsular and cortical sinuses of thoracic lymph nodes, and by 30 minutes these deposits were heavy and extended into the medullary sinuses. At 3 months these heavy deposits in medullary zones were unchanged, though they had decreased in subcapsular and cortical zones.

Within 30 minutes of injection, the Kuppfer cells of the liver were quite heavily laden with carbon and by 24 hours they were completely "engorged", and remained so up to 28 days. The amount of carbon within the liver was markedly reduced at 3 months in all but the BALB/c mice, in which there was no apparent diminution.

In all strains of mice at all times, carbon deposits occurred in perifollicular zones of the spleen, and in a diffuse manner throughout the red pulp, with occasional traces in the white pulp around the central arterioles. There was marked inter-strain variation in the

TABLE 3a

Results of Intravenous Carbon Clearance Studies

Group	$\frac{W^*}{wls}$	Phagocytic Index (K_{16})	Corrected Phagocytic Index (α)
Young NZB	18.7 ± 1.5	0.008 ± 0.002	4.67 ± 0.38
Young BALB/c	16.2 ± 2.1	0.012 ± 0.003	4.73 ± 0.32
Young CBA	14.5 ± 0.5	0.017 ± 0.002	4.65 ± 0.31
Old NZB	16.4 ± 3.5	0.012 ± 0.006	4.70 ± 0.57
Old BALB/c	14.4 ± 1.5	0.017 ± 0.005	4.74 ± 0.34
Old CBA	16.6 ± 1.1	0.015 ± 0.003	4.85 ± 0.22

* For explanation see text.

amounts of carbon in perifollicular zones over the first 24 hours after injection. Up to this time the most rapid carbon accumulation occurred in CBA and C3Hf mice. After this time there was no real differences between strains in splenic accumulation of carbon. By 3 months, there was little carbon remaining in the spleens of all strains except BALB/c, in which significant deposits were still present.

Carbon clearances (Table 3a)

The relationship of the total body weight to the weights of liver and spleen $\frac{W}{wls}$, shows striking interstrain variations. NZB mice had significantly higher values for $\frac{W}{wls}$, (showing that their livers and spleens are smaller in proportion to their total body weight), than either young or old BALB/c ($t = 3.2406$, $p < 0.0025$ for young mice; $t = 1.7456$, $p < 0.05$ for old mice) or young CBA mice ($t = 7.4770$, $p < 0.0005$ for young mice). $\frac{W}{wls}$ for old NZB mice did not differ significantly from that calculated for old CBA mice. Young BALB/c mice had higher $\frac{W}{wls}$ values than CBA mice (16.2 ± 2.1 and 14.5 ± 0.5 respectively, $t = 2.1542$, $p < 0.025$), whereas old BALB/c mice had lower values than CBA mice (14.4 ± 1.5 and 16.6 ± 1.1 respectively, $t = 4.0961$, $p < 0.0005$). The phagocytic indices (K_{16}) calculated for the six groups of mice again showed marked interstrain variations, with NZB mice having significantly lower values (cf. young BALB/c mice $t = 3.3725$, $p < 0.0025$; old BALB/c mice $t = 2.4355$, $p < 0.02$; young CBA mice $t = 9.6250$, $p < 0.0005$; old CBA mice $t = 1.7109$, $p < 0.05$).

Although K_{16} old CBA and BALB/c mice did not differ significantly, young CBA mice had higher values than young BALB/c mice ($t = 4.9273$, $p < 0.0005$).

When corrected for body weight against liver and spleen weights, the corrected phagocytic indices (λ) showed little interstrain variation.

DISCUSSION

The overall patterns of peritoneal cellular responses to carbon, and its distribution within the tissues have been studied, and were not shown to be significantly different in the six strains of mice studied. In particular the changes in NZB and BWF_1 mice were not shown to differ significantly from control strains; although the total peritoneal cell counts tended to be higher in these two strains, their differential cell counts were similar to those of the other four strains. Apart from the kinetics of the peritoneal cellular response, this experiment also provides an index of phagocytosis of colloidal carbon by polymorphonuclear leucocytes, peritoneal macrophages, and the fixed macrophage of thoracic lymph nodes, liver and spleen; no significant abnormality of phagocytosis in NZ mice was detected. Studies of interstrain differences of phagocytosis by polymorphonuclear leucocytes and peritoneal macrophages have to my knowledge not been performed, but studies of phagocytosis by the fixed macrophages of liver and spleen by the clearance of intravenous colloidal carbon have shown little or no interstrain variation of the corrected phagocytic indices (α) (160-162). Studies by Morton and Siegel (163) showed normal intravenous carbon clearance by NZ mice, and this agrees with my results, which have shown that although NZB mice have lower K_{16} values than the other two strains, once correction is made for body weight in relation to liver and spleen weights then there is no significant interstrain difference. The high $\frac{W}{wls}$ value in old

NZB mice must be commented on as one would expect large spleen weights as a result of the haemolytic anaemia which they develop. However, old NZB mice in our colony also became markedly obese with age and this offsets the increased spleen weights.

Although one must conclude from these experiments that there is no evidence of abnormality in the cellular response to, and the phagocytosis of, administered colloidal carbon, one must exercise caution before concluding that phagocytosis is normal in these animals. Carbon is relatively non-toxic and is not immunogenic; it is conceivable that the macrophages of NZ mice might be shown to respond abnormally to sheep erythrocytes or the soluble protein antigens to which these mice are known to produce exaggerated immune responses. More accurate assessment of peritoneal macrophage function might be obtained by in vitro studies of phagocytosis, which may disclose functional defects of relevance to the pathogenesis of auto-immune disease in NZ mice.

CHAPTER 4.

IN VITRO STUDIES ON THE PHAGOCYTOSIS OF *STAPHYLOCOCCUS AUREUS* BY PERITONEAL MACROPHAGES OF NEW ZEALAND MICE

INTRODUCTION

As the phagocytosis of colloidal carbon was shown to be normal in the previous chapter, it was decided to study the function of mouse peritoneal macrophages *in vitro*. In this chapter I describe a series of experiments on the ability of peritoneal macrophages of six strains of mice to phagocytose and subsequently kill *Staphylococcus aureus* in a supravital system.

MATERIALS AND METHODS

Mice aged 6-8 weeks of the inbred strains NZB, NZW, CBA, C3Hf and BALB/c, and BWF_1 mice were investigated. Older NZB and CBA mice, aged 9-10 months, were also studied.

The method used was based on that described by Holmes, Quie, Windhorst, Pollara and Good (164) for studying phagocytosis by polymorphonuclear leucocytes. The principle of the method is to incubate a known number of peritoneal macrophages with a known number of bacteria and to determine the number of bacteria which had been phagocytosed at a given time. Cellular bactericidal activity was estimated by adding antibiotics to kill extracellular bacteria, washing and lysing the cells, and determining the number of viable bacteria released. This number was then subtracted from the total number phagocytosed to give the number killed by macrophages.

Preparation of suspensions of peritoneal macrophages

Peritoneal exudation was stimulated by the intraperitoneal injection of 1 ml. of 0.1% glycogen in 0.15 M saline. Four days later the mice were killed by cervical dislocation and the peritoneal cavity was exposed aseptically. Two ml. of Hank's solution, containing 10 units of preservative free heparin was injected into the peritoneal cavity and the peritoneal fluid immediately withdrawn and placed in sterile siliconised test tubes. The aspirates from 4 mice of the same strain were pooled in each tube. The peritoneal aspirates were centrifuged at 250 g. for 10 minutes at $4^{o}C$, and the cell button obtained was washed thrice in Hank's solution and finally resuspended

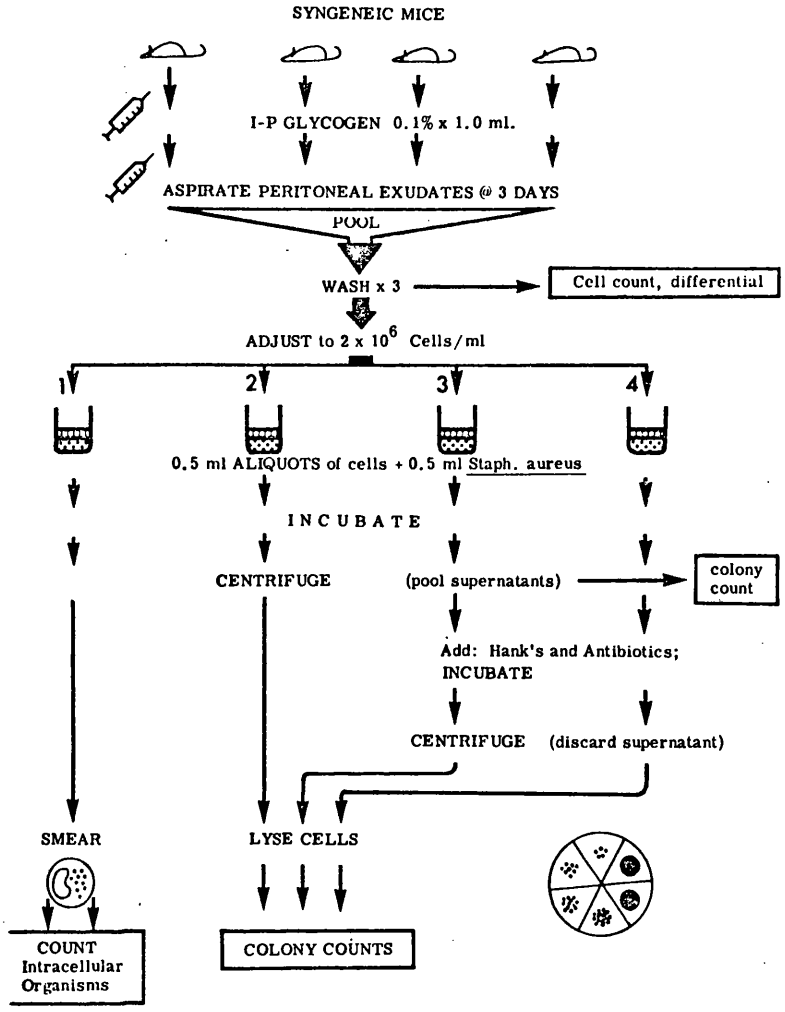


Fig. 16. Flow diagram of method of measuring phagocytic indices and bactericidal activity of peritoneal macrophages.

in 1 ml. of Hank's solution containing 10% foetal calf serum. A total cell count was performed and the volume then adjusted to give 2.0×10^6 cells/ml. A smear of the cell suspension was made and stained with May-Grunwald-Giemsa for a differential cell count. Sixty-five to 80% of the cells in the peritoneal exudates were macrophages and 2-4% were polymorphonuclear leucocytes, the remainder being lymphocytes.

One-half ml. of the cell suspensions, containing 1.0×10^6 cells, was placed in 2 series each of 4 test tubes (tubes 1-4).

Staphylococcus aureus

An overnight culture in nutrient broth of the Oxford strain (NCTC6571) of Staphylococcus aureus was washed thrice in 0.15 M saline, and then made up to its original volume in Hank's solution, containing 10% foetal calf serum. The bacterial concentration of $12 - 24 \times 10^6$ organisms per ml. One-half ml. of this final suspension was added to the cell suspensions of both series and to a control tube containing 0.5 ml. of Hank's solution with 10% foetal calf serum.

Determination of the phagocytic indices and bactericidal activity of peritoneal macrophages (Fig. 16).

All the tubes were incubated in a water-bath for 30 minutes at 37°C, and then centrifuged at 250 g. at 4°C for 10 minutes in order to separate free bacteria from those phagocytosed or adherent to the macrophage cell membranes. The supernatants from each series

of 4 tubes were pooled, and together with the control tube, kept in an ice-bath until the end of the experiment. The cells were washed three times in Hank's solution, and those from tubes 3 and 4 were resuspended in 0.5 ml. of Hank's solution containing 10% foetal calf serum and antibiotics (penicillin 100 units/ml., streptomycin 100 µg/ml.). These tubes were reincubated at 37°C, tube 3 for a further 30 minutes and tube 4 for a further 90 minutes, after which they were centrifuged as before and the cells washed three times in Hank's solution.

The cells from tube 1 were smeared on a microscope slide, and stained with May-Grunwald-Giemsa in order to count the number of bacteria ingested by individual macrophages.

The cells from tubes 2, 3 and 4 in each series were lysed by the addition of 1 ml. sterile distilled water. Serial ten-fold dilutions of the cell lysates, and of the supernatants of all tubes, including the control tubes, were made; samples were incubated on blood agar at 37°C for 16 hours, and bacterial colonies counted.

The number of bacteria phagocytosed was calculated from the difference between the bacterial count in the control tube and those of the supernatants (of tubes 1-4) at 30 minutes. The phagocytic indices were determined by dividing the number of bacteria phagocytosed by the number of macrophages in each tube, the latter being estimated from the total and differential cell counts (165). This method was adopted as attempts to determine the phagocytic indices by counting the number of bacteria phagocytosed per cell in stained

smears proved impossible due to the large number of bacteria engulfed by some of the cells. The numbers of viable intracellular bacteria at 30, 60 and 120 minutes were expressed as a percentage of the number of bacteria phagocytosed, as previously mentioned.

Day to day variation in the method

A marked day to day variation was found in the phagocytic indices, values varying by up to 30% in each strain. This appears to be due to the size of the bacterial inoculum, larger inocula being associated with higher phagocytic indices. For this reason, on each day, macrophages from all strains of mice and of the same sex were studied simultaneously, and the observations repeated on 4 occasions. The only exception to this was the experiment comparing the function of old and young NZB macrophages, which, due to animal availability, was performed on 2 occasions only. The results and studies of bactericidal activity showed a day to day variation of less than 10% for each strain.

TABLE 4.

PHAGOCYTIC INDICES OF MOUSE PERITONEAL MACROPHAGES IN CALF SERUM

	Young mice		Old mice	
	male	female	male	female
NZB	13.2	14.9	14.3	12.4
NZB/NZW F1	14.7	16.1	-	-
NZW	14.0	14.1	-	-
CBA	15.3	13.2	14.2	14.7
C3Hf	12.2	15.1	-	-
BALB/c	13.6	12.7		-

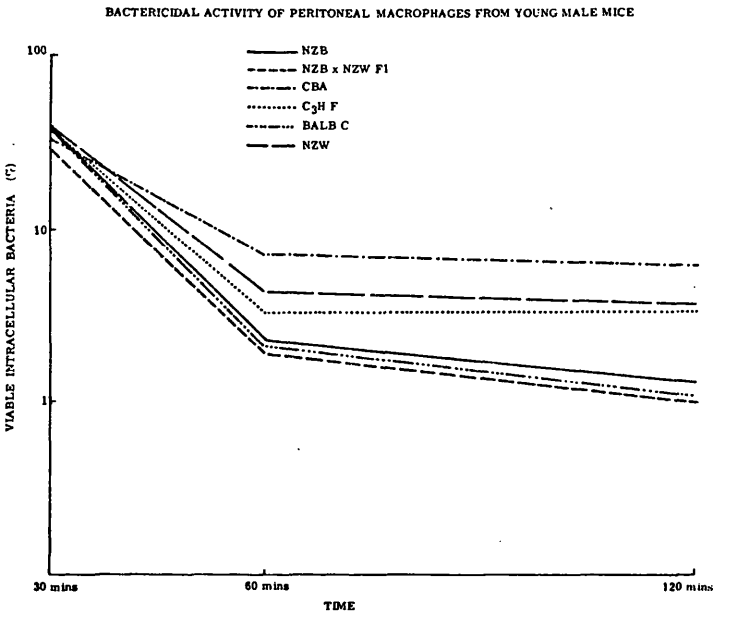


Fig. 17. A comparison of the bactericidal activity of peritoneal macrophages.

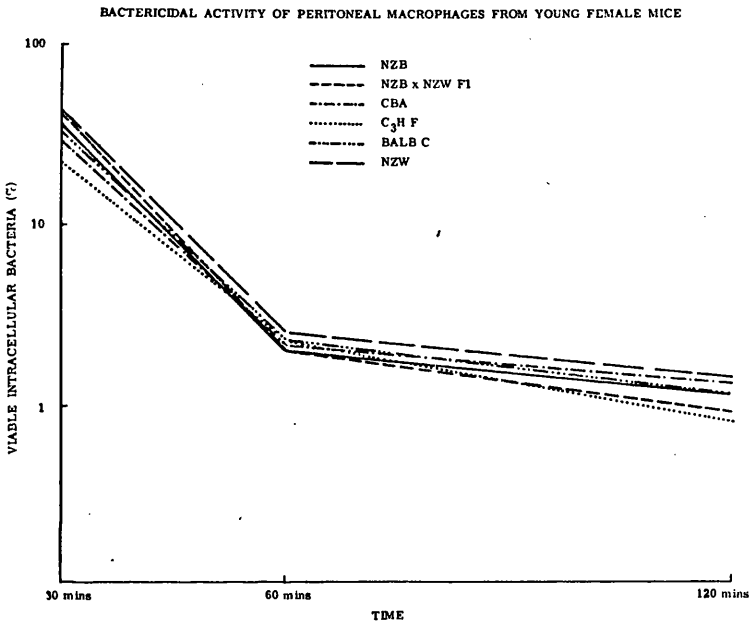


Fig. 18. A comparison of the bactericidal activity of peritoneal macrophages from young female mice.

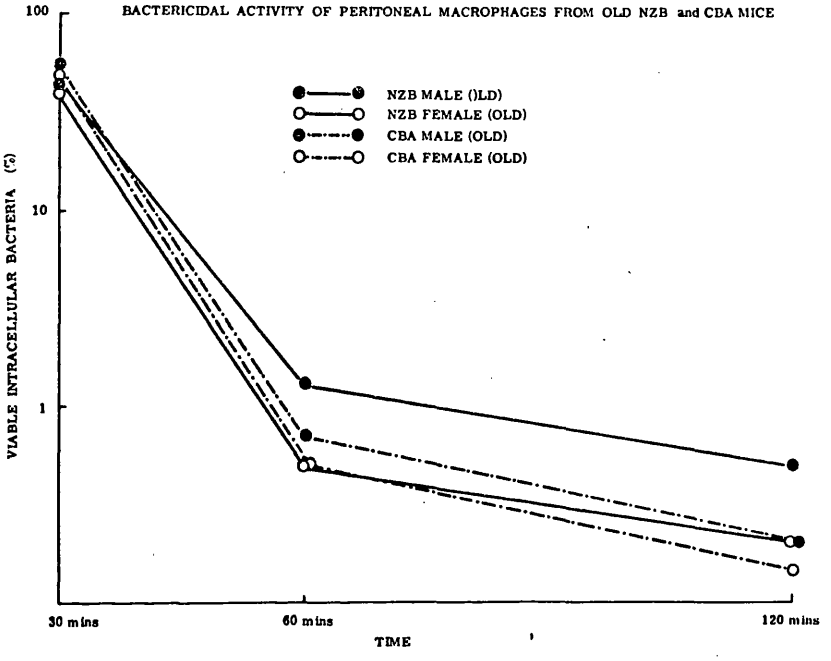


Fig. 19. A comparison of the bactericidal activity of peritoneal macrophages from old NZB and old CBA mice.

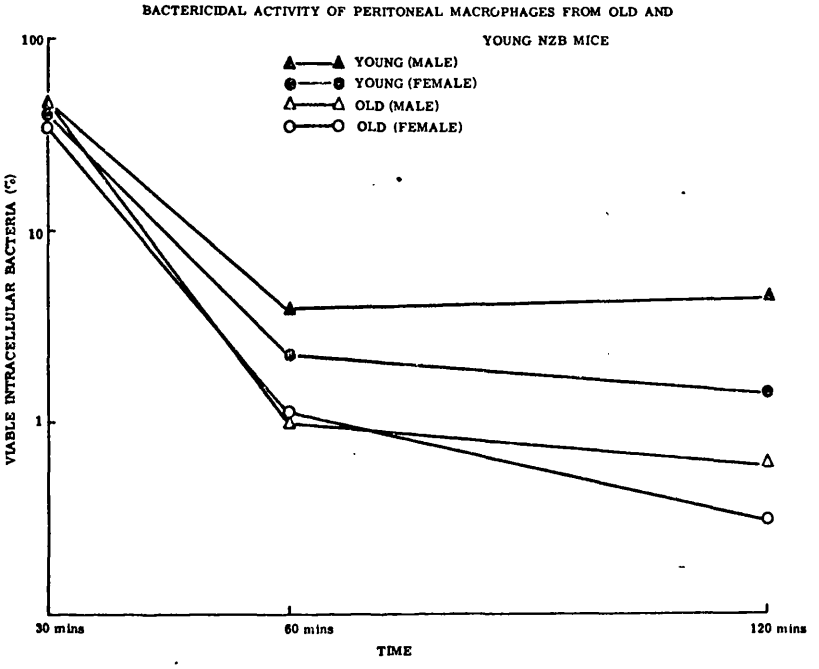


Fig. 20. A comparison of the bactericidal activity of peritoneal macrophages from young and old NZB mice.

RESULTS

(1) Phagocytic indices. The macrophage phagocytic indices in the six strains of mice are shown in Table 4. The mean values ranged between 12.1 and 16.1 for young mice and between 12.4 and 14.7 for old mice. There were no significant sex, age or interstrain differences.

(2) Bactericidal activity of macrophages. The bactericidal activities of peritoneal macrophages of young male and female mice are shown in Figs. 17 and 18. The mean percentages of viable organisms at 120 minutes for young male mouse macrophages ranged from 1.0% for NZB to 6.4% for CBA mice, and for young female mouse macrophages between 0.8 and 1.4%, the mean value for both male and female NZB mice being 1.1%. The mean percentages of viable organisms in old NZB and old CBA mouse macrophages at 120 minutes were 0.5% and 0.15% respectively. The highest number of viable bacteria was found in macrophages from old NZB male mice, the counts in old NZB females and old CBA males and females being closely grouped (Fig. 19).

Comparison of the bactericidal activities of macrophages from young and old NZB mice is shown in Fig. 20. At 120 minutes there were less viable bacteria in macrophages of old mice than in those from young mice on the two occasions that the experiment was performed. As only two experiments were performed, due to mouse availability, it was felt that statistical analysis could not reasonably be applied to such a small number of observations.

(3) Examination of NZB serum for factors affecting phagocytosis.

In similar experiments, heat-inactivated sterilised serum from

TABLE 5.

EFFECT OF SERUM FROM OLD AND YOUNG NZB MICE ON PHAGOCYTIC INDICES OF NZB PERITONEAL MACROPHAGES

Macrophages	Phagocytic index		
	Calf Serum	Young NZB Serum	Old NZB Serum
Young NZB	14.9	13.2	14.7
Old NZB	12.4	15.9	12.1

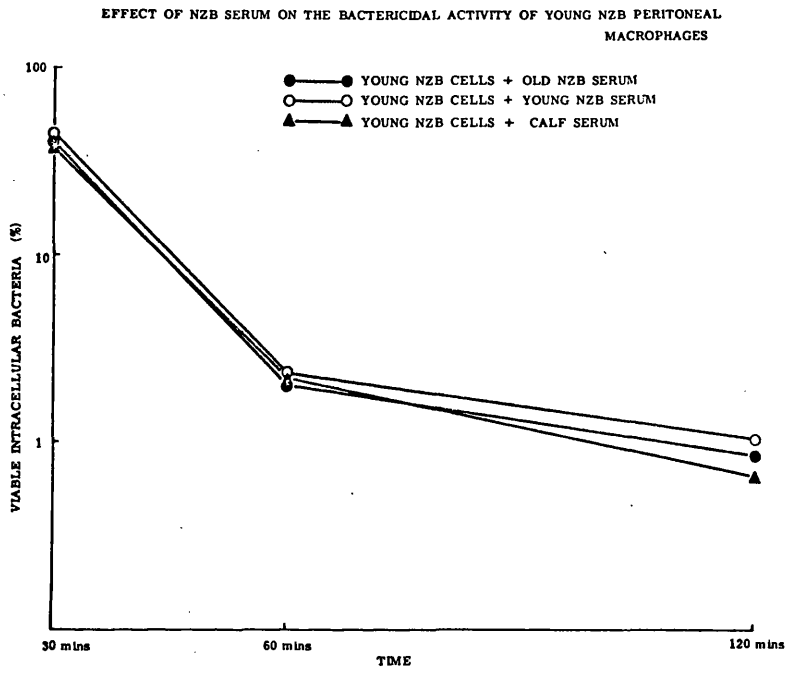


Fig. 21. A comparison of the effect of serum from young and old NZB mice and the bactericidal activity of peritoneal macrophages from young NZB mice.

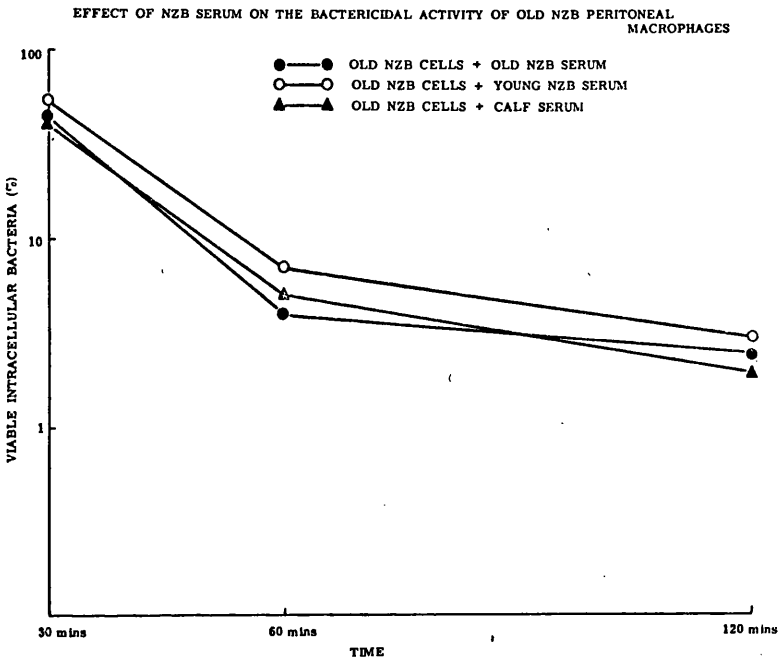


Fig. 22. A comparison of the effect of serum from young and old NZB mice on the bactericidal activity of peritoneal macrophages from old NZB mice.

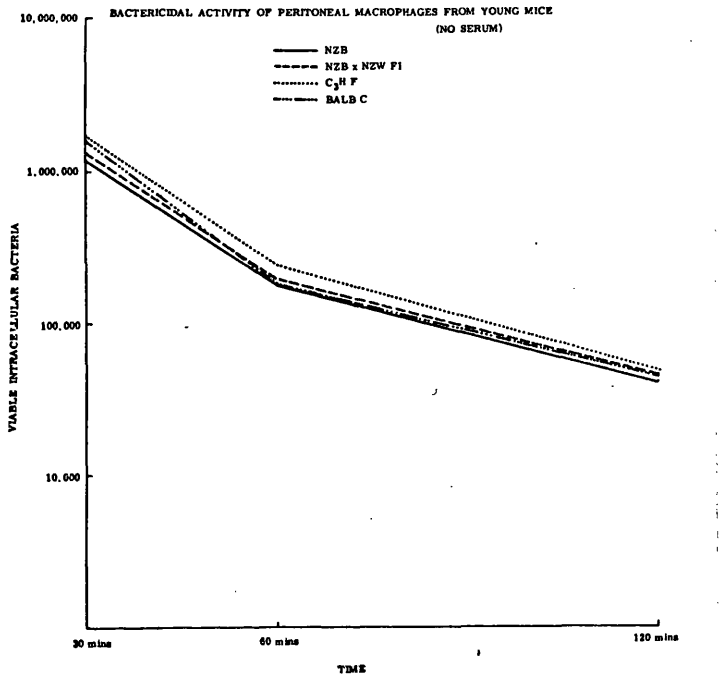


Fig. 23. A comparison of the bactericidal activity of peritoneal macrophages from young mice, in the absence of serum.

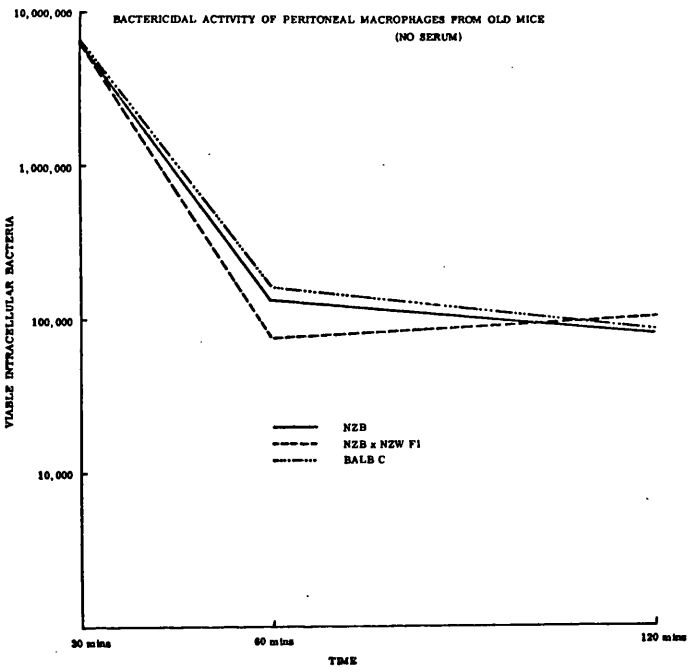


Fig. 24. A comparison of the bactericidal activity of peritoneal macrophages from old mice, in the absence of serum.

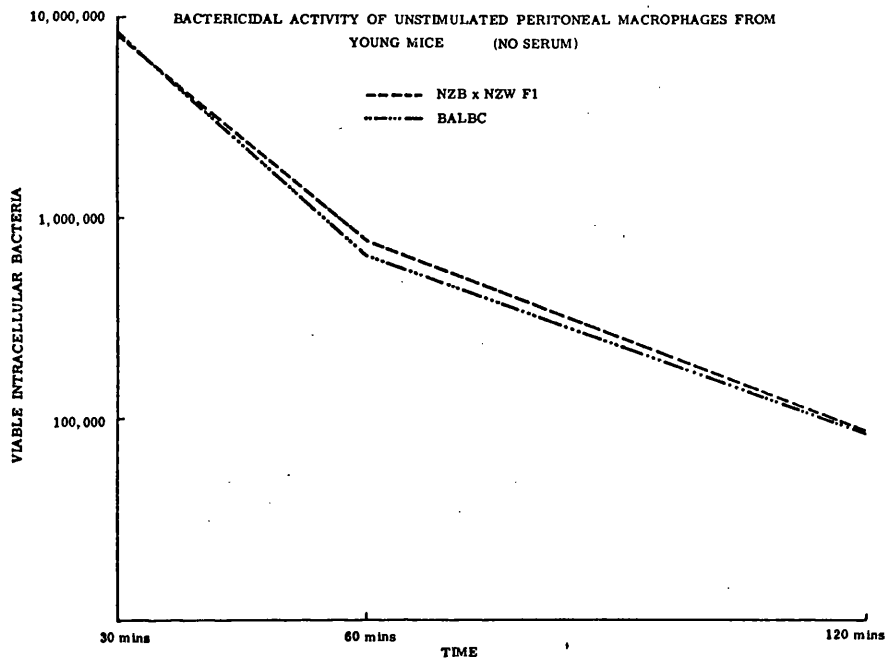


Fig. 25. A comparison of the bactericidal activity of unstimulated peritoneal macrophages from young mice, in the absence of serum.

young and old NZB mice was added to the Hank's solution in place of foetal calf serum. The phagocytic indices of macrophages incubated with NZB serum are shown in Table 5, and the bactericidal activity of young and old NZB macrophages in the presence of NZB serum is shown in Figs. 21 and 22. It can be seen that neither young nor old NZB serum affected either the phagocytic indices or bactericidal indices of NZB macrophages, whether from young or old mice.

(4) Phagocytosis in the absence of serum In the absence of serum, the bacteria in the control tube were present in greatly reduced numbers, although at 30 minutes the numbers of bacteria in supernatants and numbers of viable intracellular bacteria were similar to those in the experiments which included serum in the culture medium. As far fewer bacteria were present in the control tube than in the tubes containing cells, it is impossible to calculate the phagocytic indices, but from the intra- and extracellular bacterial counts at 30 minutes it may be concluded that neither phagocytosis nor bactericidal activity was affected by the absence of serum (Figs. 23 and 24).

To exclude the possibility that serum factors in the glycogen-stimulated exudates might have activated macrophages, an experiment was performed on unstimulated peritoneal macrophages obtained without use of glycogen. Although the phagocytic indices could not be calculated, the bactericidal activities were clearly unaltered (Fig. 25).

DISCUSSION

The peritoneal macrophages from six strains of mice have been studied for their ability to phagocytose and to kill Staphylococcus aureus. A major criticism of this method is that centrifugation and extensive washing of the peritoneal cells do not remove bacteria adherent to the macrophage cell membranes. However, as adherence of bacteria to the macrophage cell membrane is an important initial step in the process of phagocytosis (166), the phagocytic indices as derived in these experiments are meaningful. Indeed, in order to differentiate between the processes of attachment and engulfment, one would have to perform the experiments at low temperatures, in the presence of metabolic inhibitors (166, 167). The incorporation of antibiotics into the culture medium will obviously kill membrane-associated bacteria, giving falsely high bactericidal activities between 30 and 60 minutes. In addition, if penetration of the macrophages by antibiotics should occur, then the bactericidal activities will probably have little meaning as indices of macrophage function. However, using the same doses of penicillin and streptomycin as used in these experiments, Holmes et al. (165) showed that penetration of polymorphonuclear leucocytes does not occur, and Thorpe and Marcus (168) have shown that streptomycin does not penetrate polymorphs or macrophages, unless cell damage has occurred. Such damage cannot be detected using a light microscope, but it is unlikely that the experimental procedure described would have sufficiently traumatised the cells studied, although the bacteria themselves may have done so. No obvious difference in either phagocytic indices or bactericidal activities of macrophages was found between control strains

of mice and NZB and BWF_1 mice, and there is thus no evidence that abnormalities of phagocytic function are responsible for the immunological abnormalities which occur in the latter mice. That the presence of autoimmune disease itself did not affect the phagocytosis or bactericidal activity was shown by the similar results obtained using macrophages from 10 month old Coombs' positive NZB mice, old CBA. and young NZB mice. Likewise serum factors of young and old NZB mice did not affect macrophage phagocytic or bactericidal functions.

In patients with systemic lupus erythematosus defective phagocytosis of polymorphonuclear leucocytes has been shown to occur (169). This appears to be due to an intrinsic defect of the polymorphonuclear leucocytes as inhibitory serum factors could not be demonstrated. However, studies of macrophage function in patients with systemic lupus erythematosus have not been reported.

Although phagocytic indices could not be determined in those experiments which did not contain serum in the culture medium, the counts of viable bacteria in supernatants and in cells at 30 minutes indicate that neither phagocytosis nor bactericidal activity of macrophages was impaired by absence of serum. The possibility that this was due to activation of macrophages following glycogen stimulation in vivo appears to have been excluded by the experiment in which glycogen was not used to obtain peritoneal macrophages. Other possibilities include the presence of serum factors bound to macrophage cell membranes which are not removed by extensive washing; or lymphocyte factors released on exposure to Staphylococcus aureus. Staphylococcal filtrate has been

shown to stimulate the release of soluble factors from lymphocytes (170) and conceivably these may have activated the macrophages. Similar results have been obtained using peripheral polymorphonuclear leucocytes in the presence of lymphocytes (171). An attempt to exclude this possibility was made by prior incubation of the peritoneal exudate cells with anti-lymphocyte serum (kindly supplied by Dr. K.G. Gray) which had been repeatedly absorbed by monolayer cultures of mouse macrophages. However the anti-macrophage activity of this preparation was not completely absorbed and thus no conclusion could be reached.

Although the in vitro phagocytosis and killing of Staphylococcus aureus by mouse peritoneal macrophages has been shown to be quantitatively similar in NZB and BWF_1 mice, and the other strains investigated, it is still possible that phagocytosis of sheep erythrocytes and soluble protein antigens, to which these mice produce exaggerated immune responses, may be abnormal. In addition, the rate at which phagocytosed bacteria is digested by macrophages is not available from these experiments. Cohn (172) has shown that the intracellular digestion of phagocytosed bacteria is relatively slow, as compared with the time taken to kill them. Thus studies specifically designed to study antigen digestion would need to be performed to answer this point.

CHAPTER 5.

ANTIGEN CATABOLISM IN NEW ZEALAND MICE

INTRODUCTION

As the phagocytosis of both carbon and Staphylococcus aureus was shown to be normal in New Zealand mice, it was decided to study the catabolism (non-immune elimination) of soluble antigens. The antigens selected for study were bovine gamma globulin, (BGG), bovine serum albumin, (BSA), and polyvinylpyrolidone (PVP). New Zealand mice have previously been shown to have heightened immune responses to BGG and BSA (107, 108, 109, 110), and thus the study of their non-immune elimination rates might provide relevant information concerning this aspect of their disease.

MATERIALS AND METHODS

Mice

Five inbred strains of mice were used - NZB, NZW, BALB/c, CBA and C3Hf, and also the BWF_1 hybrid, in some studies NZB x BALB/c F1 hybrids were included. All mice were aged 6-8 weeks at the onset of the studies, and were housed under standard conditions and fed standard mouse diet. Fluid was supplied as half-strength physiological saline containing 0.01 per cent potassium iodide, beginning two days before the administration of antigen (see below) and continuing throughout the duration of the experiment. Saturation of the mice with iodide ensures the rapid excretion of ^{131}I released during antigen catabolism.

Antigens Used

Highly purified bovine gamma globulin, BGG (Cohn Fraction 11; Koch-Light Laboratories) and bovine serum albumin, BSA (Armour Pharmaceuticals Ltd.) were labelled with I^{131} tracer (I.B.S.3; Radiochemical Centre, Amersham) by the chloramine-T method of Hunter and Greenwood (173). Protein aggregates were removed from the antigen solutions by centrifugation at 30,000 g. for 30 minutes at $4^{o}C$, and discarding the lower one-third of the supernatant. Protein concentrations of the supernatants were then ascertained using a Unicam SP 500 Ultraviolet spectrophotometer at 280 mμ. the final protein concentrations then being adjusted to 2 mg. per ml., Aggregates are removed as they are phagocytosed much more rapidly than the soluble protein which is catabolised relatively slowly. The labelled antigens had specific activities of

approximately 7 μCi per mg.

The third antigen used polyvinylpyrrolidone, PVP (^{131}I) (I. B. 33P, Amersham), which has an average molecular weight of 30,000-40,000 and a specific activity of 10-50 μCi per mg. The concentration was adjusted with phosphate buffered saline to 2 mg. per ml.

Mice were injected intraperitoneally on Day 0 with 1.0 mg. of antigen.

Counting procedure

The whole body radioactivity of each animal was determined at intervals following injection by placing the mouse in a centred plastic well scintillation counter (Nuclear Enterprises). In addition mice were bled at intervals from the retro-orbital venous plexus into 20 μl. capillary tubes (Drummond Microcaps, Shandon Ltd.); each specimen was lysed in 1.0 ml of 0.1 per cent sodium carbonate solution in glass counting tubes, and radioactivity counted in an automatic gamma-counter (Nuclear Chicago). Counts were corrected for isotope decay, and the half-life ($T\frac{1}{2}$) of antigen elimination was calculated preliminarily by plotting the corrected counts semi-logarithmically against time, and then for the purposes of statistical analysis, by computing them by the least squares method.

Counting Schedules

With BGG only the whole body disappearance was studied, animals being counted on alternate days beginning 48 hours following injection. Removal of residual BGG aggregates is rapid, and by 48 hours the mono-exponential clearance of the soluble protein is observed (177).

Animals receiving BSA were counted and also bled at the following times after injection of antigen: 18, 29, 42, 52 and 66 hours. The counts were not performed until 18 hours, as after this time elimination of BSA oligopolymers is complete and the elimination curve thereafter is mono-exponential, representing clearance of monomer alone (174).

Animals receiving PVP were counted and bled at 10, 24, 32 and 48 hours. As a marked divergence between whole body and blood elimination rates became apparent during the course of this experiment, a further whole body counts were performed at 145 and 290 hours, and the mice were also bled at 73, 120, and 290 hours. Mice receiving PVP were exsanguinated after 4 weeks, killed by cervical dislocation, and the radio-activity present in their livers, spleens, kidneys and thymuses was determined.

Statistical analysis

Inter-strain differences and intra-strain sex differences in antigen catabolism rates ($T\frac{1}{2}$ values), and for the PVP experiment inter-strain variation in organ content of antigen, were compared using the Student's "t" test.

TABLE 6 - STRAIN DIFFERENCES IN THE WHOLE BODY ELIMINATION OF BGG

Mouse Strain	No. In Group	$T.\frac{1}{2}$ (hrs.) sex difference	Significance of sex difference	$T.\frac{1}{2}$ (hrs) whole group	Significance of inter-strain differences				
					C3Hf	CBA	BALB/c	BWF_1	NZ
NZB	8 M	49.7 ± 9.2	N S	50.09 ± 8.51	t=5.8124	t=6.1769	t=4.5698	t=2.7803	t=6.8
	7 F	50.5 ± 8.4			p<0.0005	p<0.0005	p<0.0005	p<0.005	p<0.00
NZW	8 M	109.3 ± 20.7	N S	99.94 ± 27.04	t=3.9062	N S	t=4.0746	t=5.6086	
	8 F	90.6 ± 30.7			p<0.0005		p<0.0005	p<0.0005	
BWF_1	8 M	62.7 ± 6.5	N S	59.46 ± 10.09	t=3.0983	t=4.6327	t=2.2466		
	8 F	56.2 ± 12.3			p<0.0025	p<0.0005	p<0.025		
BALB/c	8 M	70.3 ± 18.2	N S	69.00 ± 13.74	N S	t=2.8253			
	8 F	67.7 ± 8.3				p<0.005			
CBA	8 M	99.1 ± 16.5	t = 2.4475	87.36 ± 21.83	t=2.8996				
	8 F	75.6 ± 20.8	p < 0.025		p<0.005				
C3Hf	8 M	66.2 ± 10.5	N S	71.25 ± 11.45					
	8 F	76.3 ± 10.6							

TABLE 7 - STRAIN DIFFERENCES IN THE WHOLE BODY ELIMINATION OF BSA

Mouse Strain	No. in Group	$T.\frac{1}{2}$ (hrs) sex difference	Significance	$T.\frac{1}{2}$ (hrs) whole group	Significance of inter-strain differences: NZB x BALB/c F_1	C3Hf	CBA	BALB/c	BWF_1	N
NZB	5 M	14.38 ± 0.34	t=2.8714	15.41 ± 1.67				t=3.1165	t=5.8459	
	4 F	16.70 ± 1.80	$p<0.01$		N S	N S	N S	$p<0.005$	$p<0.0005$	N
NZW	6 M	13.86 ± 0.78		14.34 ± 0.92	t=6.1160	t=4.4954	t=4.8007	t=6.4291	t=9.0094	
	5 F	14.80 ± 0.96	N S		$p<0.0005$	$p<0.0005$	$p<0.0005$	$p<0.0005$	$p<0.0005$	
BWF_1	4 M	20.09 ± 3.17		20.80 ± 2.13	t=3.7739	t=4.7110	t=6.7969	t=4.1527		
	5 F	21.22 ± 1.53	N S		$p<0.005$	$p<0.0005$	$p<0.0005$	$p<0.0005$		
BALB/c	6 M	18.17 ± 1.32		17.50 ± 1.34			t=3.2084			
	5 F	16.70 ± 0.91	N S		N S	N S	$p<0.005$			
CBA	6 M	16.26 ± 0.51		16.01 ± 0.62	t=3.3311					
	4 F	15.65 ± 0.63	N S		$p<0.005$	N S				
C3Hf	5 M	15.33 ± 1.02	t=4.6715	16.85 ± 1.62						
	7 F	17.94 ± 0.91	$p<0.0005$		N S					
NZB x BALB/c F_1	1 M	16.88	-	17.08 ± 0.50						
	4 F	17.00								

TABLE 8 - STRAIN DIFFERENCES IN THE BLOOD ELIMINATION OF BSA

Mouse Strain	No. in Group	$T.\frac{1}{2}$ (hrs) sex difference	Significance	$T.\frac{1}{2}$ (hrs) whole group	Significance of inter-strain differences				
					NZB x BALB/c F_1	C3Hf	CBA	BALB/c	BWF_1
NZB	5 M 4 F	12.65 ± 0.42 15.56 ± 0.71	$t=7.7177$ $p<0.0005$	13.94 ± 1.62	$t=3.7402$ $p<0.001$	N S	$t=4.1863$ $p<0.0005$	$t=5.5174$ $p<0.0005$	$t=7.5893$ $p<0.0005$
NZW	6 M 5 F	13.03 ± 1.13 13.91 ± 0.77	N S	13.42 ± 1.04	$t=6.6804$ $p<0.0005$	N S	$t=7.5907$ $p<0.0005$	$t=8.4663$ $p<0.0005$	$t=10.0492$ $p<0.0005$
BWF_1	4 M 5 F	19.39 ± 2.84 21.97 ± 1.16	N S	21.00 ± 2.20	$t=4.1103$ $p<0.001$	$t=9.4763$ $p<0.0005$	$t=6.6874$ $p<0.0005$	$t=4.7694$ $p<0.0005$	
BALB/c	6 M 5 F	18.05 ± 0.44 16.47 ± 1.10	$t=3.2422$ $p<0.005$	17.33 ± 1.12	N S	$t=7.1493$ $p<0.0005$	$t=2.8764$ $p<0.005$		
CBA	6 M 4 F	16.36 ± 0.55 15.97 ± 0.47	N S	16.21 ± 0.54	N S	$t=5.7881$ $p<0.0005$			
C3Hf	5 M 7 F	13.20 ± 0.52 14.86 ± 0.54	$t=5.3019$ $p<0.0005$	14.17 ± 1.00	$t=5.4288$ $p<0.0005$				
NZB x BALB/c F_1	1 M 4 F	17.01 16.74	-	16.79 ± 0.60					

TABLE 9 - A COMPARISON OF WHOLE BODY AND BLOOD BSA ELIMINATION RATES

Mouse	T. $\frac{1}{2}$ hours whole body	T. $\frac{1}{2}$ hours Blood	Significance
NZB	15.41 $\pm$ 1.67	13.94 $\pm$ 1.62	N S
NZW	14.34 $\pm$ 0.92	13.42 $\pm$ 1.04	t = 2.1901 p $<$ 0.025
BWF_1	20.80 $\pm$ 2.13	21.00 $\pm$ 2.20	N S
BALB/c	17.50 $\pm$ 1.34	17.33 $\pm$ 1.12	N S
CBA	16.01 $\pm$ 0.62	16.21 $\pm$ 0.54	N S
C3Hf	16.85 $\pm$ 1.62	14.17 $\pm$ 1.00	t = 4.8826 p $<$ 0.0005
NZB x BALB/c F_1	17.08 $\pm$ 0.50	17.69 $\pm$ 0.60	N S

RESULTS

BGG: (Table 6). Significantly shorter $T\frac{1}{2}$ values were found for NZB (50.1 hours) and BWF_1 (59.5 hours) since compared to the other strains. NZW mice had the longest $T\frac{1}{2}$ value (99.9 hours) and of the three "control" strains BALB/c the shortest half-life, at 69.0 hours. A significant sex difference was present only in CBA mice, males having longer half-lives.

BSA:

(a) Whole body (Table 7). The shortest $T\frac{1}{2}$ was found in NZW mice (14.3 hours), which differed significantly from all but NZB mice whose mean $T\frac{1}{2}$ was the second shortest. The slowest rates of antigen elimination occurred in BWF_1 mice (20.8 hours). Significant sex differences were present in NZB and C3Hf mice, in both instances the males having shorter half-lives than the females.

(b) Blood. (Table 8). NZW (13.4 hours) NZB (13.9 hours) and C3Hf (14.2 hours) mice had the shortest $T\frac{1}{2}$ values, and again the longest $T\frac{1}{2}$ occurred in BWF_1 mice (21.0 hours). Significant sex differences were again evident in NZB and C3Hf mice, and also in BALB/c mice, though in this latter strain the females had faster rates of elimination.

A comparison of whole body and blood BSA elimination rates (Table 9) showed that no statistically significant difference, except for NZW ($p < 0.025$) and C3Hf ($p < 0.0005$) mice, in which the whole body $T\frac{1}{2}$ was significantly longer than the blood $T\frac{1}{2}$.

TABLE 10 - STRAIN DIFFERENCES IN THE WHOLE BODY ELIMINATION OF PVP

Mouse Strain	No. In Group	$T.\frac{1}{2}$ (days) sex difference	Significance	$T.\frac{1}{2}$ (days) whole group	Significance of inter-strain differences				
					NZB x BALB/c F_1	C3Hf	CBA	BALB/c	BWF_1
NZB	6 M 5 F	46.5 ± 10.1 23.5 ± 2.6	N S	36.1 ± 21.3	N S	N S	N S	t=2.2364 p<0.025	N S
NZW	6 M 6 F	22.2 ± 6.0 23.9 ± 3.8	N S	23.1 ± 4.8	t=5.9846 p<0.0005	t=4.006 p<0.0005	t=6.5139 p<0.0005	N S	t=5.5490 p<0.0005
BWF_1	6 M 6 F	39.8 ± 4.8 33.2 ± 7.3	N S	36.5 ± 6.8	N S	N S	N S	t=5.7247 p<0.0005	
BALB/c	6 M 6 F	17.6 ± 4.4 26.1 ± 2.6	t=4.0776 p<0.005	21.8 ± 5.7	t=5.9518 p<0.005	t=5.5672 p<0.0005	t=6.5186 p<0.0005		
CBA	6 M 6 F	36.5 ± 4.1 32.9 ± 2.8	N S	34.7 ± 3.8	N S	N S			
C3Hf	4 M 8 F	32.7 ± 4.6 35.9 ± 6.2	N S	34.8 ± 5.7	N S				
NZB x BALB/c F_1	4 M 3 F	41.1 ± 8.2 36.5 ± 4.6	N S	39.1 ± 6.9					

TABLE 11 - STRAIN DIFFERENCES IN THE BLOOD ELIMINATION OF PVP

Mouse Strain	No. in Group	$T.\frac{1}{2}$ (hrs) sex difference	Significance	$T.\frac{1}{2}$ (hrs) whole group	Significance of inter-strain differences					
					NZB x BALB/c F_1	C3Hf	CBA	BALB/c	BWF_1	NZ
NZB	60 M 5 F	28.9 ± 2.3 29.8 ± 2.2	N S	29.3 ± 2.2	$t=5.0033$ $p<0.0005$	$t=5.5145$ $p<0.0005$	$t=6.3868$ $p<0.0005$	$t=3.4028$ $p<0.0025$	N S	N
NZW	6 M 6 F	27.9 ± 1.2 36.1 ± 0.7	$t=14.0217$ $p<0.0005$	32.0 ± 4.4	N S	N S	$t=2.9896$ $p<0.005$	N S	$t=2.0842$ $p<0.025$	
BWF_1	6 M 6 F	30.8 ± 1.5 25.7 ± 2.0	$t=2.3924$ $p<0.025$	28.3 ± 4.4	$t=3.5455$ $p<0.0025$	$t=4.5244$ $p<0.0005$	$t=5.3719$ $p<0.0005$	$t=3.5745$ $p<0.0025$		
BALB/c	6 M 6 F	32.8 ± 7.2 40.4 ± 3.8	$t=2.2755$ $p<0.025$	36.6 ± 6.8	N S	N S	N S			
CBA	6 M 6 F	37.3 ± 2.8 36.1 ± 3.7	N S	36.7 ± 3.2	N S	N S				
C3Hf	4 M 8 F	35.0 ± 1.4 35.0 ± 1.2	N S	35.0 ± 2.7	N S					
NZB x BALB/c F_1	4 M 3 F	30.8 ± 1.5 25.7 ± 2.0	$t=2.3924$ $p<0.025$	28.3 ± 4.4						

TABLE 12 - STRAIN DIFFERENCES IN PVP RETENTION BY THE LIVER

Mouse Strain	No. in Group	% of dose in liver sex difference	Significance	% of dose in liver whole group	Significance of inter-strain differences				
					NZB x BALB/c F_1	C3Hf	CBA	BALB/c	BWF_1	NZ
NZB	6 M 5 F	26.78 ± 2.48 27.96 ± 9.38	N S	27.32 ± 6.22	N S	$t=4.2025$ $p<0.0005$	N S	$t=3.8411$ $p<0.0005$	$t=2.8203$ $p<0.005$	N
NZW	6 M 6 F	24.55 ± 2.59 24.02 ± 1.87	N S	24.26 ± 2.12	$t=2.8606$ $p<0.0125$	$t=9.8500$ $p<0.0005$	$t=3.1099$ $p<0.005$	$t=7.9719$ $p<0.0005$	$t=2.2802$ $p<0.025$	
BWF_1	6 M 6 F	21.74 ± 1.65 22.23 ± 4.32	N S	21.96 ± 2.98	$t=3.7536$ $p<0.0025$	$t=10.5120$ $p<0.0005$	$t=4.3085$ $p<0.0005$	$t=8.8218$ $p<0.0005$		
BALB/c	6 M 6 F	38.56 ± 3.34 34.07 ± 5.07	N S	35.86 ± 4.83	$t=2.3067$ $p<0.025$	N S	$t=3.3508$ $p<0.0025$			
CBA	6 M 6 F	26.99 ± 5.71 31.55 ± 4.31	N S	29.17 ± 5.33	N S	$t=3.6778$ $p<0.0025$				
C3Hf	4 M 8 F	38.73 ± 3.37 34.48 ± 3.14	$t=2.4521$ $p<0.025$	35.64 ± 3.62	$t=2.5577$ $p<0.0125$					
NZB x BALB/c F_1	3 M 4 F	26.91 ± 8.57 32.89 ± 4.83	N S	29.90 ± 7.03						

TABLE 13 - STRAIN DIFFERENCES IN PVP RETENTION BY THE SPLEEN

Mouse Strain	No. in Group	% of dose in spleen sex difference	Significance	% of dose in spleen whole group	NZB x BALB/c F_1	Significance of inter-strain differences C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	6 M 5 F	2.44 ± 0.34 2.36 ± 0.37	N S	2.40 ± 0.34	t=7.1905 p 0.0005	t=9.0704 p 0.0005	t=3.4606 p 0.0025	t=8.1631 p 0.0005	N S	N S
NZW	6 M 6 F	2.30 ± 0.10 2.58 ± 0.21	t=3.2052 p 0.005	2.45 ± 0.22	t=9.1855 p 0.0005	t=10.0946 p 0.0005	t=3.4482 p 0.0025	t=8.3567 p 0.0005	N S	
BWF_1	6 M 6 F	2.28 ± 0.32 2.48 ± 0.64	N S	2.37 ± 0.47	t=5.6458 p 0.0005	t=7.8207 p 0.0005	t=3.2695 p 0.0025	t=7.7341 p 0.0005		
BALB/c	6 M 6 F	4.05 ± 0.95 4.66 ± 0.71	N S	4.41 ± 0.83	t=3.2172 p 0.005	t=2.8789 p 0.005	t=5.1495 p 0.0005			
CBA	6 M 6 F	2.76 ± 0.57 3.28 ± 0.45	N S	3.02 ± 0.56	N S	t=3.6019 p 0.0025				
C3Hf	4 M 8 F	3.25 ± 0.13 3.85 ± 0.31	t=3.9741 p 0.0025	3.69 ± 0.39	N S					
NZB x BALB/c F_1	3 M 4 F	3.28 ± 0.28 3.57 ± 0.18	N S	3.42 ± 0.27						

TABLE 14 - STRAIN DIFFERENCES IN PVP RETENTION BY THE THYMUS

Mouse Strain	No. in Group	% of dose in thymus sex difference	Significance	% of dose in thymus whole group	Significance of inter-strain differences					
					NZB x BALB/c F_1	C3Hf	CBA	BALB/c	BWF_1	NZ
NZB	6 M 5 F	0.48 ± 0.15 0.26 ± 0.13	$t=3.0495$ $p<0.0125$	0.38 ± 0.18	N S	N S	N S	N S	N S	$t=2.3$ $p<0.0$
NZW	6 M 6 F	0.19 ± 0.06 0.30 ± 0.10	$t=2.6846$ $p<0.0125$	0.25 ± 0.10	$t=3.7381$ $p<0.0025$	N S	N S	N S	N S	
BWF_1	6 M 6 F	0.40 ± 0.17 0.31 ± 0.08	N S	0.36 ± 0.14	N S	N S	N S	N S		
BALB/c	6 M 6 F	0.25 ± 0.07 0.38 ± 0.05	$t=4.4888$ $p<0.0025$	0.33 ± 0.09	N S	N S	N S			
CBA	6 M 6 F	0.23 ± 0.10 0.29 ± 0.03	N S	0.26 ± 0.08	$t=4.0827$ $p<0.0005$	N S				
C3Hf	4 M 8 F	0.34 ± 0.13 0.24 ± 0.09	N S	0.27 ± 0.10	$t=3.2443$ $p<0.0025$					
NZB x BALB/c F_1	3 M 4 F	0.33 ± 0.04 0.52 ± 0.06	$t=7.0162$ $p<0.0005$	0.43 ± 0.11						

TABLE 15 - STRAIN DIFFERENCES IN PVP RETENTION BY THE KIDNEY

Mouse Strain	No. in Group	% dose in kidney sex difference	Significance	% dose in kidney whole group	Significance of inter-strain differences					
					NZB x BALB/c F_1	C3Hf	CBA	BALB/c	BWF_1	NZ
NZB	6 M 5 F	0.85 ± 0.07 0.85 ± 0.16	N S	0.84 ± 0.11	$t=6.7362$ $p<0.0005$	$t=5.8239$ $p<0.0005$	$t=5.0405$ $p<0.0005$	$t=6.0020$ $p<0.0005$	N S	$t=3.8$ $p<0.0$
NZW	6 M 6 F	0.98 ± 0.20 1.62 ± 0.35	$t=12.0458$ $p<0.0005$	1.33 ± 0.44	N S	N S	N S	N S	$t=3.2196$ $p<0.0025$	
BWF_1	6 M 6 F	0.80 ± 0.13 0.94 ± 0.42	N S	0.87 ± 0.29	$t=3.5326$ $p<0.0025$	$t=3.5035$ $p<0.0025$	$t=3.6879$ $p<0.0025$	$t=3.6879$ $p<0.0025$		
BALB/c	6 M 6 F	1.04 ± 0.14 1.37 ± 0.12	$t=13.5083$ $p<0.0005$	1.24 ± 0.21	N S	N S	N S			
CBA	6 M 6 F	1.12 ± 0.28 1.27 ± 0.14	$t=3.7543$ $p<0.0025$	1.19 ± 0.22	N S	N S				
C3Hf	4 M 8 F	1.27 ± 0.25 1.18 ± 0.18	$t=3.6269$ $p<0.0025$	1.20 ± 0.19						
NZB x BALB/c F_1	3 M 4 F	1.24 ± 0.07 1.33 ± 0.03		1.29 ± 0.19						

PVP: The PVP used in this study is a mixture of polymers different molecular weights, and although the average molecular weight is 30,000-40,000, there is a range from less than 10,000 to over 80,000 (175). By the time counts were performed 10 hours after injection, 40-50% of the PVP had already been eliminated in all mice; this is due to the rapid renal clearance of low molecular weight PVP, (176).

(a) Whole body (Table 10). The whole body half-life of PVP was very long in all strains of mice, the longest being found in NZB x BALB/c F1 (39.1 days) BWF_1 (36.5 days) and NZB mice (36.1 days). BALB/c mice had the shortest $T\frac{1}{2}$ (21.8 days) which was not significantly different from that found in NZW mice (23.1 days). A significant sex difference was only present in the BALB/c strain, male mice having a shorter $T\frac{1}{2}$ (17.6 days) than females (26.1 days).

(b) Blood (Table 11). In contrast to the extremely long whole body half-lives for PVP, the mean blood elimination $T\frac{1}{2}$ values ranged from 28.3 hours for BWF_1 mice to 36.7 hours for CBA mice. BWF_1 and NZB ($T\frac{1}{2}$ = 29.3 hours) mice had significantly shorter elimination rates than all other strains except NZW mice, which occupy an intermediate place.

(c) Organ distribution of PVP. The obvious retention of this antigen in the animal led us to evaluate its distribution. Tables 12, 13, 14 and 15 show the percentage of administered PVP dose remaining in livers, spleens, thymuses, and kidneys at 14 days. It can be seen that between one quarter

and one-third of the administered dose is found in the liver and spleen. Those strains having the fastest blood clearance have the least remaining PVP in the organs counted viz. BWF_1, NZB, and NZW. The whole body half-lives for PVP do not show this correlation; probably reflecting the relative inaccuracy of whole body half-lives, which were based on counts obtained over the first half to one-third of the biological half-life found for PVP.

DISCUSSION

In these experiments I have investigated the rates of elimination of three soluble antigens BGG, BSA and PVP in seven strains of mice. Marked inter-strain variations were found in the rates at which each antigen was eliminated. The relative rates at which these different antigens were catabolised also varied considerably within each strain of mouse, although in general New Zealand mice catabolised all antigens more rapidly than other strains. Exceptions were the slow rate of BGG catabolism in NZW mice, and of BSA catabolism in BWF_1 mice. It is perhaps of interest to note that with the exception of BSA catabolism in BWF_1 mice, the $T\frac{1}{2}$ values for the F1 hybrid mice studied is intermediate between the values found for the parent strains. This probably indicates a degree of genetic control over antigen catabolism, the extent of which could be easily evaluated by also studying the F2 generation, and the back cross of the F1 generation with parent strains. If antigen catabolism is genetically controlled, it is difficult to explain why the BWF_1 hybrid catabolised BSA more slowly than either parent strain. On the other hand, the nephritis they develop is far more florid and appears earlier than in either parent strain (78, 80, 87) and the response of BWF_1 mice to immunisation with heat-denatured DNA-methylated BSA complex also exceeds that of either parent strain (103). These observations show that the phenotype of the F1 progeny cannot always be predicted from knowledge of the parents' genotypes.

Dresser (177) showed that the catabolism of BGG could be studied effectively by whole body counting, as he found no significant difference between whole body and blood antigen elimination rates. Similarly it has been said that BSA catabolism can be studied by either method (177a). However, in this study I found that in C3Hf and to a lesser extent NZW mice the whole body antigen catabolism rates were significantly slower than those of the blood; this suggests that these two strains of mice are unable to digest BSA as rapidly as it can be phagocytosed. Indeed the observations on PVP catabolism are important as they illustrate the extreme example of an "indigestible" antigen which is efficiently phagocytosed but poorly broken down intracellularly. It must therefore be concluded that when antigen catabolism is to be studied in laboratory animals, blood as well as whole body half-lives should be determined initially in the strains of animals to be studied.

The relationship between antigen catabolism and immunogenicity is complex. It has been suggested that very rapid antigen catabolism may predispose to, or be related to, a subsequent immune response (178); conversely, slow rates of BSA elimination have been found in unresponsive mice of the Sobey strain (179). Accordingly, my finding of rapid rates of antigen catabolism in New Zealand mice may be an important factor in some of the immunological peculiarities of these mice, e.g. immunological hyperresponsiveness, and resistance to the induction of tolerance.

BALB/c mice have been shown to be relatively resistant to the induction of immunological tolerance to BGG (107, 107a, 108, 180), and of the "control" mice studied had the fastest rate of BGG catabolism, although they did not differ significantly from C3Hf mice.

Various factors have been shown to modify antigen catabolism, including the administration of such stimulatory factors as Freund's adjuvant (177, 178) Corynebacterium rubrum, Mycobacterium phlei and the peptidoglycolipid Wax D fraction WL 52 of Mycobacterium tuberculosis (181); conceivably an endogenous nucleic acid adjuvant present in NZB mice could be responsible for the rapid antigen catabolism. Such adjuvant activity has been suggested as being responsible for their exaggerated immune responsiveness (181a). Pharmacological doses of thyroxine have also been shown to increase BGG catabolism (181) and this latter observation may explain strain differences in antigen catabolism, as thyroxine secretion rates in mice have previously been found to show considerable interstrain variation (182-184). So far however, no such studies have been reported. The interstrain differences in antigen catabolism could also reflect fundamental variations in the ability of macrophages to phagocytose and process antigens. If this is so, then the differences between the catabolic rates for the three antigens studied within each mouse strain suggests that properties of both antigens and macrophages may affect the rate of antigen elimination. Although strain differences in the elimination

of low molecular weight soluble antigens do occur, the phagocytosis of DNA, a high molecular weight soluble antigen (chapter 8), colloidal carbon (163, chapter 3) and Staphylococcus aureus (chapter 4) were not found to show interstrain variations. However, as colloidal carbon is inert, and exaggerated immune responses to native DNA and Staphylococcus aureus have not been shown to occur in New Zealand mice, these findings do not exclude the possibility that hyperphagocytosis may be responsible for some of the many immunological peculiarities present in these mice.

CHAPTER 6.

ANTIGEN CATABOLISM IN NEW ZEALAND MICE: ITS RELATIONSHIP TO ANTIBODY RESPONSES

INTRODUCTION

In chapter 5 it was shown that New Zealand mice generally catabolised soluble antigens faster than other strains of mice. It has previously been suggested that more rapid rates of antigen catabolism (non-immune elimination) may contribute to immunogenicity (178). As New Zealand mice produce heightened circulating antibody responses (107-110) it is relevant to examine the relationship between antigen catabolism and antibody responses in these, and other strains of mice. In addition, the affinity of BSA antibody in these mice has been measured.

MATERIALS AND METHODS

The mice studied were those described in chapter 5. All had had antigen catabolism rates determined for BGG, BSA or PVP. All the mice in which a particular antibody response was being studied received the initial ^{131}I-labelled antigen from the same batch.

BGG antibody responses

Mice were bled from the retro-orbital venous sinus (185) 2 weeks following the administration of ^{131}I-BGG, challenged with 100 ug. of BGG in Freund's complete adjuvant (FCA) at 3 weeks, and bled again at 1, 2 and 4 weeks post-challenge. Sera were separated and stored at -20^{o}C until investigated. Antibodies to BGG were detected by passive haemagglutination (the agglutination of sheep erythrocytes coated with BGG).

Preparation of BGG coated sheep red cells

Sheep erythrocytes in Alsever's solution were obtained from Burroughs-Wellcome (Beckenham, Kent) and stored at 4^{o}C until used. In preparation for formalinisation they were washed in physiological saline until no further lysis occurred. The cells were then formalinised by Herbert's modification (186) of Csizmas' method (187). Twenty-five millilitres of packed cells were resuspended in 200 ml. of phosphate buffered saline (PBS 5%; 0.15M, pH 7.2), and placed in a 500 ml. conical flask. Fifty millilitres of commercial formalin (40% formaldehyde) were introduced into a length of dialysis tubing which was tied off in such a fashion that air was completely

excluded from the tube, which was only two-thirds full. The dialysis tubing was submerged in the sheep red cell suspension, and gently stirred at room temperature. After 3 hours the now-swollen dialysis sac was punctured, and the process of formalinisation continued overnight. The dark brown suspension was then filtered through gauze to remove debris, and the cells washed 5 times in physiological saline to remove the formalin. The cells were made up to a 25% suspension in physiological saline and stored at $4^{o}C$ until used.

The formalinised sheep erythrocytes were washed once and then coated with BGG by the following method (186). One millilitre of packed cells was added to each of 2 universal containers, and each made up to 10 ml. with phosphate buffered saline (PBS). Ten millilitres of tannic acid solution (5 mg. in 50 ml. of PBS), were added to each universal container, which were then agitated rapidly. The cells were allowed to stand at room temperature for 15 mins., separated from the tannic acid by centrifugation at 750 g. for 5 mins. at room temperature, after which they were washed twice and finally resuspended in 10 ml. of PBS. One universal container contained the control cells, and this was simply made up to 20 ml. by the addition of PBS, whereas the other cells were coated by the addition of 10 ml. of PBS containing

20 mg. of BGG (Cohn Fraction II. Koch Light Laboratories). Both containers were incubated at 37°C for 30 mins., with occasional shaking, after which the cells were separated from the supernatant by centrifugation at 750 g. for 5 mins. at room temperature. The cells were then washed three times in PBS containing 1% heat inactivated rabbit serum, which had previously been absorbed against sheep red cells, and finally resuspended to 100 ml. in this solution (1% suspension). Coated and control sheep red cell suspensions were kept at 4°C.

Estimation of BGG antibody titres

Sera were heat-inactivated at 56°C for 30 mins. and then absorbed against sheep red cells (1 drop of serum to 9 drops of control sheep red cells, final dilution 1 in 10) for 30-60 mins. at room temperature. The sera were then titrated in doubling dilution, starting at 1 in 10, using a standard micro-titer kit (Cooke Engineering Co., Alexandria, Virginia, U.S.A.). The results were read after overnight incubation at room temperature, and expressed as $\log_2$.

The titres of 7S mercapto-ethanol resistant antibody were determined by incubating one drop of undiluted serum with one drop of 0.2M 2-mercapto-ethanol at 37°C for 60 mins. (114), after which 8 drops of the control sheep red cell suspension were added to each tube (final dilution 1 in 10), and allowed to

stand for 30-60 mins. at room temperature. Titration of sera and the reading of the antibody titres was performed as before.

Controls were rabbit anti-BGG serum, and normal rabbit serum, and were included in every group of tests. All sera obtained on the same day were tested at the same time.

BSA antibody responses

Mice were bled 7 days after the administration of 1.0 mg. of ^{131}I-BSA, injected intraperitoneally with 100 µg. of BSA in physiological saline, bled on day 7 of the secondary response, then challenged with 100 µg. of BSA in FCA and bled 10 days later. Sera were separated and stored at -20°C until used. Antibody to BSA were detected using the ammonium sulphate precipitation technique of Farr (188, 189), which is based on the knowledge that at a 40% saturation of ammonium sulphate, the globulin fraction is completely precipitated from serum whereas albumin remains in solution (190). If antibody to BSA is present in a particular serum and BSA is added, then that part of the BSA complexed with antibody is precipitated with the globulin fraction, whereas the unbound BSA remains in solution. The amount of antigen precipitated is proportional to the total antibody present.

Labelling of BSA with ^{131}I

Two milligrams of BSA dissolved in 1 ml. of PBS, and 0.25 mg. of chloramine T in 0.25 ml. of PBS were added to a vial containing 3mCi of ^{131}I (IBS 3, Radiochemicals Centre, Amersham) in 0.2 ml. volume. The contents were mixed carefully and the reaction allowed to continue for 3 mins., after which time 120 µg. of sodium metabisulphite in 50 ul. of PBS, and 2.0 mg. of potassium iodide in 0.2 ml. were added, with gentle shaking, to stop the reaction. The contents of the vial were pipetted carefully onto the top of a sephadex column (G25 coarse) and eluted with PBS. The eluate was collected in test tubes in 1 ml. volumes, and the distribution of the radioactivity determined using a hand monitor. The three 1 ml. samples containing the most radioactivity were pooled, ultracentrifuged to remove aggregates, as described in the previous chapter, and the final protein concentration determined spectrophotometrically. The antigen was kept in a lead container at $4^{o}C$ when not in use.

Estimation of BSA antibody titres

The antigen binding capacities of the sera were determined by a modification of the ammonium sulphate precipitation method of Farr (188, 189). Sera were initially diluted 1 in 4 in physiological saline containing 10% normal rabbit serum, and then by quadrupling dilution over four tubes. Sera having

high antibody titres were re-titrated beginning at a 1 in 30 dilution. Each test tube contained 0.1 ml. of diluted serum, to which 0.2 ml. of labelled BSA (1.0 µg/ml) were added. The contents of each tube were thoroughly mixed and allowed to stand at 4°C overnight. To all the tubes except the TCA controls, 0.2 ml. of saturated ammonium sulphate were added, and to the TCA controls, 0.3 ml. of 10% TCA were added. The tube contents were thoroughly mixed, and allowed to stand at 4°C for 2 hours to allow for complete precipitation. The precipitates were then separated by centrifugation at 2,300 r.p.m. for 30 mins. at 4°C, after which the supernatants were carefully removed and discarded. The precipitates which were retained were washed in 0.5 ml. of 40% saturated ammonium sulphate, except the TCA controls which were washed in 0.6 ml. of 10% TCA, and the tubes recentrifuged at 2,300 r.p.m. for 30 mins. at 4°C. The supernatants were again discarded, and the precipitates dissolved in 0.5 ml. of physiological saline. The test tubes were then inserted into counting tubes, and the radioactivity present in each tube determined using a Nuclear Chicago Automatic gamma-counter. The amount of antigen remaining in each tube was then calculated, using the TCA controls as 100% and the negative controls as background. The percentage of BSA in each tube was then plotted against dilution, and the curve drawn. The ABC 30 (µg/ml) value is calculated from the dilution of the test serum at which 30% of

the BSA is bound and expressed as $\log_{10}$. As many of the sera were of very low titre in the primary response, many of the ABC 30 values had to be calculated by extrapolation, and so the ABC 30 values were also calculated on a Hewlett-Packard desk calculator programmed with a standard Farr test curve, (Mackay - unpublished data).

A standard BSA antibody serum and normal rabbit serum were used as controls throughout the study.

All sera from the same bleed were assayed on the same day. For the purposes of statistical analysis, sera not containing measurable antibody were arbitrarily assigned the lowest ABC 30 value of the particular batch in which they were tested.

Estimation of affinity of BSA antibody and the concentration of antibody combining sites in the serum.

The antigen-antibody reaction is a reversible reaction which can be quantitatively represented by the equation:-

$Ag + Ab = AgAb$

Ag = free antigen

Ab = free antibody

AgAb = antigen-antibody complex.

The ability of antibody to bind with antigen is termed affinity, and is the equilibrium constant (K) for the reaction. Antibody affinity may be measured by several different techniques including equilibrium dialysis (396), fluorescence quenching (397), fluorescence

polarisation (398) and fluorescence enhancement (399). The first three of these methods require the purification of specific antibody, and the fluorescence quenching method is limited to antigens which exhibit certain fluorescence properties. Antibodies can only be purified by association with antigen, followed by dissociation, and therefore methods requiring the use of pure antibody obviously select antibody of high affinity, as low affinity antibody probably is not purified by this method. In order to circumvent these problems and measure antibody affinity in whole serum for a range of antigens, Soothill and Steward adapted the Farr technique with good results (206). In this study I used the method of Mackay (191) to determine both the antibody affinity and the absolute concentration of antibody in the serum. This method, like that described by Soothill and Steward (206), is a modification of the Farr technique.

The formula is derived from the general equilibrium equation for multivalent antigen and heterogeneous antibody:-

$$D \log_e F = - \int_0^{\infty} \frac{A(K)\, dK}{C + \frac{1}{nK}}$$

Where D = The final dilution factor of antiserum in the reaction mixture

F = The proportion of antigen remaining free at equilibrium.

K = The intrinsic association constant, i.e. the hypothetical association constant for the reaction between isolated antigenic determinants and isolated antibody combining sites.

A(K)dK = The serum concentration of antibody combining sites with the intrinsic association constant between K and K + dK.

C = The total concentration of antigen in the reaction mixture (in moles).

n = The number of antigenic determinants per determinant type per antigen molecule (probably n = 1 for BSA and HSA, since the molecules do not contain any duplicated polypeptide chains).

Assuming that the antibody is homogeneous, the equation thus becomes

$$D \log_e F = \int_o^{\infty} \frac{-A(K)dK}{C + \frac{1}{nK}} = \frac{-A}{C + \frac{1}{nK}}$$

i.e.

$$A = -D \left(C + \frac{1}{nK}\right) \log_e F$$

Since the antigen binding capacity (ABC 30) is equal to (1-F) CD_{30}, the following formulae for A and nK may be derived.

$$A = \frac{-\log_e (1-P)}{P} \times \frac{C_1 - C_2}{\frac{C_1}{a_1} - \frac{C_2}{a_2}}$$

and

$$nK = \frac{\frac{a_1}{C_1} - \frac{a_2}{C_2}}{a_2 - a_1}$$

Where A = The concentration of antibody combining sites in the serum (moles per ℓ), i.e. the absolute quantity of antibody present.

a_1 and a_2 are the antigen binding capacities as defined by Farr, at 2 antigen concentrations C_1 and C_2.

P = The fractional binding to which one interpolated when calculating the antigen binding capacity (for ABC 30, P = 0.3).

nK = The affinity index ($\ell . M^{-1}$).

In order to examine the sera for antibody affinity and the concentration of antibody combining sites, (the absolute concentration of antibody in the serum), Farr tests were performed as previously described, the only difference being that a lower concentration of antigen (0.1 µg/ml) was used in the second test. Thus a_1 and a_2 were measured at antigen concentrations C_1 and C_2. For the purposes of statistical analysis sera not containing antibody were arbitrarily assigned the lowest affinity/concentration of antibody combining sites value of the particular batch in which they were tested.

PVP antibody responses

Animals were bled on only one occasion, 4 weeks following the administration 1.0 mg. of ^{131}I-PVP. After this bleed the animals were killed and the organ distribution of PVP determined. Sera were stored at -20°C until used. Antibody to PVP was detected by passive haemagglutination.

Preparation of PVP coated sheep erythrocytes

Sheep red cells were formalinised and tanned as described previously, and then coated with PVP (mol. wt. 10,000; General Aniline and Film Corporation, New York, N.Y.) as described by Andersson (192). Essentially the method is that described for coating sheep red cells with BGG, the only differences being that the cells were incubated with antigen at room temperature rather than at 37°C.

Estimation of PVP antibody titres

Sera were absorbed with control sheep erythrocytes as previously described, and then titrated in doubling dilution starting with a dilution of 1 in 10. Standard microhaemagglutination plates were used (Microtiter, Cooke Engineering Co., Alexandria, Virginia, U.S.A.). Control sera consisted of rabbit anti-PVP serum and normal rabbit serum. All sera were tested on the same day.

Statistical analysis of data was made using Student's "t" test.

TABLE 16

Frequency with which immune elimination of BGG was observed in 6 strains of mice at its time of onset*

Strain of Mouse	Number with immune elimination		Time of onset of immune elimination
NZB	15/15	(100%)	All 6 - 7 days
NZW	3/16	(18.5%)	1 at 6 days 1 at 10.5 days 1 at 11 days
BWF_1	11/16	(68.8%)	1 at 7 days 2 at 8 days 4 at 9 days 3 at 10 days 1 at 12 days
BALB/c	16/16	(100%)	All 6 - 8 days
CBA	6/16	(37.5%)	1 at 6.5 days 4 at 10 days 1 at 11 days
C3Hf	0/16	(0%)	-

*These data are obtained from the mice studied in chapter 5. The time of onset of immune elimination of antigen is taken as the point at which the extrapolated lines of non-immune and immune elimination intersected.

TABLE 17

BGG antibody titres 10 days after challenge with BGG (100 µg) in FCA

Strain of Mouse	Number Tested	Antibody titre Mean ± SD (log_2)	Significance of interstrain difference				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	4 M 4 F	6.50 ± 0.93	t = 11.4215 p < 0.005	t = 8.2871 p < 0.005	t = 2.7495 p < 0.01	t = 0.7071 N S	t = 3.1769 p < 0.005
NZW	4 M 3 F	5.14 ± 0.69	t = 9.3967 p < 0.0005	t = 5.8860 p < 0.0005	t = 1.0612 N S	t = 1.1977 N S	
BWF_1	4 M 4 F	6.00 ± 1.77	t = 7.1204 p < 0.0005	t = 4.9630 p < 0.0005	t = 1.7904 p < 0.05		
BALB/c	4 M 4 F	4.25 ± 2.12	t = 4.2513 p < 0.0005	t = 3.4344 p < 0.005			
CBA	3 M 4 F	2.38 ± 1.06	t = 2.2361 p < 0.05				
C3Hf	4 M 4 F	0.43 ± 1.13					

RESULTS

Onset of immune elimination of antigen. The antigen catabolism (non-immune elimination) studies were reported in the previous chapter. BGG was the only antigen with a sufficiently slow rate of catabolism to allow the onset of immune elimination to be observed. As shown in Table 16, immune elimination occurred in all NZB and BALB/c mice and was of relatively rapid onset (6 - 7 days for NZB, and 6 - 8 days for BALB/c). In addition, 11 of the 16 BWF_1 mice showed immune elimination of BGG, the onset of which occurred between 7 and 12 days. Mice with slower rates of antigen catabolism showed immune elimination less frequently (6 of 16 CBA; 3 of 16 NZW; none of 16 C3Hf) and when it was observed, it occurred later than in the two inbred strains with rapid antigen catabolism. There were two exceptions: one female CBA developed immune elimination at 6.5 days, and one male NZW at 6 days. This latter mouse also showed an unusually rapid antigen catabolic rate for NZW mice ($T_{\frac{1}{2}}$ = 45.5 hrs. cf. 99.8 hrs. for whole group).

BGG antibody responses. There were no significant antibody levels in any mouse strain at 14 days following first injection. The BGG-antibody titres after challenge with BGG-FCA are shown in Table 17. The highest titres were found in the three New Zealand strains, and BALB/c mice had higher titres than

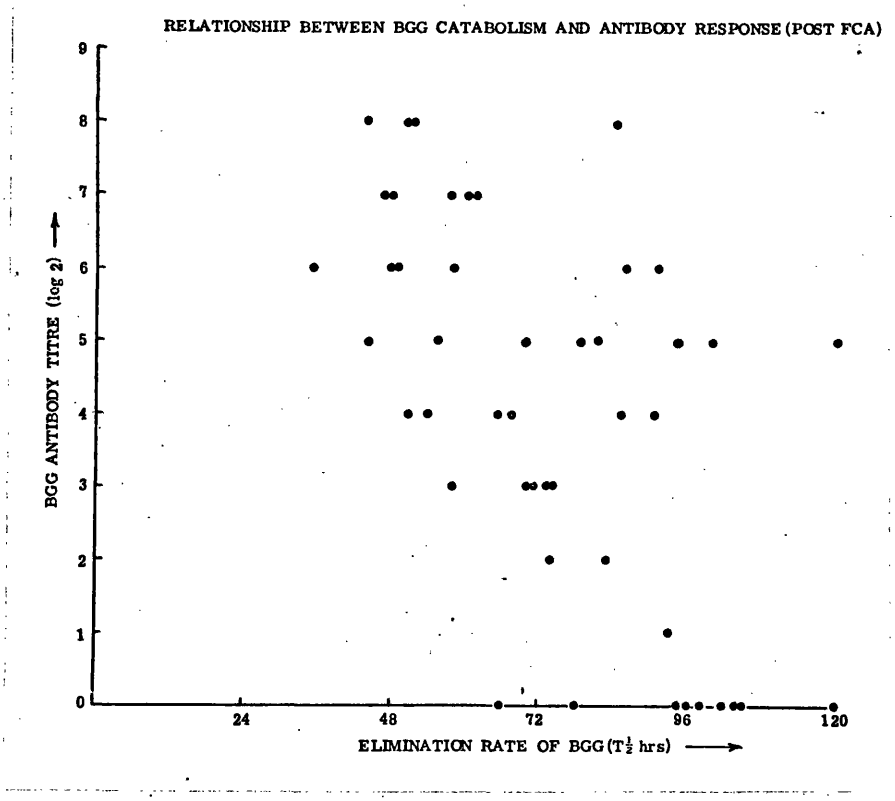


Fig. 26. Relationship between rates of BGG catabolism and BGG antibody titres 14 days following challenge with BGG in FCA.

TABLE 18a

Titre of BSA antibody (ABC 30; µg/ml; $\log_{10}$) on day 7 of the primary response

Antigen concn. 1.0 µg/ml.

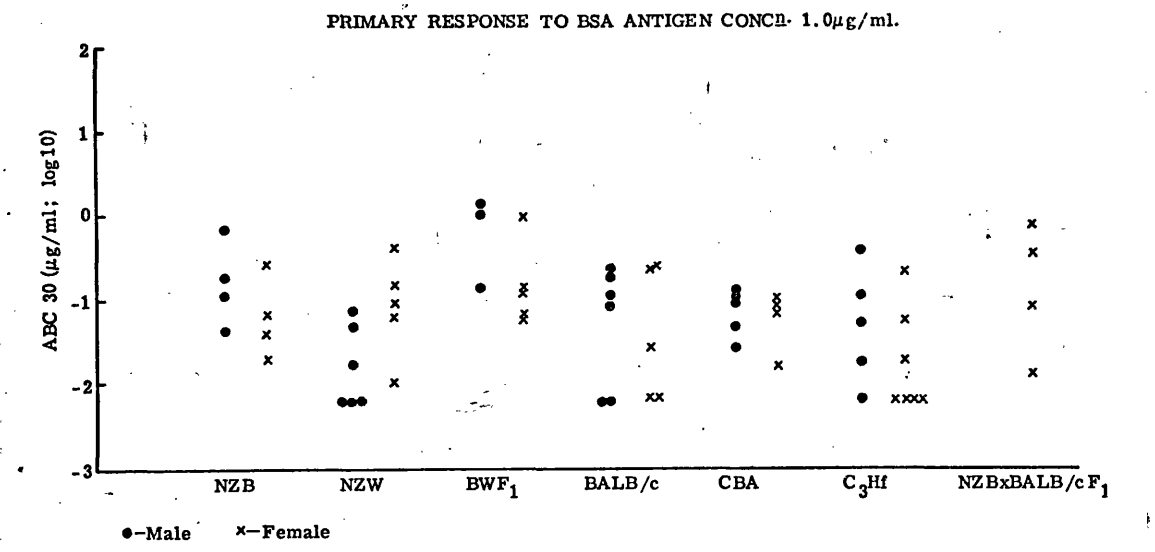
Strain of Mouse	Number in Group	ABC 30 (ug/ml) ($\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 5 F	-0.9899 ± 0.5073	t = 2.1430 p<0.02	t = 1.3330 N S	t = 1.2587 N S	t = 1.4202 N S	t = 1.7664 p<0.05
NZW	6 M 5 F	-1.4710 ± 0.6356	t = 0.4006 N S	t = 0.5155 N S	t = 0.3864 N S	t = 3.0498 p<0.005	
BWF_1	3 M 5 F	-0.6054 ± 0.5734	t = 3.4129 p<0.005	t = 2.8677 p<0.01	t = 2.4763 p<0.02		
BALB/c	6 M 5 F	-1.3602 ± 0.7080	t = 0.7700 N S	t = 0.2872 N S			
CBA	6 M 4 F	-1.2856 ± 0.4344	t = 1.2090 N S				
C3Hf	5 M 7 F	-1.5791 ± 0.6558					
NZB x BALB/c F_1	4 F	-0.8768 Range -1.8962 - 0.0762					

TABLE 18b

Titre of BSA antibody (ABC 30; μg/ml; $\log_{10}$) on day 7 of the primary response

Antigen concn. 0.1 μg/ml.

Strain of Mouse	Number in Group	ABC 30 (ug/ml $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 5 F	-1.8348 ± 0.4805	t = 1.4221 N S	t = 0.3802 N S	t = 0.7296 N S	t = 1.7101 N S	t = 1.3146 N S
NZW	6 M 5 F	-2.1702 ± 0.5924 N S	t = 0.2000 N S	t = 1.0814 N S	t = 0.5996 p 0.01	t = 2.8809	
BWF_1	3 M 5 F	-1.3539 ± 0.6339	t = 2.9244 p 0.005	t = 2.2325 p 0.05	t = 2.3616 p 0.02		
BALB/c	6 M 5 F	-2.0196 ± 0.5861	t = 0.7766 N S	t = 0.4363 N S			
CBA	6 M 4 F	-1.9189 ± 0.4552	t = 1.2280 N S				
C3Hf	5 M 7 F	-2.2228 ± 0.6616					
NZB x BALB/c F_1	4 F	-1.7291 Range -2.7959 - -1.0079					



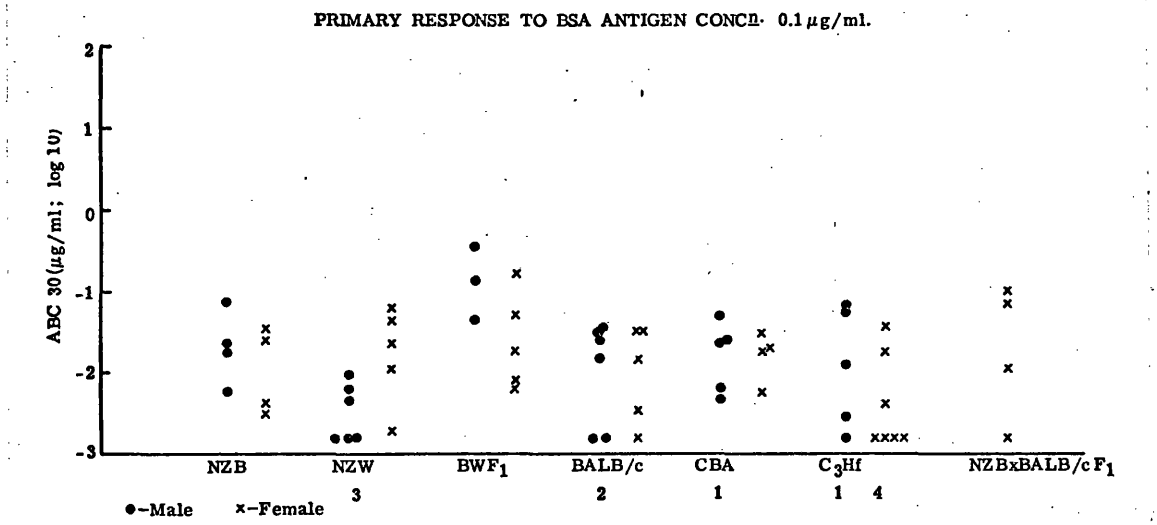


Fig. 27. BSA antibody response (ABC 30; μg/ml $\log_{10}$) on day 7 of primary response.

(a) Antigen concentration 1.0 μg/ul.

(b) Antigen concentration 0.1 μg/ul.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

TABLE 19a

Titre of BSA antibody (ABC 30; μg/ml; $\log_{10}$) on day 7 of the secondary response

Antigen concn. 1.0 μg/ml.

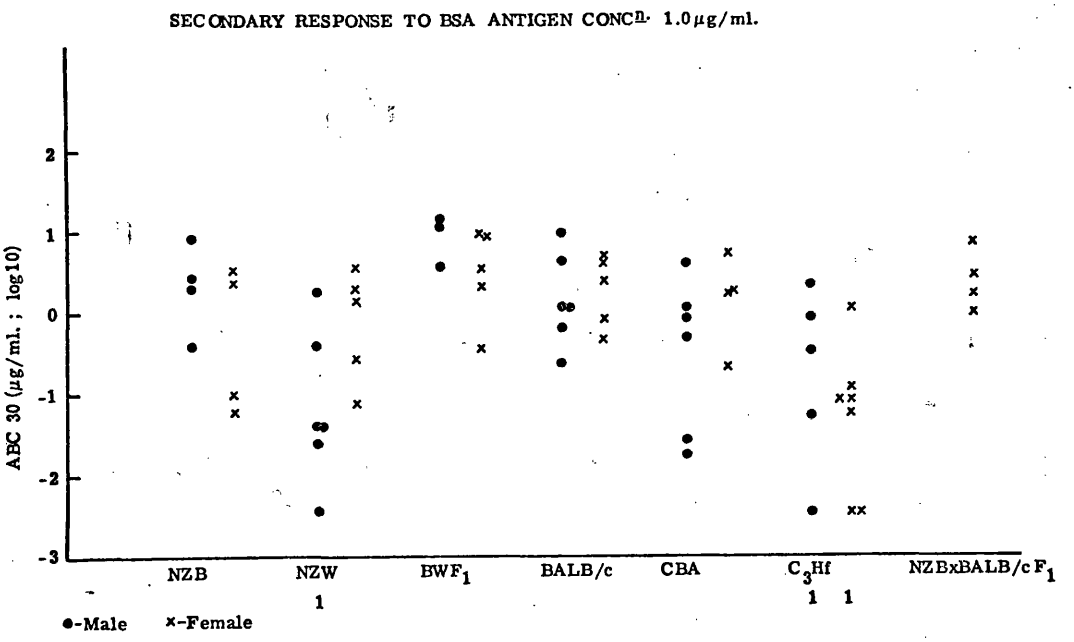
Strain of Mouse	Number in Group	ABC 30 (μg/ml $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 5 F	0.0127 ± 0.7884	t = 2.5898 p < 0.01	t = 0.4059 N S	t = 0.7391 N S	t = 1.9529 N S	t = 1.4304 N S
NZW	6 M 5 F	-0.5890 ± 0.9787	t = 1.1610 N S	t = 1.0915 N S	t = 2.4681 p < 0.02	t = 3.2843 p < 0.005	
BWF_1	3 M 5 F	0.6681 ± 0.5288	t = 4.5474 p < 0.0005	t = 2.3359 p < 0.02	t = 1.8111 p < 0.05		
BALB/c	6 M 5 F	0.2323 ± 0.5101	t = 3.9196 p < 0.0005	t = 1.2413 N S			
CBA	6 M 4 F	-0.1474 ± 0.8635	t = 2.3020 p < 0.02				
C3Hf	5 M 7 F	-1.0638 ± 0.9805					
NZB x BALB/c F_1	4 F	0.3265 Range 0.0486 - 0.9007					

TABLE 19b

Titre of BSA antibody (ABC 30; µg/ml; $\log_{10}$) on day 7 of the secondary response

Antigen concn. 0.1 µg/ml.

Strain of Mouse	Number in Group	ABC 30 µg/ml $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 5 F	-0.7947 ± 0.8843	t = 2.0401 p < 0.05	t = 0.2329 N S	t = 0.9529 N S	t = 2.0087 p < 0.05	t = 0.9856 N S
NZW	6 M 5 F	-1.2260 ± 0.9799	t = 1.1157 N S	t = 1.3143 N S	t = 2.2110 p < 0.02	t = 3.0358 p < 0.005	
BWF_1	3 M 5 F	-0.0705 ± 0.5078	t = 4.1851 p < 0.0005	t = 1.8576 p < 0.05	t = 1.7715 p < 0.05		
BALB/c	6 M 5 F	-0.4896 ± 0.5100	t = 3.5547 p < 0.001	t = 0.6996 N S			
CBA	6 M 4 F	-0.6997 ± 0.8403	t = 2.4713 p < 0.02				
C3Hf	5 M 7 F	-1.6885 ± 1.0050					
NZB x BALB/c F_1	4 F	-0.2797 Range -0.7282 - 0.2446					



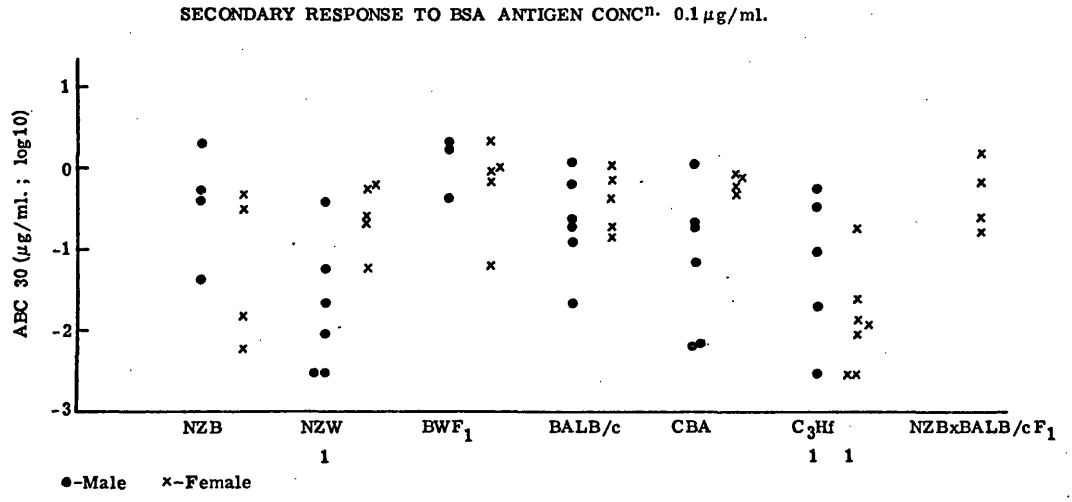


Fig. 28. BSA antibody response (ABC 30; µg/ml; $\log_{10}$) on day 7 of the secondary response.

(a) Antigen concentration 1.0 µg/ul.

(b) Antigen concentration 0.1 µg/ul.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

TABLE 20a

Titre of BSA antibody (ABC 30; μg/ml; Log_{10}) 10 days following challenge with BSA in FCA

Antigen concn. 1.0 μg/ml.

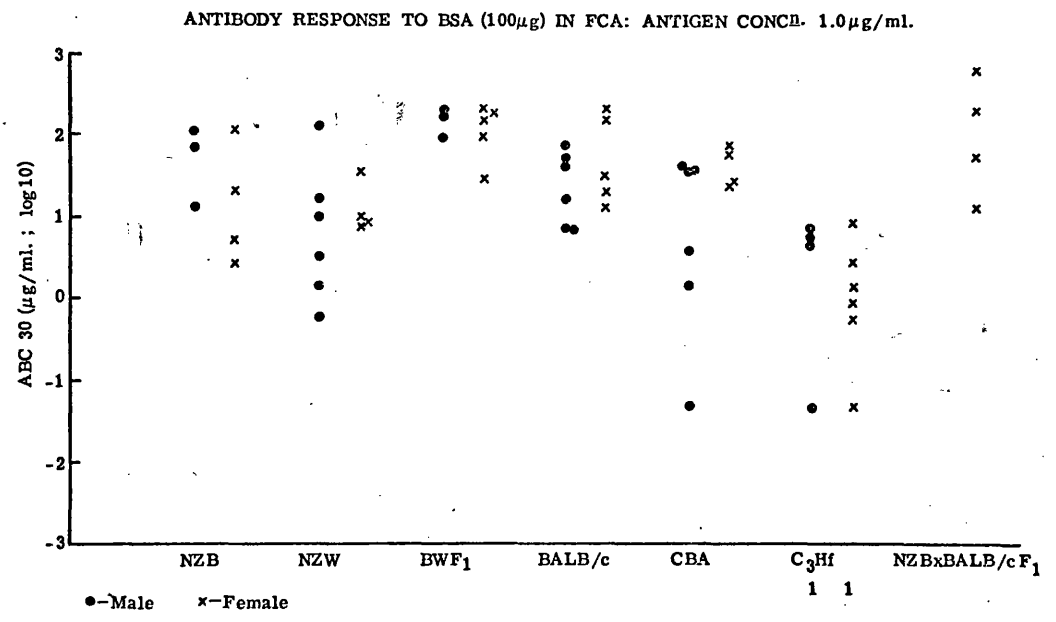
Strain of Mouse	Number in Group	ABC 30 (μg/ml log_{10})	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 4 F	1.3702 ± 0.6637	t = 3.7652 p<0.001	t = 0.6343 N S	t = 0.5981 N S	t = 2.8314 p<0.01	t = 1.3588 N S
NZW	6 M 4 F	0.9223 ± 0.6724	t = 2.2872 p<0.02	t = 0.4420 N S	t = 2.3806 p<0.02	t = 4.6073 p<0.0001	
BWF_1	3 M 5 F	2.0964 ± 0.2801	t = 9.1061 p<0.0005	t = 2.7087 p<0.01	t = 2.8519 p<0.01		
BALB/c	6 M 5 F	1.5343 ± 0.5009	t = 5.5818 p<0.0005	t = 1.2877 N S			
CBA	6 M 4 F	1.0922 ± 1.0124	t = 2.1692 p<0.05				
C3Hf	5 M 7 F	0.3209 - 0.4893					
NZB x BALB/c F_1	4 F	1.9913 Range 1.0774 - 2.8075					

TABLE 20b

Titre of BSA antibody (ABC 30; μg/ml; $\log_{10}$) 10 days following challenge with BSA in FCA

Antigen concn. 0.1 μg/ml.

Strain of Mouse	Number in Group	ABC 30 (μg/ml $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 4 F	0.7012 ± 0.8254	t = 3.2778 $p < 0.005$	t = 0.2058 N S	t = 1.2354 N S	t = 3.4595 $p < 0.005$	t = 0.7746 N S
NZW	6 M 4 F	0.4072 ± 0.7312	t = 2.7272 $p < 0.01$	t = 1.0847 N S	t = 2.4520 $p < 0.02$	t = 4.9640 $p < 0.0005$	
BWF_1	3 M 5 F	1.7753 ± 0.2905	t = 10.2507 $p < 0.0005$	t = 3.3614 $p < 0.005$	t = 3.2723 $p < 0.005$		
BALB/c	6 M 4 F	1.1021 ± 0.5184	t = 6.2859 $p < 0.0005$	t = 1.0518 N S			
CBA	6 M 4 F	0.7844 ± 0.7850	t = 3.7986 $p < 0.001$				
C3Hf	4 M 6 F	-0.3717 ± 0.5301					
NZB x BALB/c F_1	4 F	1.4403 Range 0.2303 - 2.3813					



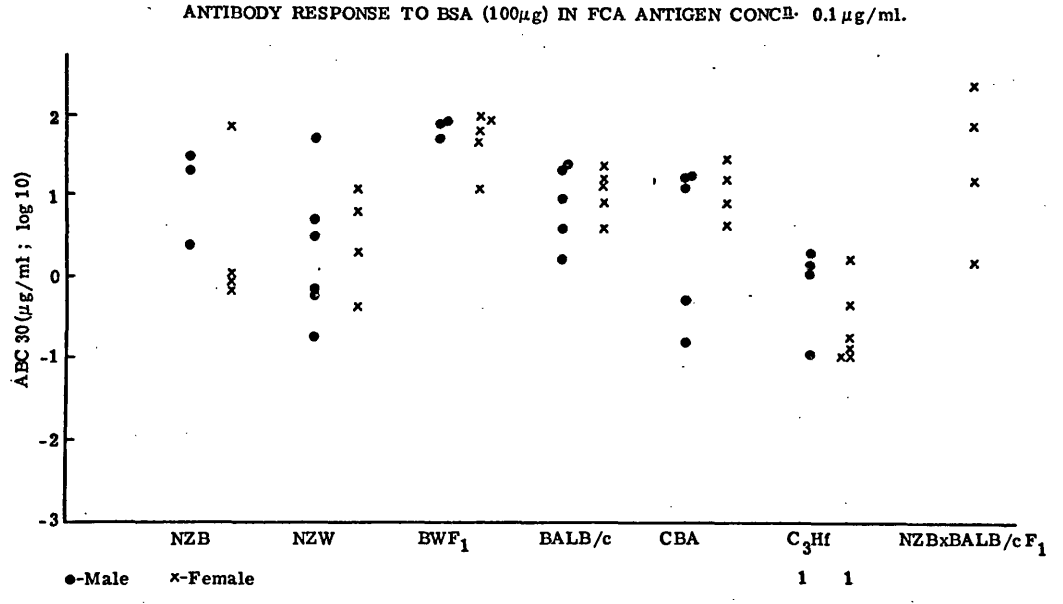


Fig. 29. BSA antibody response (ABC 30; μg/ml; $\log_{10}$)

10 days following challenge with BSA in FCA.

(a) Antigen concentration 1.0 μg/ul.

(b) Antigen concentration 0.1 μg/ul.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

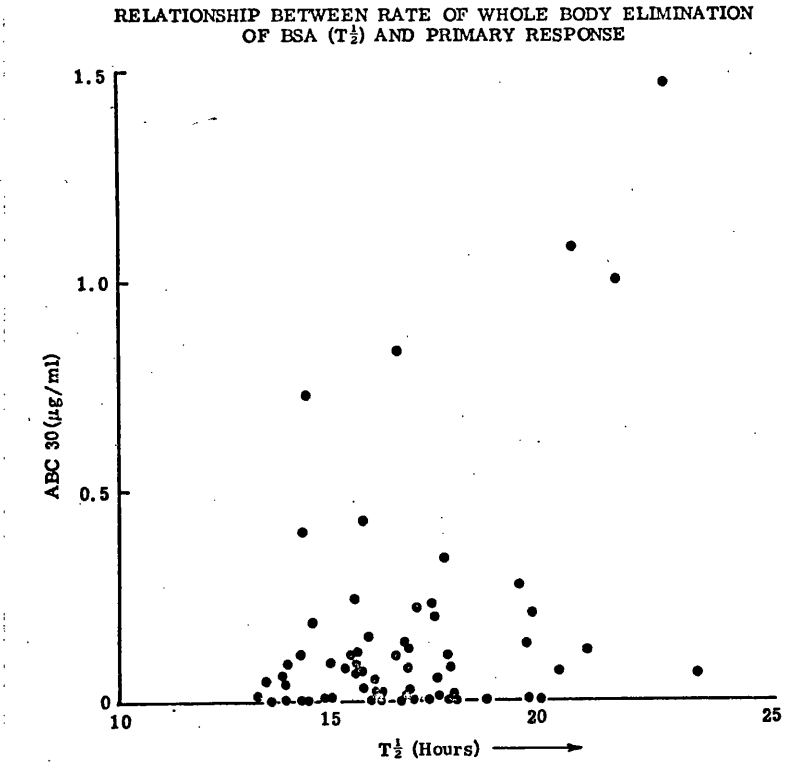


Fig. 30. Plot of BSA antibody titres (primary response) against BSA catabolism rates.

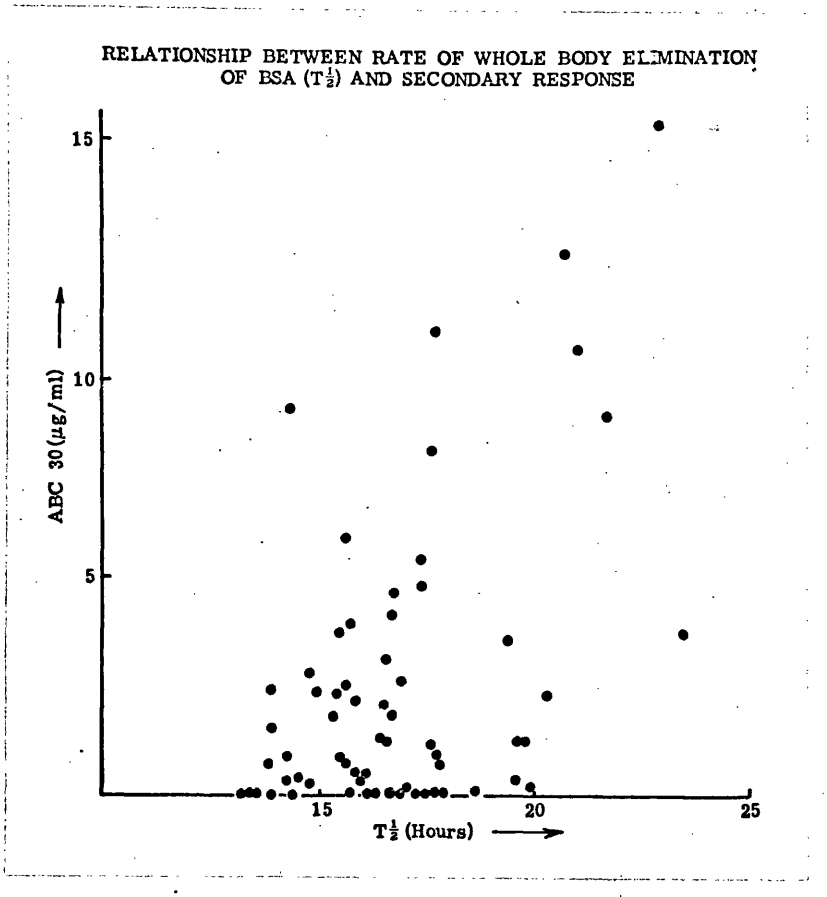


Fig. 31. Plot of BSA antibody titres (secondary response) against BSA catabolism rates.

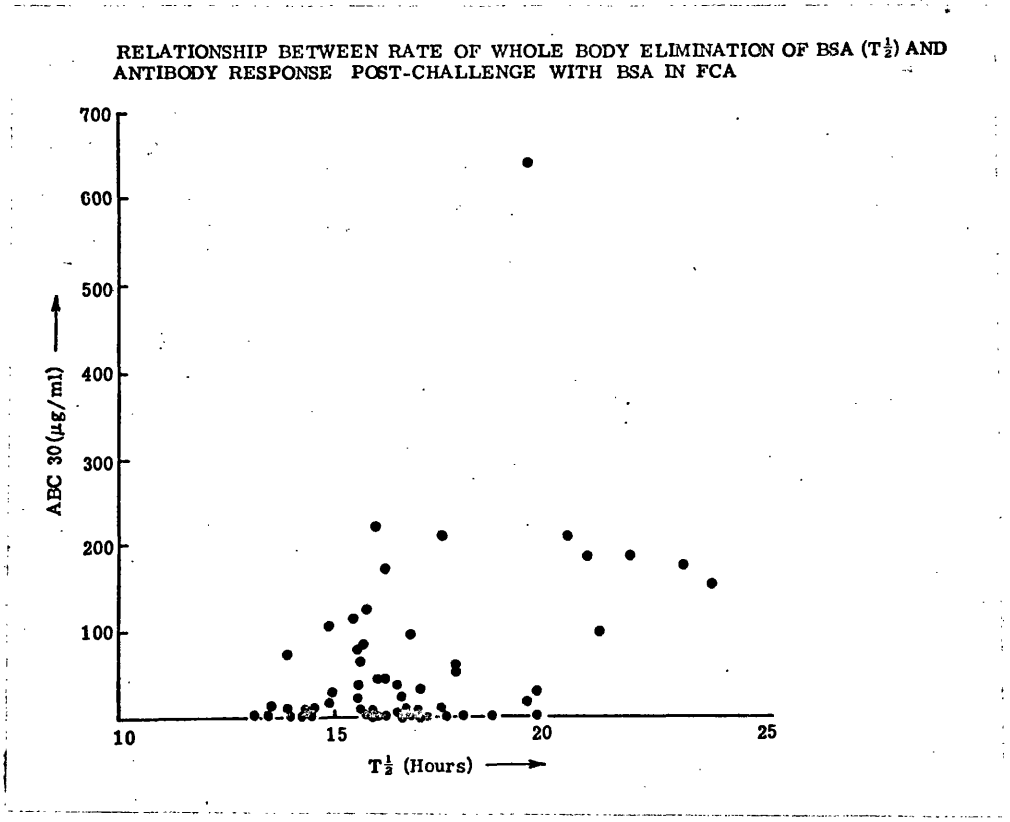


Fig. 32. Plot of BSA antibody titres (post-challenge with BSA in FCA) against BSA catabolism rates.

TABLE 21

Concentration of antibody combining sites (M/l; $\log_{10}$) in the primary response to BSA (1.0 μg) on day 7.

Strain of Mouse	Number and Sex	Concentration of antibody combining sites (M/l; $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 5 F	-8.0900 ± 0.8841	t = 2.8317 $p < 0.01$	t = 1.9237 N S	t = 1.6621 N S	t = 0.8399 N S	t = 2.0607 $p < 0.05$
NZW	6 M 5 F	-8.9156 ± 0.8466	t = 0.7259 N S	t = 0.6869 N S	t = 0.2911 N S	t = 3.2523 $p < 0.005$	
BWF_1	3 M 5 F	-7.7716 ± 0.6064	t = 4.1916 $p < 0.0005$	t = 3.7435 $p < 0.001$	t = 2.6862 $p < 0.01$		
BALB/c	6 M 5 F	-8.8038 ± 0.9514	t = 0.9885 N S	t = 0.2877 N S			
CBA	6 M 4 F	-8.7085 ± 0.4570	t = 1.6006 N S				
C3Hf	5 M 7 F	-9.1641 ± 0.7955					
NZB x BALB/c F_1	4 F	-8.0697 Range -9.0072 - -7.0361					

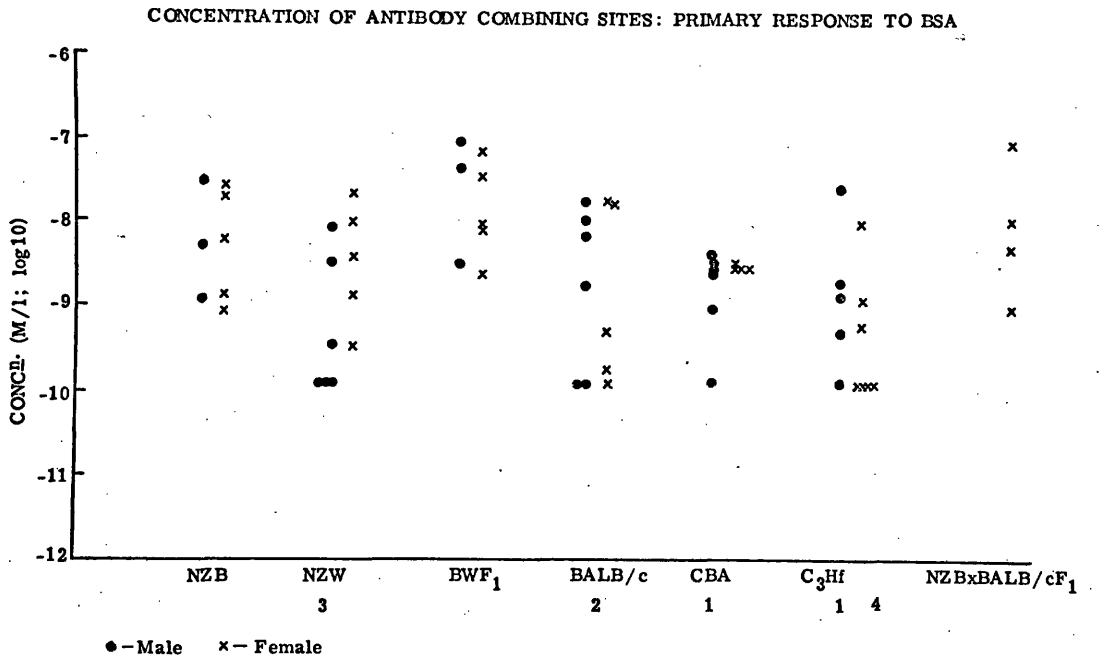


Fig. 33. Concentration of antibody combining sites in the serum (M/1, $\log_{10}$) on day 7 of the primary response to BSA.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

TABLE 22

Concentration of antibody combining sites (M/l; $\log_{10}$) in the secondary response to BSA (100 µg) day 7.

Strain of Mouse	Number and Sex	Concentration of antibody combining sites (M/l; $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 5 F	-7.1434 ± 0.7183	$t = 2.8670$ $p < 0.01$	$t = 1.4426$ N S	$t = 0.0016$ N S	$t = 1.3684$ N S	$t = 1.7635$ $p < 0.05$
NZW	6 M 5 F	-7.8175 ± 0.8678	$t = 1.0788$ N S	$t = 0.2690$ N S	$t = 2.1905$ $p < 0.05$	$t = 3.0672$ $p < 0.005$	
BWF_1	3 M 5 F	-6.6625 ± 0.6872	$t = 4.1167$ $p < 0.0005$	$t = 2.7034$ $p < 0.01$	$t = 1.7529$ $p < 0.05$		
BALB/c	6 M 5 F	-7.1439 ± 0.5131	$t = 3.5286$ $p < 0.001$	$t = 1.7866$ $p < 0.05$			
CBA	6 M 4 F	-7.7108 ± 0.9060	$t = 1.3365$ N S				
C3Hf	5 M 7 F	-8.2231 ± 0.8863					
NZB x BALB/c F_1	4 F	-7.0391 Range $-7.3517 - 6.6340$					

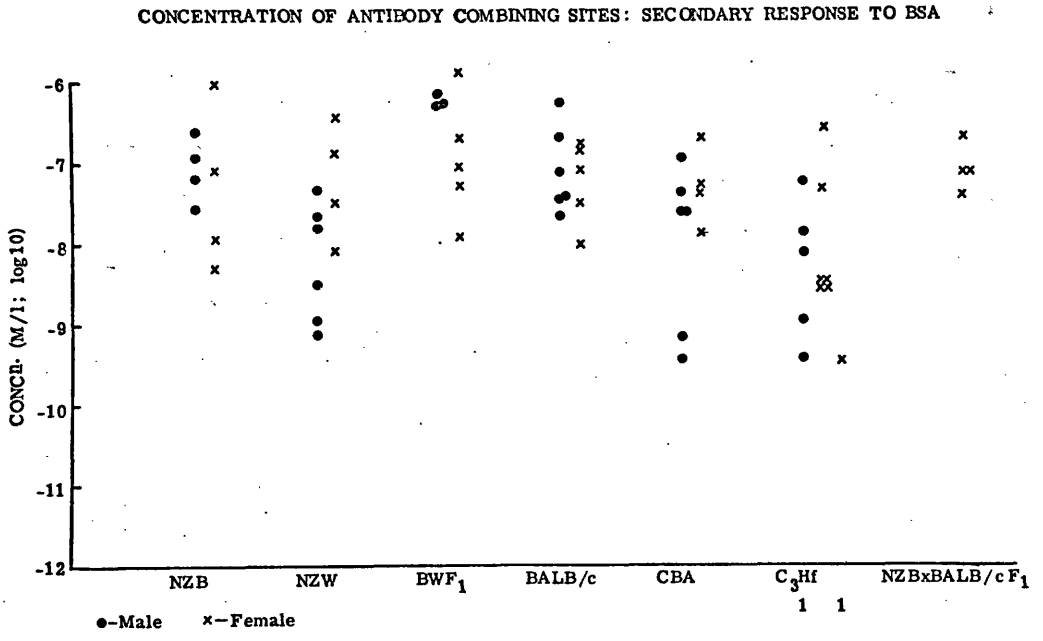


Fig. 34. Concentration of antibody combining sites in the serum (M/l) on day 7 of the secondary response to BSA.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

TABLE 23

Concentration of antibody combining sites 10 days after challenge with BSA (100 μg) in FCA.

Strain of Mouse	Number and Sex	Concentration of antibody combining sites (M/l; $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 4 F	-6.1265 ± 0.6180	$t = 2.6284$ $p < 0.01$	$t = 0.9416$ N S	$t = 0.4519$ N S	$t = 2.1892$ $p < 0.05$	$t = 1.9297$ $p < 0.05$
NZW	6 M 4 F	-6.9895 ± 1.0573	$t = 0.8823$ N S	$t = 1.1130$ N S	$t = 2.8075$ $p < 0.01$	$t = 3.6101$ $p < 0.005$	
BWF_1	3 M 5 F	-5.5979 ± 0.2775	$t = 4.2264$ $p < 0.0005$	$t = 2.7248$ $p < 0.01$	$t = 2.3877$ $p < 0.02$		
BALB/c	6 M 5 F	-6.0152 ± 0.4320	$t = 3.6759$ p 0.001	$t = 1.5926$ N S			
CBA	6 M 4 F	-6.4951 ± 0.8926	$t = 1.9714$ $p < 0.05$				
C3Hf	4 M 6 F	-7.4214 ± 1.1878					
NZB x BALB/c F_1	4 F	-5.6543 Range -6.5546 - -4.8544					

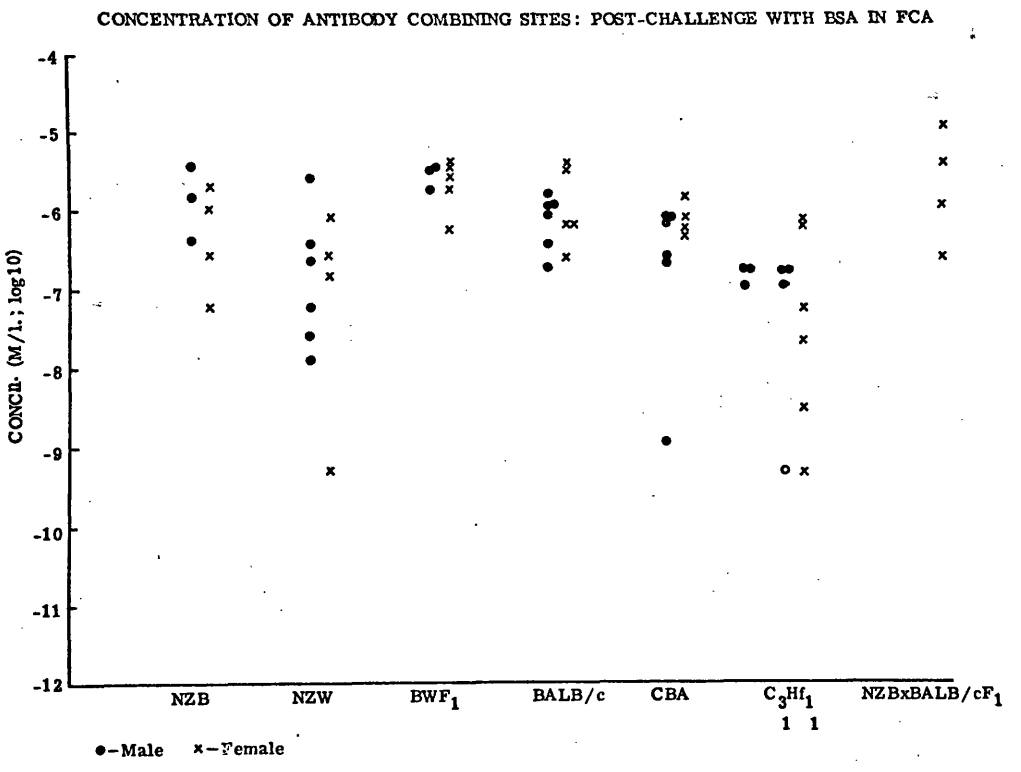


Fig. 35. Concentration of antibody combining sites in the serum (M/l) 10 days following challenge with BSA in FCA.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

the other two 'control' strains. Indeed, C3Hf mice, with one exception, produced no antibody. No correlation could be demonstrated between rates of BGG-catabolism and BGG-antibody titres (Fig. 26).

BSA antibody titres. BSA-antibody levels on day 7 after the first injection of antigen (Table 18 a + b; Fig. 27 a + b) were highest in BWF_1 and NZB x BALB/c F_1 mice and this was also the case for the secondary response (Table 19 a + b; Fig. 28 a + b), and following challenge with BSA-FCA (Table 20 a + b; Fig. 29 a + b). C3Hf mice consistently had the lowest antibody titres. There was no correlation between antibody titres and BSA catabolism (Figs. 30, 32).

PVP antibody titres. Antibody to PVP was not detected in any serum by the haemagglutination technique used.

The absolute concentration of antibody in the serum as measured by the concentration of serum BSA antibody combining sites.

The absolute concentration of BSA antibody in the serum as opposed to the ABC 30 value which depends both on quantity and quality of antibody (Tables 21-23; Figs. 33-35) was highest in BWF_1 and NZB x BALB/c F_1 mice during the primary and secondary responses, and following challenge with BSA in FCA C3Hf mice had the lowest antibody concentration on all three occasions. At all times NZB mice had significantly higher antibody concentrations than C3Hf and NZW, but not BALB/c or CBA mice.

TABLE 24

Affinity of BSA antibody in the primary response - day 7

Strain of Mouse	Number in Group	Affinity Index ($1.M^{-1}$; $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 5 F	7.5819 ± 0.6904	t = 0.6148 N S	t = 1.1699 N S	t = 0.3630 N S	t = 0.3797 N S	t = 0.4321 N S
NZW	6 M 5 F	7.8274 ± 1.4864	t = 0.9496 N S	t = 0.2756 N S	t = 0.1869 N S	t = 0.1721 N S	
BWF_1	3 M 5 F	7.7268 ± 0.8301	t = 0.9077 N S	t = 0.6718 N S	t = 0.0017 N S		
BALB/c	6 M 5 F	7.7276 ± 0.9663	t = 0.9587 N S	t = 0.6502 N S			
CBA	6 M 4 F	7.9699 ± 0.7059	t = 1.6387 N S				
C3Hf	5 M 7 F	7.3154 ± 1.0827					
NZB x BALB/c F_1	4 F	7.6136 Range 7.2879 - 8.0729					

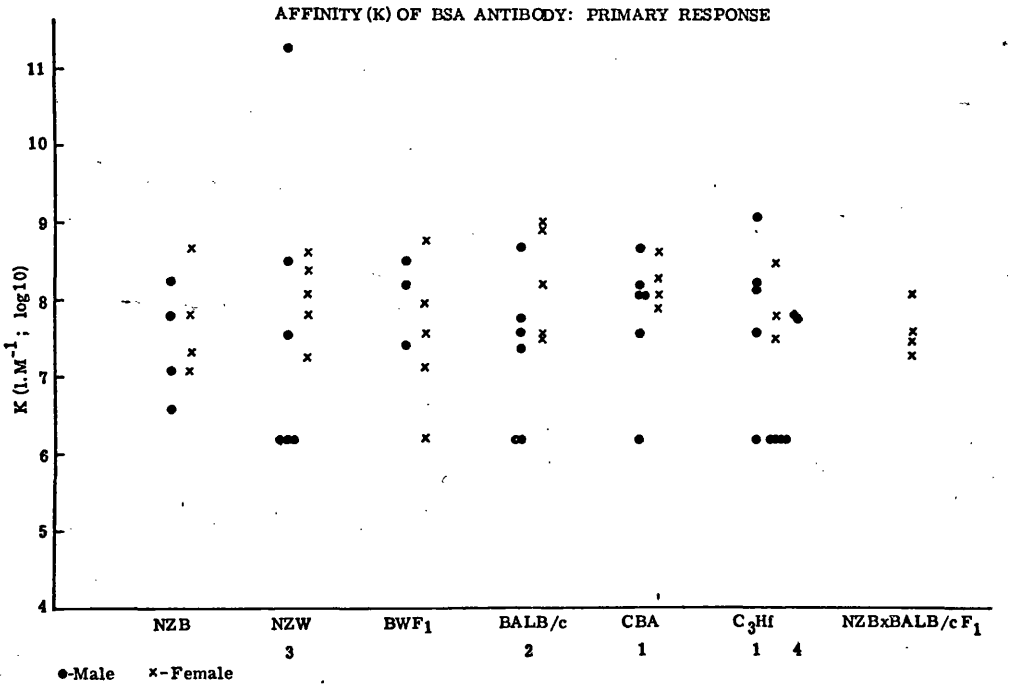


Fig. 36. Affinity of BSA antibody on day 7 of the primary response.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

TABLE 25

Affinity of BSA antibody in the secondary response - day 7

Strain of Mouse	Number in Group	Affinity Index ($1.M^{-1}$; $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 5 F	7.6330 ± 0.6083	$t = 0.8632$ N S	$t = 2.9936$ $p < 0.005$	$t = 1.5866$ N S	$t = 0.9185$ N S	$t = 1.1736$ N S
NZW	6 M 5 F	8.0025 ± 0.7222	$t = 0.3848$ N S	$t = 1.4834$ N S	$t = 0.0520$ N S	$t = 0.3610$ N S	
BWF_1	3 M 5 F	7.8936 ± 0.5243	$t = 0.0132$ N S	$t = 2.1410$ $p < 0.05$	$t = 0.5472$ N S		
BALB/c	6 M 5 F	8.0158 ± 0.4465	$t = 0.5192$ N S	$t = 1.9122$ $p < 0.05$			
CBA	6 M 4 F	8.4061 ± 0.4891	$t = 2.0051$ $p < 0.05$				
C3Hf	5 M 7 F	7.8901 ± 0.6791					
NZB x BALB/c F_1	4 F	8.0634 Range 7.7992 - 8.3531					

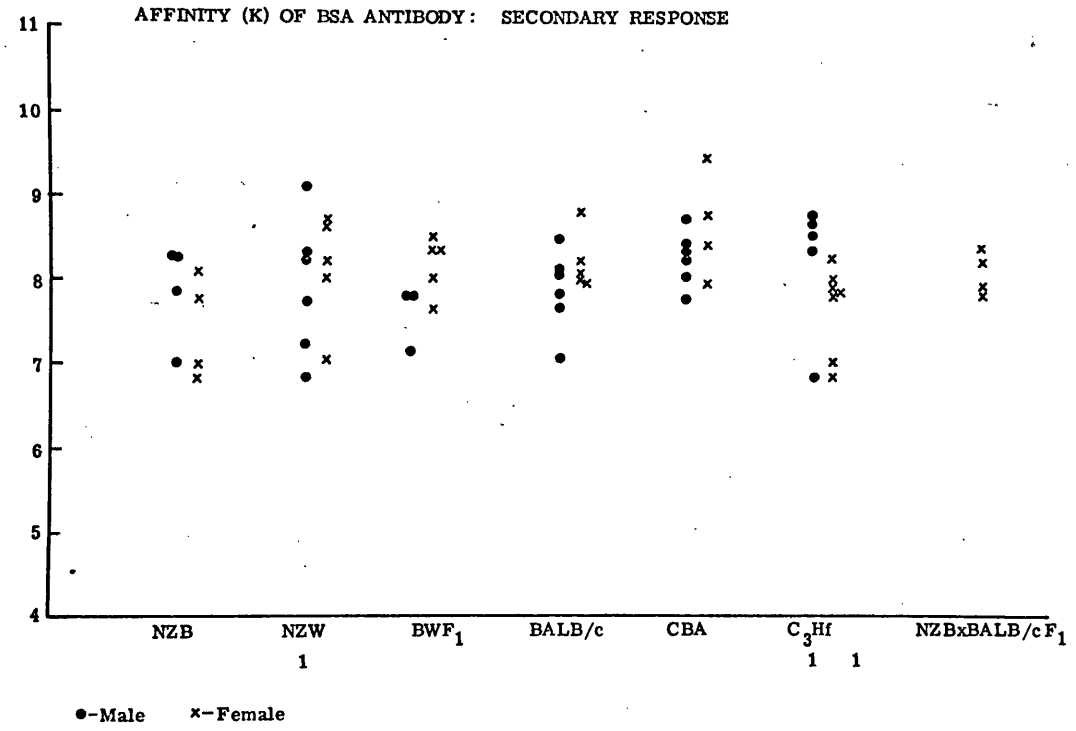


Fig. 37. Affinity of BSA antibody on day 7 of the secondary response.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

TABLE 26

Affinity of BSA antibody 10 days following challenge with BSA (100 ug) in FCA

Strain of Mouse	Number in Group	Affinity Index ($1.M^{-1}$; $\log_{10}$)	Significance of interstrain differences				
			C3Hf	CBA	BALB/c	BWF_1	NZW
NZB	3 M 4 F	8.2428 ± 0.4621	$t = 1.4649$ N S	$t = 0.0781$ N S	$t = 1.5545$ N S	$t = 3.7532$ $p < 0.005$	$t = 2.2999$ $p < 0.02$
NZW	6 M 4 F	8.6683 ± 0.3231	$t = 3.0579$ $p < 0.005$	$t = 1.9589$ $p < 0.05$	$t = 1.2892$ N S	$t = 1.6602$ N S	
BWF_1	3 M 5 F	8.8622 ± 0.0659	$t = 3.4510$ $p < 0.002$	$t = 2.9414$ $p < 0.005$	$t = 3.7213$ $p < 0.001$		
BALB/c	6 M 5 F	8.5026 ± 0.2655	$t = 2.6952$ $p < 0.01$	$t = 1.6692$ N S			
CBA	6 M 4 F	8.2623 ± 0.5704	$t = 1.5911$ N S				
C3Hf	4 M 6 F	7.7107 ± 0.9361					
NZB x BALB/c F_1	4 F	8.6648 Range 8.4576 - 8.8786					

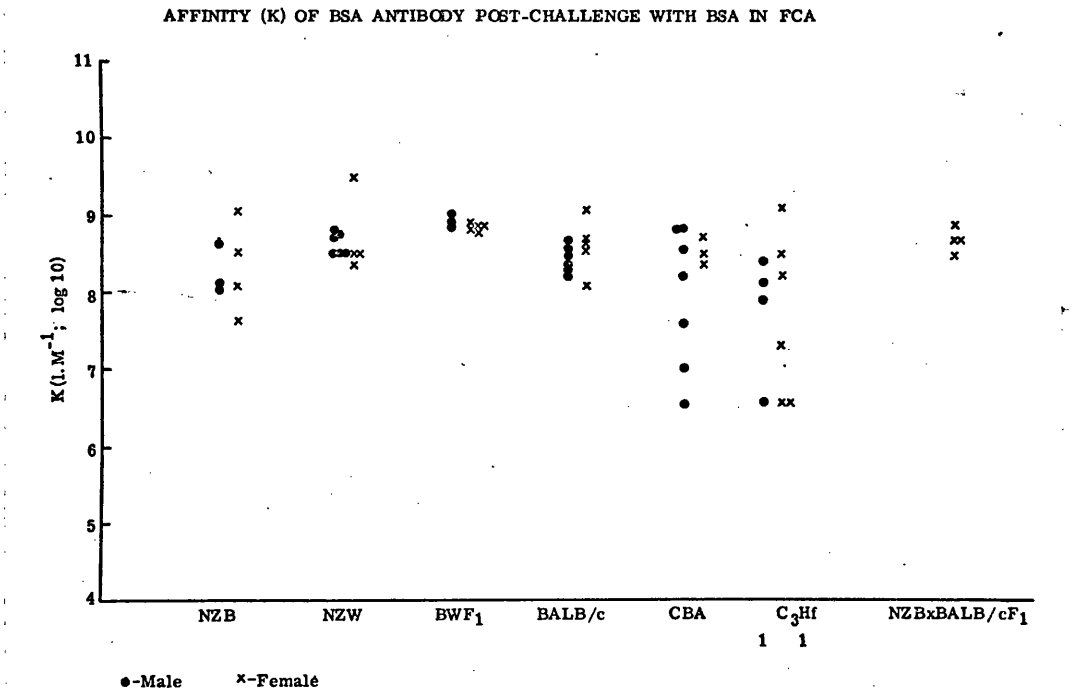


Fig. 38. Affinity of BSA antibody 10 days following challenge with BSA in FCA.

Numbers under mouse strains are the number of mice per strain without detectable antibody, but are represented as the lowest recorded value.

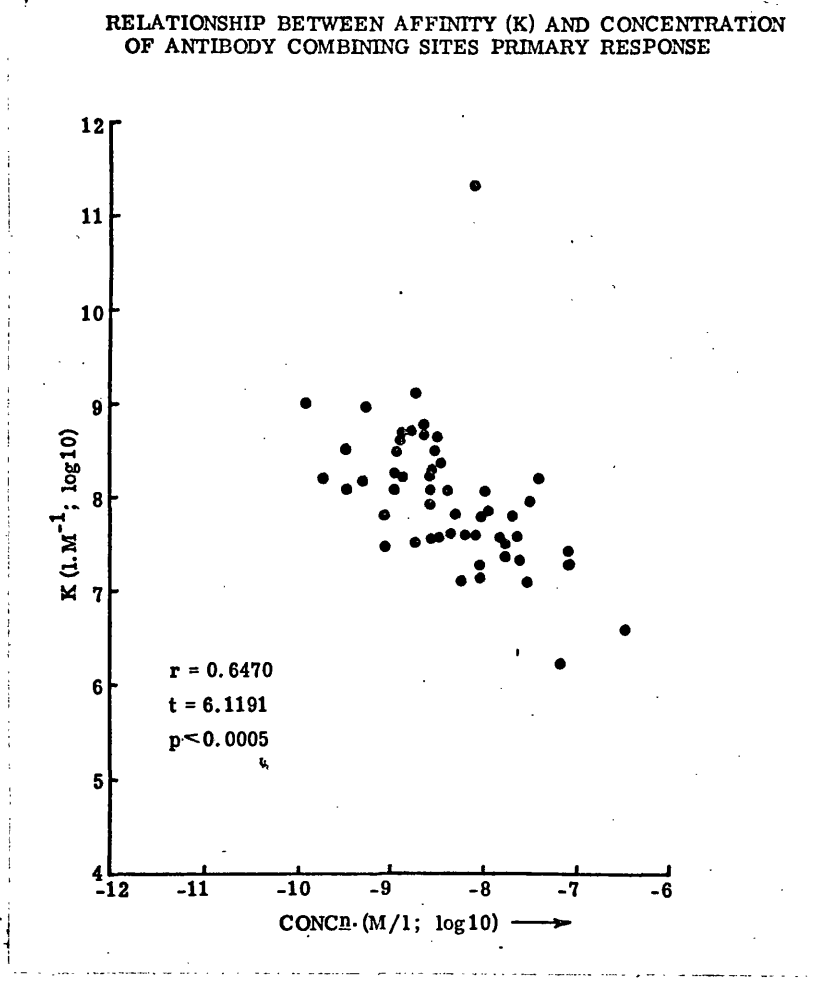


Fig. 39. Relationship between affinity of BSA antibody and the concentration of serum antibody combining sites during the primary response.

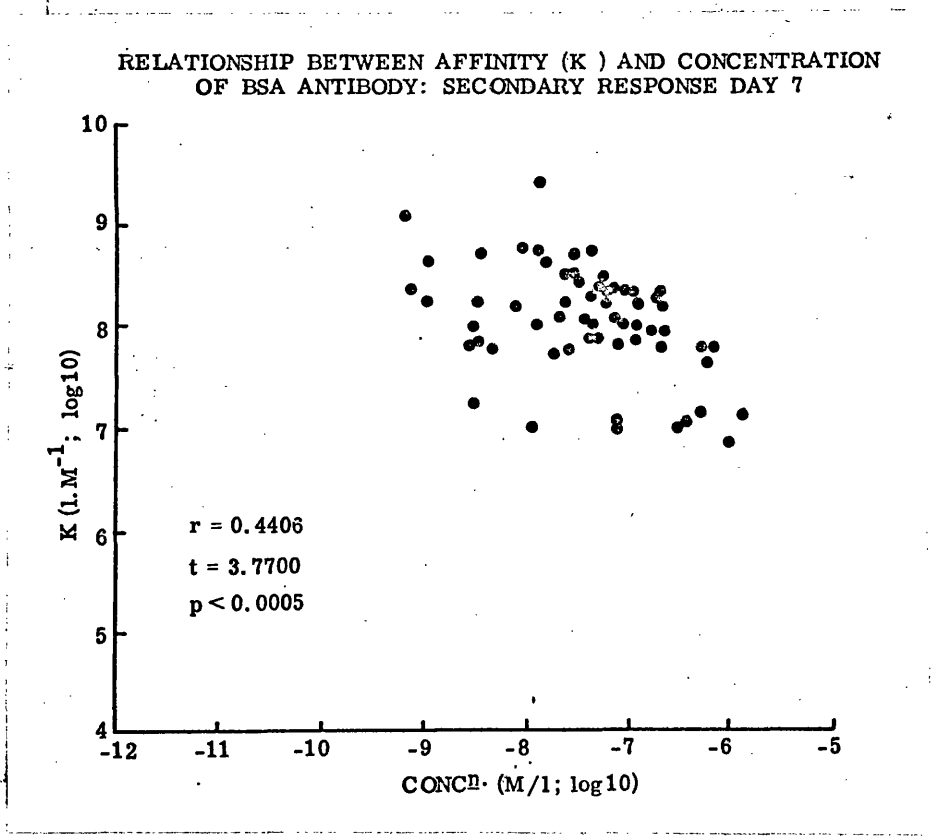


Fig. 40. Relationship between affinity of BSA antibody and the concentration of antibody combining sites during the secondary response.

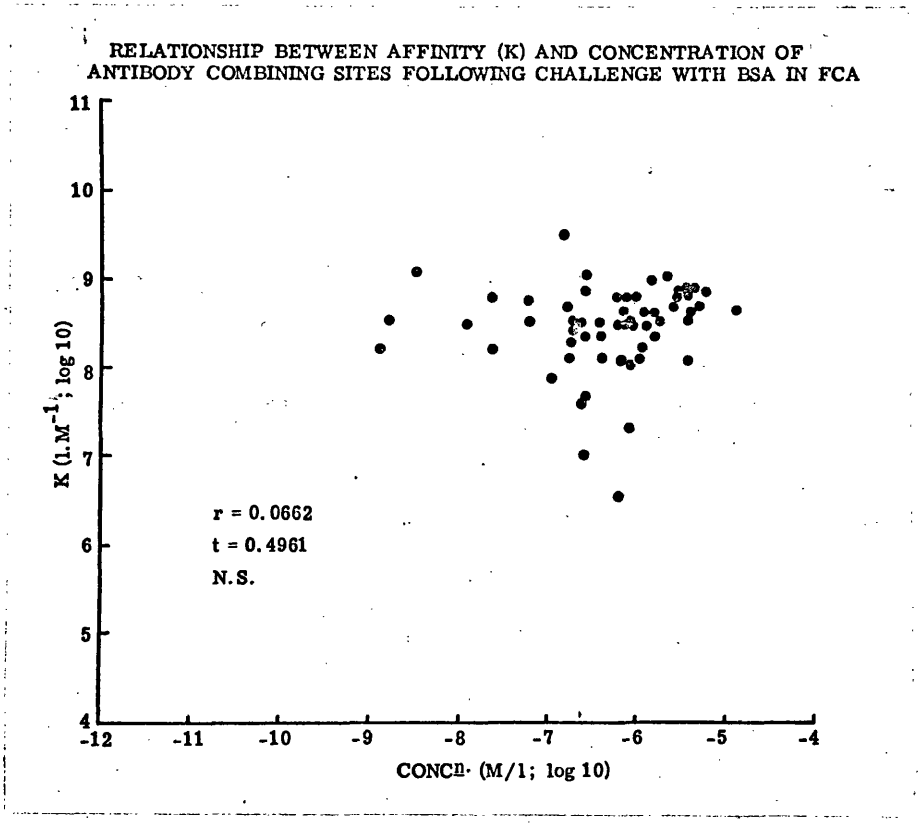


Fig. 41. Relationship between affinity of BSA antibody and the concentration of antibody combining sites 10 days following challenge with BSA in FCA.

Affinity of BSA antibody. The affinity of BSA antibody increased with progressive immunisation. During the primary response (Table 24; Fig. 36) interstrain differences were not apparent, whereas during the secondary response (Table 25; Fig. 37), CBA mice produced antibody of significantly higher affinity than all but NZW mice. Interstrain variations were most obvious following challenge with BSA in FCA (Table 26), and there was a reduction in the overall scatter of the results, with a marked reduction in intra-strain variations (Fig. 38). BWF_1 mice at this stage produced antibody of higher affinity than other strains, whereas C3Hf mice produced antibody of the lowest affinity. During the primary (Fig. 39) and secondary (Fig. 40) immune responses, an inverse correlation between the concentration of serum antibody combining sites and the affinity of the antibody produced was present ($r = 0.6470$, $t = 6.1191$, $p < 0.0005$; and $r = 0.4406$, $t = 3.7700$, $p < 0.0005$ respectively). No such correlation existed following challenge with BSA in FCA (Fig. 41).

DISCUSSION

Like Staples and Talal (107) I have found increased titres of antibody to BGG following challenge with BGG-FCA in NZB and BWF_1 mice compared to control strains. Contrary to their findings, NZW mice in the present study were also found to develop high BGG-antibody titres. BALB/c mice also produced high titres of antibody to BGG, a finding which has been previously noted (107, 180), and is thought to be their hyperphagocytosis of BGG aggregates (180).

During the primary and secondary immune responses to BSA and following challenge with BSA-FCA, antibody levels were always highest in BWF_1 and NZB x BALB/c F_1 mice. NZB and BALB/c mice also had relatively high BSA-antibody titres, but NZW mice consistently produced the lowest titres except for C3Hf mice, in contrast to their good antibody response to BGG. There has been accumulating evidence that the immune response is under genetic control (193) and it was therefore surprising to find that the BSA-antibody responses in BWF_1 mice were greater than those of either parent strain. However, the nephritis of BWF_1 mice is far more florid than that of either parent strain (78, 80, 87) and their antibody response to heat-denatured DNA-methylated BSA compex is also higher than that of either parent strain (102). The high antibody responses to BSA in BWF_1 mice, like the slow rate of BSA catabolism, again show that the phenotype of the F1 hybrid cannot be predicted from knowledge of the parents' genotypes.

The absence of PVP antibody in any of the sera of these mouse strains is in keeping with the report of Andersson (192) who found that 100 μg of PVP was a tolerogenic dose in mice. However, in this study, one month after administration of PVP, spleen weights were increased and their histological appearances were consistent with an ongoing immune response. Together with the extremely long whole-body half-life of PVP (chapter 5), this suggests that the mechanism of tolerance induction to PVP may be similar to that proposed for Type III pneumococcal polysaccharide (194-196). This form of tolerance depends on the relatively indigestible nature of the antigen. Following its ingestion by macrophages, the antigen persists intracellularly and is gradually released from the cells, binds with antibody, and is re-phagocytosed. Therefore antibody cannot be detected in the peripheral blood. That an immune response is occurring can be shown by demonstrating antibody producing cells by localised haemolysis in gel (197) or immunocytoadherence (198, 199). So far I have been unable to detect PVP antibody producing cells using these techniques on a number of occasions.

Although the relationship between antigen catabolism (non-immune elimination) and immunogenicity is complex, Stark (178, 181) suggested that rapid antigen catabolism may "predispose to, or be related to, a subsequent immune response. Mice of the Sobey strain which were unresponsive to BSA, also eliminated this antigen slowly (179), which suggested an inter-relationship between

non-immune antigen elimination and subsequent immune response. However, I was unable to demonstrate any correlations between antigen catabolism and the height of the immune response in the present study. On the other hand, those inbred strains of mice which had the more rapid rates of BGG catabolism, did develop immune elimination of this antigen at an earlier time than those with slower catabolic rates. This trend was observed to a lesser extent in the BWF_1 hybrid mice, suggesting the operation of genetic factors which differ from those controlling either antigen catabolism or immune responses.

The induction of an immune response is a complex sequence of cellular interactions any of which may be rate-limiting. However, the results of this study suggest that the process of antigen catabolism is not rate-limiting in mice with the antigens studied. Indeed, it has been shown by Mitchell, Grumet and MacDevitt (200) that C3H SW mice, which are high responders to the synthetic polypeptide antigen poly-L-(tyrosine, glutamic acid)-poly-DL-alanine-poly-L-lysine((TG)-A--L), are high responders not because of antigen processing factors, but because of an effect mediated by thymus cell interaction with antigen. In this respect, other workers have shown that there are changes in thymus-bone marrow cell co-operation in NZB mice (108, 119, 130, 131, 131a) which may explain many of the immunological phenomena peculiar to the New Zealand strains of mice.

It is interesting to speculate as to the reason why mice having rapid rates of antigen catabolism (non-immune elimination) have earlier immune elimination. The rate of catabolism represents the rate at which antigen is digested by the macrophages of the liver, bone marrow and red pulp of spleen, thus becoming unavailable for immunogenesis. Therefore mice having a rapid rate of antigen catabolism must respond rapidly, or not at all, whereas mice with slow catabolic rates are able to respond more slowly.

The relevance of the finding of rapid rates of antigen catabolism in NZ mice is uncertain. Although previous data has suggested that increased rates of antigen catabolism may contribute to immunogenicity (178), the data presented in this chapter show that the immunological hyper-responsiveness of NZ mice is not directly related to their rapid rates of non-immune antigen elimination. However, rapid removal of antigen by macrophages may at least contribute to their relative resistance to the induction of tolerance, by reducing the possibility of significant contact between the unprocessed antigen and antigen sensitive lymphocytes.

Dixon and his colleagues (201) have shown that a small number of rabbits repeatedly immunised with soluble heterologous serum proteins develop an immune complex nephritis and suggested that this could be due to variations in the quantity of precipitating

antibody formed. Christian's group have shown that the development of glomerulonephritis depends upon the formation of predominantly non-precipitating antibodies (202-204). Such antibodies would recognise only a limited number of multiple antigenic determinants, and thus favour the formation of soluble complexes, rather than the elaborate lattice structure of precipitating antigen-antibody complexes. This view has since been applied to inbred strains of mice, several of which develop chronic glomerulonephritis on exposure to infection with the lymphocytic chorio-meningitis (LCM) virus (205). It has been shown that those strains of mice prone to develop immune complex nephritis, produce antibody of low affinity compared to other strains following repeated immunisation with several soluble serum proteins and other antigens (206, 207). NZB and BWF_1 mice spontaneously develop immune complex nephritis, (82, 83) which may be aggravated by LCM or polyoma virus infections (102). It thus seemed relevant to study the affinity of antibody produced in response to BSA immunisation in these, and other strains of mice. The results of this study demonstrate that in the primary response to BSA, interstrain differences in antibody affinity do not occur. However, following a secondary antigenic challenge, NZB and BWF_1 mice produce antibody of somewhat lower affinity than that of the other strains studied, with the exception of C3Hf mice.

mice may explain this difference. Cerottini and his colleagues (110) have previously studied the affinity of BSA antibody in New Zealand and other strains of unimmunised mice, following the injection of BSA in FCA, like Soothill and Steward (206), they detected no interstrain differences.

A further difference between these results and those of Soothill's group (206, 207, 208), lies in the range of values calculated for the affinity indices (K). Those authors reported values for $\log_{10}K$ ranging between 5 and 7 1/M, which are considerably lower than those found in the present experiment (6.4 to 9.5). The affinities of antibodies produced by various animals and humans have been reported to range between 1×10^4 and 1.8×10^{10} (209), and the results of both this study, and those of Soothill's group lie well within this range. The affinity of antibody has been shown to decrease with increasing doses of antigen, and this appears to be related to the onset of immunological tolerance (210). The more prolonged antigen administration schedule of Soothill's group may have resulted in the formation of antibody of lower affinity because the mice were being rendered partially tolerant, with deletion of cell clones producing high-affinity antibody (211). However, this explanation is difficult to accept as Steward (208) has shown that New Zealand mice produce antibody of far lower

affinity than other mouse strains, yet other workers (107, 108, 109) have shown them to be relatively resistant to the induction of immunological tolerance.

The concentration of antibody combining sites in the serum showed an inverse correlation with antibody affinity in both the primary and secondary responses, but not following challenge with BSA in FCA. Steward and Petty (207), were unable to demonstrate such a correlation, and again this could be due to the prolonged course of antigen administration they employed. In the present experiment the correlation coefficient was much lower for the secondary response than for the primary response. McKay (191) has observed a similar correlation in the sera of chickens immunised with HSA.

Although differences in the immunisation schedules employed in these two studies do exist, and may account for some of the differences in the results obtained, I am unable to confirm Steward's observation (208), that New Zealand mice produce antibody of lower affinity than other strains.

CHAPTER 7.

A STUDY OF THYROXINE SECRETION RATES IN MICE

INTRODUCTION

Stark (181) showed that administration of thyroxine in pharmacological doses increased the rate of catabolism of bovine gamma globulin in mice. It thus appeared possible that the rapid rates of antigen catabolism encountered in New Zealand mice could reflect strain differences in endogenous thyroxine secretion. In this chapter I have applied the occupancy principle (212,213) to study thyroxine secretion rates in 5 strains of mice.

The Occupancy Principle

The occupancy principle is an obvious corollary of the law of the conservation of matter. The body of all animals ultimately consists only of matter which has entered from outside, since no matter is created within the body. There must therefore be a precise and definite relationship between the quantity of any kind of material in the body, or any part of the body, and the net flow of the relevant material into the body. The ratio of any quantity to its flow has the dimension of time.

Thus:

$$\frac{W}{W/T} = T$$

Where W = Quantity

T = Time

and W/T = Flow

The time found by dividing the quantity of material in any part of the body by the flow of that material either entering the body, or synthesised within the body, is defined as the occupancy of that material in the specified part.

MATERIALS AND METHODS

(a) Mice

Four inbred strains of mice; NZB, BALB/c, CBA and C3Hf; and the NZB x NZW F1 (BWF_1) hybrid were used for the study, which included six males and six females of each strain, all being aged between six and eight weeks.

(b) Environment and diet

Animals were housed in a constant temperature enclosure (18 - 20°C) for the duration of the study. For three days prior to the onset and throughout the duration of the experiment, mice were fed a thyroxine-free diet, GR 31 (214). Water was supplied ad libitum.

(c) Radiothyroxine

^{125}I-thyroxine (Radio-Chemical Centre, Amersham) with a specific activity of 20-50 mCi/mg. was used. Before use, a sample of the tracer was mixed with carrier thyroxine and sodium iodide and subjected to thin-layer chromatography on silica gel (MN-GHR), using methanol, chloroform and 0.88 ammonia (25:35:2.5, v/v/v). The position of the thyroxine spot was determined by spraying the TLC plate with ninhydrin, whilst iodide was located by spraying with 0.5% palladium chloride solution. The relative amounts of T4 and iodide were then determined by scraping the spots into glass tubes and counting the radioactivity in

a well gamma-counter (Nuclear Chicago). Isotope containing more than 5% free iodide was not used.

(d) Procedure; theoretical considerations

The standard method for obtaining thyroxine secretion rate (T4SR) is to administer radioactively labelled thyroxine (radio T4) and subsequently estimate the amount of radioactivity in serial plasma samples. T4SR is simply estimated by dividing the hormone concentration by the area under the curve of plasma activity against time(occupancy) when the activity is expressed as a fraction of the dose (215). Since the disappearance of radio T4 from the plasma occurs mono-exponentially, the area under the curve can be calculated by the simple regression method of dividing the extrapolated zero-time activity by the fractional disappearance rate. However, Gillespie et al. (216) have argued that an alternative approach is valid. They showed that when multiple doses of tracer hormone are administered over several days, the total activity remaining in any organ expressed as a fraction of the average daily dose, is equivalent to the total area under the activity-time curve following a single tracer hormone injection. This procedure has since been validated in many experimental situations (217) and has been used in this study.

(e) Experimental procedure

Each injection of ^{125}I-T4 was given in a final volume of 0.5 ml. using physiological saline as diluent. The dosage and

timing of ^{125}I-T4 administration is shown in the schedule below:

Day	Time	Dose of ^{125}I-T4
1	4.00 p.m.	0.1 uCi
2	4.00 p.m.	0.1 "
3	4.00 p.m.	0.1 "
4	4.00 p.m.	0.133 "
5	10.00 a.m.	0.0167 "
	2.00 p.m.	0.0167 "
	4.00 p.m.	Animals killed

All activities were extrapolated to zero-time and standard solutions prepared as described by Harland & Orr (215). At 4.00 p.m. on the final day the animals were weighed, and then 20 ul. of blood was withdrawn from the retro-orbital venous plexus into glass capillary tubes (Drummond Microcaps Shandon Ltd.). The blood was lysed in 1 ml. of 0.1% sodium carbonate solution. The animals were next exsanguinated under ether anaesthesia. The liver, spleen, intestines including contents, kidneys, thymus and heart were removed. The amount of radioactivity in each organ and in the 20 µl. blood sample was measured and expressed as a fraction of the counts in 0.1 µCi ^{125}I-T4.

The activity of plasma could not be determined directly due to the small volume (20 µl) of blood sample. I have found in a large number of mice of various strains that the haematocrit in health ranges between 47 and 53%, and that less than 6% of the

total blood T4 radioactivity is located in the packed cell fraction of haematocrit tubes. In the present instance, therefore, an approximate estimation of plasma activity was derived by multiplying the whole blood activity by two.

The plasma samples were pooled for each strain of mice and protein bound iodine (PBI) concentrations determined by the Technicon autoanalyser method (218). Plasma T4 concentrations (µg/100 ml) were obtained by multiplying the PBI concentrations by 1.53, as iodine contributes $^{1}/_{1.53}$ of the mass of a thyroxine molecule.

The T4SR values were then calculated by dividing the T4 concentration by the plasma activity. Thyroxine content of the various organs were calculated using the equation:-

Tissue T4 = T4SR x Tissue Activity (Occupancy).

(f) Statistical analysis of results for sex differences within each strain and for interstrain differences was made by Student's "t" test for unpaired variables.

TABLE 27: Serum protein bound iodine levels (P. B. I.) and weights (mean ± S. D.) of whole body and organs in the five mouse strains

Strain	P. B. I. (ug/100 ml)	Sex	Whole body	Intestines g	Liver g	Spleen g	Kidneys g	Thymus g	Heart g
NZB	2.2	M	25.5 ± 0.72	3.17 ± 0.28	1.22 ± 0.06	0.112 ± 0.017	0.33 ± 0.13	0.066 ± 0.010	0.14 ± 0.02
		F	19.4 ± 1.63	2.82 ± 0.30	0.85 ± 0.11	0.087 ± 0.010	0.24 ± 0.02	0.067 ± 0.016	0.11 ± 0.02
BWF_1	3.2	M	26.3 ± 2.27	2.86 ± 0.32	1.29 ± 0.09	0.103 ± 0.016	0.39 ± 0.04	0.058 ± 0.012	0.17 ± 0.03
		F	22.0 ± 1.56	2.61 ± 0.19	0.96 ± 0.13	0.082 ± 0.012	0.29 ± 0.03	0.073 ± 0.008	0.13 ± 0.02
CBA	6.0	M	23.5 ± 1.91	2.51 ± 0.20	1.06 ± 0.14	0.070 ± 0.021	0.35 ± 0.04	0.020 ± 0.020	0.12 ± 0.03
		F	18.7 ± 1.39	2.38 ± 0.46	0.93 ± 0.14	0.078 ± 0.002	0.22 ± 0.04	0.028 ± 0.013	0.10 ± 0.05
C3Hf	3.7	M	19.8 ± 1.18	2.35 ± 0.12	1.23 ± 0.12	0.087 ± 0.012	0.29 ± 0.03	0.038 ± 0.013	0.11 ± 0.02
		F	15.8 ± 0.94	2.01 ± 0.77	0.88 ± 0.06	0.065 ± 0.015	0.20 ± 0.01	0.052 ± 0.017	0.11 ± 0.04
BALB/c	4.0	M	23.0 ± 1.02	2.94 ± 0.40	1.14 ± 0.09	0.111 ± 0.013	0.36 ± 0.04	0.034 ± 0.011	0.12 ± 0.01
		F	19.9 ± 1.09	2.81 ± 0.93	0.91 ± 0.06	0.101 ± 0.014	0.23 ± 0.03	0.047 ± 0.008	0.09 ± 0.11

TABLE 28: Thyroxine secretion rates (T4SR) and organ thyroxine concentration (mean ± S.D.) in both sexes of the five mouse str

Strain	Sex	T4SR (ug./g. body-weight/day)	ORGAN THYROXINE CONTENTS (ug./g. of tissue)					
			Intestines	Liver	Spleen	Kidneys	Thymus	Heart
NZB	M	0.0060 ± 0.0013	0.0058 ± 0.0015	0.0200 ± 0.0023	0.0040 ± 0.0001	0.0985 ± 0.0240	0.0042 ± 0.0015	0.0056 ± 0.
	F	0.0063 ± 0.0008	0.0063 ± 0.0010	0.0270 ± 0.0021	0.0048 ± 0.0015	0.0138 ± 0.0017	0.0040 ± 0.0008	0.0069 ± 0.
BWF_1	M	0.0106 ± 0.0027	0.0092 ± 0.0015	0.0230 ± 0.0044	0.0073 ± 0.0021	0.0969 ± 0.0335	0.0075 ± 0.0008	0.0088 ± 0.
	F	0.0106 ± 0.0027	0.0108 ± 0.0015	0.0285 ± 0.0050	0.0085 ± 0.0015	0.0196 ± 0.0033	0.0079 ± 0.0019	0.0113 ± 0.
CBA	M	0.0150 ± 0.0017	0.0215 ± 0.0044	0.0548 ± 0.0083	0.0170 ± 0.0040	0.6830 ± 0.1960	0.0335 ± 0.0121	0.0165 ± 0.
	F	0.0202 ± 0.0050	0.0219 ± 0.0029	0.0598 ± 0.0021	0.0017 ± 0.0021	0.0490 ± 0.0079	0.0223 ± 0.0085	0.0194 ± 0.
C3Hf	M	0.0071 ± 0.0008	0.0129 ± 0.0017	0.0313 ± 0.0031	0.0063 ± 0.0008	0.1923 ± 0.1144	0.0090 ± 0.0033	0.0098 ± 0.
	F	0.0090 ± 0.0022	0.0117 ± 0.0027	0.0390 ± 0.0052	0.0070 ± 0.0010	0.0190 ± 0.0015	0.0079 ± 0.0029	0.0090 ± 0.
BALB/c	M	0.0115 ± 0.0006	0.0131 ± 0.0025	0.0463 ± 0.0056	0.0088 ± 0.0013	0.5827 ± 0.0331	0.0140 ± 0.0031	0.0115 ± 0.
	F	0.0133 ± 0.0013	0.0142 ± 0.0019	0.0490 ± 0.0040	0.0088 ± 0.0015	0.0327 ± 0.0069	0.0083 ± 0.0025	0.0117 ± 0.

TABLE 29: Significance of interstrain differences of thyroxine secretion rates (T4SR) and of organ thyroxine contents in five mouse strains indicated by Student's t value (upper figures) and the computed probability (p) (lower figures).

Strains Compared	T4SR	ORGAN THYROXINE CONTENTS						
		Intestines	Liver	Spleen	Kidneys		Thymus	Heart
					M	F		
NZB	5.66	6.49	2.07	5.48	0.69	3.73	6.81	5.04
BWF_1	5×10^{-6}	8×10^{-7}	3×10^{-2}	8×10^{-6}	NS	2×10^{-3}	4×10^{-7}	2×10^{-5}
NZB	8.70	9.84	14.16	18.52	7.25	10.59	7.09	12.98
CBA	7×10^{-9}	8×10^{-10}	1×10^{-11}	1×10^{-11}	1×10^{-5}	5×10^{-7}	2×10^{-7}	3×10^{-11}
NZB	3.12	8.38	7.60	5.21	1.96	5.38	4.63	6.55
C3Hf	3×10^{-3}	1×10^{-8}	7×10^{-8}	2×10^{-5}	NS	2×10^{-4}	7×10^{-5}	7×10^{-7}
NZB	12.47	10.35	16.06	8.32	29.03	6.60	5.91	6.85
BALB/c	1×10^{-11}	3×10^{-10}	1×10^{-11}	2×10^{-8}	3×10^{-11}	3×10^{-5}	3×10^{-6}	4×10^{-7}
BWF_1	4.64	10.09	13.13	8.46	7.22	8.33	5.90	7.64
CBA	6×10^{-5}	5×10^{-10}	1×10^{-11}	1×10^{-8}	1×10^{-5}	4×10^{-6}	3×10^{-6}	6×10^{-8}
BWF_1	2.89	2.77	4.35	1.74	1.96	0.43	0.73	0.87
C3Hf	4×10^{-3}	6×10^{-3}	1×10^{-4}	NS	NS	NS	NS	NS
BWF_1	1.94	4.49	10.50	1.27	25.26	4.23	2.82	0.73
BALB/c	3×10^{-3}	9×10^{-5}	3×10^{-10}	NS	1×10^{-10}	9×10^{-4}	5×10^{-3}	NS
CBA	6.88	7.62	8.98	10.29	5.30	9.07	5.63	9.89
C3Hf	3×10^{-7}	7×10^{-8}	4×10^{-9}	4×10^{-10}	2×10^{-4}	2×10^{-6}	6×10^{-6}	7×10^{-10}
CBA	3.90	6.54	4.20	8.12	1.23	3.81	4.71	5.90
BALB/c	4×10^{-4}	7×10^{-7}	2×10^{-4}	2×10^{-8}	NS	2×10^{-3}	5×10^{-5}	3×10^{-6}
C3Hf	6.46	1.49	5.74	3.29	8.00	5.38	1.90	6.55
BALB/c	8×10^{-7}	NS	5×10^{-6}	2×10^{-3}	6×10^{-6}	2×10^{-4}	NS	7×10^{-7}

RESULTS

The mean values for total body weight, organ weights and PBI in the strains of mice studied are shown in Table 27. It can be seen that there are marked interstrain differences in PBI concentrations, CBA mice having the highest (6.0 µg/100 ml) and NZB the lowest (2.2 µg/100 ml). The PBI levels represent the values for the pooled sera from each of these groups of mice. In previous collaborative work (unpublished) I have found little intra-strain variation in the PBI values between the strains of mice studied and between the sexes within each strain.

Table 28 shows the mean thyroxine secretion rate per g. body weight/day, and the mean thyroxine content/g. of tissue for the various organs. The statistical significance of the interstrain differences for these values are shown in Table 29. Three strains were found to have significant sex differences in T4SR which was most marked for BALB/c mice ($p = 0.004$). Neither NZB nor BWF_1 mice showed a significant sex difference in T4SR. Male mice of all strains were found to have higher kidney T4 concentrations than females, but otherwise there were only occasional sex differences in T4 concentrations of the other organs.

DISCUSSION

Although for many purposes mice provide excellent experimental models, thyroxine metabolism has been little studied because of technical difficulties related to their small size. Other workers have used indirect techniques and have required to give graded doses of exogenous thyroxine in order to prevent propyl-thiouracil goitre formation (182) or to suppress thyroidal radio-iodine release (183). More recently Wills & Schindler (209) employed a method involving the turnover of radiothyroxine. The method employed in the present study provides a direct estimate of T4SR based on radiothyroxine turnover, and also measures the thyroxine content of various organs.

Like other workers (182-184), I have demonstrated marked strain differences in thyroxine secretion rates. However in contrast to the findings of Mendoza et al. (182) CBA mice were found to have the highest T4SR. Mice of this strain are well known to demonstrate an excessive degree of physical activity. On the other hand, mice of the C3Hf strain which also exhibit excessive activity were found to have a comparatively low T4SR. Thus we cannot support the general conclusion of Mendoza et al. (182) that mice with active behavioural patterns have lower thyroid secretion rates than those of more placid strains.

My interest in the thyroid secretion rates in different strains of mice arose from the observation that administration of

TABLE 30

A comparison between the rates of BGG catabolism and thyroxine secretion rates in 5 strains of mice

Mouse Strain	Rank Regarding Rate of BGG catabolism	Rank Regarding T4SR
NZB	1	5
BWF_1	2	3
BALB/c	3	2
C3Hf	4	4
CBA	5	1

pharmacological doses of T4 to mice accelerates the catabolism of a soluble protein antigen, bovine gamma globulin (181). A study of bovine gamma globulin catabolism in these five strains of mice was undertaken to investigate the role of antigen catabolism in the development of autoimmune disease of NZB and BWF_1 mice (chapter 5). Rapid rates of antigen catabolism were found in NZB and BWF_1 mice whereas CBA mice had the slowest rate. The results of the present study show no obvious relationship of rates of antigen catabolism to either thyroxine secretion rates or thyroxine content of various organs, in these strains of mice (Table 30).

Using this technique for estimating T4SR, it should be possible to investigate the genetic control of thyroxine secretion in mice by cross-breeding experiments.

CHAPTER 8.

CLEARANCE OF DEOXYRIBONUCLEIC ACID FROM BLOOD IN NEW ZEALAND MICE

INTRODUCTION

New Zealand Black (NZB) mice spontaneously develop auto-immune haemolytic anaemia (77). When crossed with New Zealand White (NZW) mice, the NZB x NZW F1 (BWF_1) hybrid progeny develop lupus-like glomerulonephritis (77, 78, 80) and this is associated with the appearance in their serum of auto-antibodies to DNA (81). As antibodies to DNA are rare in other strains of mice (81) it seems possible that BWF_1 mice have an inborn error of DNA catabolism. This chapter describes an investigation into this hypothesis by studying the rate and mechanism of DNA clearance after its intravenous administration in five strains of mice.

MATERIALS AND METHODS

(i) Mice. Mice from the inbred strains NZB, CBA, BALB/c, and C3Hf, and BWF_1 hybrid stock, were aged 6-8 weeks at the time of the experiments. At this age, BWF_1 mice do not have anti-DNA antibodies in their sera (81) and thus formation of DNA - anti-DNA complexes with consequent increased clearance rates of DNA can be excluded. The normal appearance of the kidneys in mice of this age (80) strongly supports this conclusion.

(ii) Deoxyribonucleic acid. Radioactive labelled E. coli (^{32}P) DNA (included below as *DNA) was generously donated by Professor M. Smellie and Dr. J. Morrison. This preparation possessed a specific activity of 100,000 d.p.m./ug and more than 99% of the radioactivity was acid-precipitable, showing that the DNA was high in molecular weight.

(iii) DNA clearance rates. Each mouse received 5 µg of *DNA in 0.1 ml phosphate-buffered saline by tail-vein injection. At intervals of 3, 6, 9, 12, 15 and 18 minutes following injection, 20 µl of blood was withdrawn into standard heparinised capillary tubes (Drummond Microcaps, Shandon Ltd.) by puncture of the retro-orbital venous plexus. Each blood sample was lysed in 0.2 ml of 0.1% sodium carbonate, decolorised by the addition of 0.1 ml hydrogen peroxide (30% w/v; British Drug Houses) and solid material was disolved by adding 0.1 ml hyamine hydroxide (Nuclear Enterprises) and incubated for 16 hours at 56°C. Ten ml of

scintillation fluid (NE 250, Nuclear Enterprises) were added to each of the samples which were then counted in a Packard Tricarb liquid scintillation counter. Counts were corrected for self-absorption and then plotted semi-logarithmically against time. From the semi-logarithmic plot, it was found that DNA was cleared from the blood in a mono-exponential pattern over the time of the experiment. The $T\frac{1}{2}$ values for DNA clearance were derived from the plot.

(iv) Organ distribution of DNA. After the last blood sample was obtained, the mice were killed by cervical dislocation and weighed. Liver, spleen, kidneys, thymus and the chain of mesenteric lymph nodes were dissected out and weighed. A weighed portion of each organ, usually 30-50 mg., was dissolved by incubating for 16 hours at 56°C in 0.5 ml of soluene (Packard Ltd.). Ten ml of toluene-based scintillation fluid (one litre of toluene, containing 5 g. of 2, 5-diphenyloxazole (Nuclear Enterprises Ltd.) and 0.4 g. of 1, 4-bis-(2 - (4-methyl-5phenyloxazolyl)) - benzene (Packard Ltd.) were added to each of the samples, which were then counted as above. The percentage of the administered radioactivity remaining in each organ was then calculated after correcting for self-absorption and allowing for total organ weight.

(v) Mechanism of DNA removal from blood. To assess the role of nuclease activity in removal of DNA from blood, the following procedure was employed. Four female BALB/c and four female BWF_1 mice were given intravenously 5 µg of DNA. Two 20 µl blood samples were taken at 5, 10 and 15 minutes after injection,

absorbed onto Whatman No. 1 filter papers and dried. One of each of the paired samples was then washed twice in 5% trichloracetic acid at 4°C, twice in absolute alcohol, twice in ether, and dried (220). The second sample was simply dried, thus allowing the total amount of radioactivity of each sample to be determined, whereas the first sample gave the acid precipitable counts, which represented undegraded DNA. Ten ml of toluene-based scintillation fluid was then added to all samples and the radioactivity was counted. The $T\frac{1}{2}$ values were determined as before except that correction for self-absorption was not applied in view of the comparable nature of each of the paired samples.

To assess the role of phagocytosis in removal of DNA from the blood, reticuloendothelial depression was induced by the intravenous injection of 20 mg emulsified ethyl stearate 24 hours prior to DNA* administration (221). The rate of DNA* clearance was then studied in four female NZB and four female BALB/c mice.

Deoxyribonuclease (DNA-ase) activity in the macrophages in sections of snap-frozen livers and spleens of all animals was also assessed using the histochemical technique of Vorbrodt (222). All tissue sections were processed simultaneously.

Sections of liver and spleen were coated with photographic emulsion (Ilford K5) and stored at 4°C for 1-4 weeks. After development and fixation of the photographic emulsion, the sections were stained with methyl-green pyronin. The distribution of radioactivity within the tissues was observed microscopically.

TABLE 31 Rate of clearance and organ localisation of intravenously administered DNA

Strain of Mouse	Number and Sex (M = male F = female)	Rate of clearance of intravenous DNA (half-life or $T\frac{1}{2}$ value, in minutes)		Percentage of DNA dose in liver and spleen
		Mean	Range	
NZB	4M, 4F	7.5	6.1 - 8.9	78.2
BWF_1	3M, 3F	5.7	5.2 - 6.2	82.2
BALB/c	3M, 4F	5.3	4.8 - 8.0	84.5
CBA	2M, 2F	5.5	5.1 - 6.2	84.2
C3Hf	3M, 4F	5.8	4.1 - 9.0	87.4

RESULTS

By plotting the blood clearance rates semi-logarithmically, DNA was found to be cleared from the blood in a mono-exponential pattern over the duration of the experiment. The clearance rates ($T\frac{1}{2}$ values) and percentage localisation in liver and spleen of injected DNA in the strains of mice studied is shown in Table 31. The mean $T\frac{1}{2}$ values ranged closely between 5.3 and 5.8 minutes except in NZB mice (mean $T\frac{1}{2}$ = 7.5 minutes). Using Student's "t" test the plasma DNA half-life in NZB mice was found to be significantly longer than in BWF_1 ($t = 4.3764$, $p = 0.00045$), BALB/c ($t = 5.7438$, $p = 0.00003$), and C3Hf mice ($t = 2.5980$, $p = 0.01104$). Comparisons using the data on CBA mice were not used in view of the small number studied. Between 78.2 and 87.4% of administered DNA was localised in liver and spleen. Thymus and mesenteric lymph nodes contained less than 0.1% while kidneys accounted for up to 3% of DNA. There was no preferential DNA localisation in kidneys of NZB or BWF_1 mice as compared to the other strains. No relationship was found between $T\frac{1}{2}$ values and total body, liver, or spleen weights. Histochemically, there was no obvious difference in the content of DNA-ase in the fixed macrophages of livers and spleens between the five strains of mice studied, although it must be admitted that the technique used is little more than qualitative.

The DNA in blood of BALB/c and BWF_1 mice at 5, 10 and 15 minutes after administration was almost 100% acid precipitable.

TABLE 32 Effect of ethyl stearate administration on the rate of DNA clearance

Strain of Mouse	DNA clearance mean $T\frac{1}{2}$ (minutes)	
	Untreated	After Ethyl Stearate
NZB	7.5	13.4
BALB/c	5.3	14.7

This indicates that the DNA remaining in blood at these times was of high molecular weight, and had probably not been significantly degraded by serum nuclease activity (220), although the technique is relatively insensitive compared with direct estimations of nuclease activity.

Administration of ethyl stearate to the mice prior to injection of labelled DNA caused a sharp reduction in the rate of DNA clearance in BALB/c and NZB mice, as indicated by the prolongation of their respective $T\frac{1}{2}$ values (Table 32). There was no significant difference in the $T\frac{1}{2}$ values after ethyl stearate.

Autoradiographic examination of tissue sections showed the radioactivity to be localised in the Kupffer cells of the liver, and mainly in the perifollicular zone, but also the red pulp, of the spleen.

DISCUSSION

Although NZB mice have the longest $T\frac{1}{2}$ values of the five strains studied, it is unlikely that slow DNA clearance is related to production of antibodies to DNA in this strain, as BWF_1 mice, which almost universally develop these antibodies, have shorter $T\frac{1}{2}$ values, similar to those in the control strains. The slow DNA clearance in NZB mice is not related to interstrain differences in liver and spleen weights, which were similar in the strains of mice used in this study, contrary to my findings described in chapter 3 in which the smaller liver and spleen weights were related to slower carbon clearances. There was no evidence of obvious DNA-ase deficiency in the fixed macrophages of livers and spleens. However the technique for DNA-ase activity employed (222) is not quantitative, and despite the fact that all tissues were processed simultaneously, caution must be exercised in interpreting these histochemical data. That intravenously administered DNA appears to be phagocytosed mainly by the fixed macrophages of liver and spleen is shown by the high percentage of DNA contained in these organs at the end of the experiment. This conclusion is further supported by the markedly prolonged DNA clearance rates following the administration of ethyl stearate, which is known to depress reticuloendothelial function (221), and the tissue distribution of the injected DNA as shown by autoradiography.

Sera from patients with systemic lupus erythematosus have been shown to contain circulating inhibitors of DNA-ase, and this may be of importance in the production of auto-antibodies to DNA in this disease (223). Serum DNA-ase activities have not been measured directly in this study, neither have inhibitors of this enzyme been sought. However, it seems highly unlikely that either of these factors are of relevance to the production of anti-DNA antibodies in BWF_1 mice, as virtually all the DNA in the blood was found to be acid precipitable in BALB/c and BWF_1 mice up to 15 minutes after injection. It might be argued that DNA degradation products are removed immediately from the blood, which would also explain the latter finding. If so, one would expect such DNA degradation products to be located in organs of high metabolic activity. The thymus, which has been shown to have a high turnover of cells (224-226) would then be expected to contain a much greater proportion of the administered radioactivity that was found in this experiment.

Gosse and his colleagues (227), who also studied the degradation of DNA administered intravenously to mice, similarly found a mono-exponential clearance of DNA from plasma although the plasma half-life was longer in their experiments. Their results differ from mine in that they found that the clearance of DNA from plasma was mainly dependant on nuclease activity, and observed that the plasma DNA half-life was closely related to this nuclease activity. However the two experiments are not comparable as Gosse et al (227)

administered much larger doses of DNA to their mice (200 μg.; cf. 5 μg. in the present experiments), which may have temporarily saturated the reticuloendothelial system, thus slowing DNA clearance and perhaps allowing serum nuclease to degrade the DNA. Although the acid precipitability of the DNA was unaltered during the period of sampling in my experiments, the possibility of some DNA degradation by nuclease cannot be ruled out. However the present results indicate that phagocytosis is more important in the clearance of DNA given in low dosage. The slower clearance of DNA in NZB mice might thus represent an abnormality of phagocytosis, although the clearance of colloidal carbon is normal in these mice when corrected for body weight (228, chapter 3), and the in vitro phagocytosis of Staphylococcus aureus by peritoneal macrophages is also normal (chapter 4). Soluble antigens such as bovine gamma globulin and bovine serum albumin and polyvinylpyrrollidone (chapter 5) however appear to be phagocytosed more rapidly by the New Zealand strains of mice. To conclude that the slow clearance of DNA in NZB mice is due to a phagocytic abnormality would be highly speculative although other explanations are difficult to conceive. The data presented in this chapter show that abnormalities of DNA breakdown are probably not responsible for the production of DNA antibodies in NZ mice.

Final discussion of Phagocytosis in NZB mice and some thoughts on their further use in laboratory research

The results of this thesis show that the phagocytosis of inert particulate material (colloidal carbon) and bacteria (Staphylococcus aureus) are normal in NZ mice, whereas the phagocytosis of soluble antigens, (BGG, BSA and PVP) is more rapid in NZ than other strains of mice. The discrepancy between the rates of phagocytosis of particulate and soluble antigens suggests that these two processes are metabolically different. Indeed, it has been shown that the ingestion of particulate material is mainly dependent upon glycolysis (394, 395) whereas pinocytosis, the ingestion of soluble material, appears to depend upon oxidative phosphorylation with the ultimate participation of adenosine 5^1-triphosphate (395a). In addition, the continuing formation of pinocytic vesicles is dependent upon new protein synthesis, and studies with actinomycin D, suggested that DNA directed RNA synthesis was involved (395a). Studies of the different metabolic processes occurring within the macrophages of NZ mice may well reveal increased activity in oxidative phosphorylation relative to glycolysis.

The role of increased non-immune elimination of soluble antigens in the production of the exaggerated immune responses, resistance to tolerance inducation, and the pathogenesis of autoimmune disease has not yet been established. Certainly no relationship could be demonstrated between the rate of non-

immune antigen elimination and the height of the subsequent antibody response. However, the rapid non-immune elimination of antigen by NZ mice could be relevant to the difficulty in tolerance induction in these mice; the rapid elimination of antigen by macrophages could reduce to a minimum the direct contact between unprocessed antigen and lymphocyte, and thereby the risk of tolerance induction (266). The rates of non-immune elimination of the three antigens BGG, BSA and PVP differed not only in their overall range (i.e. days for BGG, hours for BSA and PVP) but also for each antigen, the relationship was not constant between different mouse strains, (e.g. BWF_1 mice second fastest for BGG, sixth for BSA and first for PVP). Thus to extrapolate from these results to the role of auto-antigen elimination in the pathogenesis of autoimmune disease; would be highly speculative, and probably incorrect, and the data from the DNA elimination studies included in this thesis show that elimination of the "auto-antigen" (E. coli DNA was used) did not support a role for defective removal in the production of DNA antibodies. Similar reservations must be made regarding the interpretations of other workers who have asserted that the immunological peculiarities present in NZ mice (see introduction) are probably responsible for the production of the autoimmune disease of these mice. In fact, the number of documented immunological peculiarities is so large, and appears to be increasing steadily, that their

combination drug regimes of possible use in the treatment of human autoimmune disease. The problem of neoplasia arising as a result of long-term immunosuppressive drug therapy could also be fully evaluated. Perhaps utilisation of these latter two suggestions might yet provide the most important contribution of NZ mice to medical science.

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