AN INVESTIGATION OF SOME OF THE IMMUNOLOGICAL AND PATHOLOGICAL ASPECTS OF THE DISEASE SYNDROME OCCURRING IN THE NEW ZEALAND BLACK MOUSE STRAIN

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Degree of Ph.D. University of Edinburgh 1970
It has previously been demonstrated that NZB mice spontaneously develop haemolytic anaemia associated with the production of autoantibodies directed against erythrocyte antigens. Abnormalities of lymphoid tissues, particularly splenic hypertrophy, and a high incidence of spontaneous tumour formation affecting primarily lymphoid tissue have also been described in members of this strain. These general findings were confirmed in the present study.

The autoantibodies of NZB mice responsible for the agglutination of papain-treated mouse erythrocytes were found to occur in close correlation with the antibody responsible for the positive direct Coombs test. Fractionation of NZB sera containing activity against papain-treated erythrocytes using G-200 Sephadex indicated that this activity could be associated with both 19S and 7S immunoglobulins, and that an individual animal might contain either or both sizes of molecule.

The serum of most NZB mice possessed high levels of complement-fixing activity against isogeneic liver homogenate. No correlation was found between the presence of autoantibody on erythrocytes and the occurrence of anti-liver activity. Ultra-centrifugation of NZB sera on sucrose density gradients indicated that the anti-liver activity was associated with a high molecular weight (19S) serum fraction.

The injection of viable spleen cells, obtained from NZB mice producing anti-erythrocyte autoantibodies, into young syngeneic recipients produced premature conversion to an autoantibody producing
state. Similar numbers of lymph node or bone marrow cells, or smaller numbers of thoracic duct cells did not have this effect.

In (NZB x T6)F1 hybrids given NZB spleen cells evidence of a severe graft-versus-host (GVH) reaction was found. If spleen cells from NZB animals producing anti-erythrocyte autoantibodies were used (NZB x T6)F1 hybrids developed signs of anaemia, gross splenomegaly and erythrocyte autoantibodies - features not found in the simple GVH reaction in this strain combination.

Anti-lymphocyte sera (ALS) were produced by giving rabbits three injections of $10^9$ viable mouse thymus cells, and the immuno-suppressive effects of such sera were tested using a mouse skin homograft system. Three other assays of the biological effects of ALS preparations were used: inhibition of a standard GVH reaction, IN VITRO cytotoxicity for lymphocytes, and IN VIVO lymphopenia production, but none of these assays gave results which correlated closely with homograft-prolonging ability. Digestion of ALS IgG to the F(ab')$_2$ fragment was associated with loss of IN VITRO cytotoxicity for lymphocytes and loss of the ability to suppress a standard GVH reaction.

The administration of ALS to NZB mice produced a reduction of lymphocyte numbers in peripheral blood, but long-term administration of this material to adult NZB mice beginning either before or after they developed signs of autoimmune disease had no significant effect on autoantibody production as measured by the direct Coombs test. ALS given to (NZB x CBA)F1 hybrids four hours prior to transfer of spleen cells from old NZB animals did, however, inhibit the development of autoantibodies in the recipients.
NZB and (NZB x CBA)F1 hybrid mice gave a marked immune response to the protein antigen bovine serum albumin (BSA). This response was much greater than that given by other mouse strains tested, and furthermore there was some indication that NZB mice were less easily rendered hypo-responsive to BSA by pre-treatment with antigen in aggregate-free form than were these other strains.
"There is every reason to believe that experimental autoimmune diseases represent promising model systems for probing an enormous array of questions and problems. There is a clear need NOT to discover more models and new experimental diseases, but to find new information and meaning within the ones close at hand and awaiting further dissection."

Philip Y. Paterson. 1966
INTRODUCTION
# INTRODUCTION

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It has been estimated that the immune response of humans can recognize at least $10^5$ different antigens and produce antibodies against them (Singer and Doolittle, 1966). Included among these antigens are the many structural molecules of the tissues of other individuals; yet it is generally observed that under normal conditions antibodies are not produced by an individual against the equivalent molecules of his own tissues. Some regulatory mechanism must therefore exist whereby such autoimmune reactions are prevented.

This ability to discriminate between intrinsic and extrinsic antigens was recognized in the early days of immunology by Ehrlich and Morgenroth (1908) who pointed out that in an individual with a considerable subcutaneous haemorrhage "the essential conditions, just as in experiment, are given for the formation by reaction of substances possessing specific injurious affinities for these blood cells", and that "the same can apply to other tissues; for every acute atrophy of the parenchyma of an organ can lead to the absorption of cell material and to the consequences." They investigated these possibilities by injecting a goat with a pool of homologous erythrocytes, and tested the recipient's serum for haemolytic activity. No activity was found when autologous erythrocytes were used, although the erythrocytes of eight out of nine other goats were lysed. From this finding they concluded that the serum contained antibodies reactive only with heterologous erythrocyte surface antigens, and they supposed that an animal possesses "certain regulating contrivances" which prevent
an injurious immune reaction against its own cell material. From these findings arose the well-known concept of a "horror autotoxicus of the organism."

The "horror autotoxicus" postulate has had two profound influences on subsequent research - it has provoked many studies designed to test its universal applicability and it has stimulated many investigations of the mechanisms whereby injurious immune responses are prevented. Its universal applicability remains unchallenged, but only if one emphasizes (as did Ehrlich) that it refers to injurious immune responses and that it applies to normal animals rather than those suffering from disease. For apparently harmless autoimmune antibodies have been found in normal animals, and in a number of diseases (mostly of humans) autoimmune responses are found to occur with features suggesting that they may contribute to the pathology and aetiology of the diseases. The mechanisms by which autoimmune reactions are normally prevented, however, are not well understood - nor are the factors which underlie the occasional breakdown of these mechanisms known to any significant degree.

2. NATURALLY OCCURRING, NON-PATHOLOGICAL AUTO-ANTIBODIES

There is abundant evidence from many sources that antibodies capable of reacting with autologous tissue antigens can be found in the sera of normal animals. Friedenreich (1929) noted that normal human serum could agglutinate autologous erythrocytes which had been exposed to bacterial enzymes, and a number of subsequent investigations have shown that the serum of normal healthy individuals may contain antibodies which will react with their own erythrocytes which have been pre-treated with enzymes (Stratton...
and Renton, 1958; Dybkjaer, 1966; Worlledge, Carstairs and Dacie, 1966). Such auto-antibodies of healthy individuals have generally been found to be IgM, whereas the antibodies found in autoimmune haemolytic diseases are mainly IgG (Dodd and Wilkinson, 1964; Dybkjaer and Kissmeyer-Nielsen, 1967).

Kidd and Friedewald (1942a and b) showed that apparently healthy rabbits had a serum antibody which fixed complement when reacted with antigen derived from a wide range of foetal and adult rabbit tissues, and from the tissues of other mammals. This activity could be destroyed by heating at 65°C, and was found to be absent from the sera of rabbits during the first month of life. Similar auto-antibodies reactive with widely-occurring, sedimentable tissue components have been described in humans (Hackett, Beech and Forbes, 1960); in rats (Digby and Loewi, 1965; Weir et al., 1966); and in other animals (Muschel et al., 1961). The findings with rabbits were confirmed by Asherson and Dumonde (1962), and extended to show that this naturally occurring activity behaves as a macroglobulin on DEAE-cellulose chromatography and zonal ultracentrifugation (Asherson and Dumonde, 1963; Asherson and Rose, 1963). The antigens with which such antibodies react have been found most often in the mitochondrial fractions of tissue homogenates (Elson, 1968), and are detected by complement fixation tests. Gusev (1966) studied the sera of 215 apparently healthy chickens, and found that 19 of them contained antibodies which formed precipitates in gel diffusion plates with extracts of autologous liver or kidney.

An increased incidence with age of antibodies reactive with
nuclear antigens (i.e. anti-DNA and anti-nucleoprotein antibodies) has been reported in humans (de Blecourt, Boerma and Vosenkamp, 1967; Camarata, Rodnan and Fennell, 1967) and in mice (Teague, Friou and Myers, 1968). The antigens used in these studies were not autologous so that strictly speaking auto-antibodies were not demonstrated, but Hildemann and Walford (1967) did show an autoimmune response when they demonstrated that large numbers of apparently healthy mice older than 2 years contained increased numbers of spleen cells capable of producing haemolytic antibody reactive with autologous erythrocytes.

3. AUTO-ANTIBODY FORMATION IN ASSOCIATION WITH TISSUE DAMAGE AND INFECTION

Milgrom et al. (1957) and Burgio and Severi (1965) have shown that the fluid of blisters raised on human skin by mechanical means may contain agglutinins which are reactive with autologous erythrocytes at 4°C and 37°C, and that such activity was not contained in the serum. Genova and Vaccaro (1964) however, using an immunofluorescence technique, demonstrated that the agglutinins from blister fluid were gamma globulins and probably antibodies. The injection of carbon tetrachloride into rats produces liver damage, and Weir (1963 and 1964) has shown that an increase in auto-antibodies reactive with liver antigens accompanies this damaging effect. Similar results have been reported by Arnason, Salomon and Grabar (1964) in germ-free rats. Auto-antibody formation against heart antigens has been found after myocardial infarction or cardiomyopathy (Heine et al., 1966; Davies and Gery, 1963; van der Geld, 1964), and obstruction of the pancreatic duct (Murray
and Thal, 1960) and of the vas deferens (Römke and Hellinga, 1959) has been associated with antibodies to pancreas and spermatozoa in humans.

Many animal species have been found to respond to infection with a variety of micro-organisms by producing antibodies capable of reacting with autologous and heterologous tissue antigens (see review by Asherson, 1968). Three particularly well-documented instances of auto-antibody formation occurring in this fashion are provided by coccidial infection of rabbits, and infection of humans with certain strains of haemolytic streptococci or Escherichia coli.

The Kidd and Friedewald naturally-occurring auto-antibody which is reactive with sedimentable tissue components of normal rabbits has already been mentioned. Asherson and Rose (1963) found that serum levels of this activity increased in rabbits infected with Eimeria stiedae. This organism is the cause of hepatic coccidiosis in rabbits, and liver damage is frequently found in animals which become infected in the first month of life. Such animals were found to have higher titres of complement-fixing auto-antibodies against autologous kidney tissue than their uninfected litter-mates. There is also evidence that rabbits receiving anticoccidial drugs have lower titres of natural auto-antibodies than untreated rabbits.

Rheumatic fever has been shown to be preceded by infection with group A streptococci in virtually every case (Stollerman et al., 1956) and between 2 and 3 per cent of such infections are associated with subsequent rheumatic fever (Rammelkamp, 1955-56).
Auto-antibodies against heart have been demonstrated in rheumatic fever patients by complement fixation (Osler, Hardy and Sharp, 1954), immunofluorescence (Kaplan, Mayeserian and Kushner, 1961) and haemagglutination tests (Ehrenfield, Cery and Davies, 1961). Gamma globulin bound to the auricle has also been detected in specimens taken at operation for rheumatic heart disease (Kaplan and Dallenbach, 1961).

The cause of ulcerative colitis in humans is not known, but in about 10 per cent of patients auto-antibodies reactive with colon and ileum can be demonstrated (Broberger and Perlmann, 1959 and 1962; Koffler et al., 1962; Harrison, 1965; Wright and Truelove, 1966). Perlmann et al. (1965) found that the sera of children with ulcerative colitis would react with a lipopolysaccharide antigen obtained from the colon of germ-free rats, and that gel precipitation lines were formed by many of the sera against this antigen. The further finding that this reaction could be inhibited by absorbing the sera with an antigen obtained from the 014 strain of E. coli raises the possibility that auto-antibody formation in ulcerative colitis is related to infection with this organism.

4. HUMAN AUTOIMMUNE DISEASES

The importance of spontaneous auto-antibody formation and auto-antibody formation associated with tissue damage or a known infective process lies in the information which they provide on that group of human disorders known as the autoimmune diseases. These diseases are of widely differing pathology but have a number of features in common:
they are of unknown or obscure aetiology, auto-antibodies or evidence of localized autoimmune reactions are found in a high proportion of cases, and evidence of hyper-reactivity of the lymphoid system is a frequent finding. Among the human disorders which have been included in this group are the following: systemic lupus erythematosus, polyarteritis nodosa, rheumatoid arthritis, polymyositis and dermatomyositis, systemic sclerosis, Sjögren's syndrome, the demyelinating diseases (encephalomyelitis and multiple sclerosis), endogenous uveitis, various forms of thyroiditis, various forms of acquired haemolytic anaemia, thrombocytopenic purpura, pernicious anaemia and gastritis, various forms of liver disease, ulcerative colitis, adrenalitis, and myasthenia gravis. The evidence on which these disorders have been related to autoimmunity is of variable reliability and it is possible to dissent from an autoimmune classification for almost all of them. To discuss this evidence in full would occupy a disproportionate amount of space, but three autoimmune diseases which are of particular relevance to the subject matter of this thesis will be examined in some detail.

(a) **Systemic lupus erythematosus (SLE)**. This is a generalized inflammatory disease with involvement of the micro-vasculature and connective tissues throughout the body. It most often occurs in middle aged females, and presents with greatly variable grades of severity. Lesions are primarily found in serous and synovial membranes, skin and kidney and blood disorders such as anaemia and thrombocytopenia are frequently associated findings. The kidney lesion typically involves thickening of the basement membrane
with hyaline thrombi and haematoxylin bodies in the capillaries (Holman, 1965 and Vaughan, 1968). The case for linking autoimmunity and SLE rests on the following 5 pieces of evidence: (i) auto-antibodies reactive with nuclear antigens, cytoplasmic constituents, and cell membranes are frequently found, (ii) gamma globulin levels are frequently elevated, (iii) gamma globulin is found localized on the glomerular basement membrane in SLE-associated kidney lesions, (iv) low levels of serum complement are found in patients with active disease, and (v) relatives of patients with SLE have been found to have a higher incidence of rheumatic disease, hyper-gammaglobulinaemia, and auto-antibody formation than that which is found in the general population. The auto-antibodies which react with nucleoprotein (and which are responsible for LE cell formation) and with DNA are of widespread occurrence in SLE patients: they have been detected by many immunological tests including complement fixation, immunofluorescence, haemagglutination, PCA, flocculation, and precipitation. The anti-tissue antibodies, which are directed against cytoplasmic, mitochondrial and ribosomal antigens, are less common and are more difficult to demonstrate. The depression in serum complement levels involves all the major complement components. Since complement can be demonstrated in SLE kidney lesions, it seems likely that the lowered complement level is due to its utilization by an immunological reaction. The vascular lesions of SLE patients have been found to contain gamma globulins, complement and DNA; and electron microscope studies of the kidney lesions have revealed electron-dense material on the basement
membranes of the glomeruli. The appearance of the kidney lesions closely resembles that which is found in the glomeruli of rabbits suffering from experimental serum sickness, and Dixon (1962-63) has suggested that the two situations are analogous, i.e. that in serum sickness and in lupus nephritis the electron-dense material represents deposition of antigen-antibody complexes.

(b) *Myasthenia gravis*. This is a long-recognized disorder which is characterized by rapid exhaustion and loss of strength of voluntary muscle. The primary defect which produces muscle weakness affects the neuro-muscular transmission mechanism, and is probably located post-synaptically at the motor end-plate (Osserman and Strauss, 1965). Since the demonstration by Strauss et al. (1960) that the sera of many myasthenic patients contained an antibody capable of binding to normal striated muscle, several other workers have reported finding similar antibody activity (Beutner et al., 1962; van der Geld, Feltkamp and Costerhuis, 1964; Osserman and Weiner, 1965; Joshua et al., 1967). Some of these investigators have claimed that a correlation exists between the presence of muscle auto-antibodies and the severity of the disease, but Nastuk et al. (1966) did not agree with this. Nastuk, Flescia and Osserman (1960) found that the serum complement levels of myasthenic patients differed markedly from normal levels, either being higher or lower than normal. There was some suggestion of a correlation between severity of the disease process and fluctuations in levels of complement activity, and these authors suggested that complement components might be consumed in myasthenic patients due to a reaction between
auto-antibodies and muscle antigens. An increased incidence of other auto-antibodies has also been reported in patients with myasthenia gravis - antinuclear activity by White and Marshall (1962); Beutner et al. (1962); Sturgill et al. (1962); Feltkamp, Geld and Oosterhuis (1963); and anti-thyroid activity and rheumatoid factor by van der Geld et al. (1963).

Burnet (1962) commented on the association between thymic hyperplasia or thymoma and myasthenia gravis, and suggested that malfunctioning of the thymus (which is known to be important in the development of immunological competence) might be correlated with the occurrence of auto-antibodies in this disease. General support for this idea was provided by the studies of Osserman and Weiner (1965) and Nastuk et al. (1966) who found that the highest levels of anti-muscle activity were found in myasthenic patients with thymomas and that after thymectomy in such patients, and also in some with thymic hyperplasia, auto-antibody titres were reduced. However, an alternative explanation of the involvement of the thymus in myasthenic patients is possible. Marshall and White (1961) injected antigen directly into the thymus of guinea pigs and found changes which were reminiscent of those found in myasthenic patients with thymic hyperplasia - these included germinal centre formation, plasma cell production and local antibody formation. They suggested that these changes might be the result of local stimulation of immunologically competent cells by antigen within the thymus. White and Marshall (1962) demonstrated gamma globulin within the medulla of thymuses from myasthenic patients with thymic hyperplasia, and van der Geld,
Feltkamp and Oosterhuis (1964) showed that the skeletal muscle-reacting antibody activity in the serum of myasthenic patients cross-reacted with antigen contained in the thymus. Strauss, Kemp and Douglas (1966) suggested that the determinants with which such antisera react might be myoid cells - cells with muscle-like appearance which had been described in the thymuses of various vertebrates many years previously - and they showed that myasthenic sera containing anti-muscle activity did react against the myoid cells contained in the turtle thymus. Subsequently, others have agreed that the human thymus determinant with which myasthenic sera react is an atrophic and non-striated derivative of the myoid cell (van der Velde and Friedman, 1966; Mackay and Goldstein, 1967). The proportion of thymic medullary cells identifiable as "myoid" varies from species to species and with age but it is generally small in mammals and particularly so in man (van der Velde and Friedman, 1966; Mackay et al., 1968).

The significance of the auto-antibody in the serum of myasthenia gravis patients which reacts with muscle is far from clear. The available evidence suggests that it plays no direct part in the symptoms of the disease - it binds to the cross-striations of muscle cells and not to the motor end-plate region, it cannot be demonstrated in all cases of myasthenia, and it can be demonstrated occasionally in patients with thymoma who do not have myasthenia gravis or any evidence of neuro-muscular block (McFarlin, Barlow and Strauss, 1966). The implications of its cross-reaction with cells in the thymus, however, remain to be determined.
(c) **Autoimmune haemolytic anaemia (AHA).** The autoimmune haemolytic anaemias are a group of disorders with the common feature that erythrocyte destruction is associated with the presence of auto-antibodies to erythrocytes. There is another major group of haemolytic anaemias in which erythrocyte destruction is associated with congenital or inherited defects of erythrocyte structure. Confusion of these two types of disease was originally resolved by studies of erythrocyte life span: the erythrocytes of patients with inherited haemolytic anaemias have been found to be intrinsically defective and incapable of surviving for normal periods, whereas the erythrocytes from patients with AHA frequently show increased survival to near-normal values on transfusion to normal recipients. A second differentiating feature was provided by the introduction of the antiglobulin test by Coombs, Mourant and Race (1945) and its application to the study of erythrocytes from patients with acquired haemolytic diseases by Boorman, Dodd and Loutit (1946). That study demonstrated, and many others have since confirmed, that the red cells of patients with acquired haemolytic anaemia not associated with a hereditary or congenital defect very frequently have a globulin coating (antibody) on their surfaces.

The various types of AHA are generally classified on the basis of the characteristics of the erythrocyte-reacting, auto-antibody activity which is present. That which is associated with "incomplete" warm antibodies is probably the most important. Patients suffering from AHA of this type have antibody at the cell surface which does not produce spontaneous agglutination but which can be detected by agglutination brought about by anti-gamma globulin antisera. These
antibodies have been referred to as "pan-agglutinins" because eluates prepared from the erythrocytes of affected individuals have been found to sensitize virtually all other human erythrocytes. However, Weiner and Voss (1963) showed that red cells which were congenitally deficient in antigens of the Rh group were not sensitized by such eluates, which suggests that the antibody specificity may be directed against a fundamental structural unit common to most or all Rh types. In most cases of AHA of the warm-antibody type no serum antibody can be demonstrated using unmodified red cells, but if cells are modified by treatment with enzymes such as trypsin or papain then serum antibodies are often found which can be detected by the antiglobulin test. More prolonged incubation of normal red cells with enzymes will frequently enable them to detect spontaneous agglutinating activity in the serum of normal individuals, while the sera of patients with AHA frequently agglutinate such cells to extremely high titres. In most cases of AHA of the warm-antibody type the auto-antibodies have been found to be "incomplete" non-agglutinating antibodies, but in certain rare instances auto-agglutinins or haemolysins active at 37°C have been described: in such cases the patients are usually severely ill. Warm-antibody activity is most commonly associated with IgG antibodies, although cases involving IgM or both types of globulin are occasionally found. Patients suffering from this disease may have a variety of symptoms depending on whether the disease occurs as a slow increase in anaemia over a long period or as an acute and rapid onset of haemolysis. However, mild to moderate splenomegaly, reticulocytosis, and variable degree
of jaundice are found in most patients at some stage.

The most dramatic form of AHA is that which is characterized by the acute and intermittent onset of massive haemolysis following exposure of the patient to cold. A complement-fixing auto-antibody is the major factor in this disease and it was first described by Donath and Landsteiner in 1904. It is demonstrated by a two-phase test in which the patient's serum is mixed with his own or another individual's erythrocytes at 4°C. The temperature of the mixture is then raised to 37°C, and intense or complete haemolysis occurs. It has been shown that attachment of antibody and complement takes place in the cold, preparing the cell for lysis when the temperature is raised. In the absence of complement, the Donath-Landsteiner antibody fails to attach firmly to erythrocytes so that on subsequent exposure to complement at 37°C there may be little haemolysis. Haemolysin titres demonstrated by this Donath-Landsteiner test are rarely high - a titre of 1/16 is usual. Erythrocytes withdrawn from paroxysmal cold haemoglobinuria patients at the time of, or soon after, an attack give a positive antiglobulin reaction of the non-gamma globulin type, although such a result is rare between attacks. The positive reaction is due to the presence on the cell surface of complement components. The Donath-Landsteiner antibody which brings about this complement fixation is invariably IgG (Hins, 1963).

A third form of AHA, known as the "cold-haemagglutinin syndrome", involves a "cold-antibody" type of reaction. It is distinguished from the two previously-described diseases by the presence of serum activity which agglutinates erythrocytes to very high titres with a
peak at 4°C. The antiglobulin test is positive, and is of the non-gamma type. Cold-type auto-antibodies are characteristically present in large quantities in patient's serum, and titration of such serum with normal erythrocytes may give extremely high values for agglutinin titres (i.e. 1/64,000 or more). Most cold-agglutinins have been found to be IgM in nature (or rarely IgA) with a specificity directed against I antigens (van Loghem et al., 1963). The I antigen is of widespread occurrence on normal adult erythrocytes but is absent or poorly represented on neonatal erythrocytes.

These three forms of autoimmune haemolytic anaemia share many features, perhaps the most important of which are the presence of auto-antibodies and the involvement of complement. In all three disorders low levels of complement may be found in patients at a time when disease is most apparent. This finding suggests that antibody-mediated complement fixation to autologous erythrocytes is the primary factor in the destruction of these cells.

Autoimmune haemolytic anaemias have been classified as idiopathic or secondary (asymptomatic) depending on whether or not their occurrence is associated with a demonstrable underlying disease process (Dacie, 1962 and 1964; Swisher and Vaughan, 1965). Associated diseases have included malignant disease of the lympho-reticular system (particularly lymphocytic leukemia and reticulo-sarcoma); collagen disorders (particularly SLE); and, more rarely, tumours, viral infections and inflammatory diseases of a chronic nature such as ulcerative colitis and sarcoidosis. The cold haemagglutinin syndrome has been found quite often in patients recovering from viral pneumonia, and the association between syphilitic infection and the occurrence of
the Donath-Landsteiner antibody is well documented. Most cases of AHA are of the idiopathic type, but a number of cases so classified have been found to develop symptoms of an underlying disorder months or years after the onset of the haemolytic process. Probably fewer than 5 per cent of secondary cases have these features (Swisher and Vaughan, 1965), but since it is extremely difficult to be sure that a patient has never suffered from a potentially significant disease, particularly an uncharacterized viral infection, the classification of AHA as idiopathic or symptomatic may be an unwarranted procedure.

Many attempts have been made to classify autoimmune diseases in such a way that emphasis can be placed on those which appear to share similar fundamental mechanisms. An autoimmune response could arise because of change in the antibody producing system or because a normal antibody producing system is presented with normally non-immunogenic substances in unusual amounts or in altered form. Diseases which show features compatible with the former mechanism have been described as "disturbed immune tolerance" diseases, and diseases with signs of the latter mechanism as "disturbed antigen" diseases (Hijmans et al., 1961). Similar ideas are embodied in the classification of Weigle et al. (1967) of autoimmune diseases into situations involving: (i) abnormal lymphoid tissue (in which a malfunctioning immune system makes an abnormal response to normal body constituents); (ii) sequestered antigens (in which body constituents, e.g. brain and testes, do not normally come into contact with lymphoid tissues, so that no control mechanism exists to prevent a possibly damaging immune response if as a result of trauma or some other means they do
reach the circulation); and (iii) cross-reacting antigens (in which either altered self-antigens or cross-reacting antigens, of e.g. bacteria, stimulate a possibly damaging immune response).

Such classification schemes are useful because they draw attention to the questions which must be answered before the occurrence of autoimmune diseases can be understood. This is particularly true of the following scheme based on the ideas of Burnet (1962a) and Asherson (1967), which divides autoimmune diseases into 5 groups - none of which is exclusive of the others.

(i) Diseases in which micro-organisms are directly associated with an autoimmune response. Possible examples include rheumatic fever and acute glomerulonephritis.

(ii) Diseases (or, at least, auto-antibody formation) which follow exposure to altered or secluded tissue components. Examples include the production of antibody to heart, spermatozoa, or pancreas after damage to the appropriate organ.

(iii) Hashimoto's thyroiditis, and diseases such as pernicious anaemia and Addison's disease which show clinical, serological and familial overlap with it. The auto-antibodies found in these diseases characteristically react with organ specific antigens in the thyroid, stomach or adrenals.

(iv) SLE and other diseases, where many systems are apparently affected, which sometimes merge clinically with SLE. In this non-organ specific group a high incidence of anti-nuclear antibodies is found, as are a number of other auto-antibodies reacting with widely distributed antigens.
Diseases associated with neoplasia. An example is the Coombs-positive haemolytic anaemia which may accompany any chronic lymphatic leukemia.

This scheme highlights the two most important questions concerning autoimmune diseases: how are autoimmune responses initiated, and are auto-antibodies damaging to the tissues of the organism in which they arise? Most of the information with a bearing on the first of these questions has been gained from experimental models of autoimmunity - a subject which is discussed in the next section.

Witebsky et al. (1957) considered the second question, and suggested that at least 4 criteria must be fulfilled to prove the role of autoimmunity in the pathogenesis of a particular human disease. These criteria were: (i) the direct demonstration of circulating or cell-bound antibodies which are active at normal body temperatures, (ii) the recognition of the specific antigen against which the immune response is directed, (iii) the production of antibodies against the analogous antigen in laboratory animals, and (iv) the appearance of pathological changes in the corresponding tissues of an actively immunized laboratory animal which are similar to those found in the human disease. In very few human autoimmune diseases is it possible to claim that these conditions are fulfilled. An exception is provided by Hashimoto's thyroiditis (Witebsky, 1968) in which the important antigen is thyroglobulin. A direct tissue damaging effect due to an autoimmune response is suggested in this disease by the histology of the affected organ which is infiltrated by lymphocytes and histiocytes. Anti-thyroid auto-antibodies occur,
and some of these are cytotoxic for thyroid cells in tissue culture. More direct evidence is provided by the demonstration that rabbits immunized with purified thyroglobulin in complete Freund's adjuvant produce auto-antibodies with anti-thyroid specificity, and in many cases clinical signs of thyroiditis result.

There are other findings which suggest that autoimmune responses may be directly responsible for tissue damage. Administration of immunosuppressive agents such as cortisone and folic acid antagonists in a number of autoimmune diseases has led to lessening of immune reactions and regeneration of normal function in the target organs (e.g. Ardeman and Charasin, 1965, described a series of patients with pernicious anaemia in which treatment with steroids produced a reduction of lymphoid infiltration, regeneration of parietal cells and increased intrinsic factor production). Target cell damage can also be produced in experimental animals by the injection of specific antibodies (e.g. the kidney damage of Masugi-type induced nephritis), or by transfer of lymphoid cells from sensitized animals (e.g. in experimental allergic encephalomyelitis). In autoimmune blood diseases the destructive role of auto-antibodies is well established. The lifespan of erythrocytes coated with auto-antibodies has been shown to be reduced by labelling studies, and examination of the distribution of the removed cells shows uptake by the reticulo-endothelial system. It has been shown by Jardl, Simmons and Castle (1961) that slightly damaged erythrocytes are largely removed from the circulation by the spleen, whereas more profoundly damaged erythrocytes are removed by the reticulo-endothelial system in general and the liver in particular. This finding agrees with studies on
the effect of splenomegaly in patients with AHA - it has been shown that this operation is of benefit in many cases involving incomplete IgG auto-antibodies (where damage is presumably slight), but is of little use where agglutinating and haemolytic auto-antibodies (usually IgM) are present. (Dacie, 1964; Engelfriet et al., 1968). Such findings leave little doubt that autoimmune responses can be directly responsible for causing tissue damage in some instances, but there is also convincing evidence that auto-antibodies may have a protective function. Paterson and Harwin (1963) demonstrated that the serum of rats, in which allergic encephalomyelitis had been induced by injection of homologous brain tissue, exerted a protective effect in that transfer of serum from such rats prevented induction of encephalomyelitis in the recipients. The protective activity of the serum was found to be due to the presence of a heat-labile complement-fixing anti-brain antibody (Paterson, Coia and Jacobs, 1965; Paterson, 1966). It was also demonstrated in these studies that the susceptibility of different strains of rats to the induction of allergic encephalomyelitis was inversely related to their ability to produce such protective antibody. Davies et al. (1964) have described a similar phenomenon in studies of myocardial damage in animals. They noted that, when myocardial damage was induced in rats and rabbits by injection of homologous heart in Freund's complete adjuvant, there was a negative correlation between the level of circulating anti-heart auto-antibody and the severity of myocardial lesions.

The heat-labile complement-fixing IgM anti-tissue auto-antibody which is found in normal rats (Weir et al., 1966) and in higher titre
in rats given CCl₄ to induce liver damage (Weir, 1963) does not itself produce liver damage. It has been suggested that antibodies of this type may have a normal physiological role, perhaps in the removal of tissue breakdown products. Support for this view has recently been provided by the work of Elson (1968), who found that mixtures of normal rat sera with particulate sub-cellular components of rat liver induced migration of polymorphonuclear leukocytes in vitro. This activity appeared to be mediated by a chemotactic agent released as a result of interaction between tissue antigens and complement-fixing anti-tissue auto-antibody.

Thus it is clear that auto-antibodies may potentiate or inhibit tissue damage but it is not at present clear what factors or properties are involved in either situation. This problem is well exemplified by the findings of Hedberg and Källen (1964). They found that a cytotoxic effect on human fibroblasts in tissue culture could occasionally be obtained by adding mononuclear cells from the synovial fluid of arthritic patients. Sometimes this cytotoxic effect was neutralized by the addition of patients' serum, but in other cases the addition of serum potentiated the cytotoxic effect, and in a few cases cytotoxicity was only obtained if serum was also present.

5. EXPERIMENTAL MODELS OF AUTO-ANTIBODY FORMATION AND INDUCED AUTOIMMUNE DISEASE

The second major problem associated with the occurrence of autoimmune responses is the elucidation of the factors responsible for autosensitization. Questions which have a bearing on this problem include: what is the relative importance of inheritance compared with
environmental influences such as infection? Is cellular immunity more or less important than humoral auto-antibody formation? Can procedures directly affecting the lymphoid tissues lead to autoimmune? And can normal body constituents be presented in such a way as to induce auto-immune responses? Attempts to answer these questions have made use of a variety of model systems, the findings from many of which are discussed below.

(a) Tissue damage. Exposure of tissues of normal animals to a variety of damaging procedures (e.g. freezing, exposure to $\text{CCl}_4$ or proteolytic enzymes, mechanical trauma) has been shown to induce short-lived bursts of auto-antibody formation (Milgrom et al., 1957; Weir, 1964). Generally, however, it has been shown that when the tissue damage is repaired then auto-antibodies disappear from the circulation.

(b) Graft-versus-host (GVH) reactions are induced by the injection of lymphoid cells into animals which are unable to reject them by a normal immune response. Such an inability is found in newborn animals, in X-irradiated animals and in F1 hybrids given cells from one of the parental strains. Cell transfers in these conditions are followed by the production of various pathological conditions which often culminate in wasting (runt) disease. The pathogenesis of such disorders is not well understood, but it is well established that the severity of the resulting disease is related to the genetic disparity between cell donor and recipient (Simonsen, 1962). Such GVH-induced diseases have been found to be associated with the occurrence of Coombs positive haemolytic anaemias in chickens (Simonsen, 1957).
and in mice (Oliver, Schwartz and Dameshek, 1961), and Lewis et al. (1968) have described the occurrence of immunologically-mediated glomerulonephritis in F1 hybrid mice given parental-strain spleen cells. The simplest explanation of such findings is that the transferred cells survive and mount an immune response against the foreign antigens of the host. If this were a complete explanation then the resulting antibodies might be held to provide a good model of autoantibody formation and its pathological effects.

The role of micro-organisms in the induction of the pathological features of GVH-induced disorders is, however, far from clear (Simonsen, 1962). Many authors have speculated on the role of infection in such situations, and Koltay et al. (1968) have reported that infection with LCM virus potentiates the runting effect seen in F1 hybrid mice given parental-strain cells. Elkstedt and Hayes (1967) claim to have produced a syndrome which was similar in most respects to GVH-induced runting by treating newborn mice with a variety of sterile bacterial vaccines.

(c) Thymectomy. The complete removal of the thymus very early in the life of most animals has been found to interfere with normal development of the lymphoid system and of immunological capacity. Although antibody formation to a few antigens (e.g. sheep erythrocytes and bovine serum albumin) may be depressed, the major effect of thymectomy is an interference with the development of cellular immunity (Miller, 1961 and 1963; Good et al., 1962). In most cases neonatal thymectomy leads to the development of "runt" disease - a wasting disease which culminates in death (Miller, 1962; Parrott,
The pathological features of runt disease in thymectomized animals show many similarities to GVH-induced runting (Sherman and Dameshek, 1963; Miller and Howard, 1964) and the condition is equally little understood. It has, however, been found that in germ-free and low pathogen strains of mice, neonatal thymectomy does not produce runt disease, although depression of the immune response is still found (McIntire, Sell and Miller, 1964). Thus it appears that reduction of immunological capacity is a primary effect of neonatal thymectomy, while runting is a secondary phenomenon due, perhaps, to infection with normally non-pathogenic micro-organisms which become established because of the reduction in competence.

Evidence from various sources has established that one of the effects of neonatal thymectomy is to produce an increased incidence of auto-antibody formation. Anti-nuclear antibodies and Coombs-positive haemolytic anaemia have been found in mice (de Vries et al., 1964; Good et al., 1966; Howie and Helyer, 1966; Thivolet, Monier and Richard, 1967), positive Coombs tests have been found in rabbits (Kellum et al., 1965; Sutherland et al., 1965), and it has been suggested that autoimmune processes form the basis of the post-thymectomy runting syndrome (de Vries et al., 1964). It remains true, however, that none of the studies mentioned demonstrates a clear correlation between auto-antibody formation and the occurrence of runting. There may be many other factors involved in the production of the wasting disease which follows neonatal thymectomy (e.g. thymic humoral factors, the trephocyte function of thymus
lymphocytes, the role of micro-organisms, etc.), so that it appears premature to suggest that the neonatally thymectomized animal provides a good model of autoimmune disease.

(d) Models involving infection. The association between micro-organisms and the induction of autoimmune responses has already been mentioned, and a vast literature exists describing observations and investigations of this relationship (see review by Asherson, 1968). It seems clear that one of the ways in which an autoimmune response can be evoked is by infection with micro-organisms possessing antigens similar to antigenic determinants of the host so that cross-reactive antibodies are produced. The formation of auto-antibodies reactive with heart tissue following streptococcal infection, and the auto-antibodies of ulcerative colitis which are related to antigens of E. coli, most likely fall into this category.

Other mechanisms exist, however, whereby infection can cause auto-antibody formation, for there has been a number of reports suggesting that infection with a variety of micro-organisms can lead to auto-antibody formation without any evidence of specificity between the infecting organism and the resulting auto-antibody. A good example of this is found in the work of Thewini Ali and Oakley (1967), who showed that roughly 25 per cent of rabbits infected with P. pseudotuberculosis or M. tuberculosis developed auto-antibodies reactive against liver, kidney, spleen, lung, lymph node and heart. These antibodies were detected by complement fixation, tanned-cell agglutination, and precipitation tests using organ extracts; and by immunofluorescence using tissue sections. It was
also shown that rabbits injected with sterile filtrates of organs from infected rabbits produced auto-antibodies sooner than infected rabbits did — an observation which suggested to the authors that auto-antibodies were produced because of alteration to the affected organs due to bacterial activity.

The experimental exploration of the possible role of viruses in auto-antibody formation has been less well studied, but has recently begun to arouse much interest. Rask-Nielsen (1964) found that the cell-free filtrate from a murine plasma cell leukemia could cause hypergammaglobulinaemia, Coombs-positive anaemia and amyloidosis on transfer to other mice. Sub-cellular particles were present in the filtrate, and the author felt that the findings could have been due to the transfer of a mycoplasma or of a virus (reovirus 3). Stanley and Walters (1966) and Bennette, Bush and Steele (1967) have described a runting disease which occurs in mice and rats which have been infected with reovirus type 3. The disease is characterized by myocardial and liver lesions, splenic atrophy, leucopenia and anaemia. Stanley attributed these signs to an autoimmune reaction initiated by the virus infection, but in fact he presented no evidence of an autoimmune response and appears to have arrived at this impression because of similarities between this syndrome and the runting syndrome which occurs in association with GVH-induced reactions. Baker and Hotchkin (1967) produced a tolerated infection with LCM virus by injecting mice with virus soon after birth. When such mice were examined at 18 months of age they were found to have impaired renal function and kidney
lesions with many of the features found in human glomerulonephritis. Similar lesions were found in mice infected for a short period with ECHO 9 virus (Burch, Chu and Sohal, 1968) and in this case deposits of viral antigen were found in the glomeruli. In neither of these latter two studies was auto-antibody formation demonstrated, but in both studies the authors suggested that the lesions were reminiscent of those which occurred in human SLE-associated glomerulonephritis, and that deposition of antigen-antibody complexes was probably involved.

Such evidence of virus participation in experimental disease with an immunological basis is at best suggestive. Much more conclusive evidence of the participation of a virus in an autoimmune situation has been gained from studies of Aleutian disease in mink. This disorder predominantly affects mink of a particular genotype, and it is characterised by anaemia, hyperglobulinaemia, plasma-cytosis and the occurrence of vascular lesions which particularly affect the kidney (Henson et al., 1962; Leader, 1964). Positive Coombs tests are found in affected animals and anti-nuclear antibodies have been reported (Barnett et al., 1969). The nature of the renal lesions is not clear - some observers claim that immune complexes are not involved, while others have suggested that the kidney lesions are very similar to those found in immune complex-mediated nephritis of SLE in humans (Kindig, Spargo and Kirsten, 1967). Very recently, however, it has been found that complexes of gamma-globulin and virus are to be found in the circulation of affected animals, and most likely localize eventually in the
kidneys (Larsen and Porter, 1967; Barnett et al., 1969). The most important feature of Aleutian mink disease is that it has proved consistently possible to transmit it experimentally, using cell-free filtrates of serum or organs, under conditions which make it virtually certain that a virus is involved. This suggests that a particular strain of mink develops autoimmune disease in a high incidence apparently because members of this strain are genetically more susceptible to infection with a peculiar virus than members of other strains.

(e) Models involving artificial immunization procedures. The injection of homologous or heterologous tissues, mainly with the use of adjuvants, has been found in many instances to induce the formation of antibodies reactive against normal tissue constituents. Only rarely, however, does such treatment result in lesions which bear a close relationship to the corresponding spontaneous human autoimmune disease. Two of these exceptional instances, in which such a relationship has been achieved, are found in experimental autoimmune thyroiditis and experimentally induced kidney diseases. THYROIDITIS. Rose and Witebsky (1956 a, b and c) reported that repeated intravenous or intradermal injection of crude rabbit thyroid extract into rabbits did not result in the formation of antibodies against thyroid. When such extracts were given in complete Freund's adjuvant, however, circulating antibodies developed with an anti-thyroid specificity demonstrable by complement fixation, precipitation, tanned-cell agglutination and passive cutaneous anaphylaxis. The antibodies were thyroid specific in that there was
no cross-reaction with other organ extracts or with erythrocytes, but they did cross-react with thyroid antigens of other species. Thyroidectomized rabbits, given injections of autologous thyroid, were found to produce auto-antibodies reactive with extract derived from their own thyroid glands.

Immunofluorescence was used to determine the localization of the thyroid antigen involved, and fluorescence was regularly found in the colloid-containing region and in the adjoining areas of the epithelial cells (Beutner et al., 1958). By subjecting rabbit thyroid extracts to immuno-electrophoretic analysis, the antigen responsible for precipitation was found to be an alpha globulin immuno-chemically undistinguishable from thyroglobulin. Injection of partially-purified rabbit thyroglobulin in Freund's adjuvant was found to be more potent, on a weight basis, in the elicitation of auto-antibody formation than whole thyroid extract (Witebsky et al., 1957).

In addition to the production of circulating antibody, immunized rabbits gave delayed type skin reactions to thyroid extract and thyroglobulin, although the intensity of skin reactions was not well correlated with antibody titre. Examination of thyroid glands from immunized rabbits revealed evidence of thyroiditis: areas of dense cellular inflammation were seen around follicles (mainly composed of lymphocytes and eosinophils), and epithelial cells and macrophages were occasionally found in the colloid region (Rose and Witebsky, 1956c). In severe cases the follicular pattern was obscured by accumulations of large and small lymphocytes forming
lymphoid follicles, or disrupted by inflammation of the interstitium accompanied by fibrosis. Hung et al. (1962) showed that experimentally induced thyroiditis in rabbits was associated with functional defects: immunized animals showed reduced uptake of labelled iodine and a reduced responsiveness to the administration of thyroid-stimulating hormone.

The injection of thyroid extract in Freund's complete adjuvant was found to lead to auto-antibody formation and signs of thyroiditis in almost all rabbits tested, but Rose, Kite and Doebbler (1962) found that omission of bacilli from the injected mixture led to lower titres of auto-antibody and a much reduced incidence of thyroid lesions. Immunization with alum-precipitated thyroid extract was also attempted and resulted in the formation of complement-fixing and tanned-cell agglutinating auto-antibodies, but skin-test reactions were weak and again there was little evidence of thyroid damage. The resulting auto-antibodies were occasionally present in high titres for long periods, and they were immunologically indistinguishable from the antibodies induced using antigen in complete Freund's adjuvant. The addition of killed acid-fast bacilli to the alum-precipitated extract strikingly increased the intensity of positive delayed-type skin reactions, and increased the incidence of thyroid lesions. Immunization of dogs and guinea pigs (Terplan et al., 1960), rats (Jones and Roitt, 1961) and mice (Netzgar and Grace, 1961) using homologous thyroid extracts in adjuvant has also been found to produce histological evidence of thyroiditis, sometimes in the absence of demonstrable circulating
anti-thyroid antibodies. McMaster, Lerner and Exum (1961) found evidence of thyroiditis in guinea pigs as early as five days after injection of homologous thyroid extract in complete Freund's adjuvant. After seven weeks, severe thyroid damage was present and the degree of thyroiditis was closely correlated with circulating antibody levels. At later stages, however, a much closer correlation was found between thyroid damage and the intensity of delayed reactions to thyroid extract.

Extracts of bovine, canine, human and porcine thyroid glands, injected into rabbits, have been found to induce the formation of organ-specific antibodies which cross-react with rabbit thyroid in tanned-cell agglutination and precipitation tests. Such antibodies did not fix complement with rabbit thyroid extract, nor did the injected animals develop delayed hypersensitivity (measured by skin tests) to rabbit thyroglobulin, although strong skin reactions were obtained using the heterologous thyroid extract used for immunization. The injected animals showed no histological evidence of thyroid damage (Witebsky and Rose, 1959). In another series of rabbits, given a more intensive course of porcine thyroglobulin injections, a small proportion of treated animals were found to develop complement-fixing antibodies to rabbit thyroid extract after several months, but no dermal sensitivity to this antigen developed. A mild lymphocyte infiltration was, however, found in the thyroid glands of these animals (Terplan et al., 1960).

Subsequently, however, others have reported successful attempts to induce auto-antibody formation and thyroiditis in rabbits injected
with heterologous thyroglobulin (Weigle et al., 1967), and in mice given a mixture of human, equine and bovine thyroglobulin (Nakamura and Weigle, 1968). Interestingly, some of the mouse strains used in the latter study lacked functional complement activity, but severe thyroiditis was induced nonetheless. Weigle (1965) has also described the formation of auto-antibodies and resultant thyroiditis in rabbits injected with aqueous suspensions of homologous thyroglobulin which had been altered by coupling to the diazonium derivatives of arsanilic or sulphanilic acids. Following a latent period, many of these rabbits responded to an injection of unaltered rabbit thyroglobulin by producing increased levels of agglutinating and precipitating antibodies and, in some cases, exacerbation of the thyroid lesions.

By intravenous injection of the immunosuppressive drug 6-mercaptopurine, at the same time as rabbits were given intradermal or intramuscular injections of rabbit thyroid extract in complete Freund's adjuvant, Rose et al. (1965) were able to suppress almost completely the development of thyroid lesions. Skin test reactions, however, were as strong as those in control animals, and circulating antibody levels were only slightly lower. Many attempts to produce thyroid lesions in recipient animals by transferring serum from actively immunized animals have failed, even when very large quantities of serum have been used. Felix-Davies and Waksman (1961) were, however, able to transfer experimental thyroiditis between members of an inbred strain of guinea pigs using cell suspensions. The cells were obtained from immunized animals before circulating
antibody could be detected, and on transfer to syngeneic animals mild to severe thyroiditis subsequently developed.

The results of these experiments on induced thyroiditis in laboratory animals show that it is possible to elicit auto-antibody formation and pathological signs, which are similar in many respects to those of the spontaneously occurring disease in man, by artificial immunization with an organ extract. Furthermore, the studies using heterologous antigens and chemically modified antigens support the possibility that autoimmune disease may arise as the result of an immune response to an altered body constituent. But many questions remain unanswered: most important, perhaps, is the elucidation of which components of the immune response result in tissue damage. The results presented indicate that the relationship between circulating auto-antibody formation, complement utilisation, delayed hypersensitivity and pathological change is not a simple one. It is also true that there are differences between chronic thyroiditis of man and the experimentally-induced disease in rabbits and most other animals. Rabbit antibodies to autologous thyroglobulin fix complement in vitro, but the auto-antibodies against thyroglobulin in humans fix complement poorly if at all. A second auto-antibody which reacts against microsomal fractions of human thyroid tissue can be found in certain human patients, and another factor is found which has a cytotoxic effect on human thyroid cells in tissue culture.

GLOMERULONEPHRITIS. The lesions of human glomerulonephritis have long been believed to be related in some way to an abnormal immune response, but only in recent years has it been clearly demonstrated,
mainly by the use of animal models, that at least two quite distinct mechanisms exist whereby antibody formation can result in kidney damage.

1. **ANTIBODIES AGAINST BASEMENT MEMBRANE.** Masugi, in 1933-34, first drew attention to the ability of heterologous anti-kidney antibodies to cause nephritis in experimental animals. He found that the serum of rabbits, which had been injected with homogenates of rat kidney, produced nephritis when injected into rats, and he attributed this effect to antibody. Using a similar model, Krakower and Greenspan (1951) found that the antigen involved was mainly located on the basement membrane. Subsequent analysis of the kidney lesions in Masugi-type nephritis using immuno-fluorescence and immuno-chemical techniques has shown that the injected antibody localizes in the glomerular basement membrane where it can be identified as donor-type immunoglobulin. These deposits also contain complement, and they are found in a linear arrangement along the epithelial side of the glomerular membrane.

The next important finding was that animals immunized with heterologous or homologous basement membrane antigens could themselves develop glomerulonephritis (Steblay, 1962). The original experiments were performed in sheep using human or other primate antigens, and a humoral factor was implicated by the finding that unsensitized sheep joined via the circulation with sensitized animals developed nephritis (Steblay, 1965). Early attempts to transfer the condition by serum were unsuccessful, but Lerner and Dixon (1966) showed that removal of the target organ (the kidney)
in immunized animals led to an abrupt increase in the level of anti-kidney antibody. Sufficiently high levels of such activity were reached for glomerulonephritis to be passively transferred by serum injection. Affected kidneys had pathological features similar to those found in Masugi-type nephritis (including linear deposition of immunoglobulins and complement along the epithelial aspect of the basement membrane). Similar findings have been reported subsequently from studies of rabbits injected with heterologous or homologous renal antigens (Unanue and Dixon, 1967; Unanue, Dixon and Feldman, 1967).

2. ANTIGEN–ANTIBODY COMPLEXES. The first suggestion that complexes of non-glomerular antigens with antibodies might be associated with kidney damage came from studies of human serum sickness. Von Pirquet (1911), impressed by an apparent co-existence of heterologous serum protein antigen and antibody in the circulation, noted the rapid disappearance of antigen associated with the appearance of measurable quantities of antibody, and suggested that antigen and antibody must be reacting in the circulation to form a "toxic compound" responsible for serum sickness. Support for this conclusion came much later from the work of Germuth and McKinnon (1957), who found an association between anaphylactic reactions, tissue inflammation, and the presence of soluble antigen–antibody complexes; and from the work of Ishizaka and Campbell (1958), who showed that antigen–antibody complexes injected directly into the skin could fix complement and produce tissue injury.

In experimentally-induced serum sickness of rabbits it has
been shown that vascular injury occurs only during the period when antigen-antibody complexes - formed at, or near, molecular equivalence - are present in the circulation (McKinnon et al., 1957; Germuth and Pollak, 1958; Dixon, 1962-63). In these studies development of disease correlated with the deposition of complexes in blood vessels of the kidney, heart and connective tissues. Serum complement levels were depressed, and immunofluorescence techniques revealed the deposition of complement and complexes in the affected organs.

A possible explanation for these findings is that antigen-antibody complexes formed in the presence of excess antigen may be soluble, and thus fail to precipitate out and be phagocytosed by cells of the reticulo-endothelial system. Lesions could then result because of the localization of soluble complexes in vessel walls where they fix complement and activate a series of inflammatory reactions leading to tissue injury. This mechanism has been proposed as an explanation of the Arthus reaction, and the available evidence supports such a proposal (Cochrane, Weigle and Dixon, 1959). The distribution of damaging complexes shows no evidence of immunological specificity, but rather appears to be a function of size and of the functional characteristics (e.g. filtration) of the affected organ or tissue.

A dramatic demonstration of the role of antigen-antibody complexes in producing glomerulonephritis similar to the kidney damage which may result from serum sickness is provided by the findings of Dixon, Feldman and Vasquez (1961). In these experiments rabbits were
injected with antigen (bovine serum albumin) and their antibody-forming ability was measured. The very good and the very poor antibody-formers were rejected, and the intermediate responders were selected for further study. These animals, which could readily be kept in a condition where their blood contained soluble antigen-antibody complexes in antigen excess, were given large daily injections of antigen. Most of the rabbits treated in this fashion developed progressive chronic glomerulonephritis which resulted in proteinuria and death due to renal failure. With each injection of antigen, serum complement levels fell sharply; and while antigen-antibody complexes were present, the serum was anti-complementary. The kidneys of affected animals showed deposits of antigen, autologous gamma globulin and complement. These deposits were mostly localized to the epithelial aspect of the basement membrane and when viewed by light and electron microscopy the pattern of deposition was found to be discrete and irregular.

There is good evidence that antibodies against glomerular basement membranes and the deposition of antigen-antibody complexes are involved in different forms of human glomerulonephritis. Lerner, Glassock and Dixon (1967) demonstrated that the serum of certain patients with glomerulonephritis contained antibodies with anti-basement membrane specificity, and that such antibodies could be eluted from kidneys removed at operation. In a few additional patients serum antibodies were detected after, but not before, total nephrectomy prior to transplantation. The pattern of fixation of immunoglobulin in a smooth linear fashion along the
glomerular capillary walls closely paralleled that found in experimental nephritis of the Masugi-type. Antibodies eluted from the kidneys of such patients were injected intravenously into squirrel monkeys where they were found to localize, together with host complement, in a linear fashion along the recipients' glomerular capillary walls.

It is not possible to detect anti-kidney antibodies in all cases of glomerulonephritis where their presence is suspected. If, however, the criteria of host immunoglobulin and complement components present in the kidney and arranged in a distinct, linear pattern along the glomerular basement membrane are accepted as an indication of their presence, then it has been claimed that such antibodies are present in all cases of Goodpasture's syndrome, somewhat less than half of the cases of sub-acute chronic glomerulonephritis of adults, a smaller proportion of the membranous glomerulonephritis cases of children, and few (if any) of the acute post-streptococcal glomerulonephritis, malarial nephroses, and lupus nephritides (Dixon, 1968).

The antigen-antibody complex mechanism has been implicated in the nephritis of SLE, however, and there is slightly less convincing evidence to suggest that it plays a part in the kidney diseases found in association with streptococcal and malarial infections. In SLE, anti-nuclear antibodies have been eluted from nephritic kidneys, and nuclear antigens, host immunoglobulin and complement have been demonstrated in the characteristic irregular pattern in the glomeruli (Kirshman and Kaplan, 1967; Koffler, Schur and Kunkel, 1967). DNA
and antibodies have also been found in the serum of lupus patients, and the level of DNA was found to drop with increased severity of kidney disease (Tan, Schur and Kunkel, 1965). In post-streptococcal nephritis and most cases of malarial nephroses the morphological signs of complex-induced nephritis are present (i.e. deposition of immunoglobulin and complement in an irregular pattern), but the evidence for the presence of antigen in such deposits is less strong (Andres et al., 1966).

The two experimental mechanisms which have been outlined provide convincing models of the pathological lesions found in the two main forms of human glomerulonephritis, but neither explains the aetiology of these diseases. Both forms could involve autoimmune mechanisms - the anti-basement membrane antibody-type virtually by definition, and the immune complex-type if the antigen involved were autologous (e.g. host DNA). The source of the antigen responsible for initial sensitization is a crucial factor - in both types of nephritis microorganisms could be involved either acting to produce an adjuvant effect for normal tissue constituents, by altering tissue constituents or by a cross-reaction mechanism.

Of interest in this respect is the recent finding of antigens cross-reactive with, and perhaps identical to, glomerular basement membrane antigens in the urine of normal members of a number of animal species (Hawkins, 1967). Using such antigens extracted from the urine of normal rabbits Lerner and Dixon (1968) immunized animals with autologous material, and were able to induce signs of nephritis similar to those observed after immunization with
homologous or heterologous material. The nature and source of these naturally-occurring antigens, however, remains to be determined.

The possibility that autoimmunization to an autologous antigen might result in immune complex type nephritis is supported by the results of a series of experiments performed by Heymann et al. (1959). Renal disease was produced in rats by repeated injection of homologous or autologous whole kidney extract in Freund's complete adjuvant. The resulting disease had the characteristics of a chronic glomerulonephritis and it was possible to transfer it passively using whole serum. Examination of affected kidneys, however, revealed that gamma globulin and complement were deposited irregularly on the epithelial side of the basement membrane, unlike the linear endothelial distribution characteristic of anti-basement membrane antibodies. Repeating these experiments, Edgington, Glassock and Dixon (1967) found that the nephritogenic antigen was a kidney constituent located in the apical portion, or brush border, of the proximal convoluted tubular cells. Small but significant amounts of a similar antigen were found to be present normally in the circulation. Immunization with small amounts of this homologous kidney antigen induced the formation of antibodies which combined with the circulating host antigen, and were then deposited in the glomerulus as antigen-antibody complexes. These studies attest to the possibility that normal body constituents may be involved in the production of an autoimmune glomerulonephritis involving antigen-antibody complexes, but it is not known whether
an analogous mechanism is ever found in human glomerulonephritis.

Studies of auto-antibody production and autoimmune diseases provoked experimentally in laboratory animals have provided valuable answers to a number of important questions. It is clear that mechanical tissue damage can provoke auto-antibody formation, but such a response is short-lived and does not lead to autoimmune disease. Nevertheless, it is established that immunization against normal body constituents can occur in this fashion, and the extension of these findings to the studies with normal antigens such as thyroglobulin and basement membrane given over prolonged periods or in adjuvants shows that disease can result from autoimmunization procedures. Presumably the effect of these immunization procedures is to alter the lymphoid tissues or the handling of normal body constituents by the lymphoid tissues. More direct evidence that alteration of lymphoid tissues can lead to auto-antibody formation and associated disease comes from the experiments involving thymectomy and the GVH reactions.

The finding that micro-organisms can provoke auto-antibody formation in experimental animals is of great significance, particularly since it is also likely that infection plays a part in the disease processes associated with GVH reactions and neonatal thymectomy, that Freund's complete adjuvant is required in immunizing animals with most auto-antigens, and that bacteria and viruses have been shown to affect animal cells so that they become antigenically altered.

One of the ways in which micro-organisms could influence the
induction of autoimmune responses might be by exerting an adjuvant effect so that the response becomes quantitatively greater or qualitatively different. Studies of experimental models, however, have not provided a clear answer to the question of what kind of immune response is required for autoimmune disease. Delayed hypersensitivity mechanisms, for instance, appear to be involved in the thyroid lesions of experimental thyroiditis, but the kidney lesions of anti-glomerular basement membrane antibody-type nephritis appear to result from the action of antibody and complement alone.

6. SPONTANEOUS OCCURRENCE OF AUTOIMMUNE DISEASE

IN ANIMALS - NZB MICE

Another approach to the study of human autoimmune diseases has been the experimental manipulation of analogous spontaneous diseases in laboratory animals. Surprisingly few instances of spontaneous autoimmune diseases have been reported in animals, but two in particular of those which have been studied quite fully have yielded much useful information on the relative importance of hereditary factors and environmental influences in the development of autoimmune disease. These two instances are Aleutian disease of mink and the multiple immunological abnormalities which are found in New Zealand inbred mice, the first of which was described by Bielschowsky, Helyer and Howie in 1959. The New Zealand mice findings will be further discussed below and the characteristics of Aleutian disease have already been mentioned, but it seems an appropriate point to re-state the most important conclusion reached after studies on Aleutian disease - that the spontaneous development...
of disease in a particular strain of mink appears to be related to a genetically-determined susceptibility to infection with a virus-like organism. A few other spontaneous diseases of animals have been described which have features reminiscent of human autoimmune diseases, including an SLE-like syndrome in dogs (Lewis, Schwartz and Henry, 1965) and hereditary autoimmune thyroiditis in fowls (Cole, Kite and Witebsky, 1968), but these conditions require further study before their full significance becomes obvious.

It is also true that the significance of the abnormalities which occur spontaneously in the various lines of New Zealand mice remains to be determined - and this despite the fact that they have been extensively studied in many laboratories over the past ten years. The main subject of this thesis is one of these strains - the New Zealand Black (NZB) strain - and the experiments to be described must obviously be discussed in relationship to the published work of others. This introductory section describes the published information available when this study was commenced towards the end of 1965, subsequently published material is discussed in a later section which follows the description of the experiments performed and the results gained.

In 1959 Bielschowsky, Helyer and Howie described the occurrence of a spontaneous disease in mice of the inbred strain NZB (one of a series of inbred strains developed from an outbred colony by selection for coat colour). Virtually all animals developed signs of progressive anaemia and a factor was found to be present in the serum, and in eluates from affected mouse erythrocytes, which
agglutinated ficin-treated erythrocytes from normal mice. This agglutinin was the first sign of abnormality; subsequently increased numbers of reticulocytes and lowered PCV values developed in affected animals. No signs of abnormality were detected before 3 months of age, but all animals gave positive tests by 10-15 months. Males tended to have splenomegaly, high reticulocyte counts and moderate anaemia, while females had mild splenomegaly, moderate reticulocytosis and severe anaemia. Signs of jaundice were occasionally found, and such signs were more marked in the terminal stages of the disease.

Subsequent studies in other laboratories have confirmed that haemolytic anaemia occurs in NZB mice (Holmes and Burnet, 1963a; East, de Sousa and Parrott, 1965; Holborow, Barnes and Tuffrey, 1965; Mellors, 1965), and that various types of anti-red cell activity occur in the serum and on the erythrocytes of affected animals. These mice were found to have a globulin at the erythrocyte surface which can be detected by agglutination with anti-globulin sera (the direct Coombs test), and serum factors which attach to homologous erythrocytes on in vitro incubation to give positive indirect Coombs test results. The antibody nature of the coating factor was demonstrated by Long, Holmes and Burnet (1963) who eluted NZB erythrocytes and showed that the eluate cross-reacted with erythrocytes from other mouse strains, gave slight cross-reaction with rat erythrocytes, and had no effect on other heterologous erythrocytes. Norins and Holmes (1964a) demonstrated by immuno-electrophoresis that such eluates were mainly 7S gamma
globulin in nature. Long, Holmes and Burnet also studied the relationships of the three types of anti-red cell activity (i.e. that detected by direct and indirect Coombs tests, and using enzyme-treated cells) using erythrocyte absorption and concluded that all three have the same specificity but are of differing avidity, i.e. all three were antibodies directed against a species-specific component of the erythrocyte surface. Giltinan, Norins and Holmes (1965) showed that direct Coombs test results obtained with NZB erythrocytes are detectable by specific anti-mouse IgG antisera, and cannot be explained by the non-specific binding of serum components to reticulocytes. (Such pseudo-positive reactions - due to the binding of ß-globulins such as transferrin - are occasionally detectable when anti-whole serum is used in direct Coombs tests). Furthermore, these workers claimed that a positive Coombs test result was always obtained in individual mice before evidence of reticulocytosis became apparent. Holborow, Barnes and Tuffrey (1965) described a different antibody which is present in the serum of old NZB mice and which reacts with an antigen found on both mouse and human erythrocytes. This antigen is not readily available or is poorly represented on the surface of intact erythrocytes, and its corresponding antibody, which fixes complement, is detected by immunofluorescence using dried red-cell preparations. Lindsey, Donaldson and Woodruff (1966) demonstrated that the survival time of labelled erythrocytes in old NZB mice with positive Coombs tests was markedly shortened, and suggested that the antibody coating could be directly responsible for this shortened life-span. They
also found, however, that erythrocytes transferred from Coombs positive to Coombs negative mice did not show reduced survival — an observation which is open to a number of interpretations, including the possibility that the globulin coating could have a protective function. It is generally concluded that anaemia is the major cause of death among NZB mice, and that members of this strain die significantly earlier than other mice (Helyer and Howie, 1963a).

The thymus glands of NZB mice have been found to contain areas of cellular proliferation with the general characteristics of germinal centres — accumulations of large pyroninophilic lymphocid cells, cells in mitosis, and pyknotic nuclei or nuclear fragments associated with macrophages (Burnet, 1962a and b; Burnet and Holmes, 1962; Holmes and Burnet, 1963a). Electron micrographs of these areas revealed the abundant presence of plasma cells within them, as well as smaller numbers of mast cells and eosinophils (Abbott and Burnet, 1964). Burnet was impressed by the absence of Hassall's corpuscles from the thymuses of NZB mice and by the similarity of their lesions to the thymic lesions found to occur in humans with myasthenia gravis. He suggested that the thymic lesions might be the visible evidence of the proliferation of "forbidden clones" (i.e. lymphocytes with the ability to react against self-antigens), and that the primary defect in NZB mice (and, by analogy, in human autoimmune disease) might be the failure of the thymus to carry out its postulated role of preventing the emergence of such self-reactive clones. Closer examination of the
association between thymic abnormalities and auto-antibody formation, however, revealed that in most NZB mice positive Coombs tests were detected before any signs of thymic change were seen, and it was concluded that germinal centre formation in the thymus merely signified the presence of abnormal lymphoid cell proliferation (Burnet and Holmes, 1964a and b). Furthermore, other workers (East et al., 1965; Siegler, 1965) claimed that NZB thymus glands contained normal numbers of Hassall's corpuscles, and Siegler pointed out that the thymic germinal centres and lymphoid follicles are very similar to those which develop in the spleen, lymph nodes and other lymphoid organs of NZB mice. Siegler also found thymic germinal centres in old Swiss mice which had no evidence of autoimmune disease, and Sainte-Marie (1965) demonstrated that the thymuses of normal rats and mice could display plasma cell infiltration similar to that described for NZB mice by Burnet et al. and claimed that such infiltration was not unique to autoimmune situations. Neonatal thymectomy of NZB mice was found not to delay the onset of autoimmune manifestations (Helyer and Howie, 1963b), and in one study may even have precipitated the early appearance of positive antoglobulin tests (East et al., 1965). Removal of thymus tissue a few days or a few weeks after birth delayed the onset of positive antoglobulin tests in another study by a few months, but did not prevent it (Holmes and Burnet, 1964a). Results presented by Helyer and Howie also suggest that transfer of the thymus from neonatal NZB mice to neonatal mice of
other strains is associated with the transfer of autoimmune disease, but Holmes and Burnet were unable to demonstrate this effect. The transfer of thymus grafts from young NZB mice to old NZB mice already showing signs of germinal centre formation was associated with the production of accelerated germinal centre formation in the grafted tissue. These studies on the role of the thymus in NZB mice are far from conclusive, but it is fairly clear that germinal centre formation is not the first sign of autoreactive tissue arising within the thymus. It appears more likely that thymic involvement is part of a more generalized lymphoid proliferation originating outside the thymus, perhaps as a response to an abnormal situation. It has not been excluded, however, that the thymus may normally exert a surveillance function in limiting such reactions which is abrogated in NZB mice.

The genetic basis of the NZB abnormality has been studied by investigating the incidence of positive Coombs tests in F1 hybrids between NZB and other inbred strains (Holmes and Burnet, 1964b; Burnet and Holmes, 1965). The median age for conversion to a Coombs positive state for each of the hybrid types studied was very much older than that of NZB mice (e.g. that for (NZB x CBA)F1 males was 730 days, and for females was 510 days). Burnet and Holmes suggested that their data were compatible with the hypothesis that the occurrence of autoimmunity could be the result of a specific somatic mutation whose expression is dependent on the genetic background against which it occurs. When NZB mice were crossed with other strains derived from the same outbred stock originally, very
different results were obtained. \((\text{NZB x NZC})^F_1\) and \(F_2\) animals displayed 100 per cent and 75 per cent respectively incidence of autoimmune haemolytic anaemia at an early age, independently of the sex of the NZB parent (Bielschowsky and Bielschowsky, 1964), a result which the authors interpreted as evidence for the presence of a dominant autosomal gene whose expression required a compatible background.

NZB mice have been found to have renal lesions - endothelial and epithelial proliferation associated with basement membrane thickening, globulin deposition and focal necrosis - but the resulting renal disease is not thought to be an important factor in the death of these animals (Helyer and Howie, 1961; Mellors, 1965). The \(F_1\) hybrids between NZB and NZY or NZW animals, however, develop a renal disease of much greater severity (Helyer and Howie, 1961 and 1963c; Aaron, 1964; Channing et al., 1965), and the majority of \((\text{NZB x NZW})^F_1\) hybrids were found to die of renal failure between eight and ten months of age. These mice have a low incidence of positive Coombs tests, but a high incidence of positive L.E. cell and anti-nuclear factor (ANF) tests. Such findings suggest that the disease which occurs in \((\text{NZB x NZW})^F_1\) hybrid mice may parallel in many respects human SLE, and its occurrence in mice containing genes derived from an NZB parent may reflect another expression of a genetic tendency to develop autoimmune responses. Howie and Helyer (1965) studied the incidence of kidney disease and haemolytic anaemia in a number of \(F_1\) hybrids between NZB and other inbred strains. They concluded from the results that a propensity
towards autoimmune disorder was inherited as a dominant feature from the NZB parent, but that its expression was modified by the genetic contribution from the "non-autoimmune" parent. However, definitive evidence of the autoimmune nature of the renal lesions was lacking at that time, and the significance of the high incidence of positive ANF tests in F1 hybrid mice is questionable since it has been shown that 15 per cent of C3H and C57 Bl mice and 85 per cent of an outbred mouse strain in one study were found to give positive ANF tests in the absence of any other abnormality (Norins and Holmes, 1964b). An interesting possibility was suggested by Norins (1965) to explain the differing incidence of kidney disease in NZB and (NZB x NZW)F1 hybrid mice. The suggestion was prompted by the finding that NZB mice lack a serum component possessed by other mouse strains and which is essential for functional complement activity (e.g. red-cell lysis). The florid kidney lesions characteristic of the F1 hybrid might be due to the presence of complement activity, since NZW and (NZB x NZW)F1 hybrids were shown to possess the necessary serum component.

Holmes, Gorrie and Burnet (1961) first demonstrated that signs of haemolytic anaemia could be transferred by means of cell suspensions. Young Coombs negative NZB mice were given spleen cells from older Coombs positive animals, and within three weeks of transfer the recipients became Coombs positive. This state lasted sometimes for 4-5 weeks, and in other cases the animals continued to give positive tests until an age when spontaneous conversion would anyway be expected. Similar spleen cell suspensions given to C3H
and C57Bl mice did not induce conversion, neither did suspensions from Coombs negative NZB mice given to Coombs negative recipients. Further experiments confirmed these findings (Burnet, 1963; Holmes, 1965), and showed that the ability to induce Coombs test conversion and splenomegaly was a property possessed only by spleen cells from old NZB mice—similar numbers of lymph node, thymus, bone marrow and buffy coat cells were ineffective. Holborow et al. (1965) were also able to transfer Coombs positivity to young, previously negative NZB recipients using spleen cells from old NZB mice, and they further showed that when the donor spleen cells came from an animal which was positive for the antibody which cross-reacts with fixed human red cells, the ability to produce this antibody was also transferred. East et al. (1965) described a series of experiments in which they injected spleen cells from old Coombs positive mice into young NZB recipients and failed to detect a positive conversion in the recipients. The results of these experiments, however, are not strictly comparable with those of the other groups since the recipients were newborn or weanlings, the number of cells transferred was smaller, and the recipients were Coombs tested only at 8 and 15 days or 5 weeks after transfer. Kaye and Hook (1964) injected Coombs negative NZB mice with a mixture of spleen, thymus and lymph node cells from Coombs positive animals, and found that two weeks after injection 40 per cent of the recipients gave positive direct Coombs tests. It is of interest that Kaye and Hook also found that Coombs positive NZB mice were more susceptible to infection with Salmonella typhimurium than their Coombs negative counterparts, and that this
increased susceptibility was shared by the mice in which positive Coombs tests had been induced by injection of the cell mixture.

Various surgical and immunosuppressive measures, designed to reduce the immunological activity and its resultant effects, have been utilised in NZB mice. The experiments showing that neonatal thymectomy or thymectomy prior to the onset of disease have little effect have already been mentioned (Helyer and Howie, 1963b; Holmes and Burnet, 1964; East et al., 1965).

Holmes and Burnet (1963b) found that early splenectomy decreased the earlier onset of severe haemolytic anaemia in males (which they had previously noted), but had no striking effect on the ultimate incidence of positive direct Coombs tests in males. It did, however, increase the incidence of lethal nephritis in both males and females. Splenectomy of mice older than 8 months, and which were already Coombs positive, had no detectable effect on the disease process. Helyer and Howie (1963a) found that splenectomy of old animals with gross splenomegaly precipitated death due to profound anaemia within a few days of the operation, whereas splenectomy of animals older than 6 months, with or without signs of haemolytic disease but without obvious splenomegaly, had no effect. Splenectomy of animals younger than 3 months, however, was claimed to reduce greatly the severity of haemolytic disease (as judged by lowered antibody levels, reduced reticulocytosis and diminished incidence of hepatomegaly and plasma cell infiltration of lymphoid tissue), but nevertheless no increase in life-span was found in splenectomized animals.
Daily injections of porcine ACTH were given by Helyer and Howie (1963a) to a group of NZB mice with established autoimmune disease. This treatment was associated with a marked improvement in general condition: splenomegaly and hepatomegaly were reduced, haematocrit and reticulocyte levels returned towards normal values, and there was a reduction in titre of auto-antibodies. These remissions did not, however, prove to be permanent – drug therapy was withdrawn after 25 days and relapse occurred in all animals by one month later. Giltinan, Holmes and Burnet (1965) treated NZB mice with cortisone acetate given as a weekly injection, and they found reversal of positive Coombs tests in Coombs positive mice and prevention of conversion to positive state in younger mice for as long as treatment was continued. This was associated with a reduction to normal values of reticulocyte counts, but no effect was seen on the low haematocrit and haemoglobin levels. Treated mice were found to be more susceptible to infection than control animals, and mortality was found to be higher among the treated group. Casey and Howie (1965) gave betamethasone to NZB mice aged 7-9 months in their drinking water, and found an improvement in PCV levels, weakening in strength of anti-erythrocyte antibody tests and a reduction of splenomegaly. Continued treatment of a few animals suggested that this beneficial effect could be maintained, but cessation of treatment was followed by relapse within a few weeks. Woodruff and Stickel (1965) reported that they had been unable to produce remission in NZB mice over 6 months of age which had been dosed regularly with the immunosuppressive drugs actinomycin C and azathioprine.
These findings with NZB mice clearly demonstrate that spontaneous anaemia occurs in association with auto-antibody formation and other signs of immunological and lymphoid hyper-activity. It seems likely that the anti-erythrocyte antibodies present may be directly responsible for the production of anaemia, and NZB mice therefore provide suitable models for the study of spontaneous auto-immune diseases of humans (particularly those in which signs of autoimmune haemolytic anaemia occur). The studies of NZB crosses with other strains suggest that genetical factors may be involved in the transmission of susceptibility to autoimmune disease - a situation which may find its parallel in the observation made of human autoimmune diseases that, among relatives of affected people, there occurs a higher incidence of such diseases and lymphoid abnormalities than is found in the general population. Thus it appears likely that close study of NZB mice (and their crosses with other strains) will provide information which will help to explain the induction of autoimmune disease in mice and humans. Furthermore, they seem likely to be excellent models for assessing some of the effects of therapeutic measures (immunosuppressive or otherwise) suspected of potential usefulness in human autoimmune diseases.

7. IMMUNOSUPPRESSIVE THERAPY OF AUTOIMMUNE DISEASE — 
ANTI-LYMPHOCYTE SERUM

The most frequently used immunosuppressive treatment of acute autoimmune disease (particularly in SLE and autoimmune haemolytic anaemia) is the administration of corticosteroids. Generally, high doses are administered initially, and when signs of remission occur
the dose of drug is reduced or it is withdrawn completely. However, in one series of SLE patients (Dubois, 1966) it was found impossible to withdraw steroid therapy in 46 per cent of patients. High doses of steroids (60 mg. of prednisone per day for 6 months) have been advocated for the treatment of SLE-associated renal disease (Pollak, Pirani and Kark, 1961), although others have since maintained that much lower doses are equally effective (Zweiman et al., 1968). The immunosuppressive effects of corticosteroids have frequently been demonstrated in laboratory animals, but not conclusively in humans. In therapeutic doses they have been found not to inhibit antibody production to bacterial and viral antigens (Berenbaum, 1967), although a reduction in titre of auto-antibodies against red cells has been claimed for steroids in autoimmune haemolytic anaemia patients (Dameshek and Komninos, 1956; Dacie, 1962). Steroids also have powerful anti-inflammatory properties, and have been shown to decrease both the vascular and cellular components of inflammatory responses. In addition, macrophage activity, lymphoid cell metabolism and cell membrane integrity have all been shown to be influenced by steroid therapy. Thus the mode of action of steroids in benefitting autoimmune conditions is not well understood, and further problems are presented by the well-documented incidence of unwanted side effects in treated patients. Such side effects, which include peptic ulceration, diabetes, oedema, muscle weakness, increased susceptibility to infection, and osteoporosis, have been found to increase in direct proportion to the dose of drug administered.
Anti-metabolite and alkylating agent therapy have been used in a variety of autoimmune disorders - mainly in patients who have proved refractory to the effects of corticosteroids. Corley et al. (1966) claimed that azathioprine administration was of benefit to autoimmune haemolytic anaemia and SLE patients whose condition had not improved on steroid therapy, and in similar patients with SLE-associated kidney disease improvement was associated with the administration of nitrogen mustard (Kellum and Haseriok, 1965). Other drugs of this type which have been used include 6-mercaptopurine, chlorambucil and cyclophosphamide, and although beneficial effects have occasionally been found the results are generally disappointing. This is due in no small measure to the harmful side effects which almost invariably result: the most important of which is, perhaps, the production of bone-marrow hypoplasia.

The harmful side effects of steroids and cytotoxic drugs are most probably a result of their non-specific action. Since there is much evidence to suggest that immune responses are mediated by lymphocytes it seems very likely that therapeutic measures designed to affect only these cells would produce immunosuppression with a minimum of side effects. The report of Woodruff and Anderson (1963) that heterologous antisera raised against rat lymphocytes could prolong homograft survival in animals of that species raised the exciting prospect that such anti-lymphocyte sera might prove to be the required specific immunosuppressive agent.

It seemed worthwhile, therefore, to prepare rabbit anti-mouse
lymphocyte serum, and to test its ability to affect the auto-immune haemolytic anaemia which occurs spontaneously in NZB mice. The results of these studies form a major part of the work reported in this thesis. When this investigation was begun, towards the end of 1965, a substantial literature existed concerning various in vitro and in vivo properties of anti-leukocytic and anti-lymphocytic sera, but few reports were concerned with investigations of this subject in mice. These early findings are outlined below, and subsequent findings which were published while this work was in progress will be discussed in a later section.

It has been known for many years that the injection of leukocytes from animals of one species into an animal of another species is followed by the production of antibodies with specificities directed against the donor-type leukocytes. Metchnikoff (1899) raised antisera in the guinea pig, against rabbit or rat lymph node and spleen cells, which were able to agglutinate and kill polymorphonuclear leukocytes. Subsequently a number of in vitro studies of the properties of similar antisera were reported. Besredka (1900) observed that the cytotoxicity of such sera was destroyed by heating at 56°C for 30 minutes; Christian and Leen (1905) produced antisera in rabbits against rat spleen and lymph nodes which inhibited the amoeboid movement of leukocytes; Ritchie (1908) found that antisera raised in ducks by the injection of guinea pig thymus tissue agglutinated leukocytes and fixed complement when reacted with thymus, spleen or bone marrow; and Pappenheimer (1917) found
that the sera of rabbits injected with rat thymus and human tonsillar tissue contained cytotoxic and agglutinating antibodies for thymus cells and leukocytes. (When Pappenheimer's sera were heated at 56° C. for 30 minutes, the cytotoxic activity was lost but agglutinating activity remained). Flexner (1902) found only minor evidence of lymphoid hypoplasia in the lymph nodes of guinea pigs and rabbits treated in vivo with antisera raised against mesenteric lymph nodes, but Chew and Lawrence (1937) demonstrated a marked fall in the circulating lymphocyte counts of guinea pigs treated with rabbit anti-guinea pig lymph node serum, and many subsequent studies have revealed a similar anti-lymphocytic tissue effect (Cruickshank, 1941; Woodruff and Forman, 1951; Thompson, 1955; Stuart, 1962; Sacks, Fillipone and Hume, 1964).

The ability of an anti-lymphocyte antiserum to suppress an immune response was first shown by Inderbitzin (1956). He found that the delayed hypersensitivity reaction to tuberculin was suppressed in guinea pigs which had been treated with rabbit antiserum against guinea pig lymph node cells. Using similar antisera Wilhelm, Fisher and Cooke (1958) confirmed these results, and Waksman, Arbouys and Arnason (1961) extended them to include suppression of delayed hypersensitivity to diphtheria toxoid and inhibition of the development of experimental allergic encephalomyelitis. Woodruff and Anderson (1963) found that the survival of rat skin homografts was prolonged by treatment with rabbit antiserum raised against rat thoracic duct lymphocytes, and a similar effect was obtained by
Nagaya and Sieker (1965) using antisera raised against lymph node or thymus cells (given with Freund's complete adjuvant). Jeejeebhoy (1965) has described an antiserum which delayed graft rejection and suppressed antibody formation to sheep red cells and tetanus toxoid, but which was only effective in animals which had been previously thymectomized.

Antisera raised in rabbits against mouse lymph node cells (incorporated in Freund's complete adjuvant) have been found to suppress the primary and, to a lesser extent, the secondary immune responses of mice to sheep red cells, and to delay the rejection of allogeneic and xenogeneic skin grafts (Gray, Monaco and Russell, 1964; Monaco, Wood and Russell, 1965). Such sera were found to lyse lymphocytes in the presence of complement, to agglutinate lymphocytes in vitro and to produce marked lymphopenia in vivo. The lymphopenia could be maintained by repeated injections of antiserum and was accompanied by depletion and destruction of the organized lymphoid tissues. Ruche and Crowle (1965) raised an antiserum, with similar in vivo effects on lymphocytes, by injecting rabbits with mouse thymus cells, but they were unable to demonstrate in vitro agglutination and cytotoxicity. The serum did, however, inhibit the development of immediate and delayed hypersensitivity to ovalbumin, and delayed hypersensitivity to tuberculin-protein.
8. AIMS OF THE PRESENT STUDY

The observations and experiments which are described in subsequent sections were planned with the following three ends in view:

1. The characterization and elucidation of some of the immunological and pathological features of the autoimmune disease occurring in the NZB mouse strain maintained in the Department of Surgical Science.

2. An understanding of the factors involved in the successful transfer of signs of autoimmune disease from old NZB mice to younger NZB mice, or to (NZB x CBA)F1 hybrid mice, by the injection of lymphoid cell suspensions.

3. The production of immunosuppressive anti-mouse lymphocyte sera and a study of the effects of such sera on the spontaneous NZB disease and on the transferred disease in F1 hybrids.
MATERIALS AND METHODS
# MATERIALS AND METHODS

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1. EXPERIMENTAL ANIMALS

NZB/B1 mice. The New Zealand Black mice used in this study were the descendants of a single pair of litter mates received in the Department of Surgical Science in 1963. These two animals were obtained from the Animal Breeding Station at the University of Otago, New Zealand, where they had formed part of the 12th inbred generation of a sub-line obtained from Dr. M. Bielschowsky which had been inbred for 44 generations from initial selection. A further 11 generations have been produced using strict brother-sister mating, making a total of 67 generations of inbreeding. The animals described in this thesis belonged to generations F61-66.

A careful record of the date of birth of each animal was kept, and after weaning at the age of 21 days individual mice were earmarked and housed in mass culture cages containing a maximum of 35 mice of one sex. Usually animals spent 1-3 months in mass culture cages, after which they were removed for Coombs testing and subsequently housed individually in small cages. Breeding pairs were retired after bearing 4-6 litters, Coombs tested and housed in single cages. Regular exchange of skin grafts between NZB mice of the same sex was performed and such grafts were invariably accepted. (This involved grafts to 48 animals and covered 5 generations).

Young mice during the few weeks following weaning appeared to be in excellent health - they had glossy coats and were at least as active as other laboratory strains. After 20 weeks of age increasing numbers of animals began to deteriorate and show scabbing
of the dorsal region, alopecia and signs of external infections particularly around the orbital regions. This was accompanied by the adoption of a hunched posture, ruffling of the remaining fur and general lethargy. The spleen usually became palpable and was often visible through the thin skin. Figure 1 shows 2 NZB/B1 female mice - the one on the left is 140 days old and the other is 480 days old.

Other mice. CBA/H-T6, C57Bl, A and C3H/He mice were members of inbred strains which have been maintained in the Department of Surgical Science for many years. CBA/H and DBA/2 mice were obtained as breeding nuclei from the Laboratory Animal Centre, Carshalton and were subsequently propagated by the "Traffic light system" recommended by the Laboratory Animal Centre. Skin grafts exchanged between CBA/H and CBA/H-T6 mice were invariably permanently accepted. The F1 hybrids between NZB/B1 females and CBA/H-T6 or CBA/H males, and between C57Bl females and CBA males were bred as required.

Shortened forms of the mouse strain designations have been used elsewhere in this thesis as follows:-

\[
\begin{align*}
\text{NZB} & = \text{NZB/B1} \\
\text{CBA} & = \text{CBA/H} \\
\text{C3H} & = \text{C3H/He} \\
\text{DBA} & = \text{DBA/2} \\
\text{C57Bl} & = \text{C57Bl} \\
\text{A} & = \text{A} \\
(\text{NZB} \times \text{T6})F1 & = (\text{NZB/B1} \times \text{CBA/H-T6})F1 \\
(\text{NZB} \times \text{CBA})F1 & = (\text{NZB/B1} \times \text{CBA/H})F1 \\
(\text{C57Bl} \times \text{CBA})F1 & = (\text{C57Bl} \times \text{CBA/H})F1
\end{align*}
\]

Rats and rabbits. Wistar strain rats were members of a colony maintained in the Department of Bacteriology, University of Edinburgh.

New Zealand White rabbits were obtained from various commercial sources.
Figure 1. A comparison of two NZB female mice to show the effect of age on obvious signs of spontaneous disease. The animal on the left is 140 days old and that on the right is 480 days old.
and were used for antiserum production when they weighed between 2.5 and 3.5 kg.

2. **SEROLOGY AND HAEMATOLOGY**

Blood was obtained for the following procedures by bleeding mice from the retro-orbital plexus using microhaematocrit tubes or finely drawn out capillary pipettes made from thick walled glass tubing. When cells were required, heparinised bleeding tubes were used and the blood was collected in 0.9% saline containing 0.38% sodium citrate. When serum was required, untreated tubes were used and the blood was collected in wax-coated tubes.

**Direct antiglobulin (Coombs) test.** The antiglobulin test was introduced by Coombs, Mourant and Race (1945) as a method for detecting "incomplete" Rh antibodies (i.e. antibodies which are adsorbed by Rh positive erythrocytes but which do not cause direct agglutination in saline dilutions of serum). Red cells coated with such non-agglutinating antibody, and freed from non-specific serum components by washing with saline, undergo agglutination when suspended in an antiglobulin serum raised in another species (usually the rabbit). This test has subsequently proved to be a sensitive method for the detection of various auto-antibodies found in human acquired haemolytic anaemias.

In the present work the direct Coombs test has been used. This involves the agglutination of erythrocytes which have been sensitised in vivo (i.e. obtained directly from the animal producing the "incomplete" antibody).

Rabbit anti-mouse globulin antiserum was prepared by immunising two rabbits at 14 day intervals over a period of 8 weeks with inactivated normal mouse serum given intravenously and subcutaneously.
One week later the rabbits were exsanguinated and the separated inactivated sera were pooled. After 3 absorptions with 10 times washed erythrocytes obtained from C57Bl, A and CBA mice this serum no longer agglutinated normal mouse erythrocytes but did agglutinate erythrocytes from old NZB mice to a titre of 1/1024. When tested against normal mouse serum by immunodiffusion and immunoelectrophoresis clear lines showing recognition of IgG and IgM were obtained. The antiserum was stored in 0.1 ml. aliquots maintained at -20°C., and used throughout this investigation.

Coombs tests were performed using 2% suspensions of erythrocytes which had been collected in citrate solution and washed 3 times in large volumes of saline. One drop of this suspension was mixed on a clean microscope slide with two drops of a 1/5 dilution of antiserum in saline, the slide was rocked gently to mix the two components, and 5-10 minutes later was examined with the naked eye and microscopically under low-power magnification (x 32). This test was performed at room temperature. The results of each test were assessed independently by two observers, and on the rare occasions when they did not agree the test was repeated. Erythrocytes from CBA mice and saline in place of antiserum were always included as controls when a series of tests were carried out on NZB erythrocytes. In the early part of this investigation antiserum was used at a dilution of 1/25 as well as 1/5, but since identical results were obtained for these two dilutions this procedure was subsequently abandoned. The extent of agglutination
was assessed in accordance with the following scale:

++++ = virtually every cell agglutinated; agglutination seen without magnification.

+++ = many 3-10 cell clumps and at least one large clump seen in each field; unagglutinated cells uncommon.

++ = 5-10 small clumps in each field; unagglutinated cells numerous.

+ = 2-4 clumps in each field; unagglutinated cells predominant.

- = negative reaction; no agglutination.

Mice whose erythrocytes gave a negative result in this test are referred to as "Coombs negative"; mice with a + reaction are referred to as "weakly positive", and mice with a ++ or greater reaction are "strongly positive."

Papain-treated red cell agglutinating activity

The sera of patients suffering from autoimmune haemolytic anaemia frequently contain antibodies which agglutinate homologous erythrocytes only if the latter have been pretreated with proteolytic enzyme (Dacie, 1962b). Such antibodies are not blood group specific, but appear to be directed against a widespread component of the Rh system (Dodd and Eeles, 1961; Dybkjaer, 1966). They are generally found in association with a positive direct or indirect Coombs test, but a few reports have appeared describing the occurrence of positive enzyme-treated red cell tests with negative Coombs tests (Lemaire et al., 1954; Payne et al., 1955). It has also been reported that enzyme-treated red cell reacting antibodies
occur quite widely in healthy individuals with no sign of haemolytic disease (Stratton and Renton, 1958; Dybkjaer, 1966; Worlledge et al., 1966). The type of antibody appears to be of major importance and may be related to their pathological significance - those associated with active haemolytic disease appear to be mainly IgG while those found in healthy individuals are mainly IgM (Dodd and Wilkinson, 1964).

The sera of NZB mice also contain incomplete antibodies which agglutinate enzyme-treated red cells (Long, Holmes and Burnet, 1963; Helyer and Howie, 1963). This activity was found to agglutinate all mouse red cells tested, to cross-react slightly with rat red cells, but to be inactive against other erythrocytes.

In the present study enzyme-treated red cell agglutinating antibodies were detected in the sera of NZB mice using a papain method based on that used routinely by the Edinburgh Regional Blood Transfusion Service.

Materials: Solution of papain activated with cysteine. (This was provided by the Edinburgh B.T.S., was of known activity against human red cells, and contained 2 g. papain per 310 ml. buffer).

Microtiter microtitration kit (Cooke Engineering Co.)

Phosphate buffered saline (pH 7.3).

Fooled red cells were obtained from CBA strain female mice, washed three times with buffered saline solution at 37°C. and packed by centrifugation at 24,000 x g for 10 minutes. To one volume of these cells were added four volumes of the activated
papain solution and the cells gently mixed. After incubation at 37°C for 30 min. the suspension was washed three times and re-suspended in buffered saline to a 2.5% concentration.

Agglutination tests were performed using Lucite plates with conical wells and the microtitration apparatus. Doubling dilutions of NZB sera were prepared in buffered saline containing 1/32 MMS, in a total volume of 0.025 ml. per well. To each well was added 0.025 ml. of the papain-treated red cell suspension. The plates were sealed, incubated for 1 hr. at 37°C, then read for the presence or absence of agglutination.

Control plates were set up in a similar fashion but to these were added red cells which had not been treated with papain.

Eleven NZB sera which were found to contain antibodies reactive with papain-treated red cells when tested by the above method were fractionated on Sephadex G200 and the distribution of activity in the fractions assessed by the same method.

Complement-fixing activity against isogeneic liver homogenate

This test was kindly performed by Dr. C.J. Elson of the Bacteriology Department, University of Edinburgh.

Sera from various groups of NZB mice and other mouse strains were inactivated at 56°C. for 30 minutes prior to carrying out dilutions in MRC pattern perspex plates. The complement fixation test was carried out using a four-drop system and 1.5 MHD guinea pig complement as described by Weir (1967). Isogeneic liver antigen homogenates were prepared in 0.25 M sucrose at 4°C. as described by
Pinckard and Weir (1966). Animals were killed by stunning and their livers immediately removed and homogenised in a Potter-Elvejhem smooth-walled glass homogeniser with a fitted Teflon pestle rotating at 12,000 r.p.m.

**PCV determination**

A low PCV value is an indication of the presence of anaemia although haemolysis is only one of the situations which produce it (Dacie, 1962a).

Blood was taken directly into a heparinised glass capillary tube, one end was heat sealed, and it was spun for 5 minutes in a microhaematocrit high speed centrifuge. The proportion of formed elements to total blood was calculated as a percentage.

**Reticulocyte count**

Since reticulocytes are immature red cells which are released from the bone marrow 1-2 days before they assume the morphology of mature cells, the numbers of such cells in the circulation is an accurate reflection of erythropoietic activity (Dacie, 1962a). Increased numbers of such cells is a common finding in human haemolytic disorders where haematopoietic compensation is taking place.

Counts were determined using blood smears prepared by mixing a drop of blood with a drop of freshly prepared brilliant cresyl blue in methanol. After spreading, the slide was allowed to dry and lightly counterstained with 0.04% Leishman's stain. Five hundred erythrocytes were counted on each slide and the percentage of reticulocytes was calculated.
Total and differential white blood cell counts

The total number of white blood cells present in a given volume of heparinised blood was determined using standard procedures utilizing a WBC pipette, diluting fluid (2% acetic acid containing a trace of gentian violet), and an improved Neubauer pattern counting chamber.

Differential counts were performed on blood smears which had been stained with Leishman's stain. For most purposes a simple proportion of polymorphonuclear (neutrophils, basophils, eosinophils) to mononuclear (lymphocytes, monocytes) cells was recorded.

3. SKIN GRAFTING IN MICE

The transplantation of skin has proved to be an immensely valuable technique in a number of areas of medical research - particularly in the fields of cancer immunology and organ transplantation. The most important factor determining whether a surgically successful skin graft survives in a recipient animal has been found to be the genetic relationship of donor and recipient. A transplant will survive (i) if the donor and recipient do not differ antigenically; or (ii) if the recipient has been rendered specifically tolerant of the donor's transplantation antigens; or (iii) if the recipient is treated with an immuno-suppressive agent, e.g. corticosteroids or X-irradiation (Billingham and Medawar, 1951). If one of these conditions is not fulfilled then grafts are rejected with varying degrees of intensity depending on the degree of antigenic difference between donor and recipient.
For these reasons the exchange of homografts between putatively inbred animals can serve to assess their degree of homozygosity, and animals of one inbred line bearing grafts from another genetically distinct line are a useful system for assaying immunosuppressive agents.

In the present study the exchange of skin homografts has been used to check that the NZB mice used were indeed inbred, that CBA and T6 mice did not differ antigenically, and also as an assay system to test the immunosuppressive potency of various batches of ALS. The NZB mice were tested using groups of animals in ring formation (i.e. each member of a group provided a pinch graft for one neighbour - grafts were placed in the beds from which the corresponding grafts had been cut). Grafts were cut from members of a group of CBA mice and placed on beds cut on T6 mice - the skin from these areas was transferred to the exposed areas on the donor CBA mice. The ALS assays were performed using skin from a single A donor transplanted to 8-12 CBA recipients which received ALS treatment.

The skin grafts used were full-thickness grafts (i.e. epidermis + dermis) from which the panniculus adiposus had been removed by pinning the skin to a board and scraping gently with a scalpel blade. In the case of A strain grafts, in which a wide band of skin was removed from a freshly killed animal, circular grafts 1 cm. in diameter were punched out using a metal cork borer. Other grafts were trimmed to roughly this size and shape. Skin was
placed in sterile Petri dishes containing a piece of filter paper which had been moistened with saline and stored at 4°C., but transplantation was never delayed more than 3 hours after skin had been removed.

Graft beds were prepared on the dorsal aspect of the thoracic region of recipient mice on areas which had been cleaned with "Cetavlon" in 70% alcohol after removing the fur with mechanical clippers. Circular beds of approximately 1.3 cm. diameter were cut using fine curved scissors. Care was taken to preserve the panniculus carnosus intact. This operation was performed under ether anaesthesia. A single graft was placed in the centre of a freshly cut bed, a small piece of tulle graf was used to cover the wound, and the graft was secured using 1 ½-2 turns of plaster of Paris bandage. This was found necessary to provide the light but firm pressure required to promote primary healing. The complete procedure was carried out using sterile technique and sterilised instruments and surgical cloths were always used.

Dressings were removed 8 or 9 days after transplantation and the fate of the grafts was checked by external appearance daily thereafter. Grafts which were accepted showed epithelial outgrowth and eventually grew hair of the donor type and orientation. In grafts undergoing rejection there was a change in colour from pink to a darker shade and this was followed by the appearance of exudation. Scab formation and loss of the dead graft eventually resulted. This process generally took 1-4 days, but grafts were scored as rejected when the first signs of rejection were present. An occasional graft was encountered in which early signs of rejection were followed by recovery and growth of donor type hair. Such grafts were scored as
accepted and to ensure that such grafts were not missed all
grafted animals were kept for at least 2 weeks after they had
been scored as "rejected."

Figure 2 shows a CBA mouse with an A strain skin graft
shortly after dressing removal on the 9th day after grafting.
Figure 3 shows a CBA mouse which had been treated with NRS and
which has rejected an A strain graft 11 days after transplantation.
Figure 4 shows a CBA mouse bearing a healthy A strain graft 44 days
after transplantation. This animal had been treated with 1.5 ml.
of a potent ALS preparation.

4. PREPARATION OF CELL SUSPENSIONS

Single cell suspensions were prepared from the spleen, lymph
nodes, thymus and bone marrow of mice for various purposes. These
were: (i) for injection into rabbits to produce rabbit anti-mouse
lymphocyte serum, (ii) to produce GVH reaction in (C57B1 x CBA)F1
hybrids by the injection of parental strain cells, and (iii) to
transfer Coombs positivity from old NZB mice to young NZB or
(NZB x T6)F1 hybrid mice. The procedure adopted in each case was
as follows. The appropriate organ was removed from a freshly
killed animal, cut into 2 or 3 pieces, and carefully broken up in
balanced salt solution (Hank's or Dulbecco's) using a ground-glass
homogeniser with a loose fitting glass plunger. The resulting sus-
pension was drawn up and down through a fine bore Pasteur pipette
to disperse clumps of cells and then passed through a fine-meshed
stainless steel sieve into a graduated centrifuge tube. The cells
were usually washed 3-4 times which involved centrifugation at
Figure 2. CBA male mouse bearing a skin graft from an A strain male. Photograph taken shortly after removal of plaster of Paris dressing on the 9th day after transplantation.
Figure 3. CBA male mouse which has rejected a skin graft from an A strain male. Animal was treated with normal rabbit serum and photograph was taken on the 11th day after transplantation.
Figure 4. CBA male mouse bearing a healthy skin graft from an A strain male. Animal was treated with 1.5 ml. of ALS. Photograph was taken 44 days after transplantation.
280 x g for 5 minutes and resuspension in fresh salt solution. Cell counts were then performed using ability to exclude 1/2000 Trypan Blue dye as an indicator of cell viability, and the volume was adjusted to give the required concentration of viable cells. Sterile precautions were used throughout, and the temperature of the cells was maintained as low as possible by using salt solution at 4°C and keeping the cell containers in an ice bath.

The lymph nodes used for preparing such suspensions were the axillary, brachial and inguinal nodes. (Whenever the term lymph nodes is used in the experimental section of this thesis it refers to these 3 pairs of nodes). Bone marrow cell suspensions were prepared by dissecting out both femurs and tibias and perfusing the medullary cavity of each bone with salt solution delivered from a syringe fitted with a No. 23 needle.

5. THORACIC DUCT CANNULATION

In one experiment a suspension of cells obtained from the thoracic duct lymph of NZB mice was used. Cannulation of thoracic ducts was kindly performed by Dr. J.L. Boak using the technique developed by Boak and Woodruff (1965). Lymph was collected overnight at room temperature in balanced salt solution containing 20 units of heparin, 2000 units of penicillin, and 2.5 mg. of streptomycin per ml. The cells in the lymph were pooled after 12 hours of collection, washed twice, and resuspended to the required concentration of viable cells.
6. GRAFT-VERSUS-HOST REACTIONS

The transplantation of viable immunologically competent lymphoid cells from an adult animal to an immature animal which differs from it genetically, but which is unable to reject the foreign cells, results in a peculiar wasting disease usually referred to as "runt disease" on descriptive grounds. There is good evidence that this syndrome follows the reaction of inoculated cells against the foreign antigens which confront them in their new host: (i) the incidence and severity of the disease are proportional to the genetic disparity between donor and host animals; (ii) the ability of a given type of cellular inoculum to cause the disease depends on its content of lymphoid cells (e.g., spleen cells are much more effective than equivalent numbers of marrow cells); (iii) the dosage contained in the inoculum is a crucial factor; and (iv) cells from sensitised donors produce more severe reactions (Billingham and Brent, 1959; Simonsen, 1962). For these reasons the term graft-versus-host (GVH) has been used to describe such reactions which can be produced in a variety of situations where the above conditions are fulfilled. GVH reactions have been produced in adult animals which have previously been rendered tolerant of donor strain histocompatibility antigens or which have been treated with immunosuppressive agents, but perhaps the most useful laboratory model of this situation is the injection of parental strain cells into F1 hybrid animals. The F1 hybrid produced by mating individuals from two inbred strains is unable to react against the genetically determined histocompatibility antigens of both
parental strains since it carries both types on its own cells and is presumably tolerant of them. Cells of either parental strain, however, if introduced into the hybrid, can react against the histocompatibility antigens of the other strain and thereby give rise to a GVH reaction. Such reactions produced in F1 hybrids of inbred mouse strains have been extensively studied, and various grades of severity have been found depending on the strain combinations used. Certain features, however, are commonly found to occur and these include hyperplasia and, in severe cases, subsequent atrophy of lympho-haemopoietic tissues. This is accompanied by weight loss and early death. In less severe cases milder symptoms are found; the lymphoid hyperplasia which is initially present subsides and complete recovery occurs. The initiating feature of GVH-induced disease appears to be a proliferation of the transplanted cells but this is rapidly accompanied by a proliferation of host cells. It is widely believed that the pathological and clinical features of such diseases are the result of a secondary process (e.g. infection), but a satisfactory interpretation of the situation is still awaited, and the actual cause of death of afflicted individuals is unknown (Simonsen, 1962; Billingham and Silvers, 1965).

In the present work, GVH reactions were produced in (C57Bl x CBA)F1 hybrid mice by the injection of C57Bl spleen cells. The suppression of such reactions by the treatment of donors prior to cell transfer was used as an assay for the immunosuppressive potency of different batches of ALS. GVH reactions were also found in
(NZB x T6) and (NZB x CBA)F1 hybrid mice given suspensions of NZB lymphoid organs in experiments to study the transfer of autoimmune disease. The occurrence and severity of these reactions were assessed using degree of splenomegaly and hepatomegaly, and the incidence of mortality in treated groups compared with untreated controls. Splenomegaly and hepatomegaly were expressed as relative organ weights calculated in the following manner:

\[
\frac{\text{Actual wt. of organ (g.)}}{\text{Body wt. (g.)}} \times 10
\]

7. THE PREPARATION OF RABBIT ANTI-MOUSE LYMPHOCYTE SERUM

Rabbit anti-mouse lymphocyte serum (ALS) was raised in New Zealand White rabbits by injection into the marginal ear vein of cell suspensions prepared from mouse lymphoid organs. Initially the "two pulse" regime recommended by Levey and Medawar (1966) was utilised. When the immunosuppressive capacity of the four antisera raised in this manner was assessed, using the ability to prolong skin homograft survival, the results were disappointing. Two other groups of rabbits were therefore used to produce antisera: one group was given three immunising cell injections, while the other was treated with Corynebacterium parvum before receiving two immunising injections as for the "two pulse" regime. Two other rabbits were given weekly injections of cells over longer periods: one for 5 weeks and the other for 11 weeks. (Table 14 contains detailed immunisation schedules for each of these groups). The term ALS is widely used to refer in a non-specific manner to heterologous antisera raised against any lymphoid tissue, and it has been so employed
in the present work. In fact all of the antisera used except one were raised against thymus cells - a point likely to be of significance since it is known that the properties of anti-thymus cell sera may differ from those of sera raised against other lymphoid cells (Nagaya and Sieker, 1965; Asakuma and Reif, 1968).

Thymus cell suspensions were prepared from the thymuses of CBA mice (as previously described), and the required doses of viable cells (usually 100 x 10^6) were injected at predetermined intervals into rabbits. The animals were exsanguinated via both marginal ear veins and the blood was collected in paraffin wax-coated sterile glass tubes. It was allowed to clot for 1-2 hours at room temperature, left overnight at 4°C for clot retraction, and the serum was decanted next day. The serum was centrifuged lightly to remove red cells and then inactivated by heating at 56°C for 30 minutes.

The red cell agglutinin titre of each serum was determined and the serum was either stored at -20°C. to await absorption or absorbed immediately against mouse erythrocytes. This was accomplished by mixing the serum with washed, packed mouse red cells in a ratio of 1 volume packed cells to 4 volumes of serum and leaving the mixture at room temperature for 1½ hours. The serum was absorbed a second time using a similar volume of cells but this time the mixture was left overnight at 4°C. Next day the serum was separated from the agglutinated cells, cleared by centrifugation, sterilised by passage through a 0.22μ Millipore filter, and stored in small quantities (1-5 ml.) at -20°C. until required. This procedure regularly reduced
red cell agglutination to a titre of less than 1/4. The erythro-
cytes used for absorption were a mixture of cells obtained from
male and female CBA, C57Bl, and DBA/2 mice, and they were washed
6-9 times in large volumes of saline taking care to remove the
buffy coat of leucocytes after each centrifugation. In a few cases
rat red cells were used for the first absorption and these were
found to be as effective as mouse red cells.

Small quantities of four horse anti-mouse thymus cell anti-
sera were obtained from the Wellcome Foundation. These and two
batches of serum from untreated rabbits (normal rabbit serum - NRS)
and one batch of normal horse serum (NHS), were processed as out-
lined above for the rabbit antisera.

8. THE USE OF Corynebacterium Parvum AS AN ADJUVANT

Injection of the heat-killed micro-organism C. parvum produces
an intense stimulation of the lympho- reticular tissues of mice
(Halpern et al., 1964). This stimulation is associated with resist-
ance to the induction of fatal GVH disease in F1 hybrid mice given
parental strain lymphoid cells (Biossi et al., 1965), and increases
the resistance of mice to the effects of transplanted carcinomas and
sarcomas (Woodruff and Beak, 1966). Recently, C. parvum pre-treatment
of rabbits has been shown to exert an adjuvant effect by preventing
the induction of tolerance to bovine serum albumin (Finckard, Weir
and McBride, 1968), and these authors consider that C. parvum may
exert its most important effect on antigens which normally are only
weakly antigenic. These findings suggested that C. parvum might prove
to be a useful adjuvant in producing ALS of increased immunosuppressive
potency.

Two heat-killed suspensions of *C. parvum* were used in the present work; one was obtained from the Wellcome Foundation, and the other from the Pasteur Institute in Paris through the courtesy of Dr. J.G. Howard. Three rabbits were treated with the Pasteur vaccine and four rabbits with the Wellcome vaccine. In each case 30 mg. of killed bacteria were administered intravenously 6 days prior to the first immunising injection of mouse lymphoid cells.

2. *IN VITRO* AND *IN VIVO* ASSAYS OF ALS ACTIVITY

**Cytotoxicity in vitro**

Cytotoxic antibodies were assayed using a modification of the technique described by Abasa and Woodruff (1966). The test was carried out in Kahn tubes to which were added 0.1 ml. volumes of doubling dilutions of ALS, 0.1 ml. of complement (fresh guinea pig serum), and 0.2 ml. of a CBA mouse spleen cell suspension containing 25 x 10^6 nucleated cells. Control tubes containing cells + normal serum + complement, cells + complement, and cell suspension alone were always included. The tubes were incubated at 37°C for 1½ hours and then stored at 4°C during the counting period (normally 20-30 min.). One person selected a tube at random, re-suspended the cells with a Pasteur pipette, performed a 1/20 dilution in a WBC pipette using 0.05% Trypan Blue in Dulbecco's solution, mixed the cells for 1 minute and finally filled a haemocytometer chamber. A second person, unaware of the identity of the tube, counted the proportion of stained and unstained cells in a sample of 200-300 cells. The dilution of the last tube showing more than 15% stained
cells was taken as the cytotoxic titre of the ALS it contained. The proportion of stained cells in the control tubes never exceeded 10%.

**Prolongation of homograft survival**

Each antiserum was tested on a group containing 8-12 male CBA mice (weight 21-25 g.) bearing A strain skin grafts. Injections of ALS were given subcutaneously as follows: 0.5 ml. on day -2, 0.25 ml. on day 0, 0.5 ml. on day +2, and 0.25 ml. on day +5 (day 0 = day of grafting). After dressing removal the grafts were scored visually until rejection became obvious. The number of days between grafting and rejection was taken as the duration of graft survival, and mean survival times (MST) were calculated for each ALS-treated group from these data. The mean survival time of grafts on 12 animals, given saline subcutaneously on the days indicated instead of serum, was 10.5 (± 0.7) days.

**Suppression of GVH reaction**

The effect of ALS treatment on a GVH reaction was assessed using (C57Bl x CBA)F1 hybrid mice given spleen cells from ALS-treated C57Bl parental strain animals. These donors were C57Bl males weighing between 18 and 22 g., and they received an intraperitoneal injection of 0.25 ml. ALS on each of the 4 days preceding cell transfer. (Total dose per mouse = 1.0 ml.). Pooled spleen cell suspensions were prepared from donors treated with a single batch of ALS: 0.5 ml. of suspension, containing 100 x 10^6 viable cells, was injected intraperitoneally into each hybrid recipient. Recipients were male F1 hybrids weighing 20-25 g.
Ten days after cell transfer they were accurately weighed and then killed. Their spleens were dissected out and weighed, and the relative spleen weight for each animal was calculated.

The various test groups and controls were as follows:

1. **Test groups** - groups of six F₁ hybrid mice given spleen cells from donors treated with ALS or NRS.

2. **GVH control group** - ten F₁ hybrid mice given spleen cells from donors which had been treated with saline instead of serum.

3. **Normal control groups** - (a) ten F₁ hybrid mice given saline instead of spleen cell suspension.
   (b) five F₁ hybrid mice given $100 \times 10^6$ viable F₁ hybrid spleen cells instead of C57B1 spleen cells.

(No significant difference in terms of spleen weight was found between members of groups (a) and (b) so they were considered as one group - the normal control).

The mean relative spleen weight (MRSW) was calculated for each of the above groups, and these values were used to compare the ability of each batch of ALS to suppress the GVH reaction in this system. The difference between the MRSW of the GVH control group and the normal control group was used as a measure of the maximum degree of GVH reaction. The differences between the MRSW of each of the test groups and the normal control group were used as measures of the reduced GVH reactions produced by ALS treatment of the donors.
The following ratio (expressed as a percentage) was calculated for each ALS treated group:

\[ \% \text{GVH reaction} = \frac{\text{MRSW treated group} - \text{MRSW normal control group} \times 100}{\text{MRSW GVH control group} - \text{MRSW normal control group}} \]

Production of lymphopenia

The ability of a single injection of ALS to produce a reduction in the numbers of blood lymphocytes was assayed in C57Bl female mice weighing 18-22 g. These animals were bled from the retro-orbital plexus, taking care that only blood sufficient for a total WBC count and a blood smear was withdrawn. Immediately afterwards each animal was given 0.5 ml. ALS (or NHS, or saline) by the intraperitoneal route. At intervals 4 and 48 hours later, second and third blood samples were removed. Each series of such observations was begun between 10 a.m. and 12 noon in order to avoid interference from the well-known rhythmical fluctuations in blood cell numbers over a 24-hour period. Groups of mice consisting of 3-6 animals were used to test each serum. The total circulating lymphocyte count of each animal at each of the three test times was calculated using the percentage lymphocyte count (obtained from a differential count performed on the blood smear) and the appropriate WBC count.

Table 1 records the mean lymphocyte counts of untreated animals, of animals treated with all batches of ALS considered as one group, of animals treated with normal sera (horse and rabbit), and of animals treated with saline. It shows that at 4 hours after injection the lymphocyte counts of animals treated with ALS differed markedly (and quite uniformly) from counts taken before injection but that a significant drop was also seen in the counts of animals given normal serum
or saline. By 48 hours, however, the counts in animals given normal serum or saline had returned to pre-injection levels while the counts in ALS treated animals showed much greater variation. This variation was a reflection of the efficacy of different batches of ALS in producing lymphopenia.

The mean lymphocyte count for each ALS treated group at 48 hours after injection - expressed as a percentage of the appropriate mean pre-injection count - was therefore used as a measure of the ability of each antiserum to produce lymphopenia.

10. SERUM FRACTIONATION PROCEDURES

Gel filtration (exclusion) chromatography

Proteins can be separated on the basis of their molecular dimensions by chromatography on a column of a gel consisting of beads of a cross-linked dextran commercially available as Sephadex (Tiselius, Forath and Albertson, 1963). For the fractionation of serum proteins Sephadex G-200, which has an exclusion limit of molecular wt. around 200,000, is used. The pore size of the beads is such that proteins of molecular wt. greater than 200,000 (e.g. IgM) are excluded from the beads and pass through the column with the void volume. Proteins of lower molecular wts. are able to enter the beads and hence their progress through the column is retarded. The number of molecules entering the beads determines the rate of progress of a particular molecule through the column (Fahey and Terry, 1967) with the result that proteins of intermediate molecular wts. (e.g. IgG) are eluted before low molecular wt. proteins (e.g. the albumin).

Materials: Sephadex G-200 (Pharmacia, Uppsala, Sweden)

Phosphate buffer (pH 7.2, 0.06M, containing 0.15M NaCl)
### TABLE 1

**THE MEAN LYMPHOCYTE COUNTS IN GROUPS OF C57B1 FEMALE MICE GIVEN 0.5 mL. ALS, NORMAL SERUM, OR SALINE INTRAPERITONALLY**

<table>
<thead>
<tr>
<th>Injected with</th>
<th>Number in group</th>
<th>Pre-injection counts (± 1 S.D.)</th>
<th>Counts 4 hrs. after injection (± 1 S.D.)</th>
<th>Counts 48 hrs. after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-lymphocyte</td>
<td>59</td>
<td></td>
<td>3042 (± 1195)</td>
<td>7068 (± 2629)</td>
</tr>
<tr>
<td>serum</td>
<td></td>
<td>12028 (± 2351)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal serum</td>
<td>11</td>
<td>7957 (± 1098)</td>
<td>9800 (± 3840)</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>6</td>
<td>8925 (± 1693)</td>
<td>13670 (± 2786)</td>
<td></td>
</tr>
</tbody>
</table>
Procedure: The procedure used to separate individual mouse sera was similar to that described by Fahey and Terry (1967). Sephadex was allowed to swell for 24 hours in excess buffer and fine particles were removed by occasionally decanting the supernatant fluid and mixing in more buffer. The resulting slurry was deaerated and poured into a column which was packed by opening the outflow tap and adding more slurry until the column was full. The column was then arranged so that buffer was drawn upwards through it at a constant flow rate which was controlled by the peristaltic pump. After buffer had been allowed to pass through the column overnight (to allow for the settling of beads) a serum sample was applied by removing the tubing from the buffer reservoir and dipping it into the sample. When sufficient serum had been drawn up (between 0.5 and 1.0 ml.) the tubing was returned to the buffer and elution proceeded. The eluate was passed through the Uvicord scanning device which measured percentage transmission at 254 mp. and provided a tracing of the protein distribution.

2 ml. volumes of the eluate were collected and the contents of groups of tubes were pooled to correspond with five fractions which can be seen in the example of such a run in Fig. 5. The fractions were concentrated by negative pressure ultrafiltration through 8/32" Visking dialysis tubing and reconstituted to the original volume of
Figure 5. Gel filtration pattern (on Sephadex G-200) of whole mouse serum. Fractions were obtained as indicated by pooling the contents of tubes associated with the peaks of activity.
serum applied to the column.

In the present work it has been assumed that the 19S fraction contained most of the serum IgM and the 7S fraction most of the IgG. The low molecular weight protein (L.M.P.) fraction was used as a form of control since it was not expected to contain any proteins with immunological activity.

Fractionation of human serum on G-200 Sephadex (Flodin and Killander, 1962; Fireman, Vannier and Goodman, 1964) provided three major protein peaks. The first contained large molecular weight proteins which included IgM and α2 macroglobulins, the second consisted largely of IgG although the leading edge of the peak also contained IgA and IgD. The third peak contained albumin and globulins with molecular weights less than 100,000.

Preparation of rabbit IgG using an anion exchange resin

The use of an anion exchange resin such as diethylaminoethyl (DEAE)-cellulose provides a simple procedure for the preparation of purified serum IgG (Stanworth, 1960; Fahey and Terry, 1967). Serum is added to the cellulose suspended in a buffer of around neutral pH and low ionic strength. Under these conditions negatively charged serum proteins adsorb onto the cellulose by electrostatic forces, the negatively charged groups on the protein presumably interact with positively charged groups on the cellulose. Only the neutral serum proteins (viz. IgG) are not adsorbed and these remain in solution in the suspending medium.

This procedure can be carried out by passing the serum through a column of the cellulose, but in the present work use was made of a
batch process similar to that described by Stanworth (1960).

**Materials:**

DRAE-cellulose (Whatman DE52 ion exchange cellulose -
degree of substitution = 1.0 m. equiv. per g.)

Phosphate buffer (pH 6.5 0.01M)

**Procedure:**

The cellulose was washed extensively with distilled
water until washings were no longer coloured by impurities and then
suspended in phosphate buffer adjusted to pH 6.5 by the addition of
N HCl or N NaOH. The mixture was then filtered through a coarse
sintered glass filter; the resulting pad was re-suspended in buffer
and re-filtered (after checking the pH). The equilibrated pad was
stored in a stoppered jar at 4°C. until used.

In the batch process approximately 500 g. of equilibrated ex-
changer were used to process 100 ml. of starting serum. The serum,
which had been dialyzed overnight against 1 litre of buffer, was
added to approximately two thirds of this amount of the exchanger
and the mixture stirred for 10 mins. at 4°C. Then 150 ml. of phos-
phate buffer was added and stirring continued for a further 15 min.
before the resulting slurry was filtered through a coarse sintered
glass filter. The pad of exchanger remaining in the funnel was
washed with 100 ml. of phosphate buffer and pooled with the original
filtrate. To this was added the remaining one third of unused ex-
changer and this was stirred for 15 mins. before re-filtering as
above. The filtrate was concentrated by lyophilization and recon-
stituted to the required volume in sterile phosphate buffered saline.

**Preparation of F(ab′)2 fragments of rabbit IgG**

It has been found possible to split one molecule of rabbit IgG
into three parts by treatment with papain (Porter, 1958, 1959). Two of these parts are identical and each contains one antibody combining site. (These univalent fragments have subsequently become known as Fab fragments). The third piece produced by such a digestion (known as the Fc fragment) is crystallizable, contains no antibody combining sites and is probably associated with a diverse range of activities possessed by the intact IgG molecule (e.g. complement fixation, membrane attachment and placental transfer). In 1960, Nisonoff et al. reported that it was possible to perform a similar digestion with pepsin but that if it was performed in the absence of a reagent capable of reducing disulphide bonds then only the Fc portion was removed (not in an intact form) leaving an intact divalent molecule. This bivalent residue is equivalent in many respects to two linked Fab pieces and is known as $F(ab')_2$. Such digests of IgG to give $F(ab')_2$ fragments have been used to investigate the biological activities associated with the binding activity of antibodies in comparison with secondary activities dependent on the presence of intact Fc. (It is found, for example, that $F(ab')_2$ fragments of antibodies generally retain agglutinating activity but have a greatly diminished complement fixing ability).

In the present study $F(ab')_2$ fragments of rabbit anti-mouse lymphocyte IgG and of normal rabbit IgG were prepared by a method based on that of Nisonoff et al. (1960).

**Materials:** Pepsin (3 x crystallized, ex hog stomach mucosa, Koch-Light Labs. Ltd.)

**Procedure:** An IgG preparation was prepared as already described
and dialyzed overnight against acetate buffer pH 4.0 0.1M. (The preparation was only used after its purity had been checked by immunoelectrophoresis using a sheep anti-rabbit whole serum).

For each 100 mg. of protein dissolved in acetate buffer, 2 mg. of powdered enzyme were added. The reaction mixture was incubated at 37°C. for 24 hours and then dialyzed against phosphate buffered saline overnight. The products were concentrated by ultrafiltration through dialysis tubing and reconstituted to a concentration of 1 g. per cent using phosphate buffered saline. The preparation was dialyzed extensively against this solution before use.

Immunodiffusion and immunoelectrophoresis

Since the introduction by Ouchterlony in 1948 of diffusion in agar gel as a tool for analysing the precipitation patterns of antisera against bacterial toxins the method of immunodiffusion has become widely used for the qualitative analysis of many antigen-antibody reactions. Its resolving power was considerably enhanced by combining it with electrophoresis of one of the components prior to the addition of the second component (Grabar and Williams, 1953). These techniques have proved of value in the recognition of antigen in mixtures using specific antisera, and in the recognition of specific antibody in a complex antiserum using purified antigen.

In the present work they have been used for two purposes: (i) to demonstrate the ability of the rabbit anti-mouse sera used to recognize the major immunoglobulin classes in mouse serum (section 2), and (ii) to demonstrate that the IgG fraction of rabbit anti-mouse ALS
prepared by DEAE-cellulose chromatography was free of contamination by other immunoglobulin classes.

Procedure: Immunodiffusion was performed on 75 x 75 mm. glass plates which had been coated with 0.85% agar, and dried. The gel layer was formed by running 10 ml. of 1% Oxoid Ion Agar dissolved in 0.85% saline from a warm pipette over the surface of the plate in a perfectly horizontal position. When the agar had solidified, 6 peripheral wells and 1 central well were punched and the agar removed from them. Serum or serum fractions were placed in the outside wells and antiserum in the centre well. Diffusion was allowed to take place for 48 hours in a humid atmosphere, the plates were washed for 48 hours in saline, and for 48 hours in distilled water.

Immunoelectrophoresis plates were prepared in a similar way except that the agar was dissolved in barbitone buffer (pH 8.6) instead of saline. Troughs and wells were cut using a Shandon Scientific Co. cutter, and agar was removed from the wells. The wells were filled with serum or serum fractions and the plate positioned in a Shandon electrophoresis tank. A potential gradient of 5-6 V per cm. was applied for 100 min., the plates were taken out and the agar was removed from the troughs. Antiserum was applied to the troughs and diffusion was allowed to proceed as described above.

After washing, plates were immersed in amido black solution for 15 min. to stain the remaining precipitated protein. Unbound stain was removed by washing in acid methanol for 48 hours, and
the plates were dried and examined for precipitation lines.

**11. THE IMMUNE RESPONSE TO BOVINE SERUM ALBUMIN (BSA)**

The preparation and use of BSA as an antigen in mice

It has been shown that the physical state of heterologous serum proteins can influence their behaviour as antigens. Dresser (1962) and Frei, Benacerraf and Thorbecke (1965) demonstrated that aggregated bovine gamma globulin and bovine serum albumin (BSA) respectively, may be immunogenic while their aggregated free counterparts tend to produce a specific unresponsive state.

In the present work unmodified BSA was found to be immunogenic in a number of mouse strains when injected as a precipitate with alum. Previous injection of BSA, which had been centrifuged to remove aggregates, was found to produce a hyporesponsive state to subsequent injections of unmodified BSA.

The unmodified BSA was a 100 mg. per ml. solution of BSA (Cohn Fraction V, Armour Pharmaceutical) in 0.085% NaCl. Centrifuged BSA was obtained by centrifuging 4 ml. quantities of the above solution at $1.6 \times 10^7 g\text{ min.}$ in an MSE Superspeed 40. At the end of the centrifugation period the rotor was allowed to come to rest without braking and the top 2 ml. of solution in each tube was carefully drawn off and used immediately. Alum-precipitated BSA was prepared as the aluminium hydroxide precipitate by mixing 50 mg. of BSA solution with 6 ml. of Alhydrogel (1.3% $\text{Al}_2\text{O}_3$) and 6 ml. of sterile 0.85% NaCl solution. This mixture was allowed to equilibrate at room temperature for 1 hour and then overnight at 4°C. The precipitate, obtained by centrifugation at 100 $x$ g for 10 min., was
suspended in 2 ml. of sterile NaCl solution and passed through a 23 gauge needle several times to break up any large aggregates (Pinckard, Weir and McBride, 1967).

**The ammonium sulphate precipitation test (Farr technique)**

Most commonly-used antibody assays measure a secondary effect of the primary union of antibody with antigen (e.g. the capacity to fix complement or agglutinate sensitised red cells) and may not reflect the total antibody content of an antiserum. Thus in attempting to assess the presence or absence of specific tolerance it is preferable to use a measure of the primary interaction of antigen with antibody.

The Farr technique depends on the differential solubility of antigen and antigen/antibody complexes in 50\% saturated ammonium sulphate (SAS/2), and is therefore, unfortunately, limited to antigens soluble in this solvent. At equilibrium the reaction between antigen and antibody can be represented by the equation:

\[
\text{Antigen + antibody} \rightleftharpoons \frac{K_a}{K_d} \text{Antigen/antibody}
\]

where \(K_a\) = association constant and \(K_d\) = dissociation constant.

The addition of SAS/2 to such a reaction mixture has been shown to bring it immediately to a halt by preventing formation and dissociation of the complex (Farr, 1958). Antibody bound antigen is precipitated by this procedure due to the insolubility of the bound globulin while unbound antigen remains in solution. A measure of the amount of antigen in the precipitate therefore provides a close approximation to the amount of antigen bound at equilibrium in solution.
The test is performed by adding constant amounts of labelled antigen to serial dilutions of antiserum. After allowing time for equilibrium to become established an equal volume of saturated ammonium sulphate (SAS) is added, and the resulting precipitate is centrifuged and washed with SAS/2. The number of radioactive disintegrations per unit time emitted by the precipitate is counted and the percentage of antigen bound (i.e. precipitated with the globulin) at each serum dilution is determined. If the percentage of antigen bound is plotted against the log of the reciprocal of antiserum dilution then the antiserum dilution which would bind 33 per cent of added antigen can be calculated by extrapolation. This value is used to calculate the amount of antigen bound per ml. of undiluted serum which is expressed in µg. N bound per ml. at a given antigen concentration, and is designated the antigen binding capacity at the 33 per cent end point (ABC-33).

The procedure used closely followed that described by Farr (1958) and Minden and Farr (1967).

**Materials:** 0.1M borate buffer, pH 8.4.

- Saturated ammonium sulphate (SAS). S.G. = 1.240 at 4°C.
- Half-saturated ammonium sulphate (SAS/2) prepared by diluting one volume of SAS with one volume borate buffer.
- Normal mouse serum (NMS) which was prepared as a pool derived from all mouse strains used in this study.
- 20 per cent trichloracetic acid (TCA) in aqueous solution.
- Trace-labelled BSA (BSA*). The stock solution was diluted with 1/100 NMS to give required concentration.
Procedure: Serial dilutions of antisera were made using volumetric blow-out pipettes - the initial antiserum dilution was 1/10 in buffer and subsequent dilutions were fourfold using 1/10 NMS. Duplicate tubes were set up containing 0.5 ml. quantities of each dilution. Control tubes were set up to determine:

(i) The total amount of radioactivity added per tube (this group - Ag add - received antigen and no further treatment.

(ii) The total amount of precipitable radioactivity (i.e. protein bound I\(^*\)) per tube (this group - TCA - received antigen, 0.5 ml. 1/10 NMS and 1.0 ml. TCA).

(iii) The amount of non-specifically bound BSA\(^*\) per tube (this group - C - received antigen, 0.50 ml. 1/10 NMS and 1.0 ml. SAS).

0.5 ml. of a solution containing 0.01 μg N BSA\(^*\) per ml. was added to experimental and control tubes using a 1 ml. automatic syringe with valve and cannula attachment and tubes were incubated overnight at 4\(^\circ\)C. (approximately 16 hrs.). 1 ml. SAS was added to each experimental tube and control tubes C, vigorous mixing was effected using an electric vibrator and they were put at 4\(^\circ\)C. for 30 mins. 1.0 ml. TCA was added to the TCA control tubes which were also incubated at 4\(^\circ\)C. for 30 mins. All tubes were then centrifuged at 1500 x g for 30 mins. in a refrigerated centrifuge, and the supernatants were discarded.

The various tubes involved and the reasons for setting them up can be more easily discerned from the following table:
<table>
<thead>
<tr>
<th>Tubes</th>
<th>Serum</th>
<th>BSA*</th>
<th>Precipitated with</th>
<th>To determine:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>Exp. Antiserum (0.5 ml.)</td>
<td>0.5 ml.</td>
<td>SAS</td>
<td>Proportion of added antigen bound by antiserum</td>
</tr>
<tr>
<td>Controls</td>
<td>Ag add - 0.5 ml. -</td>
<td>Total amount of added radioactivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCA NMS (0.5 ml.)</td>
<td>0.5 ml.</td>
<td>TCA</td>
<td>Total amount of precipitable radioactivity</td>
</tr>
<tr>
<td></td>
<td>C NMS (0.5 ml.)</td>
<td>0.5 ml.</td>
<td>SAS</td>
<td>Amount of non-specifically bound antigen</td>
</tr>
</tbody>
</table>

The number of radioactive disintegrations per unit time in each tube was counted using a Nuclear Enterprises "Gamma-matic" scintillation counter, the counts for each of the duplicate tubes were averaged and the following calculations performed:

(i) To check that the I\(^{131}\) being counted was bound to BSA the ratio of counts in TCA to counts in Ag. add was found:

\[
\text{Percent protein bound iodine} = \frac{\text{counts in TCA} \times 100}{\text{counts in Ag. add}}
\]

In each test this ratio lay between 99 and 100% (otherwise the BSA* preparation would have been discarded).

(ii) To estimate the percentage of the total BSA* added which was bound at each antiserum dilution the following ratio was worked out in each case:
per cent BSA* bound = \( \frac{\text{counts in Exp.} - \text{counts in C}}{\text{counts in TCA} - \text{counts in C}} \times 100 \)

The antigen binding capacity of an antiserum is the commonest method of expressing a Farr test result, and it can be calculated by plotting the above ratio (per cent BSA* bound) against the reciprocal of the antiserum dilution on semi-log graph paper. The best straight line is drawn between points where per cent bound is less than 85% but more than 15%, and the antiserum dilution which would bind 33 per cent of the added antigen is found by interpolation. The antigen binding capacity at the 33 per cent end point (ABC-33) is then given by:

\[
\text{ABC-33 (at particular Ag. conc.)} = \text{Reciprocal of end point dilution} \times \mu g. \frac{N \text{BSA}^*}{2 \times 0.33}
\]

An example of such a graph and calculation using data from the present work is given in Figure 6. The majority of antigen binding capacities, however, were estimated using the KDF9 computer located at the Department of Computer Science at this university, using a programme prepared by Mr. W. McBride of the Bacteriology Department.

Radio-iodination of bovine serum albumin

Proteins can be trace labelled by substitution of radioactive iodine in their tyrosine residues. If iodide is oxidised by a suitable oxidising agent in the presence of protein then cationic iodine is formed which, in alkaline solution, substitutes predominately in the meta position of a tyrosine group. Iodination of other amino acids, e.g. tryptophan, can occur but in the case of BSA it is thought to take place exclusively in the tyrosine
Figure 6. The Farr test - calculation of antigen binding capacity of an anti-BSA antiserum

By interpolation from the graph 33 per cent of the antigen would have been bound by a 1/620 dilution of the antiserum.

\[ \text{ABC} = 33 = 620 \times 2 \times 0.33 \times 0.01 \]

\[ = 4.09 \text{ } \mu g \text{ N BSA bound/ml, undiluted serum.} \]
residue (Hunter, 1967). In the method of Hunter and Greenwood (1962) which was used in this investigation, the oxidising agent is hypochlorous acid formed by the slow dissociation of chloramine T in aqueous solution. By this method small quantities of protein can be labelled with high efficiency, but without recognizable deleterious effect on the protein.

**Materials:**
- Borate buffer, pH 8.4, ionic strength 0.1
- 2 m.c. carrier-free sodium iodide (NaI$^{131}$) free of reducing agent (Radiochemical Centre, Amersham, Bucks.)
- Potassium iodide (KI)
- Chloramine T (sodium p-toluenesulphonchloramide) 50 mg. per 100 ml. distilled water
- BSA solution: 20 mg./ml.

**Procedure:** The iodination reaction was performed in a fume cupboard behind lead shielding. A small reaction flask containing 2 m.c. NaI$^{131}$ was placed on a magnetic stirrer; 0.2 ml. borate buffer and 0.2 ml. BSA solution were added followed immediately by 0.2 ml. of Chloramine T solution. The mixture was stirred and allowed to react for 60 seconds. 4 ml. of borate buffer was added and the mixture transferred via a hypodermic syringe to a dialysis sac. The mixture was dialysed against numerous changes of borate buffer containing a few carrier crystals of KI, to remove unbound iodine, until the radio-activity of the dialysate remained constant. The absorbance of the $^{131}$-BSA solution was measured at 280 μm in a 1 cm. silica cell in a Unicam SP-500 spectrophotometer. This value was used to calculate the protein concentration by reference to a
standard preparation of BSA whose protein concentration had been measured by the micro-Kjeldahl method (Kabat and Mayer, 1961). This preparation was only used when it was found that greater than 99% of the radio-activity was precipitated by the addition of 10% trichloracetic acid (TCA).
RESULTS
RESULTS

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1. SOME FEATURES OF THE PATHOLOGY OF UNTREATED NZB AND (NZB x T6)F1 HYBRID MICE

Thirty-two NZB mice (16 males and 16 females) and 37 (NZB x T6)F1 hybrids (21 males and 16 females) were studied closely from shortly after weaning until they died naturally or were killed when moribund. At regular intervals (approximately once per month for the NZB mice and every 2 months for the hybrids) each animal was bled and a direct Coombs test, a reticulocyte count and a PCV determination performed on the sample. The age at which each animal died was recorded, and whole body, spleen, lymph node, thymus and liver weights were determined. Sections from these organs and from one kidney were prepared and stained with haematoxylin and eosin or methyl green pyronin.

Figure 7 is a comparison of the survival times of these animals. There was little difference between male and female NZB mice: individual animals of both sexes began to develop terminal illness at around 300 days of age and all were dead by 680 days. In marked contrast, at this age (680 days) more than 70 per cent of the hybrids were still alive and in apparently good health.

Figures 8-11 show the incidence of positive direct Coombs tests among the NZB mice in relation to changes in PCV levels and in reticulocyte counts. Coombs tests first became positive in both sexes around 225 days of age. After this time the incidence among females increased rapidly and all were positive at 310 days. The rate of conversion was slower among males and 100 per cent incidence was not reached until the animals were 410 days of age.
(There was an interesting tendency among very old NZB males towards a decrease in strength of the Coombs test, and the 4 males which survived to 600 days all became negative before death). The percentage of reticulocytes to be found in the blood began to rise about 60 days before the first positive Coombs tests were detected in both sexes, and thereafter continued to rise. The highest values tended to occur among females although there was marked individual variation in both sexes. PCV values began to fall shortly before Coombs tests became positive, but in this case the values for males tended to be lower and more variable than those of the females.

The mean reticulocyte counts and PCV values among the F1 hybrid mice are shown in Figure 12. This graph covers the same age range as that for the NZB mice but observations were made at 2 monthly intervals. There was no evidence of a significant change in either PCV values or reticulocyte counts of the female hybrids, and only slight evidence among the males in that individual values became more variable after 400 days of age. Observations were continued on these mice until death, and occasional high reticulocyte counts and low haematocrit values were found in very old animals, but the majority died without any sign of anaemia. No F1 hybrid developed a persistently positive Coombs test, although a weakly positive test (1+ reaction) was occasionally found in an old animal on a single occasion. Again most animals died without ever having given such a positive result. The highest incidences
recorded in the two groups were 3 positive out of 8 surviving males at 952 days of age, and 3 positive out of 8 surviving females at 728 days of age.

More than 300 NZB mice comprising discarded breeders, animals used as controls in experiments and given only saline or untreated, and a few strongly positive animals which were used as controls for Coombs tests were studied over shorter periods or less fully than the above mice. No data which conflicted with the above results were found. The same was true for 86 (NZB x T6)F1 hybrids. One hundred and fifty-six of the NZB mice died natural deaths and the lifespans of these animals are recorded in Table 2. Mean age at death was calculated for each group depending on sex and whether the animal had been used for breeding. No significant differences in lifespan were found between any of these groups. The mean age at death for all NZB mice was 515.0 (± 130.6) days.

NZB mice of both sexes tended to have enlarged lymphoid organs and this is illustrated by the data recorded in Figures 13, 15 and 17. These show the relative organ weights of 170 untreated NZB mice which died naturally and were found in good condition, or animals which were killed to provide cell suspensions or for other reasons. Figures 14, 16 and 18 record the relative organ weights of untreated mice of other normal strains. This group contains data from 70 (NZB x T6)F1 hybrid, 20 C57Bl, 20 CBA, 20 C3H and 20 (C57Bl x CBA)F1 hybrid mice and, since there were no obvious differences between these strains in terms of
relative organ weights, they have been considered as one group.

The differences between NZB mice and normal mice were most marked in respect of spleen weights (compare Figures 13 and 14). After 200 days of age more than half of the NZB animals showed evidence of splenomegaly, and there was a continued increase in absolute spleen weights and in the proportion of mice affected with age. Few normal mice were found with relative spleen weights greater than 70 mg. per 10 g. body weight and none at all greater than 100. Similar differences were found between NZB and normal mice in respect of lymph node (Figures 15 and 16) and liver (Figures 17 and 18) weight, although there was greater variation in normal values than was found for spleen weights, and fewer NZB mice had grossly enlarged organs. NZB mice which had enlarged lymph nodes and livers also tended to have enlarged spleens, although the reverse was not invariably found.

Figures 19 and 20 record the relative thymus weights of 56 untreated NZB mice and 98 untreated mice of other strains respectively. There is no evidence of early thymic hypertrophy among the NZB animals, neither is there a difference between the two groups in terms of the well-known pattern of diminution in relative thymus weight with increasing body weight and age. It does, however, appear that the relative thymus weights of NZB mice older than 200 days were often lower than those of normal mice, and it is interesting that on occasion it proved impossible to isolate any thymic tissue whatsoever from old NZB mice.

Histology: The lymphoid and reticulo-endothelial organs of NZB
mice younger than 100 days of age were microscopically indistinguishable from those of mice of other strains. After that age a proportion of NZB mice were found to have germinal centres in the thymus, spleen and lymph nodes. Such germinal centres typically consisted of circular aggregations of large pyroninophilic lymphoid cells, plasma cells and small lymphocytes. Occasionally large germinal centres showed evidence of cells in mitosis and what appeared to be nuclear breakdown products. The numbers of germinal centres increased with age and reached a peak at 200-300 days (at which time these animals were beginning to give positive Coombs tests and show signs of anaemia). The atrophied thymuses of older mice often appeared to contain very active germinal centres and little else. The spleens of most mice eventually showed evidence of increased haemopoietic activity in the form of hypertrophy of the red pulp, and the gross splenomegaly found in a few animals (relative spleen weight greater than 300 mg. per 10 g. body weight) was directly attributable to this process. Pyroninophilic cell infiltration, and occasional localisation to form structures similar to germinal centres, were also found around blood vessels of the liver, lungs and kidneys of all NZB mice greater than 300 days of age.

Incidence of tumours: Complete post mortem examinations were carried out on 22 male and 30 female NZB mice, particular attention being paid to signs of malignant change. These animals were killed when moribund or died naturally and were found in good condition. In this group 9 tumours were found among the females and none among
the males. Of these tumours 2 were confined to ovarian tissue, 3 involved lymphoid tissues of the abdominal region and were associated with ascitic fluid, 2 were widely disseminated and involved lymphoid organs, lungs and liver, and 2 were confined to the thymus. All the mice involved were over 300 days of age and had enlarged spleens. Except for the two thymomas, thymus tissue was not involved.

These data would suggest a figure of around 33 per cent for the number of female NZB mice which show gross evidence of malignant change, and an absence of such change among NZB males. In fact a few NZB male mice were found which developed obvious signs of malignant change, but since the other members of these groups were not thoroughly examined to exclude the presence of tumours it is not possible to assign a meaningful figure to the incidence of such change in males. It appears justified, however, to state that the incidence is much lower than among females.
Figure 7. The survival times of 16 NZB female (●●), 16 NZB male (○○), 16 (NZB x T6)F1 hybrid female (▲▲), and 21 (NZB x T6)F1 hybrid male (■■) mice. The number of mice alive in each group at regular intervals is expressed as a percentage of those initially present.
Figure 8. The relationship between reticulocyte counts and the incidence of positive direct Coombs tests among 16 untreated NZB female mice bled at monthly intervals. The mean percentage of reticulocytes + range is given, together with the number of mice (expressed as a percentage) which had a positive test at that time.
Figure 9. The relationship between reticulocyte counts and the incidence of positive direct Coombs tests among 16 untreated NZB male mice bled at monthly intervals. The mean percentage of reticulocytes + range is given, together with the number of mice (expressed as a percentage) which had a positive test at that time.
Figure 10. The relationship between PCV values and the incidence of positive direct Coombs tests among 16 untreated NZB female mice bled at monthly intervals. The mean PCV value + range is given, together with the number of mice (expressed as a percentage) which had a positive test at that time.
Figure 11. The relationship between PCV values and the incidence of positive direct Coombs tests among 16 untreated NZB male mice bled at monthly intervals. The mean PCV value + range is given, together with the number of mice (expressed as a percentage) which had a positive test at that time.
Figure 12. The mean PCV values and reticulocyte counts + ranges of untreated (NZB x T6)F1 hybrid male and female mice bled at 2 monthly intervals.
**Table 2**

**The Mean Age at Death for Various Groups of Normal NZB Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>No. in group</th>
<th>Mean age at death (days) (± 1 S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discarded breeders</td>
<td>Female</td>
<td>23</td>
<td>461.3 (± 80.1)</td>
</tr>
<tr>
<td>Discarded breeders</td>
<td>Male</td>
<td>28</td>
<td>510.5 (± 151.4)</td>
</tr>
<tr>
<td>Non-breeders</td>
<td>Female</td>
<td>44</td>
<td>540.8 (± 117.6)</td>
</tr>
<tr>
<td>Non-breeders</td>
<td>Male</td>
<td>61</td>
<td>518.6 (± 138.2)</td>
</tr>
<tr>
<td>Discarded breeders</td>
<td>Male</td>
<td>51</td>
<td>488.3 (± 126.8)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-breeders</td>
<td>Male</td>
<td>105</td>
<td>527.9 (± 130.4)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All females</td>
<td>-</td>
<td>67</td>
<td>513.5 (± 98.8)</td>
</tr>
<tr>
<td>All males</td>
<td>-</td>
<td>89</td>
<td>516.0 (± 144.8)</td>
</tr>
<tr>
<td>All NZB mice</td>
<td>Male</td>
<td>156</td>
<td>515.0 (± 130.6)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 13. The relative spleen weights of untreated NZB mice.
Figure 14. The relative spleen weights of various "normal" mouse strains (NZB x T6)F1, C57Bl, CBA, C3H and (C57Bl x CBA)F1.
Figure 25. The relative lymph node weights of untreated NZB mice.
Figure 16. The relative lymph node weights of various "normal" mouse strains (NZB x T6)F1, C57BL, CBA, C3H and (C57BL x CBA)F1.
Figure 17. The relative liver weights of untreated NZB mice.
Figure 18. The relative liver weights of various "normal" mouse strains (\((NZB \times T6)F1, C37Bl, CBA, C3H and (C57Bl \times CBA)F1\)).
Figure 19. The relative thymus weights of untreated NZB mice.
Figure 20. The relative thymus weights of various "normal" mouse strains (NZB x T6)F1, C57Bl, CBA, C3H and (C57Bl x CBA)F1.
2. PAPAIN-TREATED RED CELL AGGLUTINATING ACTIVITY IN
THE SERUM OF UNTREATED NZB MICE

Occurrence: Seventy-two NZB mice of known Coombs test state were
bled and the sera were tested for activity against papain-treated
CBA red cells. The incidence of such activity in individual mice
is plotted against age in Figure 21 and it can be seen that mice
younger than 300 days had low levels of antibody (titre < 1/2) while
animals greater than this age tended to give positive reactions
although there was wide variation between the titres for individual
mice. The mean age of animals with titres less than 1/2 was 263.2
(± 136.1) days and of animals with titres greater than 1/2 was 505.5
(± 139.6) days. No sex difference was noted in the present study;
both male and female mice were encountered which had high titres
of antibody activity. As can be seen from Table 3, there was a
strong positive correlation between the presence of antibody against
papain-treated red cells and a positive direct Coombs test.

Antibody type: Eleven sera which gave high titres against papain-
treated red cells were fractionated on Sephadex G-200 and the dis-
tribution of activity in the resulting fractions was determined.
It is likely that none of the fractions contained only one type of
antibody; nevertheless the results which are expressed in Table 4
suggest that the activity found in the sera of NZB mice was due to
either 19S or 7S antibody, and in some animals both types were of
equal importance.
Figure 21. The relationship between age and the occurrence of papain-treated red cell agglutinating activity in NZB mice.
TABLE 3

THE RELATIONSHIP BETWEEN THE PRESENCE OF GLOBULIN ON THE RED CELLS (DC) AND THE OCCURRENCE OF PAPAIN-TREATED RED CELL ACTIVITY (PRCA) IN THE SERUM OF NZB MICE

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals in group</th>
<th>No. in group as a percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRCA negative DC negative</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>PRCA negative DC positive</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>PRCA positive DC negative</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>PRCA positive DC positive</td>
<td>37</td>
<td>51</td>
</tr>
<tr>
<td>No. of animals tested</td>
<td>72</td>
<td>100</td>
</tr>
</tbody>
</table>
## Table 4

The distribution of antibody activity against papain-treated red cells (PRCA antibody) in the serum of NZB mice fractionated on G-200 Sephadex

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>PRCA antibody titres in Sephadex fractions</th>
<th>Probable major antibody type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19S</td>
<td>10S</td>
</tr>
<tr>
<td>1</td>
<td>1/64</td>
<td>1/64</td>
</tr>
<tr>
<td>2</td>
<td>1/16</td>
<td>1/16</td>
</tr>
<tr>
<td>3</td>
<td>1/8</td>
<td>1/4</td>
</tr>
<tr>
<td>4</td>
<td>1/16</td>
<td>1/8</td>
</tr>
<tr>
<td>5</td>
<td>1/16</td>
<td>1/8</td>
</tr>
<tr>
<td>6</td>
<td>1/16</td>
<td>1/4</td>
</tr>
<tr>
<td>7</td>
<td>1/16</td>
<td>1/8</td>
</tr>
<tr>
<td>8</td>
<td>1/32</td>
<td>1/2</td>
</tr>
<tr>
<td>9</td>
<td>1/16</td>
<td>1/4</td>
</tr>
<tr>
<td>10</td>
<td>1/8</td>
<td>1/4</td>
</tr>
<tr>
<td>11</td>
<td>1/4</td>
<td>1/16</td>
</tr>
</tbody>
</table>
2. COMPLEMENT FIXING ACTIVITY AGAINST LIVER HOMOGENATE IN THE SERUM OF UNTREATED NZB MICE

The sera from 70 NZB, 10 CBA, 10 C57Bl, and 10 DBA/2 mice and 18 Wistar rats were tested for the presence of activity against isogeneic liver homogenate using a complement fixation test. The results are recorded in Table 5, which shows that the sera from a large proportion of NZB mice fix complement to a high titre in the presence of isogeneic liver antigens.

The relationship between age and the presence of complement fixing anti-liver activity in NZB mice is shown in Figure 22. It is evident that the lowest titres occurred in older mice. No such tendency was found among the control animals, although they had an age distribution comparable to that of the NZB mice.

In Table 6 the NZB animals have been grouped according to the presence or absence of complement fixing activity in their sera, and of globulin on their erythrocytes. There is no apparent correlation of these two parameters. Moreover, some mice which gave strongly positive Coombs tests were found to be negative in the complement fixation test and the converse was also occasionally found.

Six sera with positive complement fixing activity - three from Coombs positive animals and three from Coombs negative - were fractionated by zonal ultracentrifugation in sucrose density gradients*. The fractions obtained were tested in the complement fixation test

* Kindly performed by Dr. D.M. Weir.
against NZB liver homogenate. Activity was recovered only in those fractions which would be expected to contain the high molecular weight (19S) immunoglobulin.
TABLE 5

THE COMPLEMENT FIXATION TEST TITRES OF SERA FROM VARIOUS
STRAINS OF INBRED MICE AND WISTAR RATS TESTED AGAINST
ISOGENSEIC LIVER HOMOGENATES

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Number tested</th>
<th>Titres in complement fixation test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>less than 1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Mouse</td>
<td>CBA</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C57Bl</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>DBA/2</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>NZB</td>
<td>70</td>
<td>16</td>
</tr>
<tr>
<td>Rat</td>
<td>Wistar</td>
<td>18</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 22. The relationship between age and the occurrence of complement fixing anti-liver activity in NZB mice.
### TABLE 6

THE RELATIONSHIP BETWEEN THE OCCURRENCE OF COMPLEMENT FIXING ANTI-LIVER ACTIVITY AND THE DIRECT COOMBS TEST IN NZB MICE

<table>
<thead>
<tr>
<th>Result of complement fixation test</th>
<th>Result of Coombs test</th>
<th>Number in group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>6</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>32</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>11</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>18</td>
</tr>
</tbody>
</table>
4. THE TRANSFER OF LYMPHOID CELLS FROM OLD (COOMBS POSITIVE) NZB MICE TO YOUNG (COOMBS NEGATIVE) NZB MICE

Twelve NZB female mice which were over one year old and had had a strongly positive Coombs test for the preceding eight weeks were selected. Six of these animals underwent thoracic duct cannulation and the issuing lymph was collected overnight (18 hours). These animals and the other six were then killed by cervical dislocation and pooled cell suspensions were prepared from their spleens, lymph nodes and bone marrow. Each of the four cell suspensions was washed twice and re-suspended in Hanks solution. Cell counts were performed and the concentration was adjusted to $150 \times 10^6$ viable cells per ml. in each case. Unfortunately the yield of thoracic duct lymph cells was very low so a further dilution was made in this case to a final concentration of $15 \times 10^6$ viable cells per ml.

Twenty-nine NZB female mice, between 120 and 131 days of age and Coombs negative the day the experiment began, were randomly divided into five groups. The members of each group were injected i.p. with either 1 ml. or 0.5 ml. of one of the above cell suspensions. The control group received Hanks solution only. These recipient animals were Coombs tested weekly thereafter for twenty weeks and the results of the tests are recorded in Table 7.

By the tenth week (when the recipients were between 190 and 201 days old) all six mice which were given spleen cells had given a positive Coombs test result on at least three occasions. None of the animals in the other groups became positive until after the
tenth week, by which time a small proportion of untreated mice would be expected to be becoming positive spontaneously. The duration of the positive Coombs state induced in the spleen cell recipients varied between animals but all subsequently returned to a negative state.

The animals in this experiment (apart from the four which died accidentally) were Coombs tested occasionally after cessation of weekly testing and all subsequently became Coombs positive. The lifespan and incidence of positive Coombs tests found in each group did not differ significantly from those of untreated female NZB mice (as recorded in section 1).

These results indicate that spleen cells from old Coombs positive NZB mice can induce the formation of anti-red cell antibodies when transferred to young isogenic mice. Comparable numbers of bone marrow or lymph node cells do not have this ability, nor do smaller numbers of thoracic duct cells.
<table>
<thead>
<tr>
<th>Mouse number</th>
<th>Source of cells</th>
<th>Volume injected (ml.)</th>
<th>No. of cells injected ( (\times 10^6) )</th>
<th>Results of weekly Coombs tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SPLEEN</td>
<td>1.0</td>
<td>150</td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>LYPH NODES</td>
<td>0.5</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7</td>
<td></td>
<td></td>
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<tr>
<td>8</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>BONE MARROW</td>
<td>0.5</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>12</td>
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<td>13</td>
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<tr>
<td>14</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>THORACIC DUCT</td>
<td>1.0</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>0.6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
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<tr>
<td>23</td>
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<td>24</td>
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<tr>
<td>26</td>
<td></td>
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<tr>
<td>27</td>
<td></td>
<td></td>
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<tr>
<td>28</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results of direct Coombs tests performed at weekly intervals on groups of young NZB mice given spleen, lymph node, bone marrow or thoracic duct cells from old Coombs positive NZB mice. ("+" indicates positive D.C.T., "-" indicates negative D.C.T., "x" indicates died accidentally).
5. THE TRANSFER OF SPLEEN CELLS FROM NZB MICE TO (NZB x T6)F1 HYBRID MICE

In the previous section evidence was obtained that spleen cells from Coombs positive NZB mice transferred to Coombs negative NZB mice can induce Coombs test conversion in the recipients. The three experiments to be described in this section demonstrate: (i) that a similar Coombs test conversion occurs when spleen cells from positive NZB mice are injected intraperitoneally into (NZB x T6)F1 hybrids, and (ii) that this result is not due to a GvH response of immunologically competent NZB lymphoid cells against the transplantation antigens of the other parental strain.

**Experiment 1.** The purpose of this experiment was to study the occurrence of positive Coombs tests in (NZB x T6)F1 hybrids injected I.V. with $100 \times 10^6$ viable spleen cells from NZB +ve, NZB -ve or T6 donors. Unfortunately, the injection of a spleen cell suspension from NZB +ve donors was found to have an immediate toxic effect so that part of this objective was frustrated.

The recipients were 40 female hybrids between six and seven months of age and weighed 21-30 gm. at the start of the experiment. They were assigned randomly to four groups of ten and each was accurately weighed.

Eight NZB females over one year of age which had given a strongly positive Coombs reaction when tested 14 and 28 days previously were used as the positive donors. Thirteen NZB female mice under 200 days of age with a negative Coombs test on the day the experiment began were used as the negative donors, and the T6
donors were twelve females weighing 22-25 gm.

Cell suspensions were prepared from the pooled spleens of each group, washed twice in Dulbecco solution and re-suspended to a concentration of $200 \times 10^6$ viable cells per ml. The intravenous injection of 0.5 ml. of NZB -ve cell suspension to each of ten recipients was accomplished without mishap, but the first three recipients of the +ve cell suspension went into convulsions and died within minutes of receiving the injection. Further washing of this cell suspension did not reduce its toxicity on intravenous injection but did result in a marked loss of viability of the cells, so this part of the experiment had to be abandoned.

The injection of the remaining animals with T6 cells and Dulbecco solution only was accomplished uneventfully.

The three resulting groups were used to compare the severity of the reaction induced in (NZB x T6)F1 hybrids by the injection of NZB or T6 cells and to study the incidence of positive Coombs tests in such animals. Ten days after the injection of cells, randomly selected animals in each group were weighed and killed, and their spleen weights were accurately determined. This procedure was repeated on day 21, and again on day 65 when all surviving animals were killed. At weekly intervals after injection all animals available for test were weighed and Coombs tested.

As can be seen from Table 8, which records the mortality in each group, the injection of NZB spleen cells appears to produce a more severe reaction than the injection of CBA cells. The recipients of NZB spleen cells began to lose weight about 10 days
after injection and died around the 21st day with classical symptoms of GVH induced wasting disease. The hybrids given T6 cells, however, did not lose weight or show any external signs of GVH reaction. The relative spleen weights of the animals in each group killed 10, 21 and 65 days after injection are recorded in Table 9. The injection of both types of cell was associated with a significant degree of splenomegaly, but there was no significant difference between the mean relative spleen weights at days 10 and 21. By day 65 the spleen weights of the recipients of T6 cells were not different from those of the control group, and the NZB cell recipients were all dead. No animal in any group gave a positive Coombs test.

Experiment 2. It was found that intraperitoneal injection of spleen cell suspensions from NZB Coombs positive donors avoided the toxic effect associated with intravenous injection which was demonstrated in Experiment 1. This experiment was therefore repeated using the intraperitoneal route for all the cell suspensions.

The recipients were forty male (NZB x T6)F1 hybrids weighing 30-35 gm. and aged 7 to 8 months. They were assigned at random into five groups and accurately weighed. Four Coombs +ve NZB mice, seven Coombs -ve NZB mice and eight T6 mice for use as spleen cell donors were selected as in the previous experiment — except that in this case they were males.

Cell suspensions were prepared from the pooled spleens of each of the donor groups, the concentration of cells was adjusted
to $180 \times 10^6$ viable cells per ml., and groups of F1 hybrid recipients were injected intraperitoneally with the various suspensions as indicated in Table 10. Twelve days after injection three animals from each group (selected at random) were weighed and killed. The weights of their spleens, livers, lymph nodes and thymuses were determined and used to calculate the respective relative organ weights. The remaining animals in each group were Coombs tested weekly thereafter until death.

Table 10 records the mean relative organ weights for each group of animals killed twelve days after injection. The recipients of the NZB cells (both Coombs +ve and -ve) showed a significant enlargement of spleen, lymph nodes, and liver and a significant reduction in thymus size when compared with the corresponding values for the control animals of groups 4 and 5. The mean relative spleen weight of the group given Coombs +ve cells, however, was also significantly greater than that of the group given Coombs -ve cells.

All the animals injected with NZB cells (other than those killed on day 12) died from a form of wasting disease; but, in terms of early mortality, the induced disease was more severe among the recipients of Coombs -ve cells (Table 11).

The recipients of spleen cells from Coombs +ve NZB donors all subsequently gave a positive Coombs test result by 14 or 21 days after transfer, although all were negative when tested on day 7. The results of Coombs tests performed weekly for the first six weeks after transfer are recorded in Table 12. Only animals which had received NZB +ve cells converted to a positive Coombs state.
Of the two long term survivors in this group, one subsequently reverted to a negative state 7 weeks after transfer and the other at 20 weeks.

No positive Coombs test results were recorded among the animals given T6 spleen cells, nor was there any evidence of a GVH reaction when these animals were compared with controls in terms of mortality or relative organ weights.

Experiment 3. This experiment was undertaken in order to ascertain whether it was possible to transfer spleen cells from Coombs +ve NZB mice to (NZB x T6)F1 hybrids and obtain Coombs test conversion without evidence of a GVH reaction.

Thirty-eight F1 hybrids between 6 and 8 months of age and weighing 28-34 gm. at the start of the experiment were assigned at random into three groups. The members of each group were given 25 x 10^6, 50 x 10^6, or 75 x 10^6 viable spleen cells in a volume of 0.5 ml. intraperitoneally, or 0.5 ml. of Dulbecco solution alone. In one group the spleen cells were obtained from 5 strongly Coombs +ve NZB males over one year old, and in the other group they were obtained from six Coombs -ve NZB males less than 180 days old. Twelve days after cell transfer every animal was Coombs tested, weighed and then killed. The weights of their spleens, livers, lymph nodes and thymuses were determined and used to calculate the respective relative organ weights.

As can be seen from Table 13, positive Coombs tests occurred among F1 hybrid mice given NZB +ve spleen cells at each of the three doses, but no positive tests occurred in animals given NZB -ve cells
or Dulbecco solution alone. It can also be seen from Table 13 that a dose of $25 \times 10^6$ spleen cells from NZB -ve animals produced significant enlargement of the spleen, lymph nodes and liver, but a similar dose of spleen cells from Coombs +ve mice produced only splenomegaly. Increased doses of NZB +ve cells are associated with increased enlargement of spleen, lymph nodes and liver, and with hypertrophy of the thymus: this is not true for increased doses of NZB -ve cells. It is interesting to note that a dose of $75 \times 10^6$ spleen cells from NZB +ve mice produced a greater degree of splenomegaly than $75 \times 10^6$ cells from NZB -ve mice, and that the degree of hepatomegaly produced by each dose of NZB -ve cells was greater than that produced by any of the doses of NZB +ve cells.
### Table 8

**The Numbers of (NZB x CBA-T6)F1 Hybrid Mice Surviving**

*At 10, 21 and 65 days after the intravenous injection of 100 x 10^6 NZB or CBA-T6 spleen cells*

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. surviving at day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>100 x 10^6 NZB cells I.V.</td>
<td>10/10</td>
</tr>
<tr>
<td>2</td>
<td>100 x 10^6 CBA-T6 cells I.V.</td>
<td>13/13</td>
</tr>
<tr>
<td>3</td>
<td>0.5 ml. Dulbecco solution I.V.</td>
<td>12/12</td>
</tr>
</tbody>
</table>
TABLE 9

THE RELATIVE SPLEEN WEIGHTS OF (NZB x CBA-T6)F1 HYBRID MICE
10, 21 AND 65 DAYS AFTER THE INTRAVENOUS INJECTION OF
100 x 10^6 NZB OR CBA-T6 SPLEEN CELLS

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Relative spleen weights at day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>100 x 10^6 NZB cells I.V.</td>
<td>47.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56.9</td>
</tr>
<tr>
<td>2</td>
<td>100 x 10^6 CBA-T6 cells I.V.</td>
<td>54.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77.1</td>
</tr>
<tr>
<td>3</td>
<td>0.5 ml. Dulbecco solution I.V.</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41.7</td>
</tr>
</tbody>
</table>

Statistical evaluation of group means

1 versus 3. Day 10 p = < 0.01, day 21 p = < 0.01.
2 versus 3. Day 10 p = < 0.01, day 21 p = < 0.01,
             day 65 = not significant.
1 versus 2. Day 10 p = N.S., day 21 p = N.S.
# Table 10

The mean relative organ weights of groups of (NZB x CBA-T6)F1 hybrid mice killed 12 days after the intraperitoneal injection of \(90 \times 10^6\) spleen cells from NZB +ve, NZB -ve, or CBA-T6 mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of cells injected</th>
<th>No. in group</th>
<th>Mean relative organ weights</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>1</td>
<td>Injected I.P. with NZB +ve spleen cells</td>
<td>(90 \times 10^6)</td>
<td>3</td>
<td>97.4</td>
</tr>
<tr>
<td>2</td>
<td>Injected I.P. with NZB -ve spleen cells</td>
<td>(90 \times 10^6)</td>
<td>3</td>
<td>53.1</td>
</tr>
<tr>
<td>3</td>
<td>Injected I.P. with CBA-T6 spleen cells</td>
<td>(90 \times 10^6)</td>
<td>3</td>
<td>29.7</td>
</tr>
<tr>
<td>4</td>
<td>Injected I.P. with 0.5 ml. Dulbecco solution</td>
<td>0</td>
<td>3</td>
<td>26.5</td>
</tr>
<tr>
<td>5</td>
<td>No treatment</td>
<td>0</td>
<td>3</td>
<td>24.0</td>
</tr>
</tbody>
</table>

**Statistical evaluation of group means**

4 versus 5: All organs = not significant

1 versus 4 + 5: All organs \(p = <0.05\)

2 versus 4 + 5: All organs \(p = <0.05\)

3 versus 4 + 5: All organs = not significant

1 versus 2: Spleen \(p = <0.05\), other organs = not significant
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of cells injected</th>
<th>No. in group</th>
<th>Number surviving at day:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Injected I.P. with NZB +ve spleen cells</td>
<td>$90 \times 10^6$</td>
<td>5</td>
<td>4 2 2 2 1 0</td>
</tr>
<tr>
<td>2</td>
<td>Injected I.P. with NZB -ve spleen cells</td>
<td>$90 \times 10^6$</td>
<td>7</td>
<td>3 0 0 0 0 0</td>
</tr>
<tr>
<td>3</td>
<td>Injected I.P. with CBA-T6 spleen cells</td>
<td>$90 \times 10^6$</td>
<td>4</td>
<td>4 4 4 4 4 4</td>
</tr>
<tr>
<td>4</td>
<td>Injected I.P. with Dulbecco, or untreated</td>
<td>0</td>
<td>9</td>
<td>9 9 9 9 9 8</td>
</tr>
</tbody>
</table>
TABLE 12

THE RESULTS OF DIRECT COOMBS TESTS PERFORMED AT WEEKLY INTERVALS ON GROUPS OF (NZB x CBA-T6)F1 HYBRIDS FOR THE FIRST SIX WEEKS AFTER THE INJECTION OF 90 x 10^6 SPLEEN CELLS FROM NZB +VE, NZB -VE, OR CBA-T6 MICE

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of cells injected</th>
<th>Mouse number</th>
<th>Results of weekly Coombs tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 2 3 4 5 6</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Injected I.P. with NZB +ve spleen cells</td>
<td>90 x 10^6</td>
<td>1 - + + + D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 - + + + + +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 - + + + + +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 - + D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 - + + D</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Injected I.P. with NZB -ve spleen cells</td>
<td>90 x 10^6</td>
<td>6 - -  D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 - -  D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 - -  D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9 - -  D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 - -  D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11 - D</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12 - -  D</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Injected I.P. with CBA-T6 spleen cells</td>
<td>90 x 10^6</td>
<td>13 - - - - -</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 - - - - -</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 - - - - -</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16 - - - - -</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Injected I.P. with 0.5 ml. and Dulbecco solution, or untreated</td>
<td>0</td>
<td>17 - - - - -</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>18 - - - - -</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19 - - - - -</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 - - - - -</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>21 - - - - -</td>
<td></td>
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<td>22 - - - - -</td>
<td></td>
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<td></td>
<td>23 - - - - -</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>24 - - - - -</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25 - - - - -</td>
<td></td>
</tr>
</tbody>
</table>

("+" = positive Coombs test; "-" indicates -ve Coombs test; D = animal dead)
TABLE 13

THE MEAN RELATIVE ORGAN WEIGHTS AND RESULTS OF INDIVIDUAL COOMBS TESTS OF GROUPS OF (NZB x CBA-T6)F1 HYBRID MICE KILLED 12 DAYS AFTER THE INTRAPERITONEAL INJECTION OF SPLEEN CELLS FROM NZB +VE OR NZB -VE MICE

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of cells injected x 10^6</th>
<th>No. in group</th>
<th>No. with +ve Coombs test</th>
<th>Mean relative organ weights</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>1a</td>
<td>Injected I.P.</td>
<td>25</td>
<td>5</td>
<td>3</td>
<td>59.1</td>
</tr>
<tr>
<td>1b</td>
<td>with NZB +ve</td>
<td>50</td>
<td>5</td>
<td>2</td>
<td>67.6</td>
</tr>
<tr>
<td>1c</td>
<td>spleen cells</td>
<td>75</td>
<td>5</td>
<td>4</td>
<td>92.1</td>
</tr>
<tr>
<td>2a</td>
<td>Injected I.P.</td>
<td>25</td>
<td>5</td>
<td>0</td>
<td>54.8</td>
</tr>
<tr>
<td>2b</td>
<td>with NZB -ve</td>
<td>50</td>
<td>4</td>
<td>0</td>
<td>45.4</td>
</tr>
<tr>
<td>2c</td>
<td>spleen cells</td>
<td>75</td>
<td>3</td>
<td>0</td>
<td>41.6</td>
</tr>
<tr>
<td>3</td>
<td>Injected I.P.</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>27.3</td>
</tr>
</tbody>
</table>

Statistical evaluation of selected group means

1a versus 3 Spleen p = <0.01, other organs = not significant
2a versus 3 Spleen, liver and lymph nodes p = <0.01, thymus = N.S.
1a versus 2a Liver and lymph nodes p = <0.01, spleen + thymus = N.S.
6. THE ACTIVITY OF ANTI-MOUSE LYMPHOCYTE ANTISERUM (ALS)

Table 14 contains details of 17 antisera raised in rabbits against mouse lymphocytes (mainly thymus cells), and the results of the four assays performed on each serum.

In terms of ability to prolong homograft survival, potent ALS was regularly produced by rabbits immunised on a three-injection schedule. Immunosuppressive ALS was also produced occasionally by animals injected only twice (with or without previous *C. parvum* treatment), but since other animals in these groups produced poor antiserum it is not possible to conclude that a two pulse schedule produces superior ALS. It is, however, true that animals given more than two injections of lymphoid cells regularly had higher titres of antibodies directed against mouse red cells.

The results of similar assay procedures performed on the 4 horse anti-mouse thymus cell sera, and on 3 batches of normal serum are to be found in Tables 15 and 16 respectively.

Figures 23-28 illustrate the relationships which were found between the results of the four assay procedures used. The ability to produce lymphopenia was more closely related to graft prolongation than was the titre of cytotoxic antibodies or the ability to prevent GVH reaction, although the correlation coefficients of these relationships were not large. There was, however, a very marked relationship between the ability to prevent GVH reaction and the level of cytotoxic antibodies. Perhaps not surprisingly, the next highest degree of correlation was found between the cytotoxic titre and ability to produce lymphopenia *in vivo*.
### TABLE 1A

**THE IN VIVO AND IN VITRO PROPERTIES OF VARIOUS RABBIT ANTI-MOUSE LYMPHOCYTE SERA**

<table>
<thead>
<tr>
<th>Serum number</th>
<th>Immunisation schedule</th>
<th>Reciprocal agglutinin titre against mouse red cells (before absorption)</th>
<th>Reciprocal cytotoxic titre against mouse spleen cells</th>
<th>Mean graft survival times (days)</th>
<th>Effect on GIVH reaction</th>
<th>Ability to produce lymphopoenia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>8</td>
<td>2048</td>
<td>20.0</td>
<td>12.0</td>
<td>Not done</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>32</td>
<td>256</td>
<td>13.9</td>
<td>14.0</td>
<td>73.9</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td>32</td>
<td>256</td>
<td>15.8</td>
<td>6.1</td>
<td>60.2</td>
</tr>
<tr>
<td>4</td>
<td>I</td>
<td>64</td>
<td>1024</td>
<td>13.4</td>
<td>11.5</td>
<td>63.6</td>
</tr>
<tr>
<td>5</td>
<td>II</td>
<td>128</td>
<td>1024</td>
<td>22.0</td>
<td>13.9</td>
<td>31.4</td>
</tr>
<tr>
<td>6</td>
<td>II</td>
<td>64</td>
<td>128</td>
<td>20.3</td>
<td>9.8</td>
<td>70.1</td>
</tr>
<tr>
<td>7</td>
<td>II</td>
<td>256</td>
<td>1024</td>
<td>17.1</td>
<td>7.1</td>
<td>55.8</td>
</tr>
<tr>
<td>8</td>
<td>II</td>
<td>32</td>
<td>1024</td>
<td>20.8</td>
<td>71.1</td>
<td>49.2</td>
</tr>
<tr>
<td>9</td>
<td>III</td>
<td>32</td>
<td>256</td>
<td>15.8</td>
<td>20.3</td>
<td>41.9</td>
</tr>
<tr>
<td>10</td>
<td>III</td>
<td>32</td>
<td>1024</td>
<td>29.1</td>
<td>0.0</td>
<td>35.0</td>
</tr>
<tr>
<td>11</td>
<td>III</td>
<td>32</td>
<td>512</td>
<td>22.6</td>
<td>21.2</td>
<td>90.8</td>
</tr>
<tr>
<td>12</td>
<td>III</td>
<td>32</td>
<td>128</td>
<td>13.4</td>
<td>28.0</td>
<td>62.6</td>
</tr>
<tr>
<td>13</td>
<td>III</td>
<td>8</td>
<td>256</td>
<td>12.6</td>
<td>0.0</td>
<td>89.5</td>
</tr>
<tr>
<td>14</td>
<td>III</td>
<td>16</td>
<td>128</td>
<td>11.3</td>
<td>13.3</td>
<td>81.9</td>
</tr>
<tr>
<td>15</td>
<td>III</td>
<td>32</td>
<td>512</td>
<td>13.3</td>
<td>11.5</td>
<td>59.4</td>
</tr>
<tr>
<td>16</td>
<td>IV</td>
<td>64</td>
<td>64</td>
<td>11.7</td>
<td>104.9</td>
<td>92.4</td>
</tr>
<tr>
<td>17</td>
<td>V</td>
<td>256</td>
<td>512</td>
<td>14.4</td>
<td>8.9</td>
<td>35.3</td>
</tr>
</tbody>
</table>

**Immunisation schedules.**

- **I** = $10^9$ thymus cells day 0, $10^9$ thymus cells day 14, exsanguination day 21.
- **II** = $10^9$ thymus cells day 0, $10^9$ thymus cells day 21, $10^9$ thymus cells day 28, exsanguination day 42.
- **III** = Corynebacterium parvum treatment day -6, $10^9$ thymus cells day 0, $10^9$ thymus cells day 14, exsanguination day 21.
- **IV** = Weekly injection of $5 \times 10^8$ lymph node cells for 11 weeks, exsanguination week 12.
- **V** = Weekly injection of $7 \times 10^8$ thymus cells for 5 weeks, exsanguination week 6.
### Table 15

**The In Vivo and In Vitro Properties of Four Horse Anti-Mouse Lymphocyte Sera Provided by the Wellcome Foundation**

<table>
<thead>
<tr>
<th>Serum number</th>
<th>Wellcome Foundation batch no.</th>
<th>Reciprocal agglutinin titre against mouse red cells (before absorption)</th>
<th>Reciprocal cytotoxic titre against mouse spleen cells</th>
<th>Mean graft survival times (days)</th>
<th>Effect on GVH reaction</th>
<th>Ability to produce lymphopenia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>6768/1/5</td>
<td>128</td>
<td>128</td>
<td>19.4</td>
<td>90.5</td>
<td>54.3</td>
</tr>
<tr>
<td>42</td>
<td>6768/9/1</td>
<td>256</td>
<td>1024</td>
<td>15.2</td>
<td>2.8</td>
<td>75.6</td>
</tr>
<tr>
<td>43</td>
<td>7273/10/9</td>
<td>32</td>
<td>1024</td>
<td>14.0</td>
<td>0.0</td>
<td>52.0</td>
</tr>
<tr>
<td>44</td>
<td>7273/8/9</td>
<td>64</td>
<td>256</td>
<td>15.1</td>
<td>N.D.</td>
<td>87.3</td>
</tr>
</tbody>
</table>
**TABLE 16**

**THE IN VIVO AND IN VITRO PROPERTIES OF NORMAL RABBIT SERUM (NRS) AND NORMAL HORSE SERUM (NHS) WHEN TESTED IN THE SAME STANDARD ASSAYS USED FOR ANTILYMPHOCYTE SERA**

<table>
<thead>
<tr>
<th>Serum number</th>
<th>Reciprocal agglutinin titre against mouse red cells (before absorption)</th>
<th>Reciprocal cytotoxic titre against mouse spleen cells</th>
<th>Mean graft survival times (days)</th>
<th>Effect on GVH reaction</th>
<th>Ability to produce lymphopenia</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRS 1</td>
<td>4</td>
<td>8</td>
<td>13.8</td>
<td>109.9</td>
<td>92.3</td>
</tr>
<tr>
<td>NRS 2</td>
<td>2</td>
<td>4</td>
<td>11.9</td>
<td>100.4</td>
<td>68.3</td>
</tr>
<tr>
<td>NHS 1</td>
<td>8</td>
<td>64</td>
<td>13.3</td>
<td>101.2</td>
<td>111.8</td>
</tr>
</tbody>
</table>
Figure 23. The relationship between ability of ALS to produce lymphopenia and ability to prolong homograft survival.

\( r = 0.483 \)
The relationship between cytotoxic titre of ALS and ability to prolong homograft survival.

\( (r = 0.391) \)
Figure 25. The relationship between ability of ALS to suppress GVH reaction and ability to prolong homograft survival. 

\( r = 0.146 \)
Figure 26. The relationship between cytotoxic titre of ALS and ability to suppress GVH reaction.

\( r = -0.835 \)
Figure 27. The relationship between ability of ALS to produce lymphopoenia and ability to suppress GVH reaction.

\[ r = 0.293 \]
Figure 26. The relationship between cytotoxic titre of ALS and ability to produce lymphopenia.

\( r = -0.491 \)
7. THE EFFECT OF ALS ON THE SPONTANEOUS DISEASE OF NZB MICE AND ON THE INDUCED DISEASE IN (NZB x CBA)F1 HYBRID MICE

ALS of known immunosuppressive potency was used to treat NZB mice at two different stages in the development of the auto-immune haemolytic anaemia which characterises this strain. The effect of giving ALS to (NZB x CBA)F1 hybrids shortly before transferring sufficient spleen cells from old Coombs positive donors to induce Coombs test conversion was also studied.

Experiment 1. Twenty-eight NZB mice (14 males and 14 females) were divided into two groups which were matched on the basis of sex, and as closely as possible for weight and age. The animals were Coombs negative on the day the experiment began and were between 187 and 268 days of age. Although NZB mice of this age would be expected to show early signs of haemolytic disease, the animals used were selected to exclude those with very high reticulocyte counts or low PCV levels. The members of one group were given 0.5 ml. ALS intraperitoneally twice weekly for 10 weeks and the others were given equivalent amounts of KRS. Four batches of ALS were used (Nos. 5, 6, 8 and 11 - see Table 14) and as one batch was exhausted another was begun. (Each of these batches had been shown to be immunosuppressive in the standard homograft and GvH assays already described). At intervals of 3 weeks individual blood samples were obtained for WBC count, direct Coombs test and PCV determination. Two blood smears were also prepared and subsequently stained for differential and reticulocyte counts.

Eighteen weeks after treatment was begun the regular sampling procedure ceased. The results obtained over this period are recorded
graphically in Figure 29. The mean lymphocyte count of the ALS-treated group was significantly lower than that of the NRS-treated group at 3 weeks, but this difference was not maintained thereafter. On any one occasion there was never more than one positive Coombs test among ALS-treated animals while the NRS-treated group contained at least 3 on each occasion and 7 at 12 weeks. This latter difference is statistically significant \((p < 0.025)\). Although the reticulocyte counts of ALS-treated animals tended to be lower than their NRS-treated counterparts, neither these differences nor the differences in PCV values were statistically significant.

After 18 weeks the incidence of positive Coombs tests in the two groups became virtually identical, and no significant differences in lymphocyte count, PCV or reticulocyte counts were found. All animals eventually developed positive Coombs tests and the other signs of haemolytic anaemia. The mean age at death of the ALS-treated group (405 days) did not differ significantly from that of the NRS-treated group (429 days).

Experiment 2. Thirty NZB mice (12 males and 18 females) were put into 3 groups (matched on the basis of sex, and as closely as possible for age and strength of Coombs test). They were all over 1 year of age and had had a ++ or stronger direct Coombs test for at least the preceding 6 weeks. Members of each group were given 0.25 ml. ALS, NRS or saline intraperitoneally every day for 6 weeks and every second day for a further 2 weeks. (The ALS batches used were Nos. 1 and 9, both of which had been shown to be immunosuppressive in the standard homograft and GvH assays previously described). At weekly intervals
individual blood samples were obtained and a direct Coombs test performed on each sample.

The results of these determinations are to be found in Table 17 where it can be seen that 6 animals in the ALS-treated group, compared with 3 receiving NRS and one receiving saline, gave a negative Coombs test on at least one occasion. (This difference between the ALS group and the saline group is significant - p = < 0.05). However, if the results are considered in terms of weakening in strength of the Coombs test (positive to negative; ++++ reaction to ++ or lower; or +++ to + or lower) then the ALS group contained 8 such animals, the NRS group 7 and the saline group 5. These differences are not significant.

Experiment 3. Twenty-four male (NZB x CBA)F1 hybrid mice aged between 6 and 8 weeks were separated at random into 3 groups. Members of the first two groups were given 0.6 ml. of ALS or NRS intraperitoneally and, 4 hours later, an injection of NZB spleen cells by the same route. This suspension was derived from the pooled spleens of 9 old NZB males known to have a positive Coombs test. Each recipient was given 50 x 10^6 viable spleen cells in a volume of 0.27 ml.

Animals in the third group were given either 0.6 ml. of ALS or 0.6 ml. of NRS followed 4 hours later by 0.27 ml. of saline. Subsequent tests revealed no significant differences between these animals so they will be further considered as a single group - the control group. On days 7, 11 and 14 after injection animals of each group were bled and direct Coombs tests, reticulocyte counts and FCV determinations were carried out on the samples obtained. On day 21 all the animals
were exsanguinated and, as well as the three tests already mentioned, the papain-treated red cell agglutinin (PRCA) titres of individual sera were determined. The ALS preparation used was No. 10 (see Table 114). The spleen cell donors were NZB males aged 14-18 months; they had a strongly positive direct Coombs test (++ or greater) on the day before the experiment began and had given such a result when tested 4 weeks previously.

As can be seen from Table 18, 7 out of 8 of the animals given NRS and spleen cells subsequently became Coombs positive while only 1 animal in the ALS-treated group became weakly positive. This difference is statistically significant (p = < 0.01). The animals in the NRS-treated group also had higher levels of activity against papain-treated mouse red cells. The control group gave no positive Coombs tests or any PRCA activity. Tables 19 and 20 record the mean values for FCV and reticulocyte counts in the three groups. The FCV values of the NRS-treated group were lower than those of the control group (p = < 0.01 at days 11 and 14). The FCV values of the ALS-treated group were also lower than the control group but they were higher than those of the NRS-treated group. The reticulocyte counts of ALS-treated and NRS-treated animals were much more variable than those of the control animals but a significant difference was only observed at day 21 and this was true for both groups.
Figure 29. The mean lymphocyte counts, incidence of positive direct Coombs tests, mean PCV values, and mean reticulocyte counts in NZB mice (aged 187-268 days at start) given 0.5 ml. ALS or NRS twice weekly for 10 weeks.
Table 17

The results of direct Coombs tests performed at weekly intervals on Coombs positive NZB mice (over 1 year of age) receiving daily I.P. injections of ALS, NRS or saline

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sex</th>
<th>Coombs test at start</th>
<th>Subsequent weekly Coombs tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>Male</td>
<td>++++</td>
<td>++ - ++ ++ ++ - - + + + + ++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td>+++ +++ +++ +++ + + D*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td>+++ +++ +++ + + + + + + + + +</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
<tr>
<td>NRS</td>
<td>Male</td>
<td>++++</td>
<td>++ + - + + + + + + + + + + + +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
<tr>
<td>Saline</td>
<td>Male</td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
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<td></td>
<td></td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td>+++ +++ +++ +++ +++ + + + + + +</td>
</tr>
</tbody>
</table>

* D = animal dead
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Results of Coombs tests (days after treatment)</th>
<th>PRCA activity (day 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>ALS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+ NZB spleen cells</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>NRS</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+ NZB spleen cells</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>ALS or NRS + saline</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The results of direct Coombs and papain-treated red cell agglutination tests in \((\text{NZB x CBA})_{\text{F1}}\) hybrid mice pre-treated with ALS or NRS and given \(50 \times 10^6\) viable spleen cells from Coombs positive NZB donors or saline.
TABLE 19

THE MEAN PCV VALUES OF GROUPS OF (NZB x CBA)F1 HYBRID MICE PRE-TREATED WITH ALS OR NRS AND GIVEN 50 x 10^6 VIABLE SPLEEN CELLS FROM COOMBS POSITIVE NZB DONORS OR SALINE

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Per cent PCV values (± 1 S.D.) (days after treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ALS + NZB spleen cells</td>
<td>45.7 (+2.7) 44.2 (+1.9) 44.7 (+1.7) 47.2 (+2.3)</td>
</tr>
<tr>
<td>2</td>
<td>NRS + NZB spleen cells</td>
<td>45.9 (+1.6) 40.0 (+5.1) 39.3 (+4.4) 46.6 (+1.2)</td>
</tr>
<tr>
<td>3</td>
<td>ALS or NRS + saline</td>
<td>46.4 (+1.7) 47.0 (+1.7) 46.7 (+1.5) 46.6 (+2.2)</td>
</tr>
</tbody>
</table>

Statistical evaluation of differences between means

<table>
<thead>
<tr>
<th>Day</th>
<th>7</th>
<th>11</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 versus 3</td>
<td>NS</td>
<td>p = &lt; 0.05</td>
<td>p = &lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>2 versus 3</td>
<td>NS</td>
<td>p = &lt; 0.01</td>
<td>p = &lt; 0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>
**TABLE 20**

**THE MEAN RETICULOCYTE COUNTS OF GROUPS OF (NZB x CBA)F1 HYBRID MICE PRE-TREATED WITH ALS OR NRS AND GIVEN 50 x 10⁶ VIABLE SPLEEN CELLS FROM COOMBS POSITIVE NZB DONORS OR SALINE**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Per cent reticulocytes (± 1 S.D.) (days after treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>1</td>
<td>ALS + NZB spleen cells</td>
<td>3.3 (+1.3)</td>
</tr>
<tr>
<td>2</td>
<td>NRS + NZB spleen cells</td>
<td>7.4 (+3.6)</td>
</tr>
<tr>
<td>3</td>
<td>ALS or NRS + saline</td>
<td>6.5 (+2.6)</td>
</tr>
</tbody>
</table>
8. THE EFFECT OF F(ab')2 FROM RABBIT ANTI-MOUSE LYMPHOCYTE IgG ON THE GRAFT-VERSUS-HOST REACTION IN (C57Bl x CBA)F1 HYBRID MICE

IgG antibody from which the Fc fragment has been removed by digestion with pepsin or papain retains its ability to combine with specific antigen, but loses a number of biological activities associated with the intact molecule. The properties lost on digestion include the ability to bind complement and initiate complement-dependent immune lysis, the ability to fix to skin and release pharmacologically active substances on reaction with antigen, and the ability to be transported across the placenta (Humphrey, 1967). ALS IgG which has been digested with pepsin to give F(ab')2 fragments no longer suppresses homograft rejection, inhibits humoral antibody formation, or exerts a cytotoxic effect in vitro (Anderson, James and Woodruff, 1967; Harris and Harris, 1966; James and Anderson, 1967; James, 1967; Ogburn, Harris and Harris, 1967), and it seems possible that the loss of these properties is associated with loss of the ability to fix complement and bring about the immune lysis of lymphocytes.

In the present work it has been shown that ALS treatment of the donor is very effective in suppressing the GVH reaction produced in (C57Bl x CBA)F1 hybrids by the injection of parental strain cells, and similar findings have been reported by others (Boek, Fox and Wilson, 1967; Brent, Courtenay and Cowland, 1967; Monaco et al., 1967). The batches of ALS used in the present study gave a very high correlation between ability to suppress GVH reaction and in vitro
cytotoxic titre \(r = 0.835\), and because the cytotoxic test is known to be complement dependent it seemed of interest to determine the effect of pepsin digestion on the ability of ALS to abolish the GVH reaction.

The IgG fraction of ALS No. 5 was obtained and digested with pepsin as described under Materials and Methods. The intact molecule had a cytotoxic titre of 1/1024 and this was reduced to 1/4 after digestion. The ability of the preparation to agglutinate mouse lymph node cells to a titre of 1/2048, however, was unaffected by this procedure. IgG and \(F(ab')_2\) fragments of NRS (which neither agglutinated mouse lymphoid cells nor exerted a cytotoxic effect) were also prepared and used to treat control animals.

The ability of these preparations to influence a GVH reaction was studied in a system similar to that previously described. C57Bl mice, weighing between 25 and 30 gms, were treated with 1 ml. of a 1 g. per cent preparation given over 4 days prior to preparation of spleen cell suspensions. Recipient \((C57Bl \times CBA)F1\) hybrids, weighing 30-35 gms, received \(100 \times 10^6\) viable spleen cells intravenously. Nine days later they were weighed accurately and killed, their relative spleen and liver weights were calculated and used to assess the degree of GVH reaction induced.

As can be seen from Table 21, ALS IgG treatment abolished the ability of C57Bl spleen cells to produce splenomegaly and hepatomegaly on injection into \((C57Bl \times CBA)F1\) hybrids, but the \(F(ab')_2\) fragments derived from this preparation did not retain this ability.
### Table 21

**The Mean Relative Spleen and Liver Weights of (C57Bl x CBA)F1 Hybrid Mice Nine Days After I.V. Injection of 100 x 10^6 Viable Spleen Cells from C57Bl Mice, or Saline**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. in group</th>
<th>Mean relative spleen weight (± 1 S.D.)</th>
<th>Mean relative liver weight (± 1 S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Cells from donors treated with ALS IgG</td>
<td>6</td>
<td>42.5 (± 7.7)</td>
<td>568.7 (±7.1)</td>
</tr>
<tr>
<td>2 Cells from donors treated with ALS F(ab')2</td>
<td>5</td>
<td>128.5 (±23.9)</td>
<td>752.4 (±55.4)</td>
</tr>
<tr>
<td>3 Cells from donors treated with NRS IgG</td>
<td>5</td>
<td>108.0 (±8.3)</td>
<td>698.6 (±54.6)</td>
</tr>
<tr>
<td>4 Cells from donors treated with NRS F(ab')2</td>
<td>5</td>
<td>120.3 (±8.1)</td>
<td>637.6 (±49.9)</td>
</tr>
<tr>
<td>5 Cells from donors treated with saline</td>
<td>5</td>
<td>120.9 (±7.3)</td>
<td>746.1 (±57.3)</td>
</tr>
<tr>
<td>6 Saline only</td>
<td>7</td>
<td>43.1 (±19.2)</td>
<td>585.7 (±31.9)</td>
</tr>
</tbody>
</table>

**Statistical Evaluation of Group Means**

1 versus 5 (spleen weight)  \( p = <0.01 \)
1 versus 5 (liver weight)  \( p = <0.01 \)
2 versus 5 (spleen weight)  \( p = N.S. \)
2 versus 5 (liver weight)  \( p = N.S. \)
2. THE EFFECT OF A SINGLE INJECTION OF ALS ON NUMBERS OF CIRCULATING LYMPHOCYTES IN 4 STRAINS OF MICE

Antilymphocyte serum IgG from which the Fc fragment has been removed by digestion has been shown to be no longer capable of suppressing homograft rejection, inhibiting humoral antibody formation, or exerting a cytotoxic effect \textit{in vitro} (Anderson, James and Woodruff, 1967; Harris and Harris, 1966; James and Anderson, 1967; James, 1967; Ogburn, Harris and Harris, 1967). In the previous section it was demonstrated that such a preparation also loses the ability to suppress a GVH reaction. One possible explanation of these findings would be that the F(ab')\textsubscript{2} fragment of ALS is unable to bind complement and is, therefore, incapable of producing complement-dependent lysis of lymphocytes. The lymphopoenia which is a general finding in normal animals treated with intact ALS may therefore be a direct reflection of \textit{in vivo} lysis produced by ALS and may provide the basis whereby ALS exerts its immunosuppressive effect.

It has been reported that NZB and DBA/2 mice lack a major component of serum complement (Cinader, Dubiski and Wardlaw, 1964; Norins, 1965) and that this is associated with a deficiency in the ability to produce complement-dependent immune haemolysis (Erickson \textit{et al.}, 1964). This finding could provide an explanation for the previously demonstrated ineffectiveness of ALS in suppressing the auto-immune disease of NZB mice. It seemed worthwhile, therefore, to test the ability of a single ALS preparation to induce lymphopoenia in NZB and DBA/2 mice in comparison with the lymphopoenia produced in mice known to possess functional complement.
Five animals from each of the strains NZB, DBA/2, CBA and C57Bl were given a single injection of ALS and individual blood samples were obtained from the retro-orbital plexus at 4, 48 and 336 hours after injection. From total WBC counts and differential counts performed on smears made from these samples, the numbers of circulating lymphocytes were calculated. Immediately prior to injection blood samples were obtained and these provided an estimate of the numbers of lymphocytes to be expected in untreated animals of each strain. Equal numbers of weight-matched animals of each strain received NRS and the counts in these animals acted as controls for non-specific reduction in lymphocyte numbers.

All mice used were males between 22 and 30 gms in weight. The NZB animals were less than 200 days of age and were Coombs negative when the experiment was begun. Rabbit anti-mouse ALS preparation Number 10 (see Table 14) was administered intra-peritoneally to each animal in the experimental group as a dose of 0.025 ml. per gm. body weight, and an equivalent volume of NRS was given to the matched animal in the control group.

Figure 30, which records the mean lymphocyte counts in each group expressed as a percentage of the appropriate mean pre-injection counts, shows that a marked fall in numbers was produced by ALS treatment in each of the 4 strains tested. The pattern of response was similar in each case: a drop to around 50 per cent or less at 4-48 hours after injection, and a trend towards normal levels by 2 weeks. As can be seen from Table 22, significant differences were observed between the lymphocyte counts of ALS-treated animals compared with counts of NRS-treated animals in all 4 strains.
Figure 50. The mean peripheral lymphocyte counts (expressed as per cent pre-injection counts) in 4 strains of mice at 4, 48 and 336 hours after a single intraperitoneal injection of ALS or NRS.
### TABLE 22

The mean peripheral lymphocyte counts in 4 strains of mice at 4, 48 and 336 hours after a single intraperitoneal injection of ALS or NRS.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment group</th>
<th>Pre-injection mean count per cu.mm.</th>
<th>Significance of difference between groups</th>
<th>4 hours mean count per cu.mm.</th>
<th>Significance of difference between groups</th>
<th>48 hours mean count per cu.mm.</th>
<th>Significance of difference between groups</th>
<th>336 hours mean count per cu.mm.</th>
<th>Significance of difference between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA</td>
<td>ALS</td>
<td>6675</td>
<td>NS*</td>
<td>4120</td>
<td>NS</td>
<td>2128</td>
<td>&lt;0.05</td>
<td>3668</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>5206</td>
<td></td>
<td>4733</td>
<td></td>
<td>4002</td>
<td></td>
<td>6401</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>4120</td>
<td>&lt;0.05</td>
<td>2128</td>
<td>&lt;0.05</td>
<td>3668</td>
<td></td>
<td>6401</td>
<td></td>
</tr>
<tr>
<td>C57BL</td>
<td>ALS</td>
<td>12954</td>
<td>NS</td>
<td>4619</td>
<td>&lt;0.05</td>
<td>4138</td>
<td>&lt;0.05</td>
<td>7746</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>10101</td>
<td></td>
<td>8098</td>
<td>&lt;0.05</td>
<td>6871</td>
<td>&lt;0.05</td>
<td>7911</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>12954</td>
<td>&lt;0.05</td>
<td>4619</td>
<td>&lt;0.05</td>
<td>4138</td>
<td>&lt;0.05</td>
<td>7746</td>
<td>NS</td>
</tr>
<tr>
<td>DBA/2</td>
<td>ALS</td>
<td>7263</td>
<td>NS</td>
<td>3893</td>
<td>&lt;0.05</td>
<td>4238</td>
<td>&lt;0.01</td>
<td>6609</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>6129</td>
<td></td>
<td>8449</td>
<td>&lt;0.05</td>
<td>7101</td>
<td>&lt;0.01</td>
<td>6478</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>7263</td>
<td>&lt;0.05</td>
<td>3893</td>
<td>&lt;0.05</td>
<td>4238</td>
<td>&lt;0.01</td>
<td>6609</td>
<td>NS</td>
</tr>
<tr>
<td>NZB</td>
<td>ALS</td>
<td>5821</td>
<td>NS</td>
<td>2621</td>
<td>&lt;0.05</td>
<td>2764</td>
<td>&lt;0.05</td>
<td>3331</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>5083</td>
<td></td>
<td>4281</td>
<td>&lt;0.05</td>
<td>3541</td>
<td>&lt;0.05</td>
<td>3787</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>5821</td>
<td>&lt;0.05</td>
<td>2621</td>
<td>&lt;0.05</td>
<td>2764</td>
<td>&lt;0.05</td>
<td>3331</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS = not significant
10. THE IMMUNE RESPONSE OF NZB MICE TO BOVINE SERUM ALBUMIN

The ability of young Coombs negative NZB and (NZB x CBA)F1 mice to respond to the injection of BSA was assessed, and the magnitude of the immune response obtained was compared with that produced in DBA/2, CBA, C57Bl and (C57Bl x CBA)F1 hybrid animals. The degree to which a prior injection of centrifuged BSA reduced a subsequent response to alum-precipitated BSA was also measured in each of these strains.

All mice were males aged between 28 and 144 days, and for each strain two groups were selected on a weight-matched basis. The NZB and the (NZB x CBA)F1 animals were Coombs tested at the start of the experiment and all were found to be negative.

One group, the "test" group, received 0.05 mg. centrifuged BSA intraperitoneally, while the other group, the "control" group, received an equivalent volume of saline. Twenty days later mice in both groups were given 5 mg. alum-precipitated BSA, also by the intraperitoneal route. At 20 and 30 days after this challenge injection each animal was bled from the retro-orbital plexus. Individual serum samples were separated and stored at -20°C. The antigen binding capacity (ABC) of each sample was subsequently measured by the Farr technique.

The levels of anti-BSA activity in the sera of NZB and (NZB x CBA)F1 hybrid mice at 20 days after challenge were comparable, and between 3-100 times greater than those found in the other strains (Table 23). A similar relationship was found at day 30. Pre-treatment of CBA and (NZB x CBA)F1 hybrid mice with centrifuged BSA resulted in a highly significant difference between test and control groups at both days 20
and 30. Among the strains DBA/2, C57Bl and (C57Bl x CBA)F1 the levels of antibody obtained were low even in the control groups—nevertheless the results suggested that pre-treatment with centrifuged antigen lowered the response still further. Among NZB animals pre-treatment with centrifuged BSA was associated with a partially diminished response at day 20, but by day 30 there was no significant difference between the test and control groups.

These results demonstrate that NZB mice give a marked immune response to alum-precipitated BSA and, since a similar response is obtained with (NZB x CBA)F1 hybrids, suggest that an inherited factor may be involved. The evidence presented also suggests that NZB mice may be less easily rendered hypo-responsive by pre-treatment with centrifuged antigen than other mouse strains. The F1 hybrid does not, however, share this property with the NZB animals.

This difference between NZB and (NZB x CBA)F1 hybrid mice may be associated with strain differences in the lymphoid organs. Table 24 records mean relative organ weights of the test and control groups of these strains. (The animals were killed on day 30 after bleeding for serum). There were no marked differences between test and control groups within strains, but the spleens and lymph nodes of NZB animals were significantly larger, and the livers significantly smaller, than those of the F1 hybrids. There was no significant difference between the real body weights of the two groups, and the relative thymus weights were virtually identical.
### TABLE 23

The mean antigen binding capacities of serum samples from mice of various inbred strains 20 days and 30 days after intraperitoneal injection of 5 mg. alum precipitated BSA.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>No. in group</th>
<th>Mean ABC * (± 1 S.D.)</th>
<th>Significance of diff. between test &amp; control values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 20</td>
<td>Day 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 20</td>
<td>Day 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Day 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZB</td>
<td>Test</td>
<td>9</td>
<td>3.59 (±2.51)</td>
<td>9.26 (± 5.86)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>10</td>
<td>8.19 (±3.77)</td>
<td>13.62 (± 5.29)</td>
</tr>
<tr>
<td>(NZB x CBA)</td>
<td>Test</td>
<td>10</td>
<td>1.79 (±0.93)</td>
<td>3.72 (± 2.25)</td>
</tr>
<tr>
<td>F1 hybrid</td>
<td>Control</td>
<td>8</td>
<td>7.54 (±3.21)</td>
<td>19.65 (±13.10)</td>
</tr>
<tr>
<td>CBA</td>
<td>Test</td>
<td>9</td>
<td>0.45 (±0.33)</td>
<td>1.17 (± 0.52)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9</td>
<td>2.33 (±1.35)</td>
<td>3.75 (± 0.51)</td>
</tr>
<tr>
<td>(C57Bl x CBA)</td>
<td>Test</td>
<td>5</td>
<td>0.56 (±0.39)</td>
<td>0.70 (± 0.63)</td>
</tr>
<tr>
<td>F1 hybrid</td>
<td>Control</td>
<td>6</td>
<td>1.30 (±0.44)</td>
<td>1.99 (± 0.83)</td>
</tr>
<tr>
<td>DBA/2</td>
<td>Test</td>
<td>5</td>
<td>0.15 (±0.23)</td>
<td>0.10 (± 0.15)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>7</td>
<td>0.66 (±0.52)</td>
<td>0.99 (± 0.71)</td>
</tr>
<tr>
<td>C57Bl</td>
<td>Test</td>
<td>8</td>
<td>0.03 (±0.05)</td>
<td>0.04 (± 0.04)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>8</td>
<td>0.08 (±0.07)</td>
<td>0.18 (± 0.18)</td>
</tr>
</tbody>
</table>

# Test groups were pretreated with centrifuged BSA, control groups with saline.

*μg N BSA bound per ml. serum.

# N.S. = not significant.
TABLE 24

THE MEAN RELATIVE ORGAN WEIGHTS OF TEST AND CONTROL GROUPS OF NZB
AND (NZB x CBA)F1 MICE KILLED 30 DAYS AFTER INTRAPERITONEAL INJECTION
OF 5 mg. ALUM PRECIPITATED BSA

<table>
<thead>
<tr>
<th></th>
<th>Strain (test + control)</th>
<th>Significance of difference between means</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NZB</td>
<td>(NZB x CBA)F1</td>
</tr>
<tr>
<td>Body wt. (± 1 S.D.)</td>
<td>31.3 (± 3.2)</td>
<td>31.9 (± 1.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean relative organ weight (± 1 S.D.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>35.5 (± 4.4)</td>
<td>26.9 (± 2.8)</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>18.7 (± 5.9)</td>
<td>5.7 (± 1.9)</td>
</tr>
<tr>
<td>Thymus</td>
<td>10.0 (± 1.2)</td>
<td>10.0 (± 1.1)</td>
</tr>
<tr>
<td>Liver</td>
<td>537.9 (± 33.6)</td>
<td>599.2 (± 31.4)</td>
</tr>
</tbody>
</table>
11. SUMMARY OF RESULTS

(1) NZB mice of both sexes developed haemolytic anaemia spontaneously. This syndrome was first detected by fall in PCV and increase in reticulocyte counts in a few animals at about 175 days of age. Positive direct Coombs tests began to occur in a few animals of both sexes at about 225 days of age. The incidence of such positive tests with increasing age was more uniform among female mice (all gave positive results by 300 days of age) than among male mice. All male mice were not positive until 400 days of age, and there was a tendency for male mice to revert spontaneously to a Coombs negative state. The mean age at death of NZB male and female mice was 515 ± 130.6 days, which is considerably earlier than that of (NZB x T6)F1 hybrids and most other laboratory strains.

NZB mice of both sexes tended to have enlarged lymphoid organs - particularly the spleen. Virtually all mice developed splenomegaly, and a smaller proportion had enlarged lymph nodes and liver. No evidence of thymic hypertrophy was found, but older mice were consistently found to have atrophied thymus tissue. Microscopic examination of the organs of mice older than 100 days when killed consistently revealed germinal centre formation in spleens, lymph nodes and thymuses. The livers, lungs and kidneys of older NZB mice frequently exhibited pyroninophilic cell infiltration around blood vessels.

Tumours were frequently found among NZB mice. One group of 30 untreated females contained 9 animals which had obvious tumour formations when examined post mortem (an incidence of 33 per cent among
females). Seven of these 9 tumours involved lymphoid tissue.

(2) A high proportion of NZB mice with a positive direct Coombs test also had a serum antibody which agglutinated papain-treated mouse erythrocytes. A few mice were found which were positive in one of these tests and negative in the other, but generally there was a good correlation between the results of these two tests. Evidence from serum fractionation studies on G-200 Sephadex suggested that papain-cell activity could be due to 19S or 7S molecules and that occasionally activity of both types was present.

(3) The serum of most NZB mice was found to possess high levels of complement-fixing activity against isogeneric liver homogenate, and the highest titres of such activity were found in younger mice. No correlation was found between the presence of anti-liver activity and the occurrence of a positive direct Coombs test. Ultracentrifugation on sucrose density gradients suggested that anti-liver activity was associated with the high molecular weight (19S) fraction.

(4) The injection of $150 \times 10^6$ or $75 \times 10^6$ viable spleen cells, obtained from old NZB mice with positive direct Coombs tests, into young syngeneic NZB recipients was associated with premature conversion to a Coombs positive state. Similar numbers of lymph node or bone marrow cells, or smaller numbers of thoracic duct lymphocytes, did not produce this result.

(5) The injection of $25-100 \times 10^6$ viable spleen cells, obtained from old NZB mice with positive direct Coombs tests, into adult (NZB x T6)F1 hybrid recipients was associated with conversion to a Coombs positive state first detected about 10 days after transfer. This
Coombs positive state was associated with a drop in PCV values, splenomegaly, lymph node and liver enlargement, and early death—all of which were found to be features of the disease which occurs spontaneously in NZB mice.

These findings are not explained simply as a GVH reaction by the transferred spleen cells against parental antigens of the F1 hybrid host since comparable numbers of spleen cells from young Coombs negative NZB mice produced classical signs of severe GVH reaction—but did not produce anaemia or conversion to a Coombs positive state. Furthermore, a dose of $25 \times 10^6$ cells from Coombs positive NZB donors produced only splenomegaly and Coombs test conversion in F1 recipients, while $25 \times 10^6$ cells from Coombs negative NZB donors produced classical GVH disease (splenomegaly, hepatomegaly, lymph node enlargement, thymic atrophy and early death). An increase in the numbers of cells transferred from NZB Coombs positive donors increased the degree of organ enlargement and mortality, but increased numbers of Coombs negative cells did not increase the severity of the (already very severe) GVH disease. The splenomegaly which was obtained using Coombs positive cells was much greater than that obtainable with negative cells.

(6) Rabbit anti-mouse lymphocyte sera (ALS), which were immunosuppressive when judged by their ability to prolong the survival of mouse skin homografts, were regularly produced by giving rabbits 3 injections of $10^9$ viable mouse thymus cells. Antisera produced by giving 2 injections of cells, whether combined with previous C. parvum treatment of the rabbits or not, were not more immunosuppressive than those produced by 3 injections.
(7) None of the three ALS assay procedures - inhibition of GVH reaction, \textit{in vitro} cytotoxicity, and ability to produce lymphopenia - was found to be highly correlated with ability to prolong homograft survival. Of the three, the ability to produce lymphopenia gave the best results. A very close association was found, however, between ability to inhibit GVH reaction and \textit{in vitro} titre of cytotoxic antibodies.

(8) A study of the effects of regular treatment with ALS on the development of positive direct Coombs tests in young NZB mice gave equivocal results. There was some indication that treatment delayed the development of positive tests by a few weeks, but this effect was not maintained and the treated group eventually developed the usual signs of haemolytic anaemia. Similar equivocal results were again found when the effects of ALS in old Coombs positive mice were studied. ALS was, however, found to exert a marked effect on the induction of positive Coombs tests in (NZB x CBA)F1 hybrid recipients given spleen cells from old Coombs positive NZB mice. Such recipients, which had been treated with ALS 4 hours prior to spleen cell injection, had a very much lower incidence of positive Coombs tests and high-titre papain-cell agglutinating activity than did recipients which had been pre-treated with NRS.

(9) The IgG fraction of ALS suppressed the GVH reaction produced in (C57Bl x CBA)F1 hybrid mice given parental strain spleen cells, but \( F(ab')_2 \) fragments of this IgG fraction were unable to suppress this GVH reaction. Digestion to \( F(ab')_2 \) fragments was also associated with loss of \textit{in vitro} cytotoxicity but not of ability to agglutinate lymphocytes.
A single dose of ALS produced a marked lymphopenia in CBA and C57Bl mice at 4 and 48 hours after injection. When counts were performed about 2 weeks later the number of circulating lymphocytes had returned to normal. A similar pattern of response was obtained with NZB and DBA/2 mice, which are believed to lack at least one of the serum components required for complement-dependent cell lysis.

NZB and (NZB x CBA)F1 hybrid mice were found to give a marked immune response to BSA. This response was between 3 and 100 times greater than that given by the other strains of mice tested. Furthermore, there was some evidence that NZB mice were less easily rendered hypo-responsive to BSA by pre-treatment with antigen in soluble form. This was not found to be the case among the F1 hybrids.
1. IMMUNOPATHOLOGY OF NZB MICE

(i) General. The findings reported in this thesis are in broad agreement with those of others who have studied NZB mice. Members of this strain develop signs of anaemia, with increasing incidence with age, associated with the development of positive direct Coombs tests and proliferative abnormalities of lymphoid tissues (Bielschowsky, Helyer and Howie, 1959; Helyer and Howie, 1963a; Holmes and Burnet, 1963a; Burnet, 1963; Mellors, 1965; East, de Sousa and Farrott, 1965; Braverman, 1968).

A number of significant differences has, however, been found in the present work. The age at which positive Coombs tests began to be detected was much later (around 225 days of age) than in most previous studies. Such a finding is more in agreement with the work of de Vries and Hiijmans (1966), who found in one investigation that only 3 out of 18 mice had given a positive Coombs test by 12 months of age, and of Polackova and Strejcek (1968), who also found a later onset and 100 per cent incidence only at 14 months of age. Holmes and Burnet (1963) found that males became Coombs positive earlier than females, but East, de Sousa and Farrott (1965) reported that in their animals the reverse was true.

In the present study no clear difference was found between males and females in the age at which positive Coombs tests began to occur, although once the conversion age was passed the females showed a more uniform progression to 100 per cent incidence. Most authors, whatever the age at which positive Coombs tests are first detected, have reported that all animals eventually give positive reactions and continue to do
so until death. East, de Sousa and Parrott (1965), however, claimed that a small proportion of animals died without ever having given a positive Coombs test result. Holborow, Barnes and Tuffrey (1965) also found this, but among the animals they studied a small number of mice were found which became Coombs positive and then reverted to a negative state later. In the present work an occasional NZB mouse tested at infrequent intervals died without ever having given a positive result, but in the experiments in which mice were Coombs-tested regularly from a young age a 100 per cent incidence was found. In a few old males, however, a reversion to a Coombs negative state was found which persisted for varying periods until death. The NZB mice used in the present study lived significantly longer than the animals described by Holmes and Burnet (1963a) or East, de Sousa and Parrott (1965), and in contrast to those studies no marked difference was detected between the lifespans of males and females.

The significance of the differences demonstrated in the present study is not clear, but other examples of variation between NZB mice in different laboratories have been documented. There have been three attempts to determine whether NZB male skin transplanted to NZB females is rejected, with results which could hardly be more divergent: Holmes and Burnet (1963) claimed that all such grafts were rejected, Polackova and Strejcek (1968) that 67 per cent of such grafts were rejected, and Stutman, Yunis and Good (1968) that NZB females permanently accepted skin grafts from NZB males. The data on the occurrence of anti-nuclear factors (ANF) in various colonies of NZB mice are also conflicting: Norins and Holmes (1964b) found positive results in 42 per cent of the
adult animals they tested; Holborow, Barnes and Tuffrey (1965) in 20 per cent; Mellors (1965) in only 6 per cent; and Polackova and Strejcek (1968) in 53 per cent. Barnes (1967) found ANF in the serum of 22 per cent of conventionally-reared 6 month old NZB mice, but in pathogen-free animals of the same age he found the incidence to be 50 per cent.

At least three different reasons can be advanced to explain these considerable variations in results with NZB mice: differences in assay techniques and experimental design, environmental differences in rearing of the mice, or small genetic differences between the various colonies. The role of environmental differences appears to be demonstrated by the finding that conventionally-reared mice differ from pathogen-free mice in their incidence of ANF (Barnes, 1967), and it must be obvious that technical factors will be of importance in the variation found for tests such as the Coombs test and ANF assays where subjective factors play a part in deciding between positive and negative reactions.

The evidence suggesting that there are genetic differences between some of the sub-lines of NZB mice is perhaps more compelling. The variation in the rejection times found for male skin on female mice would support this idea, as would the studies of de Vries and Hijmans (1967) which showed that among NZB mice of 3 weeks of age obtained from 9 different laboratories there were very marked differences in the numbers of epithelial cells to be found in the thymuses and in the times at which degeneration and subsequent disappearance of the epithelial cells occurred. Thus it seems sensible to recognize that genetic differences may exist between the various NZB mouse colonies.
All these animals have been derived from an original colony whose members were homogeneous with respect to the genetic loci determining histocompatibility antigens, but not necessarily for other loci which could include those genetic factors responsible for the expression of the various immunological abnormalities which characterize this strain. Segregation of such factors within a colony could explain the variation between individuals concerning age of onset of Coombs-positivity, etc., and quantitative differences in the original distribution of such factors could be responsible for the divergent results reported from different laboratories.

(ii) The nature of anti-erythrocyte auto-antibodies. At least three antibodies which have specificities directed against antigens present on the surface of normal mouse erythrocytes, or erythrocytes which have been lightly treated with proteolytic enzymes, have been found in NZB mice (Long, Holmes and Burnet, 1963). In an extensive series of experiments which involved absorption with washed mouse erythrocytes those authors found that all anti-erythrocyte activity in eluates from Coombs-positive erythrocytes and in NZB sera was directed against one antigen—a species specific component of the mouse erythrocyte surface. The three antibodies did, however, differ in avidity. It was also found that the activity detected by agglutination of papain-treated mouse erythrocytes (PRCA antibody) occasionally occurred in the serum of mice which gave negative direct Coombs test results, but that in general PRCA antibody was only found in high titres in Coombs-positive animals. Similar results were obtained in the present work: a high proportion of the NZB mice tested and found to give a positive direct Coombs test
result also had a serum antibody which agglutinated papain-treated mouse erythrocytes. A few mice were found which were positive in one of these tests and negative in the other, but in general a very good correlation was observed between the results of these two tests.

Early attempts to determine the immunoglobulin class to which NZB anti-erythrocyte auto-antibodies belong suggested that the antibody responsible for the direct Coombs test was a 7S gamma globulin (Norins and Holmes, 1964a; Giltinan, Norins and Holmes, 1965) and that the serum antibody responsible for the indirect Coombs test was a macroglobulin (19S) antibody (Mellors, 1965). A later study, however, which made use of antisera specific for 4 of the 5 major mouse immunoglobulin types (IgM, IgG1, IgG2a, IgG2b and IgA), demonstrated that the coating present on Coombs-positive NZB erythrocytes could be due to the presence of antibody of any one (or more) of these immunoglobulin types (Warner and Wistar, 1968). Almost all of the mice tested gave a positive reaction with the antiserum against IgG1, and most of the mice also gave a positive reaction with one of the other antisera of a different specificity.

In the present study a number of NZB sera, which had been shown to agglutinate papain-treated normal mouse erythrocytes, were fractionated into higher and lower molecular weight batches using the relatively crude technique of G-200 Sephadex chromatography. In some sera higher titres of PRCA activity were found in the high molecular weight fraction, in some sera higher titres were found in the low molecular weight fractions, and in a few sera the distribution of activity was roughly equal. This finding suggests that the antibody activity responsible
for the agglutination of papain-treated erythrocytes, like that responsible for positive direct Coombs tests, may be found in different immunoglobulin classes in different animals, and that more than one immunoglobulin class may be involved in the same animal.

(iii) Neoplasia. The spontaneous occurrence of neoplasms (thymomas, reticulum cell sarcomas, and lymphatic leukemias) involving lymphoid tissues of NZB mice was first described by Bielschowsky and Bielschowsky (1962). External application of the carcinogen 2-aminofluorene over a long period increased the incidence of such tumours five-fold to 19 per cent, and NZB mice were much more susceptible in this respect than two other inbred mouse strains used as controls. Holmes and Burnet (1963a) found the incidence of spontaneous "lymphoid hyperplasia or tumour" to be 33 per cent in NZB males, and 50 per cent in females, over 400 days of age. East, de Sousa and Parrott (1965) found that 7 of 43 NZB mice dying naturally between 12 and 59 weeks of age had thymomas, and that injection of cell suspensions prepared from these thymomas into 1 or 28 day old NZB mice caused lethal, generalised leukemia 14 to 18 days later. East, de Sousa and Farrott also suggested that the gross splenomegaly and lymphadenopathy of NZB mice could be due to uncontrolled proliferation of plasma or reticulum cells, and this was associated with the macroglobulinemia which they detected. In a study of 20 old NZB mice Mellors (1966) found 4 females with malignant lymphomas and he recognised two types: those composed of malignant reticulum cells unassociated with a quantitative serum globulin abnormality, and those composed of malignant lymphoid cells
"pleomorphic malignant lymphoma") associated with hyper-gammaglobulinemia.

In one closely studied group of female mice in the present work 33 per cent of the animals developed tumours, most of which involved lymphoid tissues. No tumours were found in a similar group of NZB males studied over the same period. Spontaneous malignancies were, however, occasionally found in other male mice but since these animals had been selected for various purposes no valid figure can be given for the incidence of lymphoid tumours in male mice. Nevertheless it appears to be justified to conclude that there is an increased incidence of spontaneous neoplasia of lymphoid tissue in NZB mice of both sexes, although males are less likely to be affected than females.

The importance of this association between the occurrence of autoimmune disease and a liability to develop lymphoid neoplasia is not clear, and could conceivably be a chance occurrence of no significance. There are, however, various pieces of evidence which suggest that a chance association is unlikely.

Mellors (1966) claimed that in the 4 NZB mice with malignant lymphoma which he described, autoantibodies directed against erythrocytes were present, and the animals showed signs of anaemia and reticulocytosis. In the present study all the animals with lymphoid tumours showed signs of autoimmune disease at some stage, although occasional reversions to a Coombs-negative state were found in old animals found at post mortem examination to have tumours. Such findings would be compatible with a hypothesis that suggested lymphoid cell proliferation of a neoplastic type is essential in the pathogenesis
of the NZB autoimmune disease - autoantibodies being formed perhaps by the neoplastic cells or as a normal response to them or their products.

Most of the available evidence, however, argues against a direct relationship between neoplasia of lymphoid cells and autoantibody formation. The findings of Warner and Wistar (1968), which are supported by similar findings in the present work, that autoantibodies may be found in different immunoglobulin classes in one animal would require the proliferation of more than one cell line. The thymomas described by East, de Sousa and Farrott (1965) were sometimes found in male mice aged between 12 and 23 weeks, although positive direct Coombs test results were not found in male members of their colony until 20 weeks of age. Furthermore, the transfer of cell suspensions from such thymomas to young NZB recipients produced lethal leukemias in the recipients without any evidence of positive Coombs tests. In a direct attempt to induce an autoantibody-producing plasma cell tumour Goldstein, Warner and Holmes (1966) gave old (NZB x BALB/c)F1 hybrid mice intraperitoneal injections of paraffin. A high proportion of the animals treated in this way developed plasma-cell tumours as expected and some also developed positive Coombs tests, but no animal developed both. Casey (1968a and b) treated NZB mice with azathioprine and found that 6 out of 8 treated mice developed malignant lymphomas which particularly affected the thymus. The treated animals had no more severe signs of autoimmune disease than the controls, and although initially the known marrow toxicity of azathioprine appeared to worsen the anaemia the haematocrit values of both groups eventually reached similar levels.
A direct causal relationship between neoplasia and the occurrence of autoimmunity in NZB mice therefore appears unlikely, but there remains the possibility of a primary defect which could underlie the development of autoimmunity or neoplasia or both. The nature of such a primary defect, and the secondary factors which would be necessary to determine which abnormality results, are to a large extent matters of speculation at present. It is interesting, however, to note that NZB mice have been found to contain virus-like particles which resemble morphologically the murine leukemia viruses which have been associated with the cell-free transfer of leukemia in other mouse strains (Mellors and Huang, 1966; East et al., 1967a; Hollmann and Verley, 1967; Yumoto and Dmochowski, 1967). The significance of this finding for NZB associated abnormalities is not yet apparent, especially since the transfer of cell-free materials from NZB mice has so far failed to produce convincing evidence of either autoimmunity or neoplasia in syngeneic and allogeneic recipients. Nevertheless, this seems likely to be an area of study which will prove rewarding in future.

(iv) Anti-liver antibody. The spontaneous occurrence of anti-tissue antibodies has not previously been described in NZB mice, so that the present finding of high levels of serum antibodies with complement-fixing activity against isogeneic liver antigens is of some importance. The lack of correlation between the presence of a positive direct Coombs test and anti-liver activity in individual mice suggests that the anti-liver antibodies are unrelated to the autoimmune haemolytic anaemia. Furthermore, the finding that when six sera containing this activity were fractionated using ultracentrifugation the activity was found in the high
molecular weight (19S) region suggests that the antibody may be of
the non-damaging type. Adult and neonatal rats have been shown to
produce IgM antibodies to liver antigens released by CCl₄ induced
liver damage (Weir, 1963; Elson and Weir, 1969) and similar findings
have been reported in germ-free mice (Arnason, Salomon and Grabar,
1964) and in NZB mice (Playfair, 1968).

Such IgM antibodies seem unlikely to potentiate tissue damage
since it has been shown that they do not release pharmacologically
active substances in PCA reactions (Weir, 1963 and 1967a), and there
is some evidence to indicate that they have a physiological role in
promoting the removal of tissue breakdown products (Elson and Weir,
1967; Elson, 1968).

These observations suggest the possibility that the NZB anti-
liver antibodies described here could arise as a result of an abnormal
stimulus due to the release of tissue breakdown products. The cause
of such tissue damage is not known, but it could conceivably be related
to the presence in various organs and lymphoid tissues of NZB mice of
the previously mentioned virus-like particles. There is, however, no
evidence that virus activity results in tissue damage, or indeed that
the widespread lesions of NZB mice are associated with the release of
antigens capable of stimulating an anti-tissue response. The evidence
(to be discussed in a later section) that NZB mice are hyper-responsive
to a number of antigenic stimuli provides an alternative mechanism
whereby increased anti-tissue activity could be produced. This could
simply be a normal physiological mechanism for dealing with tissue
breakdown products which reaches higher levels in a mouse strain in
which all (or most) humoral antibody responses are enhanced.

(v) The transfer of autoimmune disease by lymphoid cells. The injection of $150 \times 10^6$ or $75 \times 10^6$ viable spleen cells from Coombs positive NZB mice into young NZB recipients was associated with the premature production of anti-red cell antibodies in the recipients. Similar numbers of lymph node or bone marrow cells, or smaller numbers of thoracic duct lymphocytes did not have this effect. Positive direct Coombs test results, and other signs of autoimmune haemolytic anaemia, were also found in adult $(NZB \times T6)F_1$ hybrid mice given $25$ to $100 \times 10^6$ viable spleen cells obtained from old Coombs positive NZB donors.

These results agree with Holmes, Gorrie and Burnet (1961) and Holmes (1965) who found that most newborn and young NZB mice injected with spleen cells - but not lymph node, thymus, marrow or buffy coat cells - obtained from 10-12 month old NZB animals became Coombs positive by 1, 2 or 3 weeks after transfer. The majority of such animals continued to have anti-erythrocyte antibody for 4-5 weeks, and a few remained positive until the age where spontaneous conversion would be expected. When cells were transferred from donors with high levels of serum antibody capable of agglutinating papain-treated mouse erythrocytes, then such antibody was also subsequently detected in the recipients. Spleen cells from young NZB mice did not induce Coombs-test conversion on transfer to other young NZB mice, nor did spleen cells from old Coombs positive animals on transfer to C3H or C57Bl animals. Holborow, Barnes and Tuffrey (1965) found that if $30-40 \times 10^6$ spleen cells from NZB donors which contained the serum autoantibody cross-reactive with human red cell antigens were transferred to young
recipients then the cross-reactive antibody was also produced as well as the antibody detected by direct Coombs tests. These workers also found signs of haemolytic anaemia (increased uptake of erythrocytes by spleen and liver, splenomegaly, reductions of haemoglobin levels and red cell counts) in the young recipients (Barnes and Tuffrey, 1966a and b). These changes were particularly marked in animals given spleen cells from old NZB donors positive for both the Coombs antibody and the cross-reactive antibody. Denman, Denman and Holborow (1967) found that one-month old NZB mice, given an injection of $200 \times 10^6$ viable spleen cells from strongly Coombs-positive NZB donors, were themselves Coombs-positive when tested 10 days later. Incubation of the spleen cells with anti-lymphocyte globulin prior to transfer prevented the development of Coombs tests in the recipients.

Holborow and Denman (1967) transferred spleen cells from 9-month old NZB mice to 6-8-week old syngeneic recipients, some of which had been exposed to a dose of sub-lethal irradiation shown to be effective in preventing an immune response to sheep red cells for the period of the experiments. Positive direct Coombs tests were found in some members of both the irradiated and non-irradiated groups: the first of these appeared 10 days after cell transfer, all the positives had appeared by 3 weeks, and all animals gave negative results when tested at 6 weeks. High reticulocyte levels were found in some of these mice. In one experiment a suspension of spleen cells which had been filtered through glass wool, so as to contain more than 90 per cent small lymphocytes, was used - this suspension also produced transient Coombs-positive reactions in irradiated and non-irradiated recipients. No Coombs test
transfer was obtained, however, when spleen cells from old NZB mice were given to irradiated NZW and C3H mice.

There have also been claims that transfer of spleen cells from old NZB mice to young syngeneic recipients can produce signs of renal disease in the recipients. In one such study (Mellors, 1966) hyper-gammaglobulinaemia, lymphoid cell hyperplasia and the structural and functional changes of membranous glomerulonephritis were found between 2 and 5 weeks after cell transfer. Barnes, Berry and Holliday (1969) transferred spleen cells from old NZB mice, with or without evidence of autoantibody production against erythrocytes, to 4-week old NZB recipients and found evidence of kidney damage only in those animals given cells from autoantibody producing donors. The damaged kidneys stained with anti-globulin serum, and elution studies suggested that this was not an antinuclear-type antibody. Spleen cell suspensions which had been subjected to freezing and thawing did not produce evidence of kidney damage on transfer to young NZB recipients.

Two other groups have transferred spleen cells from old to young NZB mice and failed to find positive Coombs test results in the recipients. Polackova and Strejcek (1968) gave newborn NZB mice 10 x 10^6 spleen cells from old Coombs-positive donors and tested them regularly throughout life without finding premature conversion to a Coombs-positive state. East, de Sousa and Parrot (1965) and East and Prosser (1967) have described a series of experiments in which newborn and weanling NZB mice were injected with cell suspensions containing between 8 x 10^6 and 17 x 10^6 viable cells obtained from the lymph nodes and spleens of old NZB mice. The recipients developed enlarged lymph nodes and spleens, and died early with evidence of widespread
invasion of many organs by sheets of pale-staining reticulum cells.
No evidence of premature production of the antibody responsible for
positive Coombs test results was found, however, among these animals.
The reason for these failures is not known, but one factor which very
probably played a part was the transfer of an inadequate number of
cells. Most of the experiments in which a successful transfer of
Coombs positivity has been demonstrated (including the experiments
described in this thesis) have required the use of spleen cell numbers
in excess of those used in the unsuccessful experiments. In one case
it may also be true that the experimental design was inadequate to
detect this often transitory phenomenon - Coombs tests being performed
only at 8 and 14, or 35 days after cell transfer.

There is at present no completely satisfactory explanation for the
transfer of autoantibody formation to young NZB and NZBF1 hybrid mice.
Perhaps the simplest possibility is that already-sensitized antibody-
forming cells are transferred and that these cells colonise the host
and continue to produce antibody in their new situation. In this case
the lag phase might represent the period required for antibody levels
to be attained great enough for present tests to be effective in
demonstrating them. Three points, however, appear to be in conflict
with such an explanation: the inability of lymphoid cell suspensions
from organs other than the spleen to give successful transfer, the
failure of spleen cells transferred to newborn or irradiated allogeneic
mice to confer Coombs positivity, and the transitory nature of the
reaction when it is obtained. It would be most unusual if suspensions
of peripheral lymphocytes obtained from blood, thoracic duct lymph or
lymph nodes were not sensitized against an antigen which had sensitized the splenic lymphocytes of the same animal. Spleen cells transferred to newborn, or irradiated allogeneic mice would be expected to survive (at least in some cases) long enough to produce antibody in their new situation – Holborow and Denman (1967) showed that spleen cells from NZB mice, immunized against sheep red cells and transferred to irradiated C3H recipients bestowed the capability to respond to subsequent challenge with sheep red cells.

An alternative explanation of the transfer phenomenon is that the splenic material injected could stimulate the recipient to premature autoantibody formation. Two things argue against this possibility – frozen/thawed or heat-killed cells are ineffective, and Holborow and Denman (1967) found that transfer of Coombs positivity by spleen cells occurred in NZB mice whose immune responses had been suppressed by irradiation.

Despite the objections already mentioned, some form of passive transfer is the most attractive possibility. The requirement for viable cells, the effect of ALS on the recipient in inhibiting the reaction as shown in this thesis, and the finding that the autoantibody-forming status of the donor (i.e. papain-treated red cell reacting, human red cell cross-reacting, etc.) influences antibody production in the host, are all in agreement with this suggestion. It may be that the transferred spleen cells require a further qualifying factor before initiating antibody formation – perhaps antigenic stimulation in their new hosts, as has been shown to be necessary for adoptive immunity to external antigens (Nakela and Mitchison, 1965). This would explain the
failure of transfers to allogeneic mice as a lack of the appropriate antigenic stimulus, a stimulus present only in old mice of the NZB genotype. Such an explanation is not supported, however, by the experiments of Lindsey and Woodruff (1968) in which adult NZB mice with positive direct Coombs tests and other signs of hemolytic disease were irradiated and repopulated with spleen and bone marrow cells from young Coombs-negative NZB or (NZB x T6)F1 hybrid mice. Most of the mice given syngeneic cells subsequently became Coombs-negative and some of them reverted to a Coombs-positive state 1-3 months later, while most of the mice given F1 cells became Coombs-negative and remained so until death (up to 1 year later). Polackova and Strejcek (1968), in a similar experiment using irradiated NZB mice repopulated with CBA cells, found that the number of mice giving positive Coombs test results gradually fell with time until by eight weeks after irradiation all had become negative. These experiments suggest that old NZB mice do not possess intrinsic antigens which automatically provide a stimulus to lymphoid cells.

The requirement that only spleen cells seem able to mediate this transfer reaction suggests a further possibility, the supporting evidence for which is at present no more than circumstantial. In mouse spleen there is a close association of lymphoid tissue and erythropoietic tissue. In NZB mouse spleen, many authors have reported evidence of virus activity (Mellors and Huang, 1966; Nowinski et al., 1968) and others have reported signs that virus activity involves budding and damage at cell surfaces in the spleen and other organs (East et al., 1967; Hollman and Verly, 1967; Yumoto and Dmochowski, 1967; Prosser, 1968). Such activity could conceivably result in damage to erythroid
cells or their precursors and perhaps the induction of an autoimmune reaction by the local splenic lymphocytes. If this were the case then most aspects of the transfer studies could be explained, particularly the transitory nature of the phenomenon in young NZB animals, by postulating that these provide unsatisfactory environments for viral replication (thus limiting further damage and perpetual sensitization). To explain the non-occurrence of the phenomenon in allogeneic mice a further hypothesis is required - not only would the environment they provide have to be unfavourable to virus replication, they would also have to reject the potential antibody-producing cells before antibody formation took place. This could happen if normal mouse strains contained antibodies directed against the responsible virus (located on the surface of at least some NZB lymphoid cells) - a not unreasonable hypothesis since normal strains do not become infected by cell-free transfers of NZB material. The spontaneous emergence of autoimmune disease with age in NZB mice might then be a reflection of the production of an increasingly favourable environment for the replication of virus, with resultant spleen cell damage producing auto-sensitization.

II. ANTI-LYMPHOCYTE SERUM

In the past few years many studies of the properties of antisera raised against heterologous lymphoid cells have been reported. It has been clearly demonstrated that such antisera can suppress most aspects of the normal immune response when administered in vivo, and also exert a variety of effects on a number of immunologically related in vitro systems.

ALS has been shown to prolong homograft survival in mice, rats, dogs, pigs, monkeys and humans (Monaco et al., 1966a and b; Levey and
Medawar, 1966a and b; Woodruff and Anderson, 1963 and 1964; Nagaya and Sieker, 1965; Guttman et al., 1967; Abaza et al., 1966; Starzl et al., 1966; Lucie et al., 1968; Balner and Dersjant, 1967; Monaco, Wood and Russell, 1967), to suppress delayed hypersensitivity reactions in mice, guinea pigs and humans (Russe and Crowle, 1965; Inderbitzen, 1956; Wilhelm, Fisher and Cooke, 1958; Waksman, Arbouys and Arnason, 1961; Turk and Willoughby, 1967; Brunstetter and Claman, 1968), and to inhibit humoral antibody production in mice, rats, rabbits and dogs (Monaco et al., 1966b; Berenbaum, 1967; James and Anderson, 1967 and 1968; Jeejeebhoy, 1965; Harris and Harris, 1966a and b; Picklmayr, Brendel and Zenker, 1967). In addition to these effects on the classical and relatively well understood components of the immune response, ALS has also been found to suppress graft-versus-host reactions in mice, rats and primates (Boak, Fox and Wilson, 1967; van Bekkum et al., 1967; Brent, Courtenay and Gowland, 1967; Levey and Medawar, 1967; Monaco et al., 1967; Field and Gibbs, 1968), to inhibit the normal and immune lymphocyte transfer reactions in guinea pigs (Levey and Medawar, 1966a and 1967), to increase the susceptibility of mice to infection with a variety of viruses (Hirsch and Murphy, 1968; Volkert and Lundstedt, 1968), and to increase the rate of tumour induction (virus induced and non-virus induced) and dissemination in mice, rats and hamsters (Allison, Berman and Levy, 1967; Philips and Gazet, 1967; Rigdon et al., 1967; Allison and Law, 1968; Hellmann, Hawkins and Whitecross, 1968; Stanbridge and Perkins, 1969; Vandeputte, 1968; Tevethia et al., 1968).

The most striking property of ALS is its interaction with lymphocytes - virtually all reports to date have demonstrated that ALS combines with lymphocytes in vitro and causes their agglutination. In the
presence of complement from a source appropriate to the antiserum type the lymphocytes are lysed. The uptake of anti-lymphocyte antibody by the surfaces of lymphocytes has been directly demonstrated by immunofluorescence (Levey and Medawar, 1966a; Russell and Monaco, 1967; Denman and Frenkel, 1968a) and by use of isotopically labelled antisera (Woodruff, Reid and James, 1967; Denman and Frenkel, 1968a). The results when ALS is injected into animals generally parallel the in vitro findings - a small quantity produces a marked, though transient, lymphopenia which is sometimes associated with a fall in serum complement level (Waksman, Arbouys and Armanon, 1961; Guttman et al., 1967). A more profound and sustained lymphopenia can be produced by repeated injections of ALS, but even with such courses a return towards normal levels of circulating lymphocytes may occur during the period of ALS administration or shortly afterwards (Woodruff and Anderson, 1964; Nagaya and Sieker, 1965; Levey and Medawar, 1966a and b; Herman and Schloerb, 1967; Clunie et al., 1968; Kalden et al., 1968). These findings suggest that the ability to produce lymphopenia is lost as animals receive repeated injections, but many workers have found that ALS-induced lymphopenia can be maintained (Denman and Frenkel, 1968a; Halpern et al., 1968; Suvatte, Githens and Colofiore, 1968; Taub and Lance, 1968; Vandeputte, 1968) and Kinne and Simmonds (1967) found a very close relationship between the ability of an antiserum to produce lymphopenia and its ability to prolong homograft survival in mice. This inconsistency is most likely due to the great variety of immunization schedules which have been employed to produce antisera and to the variation in courses of treatment which have been given. The differing abilities of antibodies to fix complement in different species, and the possibility of species and strain differences in response to ALS treatment
must also play a part in this respect. Although the production of prolonged lymphopenia does not appear to be essential for the demonstration of immunosuppression, no immunosuppressive ALS preparation has been reported which does not produce at least an initial drop in circulating lymphocyte numbers on injection, and a demonstrable cytotoxic effect on lymphocytes in vitro. Furthermore, various procedures which increase the lymphopenia-producing effects of ALS — use of thymocytes in its production (Nagaya and Sieker, 1965), use of adjuvants in its production (Gray et al., 1966; Kinne and Simmonds, 1967), administration to thymectomized animals (Monaco, Wood and Russell, 1965), and administration to animals rendered tolerant of the appropriate serum protein (Denman and Frenkel, 1967) — have been claimed to enhance its immunosuppressive properties.

The potential importance of ALS as an agent in the treatment of human transplant patients has inspired many attempts to find in vitro properties of ALS preparations which correlate well with their immunosuppressive abilities in vivo. Assays which have been recommended include: determination of agglutinating and cytotoxic titres, suppression of plaque formation against sheep erythrocytes by lymphocytes in gel (Berenbaum, 1967; Harris and Harris, 1966; Barth, Southworth and Burger, 1968), suppression of natural antibody formation against sheep erythrocytes by lymphocytes (Bach and Antoine, 1968), and inhibition of lymphocyte transformation induced by the mixed leukocyte reaction (Schwartz, Tyler and Everett, 1968) or by non-specific stimulants such as PHA (Mosedale, Pelstead and Parke, 1968). Most studies of the effects of ALS on these phenomena have failed to demonstrate a clear quantitative relationship between activity in the test system and
in vivo immunosuppression. It appears possible that the conclusion reached by one group regarding in vitro cytotoxicity, that all active sera are cytotoxic but not all cytotoxic sera are active (Jooste et al., 1968) may prove applicable to all of them. The only in vitro property of ALS which has so far given consistently good correlation with in vivo immunosuppression is its ability to promote the opsonization of lymphocytes by monolayer cultures of peritoneal macrophages (Greaves et al., 1969). That study, which holds great promise, was performed with a mouse cell system but so far no results have been reported from other systems.

It has been claimed (Levey and Medawar, 1966b; Jooste et al., 1968) that a "two pulse" immunization schedule, which involves two injections of antigen without adjuvant given 14 days apart followed by serum collection 7 days later, provides the best means of raising immunosuppressive ALS. The advantage claimed for this procedure is the production of potent antisera without production of the toxic side effects which these authors have found to be associated with multiple injections of antigen or the use of adjuvants. It is true, however, that effective ALS has been raised by many groups using multiple injection schedules and a variety of adjuvants (e.g. Abaza et al., 1966; Gray et al., 1966; Denman and Frenkel, 1967).

In the present study it was found that the "two pulse" injection schedule did not regularly produce effective ALS. The use of Corynebacterium parvum as an adjuvant did not improve the potency of "two pulse" ALS, neither did its use result in ALS with toxic side effects. ALS preparations raised by giving three injections of lymphoid cells without adjuvant proved to be effective in immunosuppression, and the
use of such preparations revealed no evidence of any toxic side effects. The titre of anti-red cell activity was higher in such antisera, but since it was also found necessary to absorb "two pulse" ALS with mouse red cells before use, this cannot be considered to be a serious disadvantage.

Because of the *in vitro* effects of ALS on lymphocytes and its ability to produce lymphopenia *in vivo*, it appears most likely that interference with lymphocyte function underlies the ability of ALS to produce immunosuppression. There are, however, several possible ways in which ALS and lymphocytes might interact, with further different possibilities concerning the results of such interactions.

Early workers suggested that ALS exerted its immunosuppressive effects by causing gross destruction of peripheral blood lymphocytes, often in association with depletion of the cells of the organized lymphoid tissues. It was subsequently observed, however, that an immunosuppressive effect could be obtained in the absence of marked or sustained lymphopenia, and that the effect of ALS treatment was often greater than that obtained by means of thoracic duct cannulation (Levey and Medawar, 1966a and b; Iwasaki et al., 1967). Thus a simple theory based on the cytotoxic effect of ALS against all lymphocytes became an inadequate explanation, and led Levey and Medawar (1967a and b) to suggest two other possibilities: the "blindfolding" theory and the "sterile inactivation" theory. The blindfolding theory recognises the ability of ALS to coat lymphocytes without necessarily bringing about their destruction. Such coated lymphocytes might have their specific receptor sites blocked, thus frustrating the interaction.
between antigen and lymphocyte to produce an immune response. There is little direct evidence in support of such a theory, however, and it is difficult to explain on this basis why antibody fragments (which bind strongly to lymphocytes in vitro and in vivo) are very much less effective than intact ALS in producing immunosuppression (Harris and Harris, 1966a and b; Anderson et al., 1967; Woodruff, Reid and James, 1967; James, 1967). The sterile inactivation theory was suggested by the observation that lymphoid hyperplasia could accompany ALS treatment (Levey and Medawar, 1966b) in vivo, and that the addition of ALS to lymphocytes in culture stimulated blast cell transformation accompanied by the increased incorporation of radioactively-labelled nucleic acid precursors (Holt, Ling and Stanworth, 1966; Humphrey, Kauffman and Dunn, 1967; Woodruff et al., 1967). According to this theory ALS is a potent agent in bringing about activation of lymphocytes and their transformation to "sterile" forms no longer capable of responding to antigenic stimulation. Again there is no direct evidence in support of such a theory, and the finding that F(ab')₂ fragments of anti-lymphocytic antibodies, which are able to transform lymphocytes in vitro, are unable to inhibit immune responses in vivo gives it no support (Anderson, James and Woodruff, 1967; James and Anderson, 1967; Riethmüller et al., 1968). Furthermore, lymphoid hyperplasia and blast cell formation are not always observed in animals undergoing ALS treatment (Russe and Crowle, 1965; Nagaya and Sieker, 1965; Gray et al., 1966; Denman and Frenkel, 1968). The ALS-induced transformation of lymphocytes in vitro is most probably misleading in this respect since studies of this effect are usually performed in the absence of
complement - in the intact animal the presence of complement would be expected to favour a destructive effect rather than transformation.

There is now a large body of evidence that the major effect of ALS is to inactivate preferentially that part of the lymphocyte population which is directly concerned with the full potentiation of the immune response. Such cells are believed to be thymus-derived and to be long-lived (Claman, Chaperon and Triplett, 1966; Davies et al., 1967). Both long-lived and short-lived lymphocytes are concerned in the initiation of a primary immune response, and both types of cell are to be found in the peripheral lymphocyte population (Miller, 1967; Davies et al., 1968). Furthermore, it has been shown that the immune response of mice to sheep red cells requires the participation of two kinds of lymphocyte: thymus-derived cells which react specifically with antigen and another class of cells which differentiate into antibody forming cells. There is evidence to suggest that the antibody forming cells are a short-lived population derived from bone marrow precursors without thymic influence (Miller and Mitchell, 1968; Mitchell and Miller, 1968). The long-lived thymus-derived cells circulate constantly within the lymphatic system, and appear to be the cells which are depleted from the thymus-dependent areas of lymphoid organs on prolonged thoracic duct drainage (McGregor and Gowans, 1965). These areas have been shown to depend on the thymus for their cellular composition (Parrott, de Sousa and East, 1966; Goldschneider and McGregor, 1968).

Martin and Miller (1967) and Denman, Denman and Bembling (1968) have suggested that the immunosuppressive effects of ALS are due to
its ability to inactivate the long-lived thymus-derived lymphocytes of the circulating lymphocyte pool. There are at least five pieces of evidence in favour of such a hypothesis: (i) the recovery of immune competence after ALS treatment is dependent on the presence of an intact thymus (Jeejeebhoy, 1965; Monaco, Wood and Russell, 1965; Russell and Monaco, 1967; Leuchars, Wallis and Davies, 1968); (ii) the thymus-dependent areas of lymphoid organs are depleted of cells in ALS-treated animals (Turk and Willoughby, 1967; Denman and Frenkel, 1968a and b); (iii) thymus-derived cells are inhibited by ALS from being stimulated by antigen to undergo mitosis (Leuchars, Wallis and Davies, 1968); (iv) peripheral blood lymphocytes, but not spleen, lymph node or bone marrow cells, can be used to restore immunological competence to ALS-treated mice (Denman, Denman and Holborow, 1968), and (v) ALS-treated mice injected with normal thymus cells give a normal immune response to sheep erythrocytes, and irradiated mice restored with spleen cells from ALS-treated animals and normal thymus cells give an adoptive immune response similar to that obtained with spleen cells from untreated animals (Martin and Miller, 1968).

A deficiency of long-lived thymus-derived lymphocytes in ALS-treated animals could conceivably arise either because ALS specifically depletes this part of the population and has little effect on the short-lived lymphocytes, or because both populations are equally affected initially but the regeneration rates of the two cell types differ. Martin and Miller (1967) and Denman, Denman and Ebling (1968) implied that they favoured the first of these hypotheses. There is, however, no evidence that heterologous ALS can recognise antigenic differences on
thymus-derived lymphocytes. If ALS could differentiate directly between thymus-derived lymphocytes and other types of lymphocyte then it could provide an explanation for the varying immunosuppressive abilities of antisera raised against different lymphoid tissues. Presumably spleen, lymph nodes, thoracic duct lymph and thymus contain different numbers of thymus-derived lymphocytes, and might be expected to vary in ability to produce immunosuppressive ALS if anti-thymic cell activity is the most important factor. ALS raised against thymus cells has indeed been claimed to be more immunosuppressive than ALS raised against similar numbers of cells obtained from lymph nodes (Nagaya and Sieker, 1965 and 1967). However, the lymphopenia which is produced on injection of potent anti-lymphocyte sera is initially so large that it must include the removal from the circulation of at least some short-lived lymphocytes (Denman and Ziff, 1964; Woodruff and Anderson, 1964; Nagaya and Sieker, 1965; Gray et al., 1966; Kinne and Simmons, 1967), and indeed in the experiments of Denman, Denman and Emling (1968) an early drop in the number of short-lived lymphocytes was directly observed. At a later stage in these experiments, while the number of long-lived lymphocytes remained low, the short-lived cells attained greater numbers than were found in untreated animals - a finding which could mean that ALS has a stimulatory effect on the production of short-lived lymphocytes. These data can also be explained, however, if ALS affects both cell types equally but short-lived cells regenerate at a much faster rate, possibly until they reach a physiologically determined optimum number for circulating lymphocytes regardless of derivation. The short-lived lymphocytes of rodents are known to have a lifespan of between 5 and 7 days (Everett, Caffrey and Riske, 1964; Denman, Denman
and Embling, 1968), and in humans (Nowell, 1965) and rats (Miller and Cole, 1968) there is evidence to suggest that the life span of long-lived lymphocytes may be months or years. Thus, although the theory that the immunosuppressive ability of ALS is correlated with its ability to inactivate thymus-derived cells specifically is attractive in many respects, such an effect remains to be clearly demonstrated. While the effect of ALS on thymus-derived cells may be an important feature, an effect on other lymphocytes is rarely absent and may ultimately prove to be significant in the suppression of at least some aspects of the immune response.

The mechanism whereby peripheral lymphocytes are inactivated by ALS has most often been assumed to be due to complement-mediated lysis. But the finding of the present and other studies that in vitro cytotoxic titres do not always correlate well with in vivo lymphopenia and immunosuppression (Jeejeebhoy and Rabbat, 1968; Jooste et al., 1968; Greaves et al., 1969) suggests that some other effect is more important. A criticism of most studies of in vitro cytotoxicity is that the complement source differs from that of the animal species against which the ALS is directed. It might therefore be expected that the ability of ALS to produce lymphopenia in vivo would give a better indication of its ability to bring about immunosuppression. Such was the finding in the present work, although the correlation was not absolute - a few sera were found which produced an initial lymphopenia but which were not markedly immunosuppressive. Similar findings have been reported by others (Woodruff and Anderson, 1964; Nagaya and Sieker, 1965; Jooste et al., 1968). Production of lymphopenia, however, need not involve
cell lysis, there are other mechanisms whereby cells can be removed from the circulation in a specific manner. Opsonisation due to the fixation of antibody and complement is one such mechanism which would result in increased removal of lymphocytes by phagocytosis, and the results of Greaves et al. (1969) support this idea. In their experiments the ability of ALS against mouse lymphocytes to promote the uptake of lymphocytes by macrophages in vitro gave a better prediction of ability to prolong skin grafts than did other in vitro tests. ALS treatment has also been shown to alter the distribution of injected syngeneic lymphocytes in rats and mice - ALS treated cells, instead of homing to the lymphoid tissues, tend to be phagocytosed in the lung and liver (Seifert, Brendel and Picklraayer, 1968; Taub and Lance, 1968). Thus it appears that the ability to produce in vitro lysis of lymphocytes may be a property of ALS which is irrelevant to its immunosuppressive function.

One of the findings of the present study was that ALS treatment of parental strain mice could abolish the ability of spleen cell suspensions obtained from such animals to produce GVH reactions on injection into appropriate F1 hybrids. Since a very high correlation was found between in vitro cytotoxic titre and ability to suppress such GVH reactions, it is tempting to suggest that ALS acted by depleting the spleen of thymus-derived cells, thus ensuring that the cells transferred were of a type incapable of mediating a cellular immune reaction. The studies showing that ALS can affect the distribution of lymphocytes, however, suggest another possible interpretation of this finding: the spleen cells may have been phagocytosed by the macrophages of the lungs and liver instead
of lodging in the lymph nodes and spleen which are the main foci of GVH reactions in the mouse. Anti-lymphocyte antibodies digested to remove the Fc portions of the molecules were not cytotoxic to lymphocytes in vitro and were unable to suppress the GVH reaction. Because of the importance of the Fc portion of the IgG molecule in bringing about complement fixation and subsequent cell lysis, these findings again suggest that cell lysis could be an important factor in suppression of the GVH reaction. However, complement activation also contributes to other biological processes including the promotion of immune adherence and phagocytosis (Humphrey, 1967), and if the cells from the F(ab')2 treated animals in this experiment were less readily phagocytosed on injection into the F1 hybrids than were the cells from animals given whole anti-lymphocyte globulin, then substantial numbers of them may have reached the spleen and lymph nodes where they mounted a normal GVH response. It is thus not possible to decide on the basis of the experiments described here whether ALS acted by killing cells in the donors prior to transfer, or by altering the distribution of cells in the recipients after transfer. The finding of Brent, Courtenay and Gowland (1967), using a GVH system which involved transfer of allogeneic lymphoid cells to newborn mice, that incubation of such cells from ALS-treated mice with trypsin prior to transfer could restore their GVH-producing ability to normal levels, suggests that the second of the two explanations is more likely to be true.

While most investigators have concentrated on the effects of anti-lymphocyte serum on lymphocytes, amassing a considerable body of evidence that interference with the functions of cells of this type is the most
important factor in its immunosuppressive properties, it remains true
that ALS as it is normally prepared interacts with a number of other
biological processes. Field and Cook (1969) have demonstrated that
in guinea pigs ALS injection may stimulate adrenal function to produce
elevated levels of plasma cortisol; Nagaya and Sieker (1967) believe,
on the basis of the increased effectiveness of ALS raised against
thymus cells, that ALS may antagonise the hypothetical thymus humoral
factor; and it has been shown in many laboratories that ALS is at
least as antigenic as, and possibly more antigenic than, non-antibody
serum proteins (Denman and Frenkel, 1967; Lance and Dresser, 1967;
Howard, Asfis and Woodruff, 1968; Jasin et al., 1968). This latter
finding raises the possibility that antigenic competition could account
for the lack of response in animals given ALS and a second antigen.
Such a mechanism, however, is unable to explain the ability of ALS to
suppress the immune response in already sensitized animals (Levey
and Medawar, 1966a), neither can it explain the immunosuppressive capacity
of rabbit anti-mouse lymphocyte globulin in mice previously rendered
tolerant of rabbit gamma globulin (Lance and Dresser, 1967). The role
of antibody to thymus humoral factor remains to be determined, but it
cannot explain the immunosuppressive effects of ALS completely since
non-thymic lymphocytes have frequently been found capable of inducing
effective antisera. The increased level of serum cortisol reported by
Field and Cook is a finding which merits further study in view of the
well-known immunosuppressive properties of steroids—again this is
unlikely to provide a complete explanation for the action of ALS, however.
Small doses of ALS and cortisone have been found to act synergistically
in producing immunosuppression (Hoehn and Simmons, 1967), an effect which is most easily explained on the assumption that ALS and cortisone produce immunosuppression by different mechanisms.

It has also been claimed that anti-lymphocyte sera contain antibodies which interfere with macrophage function (Field and Hughes, 1969; Loewi et al., 1969), and that anti-macrophage sera have immunosuppressive (Unanue, 1968; Panjel and Cayeux, 1968; Loewi et al., 1969) and phagocytosis-inhibiting properties which might affect the initiation of an immune response (Jennings and Hughes, 1969). The dendritic macrophages of lymphoid follicles are thought to be important in the retention of antigen and the initiation of some immune responses (Hanna and Szakal, 1968; Nossal et al., 1968), so that if ALS also commonly affects macrophages then interference with the function of these specialized antigen-trapping cells might well prove to be of major importance in producing immunosuppression.

At present, however, the evidence is very much in favour of a direct effect of ALS on the lymphocyte as the mechanism of its immunosuppressive action. Each of the secondary effects discussed above may play a supporting role, but it seems most likely that their usefulness lies in the explanation which they may provide for the great variation which has been found in the properties of ALS batches raised in different ways.

III. ANTI-LYMPHOCYTE SERUM AND NZB MICE

In the present study it was found that the regular administration of ALS, known to be immunosuppressive in other situations, failed to have a marked effect on the development or maintenance of autoimmune
disease in NZB mice. When given to young adults in which no signs of disease were obvious, but which were expected to develop such signs within a few weeks, ALS was associated with a slight delay in onset, but the treated animals eventually developed signs of disease indistinguishable from those occurring in control animals. In older animals, in which signs of disease were already apparent when ALS treatment was begun, a similar slight effect which was not maintained was also observed.

If ALS exerts its immunosuppressive effect by antibody-dependent complement-mediated lysis of circulating lymphocytes, then failure of this process to occur in NZB mice might explain the ineffectiveness of ALS in suppressing auto-immune disease in these animals. Such an explanation would agree with the finding of Norins (1965) that NZB mice lack one of the serum components of complement and that NZB serum is ineffective as a complement source in the \textit{in vitro} lysis of heterologous erythrocytes. It was, however, demonstrated in the present study that the administration of ALS to NZB mice (and to mice of the DBA/2 strain which also are complement deficient) produced a lymphopenia comparable to that produced in normal mouse strains. It seems, therefore, that ALS can reduce the numbers of circulating lymphocytes in the absence of complement-mediated lysis - a finding which is in agreement with the previously-discussed report of Greaves \textit{et al.} (1969) showing that the most important feature in immunosuppression by ALS may be its ability to promote opsonisation of lymphocytes leading to their phagocytosis. There is no evidence that NZB mice are deficient in the complement components which are required to bring about the
activation of C', at which stage in the complement sequence the phenomenon of immune adherence leading to increased phagocytosis can be demonstrated (Humphrey, 1967).

Denman, Denman and Holborow (1967) found that the development of Coombs-positive haemolytic anaemia in NZB mice could be prevented by regular administration of the globulin fraction of ALS if treatment was begun before 2 months of age. They were, however, unable to influence the disease process by giving ALS to 10-month old NZB mice in which positive Coombs tests were well established. The findings of that group, together with those in the present study, suggest that the events responsible for autoimmune disease production in NZB mice take place early in life, long before disease symptoms become apparent, and can only be influenced by ALS treatment given at an early stage.

A requirement for early treatment has also been demonstrated in a number of studies in which the administration of ALS has been found to influence the development of experimentally induced autoimmune lesions. ALS has been found to suppress the development of auto-allergic encephalomyelitis in guinea pigs (Waksman, Arbouys and Arnason, 1961; Leibowitz, Lessof and Kennedy, 1968), adjuvant-induced arthritis in rats (Curry and Ziff, 1966 and 1968), and thyroiditis in rats (Kalden et al., 1968). In the arthritis and the thyroiditis situations, however, ALS was only effective if treatment was begun before the diseases became established, and Leibowitz, Lessof and Kennedy found that in guinea pigs with already established encephalomyelitis although ALS could suppress clinical symptoms of the disease and slightly prolong survival times it had no effect on the inflammatory lesions found in the brain.
It is tempting to suggest that an immunological sensitization to auto-antigens is involved, both in the NZB mice and the experimental diseases, and that once sensitization has taken place ALS is much less effective as an immunosuppressive agent, perhaps because the effector cells which result no longer have lymphocyte antigens. Such an explanation would agree with the numerous reports that ALS is most effective in suppressing immune responses if administered prior to antigens such as heterologous erythrocytes or serum proteins (Berenbaum, 1967; Ficklmayr, Brendl and Zenker, 1967; James, 1967), and that ALS is much less effective against secondary immune responses than it is at suppressing primary responses (Monaco et al., 1966b; James and Anderson, 1967; Levey and Medawar, 1966a).

There are, however, two pieces of evidence suggesting that ALS can affect the cells responsible for autoantibody production in mature NZB mice. In the experiments of Denman, Denman and Holborow it was found that spleen cells from old Coombs-positive NZB mice lost the ability to transfer Coombs-antibody production to young NZB mice if the cells were previously incubated with ALS in vitro, and the present study showed that (NZB x CBA)F1 hybrid mice given ALS 4 hours prior to transfer of spleen cells from old NZB mice did not subsequently become Coombs-positive or produce antibodies capable of agglutinating papain-treated homologous erythrocytes.

The possibility has already been advanced in this discussion that the autoimmune disease of NZB mice is composed of two components: tissue destruction, possibly due to viral activity in spleen cells, followed by sensitization to erythrocyte antigens. If tissue
destruction reaches higher levels with age then it may be that the resulting continuous production of newly sensitized cells masks any effect that ALS may have on those cells already producing autoantibodies. Thus it may be that the control of already established autoimmune disease in NZB mice requires a combination of immunosuppressive treatment with the administration of anti-viral agents. Although there has been at least one unsuccessful attempt to influence the autoimmune disease of NZB mice using anti-viral therapy designed to raise the level of interferon activity in such animals (Russell, 1968), it would be very interesting to test the effect of a combination of ALS and an interferon inducer in this situation. It is of interest in this respect that in the kidney disease of (NZB x NZW)F1 hybrids, where there is good evidence that an auto-antigen may not be involved and that the target for the immune response leading to lesion formation is a virus or virus product (Mellors, 1968), ALS therapy has been found unable to influence the development of kidney disease (Denman, Denman and Holborow, 1966; Strom et al., 1968).

IV. IMMUNOLOGICAL HYPER-REACTIVITY OF NZB MICE

The findings that young NZB mice have a higher than normal background number of spleen cells capable of forming antibody against sheep erythrocytes (Diener, 1966), and that NZB mice can respond to stimulation with sheep erythrocytes a few days after birth when the response in normal mice is absent or very limited (Playfair, 1967; Evans, Williamson and Irvine, 1968) suggest the possibility that the underlying abnormality of NZB mice may be a hyper-reactivity to all antigenic stimuli. If as a result of such hyper-reactivity NZB mice are
correspondingly more difficult to render tolerant of specific antigens, then a defect in tolerance induction to self-antigens might result in the emergence of autoimmune disease. Thus it seemed worthwhile to examine the immune response of NZB mice to the soluble protein antigen bovine serum albumin (BSA) since the ability to render preparations of BSA free of aggregates makes it possible also to assess the ease with which animals can be made immunologically tolerant of this antigen (Dresser and Mitchison, 1968).

It was found that young NZB mice and (NZB x CBA)F1 hybrid mice responded to immunization with BSA by producing much larger amounts of antibody than four normal strains. When ultra-centrifuged BSA was administered prior to challenge with an immunizing dose of BSA, the immune responses of the normal strains and of the (NZB x CBA)F1 hybrid animals were found to be much reduced, whereas a significant proportion of the NZB animals was unaffected by such pretreatment. Similar results have been reported by others using bovine gamma globulin (BGG) as antigen: NZB mice and (NZB x NZW)F1 hybrid mice produced higher titres of antibody than normal non-autoimmune strains, and pretreatment with ultra-centrifuged BGG failed to induce tolerance (Braverman, 1968; Staples and Talal, 1968 and 1969). NZB mice, however, do not respond abnormally to all antigens. Playfair (1968) has reported that the response to pig or chicken erythrocytes does not differ markedly from that found in Balb/c and C57Bl strains, while McDevitt and Chinitz (1969) found that the NZB response to a synthetic polypeptide antigen was neither much higher or lower than the responses given by eight other inbred mouse strains.
Nevertheless it seems that NZB mice and mice which have one NZB parent give an enhanced immune response to a number of extraneous antigens and are more difficult to render immunologically hypo-responsive than other strains. The enhanced responsiveness could be due to a more efficient processing mechanism for antigen in NZB mice since it has been shown that the ability of antigens to produce tolerance or immunity is related to the degree to which they are phagocytosed by cells of the reticulo-endothelial system (Frei, Benacerraf and Thorbecke, 1965). There is no direct evidence bearing on this possibility in NZB mice, but it seems unlikely to be important since it has been shown in a number of ways that factors directly affecting lymphoid cells are of prime importance in tolerance studies - Golub and Weigle (1967), for instance, have shown that treatment with bacterial endotoxin may prevent tolerance induction independently of the activity of phagocytic cells.

If the enhanced response of NZB mice to certain antigens is indeed an inherent property of lymphoid cells the exact nature of the abnormality involved is a matter for speculation at this point. The possibilities include an increased number of antigen reactive precursor cells or a defect in the regulatory system which presumably controls the proliferation of immune cells in response to antigenic stimuli. It is interesting to speculate that the intimate association of murine leukemia virus with lymphoid cells (including the budding of virus at cell surfaces), which has been found in NZB mice and related strains (Mellors and Huang, 1966 and 1967; East and Prosser, 1967; Hollman and Verly, 1967; Prosser, 1968), could influence the behaviour of such cells in
their response to antigenic stimulation. Weir (1967) has suggested that the interaction of virus with lymphoid cell surfaces could interfere with normal tolerance induction processes to tissue antigens. It is, however, true that in the overwhelming majority of instances viral infection is associated with an immunosuppressive effect tending to make tolerance induction easier rather than more difficult (Peterson, Hendrickson and Good, 1963; Dent, Peterson and Good, 1965; Salomon and Wedderburn, 1966; Cremer, 1967; Siegel and Morton, 1966). Nevertheless it has been shown that infection of mice with lactic dehydrogenase virus can increase antibody formation and prevent the induction of tolerance to protein antigens (Notkins et al., 1966; Mergenhagen, Notkins and Dougherty, 1967), and it may be that the virus of NZB mice has similar properties.

The nature of the mechanism whereby young NZB mice, and mice which have an NZB component in their genotype, can give an enhanced immune response remains to be elucidated. Effort expended in this area will surely prove rewarding since it seems most unlikely that the association of heightened responsiveness to exogenous antigens is unrelated to the heightened immune responsiveness to auto-antigens which must constitute, at least in part, the phenomenon of autoimmune disease.
CONCLUSION
The findings described and discussed in this thesis, together with the related findings of others, clearly indicate that mice of the NZB strain develop a disease which has a number of features in common with human autoimmune conditions. Most important among these features are the occurrence of antibodies reactive with autologous constituents and the presence of proliferative abnormalities of lymphoid tissues. Early suggestions that the disease of NZB mice might be due to an inherited predisposition to develop clones of lymphoid cells with self-reactivity, or alternatively to a genetically-determined inability to eliminate such clones as they arose, while not formally excluded as possible explanations, are difficult to reconcile with a number of more recent observations. Of particular significance here are the inability to transfer signs of the disease with cells other than spleen cells, the lack of beneficial effect from neonatal thymectomy, and the significance of the relationship between neoplasia and autoimmunity. While there can be no doubt that there is a genetic component in the development of autoimmunity in NZB mice it seems likely that an interaction between genetic factors and some other factor underlies the occurrence of abnormalities in this strain. The most likely candidate as this secondary component is a virus.

Virus particles with morphological features reminiscent of type 'C' murine leukemia virus have been found frequently in NZB mice. Such particles have been found within cells and budding from cell surfaces; in cells of lymphoid organs, liver, pancreas, kidney and lungs; in embryos and in mice of all ages; and in fostered germ-free as well as conventionally reared animals. Thus there is good evidence
that virus particles are ubiquitous within this strain and are very likely transferred from generation to generation via the germ cells or the placenta. It cannot be ignored that virus particles of similar morphology in the electron microscope have been shown to be associated with the malignant proliferation of reticulum cells and the development of lymphocytic, erythroid and myeloid leukemias in other mouse strains and in other species.

In the introductory section of this thesis various ways were discussed whereby infection with micro-organisms might conceivably provoke autoantibody production. Those most likely to concern virus infection of NZB mice include the possession by the virus of antigens similar to normal tissue antigens (such as are found in the autoantibody production which may occur in association with streptococcal or E. coli infection of humans), or the exertion of an adjuvant effect which presumably would alter the handling of autoantigens so as to produce an autoimmune response.

If the virus of NZB mice provokes autoantibody formation by means of cross-reaction between antigens of the virus and NZB antigens, then it must be supposed that the virus does not induce immunological tolerance despite its presence during embryonic life or that such tolerance breaks down at a later stage. A possible parallel for such a situation is provided by recent findings concerning the infection of mice with lymphocytic choriomeningitis virus - if mice are injected in utero or shortly after birth with this virus then choriomeningitis does not develop but an apparently symptomless infection results and virus can be isolated from blood and organs of adult mice. This has been supposed
to be due to the induction of immunological tolerance to the virus, but Oldstone and Dixon (1967) have shown that antibody to virus can regularly be demonstrated in such infected mice and that tolerance, if present, is only partial. Indeed in a high proportion of old infected mice glomerulonephritis, most probably caused by the deposition of virus–antibody complexes in the kidney, is found (Hotchin and Collins, 1964; Baker and Hotchin, 1967). Such a mechanism might be involved in the kidney disease of NZB mice, but it is difficult to see how an immune response of NZB mice against a contaminating virus could induce antibodies which would react with tissue antigens of all mouse strains – unless the virus possessed an antigen which was widespread in mice or unless all mouse strains possess small amounts of virus antigens, possibilities for which there is no supporting evidence at present.

There are a number of ways in which virus could theoretically alter the handling of NZB antigens so as to provoke an abnormal immune response. Viruses have been shown in a variety of systems to induce the formation of new antigens at the surfaces of cells (Rubin, 1961; Hamburg and Svet-Moldovsky, 1964; Roisman and Spring, 1967; Trevethia et al., 1968) and this could provide a method whereby normally non-antigenic components of NZB cells are presented to the lymphoid system in a way which results in autoantibody formation. It is also possible that virus infection might have a direct effect on lymphocytes so as to alter their ability to produce an immune response – the evidence that virus infection can alter immune responses in a number of species, usually towards immunosuppression but occasionally towards a heightened
response, has already been discussed. Infection with C. parvum or E30, or treatment with endotoxin have been shown to affect lymphocytes in such a way that normally tolerogenic antigens produce immunity (Dresser, 1962; Glaman, 1963; Pinckard, Weir and McBride, 1967), and it is possible that persistent infection of NZB mice with virus might produce a similar effect.

There are thus many ways in which virus infection could be involved in the disease process of NZB mice - but little evidence at present to indicate which are the most important. The first priority in continuing the study of NZB mice ought to be an attempt to discover what part, if any, virus infection plays in the production of tissue damage (to kidneys, erythrocytes, perhaps liver) in members of this strain. It may be that a full understanding of the role of virus in NZB mice will not be obtained until the virus can be isolated and studied in an in vitro system, or until NZB mice free of virus can be obtained. However, it appears clear that the presence of virus alone cannot be the only factor in autoimmunity in NZB mice, for a number of other mouse strains contain morphologically identical viruses but do not develop autoimmune disease. An explanation of the part that genetics plays in NZB mice is also required - it may be (as suggested by the presence of immunological hyper-reactivity) that NZB mice inherit an inability to develop or maintain tolerance, or that NZB cells (particularly erythropoietic cells) are inherently more easily damaged by virus infection than cells of other mouse strains.

Whatever the answers to these questions ultimately prove to be they will doubtless increase our understanding of many human autoimmune diseases.
ACKNOWLEDGEMENTS
I would like to thank Sir Michael Woodruff, my principal supervisor, and Dr. James Howard for the encouragement, help and wise counsel which they so generously provided while this study was in progress. I am particularly grateful to Sir Michael for giving me the opportunity to work in his laboratory.

Dr. Keith James and Dr. Norman Anderson of the Department of Surgical Science, and Dr. Donald Weir, Dr. Chris Elson and Mr. William McBride of the Department of Bacteriology, helped me with many aspects of this work and I would like to express my gratitude to them.

I owe much to the members of the Technical Staff of the Department of Surgical Science, but to Miss Valerie Curran, who worked with me throughout this investigation, special thanks are due. I shall always be grateful to her for the way in which she carried out her duties.

The Distillers Company Limited provided the major part of the financial support required for this investigation.
REFERENCES


Asherson, G.L. and Dumonde, D.C. (1963) Immunology, 6, 19. Autoantibody production in rabbits. II. Organ-specific auto-antibody in rabbits injected with rat tissue.


Brunstetter, F.G. and Claman, H.N. (1968) Transplantation, 6, 485. Impairment of delayed hypersensitivity in uremic patients by ALS.


Studies on the effect of induced immune lymphopenia. I. Enhanced effects of rabbit anti-rat lymphocyte globulin in rats tolerant to rabbit immunoglobulin G.


Diener, E. (1966) Int. Arch. Allergy, 30, 120. The immune response in NZB and NZB x C3H F1 hybrid mice as measured by the haemolysin plaque technique.


Dodd, B.E. and Eeles, D.A. (1961) Immunology, 4, 337. Rh antibodies detectable only by enzyme technique.


   The mechanism of immunological paralysis.


   Enzyme reacting specific auto-antibodies in individuals showing no signs of auto-immune haemolytic anaemia.


   Autoimmunity and malignancy in New Zealand Black mice.


East, J., de Sousa, M.A.B., Prosser, P.R. and Jaquet, H. (1967a) 


East, J., Prosser, P.R., Holborow, E.J. and Jaquet, H. (1967b) 

   Lancet, 1, 755. Autoimmune reactions and virus-like particles in germ-free NZB mice.


mouse spleen: histologic and ultrastructural autoradiographic studies of the secondary immune reaction.

Suppression of rabbit lymph node cells by rabbit anti-leucocyte serum demonstrated in vitro by the antibody plaque test.


Holmes, M.C. and Burnet, F.M. (1964b) Heredity, 12, 419. The inheritance of autoimmune disease in mice: a study of hybrids of the strains NZB and C3H.


In vivo antibody production by spleen cells after incubation in vitro with heterologous antilymphocyte plasma.


Experimental auto-immune thyroiditis in the rat.


Notes on the preparation and assay of anti-lymphocytic serum for use in mice.


II. Antinuclear reaction of Y-globulin eluted from homogenates and isolated glomeruli of kidneys from patients with lupus nephritis.


van Loghem, J.J. et al. (1963) Vox Sang., 3, 33. Serological and immunochemical studies in haemolytic anaemia with high titre cold agglutinins.


The antibody response of rats depleted of lymphocytes by chronic drainage from the thoracic duct.


Makela, O. and Mitchison, N.A. (1965) Immunology, 8, 539. The effect of antigen dosage on the response of adoptively transferred cells.


Panjiel, J. and Cayeux, P. (1968) Immunology, 14, 769. Immunosuppressive effects of macrophage antiserum.

Pappenheimer, A.M. (1917) J. exp. Med., 26, 163. Experimental studies upon lymphocytes. II. The action of immune sera upon lymphocytes and small thymus cells.


Playfair, J.H.L. (1968b) Immunology, 15, 815. Strain differences in the immune response of mice. II. Responses by neonatal cells in irradiated adult hosts.


Production of specific rabbit thyroid antibodies in the rabbit.

Studies on organ specificity. IV. Production of rabbit thyroid antibodies in the rabbit.

Studies on organ specificity. V. Changes in the thyroid glands of rabbits following active immunization with rabbit thyroid extracts.


Autoantibodies against spermatozoa in sterile men.


Heterologous anti-lymphocyte sera and some of their effects.

I. Lymphocytotoxic effect of rabbit-anti-rat lymphocyte antiserum.


The immunodepressive effect of Friend virus.


Pathological changes of thymic epithelial cells and autoimmune disease in NZB, NZW and (NZB x NZW)F1 mice.


Weir, D.M. (1967a) Immunological methods applied to the study of


The effect of lymphocyte depletion by thoracic duct fistula and the administration of anti-lymphocytic serum on the survival of skin homografts in rats.


