THE DEVELOPMENT OF THE IMMUNE RESPONSE IN MICE

by

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In order to establish whether the autoimmune phenomena found in NZB mice originate from a disease process involving the antigen or the antibody forming system, the immune response to heterologous erythrocytes was studied in this and in control (Ju and CBA) strains of mice. The response of adult NZB to sheep erythrocytes showed little difference from the adults of the control strains although the lag period between the injection of antigen and the appearance of the antibody forming cells is longer in the NZB than in the controls.

The ontogeny of the immune response to sheep erythrocytes in the neonatal period was then investigated since it is probably in the neonatal period that the immunological recognition of autologous components is established. A good antibody forming cell response was found in the spleens of NZB mice at an age when the control strains were showing a negligible response. In addition, the response of the immature NZB had a shorter lag period between antigen injection and antibody response than the adult NZB while the response of the immature control strain babies had a considerably longer lag period than that of the adults. The response of the limb-draining lymph nodes appeared at the same age as did the response in the spleen of the NZB, but in control strains no response was detectable in the lymph nodes more than 2 weeks after the spleen was showing a good response.
No difference in the growth rate of the animals or their lymphoid organs nor of the structural maturation of the lymphoid organs could be found to account for these differences in the immune responses of the baby mice.

The anamnestic response following a single priming exposure to antigen was not very different in control and NZB mice, suggesting that the difference in the primary response occurs in the early part of the response prior to memory cell production. When the animals were injected with sheep erythrocytes every 2 days beginning at 1 day of age there was a transient sensitization in both NZB and Ju, followed by decrease of the response. This tolerance was profound in the Ju, but only partial in the NZB over a period of 10 weeks.

When the clearance of labelled SRBC was compared in NZB and controls at an age when NZB were showing an antibody response and the Ju were not, neither strain showed an immune type of elimination but the control strain may have eliminated the antigen slightly faster than the NZB.

Comparison of the responses of the NZB and Ju to chicken erythrocytes showed no difference between them, but since neither strain showed any significant response to the chicken erythrocytes until an age beyond that at which the differences of the responses to sheep erythrocytes were most in evidence it is possible that the function responsible for the abnormal response to the sheep erythrocytes is not involved in the response to the chicken erythrocytes.

The possible relationships of the early
hyperresponsiveness and autoimmunity were considered and it was suggested that all the observed effects could be explained by the presence in the NZB of a population of cells that is able to produce an immune response to certain antigens given in a form which in most animals induces tolerance.
# CONTENTS

1. Introduction  
   1.1 Immunoglobulins and antibodies  
   1.2 The Immune Response  
      1.2.1 Antigen uptake  
      1.2.2 The Function of Macrophages in the Immune Response  
      1.2.3 Interactions of lymphocytes  
      1.2.4 Immunoglobulin sequence in the immune response  
      1.2.5 The origin and fate of the cell populations  
      1.2.6 Cell mediated immunity  
   1.3 Tolerance  
   1.4 Self Recognition  
   1.5 Autoimmunity  

2. Review of the literature on the development of the immune response  
   2.1 Introduction  
   2.2 Morphological development of the immunological system  
   2.3 Maturation of antibody forming capacity  
      2.3.1 The synthesis of immunoglobulins  
      2.3.2 Production of specific antibody  
   2.4 Maturation of cell mediated immunity  
   2.5 Maturation of antigen-trapping mechanism  
   2.6 Transfer experiments  
   2.7 Conclusion  

3. Review of the literature on NZB mice  
   3.1 Clinical aspects of the disease syndrome  
   3.2 Genetic studies  
   3.3 The effects of treatment  
   3.4 The role of the thymus  
   3.5 Adoptive transfer of the disease  
   3.6 The role of the virus
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7 The immune response to heterologous antigens</td>
<td>92</td>
</tr>
<tr>
<td>3.8 Artificial induction of autoimmunity</td>
<td>95</td>
</tr>
<tr>
<td>3.9 Conclusions</td>
<td>96</td>
</tr>
<tr>
<td>4. Materials and Methods</td>
<td>98</td>
</tr>
<tr>
<td>4.1 Materials</td>
<td>98</td>
</tr>
<tr>
<td>4.2 Animals</td>
<td>99</td>
</tr>
<tr>
<td>4.3 Antigen challenge</td>
<td>99</td>
</tr>
<tr>
<td>4.4 Detection of the immune response</td>
<td>100</td>
</tr>
<tr>
<td>4.5 Iodination of antigen</td>
<td>102</td>
</tr>
<tr>
<td>4.6 Antigen clearance</td>
<td>103</td>
</tr>
<tr>
<td>4.7 Detection of autoimmune phenomena</td>
<td>103</td>
</tr>
<tr>
<td>4.8 Histology</td>
<td>105</td>
</tr>
<tr>
<td>4.9 Statistical analysis</td>
<td>105</td>
</tr>
<tr>
<td>5. Autoimmune phenomena in NZB</td>
<td>106</td>
</tr>
<tr>
<td>5.1 Introduction</td>
<td>106</td>
</tr>
<tr>
<td>5.2 Relationship of age, anaemia and erythrocyte autoantibody</td>
<td>106</td>
</tr>
<tr>
<td>5.3 Gel filtration of autoimmune sera</td>
<td>107</td>
</tr>
<tr>
<td>5.4 Staining for globulin in kidneys and for antinuclear factor</td>
<td>108</td>
</tr>
<tr>
<td>5.5 Discussion</td>
<td>108</td>
</tr>
<tr>
<td>6. The immune response of adult mice to SRBC</td>
<td>110</td>
</tr>
<tr>
<td>6.1 Introduction</td>
<td>110</td>
</tr>
<tr>
<td>6.2 The response of animals at various intervals after injection</td>
<td>112</td>
</tr>
<tr>
<td>6.2.1 The spleen</td>
<td>112</td>
</tr>
<tr>
<td>6.2.2 The lymph nodes</td>
<td>115</td>
</tr>
<tr>
<td>6.2.3 The thymus</td>
<td>116</td>
</tr>
<tr>
<td>6.2.4 The relationship of the response in the spleen and the lymph nodes</td>
<td>116</td>
</tr>
<tr>
<td>6.3 The relationship of dose and response</td>
<td>117</td>
</tr>
<tr>
<td>6.4 Discussion</td>
<td>118</td>
</tr>
</tbody>
</table>
7. The development of the immune response in baby mice
   7.1 Introduction
   7.2 Background plaques in uninjected mice
   7.3 The immune response of mice of various ages
      7.3.1 The spleen
      7.3.2 The lymph nodes
      7.3.3 The relationship between the response of the spleen and lymph nodes
   7.4 The maturation of the form of the immune response
      7.4.1 The spleen
      7.4.2 The lymph nodes
      7.4.3 The thymus
   7.5 Test for a 'hypersensitivity factor'
   7.6 Discussion

8. The growth of the body and lymphoid organs
   8.1 Introduction
   8.2 Weight
   8.3 The size of the spleen
   8.4 The size of the lymph nodes
   8.5 The structure of the thymus
   8.6 The structure of the spleen
   8.7 The structure of the lymph nodes
   8.8 Discussion

9. The secondary response and tolerance
   9.1 Introduction
   9.2 The effect of the dose of the primary injection on the secondary response
   9.3 The effect of the time of the primary injection on the secondary response
   9.4 The shape of the secondary response
   9.5 The effect of multiple primary injections
1. INTRODUCTION

An antigen is a substance which when introduced into the body of a vertebrate induces an immune response. This response has two, possibly separate, aspects (see Cooper, Gabrielson, and Good, 1967). Cell-mediated immunity results in the appearance of large numbers of cells specifically sensitized and able to respond to the introduced antigen, and it is represented in such immunological functions as the destruction of foreign tissue (graft rejection), the destruction of an immunologically incompetent host by an immunologically competent graft (graft versus host reaction) or delayed hypersensitivity type skin reactions. The humoral antibody response results in the appearance, in the serum of the reacting animal, of immunoglobulins able to react specifically by binding with the introduced antigen; these are the antibodies.

1.1 Immunoglobulins and antibodies

Immunoglobulins are an extremely heterogeneous group of proteins and they comprise about 15-25% of the total serum proteins. To understand how these proteins are able to react specifically with an antigen, it is necessary to know something about their structure. The following account has been extracted from reviews by Cohen and Milstein (1967a,b) and Putnam (1969).

The basic immunoglobulin unit consists of a complex of two 'heavy' chains each with a molecular weight of about
50,000, and two 'light' chains each with a molecular weight of about 20,000. These four polypeptide chains are joined together into a unit as shown in Fig. 1.1.1. When the immunoglobulin molecule is cleaved with pepsin or papain, the antibody combining site is found to be situated at the 'N' terminal end. There are two such combining sites for each antibody molecule and each site contains one light and one heavy chain.

An individual animal is able to produce three main classes of immunoglobulin, and some minor ones. The main classes, IgG, IgA and IgM may be differentiated by antigenically recognisable isotypic differences in the heavy chains (\(\gamma\), \(\alpha\), and \(\mu\) respectively). IgG and IgA both consist of the basic four chain molecule as illustrated in Fig. 1.1.1; they have a molecular weight of about 150,000, and a sedimentation coefficient of about 78. IgM consists of polymers of five of these basic units; it has a molecular weight of about 900,000 and a sedimentation of about 198.

There are, in addition to these heavy chain isotypes, two main classes of antigenically recognisable light chains. These are the kappa and the lambda chains. Within these major classes there are many minor subclasses producing isotypic differences within the individual and allotypic differences between individuals within the species. The amino acid sequences for many of these chains have been determined and as might be expected differences are found between the classes and the subclasses. Within each of the subclasses the 'C' terminal halves of the chains are
Fig. 1.1.1. Diagrammatic representation of an immunoglobulin adapted from Cohen and Milstein (1967) and Green (1969). The stippled areas indicate the variable portions of the chains.
relatively constant, but the 'N' terminal halves show considerable variation in many positions.

An antibody combining site is made up of the combined 'N' terminal ends of a light and a heavy chain. Any light chain can combine with any heavy chain although separated heavy chains from myelomas of different origins are found to recombine with their own light chains in preference to those from other myelomas (Grey and Mannik, 1965). Light and heavy chains from antibodies of a single specificity also seem to have a preferential affinity for one another (Roholt, Radzimski and Presman, 1965, 1966). Since antibodies can be made to resume their specific activity after complete unfolding it seems that antibody specificity is determined by the amino acid sequence of the light and heavy chains. This variation of the 'N' terminal end of the polypeptide chains could be innate to the animal in which case the antigen would act by selecting and stimulating the production of immunoglobulin of best fit, or the variation could be produced by the antigen itself providing some kind of template to induce the synthesis of a complementary immunoglobulin. Nossal, Williams and Austin (1967) and Nossal, Ada and Austin (1965) have shown that antibody forming cells generally do not contain antigen so that if instruction occurs it must be by means of some intermediary such as the RNA from macrophages suggested by Fishman (e.g. 1969).

Selection of antibody could occur on a subcellular or on a cellular level. Selection on a subcellular level
requires that the antigen discriminate among the different immunoglobulin polypeptides that a cell could produce and de-repress production of that which is the best antibody. Selection on a cellular level requires the antigen to stimulate the proliferation of any cell that is producing a suitable immunoglobulin. Instructive theories and sub-cellular selection both imply that a cell is multipotent until it makes contact with the antigen and becomes 'committed'. Selection on a cellular level implies that the cell is limited to the production of one immunoglobulin before the exposure to antigen, though it does not imply that the cell or its progeny should not change their specificity with a limited frequency. Osoba (1969), by transferring limiting numbers of spleen cells to irradiated recipients has shown that the cell populations responding to chicken and to sheep erythrocytes are different. Serial transfers of small numbers of cells through irradiated recipients however show that the determination is not permanent (Feldman and Mekori, 1966; Trentin, Wolf, Cheng, Pahlberg, Weiss and Bonnag, 1967). Cells able to respond to Salmonella flagellin could be selectively destroyed with highly radioactive Salmonella flagellin without affecting the response of the cell suspension to other antigens (Ada and Byrt, 1969). Cells able to respond to antigen may be removed from cell suspensions by passing the suspensions through an antigen-coated glass column (Abdou and Richter, 1969d; Daguillard and Richter, 1970; Singhal and Wigsell, 1970). Furthermore, tolerance, where the exposure to an
antigen results in the inhibition of the response on subsequent exposure to that antigen, is best explained by the specific inactivation of the cells able to respond to that antigen (see Section 1.3). Thus it appears that the antibody specificity of immunoglobulin-producing cells is determined prior to antigen exposure. There have been reports that individual antibody-forming cells produce antibody of only one specificity at one time (Green, Vassalli, Nussenzweig and Benacerraf, 1967; Näkel, 1967; Germon, Bauminger, Sela and Feldman, 1968) and to the contrary (Schwartzman, 1967; Michael and Marcus, 1968; Hiramoto and Hamlin, 1965), but the interpretation of these as evidence for or against cell selection is complicated by the fact that the cell that reacts initially with the antigen is not the same cell that produces the antibody (see Section 1.2.3). The evidence and current thought seem to favour a cell selection mechanism and the subject of selection of cells by antigen has been reviewed by Siskind and Benacerraf (1969). It thus appears that variation of the 'N' terminal ends of the amino acid chains is intrinsic to the organism. This variation probably originates from a large number of cistrons coding for alternative 'N' terminal sequences for the polypeptide chains rather than from somatic mutation in the variable portions of the chains, since the ability of an animal to respond to an antigen seems to be genetically controlled. Where the antigen is simple enough, the ability to respond to it can frequently be seen to be under the control of a single gene (see
McDevitt and Benacerraf, 1969). It is possible that in addition, somatic mutation may be superimposed on this genetic variation.

An individual cell will only produce one type of immunoglobulin at any one time. This applies both to the isotypes (Shearer, Cudkowicz, Connell and Priore, 1968; Merchant and Brahmi, 1970) and to allotypes (Bosma and Weller, 1970). This also explains the uniformity of the immunoglobulin produced in myeloma type diseases which are supposed to be caused by the uncontrolled proliferation of one aberrant cell.

1.2 The Immune response

For the production of an immune response, two separate events must occur. First, the antigen must be detected as foreign and its specific structure identified and it is the ability to be so recognised that gives a substance its property of antigenicity. Second, the processes of sensitization and antibody production must be initiated and it is the ability to do this that gives an antigen the property of immunogenicity. A substance cannot be immunogenic without being antigenic, but, since in particular circumstances, exposure to an antigen will not produce an immune response but will nevertheless alter the response of the animal on subsequent exposure to the same antigen it is possible for a substance to be antigenic without being immunogenic. The antigenicity and immunogenicity of a substance will depend not only on its own properties but also on the state of the animal to which it is given.
1.2.1 Antigen uptake

When an animal is injected with an antigen the first response of the recipient is phagocytosis of the antigen by the macrophages and phagocytes of the reticuloendothelial system. The antigen is accumulated in organs containing reticuloendothelial cells and macrophages such as lung, liver, spleen and lymph nodes, but the proportion of antigen in different organs will depend on the route of injection. Intradermally or intramuscularly injected antigen is drained by the lymphatics and accumulates in the regional lymph nodes (Stuart, 1970; Ada, Nossal and Pye, 1964). Intravenously injected antigen is distributed generally throughout the body and accumulates in the spleen rather than in the lymph nodes, since the reticuloendothelial structures of the spleen are in contact with the blood, while those of the lymph nodes are in contact with the draining lymphatics (Stuart, 1970; Horiuchi, Gery and Waksman, 1968). Intraperitoneal injection has a result similar to intravenous injection since the antigen is taken up by the lymphatics and emptied into the blood stream by way of the thoracic duct (Stuart, 1970). Different antigens or different forms of the same antigen will also show differences in their uptake patterns (Horiuchi et al., 1968; Ada, Nossal and Pye, 1964).

The uptake of antigen into regional lymph nodes and into the spleen has been traced in various ways. It is found that in lymph nodes the antigen localizes first in the medullary sinuses where it is taken up by pinocytosis.
into vacuoles and lysosomes of the medullary macrophages (Wellensiek and Coons, 1964; Nossal, Ada and Austin, 1964b; Ada, Parrish, Nossal and Abbott, 1967; Nossal, 1967; Nossal, Abbott and Mitchell, 1968; Diamantstein, Wagner and Graf-Keiserlingk, 1969). Following this, the antigen localizes in the cortical follicles where it is found mainly at the surface of the cells and in contact with the lymphocytes and macrophages (Nossal, Ada and Austin, 1964b; Ada, et al., 1967; Nossal, Abbott, Mitchell and Lummus, 1968). In the spleen, injected antigen localizes first into the red pulp, particularly in the areas surrounding the follicles (Diamantstein, et al., 1969), after which it becomes concentrated in the germinal centres of the follicles (Nossal, 1967; White, 1969). Antigen taken up by peritoneal macrophages is found in cytoplasmic vacuoles or lysosomes (Catanzaro, Graham and Schwartz, 1969; Rhodes, Lind, Birch-Andersen and Ravn, 1969) and on the surface of the macrophages (Unanue, Cerottini and Bedford, 1969).

The extent of the localization of the antigen in the lymph node follicles, although not the extent of its localization in the medulla, is dependent upon its ability to induce an immune response (Nossal, 1967) and not on its size (Lang and Ada, 1967b). Non-antigens such as carbon (Miller and Nossal, 1964) and also autologous proteins (Ada, Nossal and Austin, 1964; Herd and Ada, 1969) show localization in the medulla of the lymph nodes but not specifically in the follicles. The exception to this is autologous gamma globulin (Ada, Nossal and Austin, 1964) and
this is particularly interesting in view of the fact that follicular localization seems to depend on the combination of the antigen with substances, particularly antibodies, that promote the uptake of the antigen by macrophages. These substances are known as opsonins. Antigens may be opsonized by the passive administration of antibody (Nossal, Ada, Austin and Pye, 1965; Lang and Ada, 1967) or previous exposure to the antigen which results in the animal itself producing opsonizing antibody (Nossal, 1965; Nossal, Ada, Austin and Pye, 1965) and these treatments result in a greater localization of the antigen in the follicles, particularly in the germinal centres which have formed as a result of a previous exposure to the same antigen.

Antibody is also found to increase the rate of phagocytosis in peritoneal exudate cells (Argyris, 1969) but to delay slightly the rate of breakdown of the ingested antigen (Cohn, 1963). Conversely, in germ-free animals, where antibody produced against environmental antigens, which may cross react with the injected antigen, is unlikely to be present, it is found that there is less uptake of injected antigens into lymph nodes and spleen than in conventional animals and that follicular localization is slower and reduced in amount (Miller, Johnson and Ada, 1968).

1.2.2 The function of macrophages in the immune response

The fact that macrophages phagocytose antigen does not itself demonstrate that they are necessary or even involved in the immune response. However there is evidence to indicate that for some kinds of antigen
processing by macrophages is necessary before the immune response will occur.

Reticuloendothelial blockade can be induced by injecting an animal with large amounts of a substance that can be phagocytosed such as cholesterol esters, colloidal carbon or colloidal iron. This treatment results in a marked reduction of the immune response to sheep erythrocytes (Sabet, Newlin, Friedman, 1968; Melnick and Friedman, 1970) and to bovine serum albumen (Cruebaud, 1968). Blockade subsequent to antigen administration is not effective in reducing the immune response (Sabet et al., 1968; Melnick and Friedman, 1970).

More precise information about the cells required for the immune response can be obtained by studying the cells necessary for the response in vitro or those necessary to restore the response to mice that have been irradiated to destroy their own immunological systems. Macrophages for these experiments may be obtained from a spleen cell suspension or from peritoneal exudate in a relatively pure form by the separation of cells that will adhere to glass or plastic: the so called 'adherent' population. That which is left, the 'non-adherent' population consists mainly of lymphocytes. Macrophages are necessary for the primary response to sheep erythrocytes in vitro (Roseman, 1969; Mosier, 1967; Pierce, 1969) and for the restoration of the response of irradiated mice to this antigen (Mosier, Fitch, Rowley and Davies, 1970). Macrophages are also necessary for the response to a hapten-carrier complex (NIP-ovalbumen)
and to bovine gamma globulin in irradiated rabbits (Pribnow and Silverman, 1967). This restoration is equally effective when the only antigen present is that taken up by the macrophages prior to their addition to the rest of the system. Indeed, treatment of normal animals with macrophages that have been sensitized in this way is as effective as or more effective than treatment with free antigen such as sheep erythrocytes (Argyris, 1967), Maia squinado haemocyanin (Unanue and Askonas, 1967, 1968a,b), bovine serum albumen (Spitznagel and Allison, 1970; Mitchison, 1969), lysozyme, ovalbumen or human serum albumen (Mitchison, 1969). With Keyhole Limpet haemocyanin on the other hand, macrophage uptake decreases rather than increases the immunogenicity of the substance (Unanue and Askonas, 1967; Unanue, 1969). The reason for this is obscure, but may become clearer when the nature of immunogenicity is better understood. Dead macrophages, even if they contain antigen, are ineffective in the induction of the immune response (Unanue and Askonas, 1968a; Mitchison, 1969). Prior irradiation of the macrophages also prevents them from restoring the immune response to irradiated animals (Gallily and Feldman, 1966, 1967; Feldman and Gallily, 1967a,b; Pribnow and Silverman, 1967, 1969; Feldman, 1969; Mitchison, 1969) without interfering with their ability to take up antigen (Gordon, Cooper and Miller, 1955; Feldman and Gallily, 1967a;
Feldman, 1969) although there is evidence to suggest that their ability to digest the antigen after it is taken up is reduced (Donaldson, Marcus and Gyi, 1954; Gordon et al., 1955; Nelson and Becker, 1959a,b,c; Donaldson, Marcus, Gyi and Perkins, 1956). On the other hand, Mitchison (1967), Unanue and Askonas (1968) and Roseman (1969) have found the function of macrophages to be irradiation resistant. The explanation for these conflicting results may be that there is a delay before irradiation produces its effect on the processing ability of the macrophages and indeed it has been found that there is a delay of about 2 days between irradiation and the suppression of the digestive or antigen processing function of the macrophages (Gordon et al., 1955; Donaldson et al., 1956; Mitchison, 1969).

It seems probable that macrophages are implicated in the response to most but not all antigens and that they have a rather more complex role to play than the simple accumulation of antigen in the vicinity of lymphocytes that are able to respond to the antigen.

The macrophages themselves are not the antibody producing cells. Individual antibody forming cells have been identified in many ways (see Noszal and Mäkelä, 1962), in particular by using antigen labelled with fluorescein (Coons, Leduc and Connelly, 1955; Leduc, Coons and Connelly, 1955) or radioisotopes (Bosman, Feldman and Pick, 1969) to locate the antibody containing cells, or by finding the cells in the centre of haemolytic plaques (Sterzl,
Microscopic examination of these cells show them to be lymphocytes or cells of the plasma series.

There is some rather dubious evidence that lymphocytes can turn into macrophages (Boak, Christie, Ford and Howard, 1966; Howard, Christie, Boak and Kinsky, 1969; Vernon-Roberts, 1969) but none, of which I am aware, to the contrary. In the in vitro induction of the immune response (Feldman, 1969; Mosier, 1967; Pierce, 1969) and the restoration of the response of lethally irradiated animals (Gallily and Feldman, 1967; Feldman and Gallily, 1967a; Feldman, 1969; Mosier et al., 1970; Unanue and Askonas, 1968a) living lymphocytes are necessary in addition to macrophages. Thus it seems certain that an interaction between macrophages and lymphocytes is necessary for the immune response to many antigens.

It is not clear how the macrophages carry out this processing function. Most antigen that has been taken up by macrophages is rapidly digested, but about 10% is retained for longer and is also found in a different subcellular fraction than is the degraded antigen (Kälish and Mitchison, 1968). This may be the 'processed' antigen. Unanue and Cerottini (1970) found that this small proportion of retained antigen was associated with the plasma membrane.

Frei, Benacerraf and Thorbecke (1965) found that the bovine serum albumen remaining in the circulation 48 hours after its injection into a rabbit was much less immunogenic than the original antigen, suggesting that the macrophages select
the most immunogenic form of antigen to present to the lymphocytes. Howard and Siskind (1969) on the other hand after similar experiments with pneumococcal polysaccharide in mice reached the opposite conclusion that there was no difference in immunogenicity between antigen taken up by macrophages and that remaining in the circulation, and that particulate and non-particulate forms of this antigen were equally immunogenic. This probably indicates that the immune responses to different antigens or of different animals have requirements for different cell populations.

Evidence has been produced for the involvement of macrophage RNA in the immune response. If spleen or peritoneal exudate cells were incubated in vitro with sheep erythrocytes (Mosier and Cohen, 1968; Bishop, Pisciotta and Abramoff, 1967), \textit{Maia squinado} haemocyanin (Askonas and Rhodes, 1965) or bacteriophage (Herscowicz and Stelos, 1968), RNA extracts from the cells were able to induce the production of antibodies to the antigen with which the cells had been incubated. Similarly, RNA extracts from mice immunized with sheep erythrocytes (Cohen, Newcomb and Crosby, 1965) or bacteriophage (Herscowicz and Stelos, 1968) were highly immunogenic for the antigen with which they had been incubated. Actinomycin D (Bishop \textit{et al.}, 1967) or chloramphenicol (Cohen \textit{et al.}, 1965) which inhibit the synthesis of RNA can prevent the production of this immunogenic RNA, and the addition of RNA from non-immunized mice reduces the effectiveness of the active RNA (Cohen \textit{et al.}, 1965). This suggests that the effect is due to an RNA
synthesised subsequent to antigen administration and this RNA is specific for the particular antigen. On the other hand, Askonas and Rhodes (1965) showed that provided sufficient haemocyanin was homogenized with the RNA in the extraction procedure, an immunogenic RNA extract could be produced, suggesting that the effect was due simply to the adsorption of antigen to RNA resulting in increased immunogenicity. This contradiction may have been resolved by Fishman and his coworkers (Fishman, Van Rood and Adler, 1965; Fishman and Adler, 1967a,b; Fishman, 1969) who incubated macrophages with T2 phage and showed two different kinds of RNA that were able to induce specific antibody formation in cultures of lymph node fragments. IgM is produced by RNA synthesised de novo after antigen exposure and the antibody is of the allotype of the macrophages suggesting that information has been transferred. IgG antibody is induced by RNA antigen complexes where the RNA is synthesised prior to antigen exposure and is of the allotype of the lymph nodes suggesting a non specific adjuvant function for this RNA.

1.2.3 Interactions of lymphocytes

It is now generally accepted that the immune response to most antigens requires the interaction of two different types of lymphocyte. When mice have been lethally irradiated their immune response may be restored by the injection of living syngeneic spleen cells; neither bone marrow or thymus cells used alone are very effective for this purpose, but a mixture of the two cell populations
is more effective than the sum of their activities when they are used alone (Mitchell and Miller, 1966a; Claman, Chaperon and Triplett, 1966a,b; Taylor, 1968; Chiller, Habicht and Weigle, 1970; Claman, Chaperon and Selner, 1968). In addition, in restored irradiated and/or thymus deprived mice where the cells of bone marrow and thymic origin can be distinguished from each other by chromosome markers (Nossal, Cunningham, Mitchell and Miller, 1966; Cunningham, 1969a) or by allotypic differences (Miller and Mitchell, 1966a,b; Mitchell and Miller, 1966b; Munroe and Hunter, 1970) the antibody forming cells are never found to be of thymic origin. Thus the bone marrow and not the thymus contains the precursors of the antibody forming cells and these cells are called antibody forming cell precursors or AFC-P.

The two populations of cells may be physically separated by the fractionation of spleen cell suspensions in an albumen density gradient when the antibody forming cells are found in the low density area and the antigen-reactive (thymus derived) cells are found in the high density area (Müller and Hiesche, 1970). Munroe and Hunter (1970) found the antibody forming cells to be more irradiation resistant than the thymus derived cells. Treatment with antilymphocyte serum does not interfere with the immune response when given after the antigen, but spleens from hyperimmune antilymphocyte-treated mice were not able to transfer a response to irradiated recipients indicating that antigen sensitive (thymus derived) cells
are sensitive to the action of anti-lymphocyte serum but antibody producing cells are not (Mölle and Zukoski, 1966).

These thymus derived cells are not macrophages since restoration of irradiated mice with the 'adherent' (macrophage) population of spleen cells from thymectomised donors plus the 'non-adherent' (lymphocyte) population from non thymectomised donors result in the recovery of the immune response, while the restoration of irradiated mice with macrophages from nonthymectomised mice plus lymphocytes from thymectomised mice does not restore the immune response (Rosenoer, Biano and Brown, 1970; Mosier et al., 1970). Mosier and Coppleson (1968) on the basis of the response obtained from combinations of different quantities of adherent and nonadherent cells in vitro suggest that the immune response requires a reaction between one adherent and two nonadherent cells of differing populations.

The cells from the thymus are the cells which initially react with the antigen and thus are termed antigen-reactive cells or ARC. It has been shown that thymus-derived cells react specifically with antigen before reacting with the bone marrow derived cells (Miller and Mitchell, 1967; Mitchell and Miller, 1968b; Shearer and Cudkowicz, 1969b) and that this reaction with antigen induces proliferation of the antigen reactive cells (Shearer and Cudkowicz, 1969b; Davies, Carter, Leuchars, Wallis and Koller, 1969; Davies, Leuchars, Wallis and Koller, 1966; Leuchars, Cross, Davies and Wallis, 1964).

When antigen and a standard dose of thymus or bone marrow cells are transferred to irradiated recipients
together with a limiting dose of the other cell type, analysis of the response obtained indicated that it is the bone marrow derived cell and not the thymus derived cell which determines the specificity of the immunoglobulin to be produced and that the thymus derived cell can react with a bone marrow cell of any specificity (Cudkowicz, Shearer and Priore, 1969; Shearer, Cudkowicz and Priore, 1969a,b). Analysis of similar limiting cell dose-response experiments suggest that one thymus cell may be able to react with varying numbers of bone marrow cells (Groves, Lever and Makinodan, 1970) although it is not clear how much of this is due to proliferation of the original thymus cell and how much is due to the genuine interaction of a single thymus cell with more than one bone marrow cell. It has been observed that clusters of spleen cells are necessary for a response to occur in vitro. These clusters form antibody to only one antigen each and when disrupted will reaggregate specifically (Mosier, 1969). If suspensions of spleen cells from donors that have been antigenically stimulated are transplanted to irradiated recipients at intervals after stimulation, the lack of adoptive antibody formation after transfer of the cells early in the response indicates that the integrity of the structure of the spleen is necessary for the induction of the response (Inchley, 1970). This suggests a requirement for a physical interaction between the two types of lymphocytes. The interaction between the thymus derived and the bone marrow derived antibody forming cell precursors results both in the differentiation of the
antibody-forming cell precursors into antibody forming cells without proliferation (Saunders, 1969; Tannenberg, 1967) and then in their proliferation (Davies et al., 1969; Saunders, 1969; Koros, Mazur and Howery, 1968; Krisch, 1969). The proliferation of the thymus cells occurs a day before that of the bone marrow cells (Davies et al., 1969).

The nature of the interaction between the ARC and the AFC-P is as yet unknown. The ARC seem to have specific combining antibody on their surface since they may be removed from cell suspensions by passage through an antigen-coated glass column (Abdou and Richter, 1969d; Wigzell and Andersson, 1969; Singhal and Wigzell, 1970; Daguillard and Richter, 1970), and they may also be selectively inactivated by mixing them with highly radioactive antigen (Ada and Byrt, 1969) or by affinity labelling with hapten (Plotz, 1969; Segal, Glcherson, Feldman, Hairovich and Givol, 1969) to block the combining sites on the surface. Autoradiography of spleen cell suspensions that have been mixed with $^{131}$I-labelled antigen show that a proportion of small and medium lymphocytes have antigen binding sites in discrete areas on their surface (Mandel, Byrt and Ada, 1969). The AFC-P are determined for immunoglobulin specificity (Shearer et al., 1968) but whether they are also determined for antibody specificity or whether there is a transfer of information between the ARC and the AFC-P is not yet certain. Experiments with hapten-carrier complexes (Rajewski, Schirrmacher, Nase and Jerne, 1969) and with isomers of lactic dehydrogenase (Rajewski and Röttlander, 1967; Rajewski, Röttlander, Peltre and Miller, 1967) have shown that
the antigen to which an animal is sensitized is not necessarily that to which it makes the antibody provided that the sensitizing site is on the same molecule as the site to which the antibody is formed. This suggests that antigen of a suitable size may be able to form a bridge between ARC and APC-P bringing them into some kind of conjunction that allows the ARC to activate the AFC-P.

The system that has been described so far has been chiefly worked out for the response of the mouse to erythrocyte antigens but the responses to other antigens do not necessarily require exactly this process. It has already been pointed out that while processing by macrophages is necessary for *Haie squinado* haemocyanin to become fully immunogenic it is not necessary for the similar but larger molecule of Keyhole Limpet haemocyanin. Howard and Siskind (1969) compared the requirements for tolerance and immunity induction to pneumococcal polysaccharide and reached the conclusion that macrophages are not required for the response to this antigen. The finding of Baker and Landy (1967) that the response to pneumococcal polysaccharide has a very brief lag period between the injection of antigen and the appearance of an increased number of cells with specific antigen combining sites on the surface also suggests that this antigen requires less processing than sheep erythrocytes. Feldman (1969) has shown that the response of irradiated mice to Shigella may be restored by the administration of macrophages alone, but the response to sheep erythrocytes cannot be restored in this way. Since the requirement for macrophages of the response of mice to SRBC is known
(Mosier, et al., 1970; Mosier, 1967; Roseman, 1969; Pierce, 1969), the sheep erythrocytes must require macrophages and in addition some other population of irradiation sensitive cells. Humphrey, Parrott and East (1964) have suggested that neonatal thymectomy of mice has different effects on the response to different antigens and Armstrong, Diener and Shellam (1969) have shown that in the response of mice to polymerized flagellin there is no synergistic effect on the number of cells forming antibody when bone marrow and thymus are injected together. When thoracic duct lymphocytes were fractionated by size, the population required to restore the response of irradiated mice to polymerized flagellin was not the same as that required to restore the response to sheep erythrocytes (Nossal, Shortman, Miller, Mitchell and Haskill, 1967) showing that different populations of cells are required for the response to different antigens.

In the rabbit also, antigen reactive cells are necessary for the response to sheep erythrocytes (Abdou, Rose and Richter, 1969) but these ARC appear to be found in the marrow rather than the thymus. When rabbits are exposed to sheep erythrocytes the cells which are capable of a specific response disappear from the bone marrow so that when this tissue is used to restore irradiated rabbits it cannot transfer the ability to respond to the sheep erythrocytes although the ability to respond to other antigens is normal (Abdou and Richter, 1969a,b). A similar effect is found when human leucocytes are used as antigens (Abdou
and Richter, 1969). These cells in the rabbit marrow are ARC rather than AFC-P since the allotype of the antibodies formed in these restored animals is that of the recipient and not that of the donor, indicating also that antibody forming cells are comparatively irradiation resistant (Richter and Abdou, 1969). Thus it can be seen that the response to a single antigen may have different cell requirements in different animals. This situation has certain similarities to that described by Armstrong et al., (1969) for the response to polymerised flagellin, particularly since the response to this antigen also requires a radiation sensitive cell with specific combining sites on the surface (Ada and Byrt, 1969). It seems possible that the response to all antigens may require the interaction of two lymphocytes but that the ARC for certain antigens are not always dependent upon the thymus for their maturation.

From the foregoing data it is surmised that the immune response requires the interaction of two and possibly three types of cell: the macrophages, the antigen-reactive ARC and the antibody forming cell precursors (AFC-P). At any one time each ARC and possibly also each APC-P is producing an immunoglobulin of specific antibody type which is found on the surface of the cell. When an antigen enters the body any ARC that is producing an immunoglobulin with an antibody combining site of sufficiently close fit will complex with the substance. If this substance is also immunogenic, a property perhaps conferred upon otherwise non-immunogenic substances by selection or processing by
macrophages, it will induce the ARC to divide. These activated ARC will then react with the APC-P and induce them to differentiate and to proliferate thus becoming the antibody forming cells (AFC) themselves.

1.2.4 Immunoglobulin sequence in the immune response

In the normal immune response, IgM antibody appears before IgG antibody (Kidinger and Pross, 1967; Adler, 1965; Uhr and Finkelstein, 1963; Bauer, Mathies and Stavitsky, 1963). The relative amounts of each type appearing in the primary or the anamnestic response can be influenced by factors such as dosage (Borel, Fauconnet and Miescher, 1964; Ivanyi and Cerny, 1969), the injection schedule of the antigen, the presence or absence of adjuvant (Barth, McLaughlin and Fahey, 1965), the form of the antigen (Nossal, Ada and Austin, 1964a), the relative proportions of macrophage bound and free antigen (Unanue and Askonas, 1964a) or by metabolic inhibitors such as methotrexate or colchicine (Blinkoff, 1966). It is not entirely certain whether the change from the production of IgM antibody to IgG antibody occurs because the progeny of one APC-P are able to change the type of antibody they are synthetising from IgM to IgG without altering the antibody specificity, or whether there are two separate APC-P, the IgM type producing its antibody more rapidly than the IgG type. Nossal, Szenberg, Ada and Austin (1964) isolated cells producing antibody in response to Salmonella flagellin and analysed the type of antibody in each individual cell,
using mercaptoethanol to inhibit IgM activity and antibody to IgG to inhibit IgG activity. They found that a considerable proportion of cells produced both types of antibody, particularly at the time when the predominant type of antibody is changing from IgM to IgG. They concluded that a single cell produces both types in sequence. Duplicate plating for direct (IgM) plaque forming cells and indirect (IgG) plaque forming cells in the haemolytic plaque assay on the other hand, has failed to show any cell synthesising both types simultaneously (Merchant and Brahmi, 1970). Eldinger (1968) and Eldinger and Pross (1967) studied the kinetics of the IgM and IgG antibody response and compared them to the cellular events occurring in the draining lymph node. They reached the conclusion that two separate precursors were involved. When irradiated mice were restored with syngeneic thymus and marrow cells the numbers of AFC-P of each immunoglobulin type varied with the dose of the marrow cells indicating that there were separate precursors for each type and that they were already differentiated before contact with the antigen (Shearer et al., 1968; Shearer and Cudkowicz, 1969a). On the other hand, Cunningham (1969b) studying the foci of plaque forming cells formed in the spleens of irradiated mice restored with syngeneic spleen found IgG producing foci associated with IgM producing foci more often than could be accounted for by a random distribution of independent precursor cells and interpreted this as evidence that the progeny of one precursor cell could change from IgM production to IgG
production. The synthesis of IgG has been found to be more sensitive to both thymectomy (Taylor and Wortis, 1968) and irradiation (Nettesheim, Williams and Hammons, 1969) than the synthesis of IgM. Thymectomised irradiated mice can recover a considerable portion of the IgM response but not the IgG response (Nettesheim et al., 1969) suggesting that IgM AFC-P may not be so dependent upon the ARC as is the IgG AFC-P. As yet, however, the question of whether IgM and IgG are produced by the same line of cells is unsettled.

This sequence in the formation of different kinds of antibody is not confined to IgM and IgG. Different kinds of IgG also show this sequence (Benacerraf, Ovary, Bloch and Franklin, 1963; Banovitz and Ishizaka, 1967) and Shearer et al. (1968) found that antibody to the same antigen but with functional differences that probably reflect differences of the 'C' terminal end of the polypeptide chain also came from different types of AFC-P each with slightly different activation and proliferation requirements and characteristics.

1.2.5 The origin and fate of the cell populations

Thymus cells are derived originally from bone marrow since thymus grafts into thymectomised or normal recipients are always repopulated by cells of recipient origin, (Leuchars, Morgan, Davies and Wallis, 1967; Hays, 1967; Schlesinger and Hurvitz, 1968; Miller and Osoba, 1963) and where irradiated mice are restored with bone marrow, the thymus becomes repopulated with cells of bone marrow origin (Order and Wakaman, 1969). In addition, if given sufficient time, bone
Marrow alone can reconstitute irradiated mice, but for this to occur a thymus must be present in the irradiated recipient (Miller and Mitchell, 1967; Doria and Agarossi, 1968, 1969; Cross, Leuchars and Miller, 1964). Thymus cells on the other hand will never differentiate into antibody forming cells since thymus cells no matter how long incubated cannot produce antibody-forming cells (Miller and Mitchell, 1967).

Embryonic liver is another major source of lymphoid stem cells and like stem cells from bone marrow it is able to differentiate into immunoglobulin forming cells in the absence of a thymus (Tyan, Cole and Hersenberg, 1967, 1968; Tyan and Hersenberg, 1968a,b) but a thymus is necessary for the development of the capacity of irradiated liver restored mice to respond normally to sheep erythrocytes (Tyan, Hersenberg and Gibbs, 1969) or to a synthetic polypeptide (Tyan et al., 1968) showing that passage of the stem cells from foetal liver through the thymus is also necessary for the development of ARC. Taylor (1965) using marker chromosomes found stem cells in bone marrow and foetal liver that were capable of differentiating into both bone marrow and thymus cells.

In birds, bursectomy prior to hatching results in the inability to produce antibody and indeed in the inability to produce immunoglobulins (Cooper, Cain, Van Alten and Good, 1969; Cain, Cooper, Van Alten and Good, 1969; Graetzer, Wolfe, Aspinall and Meyer, 1963; see also Cooper, Gabrielsen and Good, 1967b) and it is suggested that the gut associated
lymphoid tissue in mammals, in particular the appendix of the rabbit may have a function similar to that of the bursa (Cooper, Perey, Mckeally, Gabrielsen, Sutherland and Good, 1966). It is thus possible that the maturation of the antibody forming cells may have a requirement for gut associated lymphoid tissue similar to the requirement of most ARC for the thymus.

It has been shown that the bone marrow or foetal liver contains the ultimate precursor for both ARC and AFC-P but passage through or exposure to the thymus is necessary for differentiation of ARC for many antigens and passage through the gut associated lymphoid tissue may be necessary for the differentiation of the AFC-P.

The thymus derived cells and the bone marrow derived cells are subsequently found in all the peripheral lymphoid organs. When thymocytes are labelled in vivo and transplanted, they are found in specific sites in lymph nodes, spleen, thoracic duct lymph, Peyer's patches and blood (Linna, 1968; Ernström and Larsson, 1969; Nossal, 1964; Weissman, 1967). If the cells from a grafted syngeneic thymus can be distinguished by a marker chromosome they will be found dividing in the spleen some time after grafting (Leuchars et al., 1964; Leuchars et al., 1967). Thymectomy causes lymphocyte depletion in areas around the arterioles in the spleen and the lymph nodes and in the blood and the thoracic duct lymph (Wakaman, Arnason and Jankovic, 1962; Goldschneider & McGregor, 1968; Parrott, De Sousa and East, 1966; Miller, 1965). Similar depletion
of these thymus dependent areas is found in a strain of hereditarily thymusless mice (De Sousa, Parrott and Pantelouris, 1968). In thoracic duct lymph, thymectomy causes selective depletion of antigen reactive cells (Miller, Mitchell and Weiss, 1967). Cells that can restore thymus deprived mice are found in most peripheral lymphoid organs (Parrott, De Sousa and East, 1966; Agnew, 1967; Isakovic, Waksman and Wennersten, 1965).

In summary therefore, the precursors of both the ARC and the APC are found originally in the bone marrow. From there some go to the thymus to become ARC and from the thymus they migrate into the peripheral lymphoid tissues, in particular the recirculating pool found in the thoracic duct lymph and periarteriolar sheaths in the solid peripheral lymphoid organs. The antibody forming cells may differentiate in the gut associated lymphoid tissue and from there they also migrate to the peripheral lymphoid tissue. Peripheral lymphoid organs therefore consist of a mixture of thymus derived ARC and bone marrow derived APC-P.

1.2.6 Cell Mediated Immunity

Less evidence exists on the sequence of events involved in the induction of cell mediated immunity than in the case of the humoral antibody response. Antibodies can be involved in graft rejection (see Mitchell, 1969) and Clark, Poker, Gewurs, Good and Varco (1967) suggest that they are involved particularly in attracting lymphocytes to the graft to be rejected. On the other hand, since in
birds removal of the bursa prevents antibody formation but has no effect on graft rejection (see Cooper et al., 1967b) and since in foetal lambs grafts neither stimulate the production of circulating immunoglobulin nor do antibodies to sheep immunoglobulin affect the rejection of grafts in any way, it seems that antibodies are not necessary for graft rejection.

The thymus appears to be involved in cell mediated immunity. Neonatal thymectomy of the chicken will prevent the development of the ability to reject grafts (Aspinall, Meyer, Gratezer and Wolfe, 1963; Warner and Szenberg, 1962, 1964a). Similarly in mammals, neonatal thymectomy prevents the development of graft rejection (Miller, 1961, 1962; Parrot and East, 1965), delayed hypersensitivity (Arnason, Jankovic and Waksman, 1962; Arnason, Jankovic, Waksman and Wennersten, 1962) and the ability of spleen cells to produce graft versus host responses in vitro (Trainin, Small and Globerson, 1969), although the effect on graft rejection seems to depend on the strain combination used (Martinez, Kersey, Papermaster and Good, 1962) and on microbial contamination from the environment since the effect of thymectomy is reduced in germ-free animals (Miller, Dukor, Grant, Sinclair and Saquet, 1967; Bealmead and Wilson, 1967). Adult mice thymectomised, irradiated and restored with bone marrow lose their ability to reject grafts only temporarily (Iyan and Cole, 1966a) but transplanted foetal liver cells do not acquire the ability to reject skin grafts or mount graft versus host responses.

It is not clear whether there is another cell population, as well as the thymus, involved in cell mediated immunity. Davis, Cole and Schaffer (1970) found no evidence of synergy between thymus and bone marrow in the restoration of the ability to reject homografts or to produce the graft versus host reaction. Stutman and Good (1969) found that neither bone marrow alone nor with thymus cells was effective in producing graft versus host splenomegaly, though Hilgard, Sosin, Martinez and Good (1965) and Sosin, Hilgard and Martinez (1966) consider that thymus cells alone can produce graft versus host reactions. Cells involved in graft versus host reactions are found to sediment with ARC rather than APC-P in an albumin density gradient (Möller and Hiesehe, 1970). On the other hand, irradiated restored mice do show a slight synergism when spleen or lymph node cells are given together with thymus cells (Cantor and Asofsky, 1970), and thymus cells transplanted into irradiated recipients never alone acquire the ability to produce graft versus host responses (Tyan and Cole, 1965). Umel, Globerson and Auerbach (1968) have found that foetal liver cells as well as thymus are necessary for the in vitro graft versus host reaction. By using allotypically recognisable thymus grafts in thymectomised, irradiated and bone marrow restored rats Williams and Waksman (1969) have shown that the cells arriving at the skin lesions caused by delayed hypersensitivity reactions
are of thymus origin in the first 24 hours but those arriving later are of bone marrow origin.

It seems possible that at least two populations of cells are involved in cell mediated immunity, but if this is true it is not known whether or not these are the same populations of cells involved in the humoral antibody response. The separation of the two functions in birds and probably in mammals too (Burnet, 1968), suggests that the AFC-P are not involved in cell mediated immunity, but whether the thymus cells involved are the same as the ARC for the humoral response is not yet known. In rabbits, Richter, Abdou and Midgley (1970) have observed that the cells required for graft rejection appear to be irradiation resistant in contrast to those required for the humoral antibody response.

1.3 Tolerance

In the normal immune response the exposure to antigen results in the production of specific antibody or of specifically sensitised cells. Under certain circumstances, the administration of antigen results instead in a lack of response to the antigen and more importantly to a lack of response when the antigen is subsequently administered in a manner that would normally evoke an immune response. This phenomenon is known as immunological tolerance. Tolerance is usually considered to be caused by the inactivation of the lymphocytes able to respond specifically to a particular antigen (see Dresser and Mitchison, 1968; Kraba, 1968).
The best evidence for the location of tolerance in the lymphocyte is that the transfer of normal lymphocytes into irradiated recipients restores their immune response, but transfer of lymphocytes from an animal in which tolerance to a specific antigen has been induced (Weigle and Golub, 1967; Miller and Mitchell, 1968a; Armstrong et al., 1969; Argyris, 1963; Smith Isakovic and Waksman, 1966; Isakovic, Smith and Waksman, 1965) or transfer of cells made tolerant in vitro (Diener and Armstrong, 1969) will not restore the ability of the irradiated recipient to respond to that antigen although response to other antigens is recovered. Furthermore, if normal lymphocytes are transferred into irradiated tolerant recipients they can restore the ability of the recipient to respond to the antigen to which it was previously tolerant. (Denman, Vischer and Stastny, 1967). Macrophages are not involved in tolerance since antigen bound in peritoneal exudate cells from tolerant donors will induce immune responses in normal recipients but antigen bound in peritoneal exudate cells from normal donors will not induce an immune response in tolerant recipients (Mitchison, 1969). Macrophages from a rabbit tolerant to bovine gamma globulin could take up bovine gamma globulin and stimulate spleen cells from a sensitized animal to increased DNA synthesis in a normal manner (Harris, 1967). In addition, the uptake of antigen in a tolerant animal is essentially similar to the uptake in animals which have not been exposed to the antigen before. (Humphrey and Frank, 1967; Azar, 1967; Ada, Nossal and Fye, 1965).
The way in which non-immunogenic antigen acts to render the cell tolerant is so far unknown. Reports of the length of time that the cells must be exposed to antigen to become tolerant range from a few hours (Uphoff, 1968; Scott and Wakaman, 1969; Mitchison, 1968b) to several days (Golub and Weigle, 1967; Britton, 1969). The systems used were all different so the variability may be related to dose, antigen, cell type involved (Mitchison, 1968b) or method of antigen administration. The delay before tolerance was induced in these experiments and the fact that Scott and Wakaman (1969) could induce tolerance in intact organs in vitro but not in separated lymphoid cells suggests that a processing step may be involved. Ada and Parrish (1968) found that extremely small quantities of antigen could induce tolerance suggesting some mechanism more complex than the simple masking of receptor sites with non-immunogenic antigen.

Another point of uncertainty is whether tolerance causes a permanent or temporary inactivation of the lymphocyte. The thymus is required for the recovery from tolerance (Pierpaoli, 1967; Aisenberg and Davis, 1968a,b; Taylor, 1964; Claman and McDonald, 1964; Claman and Talmage, 1963), suggesting that the production of new, non-tolerant ARC are necessary. On the other hand, the fact that tolerance can be broken by the administration of cross-reacting antigens (see Dresser and Mitchison, 1968) and the finding that tolerance can be terminated by a dose of antigen larger than the repeated doses used to maintain it
(Gras and Dalmau, 1966) suggests that the inhibited cell can be reactivated under suitable circumstances. It seems quite likely that in the recovery from tolerance both the recruitment of new cells and the recovery of old ones may occur (Dowden and Sercarz, 1967; Nachtigal, Greenberg and Feldman, 1968), and that while the tolerant cell may not be irreversibly inactivated, it may not become resensitized readily.

It is not certain whether the lymphocyte inactivated in tolerance is the thymus derived ARC or the bone marrow derived AFC-P. The thymus is necessary for the recovery from tolerance (Pierpaoli, 1967; Aisenberg and Davis, 1968a,b; Taylor, 1964; Claman and McDonald, 1964; Claman and Talmage, 1963) which suggests that the production of new non-tolerant ARCs is required. Thymus or thymocytes from a tolerant animal are not able to restore a normal response to the tolerance inducing antigen to irradiated recipients (Smith et al., 1966; Isakovio, Smith and Waksman, 1965; Taylor, 1968; Abdou and Richter, 1969a) nor were thoracic duct cells from a donor tolerant to sheep erythrocytes able to restore the response of neonatally thymectomised recipients to this antigen (Miller and Mitchell, 1968a). On the other hand, Playfair (1969) found that tolerant thymus was able to restore a normal response to irradiated recipients and Denman, Vischer and Stastny (1967) found that normal thymocytes were unable to terminate tolerance suggesting that other populations of cells must be responsible. The bone marrow has also been specifically
implicated in tolerance (Playfair, 1969) although Taylor (1968) and Isakovic, Smith and Waksman (1965) state that the bone marrow is not involved. Miller and Mitchell (1970) found that in mice made tolerant to SRBC by a large dose of sheep erythrocytes and cyclophosphamide, it was the ARC outside the thymus that were the population of tolerant cells, and they were unable to produce tolerance in the thymus cells themselves or in the non-thymus derived population. However, since Argyris (1968a) and Chiller, Habicht and Weigle (1970) have shown that both non-tolerant bone marrow and non-tolerant thymus are necessary for the restoration of the immune response to irradiated recipients the explanation is probably that either one or both populations of cells may become specifically tolerant and that the tolerance of either population prevents the immune response. The population of cells that is made tolerant under any particular circumstances will depend on such things as the nature and form of the tolerogenic antigen and the method of its administration.

In their review, Dresser and Mitchison (1968) point out that tolerance occurs under circumstances in which an animal meets a substance which can be recognised by the immunological system, i.e. is antigenic, but which, for some reason or another, does not evoke an immune response, i.e. is not immunogenic. Factors which inhibit the immune response and promote the production of tolerance may be related either to the state of the animal or the state of the antigen. Factors that decrease the response of the
immunological system include irradiation (Nachitigal, et al., 1968) anti-lymphocyte serum (Shellam, 1969) immunosuppressive drugs (Dietrich and Dukor, 1968; Aisenberg and Davis, 1968a; Schwartz and Dameshek, 1963), thoracic duct drainage to deplete the number of recirculating lymphocytes (Shellam, 1969) or immaturity (Billingham, Brent and Medawar, 1953, 1956; Hanan and Oyama, 1954; Mitchison, 1968a; and see Dresser and Mitchison, 1968). Factors which alter the immunogenicity of the antigen include antigen size since smaller units whether monomers as opposed to polymers or aggregates (Soriuch and Wakeman, 1968; Golub and Weigle, 1968; Ada, Nossal and Austin, 1965) or degraded pieces produced by enzyme digestion (Parish and Ada, 1969) as opposed to undegraded antigen, are less immunogenic than the larger units. Antigen in macrophages is often more immunogenic than the same antigen in the free form (Spitznagel and Allison, 1970).

Tolerance may be induced by particular doses of some antigens while at other doses the same antigens are immunogenic (Mitchison, 1968a; Shellam and Nossal, 1968). For most antigens the tolerogenic dose is larger than the immunogenic dose and in some antigens a dose smaller than the immunogenic dose will also produce tolerance. This effect may be due to a balance between immunogenic and non-immunogenic forms of the antigen present either when it is given to the animal or after macrophages have processed a small proportion of a relatively non-immunogenic form into a more immunogenic one. If the process of tolerance induction does not involve exactly the same mechanism as the
process of immune induction and there is no reason that it should, the kinetic values for the two processes will be different and the dose response curves will also be different, thus tolerance induction will predominate at some doses of antigen and immune induction at others. It might be interesting to test this hypothesis in its simplest form by attempting to inhibit the immune response to an antigen by the addition to the sensitizing dose of large amounts of a non-immunogenic form of the same antigen.

It is possible that the reaction between a particular population of cells and antigen in a particular form is not either totally immunogenic or totally tolerogenic but will have a tendency to produce both responses in the cells, the relative proportions of each response determining what the overall effect on the immune response will be.

In this situation also the tolerogenic mechanism and the immunogenic mechanism may have different dose response relationships resulting in the predominance of different functions at different doses.

1.4 Self-recognition

I have defined an antigen as a foreign substance of sufficient complexity to induce an immune response but have not yet discussed what it is that allows the animal to recognise the substance as 'foreign'. In other words, why it is that an animal does not produce an immune response to its own body constituents although these are quite capable of inducing an immune response in other individuals, even of the same species. This problem of self recognition is
one of the central problems of immunology, both in theoretical terms and also for clinical applications in tumour immunity, organ or tissue grafting and autoimmune disease.

In order to differentiate between autologous components and foreign components the animal must have a mechanism either for recognising 'self' in order not to respond to it or for recognising 'foreign' in order to initiate a response to it. In view of the fact that animals can respond to a great many entirely artificial substances to which they could never have been exposed in the course of their evolution it seems necessary that the animals have some means of recognising their own components.

If the mechanism that has been described for the immune response is fundamentally correct, it means that the immune response operates on the basis of a cell selection mechanism. The lack of response to autologous components must therefore be due either to a genetic inability to produce autoreactive immunoglobulins or to some method of elimination of cells producing autoantibodies. If an animal did not produce autoantibodies simply because it lacked the genetic equipment to do so, the production of autoimmunity in experimental animals would require a specific somatic mutation and its disappearance after the cessation of the treatment would be very difficult to explain. In addition, evolutionary change resulting in a change in any of the autologous antigens of the body would require a prior or simultaneous change in the immunoglobulin that might have reacted with the changed antigen. It is
possible that there might be a mechanism whereby a cell is prevented from producing an immunoglobulin that will be complementary to any of the other proteins present on its genome. Unless lymphocytes synthetise a small sample of every protein in the body this system would require that the information be obtained from the DNA itself. Since antibodies are produced to conformational rather than sequential determinants (Sela, Schechter, Schechter and Borek, 1967), and since proteins may be made up of subunits from different areas of the genome, it is hard to see how suppression at this level could be achieved. It seems more likely that self-recognition works by inhibiting those cells producing immunoglobulins that will react with autologous components. This requires some system for the cell to differentiate 'self antigens' from 'foreign antigens' present in the body as a result of infection or tissue grafting. Since there are no essential structural differences common to self antigens and excluded from 'foreign' antigens it would seem that the cell must be exposed to autologous components in some way differently to one way in which it is exposed to heterologous antigens. This might suggest a role for the prenatal period when the animal is exposed to its own antigens but is protected from those of the environment by the uterus or the egg. If this were the case, then exposure of the animal to heterologous antigens during the prenatal period should induce the animal to regard them as autologous components. Mixed blood types are found in adult dizygotic cattle twins, and Owen (1945) explained this as being due to the mixture of the blood stem cells in the
shared placenta during the prenatal period. If live allogeneic cells are inoculated into mice even as late as the neonatal period, a permanent chimera will often result allowing the acceptance of skin grafts from the donor strain (Billingham, Brent and Medawar, 1953, 1956; Galton, Reed and Holt, 1964). Similarly, the neonatal injection of LCM virus results in lifelong viraemia and lack of response to the virus (Hannover-Larssen, 1969).

The induction of specific non-reactivity during the pre- and neonatal period before the animal is able to respond is the same as the phenomenon of tolerance that has already been discussed and it seems likely that in fact self-recognition and tolerance are the same. It is possibly significant that the immunological deficiency of neonatal animals appears to be at least partly due to macrophage immaturity (Martin, 1966; Braun and Lasky, 1967; Argyris, 1968b, 1969b). This would result in the presentation of antigen to the immunological system of the animal in comparatively non-immunogenic forms and would explain why tolerance is so easily induced at this time. Other explanations must of course apply in the case of antigens for which macrophage processing is not required. Tolerance is not permanent, but once it has been established it is normally maintained while the antigen persists in the body (Mitchison, 1965; Aust, Rogers and Gutman, 1965). Continual injection of antigen will maintain tolerance indefinitely (Mitchison, 1968a; Smith and Bridges, 1958) and thus the continual exposure of the immunological system to autologous components will maintain a state of tolerance to the auto-antigens.
There are other similarities between tolerance and self-recognition. Acquired tolerance may be broken by the exposure of the animal to another antigen that cross-reacts with the one to which the animal is tolerant. If the degree of cross-reaction and the dose are right, antibodies are produced to those sites on the original antigen that cross-react with the tolerance breaking antigen (see Dresser and Mitchison, 1968). Therefore if tolerance and self-recognition were the same phenomenon the presentation to animals of antigen cross-reacting with their own body constituents should induce a state of autoimmunity. Rats and rabbits grafted with homologous kidneys produce antibodies to their own kidneys (Milgrom, Klassen and Kano, 1970) and indeed in general it is easier to produce experimental autoimmunity with heterologous than with isologous or autologous material. In diseases such as rheumatic fever also, where there is a cross reaction between group A streptococcus and certain cardiac and connective tissue antigens (Zabriskie, 1967), infections with this streptococcus can lead to immunological reactions against body constituents in susceptible individuals. The subject of the breakdown of self-recognition by antigens that cross react with body constituents has been discussed by Mitchison (1966), Asherson, (1968), Perlmann, (1969) and Weigle, Nakamura, Spiegelberg, Golub and High (1967).

Burnet (1962) has suggested a role for the thymus in the elimination of the self-reactive 'forbidden clones', and suggests that since it is in the thymus that the differentiation of antibody specificity most probably occurs
it is likely to be here also that the immature self reactive clones are destroyed. It is argued that the entry of antigens into the thymus occurs more readily in the foetus and neonate than in adults (Horiuchi et al., 1966; Mitchell and Nossal, 1966) and it is perhaps antigens which have entered the thymus at this age that are subsequently identified as 'self' antigens by cells as they go through the thymus on their way from the bone marrow to the peripheral lymphoid tissue. This idea is supported by the thymic abnormalities that are found in the autoimmune NZB strain of mice (Abbot and Burnet, 1964; Holmes and Burnet, 1963a; Burnet and Holmes, 1964). If this were the only mechanism of self-recognition it would suggest that thymectomy, by removing the mechanism that destroys the self-reactive cells might result in the development of autoimmunity, unless autoreactive clones could only be produced in the thymus. This seems unlikely since NZB produce their autoimmunity normally even when thymectomised at a very early age. The consequences of neonatal thymectomy do indeed have many similarities to graft versus host syndrome and autoimmune disease but these may also be accounted for as the effects of infection with normally non-pathogenic organisms (Yunis, Hong, Grewe, Martinez, Cornelius and Good, 1967; Yunis, Teague, Stutman and Good, 1969) and indeed neonatal thymectomy of germ-free mice (Wilson, Sjodin and Bealmeart, 1964; Dukor, Miller and Secquet, 1968) or rats (Skelly, Celozzi and Boeninghaus, 1968) does not produce these effects, and neonatal thymectomy actually suppresses the ability to develop artificially induced autoimmune allergic
encephalomyelitis (Arnason, Jankovic, Waksman and Wennersten, 1962; Arnason, Jankovic and Waksman, 1962). Since tolerance may be induced in the absence of a thymus (Follet, Battista and Bloom, 1966) and seems more easily produced in cells outside than within the thymus (Miller and Mitchell, 1970), it seems unlikely that the thymus is of unique importance in either self-recognition or tolerance.

1.5 Autoimmunity

The breakdown of self recognition by the exposure of the immunological system to cross-reacting antigens has already been mentioned. The experimental induction of autoimmune phenomena by the injection of autologous homologous or heterologous components in adjuvant may be explained on the same basis if it is assumed that the antigens may be altered by the adjuvant or by autolysis when the enzymes contained in the tissues are released by homogenization. On the other hand if animals are maintained in a tolerant state by the repeated injection of a particular dose of antigen a response may be induced by challenging them with a dose larger than that used to induce tolerance (Gras and Dalmau, 1966) suggesting that qualitative change in the antigen may not be necessary to break normal tolerance. A similar effect might be found when unusually large quantities of autologous antigens are released into the circulation. Idiopathic autoimmune disease may be considered as being either antigen-oriented or antibody-oriented. Antigen-oriented autoimmunity may be due to qualitative changes in the antigen or changes of the quantity to which
the immunological system is exposed, resulting in normal breaking of tolerance, or even in an immune response to autologous antigens such as lens protein which have previously been sequestered from the immunological system. Antibody-oriented systems suggest an abnormality in the relevant populations of cells, preventing them from becoming or remaining tolerant. In most idiopathic autoimmune diseases there is a strong hereditary factor, but hereditary factors could influence either alterations in antigens or abnormalities in lymphoid cells. It is unlikely that the same mechanism applies to all forms of autoimmune disease but the understanding of some forms of idiopathic breakdown of self-recognition may help in understanding its mechanism. NZB mice are very useful in this kind of investigation as they are the only well studied experimental animal producing spontaneous and invariable autoimmunity - in this case autoantibodies to the red blood cells.

Self recognition is probably established during the development of the immunological system in the pre and neonatal period. Any abnormality in the immune system of the NZB mice at this age might be related to the subsequent breakdown of self recognition in this strain. It would also suggest that the abnormality was in the immune system rather than in the antigen. Therefore the maturation of the immune response of NZB mice to foreign antigens was compared to that of two other non-autoimmune control strains. Because of the different cell requirements of the response to different antigens, heterologous erythrocytes were chosen as the test antigen. It was hoped by presenting foreign
antigen to these animals in the same form as the autologous antigen to which they later become autoimmune to involve as many of the same populations of cells as possible and so maximise the chances of detecting any abnormalities.

After determining that the NZB mice are showing the type of autoimmune phenomena reported for them in the literature the response of the adults to sheep erythrocytes will be studied in order to detect differences between the strains and between organs in the same strain. Following this, the immune response of baby mice of the three strains will be investigated to compare the age at which immunological responsiveness first appears in spleen and lymph nodes and to compare the immature response with the mature response in each case. This will be followed by a comparison of the growth curves for the body weight and for the spleen and lymph nodes. Because self-recognition is probably a tolerance phenomenon, the tendency of NZB and 'normal' strains to become sensitized or tolerant after exposure to the antigen at an early age will be investigated. The response of adult and baby mice to chicken erythrocytes will be compared to their response to sheep erythrocytes to find whether any differences detected are applicable to all antigens. Finally, the clearance rates of $^{131}$I-labelled SRBC in Ju and NZB will be determined in an attempt to develop an economical method for the survey of the response of baby mice to a large number of antigens.
2. REVIEW OF THE LITERATURE ON THE ONTOGENY OF THE IMMUNE RESPONSE

2.1 Introduction

The ontogeny of the immune response involves the morphological development of the lymphoid organs and tissues of the embryo and the maturation of the function of the cells. The age at which an animal is first able to produce a response to any particular antigen and the type of response that the animal produces before it is immunologically mature will depend on the functional maturation of all the cell types involved in the response. The maturation of the structure of the organ and the maturation of the function of its cells are not necessarily the same thing so they will be considered separately.

2.2 Morphological development of the immunological system

Much of the work to be described here refers to human embryos, but the patterns of development are similar in the young of all mammals.

In the early stages of embryonic life the cells which are to become myeloid blood forming organs and those which are to become lymphatic immunological organs have not yet differentiated from one another. Mesenchyme arises from embryonic mesoderm by the isolation of cells which become distributed singly or in groups in the spaces between the three germinal layers. Some of these mesenchymal cells round up to form free basophilic stem cells and these will later give rise to all the types of blood cells. This
process first occurs in the walls of the yolk sac and later in the body mesenchyme. In the yolk sac most of the primitive stem cells become red blood corpuscles, granulocytes and megakaryocytes. The primitive blood cells congregate in groups forming blood islands and between these, strands of endothelial cells transform into endothelial tubes which are the primitive blood vessels. Cells from the inside of these tubes detach and become phagocytes. Macrophage type cells are found in the diffuse mesenchyme also (Bloom and Fawcett, 1968).

The thymus is the first lymphoid organ to develop. It appears initially as epithelial outgrowths of the third and perhaps the fourth branchial pouch. In each outgrowth layers of epithelial cells surround a long thin lumen so that the thymic anlage is in effect a series of hollow cylinders. These become detached from the branchial pouches, the lumens disappear and the thymus primordium migrates downwards to the upper mediastinum. This thymus anlage remains epithelial until the 9th week in human gestation, the 14th day in the mouse, the rat and the rabbit and to birth in the opossum. Strands of the epithelium then begin to extend into the surrounding mesenchyme and lymphocytes appear (Osoba, 1968; Sterzl and Silverstein, 1967; Ham, 1965). It is probable that the lymphocytes arise in the epithelial portion of the rudiment, rather than the mesenchymal portion. Experiments by Auerbach (1960, 1961, 1964a, 1966) and Ball and Auerbach (1960) have shown that 12 day mouse thymus rudiments may be grown in tissue culture where they will differentiate. If they are separated into
epithelial and mesenchymal elements neither will differentiate alone, but when they are recombined differentiation and lymphogenesis occur. The mesenchyme from a variety of embryonic rudiments is effective in stimulating lymphogenesis, and separation of the epithelial and mesenchymal elements by a 20µ millipore filter results in differentiation of lymphocytes in the epithelial, but not the mesenchymal portion of the culture. It seems therefore that lymphocytes are induced to develop in the epithelial part of the thymus by the inductive effect of the mesenchyme. The addition of splenic rudiments to thymus cultures results in marked synergism and increase of thymic differentiation (Auerbach, 1963, 1966). Thymic rudiments may also be cultivated in the anterior chamber of adult mouse eyes where they will differentiate (Auerbach, 1960) even if the recipient mouse has been irradiated. Labelling experiments show that the lymphocytes are of donor origin (Auerbach, 1964b). Early mouse or chick thymic anlage grafted in diffusion chambers onto chick chorioallantoic membrane do not become lymphoid, but this may be because suitable induction is not found in this area. Chick thymic anlage grown in the same place without a diffusion chamber do become lymphoid suggesting that immigration of cells is also important (Owen and Ritter, 1969). Ackerman and Knouff (1965), from cytological studies of the hamster thymus, decided that the epithelial cells may differentiate into lymphocytes or stellate reticular epithelial cells, although Moore and Owen (1967) on the basis of experiments using chromosomally marked parabiotic chick embryos have reached the conclusion that the
epithelial component acts as an inducer for stem cells that enter from elsewhere. Since in the adult animal thymic lymphocytes appear to be of bone marrow origin (see Miller and Osoba, 1967) it is possible that in early embryonic life, thymus lymphocytes can both arise in situ and migrate in from exogenous stem cell areas.

After the appearance of lymphocytes in the thymic rudiment, the epithelium is converted to a reticular framework and the medulla arises on the main stem and lobules by hypertrophy of the epithelium and reduction of the number of lymphocytes. Hassal's corpuscles appear at about this time (Sterzi and Silverstein, 1967). In older embryos (about 16 days in the mouse) the lymphocyte count increases rapidly for 2 days, remains constant until 2 days after birth and then begins to rise again more slowly. Size analysis shows there is a rapid change from large to small lymphocytes between 16 and 18 days of gestational life. (Ball, 1963). In newborn animals there is a rapid seeding out of thymocytes to peripheral organs (Nossal, 1964; Michalke, Hess, Riedwyl, Stoner and Cottier, 1969; Hess, Stoner and Cottier, 1967).

As the long bones develop mesenchyme migrates to the interior of the shaft where it resorbs the calcified cartilage and becomes bone marrow, it then functions as a source of stem cells for the myeloid and lymphoid series (Bloom and Pauwett, 1968).

The spleen first appears as a thickening of the mesenchyme in the dorsal mesentary of the stomach. Some of the cells begin to differentiate into stem cells of the
blood forming series. The spleen is initially totally myeloid and only later do lymphocytes appear (Bloom and Fawcett, 1968). These lymphocytes probably migrate in from the thymus and perhaps from other central tissues rather than arising in situ since cultures of splenic rudiments in vitro or in the eye chambers of irradiated recipients do not become lymphoid unless cultured together with thymic rudiments (Auerbach, 1963, 1964a). If the thymic rudiment is separated from the splenic rudiment by a millipore filter no splenic lymphocyte development occurs (Auerbach, 1964a, 1966). Cultivation of aplenic rudiments in the anterior chamber of the adult mouse eye results in the migration of recipient cells into the splenic rudiment (Auerbach, 1963, 1964a). In a thymusless mouse mutant De Sousa, Parrot and Pantelouris (1969) have shown that only the thymus dependent areas are without lymphocytes suggesting that lymphocytes for the other areas are derived from a central source other than the thymus.

Lymphatic vessels arise as fluid-filled spaces in the mesenchyme which join to form continuous vessels or lymphatic sacs. In some places primary lymph nodes are formed by the invagination of the surrounding mesenchyme into the lumen of the sac resulting in bars of mesenchyme between lymphatic spaces. The medulla of the lymph nodes develops first, then medullary cords on the peripherae of the nodes begin to bulge into the marginal sinus and these bulges proliferate to produce the cortex. Later in development, other lymph nodes arise similarly along the
course of the lymphatic vessels (Bloom and Fawcett, 1966). Lymph nodes from neonatal and 5 day old rabbits are very small and poorly organized with few primary nodes, no secondary nodules and no cortex. The medulla contains reticular cells but not medullary cords and there are no plasma cells. Maturation continues slowly with an increase in the size of the cortex and the general complexity of the structure until about 20 days after birth (Good, Condie and Bridges, 1960).

In birds, the bursa of Fabricius arises as an epithelial outpouching from the junction of the endoderm and ectoderm in the cloacal region. Lacunae form and coalesce to form a canal. Plicae project into it and the bursa thus becomes a canal lined with epithelium, which proliferates in places to form nodules of epithelium in which lymphoid cells appear. Lymphoid cells can probably arise from the epithelium but experiments with anastomosing twins show that cells are also derived from the circulation (Osoba, 1968; Warner and Szenberg, 1964b).

The rabbit appendix is a mammalian organ which is probably analogous to the avian bursa (Archer, Sutherland and Good, 1963). Its structure is similar to that of the bursa but it develops comparatively late in the ontogeny of the animal.

The embryonic liver is an important source of stem cells for the immune system from about the 9th day of gestation in the mouse embryo, but this is better demonstrated experimentally than morphologically. Transplantation of embryonic liver cells into irradiated recipients results in
the appearance of proliferating donor lymphocytes in the bone marrow and thymus of the recipient (Taylor, 1965), and transplantation to thymectomised or non-thymectomised recipients results in the production of donor type immunoglobulins (Tyan and Herzenberg, 1966b; Tyan et al., 1967, 1968). These stem cells are also found in embryonic thymus at 11-14 days of gestation, in the lung at 15 days and near the end of pregnancy in foetal bone marrow and spleen. They are not found in the gut. Subsequently they are found in many organs up to 6 weeks of age and then in the bone marrow only (Tyan, 1968). Thymus cells of adult animals are not capable of differentiating into all the cells necessary for the antibody response, so the thymus cells of young animals must have less restricted potential than those of adults.

Immunological stimulation can hasten the maturation of the lymphoid organs since plasma cells and precocious splenic lymphoid nodules are found in human embryos congenitally infected with syphilis or toxoplasmosis (Silverstein and Lukas, 1962).

2.3 Maturation of antibody-forming capacity

In the study of the maturation of the immune response it is of importance to know both when an animal first acquires the ability to produce antibody to a particular antigen and when the production of this antibody becomes mature in all its aspects. The way in which the response of the immature animals differs from that of the adult should also be determined. Problems associated with the
detection of the first response that an animal can make, lie in the small amounts of relatively low affinity antibody that may be produced and in the choice of correct dosage schedules (Sterzl and Trnka, 1957) and times of antibody assay to induce and detect a potential response. The presence of antibody transmitted from the mother through the placenta or the milk further complicates the interpretation of the results since it not only confuses the assay of the response, but may interfere with the response of the baby itself (see Uhr and Möller, 1968). It would eventually be desirable to be able to describe the maturation in terms of the different cell populations involved and of their particular functional or numerical inadequacies in the young animals, but this is as yet very far from being realised.

For an animal to be able to produce an antibody response to an antigen it must be able to produce immunoglobulins. On the other hand, the capacity to produce immunoglobulin does not necessarily indicate that the animal has the capacity to respond to a particular antigen. In germ-free animals the level of immunoglobulins in the serum is low although not absent (Gustafsson and Laurrell, 1958) suggesting that most immunoglobulins are produced in response to some antigenic stimulus. This would also mean that the absence of immunoglobulins in foetal sera may be due to lack of immunological stimulation rather than an inability to produce antibody or immunoglobulin. The times of the first appearance of immunoglobulin, autonomously or in response to natural or artificial antigenic stimulation, will
be considered first, together with the sequence in which the different classes appear, followed by a consideration of the ages that various species acquire the ability to respond to different antigens.

2.3.1 The synthesis of immunoglobulins

In chickens there is minor transfer of maternal immunoglobulins from the yolk, but the development of their immunoglobulins has not been studied in great detail. There are changes in the electrophoretic pattern of the serum of unstimulated chickens throughout embryonic development and gammaglobulin does not appear until the time of hatching (Weller and Schechtman, 1957; Amin, 1961). The removal of the bursa of Fabricius has the effect of arresting maturation of the immunological system at the age when the operation was performed, and if chickens are bursectomised early in life subsequently they can produce IgM only suggesting that the ability to produce IgM matures before the ability to produce IgG (Cain et al., 1969).

Opossums are marsupials and as such there is no placenta. Their young are born in a very immature state after 12.5 days gestation. They then attach themselves to the teats in the pouch where they develop for a further 60 days. The development of the blood forming and lymphoid tissues at birth is equivalent to that of an 8 weeks human or 10 day mouse embryo, and is thus very useful for the studies on the functional development of these organs (Block, 1967). In unstimulated pouch young IgM is more prominent than IgG which is present in small and varying amounts.
Adult values of all serum proteins are not attained until 80 days after birth. When antigenically stimulated with bacteriophage, IgM antibodies were the most prominent (Rowlands and Dudley, 1969).

Piglets have an epitheliocorial placenta and do not receive immunoglobulin from the mother before birth (Sterzl and Silverstein, 1967). After birth they can be maintained on an artificial colostrum-free diet so that all gammaglobulins found in the animals must be produced by the embryos or neonates themselves. At birth, unstimulated pigs are synthesising very small quantities of a substance which has the electrophoretic and chromatographic characteristics of gammaglobulin, which sediments at 5S and which has no detectable antibody activity (Franěk, Říha and Šterzl, 1961; Šterzl, Kostka, Říha and Mandel, 1960; Šterzl, Kostka, Mandel, Říha and Holub, 1960; Franěk and Říha, 1964). Kim, Bradley and Watson (1967a) maintain that it is antigenically distinct from all known immunoglobulins but Prokešová, Rejnek, Šterzl and Trávníček (1969) found it contained antigenic determinants for both light and IgG heavy chains. Franěk and Říha (1964) subjected this immunoglobulin to fingerprinting analysis and found relationships to heavy chains only. In colostrum-free piglets, the first immunoglobulin to appear without specific antigenic stimulation were IgG and IgA, followed by IgM (Porter and Hill, 1970). When stimulated with sheep erythrocytes the first immunoglobulin with antibody activity to be produced is a macroglobulin, said by Prokešová et al. (1969) to be a normal
IgM. In piglets stimulated with actinophage or Keyhole Limpet haemocyanin Kim, Bradley and Watson (1966b, 1968) found it to be an IgG sedimenting as a macroglobulin. Following this, IgG and IgA are synthetised. Early macroglobulins have different binding capacities from those synthetised later (Sterzl, Mandel, Miler and Riha, 1965). In piglets fed colostrum the quantities of IgM, IgA and IgG decline after the first day, but IgM begins to increase again after the first week. The quantities of IgG and IgA in the serum do not begin to increase until after the 4th week (Porter and Hill, 1970).

Foetal lambs have a syndesmochorial placenta which, as in pigs, prevents the passage of gammaglobulin from mother to foetus (Sterzl and Silverstein, 1967). These foetuses produce an atypical gamma globulin before antigenic stimulation which is perhaps analogous to the SS immunoglobulin produced by piglets. The serum of unstimulated lambs contains small amounts of IgM and SS IgG in the latter half of gestation produced mainly by the spleen (Silverstein, Thorbecke, Kraner and Lukes, 1963). Stimulation by antigen mixtures containing ovalbumen, ferritin, Salmonella and BCG causes an increase in the IgM fraction followed more slowly than in adults by IgG (Silverstein and Kraner, 1965). Stimulation by adjuvant causes increase in the IgG fraction early in the response but this IgG appears to be without activity to the injected antigens (Silverstein, Thorbecke, Kraner and Lukes, 1963).

Rats have a haemoendothelial placenta and although some immunoglobulins are transmitted prenatally, most are
transmitted through the milk (Sterzl and Silverstein, 1967). The injection of labelled amino acids into rat foetuses shows that they are actively synthesising at least some globulins three days before birth (Kelleher, Kenyon and Villee, 1963) and the antibody present in baby rats actively immunised with Salmonella pullorum is found in the IgM fraction in contrast to the IgG antibody found in adults or in babies passively immunised through their mother's milk (Halliday, 1957).

Mice have placentation similar to that of rats (Sterzl and Silverstein, 1967) and they can absorb IgG but not IgM globulin through the gastrointestinal tract. IgM, which must therefore be synthesised by the babies themselves, first appears in the serum of unstimulated babies at 1-3 weeks of age. Normal levels are reached by 2-3 months of age (Fahey and Barth, 1965). Takeya and Nomoto (1967b) found that 3 day old mice injected with sheep erythrocytes produced only mercaptoethanol sensitive antibody (IgM) for 31 days or longer after injection. As mice grew older the interval between the appearance of IgM and IgG became shorter. Transplantation of spleen cells from 4 day old mice into irradiated adult recipients show that they contain cells able to produce both IgM and IgG (Shearer et al., 1968) so perhaps baby mice are not deficient in IgG AFC-P so much as in the requisite pathways to stimulate their activity. Cells that can differentiate into immunoglobulin-producing cells in lethally irradiated allogeneic recipients are found in the liver, the yolk sac and the caudal half of 9 day embryos. In older embryos they are found in thymus,
gut, lung, spleen, femur and peripheral blood (Tyan and Herzenberg, 1968a,b; Tyan, Cole and Herzenberg, 1968).

Rabbits have a haemoendothelial placenta in which nearly all of the gammaglobulin transfer occurs before birth (Sterzl and Silverstein, 1967). Incorporation of radioactively labelled amino acids into gammaglobulin identified by immuno-electrophoretic techniques showed a slight synthesis by 24 day foetuses (Kulingara and Schjeide, 1962) and neonatal rabbits (Deitchmiller and Dixon, 1960; Wainer, Robbins, Bellanti, Eitzman and Smith, 1963). When IgG synthesised by the mother may be differentiated from that synthesised by her offspring by allotypic differences the first appearance of IgG synthesised by the offspring is at 3 weeks old in unstimulated animals and it does not reach adult levels until about 24 weeks of age (Dray, 1962). Adler, Curry and Smith (1967, 1969) stained the cells of lymphoid organs of unstimulated rabbits with fluorescin-conjugated antibody specific for IgG or IgM heavy chains. They found no antibody containing cells in animals under 5 days of age but in animals of 5 days old a few cells producing either IgM or IgG are found, mainly in the spleen. Cells producing IgG appear in the appendix after 2 weeks and cells producing IgM after 3 weeks. Analysis of fragments of the organs of baby rabbits in tissue culture shows no synthesis of gammaglobulin in neonates and synthesis largely in the appendix in rabbits between 1 and 3 weeks old (Thorbecke, 1960).

When neonatal rabbits are stimulated with *Salmonella* flagellin 198 antibodies appear first followed by 7S anti-
bodies only after 3-4 weeks, compared with adults in which the delay between the appearance of 198 and 73 antibodies is only 3-5 days (Bellanti, Eitzman, Robbins and Smith, 1963). The first antibody formed to foreign erythrocytes is also found to be IgM (Rīha, 1962) but Adler et al., (1967) found that in rabbits immunized at birth with *Salmonella paratyphi* B IgM synthesising cells appeared after 16 hours and IgG synthesising cells only 20 hours later. Adjuvant can stimulate the production of apparently non-antibody IgG in baby rabbits as it can in lambs (Pernis, Cohen and Thorbecke, 1963).

Guineapigs have a placenta similar to that of the rabbit (Sterzl and Silverstein, 1967). Autoradiography and immunoelectrophoresis shows that IgM is produced by foetal spleen and newborn lymph nodes. ν₂ production begins 2 weeks after birth and ν₁ at 1 month of age (Thorbecke, 1964).

Man has a haemochondral placenta, and here too, gammaglobulin transmission occurs before birth (Sterzl and Silverstein, 1967). Levels of IgG approximate that of the adult at birth but then decline until 12-16 weeks of age when presumably synthesis in the baby itself begins. Adult levels are reached by 12 months of age and IgM is present at birth and rises rapidly to reach adult levels at 20-24 weeks of age (Haworth, Norris and Dilling, 1965). Smith and Eitzman (1964) have found a gammaglobulin in the infant that is not found in the adult. Normal children lack antibody-producing plasma cells until the 4th to the 6th week of life (Bridges, Condie, Zak and Good, 1959). In newborn infants
that have been immunized with *Salmonella* (Smith and Eitzman, 1964) or typhoid and paratyphoid vaccine (Pink, Lo Spalluto, Miller and Dorward, 1961) or in 28 week foetuses that have contracted congenital toxoplasmosis (Eichenwald and Sobinefield, 1963) IgM antibody is the first produced. Smith and Eitzman (1964) find that there is a long delay between the production of IgM and IgG in young babies, but Fink et al. (1961) claim that the change from 198 to 73 is faster in the infants than in the adults. Bridges et al., (1959) studied a normal child born to an agammaglobulinaemic woman and thus without maternally acquired antibodies. They found little gammaglobulin until the 6th week of life in spite of stimulation with TAB vaccine (*Salmonella typhosa*, *S. paratyphi* A and B).

The exact time of the appearance of the gammaglobulins in baby animals may be of little real significance. Thorbecke and Van Furth (1967) have suggested that the age at which gammaglobulin synthesis first occurs may be correlated with the length of gestation in a species, in that the longer the gestation period the earlier proportionately do the immunoglobulins appear, but there are so many other factors involved, such as the time of the animal's first exposure to antigen, the type of antigen to which it is exposed and the effect of the antibody transferred through the milk or the placenta, that without carefully replicated experiments it is impossible to tell whether the earliest reported appearance of immunoglobulins is really the first age at which the animal can produce these proteins. Perhaps of more significance is the common observation that the
ability to produce IgM seems to appear earlier than the ability to produce IgG although there are exceptions to this. In the response of adult mice IgM also appears before IgG, but in most cases the delay between the appearance of IgM and IgG in baby animals is a great deal longer than in the adults. This could be due to immaturity of the antibody-forming cells, but the ability of adjuvant to induce IgG formation - either non-antibody or antibody towards some component other than that which induced IgM - and the ability of cells from baby mice to produce IgG when transplanted to adults suggest that the antibody forming cells are able to produce IgG but that the immature system cannot stimulate them to produce IgG.

2.3.2 Production of specific antibody

Chickens show their first response to goat erythrocytes when injected at 15 to 17 days of the 21 day incubation period (Solomon, 1965). Injected with bovine serum at hatching they showed a small and variable response and the mature quantities of precipitins did not appear until they were injected at 5 weeks of age (Wolfe and Dilks, 1948). Chickens injected with Salmonella pullorum after the 17th day of incubation showed no antibody until 20-40 days after hatching. Injection before the 15th day of incubation induced tolerance (Buxton, 1954).

Opossum pouch young of 5 days or older would respond to bacteriophage F2 and at 15 days and over to DNP hapten (Rowlands and Dudley, 1969). Antibody to Salmonella
typhi was produced in 8 day old pouch young (La Via, Rowlands and Block, 1963; Rowlands, La Via and Block, 1964). It is at about 8 days after birth also that the lymphocytes appear in the thymus (Block, 1967). When the opossum young are injected with φX phage at 11 days of age accelerated clearance of the antigen is induced although it does not occur until 11 days later (Kalmutz, 1962).

When piglets were immunized with sheep erythrocytes after 2 of the 3 month gestation period, antibodies were found five days later (Sterzl, Mandel, Miler and Říha, 1965). Piglets challenged with MSP-2 antinophage 2 days after a 2-5 day premature delivery could produce antibodies within 48 hours (Kim, Bradley and Watson, 1964, 1966a). At one month of age the response to the antinophage was indistinguishable from that of the adults (Kim, Bradley and Watson, 1966a). Injection of newborn colostrum-free piglets with φX174 or T2 phage resulted in the appearance of antibody by the third day after immunization (Hajek and Mandel, 1966). When the piglets were injected with Brucella suis at 2 months of gestation they showed no response when tested at birth (Sterzl, Mandel, Miler and Říha, 1965), but when injected after slightly premature delivery they showed a response 2 weeks later (Sterzl, Kostka, Mandel, Říha and Holub, 1960). 2 month old foetuses could not respond to Salmonella paratyphi B and even when they were injected at birth there was very little response (Sterzl, Mandel, Miler and Říha, 1965). Under the right conditions antibody to both tetanus and diphtheria toxoid
can be produced after injections at birth (Segre and Kaeberle, 1962b). Injection of bovine serum albumin, E. coli or Keyhole Limpet haemocyanin into 2 day old prematurely delivered pigs can interfere with the response to simultaneously injected actinophage, presumably by antigen competition, but antibodies to these antigens were not tested for (Kim, Bradley and Watson, 1966a).

Pigs also provide evidence on the importance of maternally derived antibodies. Segre and Kaeberle (1962a,b) have shown that newborn colostrum deprived piglets produce antibodies to tetanus, an organism the mother may quite well have been exposed to in the normal environment, but not to diphtheria, an antigen which the mother is unlikely to have been exposed to. When fed colostrum from their mothers they could produce antibodies to diphtheria but not to tetanus. Similarly, colostrum-deprived piglets could not respond to Brucella abortus bacterin, bovine red blood cells, bovine serum of ovalbumen until they were 8 weeks old, but 3 weeks old colostrum fed piglets could produce a response to these antigens. If, however, they were fed colostrum from immune mothers the responses were suppressed (Hoerline, 1957). On the assumption that there is an extremely small transplacental transfer from sow to piglets and that a mature sow will have in her colostrum 'natural' antibodies or antibodies produced to cross reacting organisms in the environment some of which will have a limited affinity for the test antigens, it seems probable that very small amounts of antibody are necessary for the immune response in these
immature animals. The transfer of larger amounts of higher affinity antibody from mothers that were producing specific antibodies inhibited the response.

When lambs were injected at 60 days of the 150 day gestation period, they could respond essentially normally to the phage X174 but horse ferritin produced only a weak response. Even in older foetuses the response to ovalbumin and to diphtheria toxoid was weak and *Salmonella typhosa* produced no response until after birth. The earliest responses appeared at about the time that the thymus was becoming lymphoid (Silverstein, Uhr, Kroner and Lukes, 1963; Silverstein and Kroner, 1965).

Bovine foetuses inoculated with leptospira at 132-166 days of gestation can produce antibody 32-62 days later (Fennestad and Borg-Peterson, 1962) and foetuses inoculated at about 220 days show high titres at birth even before the ingestion of colostrum (Fennestad and Borg-Peterson, 1957).

Rats injected with *Brucella abortus* between birth and 4 days of age can produce high titres of agglutinins in 10-14 days although the response does not appear in its adult form in animals injected before 1 month of age (Halliday, 1964). Rats can produce antibodies to *Salmonella pullorum* in the first week of life (Halliday, 1957) but there is no response to sheep erythrocytes in animals up to 7 days old and even injected at 17 days of age they produce a good but not yet adult response (Rowley and Pitch, 1965).

Experiments with mice show clearly both the
differences in the responses of the same strain to different antigens and in the responses of different strains to the same antigen. Mice injected at 1 day of age with sheep, rabbit, or rat erythrocytes show slight and very delayed antibody production to the sheep and to the rabbit erythrocytes but none to the rat erythrocytes. When they are injected at 2 weeks old they respond to all three antigens, but the greatest response is to sheep; there is less to rabbit and least of all to rat. The response of 12 week old mice is the same to all three antigens (Makinodan and Gengozian, 1960; Gangozian and Makinodan, 1958).

Production of haemagglutinins to allogeneic mouse thymus cells does not appear until after 25-30 days of age in C57 and DBA mice (Moulton and Storer, 1962). CFW mice injected at birth with sheep erythrocytes with pertussis as adjuvant showed antibody forming cells at 4 days old. Similar neonatal injection with horse serum did not result in antiserum production as much as 7 weeks later, but an anaphylactic response could be obtained at 7 weeks old (Hargis and Malkiel, 1969). When neonatal mice were injected with bovine serum albumin they were found to develop the response to different determinants of the molecule at different ages (Habicht and Terres, 1966). The development of the immune response to sheep erythrocytes in germ-free mice was found to be the same as in conventional mice (Booma, Makinodan and Walburg, 1967), but since natural antibodies to staphylococcal antigens (Cohen, Newton, Cherry and Updyke, 1963) and to sheep erythrocytes (Nordin, 1968) are present
in germ-free mice this does not indicate that baby mice do not require antibody to produce an immune response.

When different strains of mice are tested with sheep erythrocytes NZB show their earliest response when they are injected between 1 and 3 days of age (Playfair, 1968a). AKR (Hechtel, Dishon and Braun, 1965b) and Balb/c (Playfair, 1968a) show a first response at 5 days of age, Ha/ICR at 7 days (Hechtel, Dishon and Braun, 1965b), SL at 8 days (Takeya and Nomoto, 1967a) and C57Bl at 10 days of age (Hechtel, Dishon and Braun, 1965b; Playfair, 1968a). The response measured as the number of antibody producing cells appears mature by 15 days of age in SL (Takeya and Nomoto, 1967a) and in Balb/c (Playfair, 1968a). The magnitude of the immune response to the sheep erythrocytes could be increased by treatment of the baby animals with deoxyribonucleotides (Hechtel, Dishon and Braun, 1965a,b).

Rabbits injected neonatally with Salmonella flagellin produced antibody in 7-10 days (Bellanti et al., 1963) though Šterzl and Truksa (1957) showed that the higher the dose of Salmonella paratyphi B given to 5 day old animals the sooner were antibodies produced. Neonatal rabbits could give a response to various kinds of foreign erythrocytes similar to that of older animals but it depended on the dose and on the presence of passively transferred antibodies from the mother (Šiba, 1961, 1962). Šterzl, Vesely, Jilek and Mandel (1965) showed that rabbits injected at 5 days of age had only low numbers of cells forming antibody against sheep erythrocytes. There was
little antibody production in lymph nodes to diphtheria toxin in neonatally injected rabbits (Pernis, et al., 1963). Rabbits will occasionally respond to bovine gammaglobulin when they are injected at 3 days of age, but they only produce a regular response after injection at 1 week (Harris, Harris, Ogburn and Farber, 1961). At 5 days old they sometimes will produce antibodies to bovine or human serum albumin injected in Freund's adjuvant (Riha, 1962). In animals older than 8 days bovine serum albumin may induce antibody production (Eitzman and Smith, 1959), but it gives a good response only in animals 21 days or older. Good et al., (1960) found that the immune response to intradermal bovine serum albumin did not appear in animals younger than 28 days old, but when 7 day old animals were injected with bovine serum albumin in Freund's adjuvant the first antibody was found 10-12 days later. The response to Shigella trypsin filtrate did not appear until rabbits were 1 month or older.

Human infants cannot be directly immunized as foetuses, but indirect evidence for an early antibody response has been obtained by studies of congenital toxoplasmosis (Eichenwald and Shinefield, 1963) and these show that antibody can occur as early as the 28th week of pregnancy and similar studies of congenital syphilis reveal that antibody production in the foetus to these organisms as early as the 6th month of gestation. (Silverstein, 1962). Infants immunized at birth with Salmonella antigens show agglutinin production 7-14 days later, but response was inhibited by transplacentally acquired agglutinins (Smith and Eitzman, 1964). A child from an agammaglobulinaemic mother was unable
to respond to *Salmonella typhosa* or *paratyphi* until 6 weeks of age (Bridges et al., 1959). Thus it appears that in human infants also small amounts of antibody are necessary for the immune response but that large amounts are inhibitory. The response to diphtheria toxoid improves during the first two months of life and the response of 3-month-old infants was similar to the response of 6-month-old infants (Osborn, Dancis and Julia, 1952). There is no difference between the response of premature or term infants to diphtheria toxoid, but premature infants immunized at the estimated time of term delivery gave a better response than the newborn at term suggesting that the exposure to environmental antigens may be of importance (Dancis, Osborn and Kunz, 1952). The response to tetanus toxoid is better after 2 months than before 1 month of age (Osborn, Dancis and Julia, 1952). The response to pertussis, tetanus, and diphtheria is potentiated by passive antibodies (Levi, Kravtzov, Levova and Fomenko, 1969). Premature and full-term infants show a good antibody response to phage <i>φX174</i> in the first week after injection (Uhr, Dancis, Franklin, Finkelstein and Lewis, 1962). Premature infants were immunized with typhoid and paratyphoid vaccine within 2 days of birth by repeated immunizations. Responses to typhoid <i>Y</i> and paratyphoid <i>A</i> and <i>B</i> were high but that to typhoid <i>0</i> was low (Fink, et al., 1961). Dwyer and MacKay (1970) have found that the thymuses of human foetuses of 20–22 days gestation already contain lymphocytes specifically binding to *Salmonella* antigen and therefore with specific immunoglobulins on their surface. This does
not mean that they are able to respond to this antigen at this age.

Foetal rhesus monkeys were able to give a PFC response to sheep erythrocytes as fast and as high as that of the adult, but the number of antibody forming cells reduced more rapidly than in the adult (Silverstein, Prendergast and Parshall, 1970).

The variation between species in the time when they can first produce a response is not unexpected. Different species are born in different states of maturity and the relative rates of the development of different organ systems also varies between species so that differences in the level of immunological responsiveness at birth is part of the general pattern. More difficult to explain are the different times of the appearance of the response to different antigens in a single species. The multiplicity of methods used by different workers to induce and to detect the response makes comparison between them difficult, but the finding that the first appearance of a response can vary from early in gestation to well after birth depending on the antigen is too general to be discounted.

The responses to different antigens have requirements for different populations of cells, and with any particular antigen there will presumably be little or no response until all the populations of cells involved are functional to some extent. Thus, for example, if the ability of macrophages to process antigen matures late in ontogeny the response to those antigens requiring macrophages will also mature late but if the antigen can be presented in such
a way that the macrophages are not necessary, the response will be seen much earlier than it would otherwise have appeared. Even when the response can be induced, it may still be undetected owing to inadequacies in the function or the number of the cells involved, giving it characteristics different from that found in the adult.

2.4 The maturation of cell-mediated immunity

Injection of chick embryos with whole allogeneic blood at 11-17 days of incubation sensitizes them so that they can reduce the amount of graft versus host splenomegaly produced after injection of blood from the same donor at 19 days incubation. The rate of the rejection of skin grafts was only increased if the chickens were sensitized with blood from the donor at 19 days of incubation or later (Solomon, 1963).

In the lamb, homograft rejection does not occur in animals younger than 77-80 days of gestation (Silverstein, Prendergast and Kraner, 1964; Silverstein and Kraner, 1965; Schinckel and Ferguson, 1953) and when the response does develop, the skin grafts are rejected as rapidly as in the adults (Silverstein et al., 1964). Schinckel and Ferguson (1953) have shown that since the embryo lamb would reject maternal skin the response could not be of maternal origin.

In colostrum-free piglets the injection of bone marrow or lymphoid cells at 60 days gestation causes tolerance to skin homografts from the cell donor, indicating immaturity but injection at 80 days of gestation causes
sensitization (Sinna, 1967). Sterzl, Kostka, Mandel, Riha and Holub (1960) have shown that colostrum-free piglets can reject homografts during the first week of post-natal life.

Lewis rats developing in BN x Lewis mothers showed sensitization rather than tolerance to BN skin grafts applied at 3 days of age, presumably due to exposure to maternal transplantation antigens during foetal life (Billingham, Palm and Silvers, 1965) although since mothers and foetuses do not normally respond immunologically to one another it seems curious that this should have occurred in this instance.

If baby mice are injected with viable lymphoid cells from an animal immunologically competent to react against the strain of the baby, the recipient mouse develops splenomegaly owing to the action of the injected cells (Howard and Michie, 1962). Older mice injected with immunologically incompatible cells do not develop splenomegaly because they can reject the transplanted cells. This indicates that neonatal mice have a reduced capacity to reject foreign tissue. Cells from C57/B1 mice first gain their ability to induce splenomegaly in B6/D2 hybrids at 6-8 days of age (Bortin, Rima and Saltstein, 1969). An anaphylactic response to horse serum can be induced in mice injected at birth with horse serum and pertussis adjuvant, although antibody to horse serum cannot be demonstrated (Hargis, and Malkiel, 1969).

Postnatal rabbits given adult spleen cells before day 22 of the 31 day gestation period were rendered tolerant of
subsequent skin grafts, but those given spleen cells after
day 26 of gestation were sensitized, and rejected subsequent
skin grafts more rapidly than did untreated rabbits
(Porter, 1960). Neonatal rabbits of less than 1 week of
age were able to reject skin grafts more rapidly than adults
(Najarian and Dixon, 1962) showing that they were already
fully able to respond by one week of age.

Newborn infants can also reject skin grafts
efficiently (Fowler, Schubert and West, 1960). Lymphocytes
taken from babies born to mothers who had had E. coli
infections of the urinary tract during gestation showed
greater proliferation on exposure to E. coli in vitro than
lymphocytes from babies whose mothers had not had E. coli
infection (Brody, Oski and Wallach, 1966).

Delayed hypersensitivity is difficult to produce
in newborn guineapigs (Uhr, 1960; Salvin, Gregg and Smith,
1962), but may be passively transferred from newborn animals
to adults (Salvin, Gregg and Smith, 1962) suggesting that
the baby guineapigs are able to react to the antigen but the
body is in some other way incapable of producing skin hyper-
sensitivity reactions at this age.

There is no evidence that the maturation of cell-
mediated immunity occurs by a process any different to the
maturation of antibody activity. The ability to reject
grafts and to produce graft versus host reactions appears
during the time when the maturation of the ability to produce
antibody to other antigens is also appearing. The response
to transplanted antigens need not be considered as maturing
in a different way or by different means to the antibody response.

2.5 The maturation of the antigen-trapping mechanism

The rate of the clearance of labelled antigen from the body or from the circulation will depend both on the activity of the macrophages and phagocytes and on the presence or absence of antibodies or other opsonizing materials. Injection of foetal rats with labelled bacteria or carbon particles showed that the elimination rate increased up to birth and then remained constant until weaning when the rate of bacterial but not carbon elimination began to increase again. Opsonization had no effect, suggesting that this represents a functional maturation of the reticuloendothelial system rather than the appearance of the ability to produce opsonizing antibodies (Reade and Jenkin, 1965). Mitchell and Nossal (1966) also showed a low rate of phagocytosis, even of opsonized materials, in neonatal rats.

The ability to degrade various antigens was studied and compared in newborn, 6 and 30 day old rabbits. The newborns could not degrade azo compounds, but the 6 day old rabbits were found to have developed this capacity (Robbins, Eitzman and Smith, 1963). It was suggested that this was due to the lack of an adequate functional enzyme degradative system, and indeed, Karthigasu, Reade and Jenkin (1965) showed that macrophages of foetal rats lacked the ability to kill certain bacteria, but that this function develops soon after birth, and Reade (1966) also found that the
bactericidal activity but not the phagocytic activity of peritoneal macrophages depended on the age of the animal from which they were taken. Macrophage uptake and organ distribution of labelled bacterial particles is similar in rat foetuses of 14 days to those of adults (Read and Casley-Smith, 1965). In chickens, the rate of uptake of $^{35}$S labelled bovine serum albumin into the spleen and its rate of disappearance is higher in neonates than in adults. The rate of disappearance from the liver is similar in the two age groups indicating that this was not entirely a reticuloendothelial system phenomenon (Hirata, Garvey and Campbell, 1962).

Perhaps the most thorough work of this kind comes from Nossal and his co-workers. In these experiments the distribution of $^{125}$I-labelled flagellin injected into rats of various ages was studied by autoradiography. The results show that newborn rats lack an antigen-trapping mechanism in particular, the mechanism that accumulates antigen in the lymphoid follicle. (Williams and Nossal, 1966; Mitchell and Nossal, 1966; Nossal, 1966, 1967). Cortical antigen localization first develops at about 10 days of age as a rim of antigen trapping tissue and after a few days the pattern changes to the follicular pattern of antigen uptake. The medullary trapping ability develops slowly between 2 and 6 weeks of age (Williams and Nossal, 1966). Mitchell and Nossal (1966) have shown that opsonins added to the antigen given to baby rats do not alter the immature pattern of trapping so that the observation of a close correlation
between the ability to localise antigen in the lymphoid follicles and the ability to produce an immune response (Williams, 1966) probably indicates that follicular localisation is necessary for the immune response and not vice versa. It seems therefore that at least one of the reasons for the immunological deficiency in the baby rats lies in the ability of the reticuloendothelial system to localise the antigen.

Further evidence that the immaturity of the macrophages is of significance in the inability of neonatal animals to respond to antigens comes from Winchurch and Braun (1969) who showed that baby C57Bl mice given endotoxin or synthetic polynucleotide to stimulate macrophage activity had a greater number of cells producing antibody to sheep, chicken or human erythrocytes than the untreated controls. More unequivocal evidence is produced by Martin (1966) who showed that adult macrophages given to neonatal rabbits together with bovine serum albumin resulted in an immune response in the babies. Braun and Lasky (1967) also showed that the injection of the glass adherent fraction (macrophages) of adult peritoneal exudate cells causes an immune response to sheep erythrocytes in C57Bl mice at an age when they do not respond to sheep erythrocytes alone. Argyris (1968b, 1969b) also found adult peritoneal macrophages could increase the response of baby C3H mice to sheep erythrocytes.
2.6 Transfer experiments

There is no doubt that baby animals are less able to respond to antigens than adults, and in order to confirm that this inadequacy is due to a cellular immaturity and not to some inadequacy of the milieu, experiments were carried out in which adult lymphoid cells were transferred to baby animals and tested for their ability to respond in the new environment. The spleen cells of adult chickens produced antibody to *Brucella abortus*, *Salmonella paratyphi B*, *Brucella suis* and bovine serum albumin after transfer to baby chickens (Trnka, 1958; Papermaster, Bradley, Watson and Good, 1959, 1962; Papermaster and Bradley, 1960; Trnka and Riha, 1959) and adult spleen cells were also able to produce an efficient graft versus host response in baby chickens (Simonsen, 1957; Trnka and Riha, 1959). Similarly, lymph node cells (Harris, Harris and Farber, 1959, 1962) or cells from the lymph (Holub, 1958; Holub and Riha, 1960) of adult rabbits can produce antibodies in baby rabbits. Harris et al., (1962) suggest that the response of adult cells in neonatal rabbits may be rather lower than the response of the same cells transferred to irradiated adult recipients, but have shown that this may be due to the response of the babies against the injected adult cells. Only Dixon and Weigle (1957) have produced conflicting results where adult lymph node cells transferred to baby rabbits become unresponsive, but since they did not use inbred strains of rabbits the babies probably produced a sufficient response against the injected adult cells to prevent their functioning.
In the alternative experiment where the cells of baby animals were transferred to irradiated adults, Harris et al., (1961) found that rabbit lymph node or spleen cells incubated in vivo or in vitro with Shigella and transferred to irradiated adults produced no antibody if the donors were less than 1 month old, and the maximum response of the lymphoid cells was found only if the donors were over 3 months old. This corresponded to the time of maturation of the baby rabbits to Shigella antigen. Makinodan and Peterson (1964) found that spleen cells from baby mice did not produce antibody to sheep or rat erythrocytes in adults and Glaman et al., (1966a) found that the spleen cells of mice of 6 days and younger were ineffective at producing antibody to sheep erythrocytes in irradiated adults. Shearer et al., (1968) found that spleens of baby mice 4 days old had fewer antigen-sensitive units than the spleens of the adults. Sterzl (1963) found that cells transferred to young animals would produce antibody against Brucella suis only when they came from donors older than 3 weeks of age. In tissue culture, peritoneal cells from mice of less than 6 weeks old were inefficient at producing a primary response to sheep erythrocytes (Bussard, 1967). Glaman et al., (1966a) transferred a combination of thymus and bone marrow cells into irradiated adult mice and tested their ability to produce antibody to sheep erythrocytes. Thymus cells from 1 day old mice would not produce a synergistic effect with adult bone marrow but 6 day old thymus cells could. MacGillivray, Mayhew and Rose (1970) on the other
hand found neonatal thymus as effective as 4 week old thymus in this respect. Dixon and Weigle (1959) again have the only dissenting data. They found that lymphoid cells from 4-14 day old rabbits could form large amounts of antibody to Shigella extract in irradiated adults after sensitization in vitro.

2.7 Conclusion

Evidence has been produced that the inadequacy of the immune response of baby animals is due to immaturity of one or more of the populations of cells involved in the response to an antigen. The concept that immunological immaturity is primarily an inadequacy of the macrophage uptake and processing system is a tempting one. It allows self-recognition to be explained on the basis of tolerance induction caused by the exposure of specific lymphoid cells to antigen that is non-immunogenic owing to the lack of macrophage processing. It is difficult to explain the sequential saturation of the response to different antigens by this hypothesis unless the ability of the macrophages to process different kinds of antigens matures at different times. Another explanation would be needed to account for the immaturity of the response to non-macrophage requiring antigens.

A further objection to the central role of macrophages in the immunological immaturity of baby animals lies in the well known effects of neonatal thymectomy in preventing the development of a functional immunological
system. The effect is almost certainly due to a lack of ARC in the thymectomised mice particularly since the AFC-P appear to be normal (Tyan, Cole and Herzenberg, 1966; Tyan, Cole and Nowell, 1966; Tyan, 1964; Tyan and Cole, 1966b; Umiel, Globerson and Auerbach, 1968). Thymectomy in adult life has little effect unless the animal has been depleted of its ARC for example by irradiation (Aisenberg and Davis, 1968b). The effect in baby animals depends on the age at which thymectomy is performed (Archer, Pierce, Papermaster and Good, 1962). This suggests that ARC are not present in the peripheral tissues at birth, and that immunological immaturity is due to other factors in addition to macrophage immaturity.

It seems most probable that different populations of cells mature at different times during ontogeny. The first appearance of the response to an antigen will appear when there are enough of the required cells in a sufficient state of maturity to be able to recognise the antigen and be induced to produce a quantity of antibody sufficient to be detected. The mature adult response will not appear until all the cells involved in the response to the antigen being tested are mature both in numbers and in function.
New Zealand Black (NZB) mice are a strain developed by Dr. M. Beilschowsky in New Zealand, and first reported by Bielschowsky, Helmer and Howie in 1959. The most interesting aspect of this strain is their development of idiopathic autoimmunity; that is they display a vertically transmitted failure of self-recognition and are therefore of great potential value in an enquiry into the nature of this phenomenon. There has been a considerable amount of investigation into these mice involving the clinical patterns and origin of the disease and also other abnormalities of the immunological system.

3.1 Clinical aspects of the disease syndrome

The clinical features of the autoimmune disease manifested in these mice has been reviewed in detail by Mellors (1966a) and Howie and Helmer (1968). The most regular feature is the appearance of autoantibodies to the erythrocytes at an age between 4 and 9 months (East de Sousa and Parrot, 1965; Helmer and Howie, 1963; Holmes and Burnet, 1963a; Howie and Helmer, 1965; Mellors, 1966a). These autoantibodies may be detected by the direct anti-globulin (Coombs) test, that is the ability of antibody to mouse immunoglobulin to agglutinate the antibody-coated cells of the NZB mice, and this measures the antibodies attached to the red blood cells of the diseased mouse. The autoantibodies may also be detected by the indirect anti-globulin test which measures the ability of red blood cells
from normal mice incubated with serum from diseased NZB to be agglutinated by antibodies to mouse immunoglobulin. Mouse erythrocytes treated with papain will also be agglutinated by the serum from diseased NZB. These last two tests measure the antibodies in the serum. According to Long, Holmes and Burnet (1963) these tests measure antibodies of the same specificity. The proportion of the total autoantibody on the cells and the proportion that is present in the serum will not necessarily be the same in every mouse since it will depend on such factors as the total amount of autoantibody present and its avidity. The erythrocyte antibodies have been analysed by the use of specific antiglobulins in the direct antiglobulin test and found to be predominantly gamma\textsuperscript{1}G although they can be of all classes (Warner and Wistar, 1968). Norms and Holmes (1964b) also found that the antibody coating the erythrocytes was a classical gammaglobulin but Mellors (1965) using density gradient centrifugation found the antibodies in the 19S fraction. These results could be due either to the autoantibody being an unusual 19S IgG, or to the fact that the IgG antibodies are more avid than the IgM and hence tend to be found complexed with the red cell antigen, while the less avid IgM remain in the serum. Alternatively it could be due to differences among the substrains found in different laboratories. An antibody that will react with dried smears of mouse or human erythrocytes may also be demonstrated using fluorescein-conjugated antibody to mouse globulin. It is considered to have a different specificity from that
detected by the other tests (Holborow, Barnes and Tuffrey, 1965).

The appearance of the erythrocyte autoantibody is related to a reduction in the haematocrit and with reticulocytosis (Mellors, 1966a; Helyer and Howie, 1963; Holmes and Burnet, 1963a) though the reticulocytosis is not responsible for the positive direct antiglobulin tests (Giltinan, Norins and Holmes, 1965). The appearance of the autoantibody is also correlated with reduced erythrocyte survival (Lindsey, Donaldson and Woodruff, 1966), and the breakdown of the erythrocytes occurs mainly in the livers and spleens of the animals (Donaldson, 1967).

Other manifestations of autoimmune disease that are found in these mice are kidney disease (Holmes and Burnet, 1963a; Comerford, Cohen and Desai, 1968; Helyer and Howie, 1963; Mellors, 1965; Howie and Helyer, 1965; East de Sousa and Parrott, 1965) which consists of thickening of the basement membrane of the glomerular and later to tubular damage. The glomeruli will fluoresce after treatment with fluorescein-labelled antibody to mouse gammaglobulin. When this antibody is eluted from the kidneys it will recombine with NZB kidneys from which the immunoglobulin has been eluted, but not with kidneys from healthy young NZB nor with kidneys from mice from other strains (Mellors, 1965). Immune complexes will localize in kidney glomeruli (see Andres, 1969) but Mellors (1965) found that the eluted antibodies had no affinity for papain-treated mouse erythrocytes. Some evidence has been produced that the immunoglobulins in the NZB and NZB/NZW kidney lesions are antibody to nucleic
acid (McGiven and Ironside, 1968; Seegal, Accinni, Andres, Beiser, Christian, Erlanger and Hsu, 1969) although Mellors (1965) found the immunoglobulin eluted from the kidney lesions had no affinity for cell nuclei, and McGiven and Ghose (1968) found the antinuclear factor of these mice was not cytotoxic for kidney cells.

The mice also develop hepato and splenomegaly fairly regularly. The spleen may show a proliferation of haematopoietic tissue (Holmes and Burnet, 1963a; Helyer and Howe, 1963; East de Sousa and Parrott, 1965). The livers may be enlarged and show necrotic areas (Helyer and Howe, 1963) and liver autoantibodies may be present (Elson and Mayesmith, 1969). Associated with this hepatosplenomegaly is an increase in the rate of carbon clearance (Morton and Siegel, 1970b). Antinuclear factor has been found in NZB (Norins and Holmes, 1964a; Barnes and Tuffrey, 1967) but the incidence may not be significantly higher than in other strains of mice.

Other abnormalities of the lymphoid system found in these mice include a tendency to develop lymphoproliferative disorders (Holmes and Burnet, 1963a; East de Sousa and Parrott, 1965; Mellors, 1966b). Thymic 'germinal centres' have been reported in association with the development of autoimmunity (Abbot and Burnet, 1964; Burnet and Holmes, 1964; Holmes and Burnet, 1963a), but Siegler (1965) considered that these are only an example of the lymphoid infiltrations which are found in other organs of the body (see Howe and Helyer, 1968; Holmes and Burnet, 1963a). The abnormalities described for the NZB are found
in this strain when they are kept in a germ-free condition, although often in a less severe form or later appearing than in conventional NZB (East and Branca, 1969; East, Prosser, Holborow and Jaquet, 1967).

The amount of serum gammaglobulin, especially the IgM, increases as the mice grow older (East, de Sousa and Parrott, 1965; Warner and Wistar, 1968; Mellors, 1965), but reports of the time of this increase varies from 3 months (Warner and Wistar, 1968) to 10 months (Mellors, 1965). Cryoglobulins were found in some mice over 4 months of age (Hijmans, Radema, Vanes Feltkamp Van Loghem and Schaap, 1969).

3.2 Genetic studies

Experiments in which NZB have been crossed with other strains of mice shows that autoimmune disease occurs in the offspring whether the NZB is the male or the female parent (Holborow and Denman, 1967; Bielschowsky and Bielschowsky, 1964) but the particular manifestations of the disease depend on the strain of mouse with which the NZB has been crossed. The results for crosses with other NZ strains are listed in Howie and Helyer (1965) and Bielschowsky and Bielschowsky (1964) have studied in detail the results of crosses between NZB with NZC, NZO or NZY. Braverman (1968b) has discussed the genetic basis of the autoimmunity of the various strains of NZ mouse and the crosses between them and suggests a model of a gene with a modifier to account for the assortment of the autoimmune factors. Holmes and Burnet (1964a) show that in NZB x C3H
positive antiglobulin tests develop much later than in the purebred NZB. Further investigations with C3H, AKR, NZW, C57Bl and T6 show that the hybrids of these strains with NZB nearly all produced autoantibodies that could be detected by direct antiglobulin tests, but at a later age than the NZB. (Burnet and Holmes, 1965; Holmes and Burnet, 1966). The NZB x NZW hybrid develops a very acute form of kidney disease (Howie and Helyer, 1965; Channing, Kasuga, Horowitz, Dubois and Demopoulos, 1965; Lambert and Dixon, 1968).

It is clear that the disease is transmitted vertically through the germ cells which suggests a genetic element as the most likely explanation. It does not rule out other vertically transmitted agents, nor does it rule out the additional participation of non-genetic factors.

3.3 The effects of treatment

To find whether the course of this disease could be influenced by the use of agents that inhibit the immune response, the effect of treatment with immunosuppressants has been studied. Casey, (1968a,b) has investigated the effects of 6 mercaptopurine and azathiopurine (Imuran) on the autoimmune haemolytic disease of NZB. He found that neither prevented the onset of autoimmunity. In NZB x NZW mice the lupus nephritis may be reduced by the corticosteroid betamethasone (Casey, 1968c) or by cyclophosphamide (Casey, 1968d; Russell and Hicks, 1968), and ACTH also had beneficial effects (Jelyer and Howie, 1963). Treatment with anti-lymphocyte globulin could suppress the development of
the haemolytic anaemia, but does not affect the established
disease (Denman, Denman and Holborow, 1967; Holborow and
Denman, 1966). Antithymocyte serum treatment of NZB x NZW
did not affect the renal disease and reduced the life span
of the mice (Strom, Levin, Dohrmann and Pollak, 1968).
Splenectomy at 1-4 months of age in NZB increased the
incidence of kidney disease and decreased the survival time
of female mice although it did delay the onset of the auto-
immune haemolytic disease (Holmes and Burnet, 1963b).
Splenectomy in older mice had no effect or caused rapid death
from anaemia depending on whether the mice had developed
autoimmune disease (Helyer and Howie, 1963). Morton and
Siegel (1970a) found that injection of young NZB with
Freund's complete adjuvant with or without Balb/c erythrocytes
accelerated the appearance of autoimmune phenomena. When
the Freund's adjuvant contained large amounts of mycobacterium
the autoimmune disease did not appear. They suggest that
there is a precursor cell that is responsible for the auto-
immunity and that the numbers of this precursor are increased
by adjuvant, and decreased by antigenic competition.
Greenwood, Herrick and Voller (1970) found that malarial
infection at 1 month of age delayed the onset of haemolytic
anaemia in NZB mice and protected NZB x NZW mice from the
development of renal disease, also perhaps suggesting
inhibition by antigenic competition.

3.4 The role of the thymus

In many of their papers mentioned in this review,
Holmes and Burnet have suggested a central role for the thymus
in the etiology of the autoimmune disease (see particularly Burnet and Holmes, 1964), and suggest that it is inadequate in the removal of 'forbidden clones' - the cells producing the antibody that reacts against autologous components. They consider that the 'germinal centres' may represent the proliferation of these forbidden clones. Indeed, if the thymus, - the organ supposedly eliminating the cells producing autoantibodies - is removed at birth certain autoimmune-like phenomena do occur but these are in any case not the same as those found in the NZB (Yunis, Hong, Grewe, Martinez, Cornelius and Good, 1967).

Neither neonatal thymectomy (East, de Sousa, Parrott and Jaquet, 1967; Howie and Helyer, 1965; Holmes and Burnet, 1964b) nor the grafting of normal thymus (Howie and Helyer, 1965) prevents the appearance of autoimmune disease. Grafting of NZB thymus into other strains (Howie and Helyer, 1965) even in a millipore diffusion chamber (Masters and Spurling, 1967) results in transmission of autoimmune disease to the recipients. This suggests that the action of the thymus is positive rather than negative; that is, it contains some autoimmunity inducing factor rather than lacking some autoimmunity preventing factor, and that this factor is present in organs other than the thymus and/or it has exerted its action before the time of birth.

DeVries and Hijmans (1966, 1967) have found a deficiency in the number of epithelial cells in the medulla of the thymus of the NZB, most strikingly apparent at the age of 3-4 weeks. This, together with the fact that the
thymus can exert its autoimmune inducing role through a millipore filter might suggest a role for the elusive thymic humoral factor.

3.5 Adoptive transfer of the disease.

The autoimmune syndrome may be transferred from old mice with red cell autoantibodies to young, clinically healthy mice by the transfer of live, but not of dead spleen cells (Holmes, 1965; Barnes and Tuffrey, 1966; Holborow and Denman, 1967, 1968) though the production of the autoantibodies in the recipients is frequently only transient. Polackova and Strejcek (1968) were unable to transfer autoantibody production to neonatal mice in this way. Holmes (1965) found that the transfer of lymph nodes, thymus, bone marrow or buffy coat from older to younger NZB was not effective in transferring the disease. Renal disease may also be transferred with living spleen cells (Mellors, 1966e; Barnes, Berry and Holliday, 1969). The transfer of spleen cells from autoimmune NZB to irradiated NZB results in the production of autoantibodies in the recipients (Holborow and Denman, 1967) but transfer of autoimmune NZB spleen (Holborow and Denman, 1967, 1968) or marrow (Polackova and Strejcek, 1968) to irradiated recipients of other strains (CBA and C3H) did not result in the transfer of autoimmunity although the transfer of the cells themselves was successful since this technique could transfer adoptive immunity to sheep erythrocytes. On the other hand, Denman, Russell and Denman (1969) found that NZB lymphoid cells could transfer disease to Balb/c recipients.
treated with anti-lymphocyte globulin and less successfully to C57/Bl recipients and Allman, Ghaffer, Playfair and Roitt (1969) were able to transfer autoimmunity with NZB bone marrow to newborn CBA and Balb/c after an appropriate delay. Survival of the grafted cells was found to be necessary for this transfer.

The alternative experiment where autoimmune NZB are irradiated and restored with CBA bone marrow, the anti-erythrocyte antibodies in the serum gradually disappeared (Poláčkova and Strejček, 1968), but if irradiated autoimmune NZB were given bone marrow from young healthy NZB they continued to produce autoantibody. On the other hand, Lindsey and Woodruff (1968) gave bone marrow or bone marrow and spleen from young healthy NZB or NZB x T6 mice to lethally irradiated autoimmune NZB and found that the red cell autoantibody generally disappeared although it could reappear later.

It is not easy to derive a coherent picture from these data. The transfer of autoimmunity appears to require living cells, but it cannot always be transferred to other strains. Harris, Harris and Farber (1967) found that the success of the adoptive transfer of normal immunity between strains will depend on the strain combinations used, even when the recipients have been lethally irradiated. This cannot explain why normal immunity but not autoimmunity can be transferred in some cases, nor why the transfer to young NZB often only results in transient autoimmunity. It seems that there may be some other factor as well as NZB cells that are required for autoimmunity and that if this
factor is not transferred with the cells or if the recipient will not accept it, the production of autoantibody will fail. It is possible that this factor could be a virus.

3.6 The role of the virus

A murine leukaemia-like virus has been detected in conventional (Prosier, 1968; Mellors, 1968; Yumoto and Dmochowski, 1967), and germ-free (East, Prosier, Holborow and Jaquet, 1967; Prosier, 1968) NZB. The particles have been found in spleen, thymus, lymph node, bone marrow, pancreas, (Prosier, 1968) kidneys and liver (Yumoto and Dmochowski, 1967) but not in serum or plasma (Prosier, 1968). The virus particles are also found in the spleen, thymus and pancreas of NZB embryos and neonatal thymectomy of the animals has no effect on the virus (Prosier, 1968). Similar sorts of virus are however frequently found in conventional or germ-free mice of other strains (de Harven, 1964) so their presence does not indicate whether they play any role in the etiology of autoimmune disease. In studies of the antibody forming cells of old and young NZB and Balb/c the only virus-like particles Siegel, Brooks and Morton (1970) found were unlike murine leukaemia virus and were in any case found in both strains.

One or more transfers of lymphoid cells from old to young NZB results in reticulum cell neoplasia and the presence of large numbers of virus particles in the lymphomas (Prosier, 1968; Yumoto and Dmochowski, 1967). Mellors, Aoki and Heubner (1969) have convincingly implicated the virus in autoimmune disease. Murine leukaemia virus (MuLV)
antigens were found in spleen, kidney and neoplasms of the NZB mice. Specific virus antigens were found in plasma at 3 months and its incidence increased with age until 9 months of age. The appearance of this antigen in the serum of individual animals corresponds with the appearance of antibody to the erythrocytes. Antibody to the viral antigen appears in the serum at about 10 months old, and the advent of the renal disease corresponds with the appearance of the antibody to the viral antigen and the elimination of the antigen from the serum. The glomerular lesions contained murine leukaemia antigens. Mice could be vaccinated with formaldehyde inactivated cell-free filtrate of older NZB spleens and this delayed the appearance of virus antigen and the onset of autoimmunity although it did not prevent it. Mellors and Huang (1967) have found some evidence that haemolytic disease can be transferred to Swiss mice by neonatal inoculation with cell-free filtrates of old NZB spleen. On the other hand, other workers (Braverman, 1968b; Denman, Russell and Denman, 1969) have been unable to transfer the disease with cell-free filtrates to Balb/c or C57/Bl treated with anti-lymphocyte globulin, and Russell, Hicks, Boston and Abbot (1970) could not transmit it to neonatal NI, CBA, Balb/c or C57/Bl. It seems unlikely that the virus is acting alone to induce autoimmunity but it may be involved as a precipitating factor for a genetic predisposition.

Zelesnick, Holm and Barnett (1969) found single stranded DNA antigen in a few NZB and antibody to single stranded DNA in many NZB over 4 months of age. Lambert and
Dixon (1968) have also related anti-DNA antibodies to the glomerulonephritis of NZB x NZW mice. If interferon was induced by injecting young mice with polyinosinic, polycytidylic acid the appearance of erythrocyte autoantibodies was accelerated and antibodies to RNA appeared (Steinberg, Baron and Talal, 1969). It is thus possible that the virus may act as a non-specific stimulus for the induction of RNA or DNA antibodies.

3.7 The immune response to heterologous antigens

If the autoimmunity in these mice were due to some general dysfunction of the immunological system the dysfunction might be expected to reveal itself in some abnormality of the response to heterologous antigens. The difficulty is to distinguish a truly 'abnormal' response among the many differences in immune response found between normal animals (see McDevitt and Benacerraf, 1969).

The response to sheep erythrocytes appears to be greater than normal in young mice (Siegel and Morton, 1967; Morton and Siegel, 1969a; Morton, Olsen and Siegel, 1967; Playfair, 1967,1968a; Baum, 1969b) but decreased and delayed in older, autoimmune mice (Diener, 1966; Morton and Siegel, 1969a; Siegel and Morton, 1967) although the secondary response appears normal (Morton and Siegel, 1969b). An unusually high response was also found in young NZB to bovine serum albumin (Weir, McBride and Naysmith, 1968) and in crosses of NZB with NZW to sheep erythrocytes and bovine serum albumin (Cerottini, Lambert and Dixon, 1969). On the other hand, the response of very young NZB to pig or chicken
erythrocytes appeared normal (Playfair, 1968a) and the response of the NZB/NZW hybrid was unusually low to Keyhole Limpet Haemocyanin (Cerottini, Lambert and Dixon, 1969). Salomon and Benveniste (1969) also observed that the responses of old NZB/NZW hybrids to Salmonella 'H' antigens, sheep erythrocytes, bovine serum albumin and allogeneic tumour cells were depressed compared with young NZB, and showed some similarities to the response of neonatally thymectomised mice.

With respect to cell mediated immunity, Stutman, Yunis and Good (1968) found that spleen cells from young NZB could produce graft versus host disease normally in NZB x A recipients but that cells of older NZB mice were ineffective in this respect. 3 month old NZB spleen cells are quite competent to produce graft versus host splenic enlargement in newborn C57/Bl. Old cells were much less efficient, but when old cells and young cells were mixed they produced a response as if they had been young cells alone (Cantor, Asofsky and Talal, 1970). Since Denman and Denman (1970) using labelling studies have shown that old NZB are depleted of the long-lived recirculating lymphocytes the lack of ARG may be the reason for the decreased response in old animals.

'Natural' antibodies to sheep erythrocytes, that is antibodies existing without prior exposure to the antigen measured as serum antibodies or as the naturally occurring plaques in the haemolytic plaque assay test, were not found to be different in C3H, Balb/c, young or old NZB (Diener, 1966; Morton and Siegel, 1969a, Siegel and Morton, 1967; Morton, Olson and Siegel, 1967). Baum (1969a) found that
NZB had a higher titre of natural agglutinins to SRBC than C57/Bl or A/Jax but no higher than Balb/c or DBA.

Experiments in which liver and thymus or bone marrow and thymus from baby NZB were used to restore the immune response to SRBC in irradiated adult NZB, showed that the high response of the young animals was a property of the liver-derived cells (Playfair, 1968b). Thymectomy of these animals had the expected result in that performance of the operation at birth severely reduced the ability to produce a response to sheep erythrocytes, but thymectomy at 7 days was without effect in this respect (Playfair, 1968a).

In view of the probable relationships between tolerance and self-recognition the ability to induce tolerance and its rate of breakdown in these mice should be of interest. Weanling NZB became tolerant to bovine gamma-globulin, but the tolerance was lost rapidly and in young animals tolerance to bovine gamma-globulin, human gammaglobulin and to egg albumen could not be induced (Staples and Talal, 1968, 1969a,b). Braverman (1968a) also found a reduced ability to develop tolerance to bovine gammaglobulin in young adult NZB and Weir, McBride and Hayamith (1968) found some individuals did not become tolerant to bovine serum albumin and some became tolerant quite normally. Hybrids of NZB with NZW have also been tested. Tolerance to human gammaglobulin (Cerottini, Lambert and Dixon, 1969) and to rabbit globulin (Russell and Denman, 1969) could be induced quite normally. On the other hand, Braverman (1968a) found NZB x NZW could not be made as tolerant to bovine gamma-
globulin as normal strains, but could be made more tolerant than pure NZB. Staples and Talal (1968, 1969a,b) found the inability of the hybrids to produce tolerance to bovine gamma-globulin, human gammaglobulin and egg albumin the same as the pure NZB strain. NZB x Balb/c with the help of cyclophosphamide could apparently develop tolerance to sheep erythrocytes normally (Playfair, 1969). Some of the confusion of these results may be explained on the hypothesis stated previously that acquired tolerance can be induced in any of the populations of lymphocytes required in the response to an antigen. Similarly the abnormality in the immunological system of the NZB mice may be in only one population of cells, and it is only when the particular method or antigen that is being used to induce tolerance acts on the population of cells that responds abnormally in the NZB mice that defects in the induction of tolerance will be observed.

3.8 Artificial induction of autoimmunity

Any kind of widespread defect of self-recognition might be expected to result in the easier induction of autoimmune phenomena. Surprisingly, attempts to produce autoimmunity in NZB to other organs have not been very numerous. 1 month old NZB produced the most autoantibody to liver after CCl₄ damage, but adult mice showed no difference (Plad, Playfair, Ghaffar and Miescher, 1969). Lambert and Dixon (1970) showed that anti nuclear factor was easy to induce in young NZB x NZW hybrids. Attempts to produce myasthenia gravis by injection with thymus or muscle were not successful (Kalden, 1969, personal communication). Playfair and Kriaikova (1969) showed that injection of
Syngeneic thymus or spleen into neonatal NZB mice could cause splenomegaly, indicating an isologous graft versus host response. The effect was also found in Balb/c but only in 1 month old spleen and 2 month old thymus, while the NZB showed it in the spleen throughout life, and in the thymus at 2 months only. They suggest a self-tolerance lapse at the cellular level.

3.9 Conclusions

There is no doubt that NZB produce antibodies to their own red blood cells and that they have haemolytic anaemia. What is in question is the relationship between these phenomena. Damage or alterations in the erythrocyte, perhaps by the murine leukaemia-like virus, could be the primary lesion and the autoantibody appear as a response to the antigen from the damaged erythrocytes. Alternatively, defects in the self-recognition mechanism of the immunological system could lead to the production of autoantibodies to erythrocytes, resulting in damage and haemolytic anaemia.

The transmission of the disease is clearly vertical, but the cause of this could be genetic or a virus transmitted by the germ cells. Transfer of virus in cell-free filtrates to other strains generally cannot transfer autoimmune disease suggesting that the virus, if it is implicated in the development of autoimmunity is not the only agent responsible and that there is probably a genetic factor involved. The autoantibodies to kidney or nucleic acid as well as the abnormalities found in the response to some
heterologous antigens and the difficulties encountered in the induction of tolerance point to the involvement of the immunological system rather than the erythrocyte antigen. The transfer of living cells from autoimmune animals does not always result in the transfer of autoimmune disease suggesting that genetic abnormalities of the immunological system are not in themselves enough to produce the autoimmune disease. It seems quite possible that a factor, perhaps the murine leukaemia-like virus, acts upon a genetically determined deficiency of the immunological system to produce a breakdown of self recognition in particular directions and thus autoimmune disease.
4. MATERIALS AND METHODS

4.1 Materials

$^{131}$I was obtained as a solution of high specific activity Na${^{131}}$I from the Radiochemical Centre, Amersham.

Chloramine T was purchased from British Drug Houses and dissolved in 0.9% NaCl, 0.05% Na Phosphate pH 7.5.

Sodium metabisulphite was purchased from May and Baker Ltd.

Papain was bought from L. Light and Co. Ltd.

Eagle's tissue culture medium (Basal) 10x was purchased from Burroughs Wellcome & Co. It was made up as required by mixing in the proportion of 8.65 ml $H_2O$, 1 ml 10x Eagle's medium and 0.35 ml 1% (w/v) Na$_2$CO$_3$ (Analar).

Antibiotics were not used.

The agar used was Special Agar - Noble from Difco Laboratories. It was made up by adding 0.06 g to 8.65 ml $H_2O$ and heating in a boiling water bath until entirely dissolved, when 10x Eagle's medium and 1% Na$_2$CO$_3$ were added in the proportions stated above and the mixture distributed in tubes in a 47°C water bath.

Guineapig complement was obtained as serum from freshly killed guineapigs and frozen at -20°C until needed for use.

Defibrinated sheep blood in Alsever solution was purchased from Stayne Laboratories Ltd.

Chicken erythrocytes were kindly supplied by Dr. A.J. Evans of the ARC poultry Research Centre. Blood
was taken from a wing vein by venepuncture and suspended in sterile Alsever solution. It was kept at 4°C until required for use.

4.2 Animals

The animals used in this study are the autoimmune NZB strain of mice and two control strains, an inbred strain Ju, and a closed colony of CBA. All three strains are maintained at the NRC Clinical Endocrinology Unit. They are allowed water and a modified oxoid diet ad libitum.

The mice were mated by placing male and female - siblings in the case of the NZB and the Ju - together in a cage. The males were not removed unless they were seen to be killing the litters. When in some cases it was necessary to have two females to one male the females were placed in separate cages and the male alternated between them. The animals were inspected each day at the same time to see whether any births had occurred, and those that had been born since the previous inspection were classed as having been born that day. Any injections of antigen were given at the same time as the inspection so that animals stated to have been injected at, say, 3 days old were a minimum of 3 days and a maximum of 4 days of age at the time of injection.

4.3 Antigen challenge

4.3.1. Sheep red cells (SRBC). Mice were injected intraperitoneally with washed packed SRBC containing approx. $2.5 \times 10^{10}$ cells per ml or with stated dilutions of
the packed cells. Adult mice, (3 months - 1 year) were given 0.2ml and baby mice (0 days - 21 days) were given 0.02ml.

4.3.2. Chicken red cells (CRBC). The packed chicken cells were diluted x2 since they pack to a more solid pellet than the SRBC. This also contained approx. 2.5 x 10^10 cells/ml. The volumes given were the same as for the SRBC.

4.4 Detection of the immune response

The mice were killed at the same time each morning so that a time stated to be '1 day' after injection, is 17 - 19 hours, '2 days after injection' is between 41 and 43 hours and so on. The animals were killed either by ether or by a blow on the head in the case of adults, and by decapitation or a blow on the head in the case of babies. Spleens, lymph nodes (which were always from the same area, the two axillary and one inguinal lymph node from each side, giving 6 altogether) and in some cases the thymus were removed into ice-cold Eagle's medium and disrupted by gentle grinding with a glass/teflon homogenizer. Viable cells were counted in a haemocytometer. They were diluted first in water and then an equal volume of 2x normal saline containing 0.2% trypan blue was added. The brief osmotic shock disrupted any red blood cells in the preparation, and the trypan blue stained the dead cells.

Antibody forming cells were detected by a modification of the haemolysin plaque assay of Jerne, Nordin and Henry (1963) and Ingraham and Bussard (1964). SRBC were washed and packed and diluted 1/15 with Eagle's medium. 1 drop of these SRBC and 0.1 ml of the lymphoid cell suspension were
added to 0.33 ml 0.6% agar in Eagle's medium and the mixture spread over the surface of a glass microscope slide. Slides were prepared in triplicate for each lymphoid cell suspension. The slides were incubated at 37° in a damp chamber for 2 hours and then 1 drop of guineapig serum diluted with 1 drop of Eagle's medium were spread over the surface of the agar and allowed to incubate for a further 20 minutes. The plaques were then counted in dark field using a dissecting microscope. Control slides without lymphoid cells were included in each experiment.

Although it has been reported (Jerne, Nordin and Henry, 1963) that the addition of DEAE dextran increases the number of plaques owing to its ability to suppress the anti-complementary effect of agar, in my system DEAE dextran was found to non-significantly decrease the number of plaques, so it was not used (Table 4.4.1.).

In some of the earlier experiments, complement was added to the melted agar at the same time as the lymphoid cells were added, but although this has certain advantages it was found that some of the samples of complement seemed to have a toxic effect and reduced the numbers of plaques appearing. In any case, by adding complement subsequent to the incubation of the lymphoid cells with the SRBC it could be ensured that the plaques were complement dependent.

The time of incubation was not critical. The maximum number of plaques appears after 90 minutes incubation, and 10 minutes after complement addition, the extra time
Table 4.4.1. The effect of 0.5mg/ml DEAE Dextran on the sensitivity of the haemolytic plaque assay.

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<th>Mouse No.</th>
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allowed being a safety margin. Beyond this time no further change occurs in the number of plaques until the growth of haemolytic microorganisms begins to damage the plate.

The limitations of this technique are that it measures only the number of cells producing antibody and not the amount of antibody produced, and that it only measures the lysing antibody, which is generally considered to mean IgM antibody (Rowley and Fitch, 1965; Moller and Wigtell, 1965; Sterzl and Riha, 1965; Dresser and Wortis, 1965; Wigtell, Moller and Andersson, 1966; Hege and Cole, 1966), but since the autoantibody of the mice is probably macroglobulin, (Mollors, 1965) and since the antibody first produced by baby mice seems also to be IgM (Takeya and Nomoto, 1967b) this did not seem a great disadvantage. The particular advantage of this technique for work with baby mice is that assay of their serum is unsatisfactory owing to the very small amounts that can be obtained and to the unknown contribution of any antibodies that may be derived from the mother.

In none of the experiments to be reported was any difference found between the results for male and female mice so the results from both sexes were pooled.

4.5 Iodination of antigen

The procedure was essentially the chloramine T method of Greenwood, Hunter and Glover (1963), described by Hunter (1967). Approximately 100μC Na¹³¹I was placed in a flat bottomed 10ml plastic tube over a magnetic stirrer.
To this was added, with stirring, 1ml of packed washed SRBC in Alsever’s solution, 0.1ml Chloramine T at 30mg/ml, and finally 0.1ml sodium metabisulphite at 72mg/ml. The mixture was made to approx. 8ml with phosphate buffered saline pH 7.5, centrifuged and resuspended in buffered saline. This process was repeated until the cells lost no more radioactivity, which usually took from 5 - 8 washes. About 5% of the original radioactivity remained attached to the SRBC. A fresh preparation of iodinated SRBC was made before each of the experiments.

4.6 Antigen clearance

Baby mice, litter mates, were injected intraperitoneally with 0.02ml packed SRBC labelled with $^{131}$I. The mice were left for 1 hour and then the whole body counted on an IDL scaler 1700 or occasionally in a TracerLab gamma/guard 150. A small volume of the red cell preparation was counted with the mice, and their counts were expressed in relation to this in order to allow for the decay of the $^{131}$I and for differences in the efficiency of counting from day to day. They were counted at daily intervals for about 10 days, together with uninjected litter mates. The average value for the uninjected mice was subtracted from the value of each of the injected animals and this corrected count calculated as a percentage of the initial count of each mouse.

4.7 Detection of autoimmune phenomena

Red cell autoantibodies were tested for by the papain-treated cell agglutination test. Mice were bled
from the tail into heparinized haematocrit tubes which were centrifuged. The length of the packed cells and the total length of cells plus plasma were measured, then the packed cells were cut off, the tube resealed and the serum frozen at -20°C. Two days later, blood from a Ju mouse was taken into a heparinized tube, the cells washed 3x in saline, diluted 3x the packed volume with saline and about 5mg papain added. The cells were mixed and incubated at 37°C for 30 min. The cells were then washed 5x and suitably diluted. The sera from the mice to be tested were thawed and each was tested at a dilution of 1/5. Those giving no agglutination were classed as negative and were not tested further. Those showing agglutination at this dilution were tested in doubling down dilutions to find the titre of autoantibody in the serum. The mice were given code numbers and the age of the mouse was not known at the time of reading the haemagglutination assays. In order to separate the macroglobulins (supposedly IgM) from the smaller globulins (supposedly IgG), samples of individual mouse sera were put through a G200 Sephadex column and the optical density of the fractions at 260μ recorded. The fractions were concentrated and dialysed and the titre of the autoantibody assayed by the papain-treated mouse erythrocyte method. Since the fractions were not all of the same volume, the volume was multiplied by the titre to give the total quantity of autoantibody in each fraction in arbitrary units.

Mouse gammaglobulin was detected in frozen sections
of NZB kidney using anti-mouse globulin conjugated with fluorescent isothiocyanate, prepared in our laboratory by Mr. W.G. Williamson. Antinuclear factor was detected in the serum of NZB mice using frozen sections of liver of non autoimmune mice which were incubated with the NZB serum and then with the fluorescent isothiocyanate conjugated anti-mouse globulin. The preparation of the sections was done by Mr. W.G. Williamson and the photographs taken by Dr. J. Kalden.

4.8 Histology

This was kindly done for me by Miss Sylvia Wheeler. Spleens, thymuses and sometimes lymph nodes from baby mice were fixed in Carnoy's fixative and paraffin sections were stained with haematoxylin and eosin, PAS, methyl green pyronin and in some cases toluidine blue.

4.9 Statistical Analysis

Where the results have been graphed logarithmically the geometric means were calculated, values of less than 1 being regarded as 1 for this purpose. Limits shown on the graphs are standard errors. The significance of differences was calculated by means of the randomization test (Siegel, 1956) which is essentially Student's 't' test. The level of significance was usually taken as \( p < 0.05 \). Regression lines and correlation coefficients \((r)\) were calculated by the usual methods (Snedecor, 1965).
5. AUTOIMMUNE PHENOMENA IN NZB

5.1 Introduction

Since the experiments described in this thesis are intended to compare the maturation patterns of the immune response of normal strains of mice with a strain in which self-recognition is defective it seemed advisable to ascertain that our own colony of NZB mice was showing autoimmune phenomena similar to that reported for this strain in the literature.

5.2 The relationship of age, anaemia and erythrocyte autoantibody

Samples of blood were taken from the tail veins of a number of mice aged between 2 and 10 months of age and the packed red cell volume of the blood was determined. The plasma was separated and tested at 1/5 dilution for agglutinins to papain-treated erythrocytes from a different strain of mice. This method detects autoantibodies to erythrocytes in the NZB serum (Long, Holmes and Burnet, 1963). Those sera showing agglutinins at this dilution were then tested in further doubling down dilutions to determine the titre of the autoantibody.

Fig. 5.2.1 shows the packed cell volumes and the serum autoantibody titres of individual mice of various ages. It may be seen that the mice begin to show autoimmune phenomena at about 5 months of age, and that all animals are showing autoantibody by 9 months of age. In these animals there is no evident differences between the incidence in
Fig. 5.2.1  Packed cell volume and reciprocal autoantibody titre of NZB mice of various ages. Each point represents the result for one mouse.
males and the incidence in females, but the sample is only small. It is also shown in Fig. 5.2.1 that concurrent with the rise in the autoantibody titre, the packed cell volume begins to decrease, although these two values were not directly related in individual animals.

Six Ju mice between 8 and 10 months old showed no agglutinins for papain treated mouse erythrocytes when the serum was tested at 1/5 dilution.

5.3 Gel filtration of autoimmune sera

To find whether the erythrocyte autoantibody was a macroglobulin the sera from several mice were put individually through a column of G200 sephadex and the titres of autoantibody in reconcentrated fractions tested in the same way as before.

Fig. 5.3.1 shows a typical curve for the absorption at 280\textmu m together with the antibody content of the fractions of sera from 3 typical NZB. It may be seen that most of the autoantibody appears at the beginning with the excluded proteins, suggesting that it is probably largely macroglobulin, which usually means IgM, thus supporting the findings of Mellors (1965). There is a slight 7S component and this may indicate that there is only a slight synthesis of 7S autoantibody, but it may indicate that 7S autoantibody is preferentially adsorbed onto the erythrocyte leaving a greater proportion of the 19S in the serum. Variable adsorption of the autoantibody onto the erythrocytes may also contribute to the variability of the autoantibody titres seen in Fig. 5.2.1.
Fig. 5.3.1. Autoantibody content of the sera of 3 NZB mice after passage through G200 Sephadex. At the bottom is shown a typical UV absorption curve, and at the top the autoantibody content of the fractions of the 3 sera, matched against the UV absorption curve. The amount of autoantibody is expressed in arbitrary units obtained by multiplying the volume of the fraction by its titre.
5.4 Staining for globulin in Kidneys and for anti-nuclear factor

Frozen sections of NZB kidney were stained with fluorescein-conjugated anti-mouse globulin to show the presence of mouse globulins in the glomeruli, and frozen sections of liver from a normal mouse were treated with NZB serum and then with fluorescein conjugated anti-mouse globulin to show the nuclear staining associated with the presence of anti-nuclear factor in the serum. Dr. J.R. Kalden has kindly allowed me to use his pictures.

Fig. 5.4.1 shows the glomerulus from the kidney of a 2-3 month old NZB. It can be seen that there is globulin deposition in the basement membrane, even at this early age. Fig. 5.4.2 shows the glomerulus from the kidney of a 15 month old NZB similarly stained for gammaglobulin. At this age the entire glomerulus is showing heavy deposition of globulin. Fig. 5.4.3 shows the section of a liver of a normal mouse. Clear nuclear staining indicates the presence of anti-nuclear factor in the serum of the NZB.

Insufficient animals were tested to allow an estimate of the incidence of the anti-nuclear factor or kidney disease, but there was no difficulty in finding animals showing either of these phenomena.

5.5 Discussion

It must be stressed that these data are not intended to provide new information about the autoimmune phenomena in the NZB mice which have already been very well documented. It is rather intended to make sure that our colony of mice
Fig. 5.4.1. Frozen section of 2 - 3 month old NZB kidney stained with fluorescein-conjugated anti-mouse globulin, and viewed by fluorescence microscopy.
Fig. 5.2. Frozen section of 15 month NZB kidney stained with fluorescein-conjugated anti-mouse globulin and viewed by fluorescence microscopy.
Fig. 5.4.3. Frozen section of normal mouse liver treated with NZB serum and then with fluorescein-conjugated anti-mouse globulin and viewed by fluorescence microscopy.
is also showing the expected syndrome. It does seem that this is the case since serum autoantibodies were found in all mice tested after the age of 8 months and the time of their appearance is similar to that reported for other colonies (East, de Sousa and Parrott, 1965; Helyer and Howie, 1963; Howie and Helyer, 1965; Holmes and Burnet, 1963a; Mallors, 1966a). The mice also show the expected kidney lesions and antinuclear factor. It seems safe therefore to conclude that the NZB mice used in the experiments reported in the following pages are showing or would have shown the failure of self recognition that makes the comparison of their immune response with that of normal strains of so much potential interest.
6. THE IMMUNE RESPONSE OF ADULT MICE TO SRBC

6.1 Introduction

Before starting to compare the development of the immune response in the different strains of mice, the mature form of the response found in the adult animals will be compared. This not only allows an understanding of the relationship of the immature form with that towards which it is developing, but also will show whether the adult NZB have any differences in their response compared with that of the adults of non-autoimmune strains.

There has already been a considerable amount of work published on the kinetics of antibody plaque-forming cells (PFC) found in the spleen after the administration of SRBC antigen to mice. After the antigen has been injected into the animal there is a lag period of between 12 (Perkins, Sado and Makinodan, 1969) and 24 (Dutton and Mishell, 1967) hours during which there is no proliferation but RNA synthesis occurs (Ortiz-Ortiz and Jaroslow, 1969). In vitro the lag phase of the primary response is 20 hours (Bussard and Lurie, 1967). After 24 hours, the first PFC appear (Perkins, Sado and Makinodan, 1969; Eidinger and Pross, 1967; Wigsell, Möller and Andersson, 1966) and these proliferate with a doubling time dependent upon the dose of antigen (Wortis, Taylor and Dresser, 1966; Wigsell, Möller and Andersson, 1966; Campbell and Kind, 1969). The increase in the numbers of PFC is due both to proliferation of the antibody forming cells themselves and to recruitment from non-antibody forming precursors (Tannenberg and Malaviya,
Perkins, Sado and Makinodan (1969) did a very careful examination in which they identified PFC and proliferating cells at 2 hour intervals after the injection of antigen and reached the conclusion that the increase in PFC occurred in a staircase manner indicating synchronous proliferation and recruitment. The maximum number of PFC is reached about 4 - 5 days after injection although both the maximum number and the time after injection depend to some extent on the dose of antigen (Wigzell, Möller and Andersson, 1966; Koros, Mazur and Mowery, 1968) and the route of injection (Wortis, Taylor and Dresser, 1966). Above a certain dose of antigen the response reaches an optimum where differences in the dose have little effect on the magnitude or time course of the response, and only at the very highest doses is some suppression observed. The maximum number of PFC reached varies between strains (Friedman, 1964) but seems to depend on some feedback from the body, probably by antibody, since transplantation to irradiated recipients (Möller, 1968) or cultivation in vitro (Dutton and Mishell, 1967) can result in much higher numbers of PFC than can be induced in the normal animal.

Friedman (1964) found that the lymph nodes of NIH or C57 mice injected with $10^9$ SRBC gave a response similar to that of the spleen but of lesser magnitude.

Ingraham and Bussard (1964) showed that the spleens of rabbits give a similar form of response to SRBC as the spleens of mice.

Diener (1966) showed that while C3H mice attained a
peak response of about $10^5$ PFC per spleen at 4 - 5 days after injection, 300 day old NZB showed a maximum response of only $10^4$ PFC per spleen and this did not occur until 6 - 7 days after injection.

The aims of this section then, are to compare the adult response of the NZB and the control strains of mice and to find whether the results of Diener (1966) can be confirmed in this laboratory and in comparison with different control strains of mice. The lymph nodes of the animals will also be tested to find how their response compares with that of the spleen and whether any differences that may be found in the response of the NZB spleen are peculiar to the spleen or are found also in the lymph nodes. In addition, the effects of differing antigen doses will be investigated.

6.2 The response of animals at various intervals after injection

Adult mice between 3 months and 1 year of age were injected intraperitoneally with 0.2ml of packed washed SRBC and killed at various times after injection. The spleen, lymph nodes and in some cases the thymus were removed, weighed and assayed for antibody plaque forming cells. The number of viable cells in each organ was also determined.

6.2.1 The spleen

Fig. 6.2.1.1. shows the number of PFC in the spleens of NZB, Ju and CBA mice at various times after antigen injection, expressed as PFC per million viable cells; in other words, as the proportion of the available lymphoid
Fig. 6.2.1.1. The number of PFC per $10^6$ viable cells in the spleens of adult NZB, Ju and CBA mice given $5 \times 10^9$ SRBC and tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
tissue participating in the immune response. It may be seen that the Ju mice reach their maximum response 4 days after injection, the CBA reach their maximum response 4-5 days after injection, but the NZB do not reach their maximum until 5-6 days after injection. The height of the Ju response at its maximum (4 days) and the height of the NZB response at its maximum (6 days) were not significantly different from one another (p > 0.08). The response of the CBA at their maximum (5 days) and the NZB at their maximum were not significantly different either (p > 0.04). Neither were the maximum responses of the Ju and the CBA significantly different (p > 0.1). At 4 days after injection the response of the NZB was significantly lower than that of the Ju (p < 0.001) and the CBA (p < 0.02), and the Ju and CBA were not significantly different from one another. Similar results were seen at 3 days indicating that the lag period before the appearance of significant numbers of antibody forming cells is about 1 day longer than that of the Ju and CBA. In contrast to the findings of Diener (1966) the slope of the initial phase of increase seem very similar in all 3 strains, suggesting a rate of proliferation and recruitment the same as that of the other strains.

Since PFC are produced by the proliferation of precursor cells which themselves must add to the total number of cells in the spleen, it could be argued that a better measure of the immune response would be obtained by comparing the total number of PFC in the spleens of the animals rather than the proportion of PFC to total spleen cells.
In addition, many of the NZB show splenomegaly, so it is possible that although the proportion of PFC is the same in the different strains, the total number of PFC is greater in the NZB. Another advantage of calculating the total number of PFC is that it eliminates variation and errors in counting the viable cells although it is subject to errors due to different cell mortality in different spleen preparations.

Fig. 6.2.1.2 shows the results for the spleen when they are calculated as PFC per whole spleen. It may be seen that the shape of the curves and their relationship to one another is very similar to the results calculated as PFC per million cells. The maximum responses occur at the same times in each of the strains, the maximum response of the NZB is not significantly different from either of the control strains, although now the CBA are lower than the Ju ($p < 0.05$). The slope of the ascending edge of the curve is still the same in all three strains. The agreement between the two methods of calculation also suggests that neither errors of counting nor variable survival of the spleen cells contribute significantly to the results.

In order to see how the reported and observed splenomegaly of the NZB can be reconciled with the similarity of the results when calculated as PFC per million cells or per whole spleen, the sizes of the spleens and the lymph nodes of the mice were measured. The CBA lymphoid organs were not weighed so they were not included in the analysis. Table 6.2.1.1 shows the results. It can be seen that while
Fig. 6.2.1.2. The number of PFC in the whole spleens of NZB, Ju and CBA mice given $5 \times 10^9$ SRBC and tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
Table 6.2.1.1. The size of the lymphoid organs of the Ju and the NZB mice.

The means are arithmetic, the limits are standard deviations. The bottom line shows the significance for the difference between the NZB and the Ju.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Lymph nodes</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>viable cells $\times 10^6$</td>
<td>wt (mg)</td>
</tr>
<tr>
<td>NZB $\bar{x} \pm SD$</td>
<td>39</td>
<td>25.1 ± 17.5</td>
<td>95 ± 75</td>
</tr>
<tr>
<td>Ju $\bar{x} \pm SD$</td>
<td>28</td>
<td>13.8 ± 7.8</td>
<td>35.7 ± 10.8</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt; 0.01</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
the NZB spleen is heavier than the spleen of the Ju, it does not contain a significantly greater number of lymphoid cells. Therefore the increase in the weight must be due to an increase of some element other than the lymphocytes, and, as would be expected there are less lymphoid cells per mg. spleen weight in the NZB mice.

6.2.2 The lymph nodes

The lymph nodes of these animals were analysed for PFC at the same time as the spleens. Fig. 6.2.2.1. shows the results when expressed as PFC per million viable cells. The maximum responses of the three strains of mice were not significantly different from one another. The Ju and CBA reach their maximum at 5 days and the NZB at 6 days. Thus the NZB are still showing a response with a peak later than that of the control strains and the lymph nodes of all three strains of mice respond slightly later than their spleens.

In contrast to the results for the spleens, the CBA show a response lower than either NZB or Ju 4 days after injection, and then rise very abruptly to their maximum so that in the lymph nodes the CBA appear to have the longest lag period followed by NZB and then Ju.

Table 6.2.1.1 shows that the lymph nodes of the NZB are significantly heavier and contain more lymphoid cells than the lymph nodes of the Ju, but since they also contain significantly fewer lymphoid cells per mg of weight some of this increase in weight must be due to non-lymphoid cell elements. The results, calculated as the number of antibody-
Fig. 6.2.2.1. The number of PFC per $10^6$ viable lymph node cells of adult NZB, Ju and CBA mice given $5 \times 10^9$ SRBC and tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
forming cells in the six lymph nodes dissected from each animal are shown in Fig. 6.2.2.2 and again little difference is seen between the three strains, although because of their larger lymph nodes the total number of PFC in the NZB has increased a little in comparison with the other strains.

6.2.3 The thymus

The thymus of some of these mice were also assayed for PFC, but in most cases, in both strains, the response was negligible (Fig. 6.2.3.1). Individual results are shown, and the response shown by a few animals may be due to the inadvertent inclusion of some of the circumaortic lymph nodes with the thymus.

6.2.4 The relationship of the response in the spleen and the lymph nodes

From these results, in agreement with the literature, it is evident that there is considerable variation in the response between individuals within a strain. In order to find whether this response varied in the same way in the spleen and the lymph nodes of an animal - whether a high response in the spleen of an animal meant a high response in the lymph nodes as well, the response of the spleen and the response of the lymph nodes in individual mice of NZB and Ju strains were graphed one against the other. The results are shown in Figs. 6.2.4.1 and 6.2.4.2. The figures within the points indicate the number of days after injection that the animal was tested. The regression lines indicate the straight
Fig. 6.2.2.2. The number of PFC in the 6 limb draining lymph nodes dissected from adult NZB, Ju and CBA mice given $5 \times 10^9$ SRBC and tested at various intervals after injection. The points are geometric means and the limits are standard error.
The number of PFC per $10^6$ viable thymus cells in NZB and Ju mice given $5 \times 10^9$ SRBC and tested at various intervals after injection. The points represent results for individual animals.

**Fig. 6.2.3.1.**
Fig. 6.2.4.1. The correlation between the number of PFC per $10^6$ spleen cells and the number of PFC per $10^6$ lymph node cells in adult NZB mice given $5 \times 10^9$ SRBC and tested at various intervals after injection. The points are results for individual mice, the figure within the point indicates the number of days since antigen injection the animal was tested, and the lines are the regression of X on Y and Y on X.
Fig. 6.2.4.2. The correlation between the numbers of PFC per $10^6$ spleen cells in adult Ju given $5 \times 10^9$ SRBC and tested at various intervals after injection. The points are results for individual mice, the figure within the point indicates the number of days after the antigen injection the animal was tested and the lines are the regressions of X on Y and Y on X.
lines of best fit for the spleen against any value for the lymph nodes, and for the lymph nodes against any value for the spleen. It may be seen that there is no close correlation between the response of the spleen and the response of the lymph nodes. The correlation coefficients for the group as a whole and for the separate days are shown in Table 6.2.4.1 together with the significance of the correlation. It may be seen that the NZB show a better correlation between the response of the lymph nodes and the response of the spleen than do the Ju, but the correlation is not strong.

6.3 The relationship of dose and response

Adult Ju and NZB were injected with 0.6ml packed washed SRBC, 0.2ml packed washed SRBC and 0.2ml SRBC diluted 1/10, 1/100, 1/1000 and 1/10,000 (v/v). The animals were killed 5 days after injection in the case of the Ju and 6 days after injection in the case of the NZB; these times being those at which a near maximum response was shown by the spleen and the lymph nodes of the respective strains of mice. Reduction of the response at this point could indicate a reduction in the magnitude of the maximum response or a change in the time after injection when the maximum occurs, or both. It may be seen in Fig. 6.3.1 that the spleen reaches its plateau of response when the mice are given between $5 \times 10^7$ and $5 \times 10^8$ SRBC. The lymph nodes on the other hand do not reach their maximum until a dose of $5 \times 10^9$ SRBC or above has been given.
Table 6.2. The correlation coefficient (r) for the response of the spleen and of the lymph nodes in individual mice expressed as PFC per million cells. Results for each day after injection and for the whole group are shown. Also shown is the number of animals in each group (n) and the level of significance of the correlation (p).

<table>
<thead>
<tr>
<th>day since injection</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>all</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NZB r</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-0.52</td>
<td>0.73</td>
<td>0.5</td>
<td>0.76</td>
<td>0.75</td>
<td>0.21</td>
<td>0.79</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.02</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Ju r</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>-0.11</td>
<td>-0.01</td>
<td>0.52</td>
<td>0.24</td>
<td>0.41</td>
<td>0.41</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>38</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Fig. 6.3.1. The number of PFC per million viable spleen or lymph node cells of NZB and Ju mice given various doses of SRBC and tested 6 and 5 days after injection respectively. The points are geometric means of 5 or more animals and the limits are standard error.
to the mice. There is no difference between the NZB and the Ju. The low response of the NZB spleen at $5 \times 10^7$ SRBC is due to two of the five mice tested giving low responses, the others show a mean very similar to that of the Ju.

6.4 Discussion

It has been shown that the immune response measured as the number of antibody-forming cells does not differ greatly between the adults of NZB, Ju and CBA mice. The magnitude of the response is not significantly different, but the NZB produce their maximum response a day or two later than the other strains in both the spleen and the lymph nodes. This delay in reaching a maximum is probably a genuine characteristic of NZB older than 3-4 months (Morton and Siegel, 1969a) since Diener (1966) has found a time of 6-7 days after injection for the maximum of the NZB splenic response compared with a time of 4-5 days for other strains of mice in his own and other published results (Wortis, Taylor and Dresser, 1966; Friedman, 1964). Other data for the lymph nodes of NZB are lacking, but Friedman (1964) has found a maximum at the same time as in the spleen in NIH and C57 mice. His mice were injected intravenously suggesting that intraperitoneally injected antigen may take longer to reach the lymph nodes.

Whether the long interval between antigen injection and the maximum response of the NZB indicates an abnormality or the upper end of a normal range, there must be an
explanation for it. Diener (1966) has suggested that it is due to a slower rate of recruitment and proliferation but my data indicate that it is more likely to be due to a delay in the first appearance of antibody forming cells compared both to published results and to my controls except for the lymph nodes of the CBA which have an even longer lag period than the lymph nodes of the NZB. The increase in the lag period may be due to an increase in the time required for antigen processing or to a low number of precursor cells so that more divisions are necessary before the number of antibody forming cells reaches a number significantly above background. The explanation is not necessarily the same for the CBA as the NZB.

In agreement with other users of this method a high variability between the responses of individual animals has been found, and this variability occurs to some extent independently in the spleen and the lymph nodes of an animal. The thymus, as might be expected, does not respond by the production of antibody forming cells in either the NZB or the Ju.

When the effect of dose on the magnitude of the response was considered, since the weight of an adult mouse is about 30gm, it is calculated that a dose of between $1.7 \times 10^6$ and $1.7 \times 10^7$ SRBC per gm of body weight produces a maximum response in the spleen of the animals, and a dose of $1.7 \times 10^8$ upwards produces a maximum response in the lymph nodes. It is possible that the response of the lymph nodes following intraperitoneal injection of antigen
occurs only when it cannot be dealt with by the spleen or the macrophages in the peritoneal cavity and so 'overflows' to the rest of the body and reaches other organs.

Considering the results for both the spleen and the lymph nodes it seemed that a dose of about $1.5 \times 10^8$ cells per gm. of mouse body weight was the most suitable for producing a consistent response in both spleen and lymph nodes.

In this section it has been shown that the only difference between the response of the NZB and the control strains of mice is a slight delay in the appearance of the maximum number of PFC and that this might be due to a lag period longer than in the other strains. The difference is slight and there is no indication that it represents an abnormality.
7. THE DEVELOPMENT OF THE IMMUNE RESPONSE IN BABY MICE

7.1 Introduction

The study of the development of the immune response of baby mice involves not only the determination of the age at which the immune response first appears but also the comparison of the shape of the response of the immature mice with that of the adults. The response of adult mice of many strains has been thoroughly studied (see Section 6) but in spite of the usefulness of the haemolytic plaque assay for studying the response of animals when very little serum is available and a low response is expected, not a great deal of work using this method on immature animals has been published. Hechtel, Dishon and Braun (1965b) immunized three strains of mice with $10^6$ SRBC and tested them between 3 and 9 days after injection. They found that the youngest animals showing a detectable response were between 5 and 10 days of age depending on the strain of mouse. Insufficient animals were tested at each interval to permit a reasonable estimation of the shape of the response of the babies, and in any case adults were not tested to allow a comparison between the immature and the mature responses. These authors also tested the pancreatic and mesenteric lymph nodes and found that the spleen was the first to gain immunological competence followed by the pancreatic lymph nodes and then the mesenteric lymph nodes. More recently, Playfair (1968a) has compared the response of Balb/c and NZB mice with one another at various ages. He finds that
the NZB are able to produce a higher response at an earlier age than the Balb/c but the lymph nodes were not tested and neither was the response of the babies compared to that of the adults.

In this section, CBA, Ju and NZB mice of various ages were injected with a standard dose of SRBC and their responses compared with one another and with that of the adults obtained in the previous section. Initially, in order to determine what constitutes a 'significant' response the number of background PFC found in uninjected animals of various ages was determined.

7.2. **Background plaques in uninjected mice**

Uninjected mice of 5, 10, 15 and 30 days of age were killed and their spleens and lymph nodes assayed for PFC. Fig. 7.2.1. shows the results for the spleens calculated as PFC per million viable cells. There is no difference in the numbers of background plaques between the three strains. The number of plaques increases in animals between 5 and 10 days old, but thereafter remains between 1 and 10 background plaques per million spleen cells. It may be seen that in mice over 5 days of age a number of plaques below 10 per million is not significantly above background.

No background plaques were detected when the lymph nodes of these mice were tested, but there were so few lymph node cells in the younger animals that a number below 10 - 20 per million would have in any case been undetectable.
Fig. 7.2.1. The number of PFC per million viable spleen cells of uninjected NZB, Ju and CBA mice of various ages. The points are results for individual mice or sometimes of spleen pooled from two littermates. The line represents the baseline of most of the graphs in this thesis.
7.3 The immune response of mice of various ages

Mice of various ages up to 21 days were injected intraperitoneally with 0.02 ml. packed washed SRBC. This dose of SRBC was chosen because it contains about $5 \times 10^8$ SRBC, and since the weight of the baby mice is usually between 2 and 8 gm. it gives them a dose of $0.6 - 2.5 \times 10^8$ SRBC per gm. of body weight which is near the plateau of optimum response determined for the adult lymph nodes and spleens. In this experiment, mice were killed at a single interval after injection, 5 days in the case of the Ju and CBA and 6 days in the case of the NZB. This time was chosen because in the adults it gave a response near the maximum for both spleen and lymph nodes.

7.3.1 The spleen

Fig. 7.3.1.1 shows the response of the spleens of mice of all three strains expressed as PFC per million viable spleen cells. It can be seen that up to 8 days of age the NZB show a higher response than either the Ju or the CBA. The differences between the Ju and the NZB are significant on days 3, 4, 5, 6, 7 and 14. The differences between the NZB and CBA are significantly different on days 4, 5, 6, 7 and 8. The Ju and CBA are significantly different from one another only on day 8. The NZB mice can be seen to have reached mature levels of response by day 4 and from then on the response is not significantly different from that of the adults. In the Ju mice, the response at day 6 is not significantly different from the adult response.
Fig. 7.3.1.1. The number of PFC per $10^6$ viable spleen cells of NZB, Ju and CBA mice injected at various ages and tested 5 days after injection (Ju and CBA) and 6 days after injection (NZB). The points are geometric means of 6 or more animals and the limits are standard error.
but considering the overall shape of the curve and the large standard error at day 6 mature levels of response are probably not reached until 7 or 8 days of age. CBA mice show a response significantly less than that of the adults at all ages up to 8 days, and mature levels are reached some time between 8 and 14 days old.

These results too, for the same reasons as the adult, may be calculated per whole organ, but since the younger animals are naturally smaller than the older, this would give an erroneous idea of their state of maturity. A better estimate may be obtained by calculating the number of PFC per mg mouse. This is shown in Fig. 7.3.1.2. It can be seen that the results calculated in this way are not greatly different from the results calculated as PFC per million cells. The difference between the NZB and the Ju babies is significant on all except day 6, and the difference between the NZB and the CBA babies is significant on all days. The Ju and the CBA are not significantly different from one another on any day. It may be seen that by this method of calculation the NZB have a greater number of PFC until they are 14 days of age or more, suggesting that they may have a larger number of cells in their spleens. This question will be gone into more fully in Section 8.

7.3.2 The lymph nodes

The differences in the responses that have been seen in the spleen are significant, but not very marked. Quite a different state of affairs is seen when the responses of
Fig. 7.3.1.2. The number of PFC in the spleens of NZB, Ju and CBA mice per gm of body weight. The mice were injected at various ages and tested 5 days after injection (Ju and CBA) or 6 days after injection (NZB). The points are geometric means of 6 or more animals and the limits are standard error.
the lymph nodes are investigated. Fig. 7.3.2.1. shows the response of the lymph nodes measured as PFC per million viable cells. It can be seen that while the response of the lymph nodes of the NZB reaches mature levels in animals injected at 4 days old, the response of the lymph nodes of the two control strains of mice gave only a very low or negligible response throughout the period of the experiment except in the adult animals. The standard error of the response of the NZB injected at 0, 2, or 3 days old is very large. This seems to be due to the fact that the animals either respond near the mature level of response or they do not respond at all. Of the 5 animals injected at 0 days of age 4 gave a negligible response and 1 gave a response indicated by the cross. Of the 6 animals injected at 2 days of age, 3 gave negligible response and 3 gave a response the mean of which is indicated by the cross. Similarly with the 3 day old animals: 3 of the 8 gave a negligible response and the mean of the others is near the adult values.

7.3.3 The relationship between the responses of the spleen and the lymph nodes

Since the lymph nodes of the Ju and CBA babies are not responding there can be no relationship between the responses of the spleens and the lymph nodes in these animals. Fig. 7.3.3.1 shows that in the NZB the maturation of the response of the lymph nodes and of the spleen follow one another very closely. However as Table 7.3.3.1. shows there
Fig. 7.3.2.1. The number of PFC per million viable lymph node cells of NZB, Ju and CBA mice injected with $5 \times 10^6$ SRBC at various ages and tested 5 days after injection (CBA, Ju) or 6 days after injection (NZB). The points are geometric means of 6 or more animals and the limits are standard error. The crosses are explained in the text.
Fig. 7.3.3.1. The number of PFC per $10^6$ viable spleen or lymph node cells in NZB mice given $5 \times 10^6$ SRBC at various ages and tested 6 days after injection. The points are geometric means and the limits are standard error.
Table 7.3.3.1. Correlation between the immune response of lymph nodes and spleen measured as PFC per million cells in NZB mice injected at various ages and tested at 6 days after injection. The table shows the correlation coefficient (r) the number of animals (n) and the level of significance (p).

<table>
<thead>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>14</th>
<th>A</th>
<th>total</th>
</tr>
</thead>
<tbody>
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<td>-0.47</td>
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<td>0.24</td>
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</tr>
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<td>7</td>
<td>9</td>
<td>6</td>
<td>10</td>
<td>66</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td>&lt;0.1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>
is no correlation between the response of the lymph nodes and of the spleen in individual mice.

7.4 The maturation of the form of the immune response

The reason that the mice in the foregoing experiments are showing a response lower than that of the adults could be due either to the reduction of the overall magnitude of the response or to a change in the time when the maximum response is reached. Therefore mice of 0, 3, 5, 7, 14 and 21 days old were injected with the same dose of SRBC as before and tested at various intervals after injection, and compared with the response obtained for the adults in Section 6.

7.4.1 The spleen

Fig. 7.4.1.1. shows the response of the spleens of Ju mice injected at 3, 5, 7, 14 and 21 days of age and as adults. The graph has been divided into two sections for the sake of clarity. In animals injected at 3 days old there is no significant response until 8 days after injection and even this is very low. Mice injected at 5 days are already showing a considerably greater response than those injected at 3 days. The maximum response occurs probably at 7 days and certainly not earlier than 6 days after injection. There is no response 4 days after injection when the adults are showing their maximum. The maximum response of the mice injected at 5 days old is significantly lower than that of the adults. Mice injected at 7 days probably reach their maximum at 6 days after injection and
Fig. 7.11.1. The number of PFC per $10^6$ viable spleen cells of Ju mice injected with $5 \times 10^8$ SRBC at 3, 5, 7, 14, or 21 days old or with $5 \times 10^9$ SRBC as adults. The animals were tested at various intervals after injection. The points are geometric means of 6 or more animals, and the limits are standard error.
thereafter maintain this at 7 and 8 days. Similarly to the 5 day response the response 4 days after injection is negligible. The maximum response is not significantly different to that of the adults. Mice injected at 14 days show a response very similar to those injected at 7 days. Animals injected at 3 weeks old show a response much more like that of the adults than the animals injected at earlier ages. There is a high response at 4 days after injection, the response continues to increase until 5 days but the maximum is not significantly higher than that of the adults. Together, these results show that the immature response of this strain of mice has both a lower maximum and a longer delay before the first appearance of antibody forming cells than the mature response. Slightly older mice do not show the reduction of the maximum, but the delay in the first appearance of antibody forming cells is found until the mice are over 2 weeks old. In these mice the maximum response seems to be maintained longer than in the adults.

Fig. 7.4.1.2 shows the response in the spleen of the other control strain, the CBA. Animals injected at 5 days of age show a maximum response at 5 days or later after injection. There is no response at 4 days after injection when the adult mice are producing a response very near their maximum. The maximum response of the mice injected at 5 days is significantly lower than the maximum response of the adults. The mice injected at 7 days of age show a higher response than the animals injected at 5 days of age. There is no response at 3 days and a negligible response only at 4 days after
Fig. 7.4.1.2. The number of PFC per $10^6$ viable spleen cells of CBA mice injected with $5 \times 10^6$ SRBC at 5, 7, 14, or 21 days old or with $5 \times 10^9$ SRBC as adults. The animals were tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
injection. The response rises to a maximum at 6 days after injection and this maximum is maintained at 7 and 8 days. The maximum of mice injected at this age is no longer significantly different from the adults. CBA injected at 14 days old are showing a good response 4 days after injection, which rises to a maximum at 5 and then gradually declines, although not as fast as in the adults. The 21 day response is very similar. Thus in the CBA, as in the Ju, the immature animals show both a maximum lower than that of the adults and a delay in the first appearance of antibody-forming cells. In slightly older animals the magnitude of this response is the same as in the adults, but the delay remains in this case until the animals are over 7 days old and the high response seems to be maintained longer in these young mice than in the adults.

Fig. 7.4.1.3 shows the response for the NZB mice injected on the first day after birth. There is no significant response even in mice tested as late as 13 and 15 days after injection (not shown on graph). Mice injected at 3 days of age are showing a good response as early as 3 days after injection, a time when the response of animals injected as adults has not yet appeared. The maximum is reached by 4 days after injection, and it is not significantly different to the maximum of the adult response. Animals injected at 5 days of age are showing a high response as early as 3 days after injection and they reach their maximum by 5 or 6 days after injection. Mice injected at 7 days of age also show the response at 3 days after injection and
Fig. 7.4.1.3. The number of PFC per $10^6$ viable spleen cells of NZB mice injected with $5 \times 10^8$ SRBC at 0, 3, 5, 7 and 14 days old or with $5 \times 10^9$ SRBC as adults. The animals were tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
it is not significantly different from the 3 day response of mice injected at 3 or 5 days old. The maximum response is still reached about 5 days after injection. In NZB injected at 14 days old the 3 day response is lower than in the younger mice and approaching adult values although the response at 4 days is still considerably higher than in the adults. It seems that the immature form of the response of NZB mice is different from that of the other two strains of mice. While the Ju and CBA show a response that is reduced and delayed compared with that of the adult, baby NZB show a response that is advanced, and as high as that of the adults in mice at an age at which the other strains were giving very little response, but by 14 days of age this early response is beginning to disappear.

This difference between the strains may be clearer if the responses of all the strains of mice are looked at simultaneously. Fig. 7.4.1.4 shows the curves plotted in this way. Comparing the Ju and the CBA, it can be seen that the progression is essentially very similar, although the CBA seem to start later and mature more quickly than the Ju. At 5 days of age the Ju are showing a higher response than the CBA, although it appears at about the same time after injection. By 7 days of age the CBA are showing a very similar response to the Ju, but at 14 days they are more mature than the Ju, which seem to continue to show an immature form of response for some time, though by 21 days the two strains are showing a response similar to each other and to the adults. In contrast, the NZB show a high
Fig. 7.4.1.1. The number of PFC per $10^6$ viable spleen cells in NZB, Ju and CBA mice injected at various ages with $5 \times 10^8$ SRBC and tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
response from the age of 3 days that only alters in the older mice by moving slightly to the right.

7.4.2 The lymph nodes

As might be expected from the results of Section 7.3.2, the results for the lymph nodes show a very much greater difference between the NZB and the control strains than do the results for the spleen. Fig. 7.4.2.1 shows the response of the lymph nodes of Ju mice injected at various ages compared with the adult form. It may be seen that even in animals as old as 3 weeks there is no significant response up to 8 days after injection. Fig. 7.4.2.2 shows the similar result for the CBA mice. Again there is no significant response up to 8 days after injection in animals of any of the ages tested.

The results for the NZB mice are very different. Fig. 7.4.2.3 shows that the lymph nodes of the mice injected within 24 hours of birth, like the spleen, show negligible response, but those of the animals injected at 3 days old show a maximum response as high as that of the adults, although in contrast to the splenic response, this does not appear until 5 days after injection and maximum occurs by 7 days after injection. Mice injected at 5 days old also show their first response at 5 days, reach their maximum by 6 days as the adults do, but the response seems to continue at a high level for longer. At 7 days and by the time the animals are 14 days old, the response follows the adult response very closely. One interesting observation is that the size of the plaques formed by the lymph node cells in these baby NZB
Fig. 7.4.2.1. The number of PFC per $10^6$ lymph node cells in Ju injected with $5 \times 10^8$ SRBC at 3, 5, 7, 14 or 21 days old or with $5 \times 10^9$ SRBC as adults, and tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
Fig. 7.4.2.2. The number of PFC per $10^6$ lymph node cells in CBA injected with $5 \times 10^8$ SRBC at 5, 14 or 21 days of age or with $5 \times 10^9$ SRBC as adults, and tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
The number of PFC per $10^6$ lymph node cells in NZB injected with $5 \times 10^8$ SRBC at 0, 3, 5, 7 or 14 days of age or with $5 \times 10^9$ as adults, and tested at various intervals after injection. The points are geometric means of 6 or more animals and the limits are standard error.
are considerably smaller than the plaques formed by the spleen cells of the same animals or the plaques formed by either the spleen or the lymph nodes of the adults.

7.4.3 The thymus

Although none of the thymuses of the adults showed any immune response it was possible that the thymuses of the baby animals might show such a response. Therefore some of the thymuses of Ju and NZB mice injected at 5 days old were assayed by the haemolytic plaque assay method. Of 6 Ju injected at 5 days old and tested at 4 and 7 days after injection, none gave any response and of 13 NZB injected at 5 days old and tested at all days between 3 and 8 none gave any response. It is thus demonstrated that the thymuses of adult and baby NZB and Ju are incapable of responding to antigen by the production of antibody forming cells.

7.5 Test for a 'hypersensitivity factor'

It seemed possible if not very probable that the hypersensitivity of the baby NZB could be caused by some non-cellular factor, for example, the murine leukaemia-like virus that has been found in these mice (e.g. Mellors, 1968). This factor might be transmissible to other mice so that if it was introduced into the Ju babies too young to respond normally a high response like that of the NZB might result. To test this idea, the spleens were removed from a 1 year old NZB and a 1 year old Ju mouse, they were homogenised in Eagle's medium, frozen and thawed and then
centrifuged. 0.3 ml. washed packed SRBC was mixed with
0.6 ml. of the extract from the NZB or Ju spleen or with
Eagle's medium, and after leaving for 10 minutes at room
temperature, 0.05 ml. of the mixtures were injected into
3 day old Ju mice from a single litter. The spleens were
tested 5 days later and the results are shown in Table 7.5.1.

The addition of adult spleen extract to the antigen
injection may cause a slight increase in the number of PFC
per spleen, but this increase is insignificant when compared
to the response of the 3 day old NZB and is in any case
found both with the Ju and NZB spleen extract. The one high
response found in the animals treated with Ju spleen extract
is probably due to the inclusion of a living Ju spleen cell
that had survived the freezing. Pooled lymph nodes from
the three groups were also tested, but none gave a significant
response.

This experiment is not, of course, conclusive. Firstly,
it is only a single experiment, secondly, the conditions
may not have been suitable for the operation of the factor
if it had been present; thirdly, the factor may not have
been present in the NZB mouse used as the donor of the
splenic extract, or fourthly, the conditions of extraction
may not have been suitable to retain the activity of the
factor. Notwithstanding, the results of this experiment
make it improbable that there is any factor present in NZB
of all ages that acts on the injected SRBC themselves to
make them more immunogenic.
Table 7.5.1. The numbers of PFC in the spleens of Ju mice injected at 3 days old with SRBC and an extract of NZB spleen, Ju spleen or Eagle's medium and tested 5 days later. The response of NZB mice injected at 3 days with SRBC and tested 5 days later are included for comparison.

<table>
<thead>
<tr>
<th>mouse</th>
<th>injection</th>
<th>PFC per spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ju</td>
<td>SRBC + Eagle's</td>
<td>13, 6, 3, &lt;3, &lt;3</td>
</tr>
<tr>
<td>Ju</td>
<td>SRBC + Ju spleen</td>
<td>710, 60, 16, 6, 6</td>
</tr>
<tr>
<td>Ju</td>
<td>SRBC + NZB spleen</td>
<td>43, 36, 13, 13, 10</td>
</tr>
<tr>
<td>NZB</td>
<td>SRBC</td>
<td>5460, 5175, 2505, 2060, 1980, 1870</td>
</tr>
</tbody>
</table>

These figures were calculated from the mean of 3 plates each with 1/10 of the total spleen, so that figures of less than 20 represent a mean of less than 2 plaques per plate and are probably not significant.
7.6 Discussion

The results in this section have shown differences in the immature patterns of the responses of NZB and the two non-autoimmune strains of mice. The spleens of the 'normal' babies produce a response with a maximum lower than that of the adults in mice below 5-7 days old and an increase in the lag period between antigen injection and appearance of PFC in mice up to 2 weeks old. The NZB produce a maximum number of PFC equal to that of the adults by 3 days of age, and in contrast to the other strains of mice they show a reduction of the lag period between antigen injection and the appearance of antibody forming cells in mice up to 2 weeks old.

Playfair (1968a) has suggested that the maximum response will not occur before the age when immunological maturity is reached, so in animals injected before this age the response at any time will depend more on the age of the animal than on the time since it was injected. The results obtained in this section were graphed as he suggests in Fig. 7.6.1 which shows that these results do not support this idea.

A further difference between the NZB and control strains of mice is found in the lymph nodes which in the control strains of mice produce no response up to 21 days old, but in the NZB produce a response as soon as the spleen.

The response of the baby JU and CBA differs from the response of the adults in two respects: it is lower, and the lag period is longer. In slightly more mature babies the lag period is still found to be longer than that of the adults.
Fig. 7.6.1. The number of PFC per 10^6 viable spleen cells of NZB, Ju and CBA mice injected with 5 x 10^6 SRBC at various ages and tested at various times after injection. The points are geometric means.
but the maximum response is no longer reduced. Argyris (1968b, 1969b) and Braun and Lasky (1967) have suggested that the inadequate response of baby mice is due to the immature processing capabilities of their macrophages. Since the lag period will be, at least in part, due to the time taken for the macrophages to process the antigen, a prolonged lag period is in accord with the idea of deficient macrophage function. In the young animals the lag period is increased over that of the adults long after the animals are well able to respond to the sheep erythrocytes, suggesting that macrophages are not fully mature until the mice are several weeks old. In support of this idea Argyris (1969b) found that while macrophages from adults were able to induce baby mice to respond to sheep erythrocytes, macrophages from mice as old as 1 month were not effective in this respect.

The reduction in the maximum response is not necessarily due to the same cause as the increased lag period and may for example be due to an inadequacy in the number or the proliferative capacity of precursor cells in the very young mice. It is not possible to say whether the lack of a response in the lymph nodes for so long after the spleen is showing a good response could be due to a lack of functional macrophages or to some other deficiency.

The baby NZB mice, in contrast to the babies of the other two strains have a shorter lag period between the injection of antigen and the appearance of antibody forming cells than do the adult NZB. This might be due to a precocious maturation of the macrophages. Since the response
of the animals changes rapidly from none in animals injected at 0 days of age to 'adult' at 3 days of age without evidence of an intermediate 'immature' response the macrophages probably would be fully mature from before birth, and the lack of response at birth due to the immaturity of some other function. Since the macrophages of the other two strains of mice do not show full maturity until the animals are over 14 days old, this would mean that the macrophages of the NZB are very premature indeed. In addition, the baby NZB spleens are showing a lag period which is shorter, not only than that of the adults where the autoimmune phenomena may be interfering with the response to heterologous antigens (Disner, 1966), but also shorter than that of 14 day old mice, long before the appearance of autoimmunity. Therefore as well as being extremely prematurely functional the macrophages of the babies must be able to process the antigen more rapidly than those of other strains or of older mice, pointing to a genuine though transient abnormality in the macrophages of baby NZB.

An alternative hypothesis to this is that the NZB are able to dispense with macrophages altogether by having a population of cells able to react directly with antigen. The reduction in the amount of processing necessary results in a reduction in the lag period. The initial unresponsiveness of the animals injected at birth would be due to the immaturity of a different population of cells, but as soon as these became functional the response would occur. Assuming that the lack of response in the lymph nodes of the
young mice of 'normal' strains is due to a lack of macrophages in these organs, and that cells able to respond to 'unprocessed' antigen were present in the lymph nodes of the NZB, the very early response in the lymph nodes of the NZB would be explained. There is a longer lag period before the response of the lymph nodes than before the response of the spleen in the baby mice, and this may be a result of the inability of immature macrophages to transfer the antigen from the peritoneal cavity to the lymph nodes. As the mice grow older their macrophages mature and become functional in the normal manner taking over the pathways of the immune response and accounting for the gradual reversion to a normal response in older animals. These abnormally reactive cells could be found in the neonatal period only, but they might persist throughout life, only becoming evident if for some reason the macrophage function of the animals is defective.

In this section, the maturation of the immune response to SRBC has been compared in NZB, Ju and CBA mice. It has been shown that while the immature response of the spleen of 'normal' babies is reduced and delayed in comparison with the adults, that of the NZB is advanced and of the same magnitude as that of the adults from a very early age. The lymph nodes of 'normal' strains are found to be showing no response by 3 weeks of age, but those of NZB are responding as soon as the spleen. It is suggested that the delay in the response of the baby Ju and CBA could be due to immature macrophages taking longer to process the antigen than the mature macrophages of the adults, and that in the NZB the
macrophages are either hyperactive or the requirement for them is absent.
8. THE GROWTH OF THE BODY AND LYMPHOID ORGANS

8.1 Introduction

In the previous section it was shown that baby NZB mice achieve immunological competence at an earlier age than the two 'normal' strains of mice. This early response of the NZB does not show the characteristic immature form of response found in the other two strains. In order to find whether this early response is reflected by differences in the growth rate of the whole animal or of its lymphoid organs, the weights of the 3 strains of animals of different ages and the size and structure of their lymphoid organs were compared.

8.2 Weight

The weights of the baby mice used in previous and subsequent sections were taken when they were killed. This means that each animal has contributed only a single value to the graphs. In case the injection of antigen might have any effect on growth rate, animals which were injected at different intervals prior to killing or with different antigens were calculated separately for each age but previous injection with antigen was found to have no observable effect on the body weight of the animals so they were combined and compared. This comparison is shown in Fig. 8.2.1. It may be seen that the growth rate of all three strains of mice is very similar. It is possible that the NZB are fractionally larger than the other two strains at ages greater
Fig. 8.2.1. Body weights of NZB, Ju and CBA mice of various ages. The points are arithmetic means of 6 or more animals and the limits are standard error.
than 2 weeks, and the differences from the closest control
strain are significant on days 13 (p < 0.02), 14 (p < 0.001),
16 (p < 0.001), 19 (p < 0.01) and 20 (p < 0.05). Since the
maximum differences in the immune response occur before 10
days of age this is unlikely to have any relationship to
the immunological differences.

8.3 The size of the spleen

The size of the spleen was measured as the number of
viable cells. For this, too, animals that had received
antigen at different times before being killed or had received
different antigens were calculated separately for each age.
As with the body weight, in none of the strains did previous
treatment with antigen have any significant effect on the
number of cells, so again all groups for each age were
combined and compared. Fig. 8.3.1 shows the results. As
with the body weights there is very little difference between
the three strains.

Comparison of the body weight curves and the spleen
size curves show a correspondence between them, suggesting
that the variation in the size of the spleen is related to
variation in the size of the body. The relationship between
these two parameters for individual 20 day old Ju and NZB
mice is shown in Fig. 8.3.2 and the correlation may be seen
to be similar in both strains of mice.
Fig. 8.3.1. The number of viable cells per spleen of NZB, Ju and CBA mice of various ages. The points are geometric means of 6 or more animals and the limits are standard error.
Fig. 8.3.2. The correlation between the body weight and the size of the spleen of 20 day old NZB (○) and Ju (O) mice. Each point represents the results for one individual mouse.
8.4 The size of the lymph nodes

Fig. 8.4.1 shows that the cell number of the lymph nodes is also very similar in all three strains of mice. This is perhaps particularly interesting in view of the fact that many of the NZB lymph nodes counted in this experiment contained large numbers of antibody forming cells and there were virtually none whatever in the lymph nodes of either the Ju or the CBA.

8.5 The structure of the thymus

Baby Ju and NZB mice of ages up to 1 week were killed without previous antigen injection, and sections of the thymus, spleen and lymphoid organs examined. The thymus shows a typical structure from birth, the thymic cortex containing closely packed thymocytes and the medulla containing lymphocytes, reticular cells and a few Hassal's corpuscles. Fig. 8.5.1 and Fig. 8.5.2 show a section of a thymus taken from a Ju mouse at the day of birth under low and high power respectively, and Fig. 8.5.3 shows the medulla and the cortex of the thymus of an NZB mouse of less than 1 day old. By 1 week of age the thymus of the NZB is beginning to show the deficiency of the number of large epithelial cells in the medulla reported by de Vries and Hijmans (1966; 1967). This is shown in Figs. 8.5.4 and 8.5.5 which show the medulla of the thymuses of 7 day Ju and 8 day NZB respectively. In both cases the fields were selected to show as many epithelial cells as possible. The thymuses of neither strain of animal showed more than minimal PAS staining and there were no pyroninophilic cells at the ages studied.
Fig. 8.1.1. The number of viable cells in the six dissected limb-draining lymph nodes of NZB, Ju and CBA mice of various ages. The points are geometric means of 6 or more animals and the limits are standard error.
Fig. 8.5.1. Thymus of Ju mouse of less than 1 day old showing the general structure of the cortex and medulla. PAS: x 180.
Fig. 8.5.2. The same section as Fig. 8.5.1. showing the cortex and medulla of thymus of less than 1 day old in more detail. A Hassal's corpuscle is also shown.  PAS: x 800.
Fig. 8.5.3. Thymus of NZB mouse of less than 1 day old showing the cortex and the medulla. H & E: x 800.
Fig. 8.5.4. Thymus medulla of 7 day old Ju mouse showing epithelial cells. H & E: x 600.
Fig. 8.5.5. Thymus medulla of 8 day old NZB mouse showing deficiency of epithelial cells relative to Ju in Fig. 8.5.4. H & E: x 800.
8.6 The structure of the spleen

The spleen of the Ju and NZB mice show few differences from one another. In newborn and 1 day old mice there is little structure to be seen and the spleen apparently consists almost entirely of red pulp with many red blood cells, reticuloendothelial cells and lymphocytes. There are no primary nodules. Fig. 8.6.1 shows a section of the spleen of an NZB within 24 hours of birth, and Fig. 8.6.2 shows the spleen of a 1 day old Ju. At 2 days of age the primary nodules are beginning to form. Fig. 8.6.3 shows the structure of a 2 day old Ju spleen and Fig. 8.6.4 a 2 day old NZB spleen. As the animals grow older the primary nodules become more numerous and more clearly defined. Fig. 8.6.5 shows a 3 day old Ju spleen, and Fig. 8.6.6 a higher power view of the same section, showing part of a lymphoid follicle and the circumfollicular space containing erythrocytes. Fig. 8.6.7 and 8.6.8 show the splenic structure of 8 day old Ju and NZB respectively. Staining with methyl green pyronin showed no or very few plasma cells in animals 8 days of age or under, but these animals had not been antigenically stimulated except by natural means. No cells containing PAS positive material were found. There were many cells containing toluidine blue positive granules in the spleen although not in the thymus (Fig. 8.6.9).

8.7 The structure of the lymph nodes

The limb draining lymph nodes were visible and could be dissected out from the time of birth, but they were so
Fig. 8.6.1. Spleen of NZB mouse of less than 1 day old showing structural immaturity, in particular lack of primary nodules. H & E: x 180.
Fig. 8.6.2. Spleen of 1 day old Ju mouse showing structural immaturity, in particular, lack of primary nodules. PAS: x 180.
Fig. 8.6.3. Spleen of 2 day old Ju mouse showing primary nodules beginning to form. PAS: x 180.
Fig. 8.64. Spleen of 2 day old NZB, showing primary nodules beginning to form. H & E: x 180.
Fig. 8.6.5. Spleen of 3 day old Ju showing typical structure. H & E: x 180.
Fig. 8.6.6. Spleen of 3 day old Ju showing part of follicle and circumfollicular space containing erythrocytes. H & E: x 800.
Fig. 8.6.7. Spleen of 8 day old Ju showing well-defined structure. H & E: x 180.
Fig. 8.6.8. Spleen of 8 day old NZB showing well-defined structure. H & E: x 180.
Fig. 8.6.9. Spleen of 3 day old NZB showing granule containing cells. Toluidine Blue: x 1200.
small that it was not possible to get good sections from animals younger than 6 days of age. Even at this age however, they showed little of the typical lymph node structure, being merely accumulations of lymphocytes and reticular cells. Fig. 8.7.1 and Fig. 8.7.2 show lymph nodes from 6 day old Ju under low and high power, and Fig. 8.7.3 and Fig. 8.7.4 show the same from 6 day old NZB.

8.8 Discussion

The results presented in this section have not shown any difference in the general maturation rates of the three strains of mice. The early immunological reactivity of the NZB is not reflected by any differences in the size or structure of the lymphoid organs and the difference must therefore lie in purely functional aspects of the antibody response.

The periarteriolar follicular areas are the 'thymus dependent' areas of the spleen (Parrott, de Sousa and East, 1966) and it seems possible that the appearance of these structures at about 2-3 days of life represents the arrival of functional thymus-derived antigen-sensitive cells in the spleen. If, as I have suggested, the NZB show a precocious macrophage development or an ability to bypass the macrophage stage in the response to sheep erythrocytes, their response would appear as soon as all the other elements involved in it were sufficiently mature. The appearance of the NZB response between 0 and 3 days when the splenic structure is also developing could be due to the
Fig. 8.7.1. Lymph node of 6 day old Ju to show the lack of structure. H & E: x 180.
Fig. 8.7.2. Lymph node of 6 day old Ju, the same as Fig. 8.7.1. H & E: x 800.
Fig. 6.7.3. Lymph node of 6 day old NZB to show the lack of structure. H & E: x 180.
Fig. 8.7.4. Lymph node of 6 day old NZB, the same as in Fig. 8.7.3. H & E: x 800
arrival of functional antigen-sensitive cells, and the delay of the response of the Ju mice in spite of the apparent structural maturity of the spleen due to a lack of macrophages to process the antigen.

The lymph nodes on the other hand are functional in the NZB in spite of a lack of mature structure. Antigen sensitive cells and antibody forming cell precursors are not morphologically distinguishable so it is probable that both populations of cells are present in the lymph nodes. The lack of a follicular structure may suggest a macrophage bypass mechanism rather than a precocious macrophage function in the NZB, but as Williams and Nossal (1966) have shown, antigen uptake mechanisms may be present in the lymph nodes of young animals without morphologically apparent structures so that the lack of visible structure is not really indicative of either alternative.

The conclusions to be drawn from this section are mainly negative. In spite of the evident differences in immune response of young NZB and young of the two control strains no differences in the body size, lymphoid organ size or structure could be found.
9. SECONDARY RESPONSE AND TOLERANCE

9.1 Introduction

The probability that self-recognition is a result of tolerance to autologous constituents has been discussed in Section 1. When an animal is exposed to an antigen the animal may become tolerant or it may become sensitized. Tolerance is more likely to occur under conditions where an animal cannot produce an immune response; one of these conditions is immaturity. Since the NZB are able to produce an immune response to SRBC at an early age, they may be more likely to become sensitized rather than tolerant after exposure to SRBC in the early postnatal period. If this early sensitivity applies to other antigens including auto-antigens it may mean that tolerance to these components is less securely established and more inclined to be broken. Even if this is not the case, differences in the tendency of animals to become sensitized or tolerant, particularly in the perinatal period when self-recognition is being established, may offer a partial explanation for the later development of autoimmunity. Several workers have reported that adult NZB show certain defects in their ability to become tolerant to various antigens (Braverman, 1968a; Staples and Palal, 1968, 1969a,b), but attempts to produce tolerance in NZB by exposure to antigen in the neonatal period, a situation more analogous to that involved in the induction of self tolerance, have not yet been reported.
9.2 The effect of the dose of the primary injection on the secondary response

For these experiments the primary injection was given to 3 day old animals since it was at this time that the NZB and control strains were showing the maximum difference in response from one another. They were injected intraperitoneally with 0.02ml of undilute SRBC (about $5 \times 10^8$ cells) or 0.02ml of SRBC diluted 1/10, 1/100, 1/1000 or 1/10,000 v/v. The animals were given a secondary challenge at 14 days old of 0.02 packed washed SRBC diluted to 1/30 v/v i.e. about $1.7 \times 10^7$ cells, and tested for their response 6 days after the secondary injection and compared with controls which had received the injection at 14 days only. The results for the spleens of NZB, Ju and CBA mice are shown in Table 9.2.1. It can be seen that primary injection tends to depress the secondary response of the Ju mice, but the difference from the controls is only significant when they were given undilute, 1/10 or 1/100 SRBC as a primary dose, and the level of this significance decreases as the dose is reduced. This could have been the result of high zone tolerance, but there was no evidence of a low zone effect. The NZB on the other hand, show a slight non-significant increase over the level of the response of the controls.

If the response of the lymph nodes of the same mice is investigated the difference between the strains in the effect of previous antigen exposure is even more obvious. As was shown in Section 6, the lymph nodes of 14 day Ju mice do not respond to the injection of SRBC and Table 9.2.2.
Table 9.2.1. The effect of primary dose on the secondary response. Mice were injected at 3 days old with various doses of SRBC, challenged at 14 days with $1.7 \times 10^7$ SRBC and tested 6 days after the secondary challenge. The table shows geometric means of the number of PFC per $10^6$ spleen cells ($\bar{x}$), the limits of standard error ($+SE$) the number of animals in each group (n) and the significance of the difference from the control that received no primary dose at 3 days old (p).

<table>
<thead>
<tr>
<th>Primary dose at 3 days old</th>
<th>$5 \times 10^8$</th>
<th>$5 \times 10^7$</th>
<th>$5 \times 10^6$</th>
<th>$5 \times 10^5$</th>
<th>$5 \times 10^4$</th>
<th>none</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{x}$</td>
<td>65</td>
<td>155</td>
<td>190</td>
<td>290</td>
<td>162</td>
<td>470</td>
</tr>
<tr>
<td>$+SE$</td>
<td>32-132</td>
<td>118-204</td>
<td>138-260</td>
<td>186-450</td>
<td>55-310</td>
<td>360-600</td>
</tr>
<tr>
<td>Ju</td>
<td>n</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.01</td>
<td>&lt;0.02</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primary dose at 3 days old</th>
<th>$5 \times 10^8$</th>
<th>$5 \times 10^7$</th>
<th>$5 \times 10^6$</th>
<th>$5 \times 10^5$</th>
<th>$5 \times 10^4$</th>
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</tr>
</thead>
<tbody>
<tr>
<td>$\bar{x}$</td>
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<td>1550</td>
<td>316</td>
<td>575</td>
<td>510</td>
<td>340</td>
</tr>
<tr>
<td>$+SE$</td>
<td>265-675</td>
<td>705-3400</td>
<td>123-810</td>
<td>288-1170</td>
<td>262-980</td>
<td>218-525</td>
</tr>
<tr>
<td>Ju</td>
<td>n</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 9.2.2. The effect of primary dose on the secondary response in the lymph nodes. Mice were injected at 3 days old with various doses of SRBC, challenged at 14 days old with $1.7 \times 10^7$ SRBC and tested 6 days after the secondary challenge. The response of the Ju was not detectable in any animal. For the NZB are shown the geometric means of the numbers of PFC per $10^6$ lymph node cells ($\bar{x}$), the limits of standard error ($\pm$SE), the number of animals in each group (n) and the significance of the difference from the control that received no primary dose (p).

<table>
<thead>
<tr>
<th>dose of primary injection</th>
<th>$5 \times 10^8$</th>
<th>$5 \times 10^7$</th>
<th>$5 \times 10^6$</th>
<th>$5 \times 10^5$</th>
<th>$5 \times 10^4$</th>
<th>none</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ju</td>
<td>$\bar{x}$</td>
<td>296</td>
<td>310</td>
<td>6.8</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>±SE</td>
<td>126-690</td>
<td>162-525</td>
<td>3.0-15.5</td>
<td>11.8-63</td>
<td>6.5-29.5</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>$&lt;0.001$</td>
<td>$&lt;0.001$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
shows that previous exposure to SRBC does not alter this. On the other hand, the NZB lymph nodes, although giving little response to the control dose, which is 1/30 of that given to the 14 day old animals in Section 6, do show a considerable enhancement of this response when they have been given a primary dose of undilute or 1/10 SRBC at 3 days of age.

From these data it would appear that Ju can be suppressed, but not greatly, and that the NZB show no suppression in the spleen and definite sensitization in the lymph nodes.

9.3 The effect of the time of the primary injection on the secondary response

For this experiment, mice were injected at various ages with 0.02ml undilute SRBC which was the dose that had had the greatest effect in the previous experiment, and again challenged with 0.02ml 1/30 SRBC at 14 days old. They were tested 6 days after injection. The results for the spleen are shown in Table 9.3.1. It can be seen that the response of the Ju injected at any age between 1 and 7 days is significantly lower than that of the controls which had an antigen injection on day 14 only. A primary injection at 1, 3, 5 or 7 days given to CBA mice produced a similar reduction although the difference was significant in animals injected only on day 7. All the NZB that had been given a primary injection showed a response higher than the controls that had had a 14 day injection only, and in animals that had
Table 9.3.1. The effect of time of primary dose on the secondary response of the spleen.

Mice were injected at various ages with $5 \times 10^8$ SRBC and challenged at 14 days with $1.7 \times 10^7$ SRBC and tested 6 days later. The table shows the geometric means of the numbers of PFC per $10^6$ spleen cells ($\bar{x}$), the limits of standard error ($\pm SE$), the number of animals in each group (n) and the significance of the difference from the control that received no primary dose (p).

<table>
<thead>
<tr>
<th>age at primary injection (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>No. 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{x}$</td>
<td>135</td>
<td>112</td>
<td>64.5</td>
<td>57.5</td>
<td>62</td>
<td>95.5</td>
<td>107</td>
<td>470</td>
</tr>
<tr>
<td>$\pm SE$</td>
<td>89-200</td>
<td>49-262</td>
<td>31.6-132</td>
<td>40-83</td>
<td>30-123</td>
<td>71-132</td>
<td>89-132</td>
<td>363-600</td>
</tr>
<tr>
<td>Ju</td>
<td>n</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>6</td>
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<td>7</td>
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<tr>
<td>p</td>
<td>&lt;0.02</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>110</td>
<td></td>
<td>72.5</td>
<td></td>
<td>69</td>
<td></td>
<td>55</td>
<td>250</td>
</tr>
<tr>
<td>$\pm SE$</td>
<td>72.5-170</td>
<td>41-132</td>
<td>34.6-138</td>
<td></td>
<td></td>
<td></td>
<td>38-78</td>
<td>151-425</td>
</tr>
<tr>
<td>CBA</td>
<td>n</td>
<td>8</td>
<td>5</td>
<td>6</td>
<td></td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>$\bar{x}$</td>
<td>2340</td>
<td>2090</td>
<td>415</td>
<td>910</td>
<td>775</td>
<td>1290</td>
<td></td>
<td>340</td>
</tr>
<tr>
<td>$\pm SE$</td>
<td>1290-4160</td>
<td>1070-4080</td>
<td>263-660</td>
<td>525-1590</td>
<td>456-1290</td>
<td>795-2140</td>
<td></td>
<td>219-525</td>
</tr>
<tr>
<td>NZB</td>
<td>n</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>
been given their primary injection on the first or second days after birth the difference was significant. A priming dose at any age sensitized the lymph nodes of the NZB (Table 9.3.2) but not the other strains. This does suggest a difference between the NZB and the two control strains in the ability to be sensitized but since the effect was not greatest when the primary injection was given to the younger animals, this apparent suppression may not be due to tolerance. It may indeed be related to the different intervals between the primary and secondary doses for animals given their primary injection at different ages which prevents them from being strictly comparable with one another, though different strains given the same treatment may of course be compared with one another. It may be worth pointing out that the alternative experiment, where the interval between primary and secondary antigen injection is kept constant, would result in the secondary injection being given to animals of different ages, and since the animals are not immunologically mature at 21.5 days or later, these results would not be comparable with one another either.

9.4 The shape of the secondary response

As I have pointed out previously, results obtained by measuring the number of antibody forming cells at only a single interval after antigen injection can be misleading so animals were injected with $5 \times 10^8$ SRBC at 3 days of age and given the secondary injection of $1.7 \times 10^7$ at 14 days of age as before. They were killed at various times after injection.

Fig. 9.4.1 shows the secondary response in the Ju
Table 9.3.2. The effect of the time of primary dose on the secondary response of the lymph nodes of NZB mice. Mice were injected at various ages with $5 \times 10^8$ SRBC and challenged at 14 days with $1.7 \times 10^7$ SRBC and tested 6 days later. The table shows the geometric means of the numbers of PFC per $10^6$ lymph node cells ($\bar{x}$), the limits of the standard error ($\pm SE$), the number of animals in each group (n) and the significance of the difference from the controls (p).

<table>
<thead>
<tr>
<th>age at primary injection (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>No.1°</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{x} )</td>
<td>425</td>
<td>340</td>
<td>296</td>
<td>435</td>
<td>456</td>
<td>1320</td>
<td>6.8</td>
</tr>
<tr>
<td>( \pm SE )</td>
<td>224-815</td>
<td>186-615</td>
<td>126-690</td>
<td>250-760</td>
<td>230-935</td>
<td>645-2700</td>
<td>4.1-11.2</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>
**Fig. 9.4.1.** The number of PFC per $10^6$ viable spleen cells of Ju mice injected with $5 \times 10^8$ SRBC at 14 days of age (primary response) or $5 \times 10^8$ SRBC at 3 days of age and $1.7 \times 10^7$ SRBC at 14 days of age (secondary response). They were tested at various times after the injection at 14 days. The cross indicates the result for animals given $1.7 \times 10^7$ at 14 days only. The points are geometric means of 6 or more animals, the limits are standard error.
spleen compared with the 14 day primary response obtained in Section 7. The cross shows the results for the control mice who were given, at 14 days of age, 1/30 of the dose given to the mice shown in the 'primary response'. It can be seen that the decrease in the 6 day response found in the mice receiving a secondary injection is not due to the induction of tolerance but to a change in the position of the maximum of the response. Animals receiving the lower antigen dose at 14 days nevertheless produced a 6 day response as great as the animals receiving the higher dose.

In order to determine how much the early secondary response owes to PFC still being produced in response to the primary SRBC injection given at 3 days of age, Ju mice injected at 3 days of age were tested 13 days later, that is at 16 days old and at a time equivalent to 2 days after the secondary injection. The geometric mean of the response was 13.5 PFC per million viable cells and the limits of the standard error were 21.4 and 8.5 PFC per million viable cells and this was significantly different (p < 0.05) from the secondary response 2 days after injection, showing that at this age the secondary response is able to induce an increase in antibody-forming cells as early as 2 days after injection in contrast to the primary response where the earliest response takes 5 days to appear.

The response of the NZB spleens is shown in Fig. 9.4.2. It can be seen that here, too, the secondary response is both earlier and higher than the primary response although the difference between the primary and secondary responses is
Fig. 9.4.2. The number of PFC per $10^6$ viable spleen cells of NZB mice injected with $5 \times 10^8$ SRBC at 14 days of age (primary response) or $5 \times 10^8$ SRBC at 3 days of age and $1.7 \times 10^7$ SRBC at 14 days of age (secondary response). The animals were tested at various times after the injection at 14 days of age. The cross indicates the result for animals given $1.7 \times 10^7$ SRBC at 14 days only. The points are geometric means and the limits are standard error.
not so marked as in the Ju probably owing to the fact that
the primary response of the NZB is, in any case, earlier
and higher than that of the Ju. The primary response
obtained at 6 days after injection with the lower dose of
antigen is not significantly lower than the primary response
following treatment with the larger amount of antigen.
Neither is the maximum of the secondary response significantly
higher than the maximum of the primary response.

The NZB mice also were tested at 16 days of age after
having been given only the 3 day primary dose. This
resulted in a mean of 13.2 PFC per million viable cells
with the limits of the standard error at 19.5 and 8.9 PFC
per million. This also was significantly different from
the secondary response 2 days after injection (p < 0.01) so
that in this strain too, previous exposure to antigen
resulted in a response within 2 days against the 4 days
required for the primary 14 day response.

It is interesting to compare the secondary responses
of the NZB and the Ju. Referring to Fig. 7.4.1.4 it can be
seen that in the primary responses of 14 day old NZB and Ju
mice the NZB produce a response earlier, but not significantly
higher than the Ju. Fig. 9.4.3 shows that the secondary
response of the NZB is significantly higher than that of the
Ju (p < 0.02), and that the maximum Ju secondary response
occurs a day earlier than that of the NZB.

The secondary response of the lymph nodes of 14 day
old NZB was also compared with the primary response, and
these results are shown in Fig. 9.4.4. It can be seen that
Fig. 9.4.1. The number of PFC per $10^6$ viable spleen cells of NZB and Ju mice injected with $5 \times 10^8$ SRBC at 3 days of age and $1.7 \times 10^7$ SRBC at 14 days of age. The animals were tested at various times after injection at 14 days of age. The points are geometric means and the limits are standard error.
Fig. 9. The number of PFC per $10^6$ viable lymph node cells of NZB mice injected with $5 \times 10^8$ SRBC at 14 days of age (primary response) or $5 \times 10^8$ SRBC at 3 days of age and $1.7 \times 10^7$ SRBC at 14 days of age (secondary response). The animals were tested at various times after the injection at 14 days of age. The cross indicates the result for animals given $1.7 \times 10^7$ SRBC only at 14 days. The points are geometric means and the limits are standard error.
in this case too, the secondary response occurs at least 2 days earlier although it is no higher than the primary response. It is also interesting that the primary response of the lymph nodes of those animals given a low dose of antigen at 14 days is considerably lower than the response of the animals given the higher dose at 14 days. It seems that the lymph node response of the NZB is much more dose dependent at the higher doses of antigen than is the spleen response and this situation corresponds to that found in the adult animal.

9.5 The effect of multiple primary injections

Since it was obvious that tolerance had not been produced in either the Ju or the NZB by any of the methods used so far, in an attempt to induce tolerance to this antigen animals were injected with 0.02ml undilute SRBC on days 1, 3, 5 and 7 of life and were then given a challenging dose of $1.7 \times 10^7$ SRBC at 14 days of age as before and tested 6 days later. Table 9.5.1 shows the result compared with the controls injected with SRBC 1/30 at 14 days only and compared with the animals injected at 3 days only and then at 14 days. It can be seen that the only significant difference observed is in the lymph nodes of the NZB where previous injection, whether one or several, causes sensitization. The Ju given multiple sensitizing injections show a higher response than the Ju given only one sensitizing injection though the difference is not significant. Whether this effect is due to further alteration of the shape
Table 9.5.1. The effect of multiple primary injections. The mice were given $5 \times 10^8$ SRBC at the times stated and then $1.7 \times 10^7$ at 14 days old and tested 6 days later. The geometric means of the number of PFC per $10^6$ viable cells are shown ($\bar{x}$), the limits of the standard error ($\pm$SE) and the number of animals in each group (n). The significance of the differences are shown between the values.

<table>
<thead>
<tr>
<th>Age at primary injection (days)</th>
<th>none</th>
<th>1,3,5&amp;7</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>Ju $\pm$SE</td>
<td>Ju $\pm$SE</td>
</tr>
<tr>
<td></td>
<td>467</td>
<td>362-604 NS</td>
<td>224-435 NS</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>NZB spleen $\pm$SE</td>
<td>NZB spleen $\pm$SE</td>
</tr>
<tr>
<td></td>
<td>339</td>
<td>219-525 NS</td>
<td>1100-2140 NS</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>NZB LN $\pm$SE</td>
<td>NZB LN $\pm$SE</td>
</tr>
<tr>
<td></td>
<td>6.7</td>
<td>4.1-11.2 0.001</td>
<td>282-467 NS</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>
of the curve or an increase in the response itself is not known.

In an attempt to produce tolerance in these mice, NZB and Ju were injected every 2 days with $2.5 \times 10^8$ SRBC until they were 3 weeks old and thereafter with $5 \times 10^8$ SRBC and were tested at weekly intervals for the number of PFC in the spleen. This is more analogous to the self-tolerance inducing situation where the animal is continually exposed to its own antigens. The results are shown in Fig. 9.5.1. It can be seen that repeated injections of antigen produce an initial sensitization in both strains of mice. This level of response decreases gradually over a number of weeks as the injections continue. At about 6-8 weeks the Ju are producing a negligible response and tolerance is complete. The NZB on the other hand, although showing a response well below the initial level, are producing a significant number of PFC and there is no sign of further reduction of this number by 10 weeks from the start of treatment. It would appear therefore that with this treatment only partial tolerance can be induced in the NZB mice.

9.6 Discussion

The immune response after a second injection may be lower (tolerance) or higher (Šterzl and Jilek, 1967) than the primary response but it is usually faster, although this will depend on the time elapsed between primary and secondary challenge (Hege and Cole, 1966). The difference between the primary and the secondary response is generally considered
The effect of repeated injection on the immune response. Ju and NZB mice were injected every 2 days with $2.5 \times 10^8$ SRBC up to 3 weeks old and thereafter with $5 \times 10^8$ SRBC and tested at intervals. The points shown are the results for individual mice.
to be due to the formation of large numbers of specifically sensitized 'memory cells' (Nossal, Austin and Ada, 1965; Hamaoka, Kitagawa, Matsuoka and Yamamura, 1969; Celada, 1967). It thus appears that the rapidity of the secondary response is due to the fact that less processing is required than in the primary response and indeed it is known that proliferation starts within a few hours of a secondary antigen injection (Byfield and Sercaro, 1969) compared with the day or longer required in the primary response. The secondary response is less susceptible to irradiation (Uyeki and Klassen, 1968) and administration of antiserum against thymus derived cells will inhibit the primary response but not the secondary response to SRBC (Davies, Leuchars, Wellis, Sinclair and Elliott, 1968), suggesting that either the ARC or their irradiation and antilymphocyte serum sensitive functions are not required for the secondary response. Labelling experiments also suggest that the memory cell transforms directly into the antibody-forming cell (Bosman and Feldman, 1968), but Simons and Fitzgerald (1969) found that small lymphocyte memory cells proliferated and underwent blast transformation in the presence of antigen but did not produce antibody, suggesting that it is the ARC that is the memory cell. Cunningham (1969a) also found that spleen cells from immunized animals mixed with chromosomally distinguishable bone marrow cells from non-immunized animals in vitro resulted in antibody production by the bone marrow cells, which also leads to the conclusion that the ARC are the memory cells. The secondary response to neither SRBC (Pierce, 1969) ferritin (Moore and Schoenberg, 1968) nor
tetanus toxoid (Ellis, Gowans and Howard, 1969) seems to require the action of macrophages, so that memory cells can probably respond to unprocessed antigen. The difference between the primary and the secondary response - the 'prememory' stage probably consists of the uptake and processing of antigen by macrophages, the sensitization of the APC and their proliferation.

The Ju mice show a long delay in the production of their primary response, but this delay is not evident in the secondary response indicating that the delay in the primary response must be due to the immaturity of some function prior to the production of memory cells. This is in agreement with the hypothesis that the long lag period of the response of immature Ju is caused by macrophage immaturity.

Comparison of the secondary responses of the spleens of the NZB and Ju shows that apart from magnitude they are not dissimilar and particularly that the response of the NZB is not advanced compared with the Ju. There is a considerable difference between the primary responses of the NZB and the Ju at this age. It seems likely therefore that the difference in the primary responses is also due to differences in a part of the response involved in the primary but not the secondary response, that is, in the prememory induction stage. The smaller magnitude of the secondary response of the Ju seems likely to be due to the presence of a smaller number of sensitized cells than in the NZB, although since repeated injections lead to increased sensitization this is probably not because of partial tolerance induction in the Ju.
It could be suggested that the NZB may have been previously exposed to the SRBC antigen perhaps as a cross-reacting antigen in the virus which has been described in their lymphoid tissues (Yumoto and Dmochowski, 1967; Mellors, 1968; Prosser, 1968) but the clear difference between the NZB primary and secondary response and the similarity in the number of 'natural' background PFC to SRBC in the spleens of unimmunized mice of both strains argue against this explanation.

Complete tolerance to SRBC was not produced in NZB mice under circumstances that produced complete tolerance in the Ju. Since both strains of mice showed sensitization before they started to become tolerant, this resistance to tolerance induction in the NZB could not have been directly caused by an increased tendency to sensitization as a result of the earlier response of the NZB. I have suggested that the particular form of the response in the immature NZB is likely to be caused either by a transient hyperactive population of macrophages or by immunocytes that are able to bypass the normal requirement for macrophages. The transient hyperactive macrophages do not offer an explanation of the resistance to tolerance induction but if the basic abnormality of the immunocyte were that it responds by sensitization rather than tolerance to antigen which in normal animals is non-immunogenic, this would explain both its ability to respond to antigen unprocessed by macrophages and its tendency to become less tolerant to antigen that produces complete tolerance in normal animals.
In this section the primary and secondary responses of 14 day old mice to SRBC have been compared. Neither the delay of the Ju nor the relative advancement of the NZB primary responses were found in the secondary responses of these strains indicating that these effects are due to some function occurring prior to memory cell production. The NZB have also been shown to produce only incomplete tolerance after repeated injections of SRBC. These results support the idea that the delay in the response of young Ju is due to macrophage immaturity, and that NZB are able to bypass this limitation by the possession of a population of immunocytes that are able to respond by sensitization to antigen which in normal animals is non-immunogenic. This explanation would also account for the relative inability of the NZB to become tolerant.
10. THE IMMUNE RESPONSE TO CHICKEN ERYTHROCYTES

10.1 Introduction

NZB mice have been found to show certain abnormalities in their response to SRBC and it has been suggested that these abnormalities are due to a certain population of cells, which are able to respond to antigen in what is usually a non-immunogenic form. This enables them to respond to SRBC which have not been processed by macrophages, although macrophage processing is necessary for the response of other strains of mice to this antigen. It would be most interesting to know whether this abnormality applies only to sheep cells or can be found in the response to other antigens also, since if it applies exclusively to SRBC it would be unlikely to have relevance to the autoimmunity produced by these mice to their own red blood cells.

Therefore the immune responses of NZB and Ju to chicken red blood cells were compared to each other and to the immune responses of both to sheep red blood cells. Chicken red blood cells were chosen because the antigen is presented to the immunological system in the same form as that of the SRBC and so was intended to minimise differences due to different processing requirements, and in addition because the haemolytic plaque assay may be used with the minimum of modification.

10.2 The immune response of the adults

Adult mice were injected with chicken cells and the spleens and lymph nodes assayed for antibody forming
cells at various intervals after injection. Fig. 10.2.1 shows the immune response of the spleens of NZB and Ju adults. The mean number of PFC produced as the peak of the response (425 and 275 per $10^6$ respectively) are not very different from the mean peak number produced to the SRBC (440 and 480 respectively) but the response appears earlier after injection and is maintained for longer. It is possible that in response to this antigen the NZB are exhibiting the same delay in the appearance of the response as they do to the sheep erythrocytes. The difference between the two strains is just significant ($p < 0.05$) at 2 days after injection and just not significant ($p = 0.1 - 0.05$) at 3 days after injection. The number of 'natural' background plaques is high, but while the NZB are clearly showing no increase above background 2 days after injection, the Ju are showing a response which is approaching significance ($p = 0.1 - 0.05$). These results were also calculated as PFC per whole spleen, but, as with the sheep erythrocytes, this produced no additional information and is not included.

Assay of the lymph nodes of these same animals showed no significant response in any of them. This may have been due to the dosage requirements of the lymph nodes for this antigen being higher than the amount of antigen provided, and not necessarily to an inherent incapacity of the lymph nodes of these mice to respond to chicken cells.
102.1. The number of PFC per million spleen cells in adult Ju and NZB mice injected with $5 \times 10^9$ CRBC and tested at various times after injection. The points are geometric means of 6 or more animals and the limits are standard error.
10.3 The development of the immune response in baby animals

Baby NZB and Ju were injected with chicken cells at 3, 5, 7, 9, 11 and 14 days of age and the response observed 4 days after injection in both strains of mice. This time interval was chosen because the adults of both strains of mice were showing a good response at this interval after injection, but it was early enough after injection to reveal any differences analogous to those occurring in the response of baby animals to SRBC.

Fig. 10.3.1 shows the result. The curves appear to be not dissimilar to those obtained with the sheep erythrocytes (Fig. 7.3.1.1) except that the Ju and the NZB are not showing any difference from one another except on day 11. Fig. 10.3.1 also shows the background numbers of PFC found in uninjected adults and 9 or 10 day old baby mice. The values are joined by dotted lines and it may be seen that it is not until the animals were injected at 11 days old that they are showing any significant response to the chicken erythrocytes.

The lymph nodes of some of these mice were also tested, but as with the adults there was no response of either strain.

If both the NZB and the Ju were showing a delay of response similar to that shown by the baby Ju to the SRBC, testing them at 4 days after injection only will give an erroneous idea of their response. Indeed if the Ju response to the sheep cells had only been tested for 4 days after injection they would have shown no response until after 14 days old. The NZB and the Ju were therefore injected at 5, 7 and 14 days of age and tested at various times after
Fig. 10.3.1. The number of PFC per million spleen cells in Ju and NZB mice injected at various ages with $5 \times 10^8$ CRBC and tested 4 days later. The results of 9 and 10 day old babies and adult uninjected mice are shown joined with dotted lines.
injection. Fig. 10.3.2 shows the results for the Ju mice of the various ages compared with the result for the adult. The animals injected at 5 days old show an initial very slight response 2 days after injection, significantly \((p < 0.01)\) higher than both the 9 day and adult uninjected levels and as high as the response of the adults at this very early time after injection, but after this, the response of the mice injected at 5 days old falls off very rapidly, and at no other time up to 7 days after injection showed any difference from the uninjected animals. Animals injected at 7 days old show a similar pattern. Their response is significantly different from the level in 9 day uninjected animals on days 2, 3 and 5 after injection, but is not significantly different from the uninjected adults at any time. When the animals are injected at 14 days old there is a good response.

Fig. 10.3.3 shows the maturation of the response of the NZB mice injected at the same ages as the Ju. Mice injected at either 5 or 7 days of age show no response at all at any of the times tested. The 14 day old animals show a response which follows the adult response as far as 3 days after injection and then rises more slowly and not as far.

10.4 Discussion

It is not certain whether Ju mice below 14 days of age are really showing any response to the CRBC since only one of the points in mice injected at 5 or 7 days old is significantly above background. If this is a real response,
Fig. 10.3.2. The number of PFC per million spleen cells in Ju mice injected at various ages with \(5 \times 10^8\) CRBC, and tested at various times after injection. The points are geometric means of 6 or more animals and the limits are standard error.
**Fig. 10.3.3.** The number of PFC per million spleen cells in NZB mice injected at various ages with $5 \times 10^8$ CRBC and tested at various times after injection. The points are geometric means of 6 or more animals and the limits are standard error.
the immature form of the response is quite different to the immature form of the response of this strain to SRBC. The response would appear to be initiated in the normal way, but the proliferation and/or recruitment fails and the response dies rapidly away. In the NZB too, there is no sign of the rapid early response found to the SRBC, and indeed no response at all until they are over 7 days old. The response of 14 day old NZB also appears to be initiated normally but to fail in its proliferation and recruitment and to fall off before the adult maximum is reached.

It is clear that the maturation of the response of these mice to chicken cells is not at all the same as the maturation of their response to sheep cells, but it is not clear whether the NZB are showing the same form of maturation as the Ju. The 14 day response of the NZB could represent a type intermediate between the 7 day and the 14 day Ju response, but it might not. If there is a difference between the two strains it is not the same kind of difference as that found in the response to the SRBC, and it seems probable that different cell populations are involved.

Since the response to CRBC is very low or absent at an age when these animals are showing a good response to SRBC it suggests that the response to CRBC requires a population of cells that is not required in the response to SRBC. In addition, since in their response to CRBC the 14 day old Ju do not show the long lag period that in the response to SRBC was considered to be indicative of macrophage immaturity, it seems likely that the response to CRBC does not require macrophages. This conclusion is supported by the fact that the adults show a shorter lag.
period in their response to CRBC than in their response to SRBC. Litt (1967) reported that the lag period found in the response of guinea-pigs to this antigen was extremely short. A correlation between short lag period (Baker and Landy, 1967) and macrophage independence (Howard and Siskind, 1969) is also found in the response of mice to pneumococcal polysaccharide, but there is no direct evidence on the requirement for macrophages of the response of mice to chicken erythrocytes. This difference in requirements for the response of two apparently similar antigens is also found with haemocyanin where the response to Haia equinado haemocyanin requires macrophages, but the response to Keyhole Limpet haemocyanin does not (Unanue and Askonas, 1967, 1968a; Unanue, 1969). It is possible that the presence of a nucleus in the chicken cells has some relationship to their lack of requirement for macrophages since deoxyribonucleotides have been shown to have a stimulating effect on the response of baby mice to SRBC (Hechtel, Dishon and Braun, 1965a).

If the lack of response of baby mice to CRBC is not due to macrophage immaturity it must be due to the immaturity of some other component of the immune response, and since there is a reasonable response to SRBC at an age when the response to CRBC is negligible, this component cannot be involved in the response to SRBC. It is possible that even if macrophages were required in the response to CRBC the effects of their immaturity on the immune response would be masked by the immaturity of this other component.

In this section, the response of adult and immature
NZB and Ju to CRBC has been studied. It was found that for both strains the adult and immature responses were quite different from the response to sheep erythrocytes of these animals. In particular the response to the CRBC appeared at a later age and did not show either the increased lag period of the Ju or the reduced lag period of the NZB that were found in the response to the SRBC. It was concluded that in spite of the apparent similarities of the two antigens the CRBC have a requirement for a different population of cells than the SRBC and in addition probably do not require macrophages for their response.
11. THE CLEARANCE OF $^{131}$I-LABELLED SRBC FROM THE BODIES OF BABY MICE.

11.1 Introduction

The question of whether the early and rapid response of the NZB to SRBC is found in the response to any other antigens still remains. The haemolytic plaque assay has many advantages, but it is time consuming and requires a large number of baby mice and it is also difficult to convert to non-erythrocyte antigens. It was hoped to develop a method by which a large number of antigens could be surveyed so that those antigens to which the NZB were showing an early response could be identified and investigated more closely.

The rate of clearance of antigen from the body of animals can be used to detect an immune response since an animal producing antibody eliminates antigen more rapidly than a non-sensitized animal (Weigle, 1960). Therefore the elimination rate of SRBC labelled with $^{131}$I was tested in order to find out whether the differences observed in the immune response of these strains to this antigen would be reflected in differences in the elimination rate.

The elimination rate was tested after a single primary dose since it has been shown in Section 9 that injection even of 1 day old Ju results in sensitization in spite of their lack of immune response. This procedure has the additional advantage that for the first few days after injection the rate of elimination will be dependent upon the rates of phagocytosis and degradation of the antigen and so should provide some indication of the level of macrophage activity.
When baby pigs were injected with M8P-2 phage the rate of clearance from the blood increased markedly after 2 days corresponding to the time of appearance of 19S antibodies (Kim, Bradley and Watson, 1967b) and it was hoped that a similar effect would be seen with the clearance of the SRBC from the body of baby NZB but not from Ju. If this had occurred, and if similar tests with SRBC had proved negative in baby NZB, it would have been reasonable to assume that the clearance rate was reflecting the immune response.

11.2 The clearance of sheep erythrocytes

Sheep erythrocytes were iodinated with $^{131}\text{I}$ and thoroughly washed. They were then injected intraperitoneally into baby mice who were whole body counted for $^{131}\text{I}$ activity between half and one hour after injection and thereafter counted at daily intervals, together with control uninjected littermates. The counts were adjusted to allow for the decay of the $^{131}\text{I}$, and the results for the uninjected littermates subtracted and expressed as a percentage of the initial count. The dose of sheep cells given to the mice was the same as that given in the experiments in Section 7 and could therefore be assumed to be inducing a similar immune response in these mice. The results were graphed logarithmically.

In the first experiment, 5 day old NZB and Ju mice were injected with the labelled sheep erythrocytes. The results are shown in Fig. 11.2.1 in which each curve represents the results for one individual animal. It may be seen that the elimination of antigen is rapid during the
Fig. 11.2.1. The percentage of injected radioactivity remaining in the bodies of NZB and Ju after injection with $5 \times 10^8 \text{I-SRBC}$ at 5 days old. The values have been adjusted to allow for radioactive decay and the values found in un.injected littermates subtracted.
first three days after injection, and then, contrary to expectation, 4 - 5 days later, or when the animals contain about 15 - 20% of the original quantity of radioactivity, the elimination rate slows down. In some cases it is possible to draw two straight lines through the points in the two halves of the curve, and these intersect at about 14½ days. When this graph is compared with the PFC curves for the 5 day old mice (Fig. 7.4.1.4) it may be seen that while this change in rate might correspond with the appearance of significant quantities of circulating antibody in the NZB mice, the Ju are producing hardly any antibody-forming cells at this time. Also, in spite of the clear differences in the immune response of 5 day old Ju and NZB mice, no differences can be observed in the elimination patterns.

Since 5 day old Ju are able to produce an immune response it was thought that the use of younger mice, where the differences in the immune response are more marked, might reveal differences in the clearance rates not seen in the previous experiment. Therefore Ju mice of three days old and NZB mice of 4 days old were injected with 131I-SRBC and the clearance of the radioactive antigen measured by whole body counting as before. Fig. 11.2.2 shows that the elimination rate is more rapid than in the previous experiment, but here too, the rate of elimination slows at about 4 days after injection. NZB show the same rates of antigen elimination as the Ju until this time and thereafter the rate of elimination is slower than that of the Ju. 3 day old Ju mice have virtually no antibody-forming cells at this time (Fig. 7.4.1.4), but 4 day old
Fig. 11.2.2. The percentage of injected radioactivity remaining in the bodies of NZB and Ju mice after injection with $5 \times 10^8$ $^{131}$I-SRBC at 4 and 3 days old respectively. The values have been adjusted to allow for radioactive decay and the values found in uninjected littermates subtracted.
NZB (presumably with a curve intermediate between the 3 day and the 5 day old animals) are producing quite high numbers of antibody forming cells.

The experiment was repeated using 3 day old NZB and Ju. Fig. 11.2.3 shows the results and here again it may be seen that the NZB are showing a slower rate of elimination of antigen than the Ju, but in this case the reduced rate appears to occur from the beginning of the experiment, and the rate of the elimination of both the Ju and the NZB is intermediate between the rates found in the first and second experiments, although the Ju are the same age as those in the second experiment and the NZB are younger than those in both the previous experiments.

Fig. 11.2.4 shows the results for all the experiments together. The mean values for each day after injection of each strain in each experiment have been calculated. It may be seen that variations with the age or the strain of the mice are masked by the much greater variations between individual experiments. The difference between NZB and Ju are fairly consistent within each experiment but it is clear that the major variation is due to differences in the different preparations of iodinated antigens. This suggests that the iodination of the SRBC has changed them in such a way that they have different characteristics for whole body elimination of the iodine. Thus, unless animals are injected with the same preparation the method is not useful for distinguishing differences in the immune response between the two strains of mice.

To investigate whether the change in the rate of
Fig. 11.2.3. The percentage of injected radioactivity remaining in the bodies of NZB and Ju mice after injection with $5 \times 10^8 \text{I}^{131}$-SRBC at 3 days old. The values have been adjusted to allow for radioactive decay and the values found in uninjected littermates subtracted.
Fig. 11.2.4. The percentage of injected radioactivity remaining in the bodies of NZB and Ju mice after injection with $5 \times 10^8 \ 131I$-SRBC. The results for each litter in the previous three experiments have been averaged and are shown here compared with one another.
elimination is due to limitations of the animals' capacity to deal with antigen in different ways the relationship between the initial dose and the amount of antigen remaining after 8 days was studied. If the animal can only eliminate a certain amount rapidly, the larger the dose the smaller the proportion of it that will undergo this rapid elimination, and the larger the proportion remaining after 8 days. Conversely, if the animal can retain a certain amount of antigen or iodide or if it is unable to eliminate antigen rapidly when its concentration falls below a certain value, the larger the dose the larger the proportion that will be eliminated rapidly, and the smaller the proportion remaining after 8 days.

The results are shown in Table 11.2.1. Since the values for the counts are in arbitrary units different for each experiment, the results may only be compared within each experiment. There are not enough results in each section for meaningful statistical analysis but it can nevertheless be seen that there is no consistent relationship between the quantity of antigen injected and the percentage remaining after 8 days although the difference between the different strains and the different antigen preparations can be clearly seen.

11.3 Discussion

The clearance of radioactive iodide from the body of baby mice will depend not only on the rate of phagocytosis and degradation of the antigen, but also on the ability of baby mice to eliminate the iodide from their body. Humphrey
Table 11.2.1. The relationship of the quantity of initially injected antigen (counts) with the percentage remaining after 8 days.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial injection</th>
<th>% remaining at 8d</th>
<th>Initial injection</th>
<th>% remaining at 8d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NZB</td>
<td>Ju</td>
<td>mean</td>
<td>1</td>
</tr>
<tr>
<td>Experiment 1 (5d)</td>
<td>300 327 249 292</td>
<td>11.9 9.6 8.4 10.0</td>
<td>490 246 276</td>
<td>337</td>
</tr>
<tr>
<td>Experiment 2 (4d NZB, 5d Ju)</td>
<td>1245 769 1443 1152</td>
<td>4.8 3.5 3.8 4.0</td>
<td>644 798 298</td>
<td>580</td>
</tr>
<tr>
<td>Experiment 3 (3d)</td>
<td>857 702 768 775</td>
<td>9.0 6.9 7.5 7.8</td>
<td>797 482 435 639</td>
<td>588</td>
</tr>
</tbody>
</table>


(1961) has suggested that in rabbits, renal excretion of iodide is inefficient until the age of 3 weeks. It is possible that this might be an explanation for the lack of the immune type of antigen clearance in NZB in spite of the fact that they are producing an immune response. On the other hand, 90-95% of the injected iodine has been cleared from the bodies of the mice by 8 days after injection which compares quite favourably, for what it is worth, with the extrapolation of the data of Staples and Talal (1969b) for whole body clearance of $^{131}$I-labelled bovine gammaglobulin from non-immune C$_2$H mice, and which does not support the idea of iodide retention. An alternative explanation for the lack of immune clearance is that antibody, by complexing with the antigen, inhibits rather than promotes its clearance from the body. This hypothesis derives slight support from the fact that in experiments with the younger mice, the NZB are eliminating antigen a little more slowly than the Ju. Since the animals are not producing antibody at the start of the experiment it would be expected that the appearance of antibody at about 4-5 days after injection would result in the reduction of the rate of antigen clearance. This reduction is found, but occurs in the Ju equally with the NZB, so unless the Ju are producing antibody of a type I have not detected or in an organ other than spleen or lymph nodes the retention of immune complexes cannot be the explanation for the effect.

The percentage of injected antigen remaining after 8 days is not dependent upon the amount injected, but varies both with the strain and with the antigen preparation. It
seems that the rate of elimination must be influenced by changes produced in the antigen by the iodination or more probably by differences in the strength of iodide attachment. If some of the iodide was only loosely attached it would be rapidly removed and eliminated from the body, accounting for the initial rapid clearance rate. The more firmly bound iodine will only be eliminated after antigen degradation and it will only contribute significantly to the clearance rates after most of the loosely bound iodine has been removed. This would account for the change in the elimination rates observed in both strains of mice at about 4½ days.

Differences in the proportions of firmly and loosely bound iodine in the different preparations would account for differences in their rates of elimination. If the very young Ju were slightly more efficient than the NZB at excreting iodine the differences between the strains would be explained.

It is possible that a method for removing the 'loosely bound' iodine from the SRBC could be developed, but since the NZB were showing no sign of immune elimination towards the ends of the experiments it seemed unlikely that this would prove a useful method for detecting immune response in the baby mice.
12. GENERAL DISCUSSION

It has been shown in this thesis that there is a difference between NZB and two non-autoimmune control strains in the maturation of the immune response to sheep erythrocytes, and that this difference is not found in the maturation of the response to chicken erythrocytes. Without data from many more strains of mice it is not possible to say with certainty whether this difference indicates an abnormality found in NZB alone or whether other non-autoimmune strains of mice also show the NZB type of maturation.

Published data for the response to sheep erythrocytes of baby AKR, AJ(ICR), C57Bl/6 (Hechtel, Dishon and Braun, 1965b) and Balb/c (Playfair, 1968a), suggest that these strains of mice, like the JU and CBA, but unlike the NZB show a delay in the appearance of the response when compared to the adults of the same strain. In addition, the ability of the lymph nodes of the NZB to respond at an age so much younger than that of the other strains and the fact that the NZB did not become fully tolerant to SRBC suggest that there is an underlying immunological abnormality in the NZB. This abnormality is not found in the response to all antigens and it may only appear in those that require the particular function or the particular cell population that is abnormal.

Even if the NZB are showing a true difference in the maturation of their response to SRBC, it has yet to be shown that this has any connection with the later appearance of erythrocyte autoantibody in this strain. The principle of
Occam's razor argues that it is unlikely that the NZB would be showing two unrelated immunological abnormalities. I have suggested that the abnormalities in the response to sheep erythrocytes are due to a population of cells that can respond to antigen, in a form which in normal mice, is non-immunogenic. Thus the requirement for macrophages in baby mice in which the processing function of the macrophages has not yet developed (Argyris, 1966b, 1969b; Braun and Lasky, 1967) may be bypassed. Because the population of cells become sensitive rather than tolerant it will be difficult or impossible to produce tolerance to antigens which require this population for their response. Tolerance induction to the sheep erythrocytes is inhibited but not totally prevented. This could represent normal tolerance to some and absence of tolerance to other sites on the antigenically complex surface of the red blood cell. More probably it represents a relative rather than absolute tendency to become sensitized so that under a particular regime of antigen administration the number of cells being made tolerant is much lower than in other strains of mice. Other antigens also show various degrees of deficiency in their ability to induce tolerance in NZB mice (Weir, McBride and Naysmith, 1968; Braverman, 1968a; Staples and Talal, 1968, 1969a,b).

Playfair (1966b) has found this early hyperreactivity to be a characteristic of the foetal liver rather than the thymus cells suggesting that the cell responding to non-immunogenic antigen is an AFC-P. On the other hand, neonatal thymectomy has the same effect on the immune response
of NZB as of other strains (Playfair, 1968a) showing that the AFC-P are not ARC independent. It cannot therefore be said for certain whether the abnormal population of cells is an ARC able to respond to a non-immunogenic antigen or an AFC-P able to respond to a complex of 'tolerant' ARC and non-immunogenic antigen. To examine the question of cell populations further it would be interesting to discover whether these abnormalities are found also in the 78 response to SRBC and whether the restoration of the response to SRBC in irradiated NZB requires the same population of cells as it does in other strains of mice.

The increased tendency of a population of cells to respond to antigen in a form which is normally non-immunogenic could be an explanation of the later development of autoimmunity, provided that the same population of cells is involved in the production of antibody to both sheep and mouse erythrocytes. It is probable that self-recognition and tolerance both occur by a mechanism in which a cell meeting an antigen which it can recognise but to which it is unable to respond is inactivated for subsequent meetings with the same antigen. Autologous antigens are not normally taken up by macrophages. The reasons for this are as yet obscure. It may be due to the lack of opsonizing materials for autologous components (see Stuart, 1970, p.191) or it may be due to a self-recognition system in the macrophages themselves. Whatever the reason is, continual exposure of the antibody forming system to autologous antigen in an unprocessed non-immunogenic form will ensure that tolerance is maintained. The lack of autoimmunity in young
NZB shows that self tolerance can be established, but it is possible that because of the tendency of the cells to become sensitized it is only precariously maintained and minor changes in the antigen may result in the breaking of the tolerance.

So far it has been assumed that the abnormality is in the tendency of a group of cells to respond to normal antigen in an abnormal way. Equally it could be in the response of a normal group of cells to an antigen which has been rendered immunogenic instead of tolerogenic by some factor, but in order for the relationship between early hypersensitivity and autoimmunity to be retained, this factor would have to act on sheep erythrocytes as well as on mouse erythrocytes. This might suggest a role for the virus that has been discussed in relation to the NZB. The NZB appear to be tolerant to this virus for several months after birth (Mellors, Aoki and Heubner, 1969) and in addition the NZB are not showing a secondary form of response when first injected with sheep erythrocytes as babies and the numbers of PFC to SRBC in un.injected mice are no different in NZB and normal strains so any hypothesis requiring viral antigens cross reacting with sites on both mouse and sheep erythrocytes is ruled out. It is known however that red blood cells can have virus receptor sites on the surface (see Burnet, 1960, pp. 66 and 113) and it does not seem improbable that the virus would be able to combine with both mouse and sheep erythrocytes and convert the antigen with which it was combined into a highly immunogenic form, perhaps producing something similar to the immunogenic
RNA-antigen complexes found after processing by macrophages (Bishop, Pisciotto and Abramoff, 1967; Askonas and Rhodes, 1965; Herskowitz and Stelos, 1968; Cohen, Newcomb and Crosby, 1965; Fishman, 1969). It has been shown that virus can act as an adjuvant and render an antigen highly immunogenic (Mergenhagen, Notkins and Dougherty, 1967).

The results of this would be very similar to the results of the hypothetical hypersensitive cell. Sheep cells injected in the neonatal period would complex with the virus and this complex would substitute for the macrophage-processed antigen, resulting in a response before the animal's own processing system was functional. The exposure to the immunogenic mouse erythrocytes from before birth would result in precariously established tolerance and an increasing tendency for the animals to respond by sensitization to the continual exposure to highly immunogenic antigen.

This hypothesis does not explain the difficulty of the induction of tolerance to other non-erythrocyte antigens in the NZB. The injection of virus from NZB tissues into other strains does not generally result in the transfer of autoimmune disease, and in addition the injection of baby Ju with sheep erythrocytes together with extracts from old NZB spleen in no way enhances their response to the sheep erythrocytes so it seems unlikely that the virus is the sole agent responsible for either the autoimmune disease or the hyperreactivity in the baby animals, but this does not mean that it is not a contributing factor.

On the whole it seems most likely that the basic defect in these mice is an abnormally sensitive population
of cells. This population of cells is involved in the response to sheep erythrocytes, mouse erythrocytes and probably many other antigens that require macrophage processing for their response. The sensitivity of these cells to antigen which has not been processed by macrophages results in the response of baby mice to antigen at an age before the macrophages have become functional and in a shortening of the lag period between antigen exposure and antibody formation indicating a reduced processing time. As the animals grow older the maturing macrophages take over the normal pathways and the response reverts towards normal. The profound tolerance that is normally present towards autologous components can be maintained provided no other factors enter into the response. In NZB however there is also a murine leukaemia like virus and this, perhaps by complexing with the erythrocytes presents the already hypersensitive population of cells with antigen in a highly immunogenic form. The result is that the next cell that can recognise the antigen is sensitized rather than made tolerant and autoimmunity ensues. Neither the cell nor the virus alone are normally sufficient to break self-tolerance, but when transferred together the recipient will eventually become autoimmune.

This model does, I think, explain most of the facts without an undue straining of probabilities, but it is based on far too many assumptions about the immune response, the nature of self recognition and the relationship between the immunological abnormalities of the NZB to allow it more
than provisional status. The model predicts that the response to a number of antigens that require macrophages in 'normal' strains of mice will prove to be non-macrophage requiring in the NZB. It also predicts that experimental autoimmunity to some but not to all autologous components will be readily induced with the aid of adjuvants. Both these predictions may be easily tested and it will be of great interest to see in the future whether the results will support the model I have suggested.
13. ACKNOWLEDGEMENTS

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