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The Ontogeny of Antigen-Binding Cells

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THE ONTOGENY OF ANTIGEN-BINDING CELLS

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ABSTRACT

In order to analyze the development of antigen-specific cells, the binding of a variety of antigens by cells in the fetal, neonatal, and adult mouse was compared.

The fiber-binding assay was used in many of these experiments, because it provides a simple and uniform method for studying the specific interactions of cells with any of a wide variety of antigens. To demonstrate the specificity of the assay, cells from the spleens of immune and nonimmune adult mice were isolated and characterized. Specifically, after removal from the fibers, these cells were assayed for their viability, their ability to rebind to fibers of the same specificity, and their in vivo response to antigen after transfer to irradiated syngeneic recipients. These experiments indicated that the fiber method yields highly enriched populations of specific antigen-binding cells that are viable and include antigen-sensitive bone marrow-derived cells capable of undergoing differentiation into antibody secreting cells.

This assay was then used to characterize cells specific for each of eleven different hapten and protein antigens. In all cases, specific antigen-binding cells were first detected in the liver, on the 15th day of the 19-day gestation period. These cells disappeared from the liver within a day of birth, but continued to increase in number in the spleen until adulthood. The proportions of antigen-binding cells of different specificities were similar in fetal, neonatal, and adult tissues. The antigen-binding cell populations from fetal livers and spleens were similar to each other and to adult spleen cell populations in the distributions of their relative avidities for several antigens. These results indicate that antigen-binding cells of various specificities arise relatively rapidly and in parallel during development, and therefore that strong positive antigenic selection is not likely to operate during ontogeny. This has several implications for theories on the origin of antibody diversity, and in particular suggests that positive selection may not be required for somatic diversification to occur. These results also suggest that the sharply restricted ability of the neonatal animal to respond to antigenic stimulation is not due to the lack of antigen-

specific cells, but rather to the absence of mature cells capable of the interactions necessary for a full immune response.

While measurements of the numbers of antigen-binding cells in the spleens of individual outbred fetal mice failed to detect subpopulations of individuals differing systematically from the fetal population as a whole, significantly more variation among individuals was found than would be expected if the actual number of cells binding a specific antigen were constant, or nearly so, among fetuses. To determine the source of this variation more precisely, the numbers of cells specific for each of two antigens in the spleens of individual outbred (Swiss-L) and inbred (Balb/c and CBA/J) fetal mice were measured as a function of spleen size. For outbred Swiss-L fetuses, the ratio of antigen-binding cells to nucleated cells varied significantly more than could be accounted for by sampling fluctuation. For each inbred strain, however, the number of cells specific for a given antigen was a constant proportion of the number of nucleated cells, within sampling error. These proportions varied from antigen to antigen, and from strain to strain. The ratio of the proportions of cells specific for the two antigens, however, differed no more from CBA/J to Balb/c mice than would be expected in repeated samples of cells from the spleen of a single fetus.

These results confirm at the level of the individual fetus the uniform pattern of development seen for populations of fetuses. They reveal a surprising precision in the proliferation of specific antigen-binding cell populations and suggest that the development of these cell populations may be subject to strong genetic controls.

TABLE OF CONTENTS

INTRODUCTION

When an animal is exposed to a foreign substance, or antigen, it responds by producing antibodies. These antibodies are protein molecules that bind specifically to the antigen, facilitating its neutralization and removal from the body. This immune response has four particularly striking features. First, an enormous range of foreign substances can cause a response. Second, these responses are highly specific, and can distinguish between closely related structures. Third, the immune system exhibits a specific "memory"--that is, later exposure to the same antigen causes a greatly enhanced response. Finally, the system can distinguish structures present in the animal itself from foreign ones, and normally responds only against the latter.

These physiological properties of the immune system have been recognized for many years, but a description of the molecular and cellular processes responsible for them has become possible only within the last fifteen years. This description has resulted form the systematic analysis of antibody structure, and the elaboration of the clonal selection theory. It is now well established that the range of antigen-binding specificities expressed by an animal corresponds to a range of antibody structures (Edelman and Gall, 1969). The overall structure of an antibody molecule is shown schematically in Figure 1. Antibody molecules are multichain structures, containing two identical heavy polypeptide chains and two identical light polypeptide chains, held together by noncovalent forces and disulfide bonds. Each chain contains two distinct regions: a variable region, whose sequence varies from one species of antibody to the next, and a constant region, whose sequence is largely conserved (Cunningham et al., 1969; Hilschmann and Craig, 1965). The variable region consists of the amino-terminal 110-120 amino acid residues of each chain. The constant region, which comprises the remainder of each chain, determines the class of the antibody. The antigen-binding site itself is formed by the interaction of the variable regions of the heavy and light polypeptide chains, (Poljak, 1975), and its antigen-binding specificity is determined by the amino acid sequences of these variable regions. On the basis of structural and genetic evidence, it appears likely that the variable and constant regions of each polypeptide chain are coded for by separate

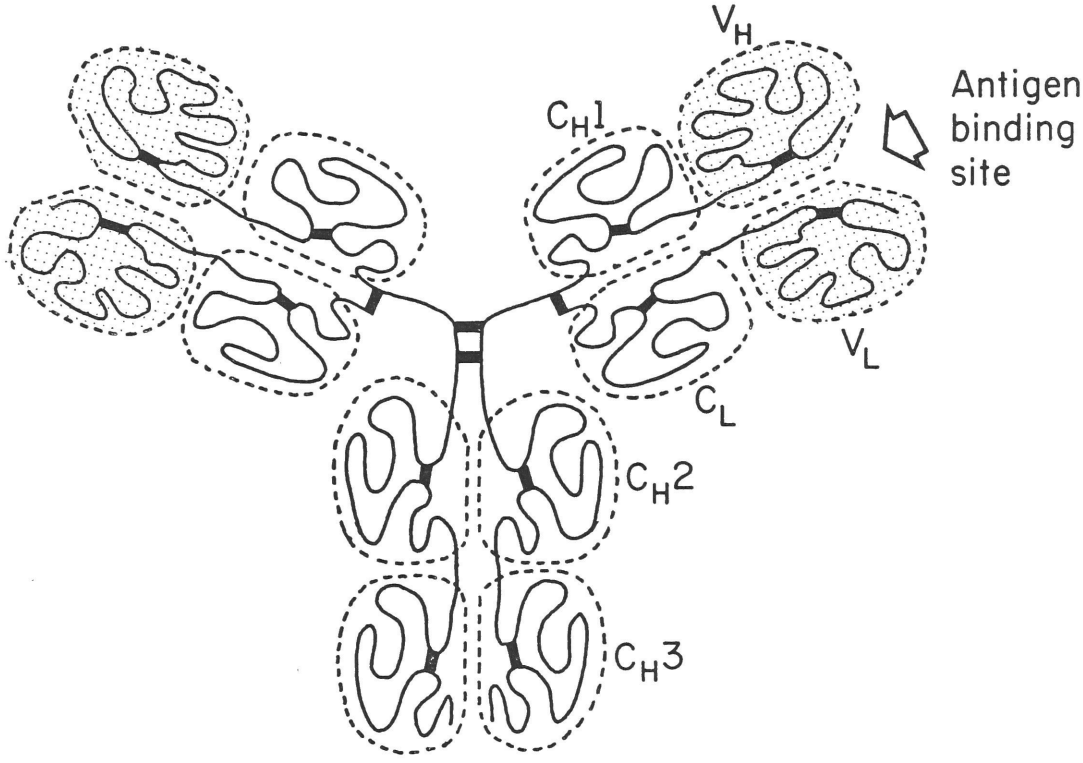

Figure 1. Diagram of the structure of a human antibody molecule of the IgG class (Gally and Edelman, 1972). V_H and V_L are the variable regions of the heavy and light chains, respectively, and are involved in antigen binding. C_L is the constant region of the light chain. C_H1, C_H2, and C_H3 are homology regions in the constant portion of the heavy chain. The interchain and intrachain disulfide bonds are indicated by the bars.

genes which are located in different parts of the genome. Gally and Edelman (1972) have postulated that in the course of development, variable-region genes are translocated and linked to constant-region genes to form genes coding for complete immunoglobulin (Ig) chains.

The expression of these molecules by the cells of the immune system is now understood in terms of the clonal selection theory. The central point of the clonal selection theory is that the full repertoire of these antibody structures exists in the normal animal before its exposure to foreign antigens, and that the exposure of an animal to a particular antigen functions only to stimulate selectively the production of more copies of those antibodies capable of binding it. Such a theory was first proposed by Jerne (1955), who suggested that antibodies of all specificities were generated continuously in the animal by a random genetic mechanism and released into the blood. An antigen introduced into the animal would then be bound by those antibodies specific for it, and these antigen-antibody complexes transported to the appropriate cells, stimulating them to produce more of the same antibody. It was suggested that a separate mechanism was required to suppress antibodies specific for self-antigens. Although Jerne recognized the selective nature of the immune response, a number of difficulties arose with respect to the specific postulates of his theory. For example, a fundamental aspect of the immune response _in vivo_ is that the amount of new antibody synthesis triggered by exposure to an antigen is in general inversely proportional to the amount of antibody against that antigen already present in the blood (Uhr and Möller, 1968).

A modification of Jerne's proposal by Burnet (1959) gave rise to the form of the clonal selection theory which is accepted at present (Figure 2). Burnet postulated that in the course of development each cell in the immune system becomes committed to the production of antibody of a single specificity, and that the initial recognition of an antigen takes place through direct binding to the cell by means of copies of this antibody on the cell surface. This event would lead to mitosis, giving rise to a clone of cells capable of synthesizing more antibody molecules of the identical specificity. By clonal expansion, Burnet's theory provides a

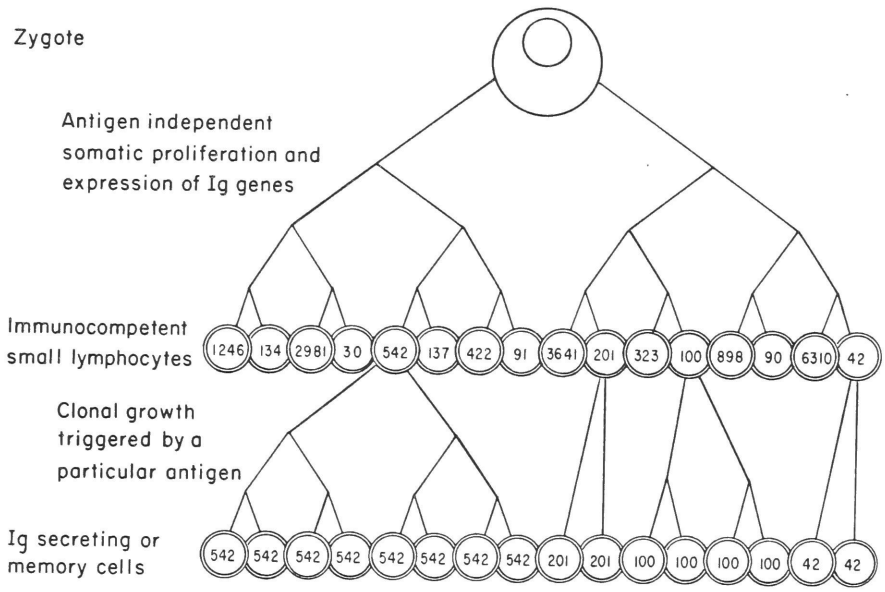

Figure 2. A model of the somatic differentiation of antibody-producing cells according to the clonal selection theory. The number of immunoglobulin (Ig) genes may increase during somatic growth to give rise to a repertoire of immunocompetent small lymphocytes, each committed to the synthesis of a structurally distinct antibody (indicated by the arabic numerals). A small proportion of these cells proliferate upon antigen stimulation to form clones of cells, each clone producing a different antibody.

basis for immunological memory and explains by a process of repeated selection, the gradual increase in avidity of specific antibody which follows immunization. Burnet suggested that the variety of antibody molecules expressed by the cells of the immune system would be generated by somatic mutation. The failure of an animal to produce antibodies against self-antigens was attributed to a specific deletion or killing of any cells that became committed to recognizing such antigens.

The clonal selection theory in this form specifies three experimentally verifiable features of the immune system. First, the full repertoire of specific antigen-binding cells should exist in the animal prior to exposure to antigen. Second, any one cell should express only one antigen-binding specificity. Third, stimulation of a cell by antigen should lead to the secretion of specific soluble antibody and to the generation of a clone of progeny cells with the same antigen-binding specificity.

These specifications have been corroborated by a wide variety of experimental evidence. Antigen-binding cells have been detected in the non-immune adult by several techniques. These have included the incubation of cell suspensions with radioactive antigen followed by autoradiography (Byrt and Ada, 1969), and the incubation of cell suspensions with antigen-coated fibers (Rutishauser et al., 1972) or red blood cells (McConnell et al., 1969) followed by direct quantitation of bound cells. In all cases, following immunization with antigen, the frequency of cells capable of binding specifically to the antigen in these assays is increased sharply, consistent with the clonal proliferation of pre-existing antigen-specific lymphocytes. Further, if the antigen-binding cells are killed or physically removed from the cell population, the ability of the remaining cells to respond to that antigen is sharply reduced, although the responsiveness of the population to unrelated antigens is unchanged (Basten et al., 1971; Wigzell and Andersson, 1969). Conversely, the subpopulation of purified antigen-binding cells has an enhanced ability to respond to stimulation with that antigen (Haas, 1975; Julius and Herzenberg, 1974), consistent with the commitment of each cell in the repertoire to a single antigen-binding specificity.

One point not explicitly anticipated in the clonal selection theory, however, is that there are in fact two functionally distinct classes of antigen-specific lymphocytes, B cells and T cells (Figure 3). The B lymphocytes correspond to the antigen-specific cells of the theory: exposure to antigen stimulates these cells to divide and produce antibody. The T lymphocytes do not secrete antibodies in response to foreign antigen, but instead exhibit a "helper" activity necessary for the full stimulation of B cells. Despite the added complexity of T cell-B cell interaction, the key event in the specific humoral immune response is the initial recognition of an antigen by those cells specific for it. Later cell-cell interactions may alter the course of the immune response, but its specificity is determined by this initial interaction.

While a wide variety of evidence has been accumulated in support of this description of the adult immune system, the ontogeny of the system remains less well understood. Although immunoglobulin-bearing cells and antigen-binding cells have been shown to appear simultaneously in the fetal mouse, shortly before birth, responsiveness to antigenic stimulation only appears after birth (Spear et al., 1973). Furthermore, responsiveness to various antigens arises in a well-defined sequence, spread over a period of days or weeks (Sherwin and Rowlands, 1974; Press and Klinman, 1974). These studies of immune responsiveness, however, cannot distinguish between the development of the repertoire of specific antigen-binding cells and, for example, the development of factors necessary for T cell-B cell interaction. It is therefore important to examine directly the development of the antigen-binding cells in the fetus, for each of a wide range of antigens. Experimental difficulties and the lack of suitable methods have prevented this approach from being widely used. To carry out such an analysis, a uniform source of fetal tissues of precisely known age is needed, as well as quantitative techniques for measuring cells specific for each of a wide variety of antigens.

The experiments in this thesis were designed to measure directly the development of antigen-binding cells in fetal mice. Mice were used because they have a relatively short gestation period (19-20 days), and

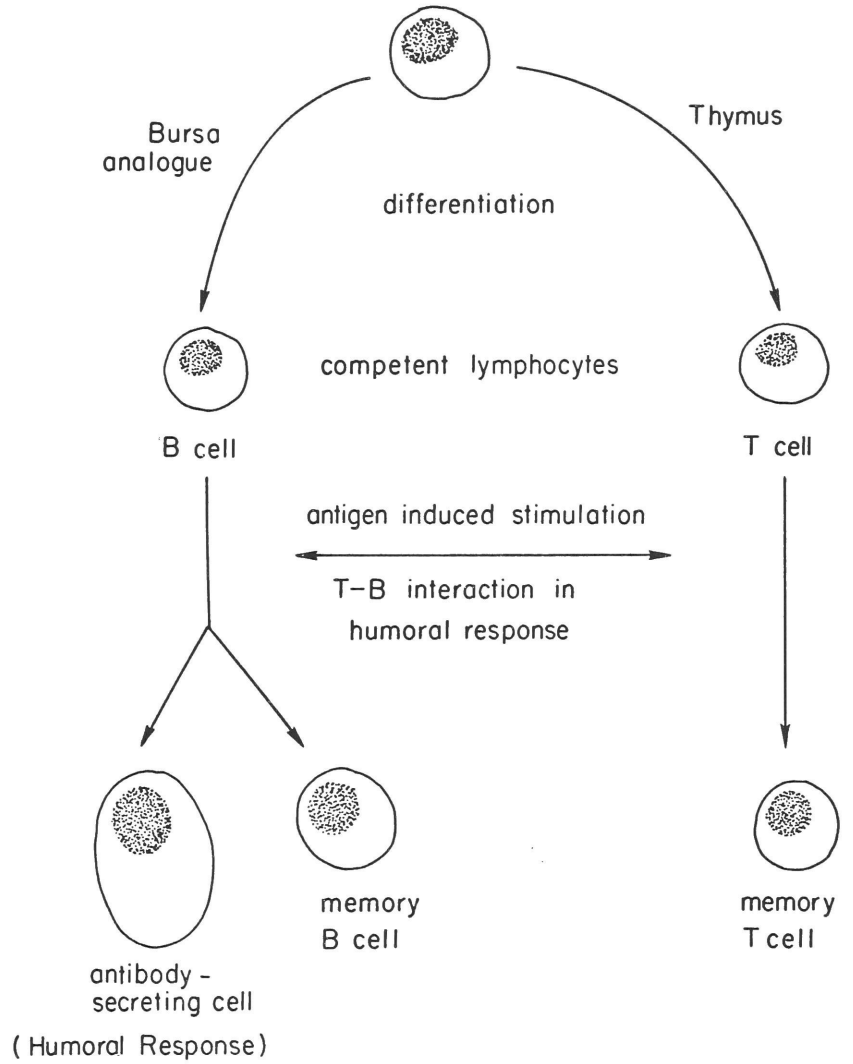

Figure 3. The humoral immune system. Antibody production by B cells results from the specific interaction of a B cell with antigen and with antigen-stimulated T cells. Both antibody-secreting cells and clones of B and T memory cells arise in the course of an immune response.

a variety of highly inbred, genetically homogeneous strains is available. These experiments were focused on four particular questions.

1) How can specific antigen-binding cells be quantitated?

2) When and where in the course of an animal's development do specific antigen-binding cells appear?

3) How do the antigen-binding properties of cells from developing animals compare with those of cells from adults?

4) To what extent does the development of the repertoire of antigen-binding cells vary from individual to individual?

The results of these experiments provide a basis for at least a preliminary description of the development of the repertoire of antigen-binding cells. Together with data from other systems, these results allow the definition of boundary conditions within which the underlying molecular processes responsible for the generation of antibody diversity and the commitment of developing cells to the production of single species of immunoglobulin must operate.

ONTOGENY OF THE HUMORAL IMMUNE SYSTEM

T and B Lymphocytes

In the course of its development, an animal acquires a large number of lymphoid cells, each committed to make a single type of antibody molecule. In a distinct but closely related maturational process, these cells acquire the ability to divide and to produce antibody in response to stimulation with foreign antigen. In order to provide a basis for a detailed consideration of these two aspects of the ontogeny of the immune system, it is necessary first to describe more precisely the lymphoid cell types involved, and the means by which they can be characterized. T and B lymphocytes are virtually indistinguishable by conventional morphological means, but can be identified on the basis of their ontogeny, their cell surface components, and their responses to mitogenic agents (Table I)

Development. Although T and B lymphocytes appear to be derived from a common stem cell, they mature separately in the body. The overall pattern of T and B cell maturation will be discussed in detail below. Here it is relevant simply to note that these distinct maturation pathways provide a means by which the two cell types can be distinguished. T cells mature in the thymus (Figure 3), and if this organ is removed from a mouse at birth, the animal fails to produce T cells (Miller, 1961). Similarly, mice homozygous for the "nude" mutation, which prevents the development of a thymus, lack functional T cells (Pantelouris and Flisch, 1972). In either case, if a normal thymus is grafted into such an animal, the graft becomes populated with host cells that develop into mature T lymphocytes (Metcalf and Wakonig-Vaartaja, 1964; Loor and Kindred, 1973; Pritchard and Micklem, 1973).

The site, or sites, at which B lymphocytes mature have not been unequivocally identified for any mammal. In birds, the bursa of Fabricius, a lymphoid sac which opens into the large intestine, has been shown to control B cell maturation: in its absence B cells do not develop (Glick, 1956) but if bursal tissue is grafted into a bursectomized animal, B cell production is restored (Moore and Owen, 1966).

Table I

T and B Lymphocytes

Property	T cell	B cell
Role in humoral immune response	helper cells	antibody production
Origin	thymus	bursa of Fabricius or equivalent
Cell surface markers		
Ig	-	+
Fc receptor	-	+
complement receptor	-	+
θ antigen	+	-
Ly	+	-
TL	+ (immature only)	-
Mitogen responsiveness		
PHA	+	-
Con A	+	-
lentil agglutinin	+	-
sodium borohydride	+	-
LPS	-	+
PPD	-	+

No bursal analog has been identified in mammals, although some evidence suggests that, at least in the mouse, B cells mature in the fetal liver and spleen (Owen et al., 1975) and in the adult bone marrow (Osmond and Nossal, 1974a,b).

Cell surface markers. Many of the components of the lymphocyte surface are themselves antigenic and therefore it has been possible to use antibodies against them to compare the surfaces of T and B cells. This usually involves detection of specific binding of these antibodies to a cell by immunofluorescence or by lysis in the presence of complement. On the basis of such experiments with various types of lymphocytes, it has been possible to define a large number of lymphocyte surface antigens, some of which can be used to distinguish T cells from B cells.

B lymphocytes are most readily identified by the large amount of Ig on their surfaces (Raff, 1970; Rabellino et al., 1971). Although Ig may in fact be present on both T and B lymphocytes (Marchalonis and Cone, 1973), it is readily detected only on B cells, and is therefore a useful marker for this cell type. In addition, surface receptors capable of binding aggregated Ig (the so-called Fc receptors--Basten et al., 1972), and complement (Bianco et al., 1970) have been demonstrated on B cells.

The most prominent marker for T lymphocytes is the Θ antigen, first described by Reif and Allen (1964) as a surface component of thymocytes, and subsequently shown by Raff (1970) to be a characteristic marker of mature T cells. Two other groups of antigens found on T cells are the TL and Ly markers (Boyse et al., 1968, 1971; Boyse and Old, 1969). Each group is thought to consist of several distinct antigens (Ly 1, 2, and 3; TL 1, 2, 3, and 4), each coded for by a separate gene. The number and specificity of these markers expressed by a given cell at any particular time appears to depend on its degree of maturation and on its functional specialization (reviewed by Medawar and Simpson, 1975).

Response to mitogens. An independent approach to the identification of T and B lymphocytes and to studies on lymphocyte stimulation, has been provided by the use of exogenous mitogenic agents. Since the original demonstration that lymphocytes treated in culture with the

phytohemagglutinin isolated from Phaseolus vulgaris (PHA) undergo striking morphological changes ("blast transformation"), synthesize DNA, and divide (Nowell, 1960), the mitogenic properties of a wide variety of substances have been studied. Most of these substances appear to stimulate T cells or B cells preferentially. For example, the jack bean protein Concanavalin A (Con A) induces blast transformation and DNA synthesis in thymocytes, and lymphocytes from normal adult spleen, but fails to stimulate spleen lymphocytes from "nude" mice, or normal spleen lymphocytes pre-treated with anti-θ serum plus complement (Greaves and Janossy, 1972). Similar T cell specificity has been demonstrated for PHA and lentil phytohemagglutinin (Greaves and Janossy, 1972). Brief treatment with sodium borohydride is also mitogenic for T cells (McClain et al., 1975). Conversely, bacterial lipopolysaccharide (Sjöberg et al., 1972), aggregated tuberculin ("purified protein derivative" - PPD) (Nilsson et al., 1973), and a variety of polyanions such as dextran sulfate (Diamantstein et al., 1973) stimulate B cells.

The Ontogeny of Lymphoid Cells

Direct analysis of the generation of specific antigen-binding cells requires characterization of the lymphoid cell precursor at the stage in development at which diversification and commitment are taking place. Lymphocytes appear to be derived ultimately from pluripotent hemopoietic stem cells. In the mouse, these stem cells can first be detected in the yolk sac, on the 7th - 8th day of gestation, and they migrate successively to the liver, the spleen, and, shortly after birth, to the bone marrow, where they remain throughout the animal's adult life (Metcalf and Moore, 1971; page 200). These stem cells appear to give rise to several classes of oligopotent "progenitor" cells each of which is restricted to one or a few related lines of development, and which differentiate further into fully committed, functional end cells in the proper environment.

Thus, if an animal whose own hemopoietic tissue has been destroyed by exposure to radiation is injected with bone marrow, hemopoietic foci appear in its spleen, each of which can be shown to be derived from a single donor hemopoietic stem cell (Wu et al., 1967). If cells from one

of these clones are injected into a second irradiated mouse, its entire hemopoietic tissue can be restored, including a full complement of functional lymphocytes (Wu et al., 1968).

These stem cells and progenitor cells can only be detected by indirect assays of their ability to give rise to hemopoietic foci. The cells themselves have not been isolated and characterized, and as a result, neither the exact number of different progenitor cell types is known, nor is the exact pathway known by which any of them arise from the stem cell.

Direct identification of the lymphocyte progenitor is first possible at the time of its migration to the central lymphoid organ where it will mature into a functional T or B lymphocyte.

The maturation of T cells. T cells appear to be derived from large basophilic cells which first appear in the thymus of the fetal mouse on the 11th day of gestation (Moore and Owen, 1967). There is substantial evidence that these cells migrate into the thymus from yolk sac and fetal liver in embryonic life and from bone marrow in adult life. Thus, thymic tissue taken from a fetal mouse, placed in a chamber sealed with a Millipore filter, and implanted on the chorioallantoic membrane of a chick embryo will survive for extended periods of time, but will develop lymphocytes only if the basophilic cells are present in the thymus at the time of its removal from the mouse (Owen and Ritter, 1969). Similarly, if an adult mouse is irradiated, thereby destroying its own lymphocytes, then injected with hemopoietic stem cells from a healthy mouse of the same strain and grafted with the thymus of a fetal mouse of a second strain, the thymus graft will gradually be repopulated by lymphocytes of the host type (Owen and Raff, 1970).

These T cell precursors possess no detectable immunological function, and their further differentiation in the thymus has been described largely in terms of the antigenic markers expressed on their surfaces. The basophilic stem cell, which expresses no known lymphocyte-specific surface markers, gives rise to cells with the morphology of lymphoblasts that express the TL and Ly antigens and a large amount of θ antigen. As these cells mature further, the amount of θ antigen decreases, and

by the time they leave the thymus, the TL antigen has disappeared entirely (Owen, 1972).

In the mouse, T cells first appear outside the thymus at about the time of birth. They have been detected in the spleen as early as three days before birth (Spear et al., 1973), and in the lymph nodes, Peyer's patches and blood shortly after birth (Raff and Owen, 1971; Chanana et al., 1973; Stobo and Paul, 1972). Although the exact time at which these cells appear may vary from strain to strain, in all cases they rapidly increase in frequency, and reach adult levels within three weeks of birth.

At the time of its departure from the thymus, the T cell is still immunologically immature. By correlating changes in the surface markers and mitogen responsiveness of T cells in the young animal with the onset of immune function, it has been possible to define distinct classes of "helper" and "suppressor" T cells. The T cells found in the spleen of the neonatal mouse express all three Ly markers (Ly-1,2,3+) (Cantor and Boyse, 1975). Cells from a neonatal spleen cannot respond to stimulation by antigen and this now appears to be due at least in part to a suppressive effect mediated by these T cells, inasmuch as neonatal T cells added to a culture of adult spleen cells with antigen, prevent the appearance of antibody-forming cells. B cells from neonatal spleens do no have this inhibitory effect on adult cells in such a culture, although they themselves appear not to respond to antigen (Mosier and Johnson, 1975).

In the three weeks following birth, the proportion of Ly-1,2,3+ T cells gradually falls, and cells having only the Ly-1 antigen (Ly-1+) or the Ly 2 and 3 antigens (Ly-2,3+) appear, in parallel with the onset of full immune responsiveness, suggesting strongly that one or both of these cell types are "helper" T cells. By killing one of these subpopulations with the appropriate anti-Ly antisera plus complement, and testing the surviving cells for immune function, it has been possible to analyze the function of these cell populations directly. If adult spleen cells are treated with antiserum against Ly-1, plus complement, the surviving cells are no longer responsive to antigenic stimulation,

suggesting that "helper" T cells are of the Ly-1 type. On the other hand, the cells surviving treatment with anti-Ly-2 show a normal or even enhanced response to the antigen, consistent with the removal of Ly-2,3+ suppressor cells (Hirst et al., 1975; Feldmann et al., 1975; Medawar and Simpson, 1975). It is important to note that, even if the identification of Ly-1+ cells as antigen-specific helper cells and Ly-1,2,3+ and Ly-2,3+ cells as antigen-specific "suppressor" T cells is shown to be generally valid, the means by which the interactions of antigen with these cell types determines at the cellular and molecular level whether the response to that antigen is to be enhanced or suppressed remains entirely unclear. In particular, it remains unclear how these various cell types interact in the course of an animal's development to modulate its immune response, and how these interactions are affected by the rapid changes in the relative numbers of these cell types known to occur during development.

An independent approach to the problem of distinguishing functional subclasses of T cells is provided by the differential responses of these cell types to the mitogenic agents Con A and PHA. Neonatal mice have few or no cells responsive to these two mitogens. The number of cells responsive to Con A increases sharply with age, however, reaching a plateau approximately three weeks after birth. On the other hand, PHA-responsive cells could not be detected in the spleen until 1-3 weeks after birth (Stobo and Paul, 1972; Spear and Edelman, 1974; Mosier, 1974). At least in Swiss-L mice, the latter cells were found to respond also to Con A, but to a dose 3 times lower than that required for the bulk of the Con A-responsive cells. Further, these cells responsive to both PHA and Con A appear in the spleen at about the time that mice acquire the ability to produce humoral antibodies (Spear and Edelman, 1974). These cells, then, would appear to be T helper cells, and consistent with this hypothesis, it has recently been shown that those adult spleen cells which survive treatment with antiserum against Ly-1 are not responsive to Con A. Treatment with antiserum against Ly-2 has no effect on Con A response (Hirst et al., 1975).

It must be emphasized that at this point it is by no means clear that all the functional subclasses of T cells have been identified, nor

is it possible to assign each T cell to a unique subclass on the basis of those properties which can be measured at present. At the same time, it clearly is possible to identify those T cells involved in the humoral immune response and to distinguish "helper" and "suppressor" cells within this population, on the basis of their developmental histories, their antigenic markers, and their responses to mitogens.

The maturation of B cells. Much less is known about the markers and developmental history of B lymphocytes than is known about T lymphocytes. Nevertheless, using the same overall approach, it has been possible to distinguish several subclasses of B lymphocytes. Although mature B lymphocytes appear to be functionally a uniform group of cells, several distinctive classes of immature B cells have been defined.

A difficult problem in the study of B lymphocyte maturation in mammals has been the identification of the site, or sites, at which the maturation occurs. The gut-associated lymphoid tissues (including the appendix (Archer et al., 1963), the tonsils (Peterson et al., 1965) and the Peyer's patches (Perey et al., 1968)), and hemopoietic tissues (Nossal and Pike, 1973) have generally been considered the main candidates for this role. No single organ equivalent to the avian bursa of Fabricius has been identified, whose removal uniformly prevents further B cell maturation.

Experiments carried by several groups within the past few years, however, indicate strongly that the fetal liver and spleen, and the adult marrow, are the sites of B cell maturation. Cells synthesizing Ig can be detected in the fetal liver as early as the 12th day of gestation by intrinsic labelling and immunoprecipiation techniques (Melchers et al., 1975; Raff et al., 1976). Furthermore, if explants of 12-day fetal liver are cultured for up to seven days, lymphoid cells bearing Ig on their surfaces gradually appear. A large proportion of these cells also possess surface Fc receptors. Similar results are obtained with cultures of 14-day spleen explants. On the other hand, thymus explants fail to develop Ig bearing lymphocytes, and explants of 10- or 12-day yolk sac fail to develop any lymphoid cells at all, although other hemopoietic cells are present (Owen et al., 1974, 1975). Because

hemopoiesis (including lymphopoiesis) is confined to the yolk sac, the liver, the spleen and the thymus, at this stage of development, (Metcalf and Moore, 1971; page 10) all these observations are most simply explained by the hypothesis that the development of progenitor cells into B cells occurs in the fetal liver and spleen. This process presumably continues in the bone marrow in the adult (Raff et al., 1975).

Unlike T cells, therefore, the key steps in the generation of B cells appear not to be confined to a single organ, but proceed in the liver and spleen of the fetus, and in the bone marrow of the adult. In all cases, the immediate precursor cell appears to be a large basophilic cell morphologically indistinguishable from the T cell precursor described above. This cell gives rise to one with the appearance of a lymphoblast which, in the case of the fetal liver, has been shown to synthesize Ig actively without, however, either secreting the material or expressing detectable amounts of it on its surface (Melchers et al., 1975). In the fetal liver, these cells first appear on the 12th day of gestation. Cells with the morphology of small lymphocytes and bearing detectable surface Ig appear three to four days later (Melchers et al., 1975; Nossal and Pike, 1973; Raff et al., 1976). Autoradiographic studies of the kinetics of appearance of labeled cells of the various morphological types in the bone marrow following the injection of [^{3}H] thymidine into adult mice suggest that the same differentiation pathway is followed there (Osmond and Nossal, 1974a, b).

These Ig-bearing cells, however, appear to be immunologically immature. Although treatment with LPS or LPS plus antigen can stimulate cells from neonatal spleen or livers to produce antibody in vitro, they do not respond to antigen alone (Spear and Edelman, 1974; Melchers et al., 1975), even in the presence of mature T cells (Mosier and Johnson, 1975). There is in fact some evidence that such cells are paralyzed or suppressed, rather than stimulated, by binding specific antigen. If cells from mouse bone marrow are cultured briefly in the presence of an antigen such as Dnp coupled to human Ig, then mixed with T cells and injected into an irradiated adult mouse, the recipient's immune response is restored, except that it remains specifically

unresponsive to Dnp. Adult spleen cell populations, on the other hand, remain responsive to all antigens when treated and assayed in the same way (Nossal and Pike, 1975). A possible mechanism by which this suppression might be brought about is suggested by recent experiments of Raff and co-workers (1975), who have shown that lymphocytes obtained from the livers of fetal mice and cultured in the presence of antibodies against mouse Ig rapidly and irreversibly lose their cell surface Ig, failing to regenerate it even on further culture in the absence of such antibodies. When adult spleen cells are treated in this way, only a small proportion of the cells are affected, and even these cells rapidly regenerate their surface Ig on further culture in the absence of antibody. In no case, however, is there any evidence that cells are killed by such treatment. It is well known that neonatal animals are more readily made tolerant to foreign antigens than adults (Dresser and Mitchison, 1968), and it has been proposed that this antigen-sensitive, unresponsive stage of B lymphocyte development provides at least one means by which cells specific for self-antigens are suppressed (Nossal and Pike, 1975; Raff et al, 1975).

The final maturation of the B lymphocyte to antigen-responsiveness appears to be associated with three further changes in the cell. First, it becomes responsive to the mitogen PPD (Gronowitz, Coutinho and Möller, 1974). Second, it acquires surface complement receptors (Gelfand et al., 1974). Finally it begins to express at least part of its surface Ig with heavy chains of the delta class (Abney and Parkhouse, 1974; Abney et al., 1976; Vitetta et al., 1975). It should be pointed out that the evidence linking these changes to the functional maturation of the B cell is indirect: in all cases, cells expressing the property in question first appear in B cell populations in mice at the same time as these populations become responsive to antigenic stimulation.

Ontogeny of Antigen-Specific Cells

In terms of clonal selection, the key problem in this lymphopoietic process is the generation of antigen-specific cells. More specifically, within this overall process, when and where is the repertoire of antigen-binding specificities (i.e. the repertoire of Ig variable regions)

generated, and how is it expressed in the course of development? This problem has been approached in two ways: first, by studying the kinetics of appearance of cells responsive to various antigens, and second, by studying the appearance of antigen-binding cells.

Antigen-responsive cells. The ability of fetal and neonatal cells to respond to antigenic stimulation has been tested both *in vivo*, by direct immunization of developing animals, and in a variety of culture and adoptive transfer systems using fetal tissue. In both groups of experiments, responsiveness to different antigens appears in a well-defined sequence.

Studies of responsiveness to direct immunization have been carried out for fetal and neonatal sheep, opossums, and mice. Fetal sheep were immunized *in utero* with one or more of the antigens, ϕX bacteriophage, ferritin, ovalbumin, diphtheria toxoid, *S. typhosa*, or BCG, between the 39th and 120th days of a 130-day gestation period. Immune responses to these antigens were detected by the appearance of serum antibodies in the fetus: responses to ϕX were detected at 39–40 days gestation; to ferritin at 90 days; and to ovalbumin at 120 days. The fetuses failed to respond to the other antigens (Silverstein *et al.*, 1963). Similarly, when opossum pouch young were immunized with a panel of nine bacteriophage, hapten, and protein antigens, a distinct hierarchy of responsiveness was observed, as measured by the appearance of serum antibody. No response to any antigen was seen in animals younger than 19 days, and response to some antigens could not yet be detected in animals as old as 50 days (Rowlands *et al.*, 1974). Playfair (1968) has examined the appearance of antibody secreting cells in the spleens of neonatal mice following immunization with red blood cells. Three strains of mice (NZB, Balb/c, and C57B1) were immunized with each of three kinds of red cells (from sheep, pig, and chicken). For each strain, responsiveness to the three cell types appeared in a well-defined sequence in the four weeks following birth.

An alternative approach to the characterization of immune responsiveness in fetal mice has been to transfer fetal cells to an irradiated adult mouse of the same strain. When an adult mouse is exposed to high

doses of X-irradiation, its own antigen-responsive cells are destroyed. If it is injected with fetal cells and then immunized with antigen, its response will reflect that of the injected cells. The results of such studies have confirmed and extended the results obtained in the direct experiments discussed above. Sherwin and Rowlands (1974, 1975) have examined the appearance of immune responsiveness in this way, using cells obtained from fetal liver. Reconstituted adults showed a sequential appearance of responsiveness, with responsiveness to some bacteriophages appearing as early as three days after reconstitution, and response to some hapten-protein conjugates not appearing until three weeks later, and this sequence was essentially invariant for different individuals from any one strain of mice. The same sequence was observed for all three strains of mice tested (Balb/c, AKR, and C3H/He), except that responsiveness to the protein myoglobin was significantly accelerated in Balb/c mice relative to the other strains.

Detailed characterization of the antibodies produced by these fetal cells indicates that, in addition to the restriction in the antigenic specificities which elicit a response, there are restrictions on the physical and chemical properties of the antibodies produced. If adult spleen cells are injected into an irradiated adult mouse and the recipient is then immunized with the hapten dinitrophenol (Dnp) conjugated to bovine gamma globulin, the average affinity of the anti-Dnp antibodies produced is high, and the range of affinities is broadly distributed about this mean. Fetal liver cells assayed in the same way give rise to antibodies with a sharply restricted range of affinities. Cell populations from young mice achieve an "adult" character with respect to heterogeneity of affinity by two weeks after birth (Goidl and Siskind, 1974).

Analogous results have been obtained in comparative studies of the isoelectric points of antibodies produced by lymphoid cells from neonatal and adult mice (Klinman and Press, 1975). In these experiments, fragments of splenic tissue from irradiated, reconstituted adult mice were placed in culture. The fragments were stimulated with antigen, and the antibodies produced were analyzed by isoelectric focusing. Antibodies

produced by cells derived originally from adult spleens showed a wide range of isoelectric points, while antibodies from fetal and neonatal spleens showed a sharply restricted range. Further, this range appeared invariant from experiment to experiment. As before, the range increased as a function of the age of the cell donor, reaching a plateau in donors about two weeks post-partum. In a parallel set of experiments, cells from neonatal mice were assayed in these fragment cultures for their response to the antigen phosphorylcholine. Cells capable of producing antibodies exhibiting the TEPC 15 idiotypic determinants characteristic of most anti-phosphorylcholine antibodies produced by adult cells in these assays did not appear in the spleen until a week after birth (Sigal et al., 1976).

The basic observation in all these experiments that immune responsiveness matures in the course of development, both in terms of the range of antigens capable of eliciting a response and in terms of the physical and chemical properties of the antibodies produced, appears well-established. Its interpretation, however, is extremely difficult. Two possible interpretations are that this maturation represents the sequential appearance of specific antigen-binding cells, or that it represents the maturation of additional cell functions required for efficient cell-antigen and cell-cell interaction in the induction of the immune response following specific antigen-binding. As discussed in the previous section, there is substantial evidence that such functional maturation does take place and that it is not complete, in the case of the mouse, until at least three weeks after birth. In particular, this functional immaturity can include the inability of an antigen-specific B cell to respond to stimulation with that antigen (Nossal and Pike, 1975). As a result, carrying out these assays under conditions in which adult lymphocytes are fully responsive to antigen still does not address the question of the composition of the repertoire of antigen-specific cells in the developing animal. The resolution of this ambiguity would appear to require that the appearance of antigen-binding cells be examined directly in the developing animal, and that the antigen-binding properties of these cells be compared with those of cells from adult

animals.

Antigen-binding cells. Only limited studies of the ontogeny of these cells have been carried out, however. Dwyer and co-workers have examined the appearance of cells specific for each of two antigens, flagellin and hemocyanin, in the human and the mouse. These cells were detected by autoradiography following incubation with radioactive antigen. Although cells resembling lymphocytes from human and mouse liver and bone marrow showed antigen binding at early gestational ages, this binding could not be blocked with species-specific antisera against immunoglobulin. Specific antigen binding to lymphocytes was first detected with thymic lymphocytes at gestational ages of 12 weeks in humans and 14 days in mice, then with spleen lymphocytes at 16 weeks in humans and 17 days in mice, and later with gut lymphocytes. The relative proportions of antigen-binding cells in the human fetal thymus were maximal at 16 - 22 weeks and decreased thereafter. In both species the initial appearance of specific antigen-binding cells closely followed the appearance of thymus cells with the morphology of lymphocytes (Dwyer and Mackay, 1970, 1972; Dwyer and Warner, 1971; Dwyer et al., 1972). Cells specific for β-galactosidase have also been detected in the human fetal thymus in parallel with the appearance of flagellin- and hemocyanin-specific cells (Hayward and Soothill, 1972).

Cells specific for each of five antigens have been detected by half term in embryonic mice, rabbits, and chickens (Decker et al., 1972, 1974). These cells were quantitated on the basis of their ability to bind fluorochrome-conjugated protein antigens (ferritin, hemocyanin, and ovalbumin) or to bind active enzymes, revealed by subsequent histochemical analysis (β-galactosidase, and horseradish peroxidase). In addition, a series of individual fetal mice was tested for antigen-binding cells, and in each case such cells were found, although the frequencies of these cells appeared to vary considerably from individual to individual. A disturbing aspect of these results is that the frequency of antigen-binding cells detected in these assays was maximal in the fetus, and fell in the course of the animals' subsequent development. Further, in the case of the fetal mouse, antigen-binding cells were first

detected at 10 days' gestation, well before the initial appearance of cells bearing surface immunoglobulin on the 14th day of gestation (Nossal and Pike, 1975). These discrepancies between the rate of appearance of cells specific for particular antigens and the population of antigen-binding cells as a whole (as judged by the appearance of Ig-bearing cells) are difficult to explain.

In contrast, Spear et al. (1973) found that cells specific for each of three antigens, as detected by the rosette assay, first appeared in the spleens of fetal mice between the 15th and 16th days of gestation, together with the initial appearance of Ig-bearing cells in the spleen. The frequency of cells specific for each antigen increased thereafter, in parallel with the increase in the frequency of Ig-bearing cells. In addition, fetal and adult spleen cell populations specific for the antigen trinitrophenol were shown to bind the antigen with the same distribution of relative avidities.

These studies, taken together, indicate that at least some antigen-binding cells are present in the developing animal well before the onset of immune responsiveness, and that in at least one case the antigen-binding properties of these cells are not detectably different from those of cells in the mature animal. Relatively few antigen-binding specificities have been examined, however, and the methods used may not have given an accurate indication of the antigen-binding properties of the cell populations examined. This results from the fact that many antigen molecules have several identical antigenic determinants and therefore can form multiple bonds with the same cell. If soluble antigens with different numbers of such determinants are compared in binding assays, they will appear to have a different binding affinity for the cell even though the cell is equally specific for both of them. For this reason, a valid comparison of different antigen-binding cells must take account of the valences of the antigens being studied. In practice, this has most readily been accomplished by analyzing the binding of cells to antigenic determinants immobilized with equivalent densities on a solid matrix.

DETECTION OF SPECIFIC ANTIGEN-BINDING CELLS

A wide variety of assays have been described for the detection of antigen-binding cells (see Greaves et al., 1973; page 128, for a review). Three of these assays, the formation of rosettes with antigen-coated red cells, the binding of cells to surfaces coated with antigen-gelatin conjugates, and the binding of cells to antigen-coated fibers, seem particularly useful in terms of the criterion discussed above. Furthermore, in each case it has been possible to demonstrate a correlation between the cells detected as antigen-binding cells and the cells in the population specifically responsive to stimulation with the antigen when a cell population known to be immunocompetent is assayed. Without this positive control, the relevance of the cells detected in the assay to the clonal selection process would remain difficult to assess.

The rosette-forming assay. The rosette assay is a rapid and sensitive method for the detection of antigen-binding cells (Biozzi et al., 1966; McConnell et al., 1969; Zaalberg et al., 1966). Lymphoid cells are mixed with erythrocytes that have (or have been coated with) surface antigens. The mixture of lymphocytes and erythrocytes is centrifuged at about 500 x g for 10 minutes and gently resuspended. Those lymphocytes that are specific for the red cell surface antigen bind the red cells to their surfaces to form a characteristic stable rosette. These rosette-forming cells are easily detected by direct microscopic examination of the cell mixture. The assay appears to be antigen-specific: rosette formation is competitively inhibited in the presence of homologous soluble antigen, and in the presence of antibodies against Ig. In addition, the frequency of rosette-forming cells specific for a given antigen is increased in cell suspensions obtained from animals immunized against that antigen. A disadvantage of the method is that it has not been possible to couple a wide variety of chemically defined hapten antigens to the red cell surface. As a result, the assay can only be used to detect cells specific for erythrocyte surface antigens, and for the hapten Tnp. A second disadvantage is that the morphology of the antigen-binding cell is usually obscured by the adherent erythrocytes. Nevertheless, the antigen-binding cells are not destroyed by the

process, and can in fact be recovered for further analysis of their immunological function.

These functional assays have confirmed the specificity and efficiency of the assay procedure (Osoba, 1970; Elliott and Haskill, 1973; Elliott et al, 1973). Following the formation of rosettes with native sheep red blood cells, it is possible to fractionate the cells which have formed rosettes from the remainder of the nucleated cells by means of the physical technique of velocity sedimentation at unit gravity. The rosette forming cells recovered by this procedure have been shown to be highly enriched in the precursors of sheep red blood cell-specific antibody forming cells. Conversely, the large population of lymphoid cells which failed to form rosettes with the antigen also was unresponsive to subsequent stimulation with it.

Binding of cells to gelatin-coated surfaces. Bovine gelatin conjugated with haptens such as Dnp or 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) can be applied as a uniform coating to the surface of a nylon fiber (Rutishauser et al., 1973) or to the surfaces of plastic tubes or petri dishes (Haas et al., 1974; Haas and Layton, 1975). In either case, if lymphoid cell suspensions are incubated with the antigen-gelatin matrix under conditions of gentle but continuous agitation, a small proportion of the cells in the suspension is bound to the matrix. The properties of cells fractionated on gelatin-coated fibers will be discussed in the experimental section of this thesis; the discussion here will focus on the characterization of cells bound to gelatin-coated surfaces.

Binding of mouse spleen cells to the antigen-gelatin surfaces appears to be antigen-specific, inasmuch as the binding was inhibited in the presence of soluble antigen, and in the presence of antibodies against mouse Ig. Binding to Dnp-gelatin surfaces was unaffected by soluble NIP-gelatin and vice versa. In addition, the frequency of binding cells in the spleen was increased following immunization with the test antigen (Haas et al., 1974; Haas and Layton, 1975).

If cells from non-immune adult mice were allowed to bind to gelatin-coated surfaces, released by melting the gelatin at 37^{o}, and treated

briefly with collagenase to remove adherent gelatin, the recovered cell could be shown to respond to stimulation with antigen in culture. NIP-fractionated cell populations were enriched as much as 300-fold over unfractionated cells in terms of their ability to generate antibody-producing cells when stimulated with that antigen *in vitro*. These cells were unresponsive to stimulation with Dnp. On the other hand, Dnp-fractionated cell populations were enriched up to 100-fold in their responses to Dnp, but were unresponsive to NIP (Haas, 1975).

This method appears to allow the detection and recovery of functional antigen-specific cells. It should be applicable to the characterization of cells specific for any of a wide variety of antigens. Its major drawbacks, however, are that direct visualization of the bound cells is difficult, that the gelatin matrix is mechanically somewhat unstable, and that at least under some circumstances adherent gelatin remaining on the fractionated cell populations interferes with subsequent assays of their function (Haas and Layton, 1975).

Binding of cells to antigen-coated fibers (Figure 4). The fiber-binding assay, originally developed by Edelman *et al.*, (1971), involves the interaction between cell surface receptors and ligands bound to the surface of a nylon fiber. These nylon fibers can be uniformly coated with any of a wide variety of antigens. They can be used to quantitate the frequency of specific antigen-binding cells in a population, and to determine the relative avidity of a cell for antigen by titrating the effect of soluble antigen on the binding of cells to antigen-coated fibers. The number of cells bound to the fibers can be determined by direct observation. Because the number of cells bound to a given length of fiber is proportional to the number of antigen-specific cells in the incubation mixture, the amount of binding observed provides an index of the number of antigen-specific cells in the incubation mixture.

The specificity of the binding has been shown by competitive inhibition and depletion experiments. Binding to Dnp-coated fibers was almost completely inhibited by soluble Dnp-BSA but was not affected by soluble toluenesulfonyl-BSA (tosyl-BSA),while binding to toluenesulfonyl-coated fibers was inhibited by tosyl-BSA and not by Dnp-BSA. Cell

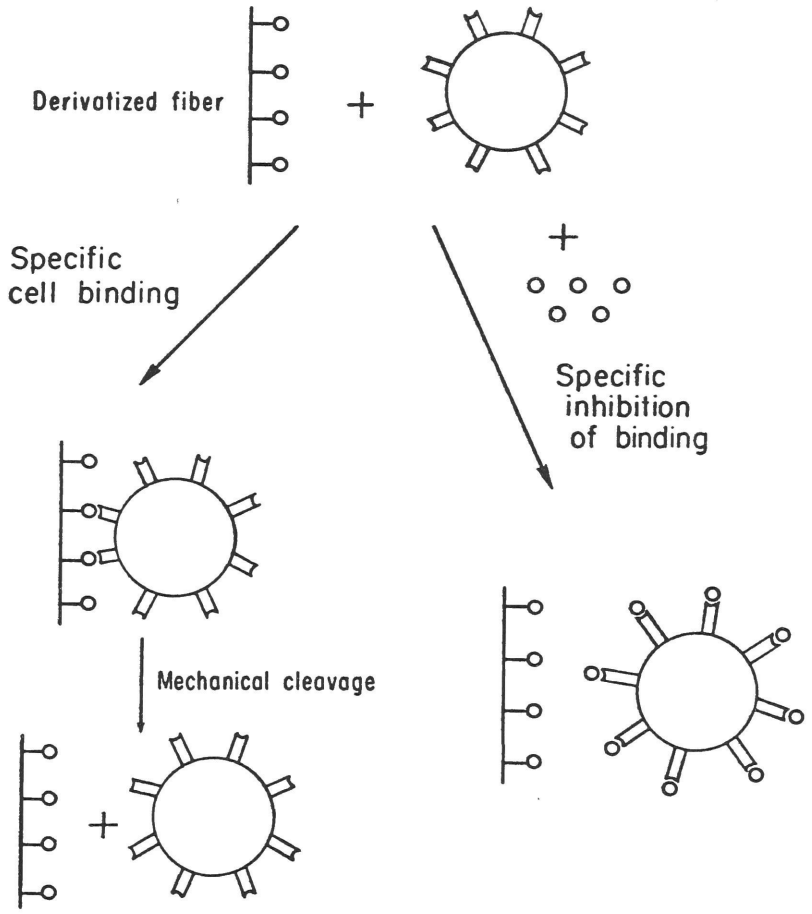

Figure 4. General scheme of fiber fractionation.

populations depleted of tosyl-specific cells by preincubation with tosyl-coated fibers contained the same proportion of Dnp-specific fiber-binding cells as untreated control cell populations.

Binding was also inhibited if the cells were preincubated with rabbit antibodies against mouse Ig, suggesting that binding takes place via specific cell surface antibodies.

Finally, as in the other assays, the frequency of fiber-binding cells in a population specific for a given antigen was increased following immunization with that antigen (Rutishauser et al., 1972).

By all these criteria, the fiber-binding assay is capable of quantitatively detecting cells specific for any of a wide variety of antigens. Because of the uniformity of the method, it should allow direct comparison of the results obtained with different cell populations, in assays for different antigenic determinants. It remains, however, to show directly that the cells bound to an antigen-coated fiber are specific for that antigen at the level of the immune response. The functional properties of such fiber-fractionated cells will be discussed in the experimental section.

The development of all of these specific methods of quantitating antigen-binding cells allows a direct experimental approach to be made to the analysis of the ontogeny of these cells. A description of this approach is the main subject of this thesis.

EXPERIMENTAL

Purpose of the Experiments

Extensive evidence is now available to support the central points of the clonal selection hypothesis: that there exists in the body, prior to contact with antigen, a repertoire of antigen-binding lymphocytes; that each of these cells is committed to the production of a single species of Ig molecule; and that the role of a foreign antigen is to stimulate the clonal expansion of those cells whose antibodies are specific for it. Much less is known, however, about the development of that repertoire. In particular, the means by which the full range of Ig molecules is generated, the process by which each lymphocyte becomes committed to the expression of only one of these molecules, and the mechanisms by which these events are controlled all remain unclear. The experiments in this thesis were designed to approach these problems at the level of the antigen-binding cell. In particular, the experiments will be directed towards four questions.

1) How can specific antigen-binding cells be quantitated?

2) When and where in the course of an animal's development do specific antigen-binding cells appear?

3) How do the antigen-binding properties of cells from developing animals compare with those of cells from adults?

4) To what extent does the development of the repertoire of antigen-binding cells vary from individual to individual?

Materials and Methods

Animals. Balb/c mice were obtained from Jackson Laboratory (Bar Harbor, Maine) or Charles River Laboratory (Wilmington, Massachusetts), or bred at The Rockefeller University from Jackson stock. All other inbred mice were obtained from Jackson or bred here from Jackson stock. Swiss-L mice were obtained from the specific pathogen-free colony maintained at The Rockefeller University (Nelson and Collins, 1961). These colony-bred mice are the descendents of nine animals used to start the colony in 1926.

To obtain fetuses of known gestational age, female mice (2-3 months old) were housed with males and inspected daily for vaginal plugs. Each breeding cage contained one male and 3-5 females. The day a plug was found was taken to be day 0 of gestation. Parturition occurred regularly on the 19th day of gestation in the Swiss mice, and on the 20th or 21st day of gestation in Balb/c and CBA mice. Cages containing pregnant females were checked daily for new births; the day of birth was taken to be the day the litter was found. Because most births occur at night, the mice could be as much as 12 hours older than reported.

Antigens. Limulus hemocyanin (Hcy) was prepared from fresh hemolymph by repeated centrifugation (Campbell et al., 1970). Lysozyme (Worthington Biochemicals, Freehold, New Jersey), sperm whale myoglobin (Miles-Seravac, Maidenhead, Berks., England), bovine gamma globulin (BGG; Hyland Laboratories, Costa Mesa, California), and bovine serum albumin (BSA; Pentex Chemicals, Inc., Kankakee, Illinois) were obtained commercially and used without further purification. p-Aminobenzoic acid, sulfanilic acid, p-toluenesulfonic acid (tosyl), dinitrobenzenesulfonic acid (Dnp), p-iodobenzoyl chloride (all from Eastman Kodak, Rochester, New York), 5-aminoquinoline (Aldrich Chemical Co., Milwaukee, Wisconsin), p-aminophenyl-β-D-lactoside (Cyclo, Los Angeles, California), fluorescein isothiocyanate (Baltimore Biological Laboratories, Cockeysville, Maryland), trinitrobenzenesulfonic acid (Pierce Chemical Co., Rockford, Illinois), and adenosine monophosphate (Calbiochem, San Diego, California) were coupled to BSA, BGG, or Hcy by standard procedures (Williams and Chase, 1967). The number of Dnp or tosyl groups per protein molecule was determined spectrophotometrically.

Immunization. Mice were immunized intraperitoneally at 8 weeks of age and at monthly intervals thereafter with 400 μg of Hcy, or 200 μg of Hcy or BGG conjugated with the haptens Dnp or sulfanilic acid (Dnp-Hcy, etc.). These antigens were adsorbed onto bentonite (Rittenberg and Pratt, 1969) before injection. Animals received 1 to 4 injections; spleens were removed 5 days after the last injection.

Preparation of anti-θ serum. Anti-θC3H antibodies were prepared by the method of Reif and Allen (1964). AKR/J mice were injected intraperitoneally with 10^7 thymocytes obtained from C3H/HeJ or CBA/J mice, at

weekly intervals for 10 weeks. Blood was collected from the retro-orbital sinus 6 days after the 6th and each succeeding injection. In the presence of complement, this antiserum was specifically cytotoxic for thymocytes and T cells from Balb/c and Swiss-L mice. At a dilution of 1:10 the antiserum killed over 90% of Balb/c thymocytes, 30-40% of Balb/c spleen cells, but less than 5% of Balb/c bone marrow cells or AKR/J thymocytes. Guinea pig complement (Grand Island Biological Co., Grand Island, New York, obtained as a lyophilized powder and freshly reconstituted before use) was absorbed with Balb/c spleen cells to remove cytotoxic antibodies and was used at a final dilution of 1:10. These concentrations of antibody and complement were found to be optimal in cytotoxicity assays with spleen cells.

Preparation of antibodies to immunoglobulin. Antimouse immunoglobulin serum (anti-Ig) was prepared in rabbits by the injection of Swiss-L mouse Ig purified by zone electrophoresis in starch (Kunkel, 1954). The protein was emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan) and injected subcutaneously at a dose of 1-2 mg per rabbit. Booster injections were given every two weeks in Freund's incomplete adjuvant. The immunoglobulin fraction of the antiserum was purified by precipitation with ammonium sulfate at 37% saturation and chromatography on DEAE-cellulose (DE52; W. and R. Balston, Maidstone, Kent, England).

Cell suspensions. The number of mice used to prepare each cell suspension varied from two for adults to as many as 50 for fetuses. The tissue was minced with scissors and the cells gently forced out into cold Eagle's minimal essential medium (Microbiological Associates, Inc., Bethesda, Maryland) supplemented with 20 μg/ml of bovine pancreatic deoxyribonuclease (MEM-DNase; Worthington). After gently pipetting the cell suspensions, cell clumps were removed by low speed centrifugation and the cells were pelleted. The cells were washed, resuspended in MEM-DNase, and the numbers of cells estimated using hemocytometers. This procedure allowed nearly complete recovery of lymphoid cells from the spleen, and when applied to fetal liver, gave good yields of hemopoietic cells (including lymphocytes) while largely destroying any parenchymal tissue present (Seller, 1972).

To prepare cell suspensions from individual fetal spleens, the organs were placed in the wells of a Falcon "Multiwell" plate (Falcon Plastics, Division of BioQuest, Oxnard, California) together with 1 ml of MEM-DNase. The spleens were teased apart with fine forceps, and the cells were forced out of the tissue fragments by repeated passage first through a syringe and 22 gauge needle and then through a syringe and 26 gauge needle.

Detection of antigen-responsive cells in vitro. Cell suspensions were prepared from fetal and adult tissues by the usual procedures, but using sterile equipment and reagents. The suspensions were transferred to sterile plastic tubes and left in an ice bath for a few minutes to allow remaining clumps to settle. After transferring the declumped suspension to another tube, the cells were pelleted by low-speed centrifugation and then resuspended in culture medium. The concentration of viable nucleated cells was determined by counting the cells that excluded trypan blue, using a hemocytometer. In these preparations, 75-90% of the cells were viable.

Twenty million viable nucleated cells were cultured in 35-mm disposable plastic dishes (Nunc, distributed by Vangard International, Inc., Red Bank, New Jersey) by the procedure of Mishell and Dutton (1967) with the addition of 2-mercaptoethanol as suggested by Click et al. (1972). Components of the culture medium, formulated exactly as described by Mishell and Dutton, were purchased from Microbiological Associates, Inc. with the exception of the serum. Normal human serum which had been heat-inactivated by incubation for 30 minutes at 56° was used in the culture medium. This particular serum was chosen from among several tested for its ability to support good primary immune responses to sheep red blood cells with low background levels of antibody-secreting cells produced in the absence of added antigen. A stock solution of 2-mercaptoethanol (0.1% vol/vol in saline) was diluted 1:6 with medium and 25 µl aliquots were added to each culture. To some cultures, a 30 µl aliquot of 1% SRBC (Microbiological Associates, Inc.) in saline was added as the antigenic stimulus. Bacterial lipopolysaccharide (LPS) prepared from Salmonella typhosa 0901 by the procedure of Westphal et al. (1952) (lot no.589254, Difco Laboratories), appropriately diluted with culture medium, was added to some cultures while maintaining the total volume at 1 ml and

the cell concentration at 2 x 10^7 viable cells per ml. Cultures were incubated at 37° in a humidified atmosphere of 10% CO_2, 7% O_2 and 83% N_2 on a rocking platform (Bellco Glass, Inc., Vineland, New Jersey) and were fed daily with a nutritional mixture as formulated by Mishell and Dutton (1967) except that, as before, human serum was substituted for fetal calf serum. After 2-6 days of incubation, duplicate cultures were pooled. The cells were washed, resuspended in a balanced salts solution, and then assayed for the presence of cells secreting antibodies to SRBC using the Jerne plaque technique (Jerne and Nordin, 1963).

Preparation of derivatized fibers. Transparent nylon monofilament (size 50 sewing nylon, Dyno Merchandise Corp., Elmhurst, New York), was strung into polyethylene collars (cut from S-6 hollow stoppers, Mallinckrodt, New York, New York). These fit snugly into 35-mm petri dishes (Nunc), holding the nylon fibers under tension. This arrangement greatly facilitated the handling and subsequent use of the fibers. Surface contaminants were removed by 10 minute extractions of the strung fibers first with carbon tetrachloride and then with petroleum ether. Proteins were coupled to the cleaned fibers by use of a water-soluble carbodimide, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate (Aldrich). Specifically, the protein and the carbodiimide were dissolved in 0.15 M NaCl, pH 6.8, at final concentrations of 0.25 mg/ml and 1.25 mg/ml respectively. The fibers were incubated with this mixture for 30 minutes at room temperature, washed extensively with phosphate-buffered saline to remove unbound material, and used the same day (Rutishauser et al., 1972).

Alternatively, in a new modification of the method, nylon fibers were coated with Dnp-derivatized bovine gelatin (Dnp-Gel). Dnp-Gel was prepared by reacting 20 g of gelatin (Fisher Scientific Co.) with 2 g of 2,4-dinitrobenzenesulfonic acid in 100ml of 2% K_2CO_3 for 1 hour at 37°. The Dnp-Gel was dialyzed at room temperature against water containing 0.1% merthiolate, and stored at 4° as a 5% gel. Each collar was dipped in a 5% solution of the Dnp-Gel at 40-50° and immediately centrifuged in a clinical centrifuge (International Equipment Co., Needham, Massachusetts) to shake off excess gelatin. Each collar containing the fibers was placed directly in a no. 325 supporting ring of a no. 215

swinging bucket head so that the fibers were horizontally and radially aligned, and centrifuged for 10 seconds at 200 RPM. After centrifugation, the coated fibers were cooled and washed with cold phosphate-buffered saline.

Cell binding to antigen-coated fibers (Figure 4). Derivatized or coated fibers strung in polyethylene collars were incubated with 5-100 x 10^6 cells in 4 ml of MEM-DNase for 60 minutes at 4° on a reciprocal shaker having a 3.3 cm horizontal stroke at 78 oscillations per minute. The fibers were aligned perpendicular to the direction of shaking. Inhibition of binding with soluble antigen or purified rabbit anti-mouse Ig was accomplished by adding the indicated concentrations of the inhibitor to the cell suspensions before incubation with the fibers. In all cases, following the incubation, unbound cells were washed away by complete immersion of the dish in a series of larger vessels containing isotonic medium. During this and all subsequent procedures care was taken not to remove the fibers from the liquid, because removal resulted in release and damage of the cells. Dishes containing derivatized fibers were washed at room temperature; those containing gelatin-coated fibers were washed at 4° to prevent the gelatin from melting and releasing the bound cells.

When fibers were incubated with liver cell suspensions under these conditions, cellular debris and small tissue fragments adhered to the fibers. Addition of BSA to the incubation mixture blocked this non-specific binding with no apparent effect on the number of lymphoid cells bound. Fiber-binding assays on liver cell suspensions were therefore routinely carried out in the presence of 400 µg/ml of BSA. In those cases where soluble inhibitors were also present in the incubation mixture, the amount of BSA was reduced so that the final concentration of soluble protein was 400 µg/ml.

The binding of mouse spleen cells to Dnp-BSA and Dnp-Gel fibers is illustrated in Figure 5. The appearance and numbers of cells bound to the two types of fibers do not differ greatly, and in both cases, 75-95% of the binding is inhibitable by soluble Dnp-BSA, and 85-95% by antibodies against mouse Ig. Over 95% of the cells bound to such fibers

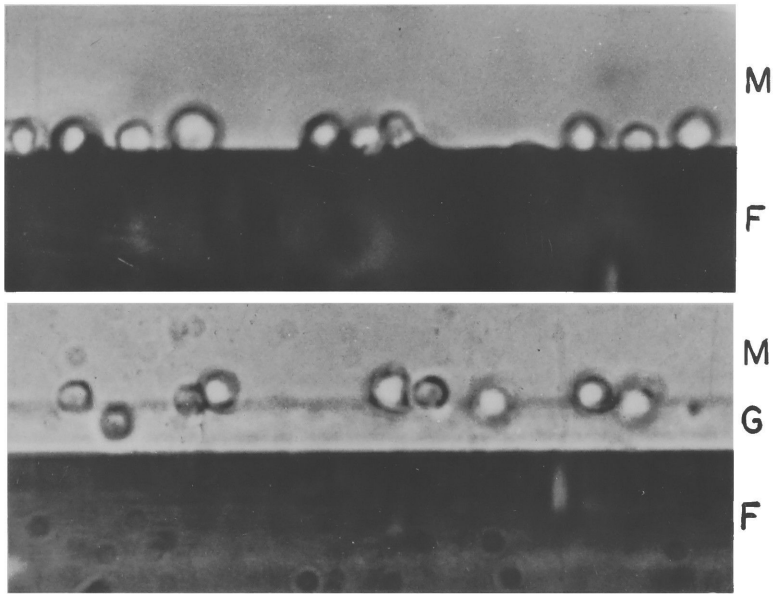

Figure 5. Mouse spleen cells bound to Dnp-BSA fibers (top), and Dnp-Gel fibers (bottom). F, fiber; M, medium; G, Dnp-Gel.

appear to be viable, inasmuch as they exclude trypan blue in situ. Assays for antigen-binding cells were carried out using collars with one row of 12 fibers each. For preparative fractionation, collars with two rows of 24 fibers each were used. After unbound cells were removed by extensive washing, such a collar yielded 2-5 x 10^4 fractionated cells.

Fractionation of cells according to their avidity for antigen. Incubation of lymphocytes with antigen-coated fibers in the presence of various concentrations of soluble homologous or cross-reacting antigens permitted the fractionation of the cells with respect to their avidity for the soluble inhibitor. The binding of cells to the fibers is generally irreversible. Under these conditions, inhibition by soluble antigen is the result of specific blockage of the receptors on the cell surface (Figure 4). Therefore, the higher the avidity of a cell, the lower the antigen concentration required for inhibition.

Quantitation of fiber-bound cells. The number of cells bound to a given length of fiber was proportional to the number of antigen-specific cells in the incubation mixture (Rutishauser et al., 1972). Knowing the number of cells bound to a 25-cm length of fiber (the amount in one dish), the number of cells in the incubation mixture, and the number of spleens or livers used to prepare the incubation mixture, the number of fiber-bound cells per organ could be calculated directly.

Cytotoxicity assays on fiber-bound cells. After all unbound cells were washed away, the cytotoxic effect of anti-θ serum and anti-Ig serum on the fiber-bound cells was determined directly.

The dishes containing the bound cells were incubated with a 1:10 dilution of anti-θ serum or anti-Ig serum in MEM-DNase at 37° for 45 minutes. The serum was then washed away and the cells were incubated with a 1:10 dilution of guinea pig complement (previously absorbed with spleen cells) for 30 minutes at 37° with gentle shaking. In most experiments, when strong antiserum and complement were used the cells bearing the target antigen were severely damaged and no longer remained bound to the fibers; with less potent reagents, some dead cells were not released. Cytotoxicity was determined by counting the number of viable bound cells

before and after treatment with complement (Rutishauser and Edelman, 1972).

Recovery and characterization of fiber-fractionated cells. Specifically bound cells were efficiently released by plucking the taut fibers with a needle (Rutishauser et al., 1972). In an alternate procedure, cells bound to Dnp-Gel fibers could be released by melting the Dnp-Gel (37°, 15 min). The released cells were quantitated by a hemocytometer count; viability of the fractionated cells was estimated by trypan blue exclusion. Specifically, the petri dish containing the suspension of released cells was pressed into a Mallinckrodt no. 8 hollow stopper which in turn was placed in a no. 320 bucket of a no. 269 swinging bucket rotor (International Equipment Co.) and centrifuged for 10 min at 1000 RPM. The supernatant fluid was removed by aspiration and replaced by a small volume of trypan blue solution, and the proportion of cells excluding the dye was determined in situ.

Rebinding studies with fiber-fractionated cells. The efficiency with which cells removed from Dnp-coated fibers could rebind the same (Dnp) or a different (tosyl) antigen was determined by the fiber-binding assay. Spleen cells were fractionated with preparative (48-strand) dishes made with Dnp-BSA or Dnp-Gel fibers. The bound cells were removed by plucking into MEM plus 10% fetal calf serum (2.5 ml per dish). This cell suspension was added without dilution to 12-strand dishes containing fibers derivatized with Dnp-BSA or tosyl-BSA. The specificity of the rebinding was determined by doing duplicate assays in the presence of 300 µg/ml of Dnp-BSA or tosyl-BSA. In addition, the binding of various concentrations of unfractionated cells was assayed at the same time.

Transfer of cells to irradiated recipients (Figure 6). Unimmunized Balb/c mice that had been exposed 24 hr previously to 630-730 rad of ^{60}Cobalt gamma irradiation were injected intravenously with 5 to 10 x 10^6 splenocytes from mice immunized with Hcy, plus either 10^4-10^5 Dnp- or tosyl-fiber-binding cells, or 10^4-10^6 unfractionated spenocytes from mice immunized with Dnp-Hcy. Control mice received only 5-10 x 10^6 cells from mice immunized with Hcy, representing the carrier specificity. The recipient mice were then immunized intraperitoneally with 200 µg of Dnp-Hcy adsorbed onto bentonite. All materials used in this procedure were

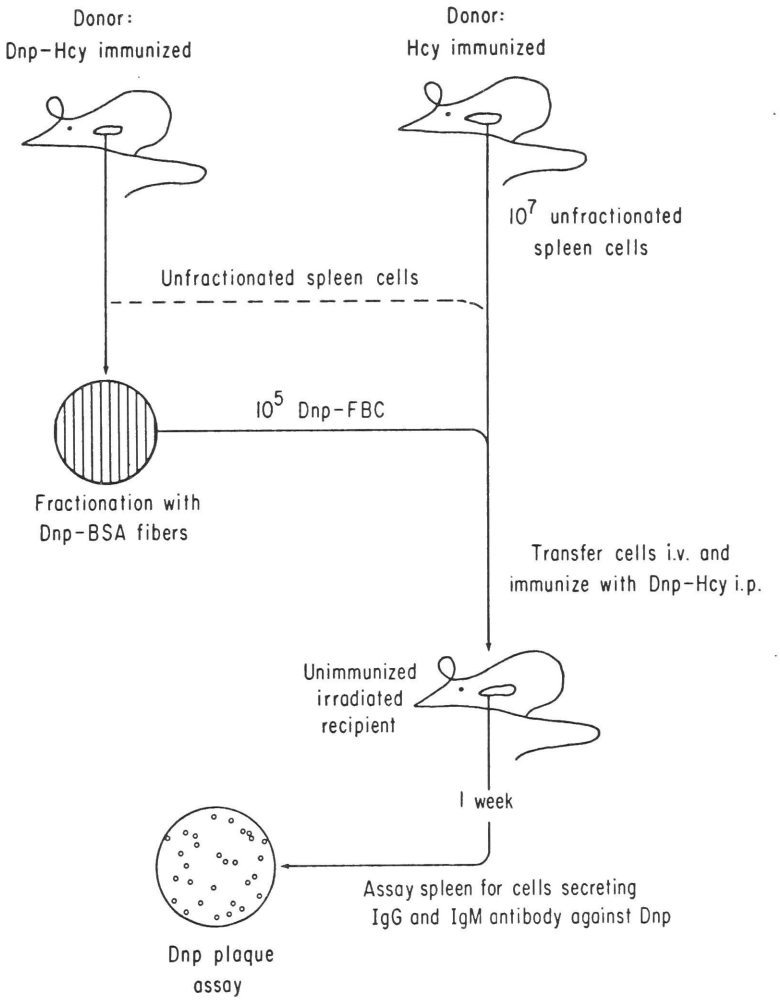

Figure 6. Protocol for transfer of Dnp fiber-fractionated cells to irradiated hosts. i.p., intraperitoneal; i.v., intravenous.

sterile. Contamination of the fractionated cells was minimized by extensively washing the fiber-bound cells with phosphate-buffered saline. Seven days after transfer, the spleen from each recipient animal was tested for cells secreting antibodies against Dnp by a modification of the Jerne plaque assay (Rittenberg and Pratt, 1969). Spleens were teased into Hank's balanced salts solution; aggregates were removed by filtering the cell suspensions through nylon mesh; finally the cell suspensions were pelleted by centrifugation and resuspended in the appropriate volume of medium for the assay. IgM-secreting cells were detected directly; IgG-secreting cells were detected by the addition of rabbit antibodies against mouse Ig to duplicate assays (Rutishauser et al., 1972).

Detection of rosette-forming cells. To detect antigen-binding cells in single fetal spleens, the rosette assay was used, because of its very high efficiency (Elliot and Haskill, 1973). The rosette assays were carried out by a slight modification of the method of McConnell et al. (1969), using native SRBC, or SRBC derivatized with the trinitrophenol (Tnp) hapten (Rittenberg and Pratt, 1969). The cell suspension prepared from a single fetal spleen was transferred to a 12 x 75 mm disposable plastic tube (Falcon) and pelleted by centrifugation. The medium was carefully removed from each tube without disturbing the cell pellet, and the cells were resuspended in 0.4 ml of MEM supplemented with 1% heat-inactivated (56°; 30 min) fetal calf serum (FCS; Microbiological Associates). Red blood cells (0.1 ml of a 1% suspension of cells in MEM-1% FCS) were added to each tube, and the mixture were centrifuged for 10 min at 200 x g, and the pellets were left undisturbed for an additional 10 min. The entire procedure was carried out at 4°. The pellets were then gently resuspended and 25 µl aliquots placed between two glass slides separated by a spacer of tape. The suspensions were scanned at a magnification of 200 diameters. Single lymphocytes with at least seven adherent erythrocytes were scored as rosette-forming cells (RFC). The bound red cells were usually uniformly distributed (Figure 7), but sometimes formed rings or caps. Rosette formation was specifically inhibited if the spleen cells were incubated for 30 min at 4° with rabbit antibodies against mouse Ig before being assayed for RFC. Pre-incubation with normal rabbit immunoglobulin did not affect rosette

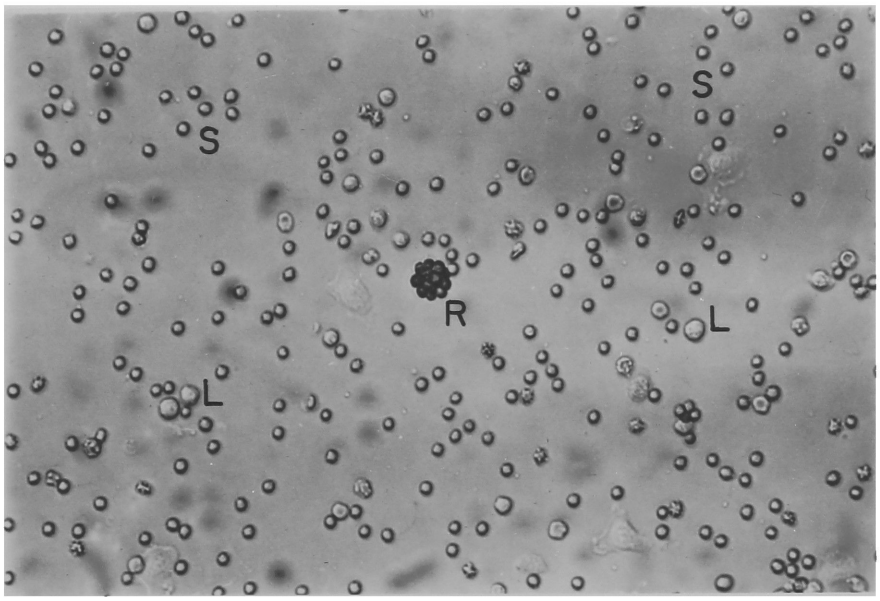

Figure 7. Fetal rosette-forming cell (R). Several unreactive lymphocytes (L) and large numbers of sheep red blood cells (S) are also visible in the field.

formation.

For each spleen, 1 to 8 25 µl aliquots were scanned for RFC. To determine the numbers of nucleated cells in the spleens, 0.2 ml of each rosette assay mixture was mixed with 0.2 ml of 1% acetic acid in water to lyse erythrocytes, and 0.1 µl aliquots of this mixture were scanned for nucleated cells using hemocytometers. To facilitate the statistical analysis of these data, equal numbers of aliquots were scanned for RFC and for nucleated cells in each case.

In those cases where a single fetal spleen was tested for RFC of two different specificities, the cell suspension was divided into two equal portions, each of which was pelleted, resuspended in 0.4 ml of MEM-1% FCS, and assayed for RFC as usual.

RESULTS

Immunological Function of Fiber-Binding Cells

Recovery of viable fiber-fractionated cells. The binding of cells to antigen coated fibers is in general an irreversible process: once bound, the cells cannot be released by the addition of competitive inhibitors (Edelman et al., 1971). It was therefore necessary to develop mechanical procedures for removing the bound cells from the fibers (Table II). Before removal from the fibers, over 95% of the bound cells were viable, as judged by their ability to exclude trypan blue. The viability of the recovered cells varied greatly depending upon the type of fiber, the removal method, and the medium into which the cells were released. Cells could be released from the fibers either by plucking the taut fibers, or, for cells bound to Dnp-Gel fibers, melting the gelatin. More cells survived removal from Dnp-Gel fibers than from Dnp-BSA fibers, especially if the cells were removed from the Gel fibers by melting the gelatin. In all cases, cell survival was enhanced by the presence of fetal calf serum in the medium. Furthermore, the proportion of viable plucked cells increased when the cells were incubated for 30 min at 37° in the presence of serum, as if the cells were repairing lesions in their surface membranes. In the absence of serum, however, the viability of both fractionated and unfractionated cells decreased. These experiments clearly indicate that the initial high viability of the fractionated cell populations can be maintained by choosing the appropriate conditions for cell removal.

Antigen-binding properties of fiber-fractionated cells. Although the cells removed from the fibers were viable, it was not known whether they retained various immunological functions. As a straighforward test of their antigen-binding function, their ability to rebind to antigen-coated fibers was determined. The fractionated cell populations appeared to rebind specifically to the antigen used for the inital fractionation. In a typical experiment (Table III), spleen cells from Balb/c mice that had been immunized with Dnp-Hcy were fractionated on Dnp-Gel fibers under preparative conditions. The cells were recovered from the gelatin fibers by plucking into MEM supplemented with 10% FCS. Four

Table II

Viability of Spleen Cells Removed from Dnp Fibers

Fiber	Medium*	Removal	Incubation‡ (min)	% Viable†
Dnp-BSA	PBS	Pluck	30	21 ± 2
Dnp-BSA	PBS	Pluck	--	46 ± 2
Dnp-BSA	FCS	Pluck	--	70 ± 2
Dnp-BSA	FCS	Pluck	30	81 ± 3
Dnp-Gel	PBS	Pluck	30	21 ± 3
Dnp-Gel	PBS	Pluck	--	62 ± 4
Dnp-Gel	PBS	Melt	30	70 ± 2
Dnp-Gel	FCS	Pluck	--	83 ± 3
Dnp-Gel	FCS	Pluck	30	91 ± 2
Dnp-Gel	FCS	Melt	30	97 ± 1
Unfractionated cells	PBS	--	30	63 ± 2
cells	PBS	--	--	84 ± 2
cells	FCS	--	30	85 ± 1

*) PBS, phosphate-buffered saline, FCS, PBS plus 10% fetal calf serum

‡) Incubation was carried out at 37^{o} in the same medium as the removal process in all cases.

†) As determined by trypan blue exclusion. Each number is the average of three determinations. Standard errors of the mean are shown.

Table III

Specific Rebinding of Fractionated
Cells to Derivatized Fibers

Rebinding Assay*	Fiber Antigen	Soluble Inhibitor†	Number of Cells Bound‡
A	Dnp-BSA	--	74§
B	"	Dnp-BSA	20
C	Tosyl-BSA	--	20
D	"	Tosyl-BSA	25

*) Each assay dish contained 5×10^4 fractionated cells in 4 ml MEM supplemented with 10% FCS.

†) Inhibitors were added to the incubation mixture to a final concentration of 300 μg/ml.

‡) Cells bound to the edges of a 10 cm segment of fiber.

§) The difference between the number of cells bound in assay A and the numbers bound in assays B, C, or D was highly significant by the variance test for homogeneity ($P < 0.005$ in each case). The differences among B, C, and D were not significant ($P > 0.25$ in each case). (Snedecor and Cochran, 1967; page 241).

milliliter portions of this suspension of fractionated cells were incubated further with Dnp-BSA or tosyl-BSA derivatized fibers, in the presence or absence of homologous soluble antigen. Under these conditions, the fractionated cells rebound to the Dnp-BSA fibers, and this binding was inhibited by soluble Dnp-BSA fibers.

To determine the extent to which fractionated cell populations were enriched in antigen-specific cells, spleen cells from normal and immune mice were fractionated using Dnp-BSA and Dnp-Gel fibers, and in each case the binding efficiency of the fractionated cells was determined. The extent of the rebinding observed in such experiments appeared not to vary with the source or method of preparation of the fractionated cell population. If binding was expressed as a ratio of the numbers of cells bound to the numbers of cells incubated, cells from both immune and nonimmune mice, fractionated initially either on Dnp-BSA or Dnp-Gel fibers were found to bind to a second set of Dnp-BSA fiber with the same efficiency (Table IV).

To quantitate the enrichment of Dnp-specific cells obtained by fiber fractionation, these binding efficiencies obtained for fractionated cells were compared with those of the original unfractionated cell populations. Standard curves were established for each of the unfractionated cell populations. As shown in Figure 8 , the number of cells bound to a 10 cm length of fiber was a linear function of the number of cells in the assay mixture. This observation suggested that, under the conditions used, the binding is proportional to the number of input cells, the binding of one cell is independent of that of another, and the fiber surface is not saturated with cells. As noted previously (Rutishauser et al., 1972), a larger proportion of the cells from immunized animals was bound to the fibers than was the case with cells from unimmunized animals.

On the basis of these standard curves, it was possible to calculate in each case the concentration of unfractionated cells required to yield a number of fiber-bound cells equivalent to that observed for a given concentration of the corresponding fractionated cells, as shown in Figure 8. The enrichment of specific antigen-binding cells obtained

Table IV

Rebinding Efficiency of Fiber-Fractionated Cells

	Fractionated Cells		Rebinding Assay		
Experiment	Immunization	Initial Fractionation	Input cell count*	Fiber-bound cells*	Rebinding Efficiency†
1	None	Dnp-BSA	42	48	45.7
		Dnp-Gel	69	75	43.5
2	None	Dnp-BSA	43	18	16.7
		Dnp-Gel	40	48	48.0
3	Primary (5 days)	Dnp-BSA	33	30	36.4
		Dnp-Gel	31	28	36.1
4	Primary (13 days)	Dnp-BSA	68	49	28.8
		Dnp-Gel	53	51	38.5
5	Secondary	Dnp-BSA	54	49	36.3
		Dnp-Gel	51	48	37.6

*) A 4 µl aliquot of each incubation mixture was counted in a hemocytometer. The number of cells bound to the edges of a 10 cm segment of fiber was counted in each case. Evaluation of a 2 x 10 contingency table in which each pair of entries consisted of these two numbers yielded $\chi^2=13.67741$ with 9 degrees of freedom; $0.10 < P < 0.25$ (Snedecor and Cochran, 1967, page 238).

†) Defined as the number of cells bound to both edges of a 10 cm fiber segment under standard conditions at 1×10^4 fractionated cells per ml.

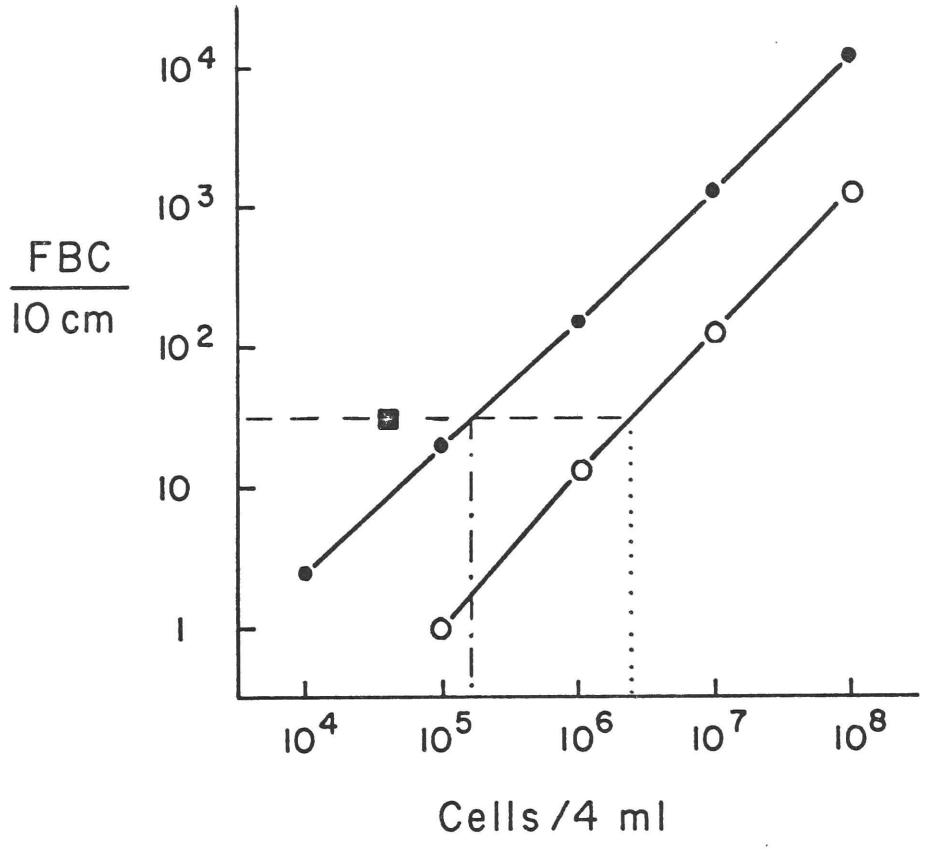

Figure 8. Binding of spleen cells to Dnp-BSA fibers. Standard curves are shown for the binding of previously unfractionated spleen cells from Dmp-immune (●) or unimmunized (O) animals as a function of the number of cells in the incubation mixture. The rebinding obtained with 4×10^4 previously fractionated cells is shown separately (■). Extrapolation of this binding value (---) shows that 60 times as many unfractionated nonimmune cells (...) or 5 times as many unfractionated immune cells (-·-) would be required to produce the same amount of binding (see Table V).

in the fractionation process was then the ratio of these two cell concentrations (Table V). In all cases the observed enrichment was nearly equal to that expected for a pure population of antigen-binding cells specific for Dnp.

Specific immune function of fiber-fractionated cells. The results of experiments designed to test the function of fractionated cells in vivo are shown in Tables VI and VII. Fractionated Dnp-binding cells from mice immunized with Dnp-Hcy were transferred to syngeneic irradiated mice and tested for their ability to produce a specific humoral antibody response when stimulated by Dnp-Hcy (Figure 6). Cells from mice immunized with Hcy were mixed with the fractionated cells both to serve as a carrier and to provide the Hcy-specific T cells required for a complete response by the Dnp-specific cells. Inasmuch as the unfractionated cells were from mice that were not immunized against Dnp, the frequency of Dnp-specific B cells was relatively low and these cells responded weakly with the production of both IgM and IgG antibodies. On the other hand, the fractionated cells from mice immunized with Dnp responded by the production of IgG antibodies only.

In order to quantitiate the enrichment obtained by fractionation, it was necessary to determine the response per cell injected. The number of cells secreting antibody of the IgG class was not a simple function of the number of transferred cells (Figure 9). Over the range from 10^5 to 10^7 injected cells, it was found that increasing the cell number by a factor x increased the response by a factor of approximately $x^{2/3}$. Fractionation by Dnp-BSA (Table VI) or Dnp-Gel (Table VII) of spleen cells from Dnp-Hcy immune mice yielded cells that produced a Dnp-specific response equivalent to that of at least 10 times as many unfractionated cells from the same animals assayed in parallel in the same experiment. In contrast, cells obtained by fractionation of these cell populations with tosyl-derivatized fibers did not yield numbers of Dnp plaque-forming cells (PFC) above control values. There was also no detectable response if the recipient animals were not simultaneously injected with Dnp-Hcy. Inasmuch as these unfractionated cell populations contained 7-15% Dnp-binding cells, the observed enhancement in the transfer experiments approached the value expected for a pure population of Dnp-specific cells, of which

Table V

Specific Rebinding of Fractionated Cells
to Derivatized Fibers

Immunization	% Specific ABC in unfractionated population	Predicted enrichment†	Observed enrichment ǂ
None	1-2	50-100 x	44-78 x
Primary (5 days)	3-5	20-33 x	23 x
Primary (14 days)	6-8	12-17 x	12-15 x
Secondary	10-17	6-10 x	6-7 x

*) Determined by the fiber and/or rosette methods with unfractionated spleen cells (Rutishauser et al., 1972). ABC is antigen-binding cells.

†) Based on enrichment of cell population for 100% specific antigen-binding cells.

ǂ) Calculated from the data shown in Table IV as outlined in Figure 8.

Table VI

Response of Cells Isolated with Dnp-BSA Fibers
After Transfer to Irradiated Recipients

Transferred cells*			Response†	
Fractionated Dnp-FBC	Unfractionated Dnp-Hcy cells	Unfractionated Hcy cells	IgM PFC per spleen	IgG PFC per spleen
0	0	10^7	4,163 ± 290	6,503 ± 752
0	7×10^4	10^7	5,077 ± 1,583	9,537 ± 2,158
0	7×10^5	10^7	4,873 ± 246	25,533 ± 2,801
7×10^4	0	10^7	4,487 ± 354	30,733 ± 3,177

*) The experimental procedure is summarized in Figure 6. The numbers shown are the numbers of cells injected into each recipient mouse.
†) Individual recipient spleens were assayed for PFC 7 days after transfer. Each number is the average of data from six mice. The standard errors of the mean calculated from the data are shown. Cells from Dnp-immune animals fractionated on tosyl-BSA fibers did not yield PFC above control values.

Table VII

Response of Cells Isolated with Dnp-gelatin fibers
After Transfer to Irradiated Recipients

Transferred cells*			Response†	
Fractionated Dnp-FBC	Unfractionated Dnp-Hcy cells	Unfractionated Hcy cells	IgM PFC per spleen	IgG PFC per spleen
0	0	5×10^6	2,240 ± 481	1,120 ± 171
0	2×10^4	5×10^6	1,299 ± 133	2,033 ± 444
0	2×10^5	5×10^6	1,390 ± 281	4,182 ± 1,188
2×10^4	0	5×10^6	2,073 ± 240	5,750 ± 1,285

8) Except for the fact that the fractionated cells were prepared with Dnp-Gel fibers, the transfer procedure was identical to that described in Table VI.

†) Individual spleens were assayed for PFC. Each number is the average of data from six mice. The standard errors of the mean calculated from the data are shown.

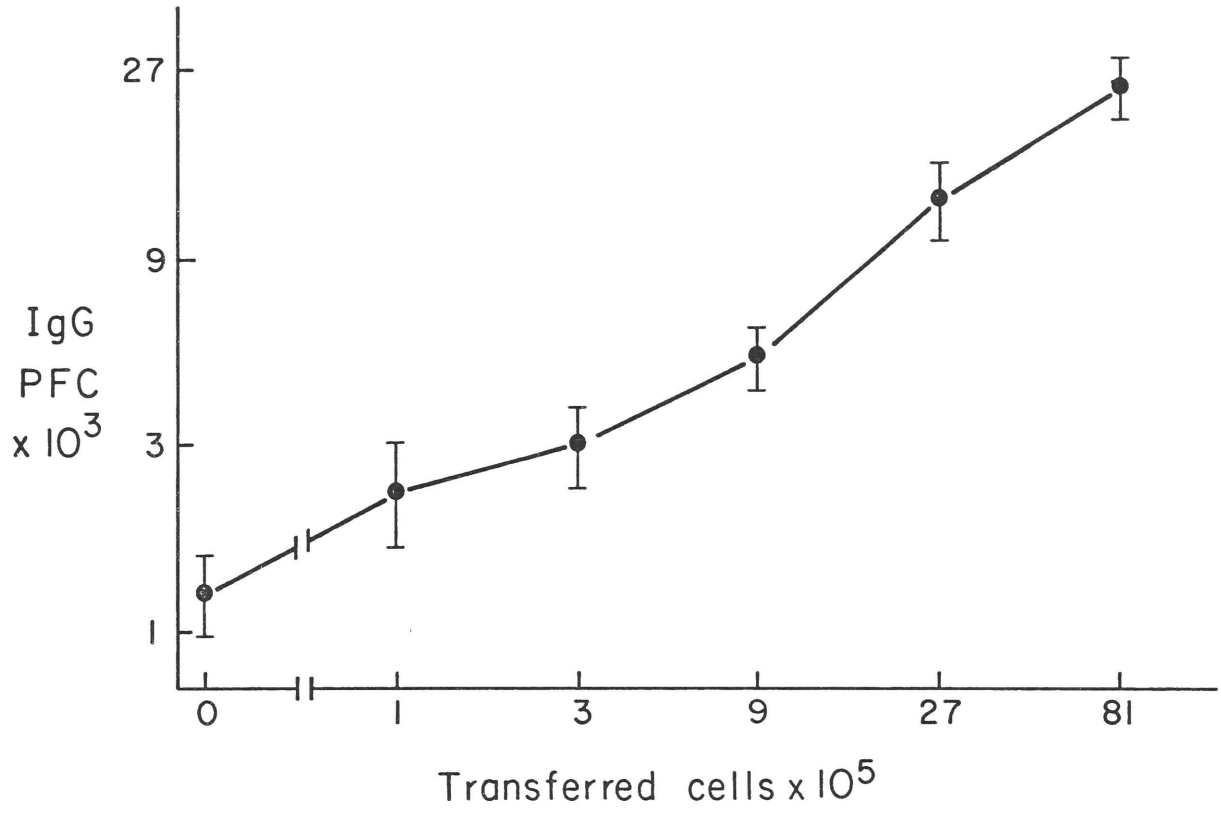

Figure 9. Efficiency of immune response of unfractionated spleen cells after transfer to irradiated recipients. The indicated number of spleen cells from mice immunized with Dnp-Hcy was mixed with 10^7 cells from mice immunized with Hcy and injected intravenously into an irradiated syngeneic recipient. The recipient mouse was then immunized with Dnp-Hcy. The spleen of each recipient was assayed for PFC 7 days later. Each point represents the average of data from 3 mice; standard errors of the mean are indicated by the bars.

at least the antigen-sensitive B cells are functioning normally. As expected of cells from immunized mice, enhancements were not observed in the IgM response. It is noteworthy that in several experiments the Dnp-specific IgM response of 5×10^6 unfractionated Hcy cells was partially suppressed in the presence of 2×10^4 unfractionated Dnp-Hcy cells, but not in the presence of 2×10^4 Dnp-fractionated cells from the same Dnp-Hcy population (Table VII).

Cross-reactivity among antigen-binding cells. One to two percent of the cells in the spleen of a non-immune adult mouse are capable of binding specifically to Dnp-coated fibers, as noted in Table V. Previous work has shown, however, that only the subpopulation of these cells that binds the antigen with relatively high avidity is stimulated to proliferate following immunization with Dnp (Rutishauser et al., 1972). In order to define more precisely the role of the remaining lower avidity cells in the repertoire, the relationship between antigen-binding specificity and avidity were examined for cells from immune and non-immune adult mice.

The relative avidity with which a lymphocyte binds to a soluble antigen can be estimated from the concentration of conjugate required to inhibit binding to a fiber coated with the antigen. If a small amount of conjugate is present, only cells with high avidity will be prevented from binding to the fiber. As the amount of soluble antigen is increased, lower avidity cells will also be inhibited from binding. The progressive inhibition of fiber-binding by increasing amounts of soluble antigen thus provides a measure of the distribution of relative avidities within the population of antigen-binding cells.

Spleen cells from non-immune adult Swiss-L mice were incubated with antigen-coated fibers in the presence or absence of soluble antigen, and the relative numbers of cells bound under these conditions were determined (Table VIII). Binding to fibers coated with a given hapten-BSA conjugate was inhibited in the presence of 100 µg/ml of soluble conjugate. In addition, however, extensive cross-inhibition was observed in several cases. Binding to arsanilate-BSA fibers, for example, was inhibited nearly as effectively by soluble sulfanilate-BSA as it was by soluble arsanilate-BSA.

Table VIII

Cross-Inhibition of Fiber-Binding

Fiber antigen	Percent inhibition of binding by soluble antigen*		
	p-aminobenzoate-BSA	arsanilate-BSA	sulfalinate-BSA
p-aminobenzoate-BSA	35%	5%	21%
arsanilate-BSA	15%	51%	24%
sulfanilate-BSA	27%	37%	45%

*) Numbers of cells bound in assays carried out in the presence of 100 µg/ml of hapten-BSA conjugate were compared with the numbers obtained in parallel assays carried out in the absence of soluble antigen.

Cross-reactivity at the level of the antigen-binding cells has been compared for immune and nonimmune animals for the antigens Dnp and sulfanilate (Figure 10). Spleen cells from normal and Dnp-immune Balb/c mice were incubated with Dnp-BSA fibers in the presence of various amounts of soluble Dnp-BSA or sulfanilate-BSA. In the case of cells from nonimmune adults, 50% of the binding to Dnp-coated fibers was inhibited by 0.6 μg/ml of soluble Dnp-BSA, while 6 μg/ml of sulfanilate-BSA was needed for comparable inhibition. Following immunization with Dnp, 0.005 μg/ml of soluble Dnp-BSA inhibited 50% of fiber binding, indicating that the average avidity of the cell population for Dnp had increased substantially. The avidity of these cells for sulfanilate, however, was indistinguishable from that of non-immune cells.

All of these experiments established that the fiber method could be used to study antigen-binding cells during development. Attention was therefore turned to an extensive analysis os antigen-binding cells in fetuses.

Ontogeny of Specific Antigen-Binding Cells

Quantitation of antigen-binding cells as a function of age. The fiber-binding assay was used to estimate the frequency of spleen and liver cells from Swiss-L mice specific for each of a variety of antigens as a function of age. Several control experiments demonstrated the specificity of this assay for the detection of antigen-binding cells in fetal and adult tissues (Table IX). If the assay was done in the presence of 400 μg/ml of a given soluble antigen-BSA or antigen-Hcy conjugate, or in the presence of 400 μg/ml of rabbit antibodies against mouse Ig, 50-95% of binding to fibers coated with that antigen was inhibited. Underivatized BSA caused less than 10% inhibition. These experiments suggest that the specificity of binding to hapten-BSA conjugates on the fibers was primarily for the hapten moieties and not for the BSA. For any given cell type, the number of cells bound in the assay was linearly proportional to the number of cells in the incubation mixture, from 10^6 to 10^8 cells/dish. Direct comparison of the numerical results obtained from the fiber assay with those of the rosette assay (Spear et al., 1973) is difficult because the two assays differ somewhat in the efficiency and sensitivity with which they detect antigen-specific cells. When cell suspensions from mice of various ages were tested for Tnp-specific cells by both methods, however, and the numbers of antigen binding cells obtained were expressed as percentages of the adult

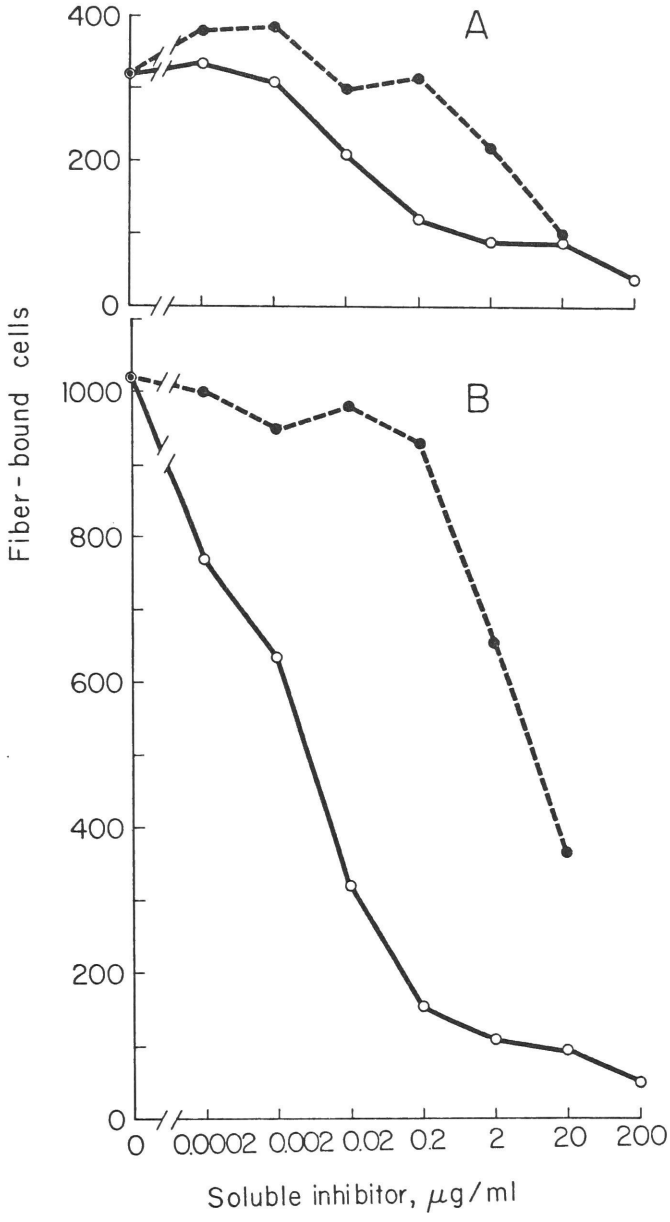

Figure 10. Cross-reactivity between Dnp-specific and sulfanilate-specific lymphocytes. 10^7 spleen cells from non-immune (A) or Dnp-immune (B) animals were incubated with fibers derivatized with Dnp-BSA. The incubations were carried out in the presence of various concentrations of soluble Dnp-BSA (-O-) or soluble sulfanilate-BSA (••●••). The number of cells bound to a 10 cm segment of fiber was determined in each case. Each point represents the results of one fiber-binding assay carried out at a particular inhibitor concentration.

Table IX

Fiber-Binding Cells in Fetal and Adult Mice

Antigen	17-day fetal liver*			18-day fetal spleen*			Adult spleen*		
	FBC/10^7 cells	Inhibition by:		FBC/10^7 cells	Inhibition by:		FBC/10^7 cells	Inhibition by:	
		Antigen‡	Anti-Ig§		Antigen‡	Anti-Ig§		Antigen‡	Anti-Ig§
		%			%			%	
p-Aminobenzoic acid	31 ± 2¶	75	74	880 ± 220¶	81	77	4,380 ± 660¶	85	81
Sulfanilic acid	23 ± 3	81	69	1,340 ± 280	84	81	4,800 ± 320	91	74
5-Aminoquinoline	—	—	—	360 ± 20	71	78	4,060 ± 860	56	89
p-Aminophenyl-β-D-lactoside	—	—	—	220 ± 40	72	79	2,860 ± 320	57	82
p-Toluenesulfonic acid	—	—	—	300 ± 120	76	85	800 ± 100	84	74
p-Iodobenzoic acid	18 ± 3	72	67	800 ± 60	80	70	4,320 ± 1,120	60	94
Fluorescein	—	—	—	280 ± 190	76	83	2,840 ± 240	56	90
Trinitrophenol	—	—	—	520 ± 120	81	68	2,620 ± 280	92	91
Adenosine monophosphate	—	—	—	540 ± 140	71	76	4,040 ± 700	80	87
Myoglobin	—	—	—	480 ± 100	66	69	2,480 ± 540	70	78
Lysozyme	—	—	—	8,280 ± 1,380	—	78	19,780 ± 2,100	—	75

*) The liver cell suspension used in any one experiment was prepared from one litter of mice (9-11 fetuses); spleen cell suspensions were prepared from one to three litters of fetal mice, or from two adult mice.

‡) Calculated from the results of parallel fiber-binding assays performed in the presence or absence of 400 µg/ml of soluble antigen-BSA conjugate.

§) Calculated from the results of parallel fiber-binding assays performed in the presence or absence of 400 µg/ml of rabbit antibodies against mouse Ig.

¶) Cells bound per 25 cm of fiber, expressed as the mean ± SEM, based on three or more independent determinations.

values, the results of the two assays were identical within experimental error.

The numbers of cells in the livers and spleens of Swiss-L mice which bound to antigen-coated fibers (fiber-bound cells) as a function of age are shown in Figure 11. For most of the 19-day gestation period, these embryos had no detectable antigen-binding cells. In all cases, specific antigen-binding cells were first detected in the liver on the 15th day of gestation (Figure 11A). Their numbers increased in parallel to a peak on the 17th day of gestation, and they disappeared again by birth or one day postpartum. This process was coincident with the transient appearance of large numbers of nonparenchymal cells in the liver (Figure 11A). In the spleen, specific antigen-binding cells were first detected on the 16th day of gestation (Figure 11A, B, C). The number of antigen-binding cells per spleen increased rapidly and in parallel for all antigens until about 1 wk after birth, and then more gradually to adult levels, reflecting increases both in the frequency of antigen-binding cells in the spleen cell population (Table IX) and in the total number of cells in the spleen (Figure 11). Neonatal thymuses and bone marrow both lacked cells capable of binding to antigen-coated fibers.

Measurements of the numbers of fiber-bound cells in the spleens of Balb/c mice as a function of age gave results closely parallel to those obtained with the Swiss mice. Cells specific for each of two antigens, p-aminobenzoate and Tnp, could first be detected three days before birth, and the numbers of these cells per spleen increased rapidly until about a week after birth, and then more gradually to adult levels.

In order to estimate the percentages of B and T cells in the population of antigen-binding cells, spleen cells from Swiss mice, bound to p-aminobenzoate-coated fibers, were treated with antisera against mouse Ig or the θ(C3H) antigen. Of the fiber-bound cells from 18-day fetuses, 20-30% were killed by anti-θ serum (T cells) and 45-50% were killed by anti-Ig serum (B cells), comparable to values determined in parallel experiments on fiber-bound adult spleen cells (20-25% θ-sensitive, 45-50% Ig-sensitive).

Response of fetal liver cells to antigenic stimulation in vitro. In order to evaluate the functional properties of the antigen-binding cells

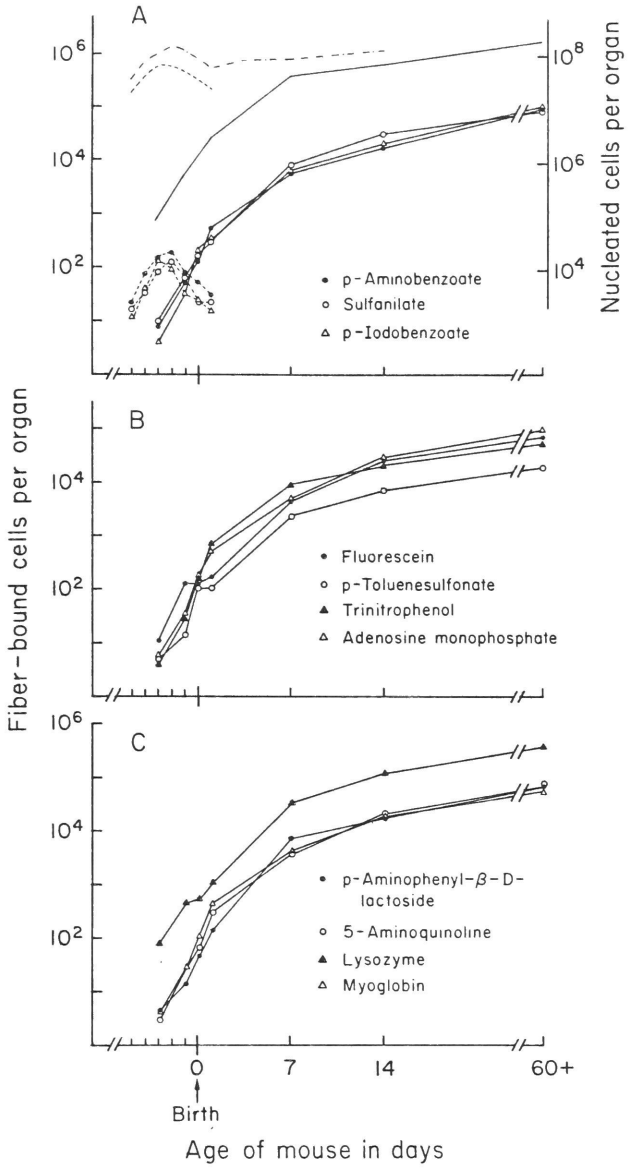

Figure 11. Numbers of fiber-bound cells per organ specific for each of 11 antigens, in the spleens (——) and livers (---) of Swiss-L mice as a function of age. These numbers were calculated from the numbers of cells bound per 25 cm of fiber and the numbers of nucleated cells per organ as described in materials and methods. Each value shown is the mean of two or more independent determinations. The numbers of nucleated cells recovered per,spleen (——) and per liver (---) as a function of age are shown, together with the total number of nucleated cells per liver (-·-·-). This last number was estimated from hemocytometer counts of liver cell suspensions which had not been washed to remove damaged cells, and therefore includes both hemopoietic and parenchymal cells.

found in fetal liver, the response of liver B cells to antigen in vitro was determined. Liver cells obtained from Swiss-L fetuses on the 17th day of gestation were cultured in the presence of antigen (SRBC), or bacterial lipopolysaccharide, or both. In the presence of both agents, cells secreting antibodies of the IgM class specific for SRBC were generated (Figure 12). The number of these cells was maximal 4 to 5 days after the initiation of the cultures. Antigen alone, or lipopolysaccharide alone, failed to elicit the appearance of antibody-forming cells.

Relative avidities of antigen-binding cells. Experiments were carried out to compare the ranges of avidities for several antigens in fetal and adult antigen-binding cell populations. As described above, the relative avidity with which a lymphocyte binds to a soluble hapten-protein conjugate can be estimated from the concentration of soluble conjugate required to inhibit binding to a fiber coated with the antigen.

To characterize the distributions of avidities among fetal and adult cells specific for sulfanilic acid, fiber-binding assays were carried out in the presence of various concentrations of soluble sulfanilate-BSA. Cells from the livers of 17-day fetuses and the spleens of 18-day fetuses were tested in parallel experiments with cells from adult spleens (Figure 13). Both fetal spleen cells (Figure 13A) and fetal liver cells (Figure 13B) gave avidity distributions that were identical within experimental error to those obtained with normal adult spleen cells. In contrast, spleen cells from an adult immunized against sulfanilate were greatly enriched in high avidity cells. As little as 0.003 μg/ml of soluble sulfanilate-BSA inhibited 50% of immune cell binding, while 0.3 μg/ml was necessary for comparable inhibition of binding by cells from unimmunized animals (Figure 13A). The relative avidity distributions of spleen cells from fetuses and adults were compared for three additional antigens, toluenesulfonate (tosyl), p-aminobenzoate, and Tnp, and for each of these three antigens the fetal and adult cells gave closely similar distributions (Figure 14).

Antigen-Binding Cells in Single Fetal Mice

All of the experiments described above were performed with pools of

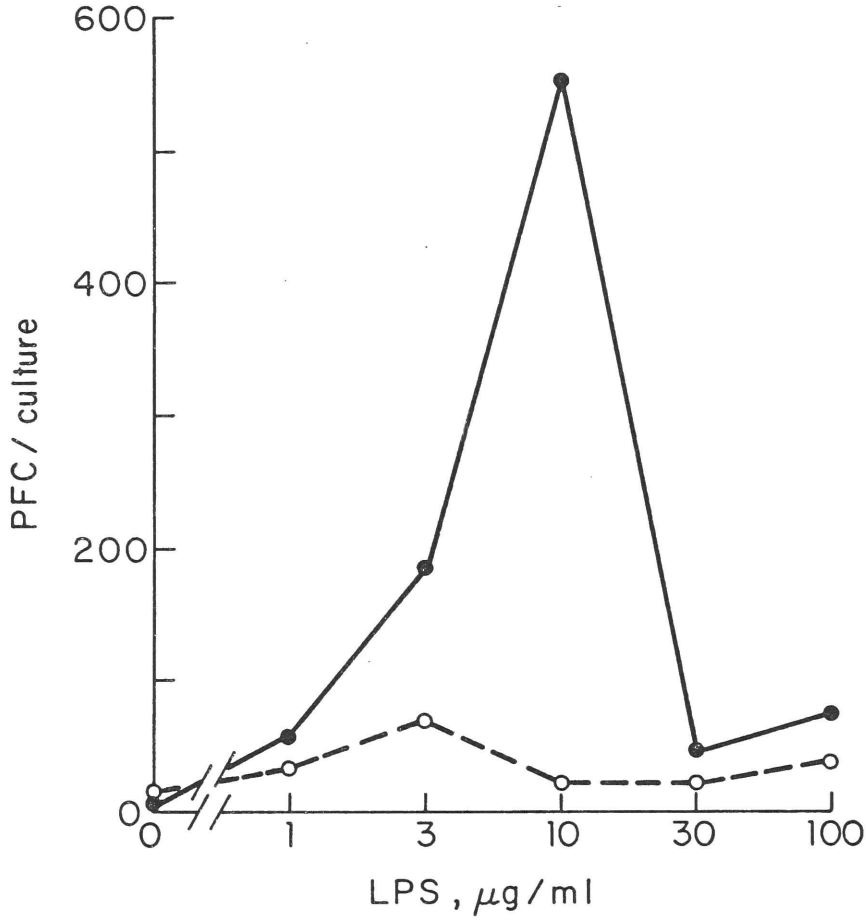

Figure 12. Effect of antigen and bacterial lipopolysaccharide on the induction of PFC in vitro. 2 x 10^7 viable cells obtained from the livers of Swiss-L fetal mice on the 17th day of gestation were cultured with various amounts of lipopolysaccharide in the presence (●) or absence (O) of SRBC as a specific antigenic stimulus. The cultures were assayed for PFC on the 5th day of incubation.

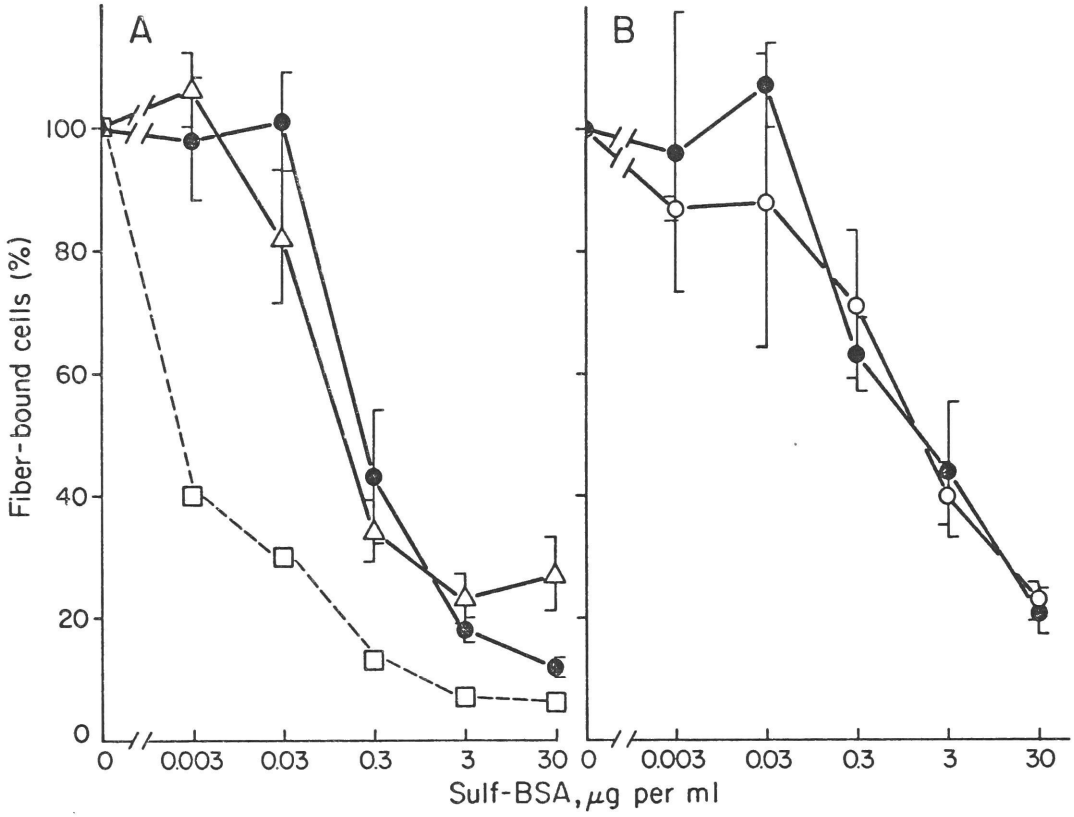

Figure 13. Number of cells bound to sulfanilate-coated fibers in the presence of sulfanilate-BSA at several concentrations, expressed as a percentage of uninhibited control values. Cells from fetal spleen (A) and fetal liver (B) were compared with cells from adult spleen in parallel experiments. Liver cells were obtained from fetuses on the 17th day of gestation (○). Spleen cells were obtained from fetuses on the 18th day of gestation (△) or from adults (●). Each value shown for these cells is the arithmetic mean of 3 or more independent determinations ± SEM. Spleen cells from adult mice immunized against sulfanilate (2 intraperitoneal injections, one month apart, of sulfanilate-Hcy adsorbed on bentonite) were assayed in the same way(□). The results of a typical experiment are shown.

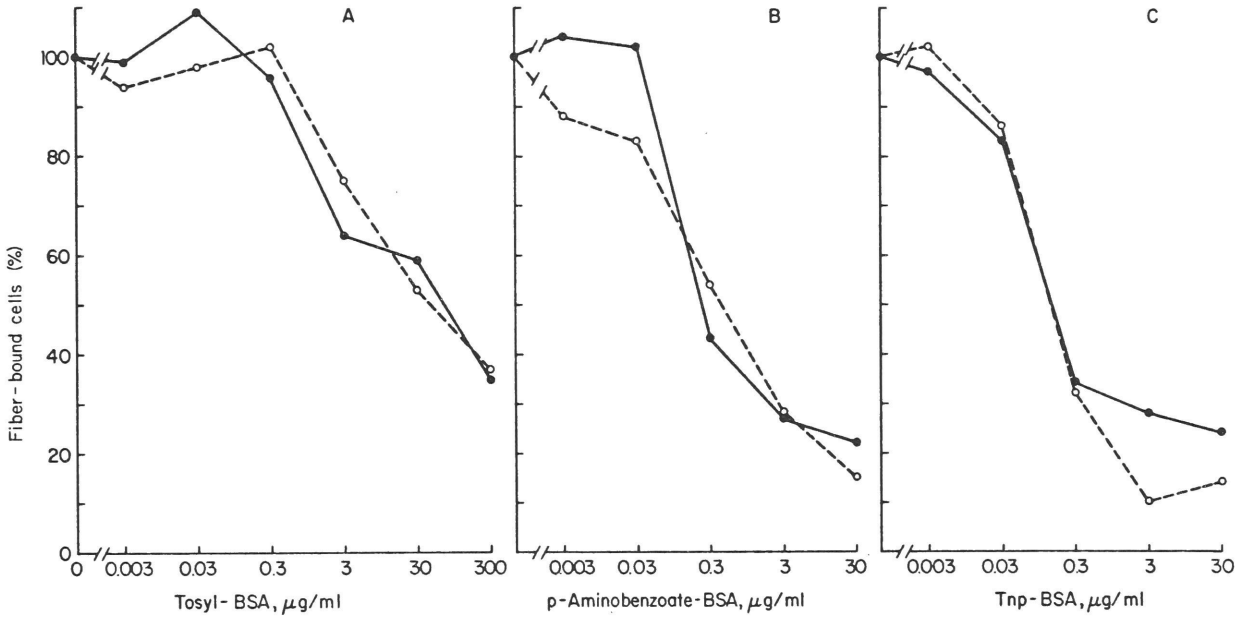

Figure 14. Numbers of spleen cells bound to antigen-coated fibers in the presence of various concentrations of soluble antigen, expressed as a percentage of uninhibited control values. Cells from fetal (●) and adult (○) spleens were compared in parallel experiments. The antigens used were tosyl-BSA (A), p-aminobenzoate-BSA (B), and Tnp-BSA (C).

cells obtained from as many as 50 mice. To determine the extent of the variation from animal to animal, which would be obscured in the large cell pools, spleen cell suspensions from individual 18-day fetuses were tested for cells specific for each of two antigens, Tnp and SRBC. The rosette assay was used for these experiments because its very high efficiency (Elliot and Haskill, 1973) allowed accurate measurements of the numbers of antigen-specific cells in these small cell populations.

Numbers of RFC in individual fetal spleens (Table X and Figure 15). One hundred and two Swiss-L fetuses, obtained from 19 different litters, were examined for Tnp-specific RFC, and 44 fetuses from 11 different litters were examined for SRBC-specific RFC. For each antigen, the mean number of RFC per spleen, calculated from these measurements on individual fetal spleens (Table X) was in agreement with the corresponding numbers previously determined for pooled fetal spleens (Spear et al., 1973). For both antigens, the numbers of RFC measured in individual fetal spleens were unimodally distributed about these mean values (Figure 15).

To measure the variation in number of antigen-binding cells from fetus to fetus within any one litter, fetuses from several litters of Swiss-L mice were assayed individually for Tnp-specific or SRBC-specific RFC (Table X). For each antigen, the mean numbers of RFC observed for each of the litters were not significantly different from each other or from the means for the population as a whole. Further, the amount of variation about these means shown by individuals from any one litter was as great as that shown by the population as a whole. Spleens from Balb/c and CBA/J fetuses were assayed for Tnp-specific and SRBC-specific RFC (Table XI). As for the outbred Swiss-L mice, for each antigen and each strain of inbred mice, the values given by the individual fetuses were broadly distributed about the means for the populations of fetuses. As much variation was observed within single litters as in the whole populations.

Frequency of RFC in individual Swiss-L fetal spleens. To determine the extent to which the variation in the number of RFC per spleen was due to variation in spleen size, individual Swiss-L fetal spleens were assayed for RFC as a function of spleen size (Figure 16). The total

Table X

Rosette-Forming Cells in the Spleens of Swiss-L Fetuses

Antigen	Litter	Mean RFC/spleen*	Standard deviation†
Tnp	#1 (10 fetuses)	198	121
	#2 (9 fetuses)	264	203
	all (102 fetuses)	265	190
SRCB	#1 (4 fetuses)	45	25
	#2 (6 fetuses)	27	18
	all (44 fetuses)	38	30

*) For each antigen, two-tailed Student's t tests indicated no significant differences between the mean values observed for the two litters, or the mean value for each litter and that for the whole population ($P > 0.1$ in each case).

†) For each antigen, two-tailed F tests indicated no significant differences between the variances observed for each litter and that for the whole population ($P > 0.05$ in each case).

Table XI

Numbers of RFC in the Spleens of Inbred Fetuses

Strain	Number of RFC per spleen*	
	Tnp	SRBC
CBA/J	99 ± 62	20 ± 5
Balb/c	70 ± 52	12 ± 8

*) Each number is the mean of the values obtained for 3 - 13 individual fetuses, ± the standard deviation of the mean.

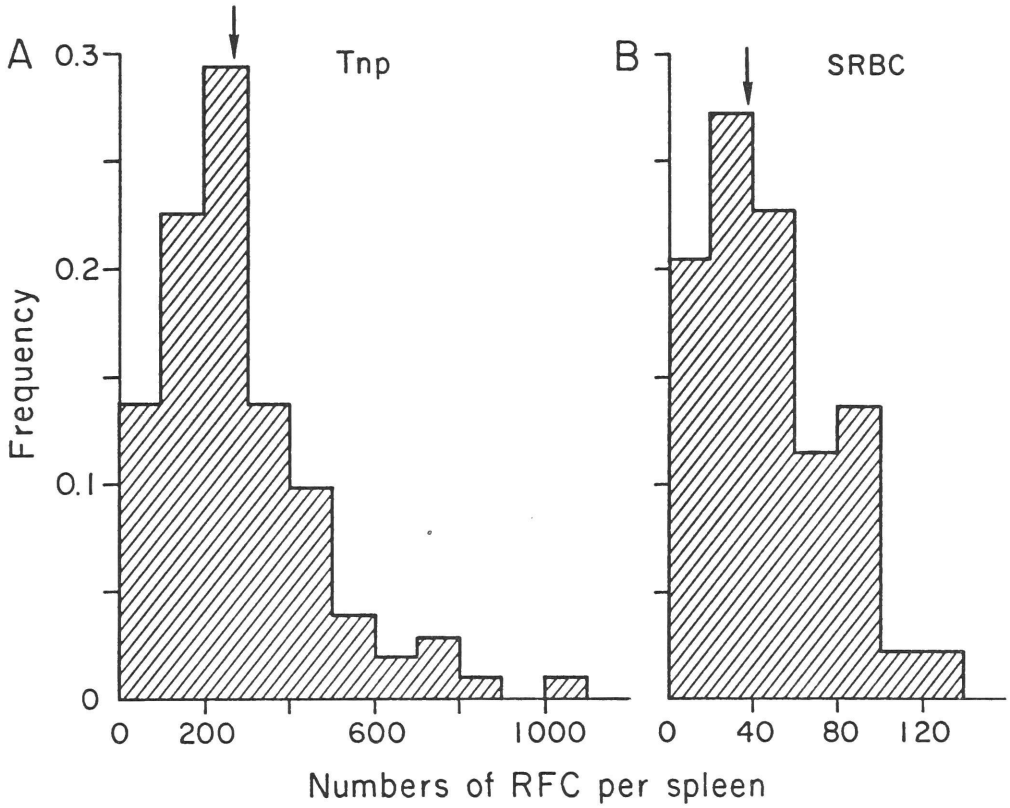

Figure 15. Frequency distributions of the numbers of RFC in single fetal spleens. Spleens from individual 18-day fetuses were assayed for RFC, using Tnp-coated SRBC (A) or plain SRBC (B) as the test antigens. The mean values observed for each antigen are shown by the arrows. See Table X.

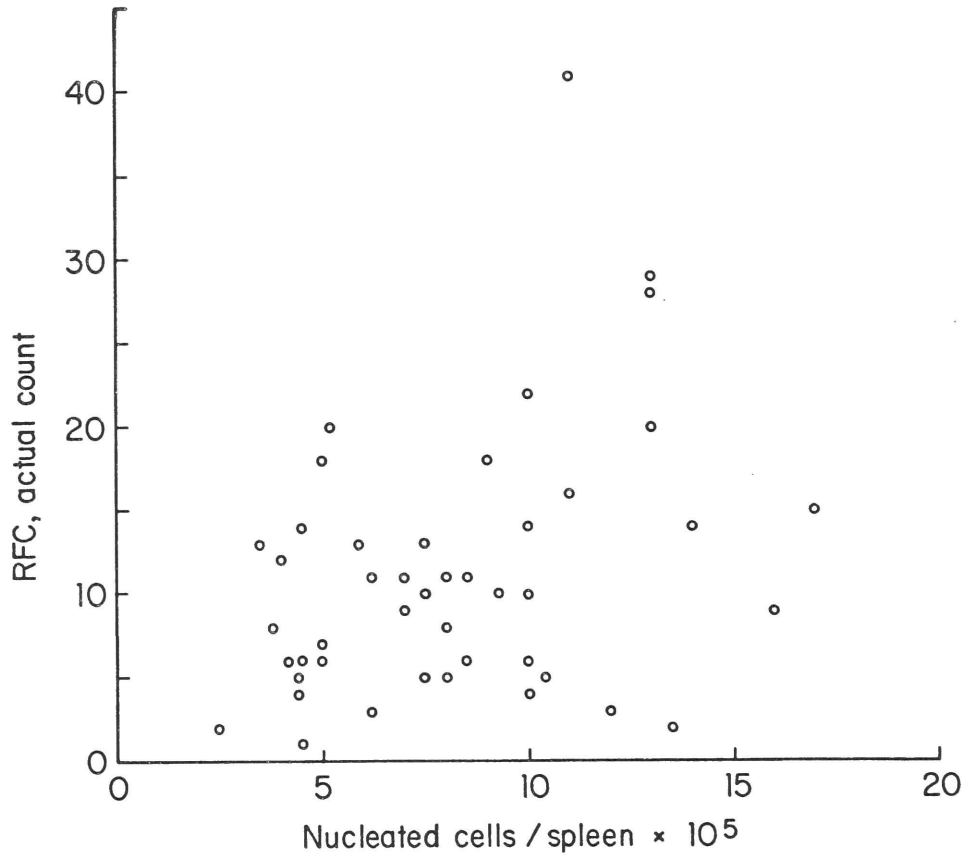

Figure 16. Frequency of Tnp-specific RFC in the spleens of individual 18-day Swiss-L fetal mice. In each case, an aliquot corresponding to 1/20 of the spleen was scanned for RFC and the number of RFC found was plotted as a function of the total number of nucleated cells in the spleen. Each point represents the results obtained for one spleen.

number of nucleated cells in the spleen was taken as an index of its size. Forty-five spleens, from a total of 9 litters, were assayed. No simple correlation could be found between the number of RFC per spleen and the number of nucleated cells per spleen. As much variation was seen between littermates as between fetuses from different litters.

Frequencies of RFC in individual Balb/c and CBA/J fetal spleens. Individual inbred fetuses of the CBA/J and Balb/c strains were then assayed in the same way for numbers of RFC and numbers of nucleated cells per spleen. Assays were carried out both for Tnp-specific and for SRBC-specific RFC (Table XII), and the individual variation in these measurements was assessed by the use of 2 x C contingency tables (Table XIII). Comparison of the frequencies of Tnp-specific RFC found for CBA/J fetuses from any one litter showed no variation from individual to individual beyond that to be expected from sampling error alone (Table XIII; tests a and b). Comparison of individuals from different litters again showed no variation beyond that expected from sampling error (Table XIII; tests c and d). The same low variation was observed when Balb/c fetuses were tested for Tnp-specific RFC, both within and between litters (Table XIII; tests e, f, and g). A highly significant difference was observed, however, when the frequency of Tnp-specific RFC in CBA/J fetuses was compared with that in Balb/c fetuses, as shown in Table XIII (test h) and in Figure 17.

Similarly, the observed frequency of SRBC-specific RFC was constant within sampling error for all individuals of each strain, but differed slightly between strains (Table XIII; tests i, j, and k).

The numbers of Tnp-specific and SRBC-specific RFC per 10^5 nucleated cells, calculated for fetuses of each strain, are shown in Table XIV. For each antigen, the frequency of RFC found in CBA/J fetal spleens was 4-5 times greater than the frequency found in Balb/c spleens. Despite this difference between the two strains in the frequencies of RFC, however, the relative proportions of Tnp-specific and SRBC-specific RFC were the same for both strains within sampling error.

Because the frequencies of RFC in individual Swiss-L fetuses specific for any one antigen were highly variable (Figure 16), it was

Table XII

Numbers of Nucleated Cells and RFC in Single Fetal Spleens

A) Tnp-Specific RFC

Strain	Litter	Fetus	(Number of aliquots examined)	RFC found*	Nucleated cells found*
CBA/J	1 (26)**	1	(2)	23	128
	2 (26)	1	(2)	18	86
		2	(2)	16	85
		3	(2)	7	33
		4	(2)	9	54
	3 (26)	1	(4)	10	59
		2	(4)	8	32
		3	(4)	6	34
	4 (26)	1	(4)	29	159
		2	(4)	15	116
	5†(5)	1	(2)	5	26
		2	(2)	6	24
	6†(26)	1	(3)	16	75
Balb/c	1 (26)	1	(6)	12	381
		2	(6)	10	289
	2†(5)	1	(2)	7	226
		2	(2)	2	80
	3†(5)	1	(4)	19	412
		2	(4)	32	582

(Table XII, continued)

B) SRBC-specific RFC

Strain	Litter	Fetus	(Number of aliquots examined)	RFC found*	Nucleated cells found*
CBA/J	1 (7)	1	(8)	4	42
	2 (7)	1	(6)	4	159
	3 (7)	1	(6)	6	112
		2	(2)	0	26
	4†(7)	1	(6)	5	157
Balb/c	1†(7)	1	(6)	7	817
		2	(6)	5	448

*) Actual counts. For detection of RFC, 25 µl portions of the rosette assay mixture were examined as described in Materials and Methods. One aliquot was taken to be one 25 µl drop. For detection of nucleated cells, 1:1 mixtures of the rosette assay suspension and 1% acetic acid were prepared and loaded into hemocytometers. One aliquot was taken to be 0.1 µl, measured by means of the hemocytometer calibrations. In each case, the number shown is the total number of RFC or nucleated cells found in all the aliquots scanned for that fetus. To estimate cells/spleen: nucleated cells/spleen = (nuc. cells found x 10^4)/(number of aliquots); RFC/spleen = (RFC found x 20)/(number of aliquots).

†) Counted blind. Labels on the assay mixtures were changed after RFC counts were done; the experimenter did not learn from which fetus each assay mixture had come until all counts were completed.

**) Number in parentheses indicates the serial number of the sheep whose blood cells were used, plain or derivatized with Tnp. The homogeneity of results for Tnp-SRBC assays using cells from different sheep indicates that the antigen detected in these assays is Tnp only.

Table XIII

Analysis of Variation in RFC Frequency

Antigen	Test*	degrees of freedom	χ^2	
Tnp	a) CBA litter 2 (fetuses 1-4)	3	0.3226	$0.95 < P < 0.98$
	b) CBA litter 3 (fetuses 1-3)	2	0.6180	$0.50 < P < 0.75$
	c) CBA litters 2 and 3	6	0.9398	$0.98 < P < 0.99$
	d) CBA litters 1-6	12	3.5478	$0.99 < P < 0.995$
	e) Balb litter 1 (fetuses 1, 2)	1	0.0467	$0.90 < P < 0.95$
	f) Balb litter 2 (fetuses 1, 2)	1	0.0698	$0.75 < P < 0.90$
	g) Balb litters 1-3	5	4.9129	$0.25 < P < 0.50$
	h) All Balb litters vs all CBA litters	18	137.8438	$P << 0.005$
SRBC	i) CBA litters 1-4	4	5.7531	$0.10 < P < 0.25$
	j) Balb litter 1	1	0.2030	$0.90 < P < 0.95$
	k) All CBA litters vs Balb litter 1	6	28.4575	$P < 0.005$

*) 2 x C contingency tables were evaluated as described by Snedecor and Cochran (1967), pages 238 ff. Test "b" from the table is shown in detail on the next page.

(Table XIII, continued)

CBA/J fetuses assayed for Tnp-RFC and nucleated cells (litter no. 3).

Null hypothesis: the ratios of RFC : nucleated cells in each case are estimates of the same quantity.

	(fetus)	(1)	(2)	(3)	Total
Observed (f)	RFC found	10	8	6	24
(from Table XII)	nuc. cells found	59	32	34	135
	Total	69	40	40	149

					Total
Expected (F)	RFC expected	11.114	6.443	6.443	24
On the null hypothesis, the ratio in each fetus would be expected to be the same as the ratio for the whole population, hence in each case:	n. c. expected	57.886	33.557	33.557	125
	Total	69	40	40	149

$$F=\frac{(\text{column total})\times(\text{row total})}{(\text{grand total})}$$

Deviations (f-F)	-1.114	+1.557	-0.443
	+1.114	-1.557	+0.443

$$\chi^2 = \sum \frac{(f-F)^2}{F} = \frac{(-1.114)^2}{11.114} + \frac{(1.114)^2}{57.886} + \ldots + \frac{(0.443)^2}{33.557} = 0.6180$$

Number of degrees of freedom = (no. rows - 1) x (no. columns - 1) = 2

$\chi^2 = 0.6180$; degrees of freedom = 2; $0.50 < P < 0.75$. That is, this much variation (or more) would be expected due to sampling error alone between 1/2 and 3/4 of the time.

Table XIV

Frequency of Rosette-Forming Cells in Fetal Spleens*

	Tnp-RFC per 10^5 cells	SRBC-RFC per 10^5 cells	Tnp-RFC: SRBC-RFC †
CBA/J	38.7 ± 6.7	8.2 ± 7.1	4.7
Balb/c	7.4 ± 2.2	2.0 ± 0.4	3.7

*) Calculated from the data in Table XII. Each entry is the mean value for all fetuses tested, ± the standard deviation of the mean.

†) Analysis of the individual data in Table XII for degree of association, as described by Fleiss (1973; page 115), indicates that there is no variation in the ratios (Tnp-RFC per 10^5 cells) : (SRBC-RFC per 10^5 cells) between CBA/J and Balb/c fetuses beyond that expected from sampling error alone.

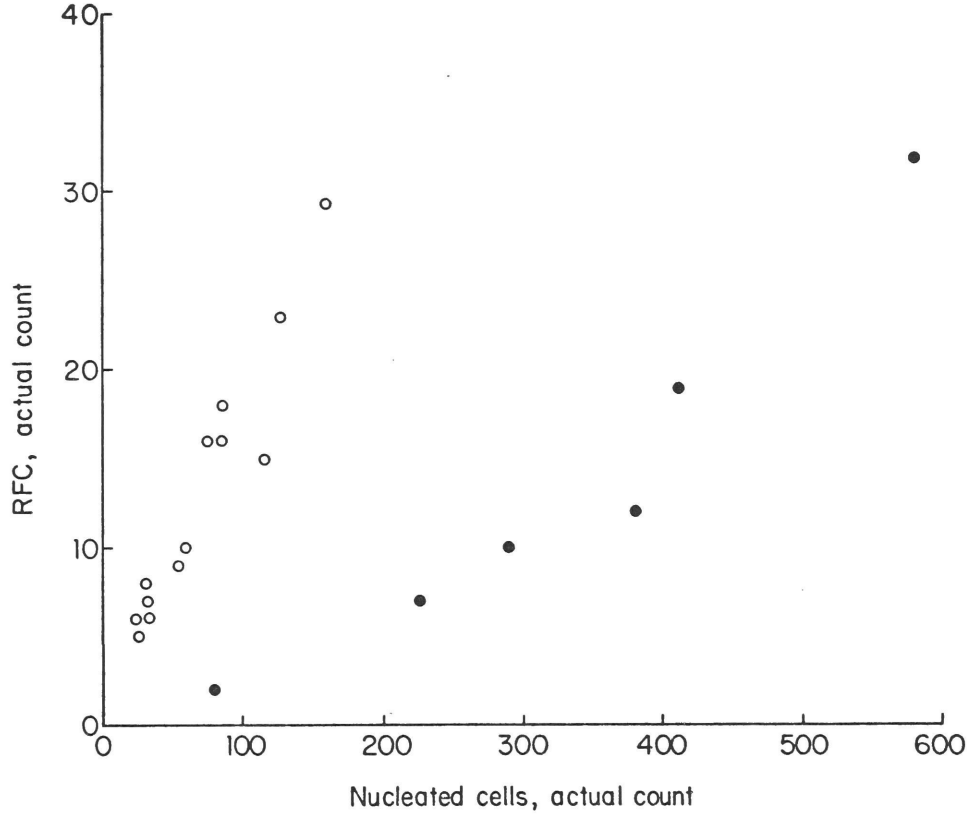

Figure 17. Frequency of Tnp-specific RFC in the spleens of individual 18-day CBA/J (O) and Balb/c (●) fetal mice. Spleens were assayed as described in Table XII; each point represents the results obtained for one spleen.

necessary to measure directly the relative frequencies of RFC specific for each of the two antigens in any one fetal spleen. In these experiments, the cell suspension from a single fetal spleen was divided into two equal portions, one of which was assayed for Tnp-specific RFC, while the other was assayed for SRBC-specific RFC. The ratio of Tnp-specific cells to SRBC-specific cells could therefore be calculated for each spleen. A total of 33 fetuses were tested in this fashion. The individual spleens contained on the average 6.3 times as many Tnp-specific cells as SRBC-specific cells, suggesting that the relative scarcity of SRBC-specific cells in the fetal population as a whole was reflected in the individual fetuses.

DISCUSSION

Because the approach to the quantitation of antigen-binding cells in ontogeny had not been attempted before on an extensive scale or at high precision, it was necessary to test the assumptions of new methodology with some care. In this discussion, I consider this subject before turning to the implications of the data on ontogeny.

Quantitation of Specific Antigen-Binding Cells

In order to analyze the ontogeny of specific antigen-binding cells, uniform methods are required for the detection of cells of each of a wide range of antigenic specificities. It has been shown that the fiber-binding assay provides a general, rapid method for isolating and characterizing antigen-binding T and B cells (Edelman et al., 1971; Rutishauser et al., 1972; Rutishauser and Edelman, 1972). Any of a wide variety of antigens can be coupled to the fibers and, inasmuch as the antigens are presented as uniform coatings on the fibers, physical differences between them are minimized, facilitating direct comparison of the results obtained with different antigens. Moreover, the detection of antigen-binding cells is not affected by the presence of small amounts of cellular debris during the assay, so that cell suspensions can be assayed without extensive preliminary fractionation. These factors make the fiber assay especially suitable for measuring rates of appearance and properties of antigen-binding cells in the course of development. Moreover, these analyses have interesting implications for understanding the nature of clonal selection of antigen-binding cells in the immune response.

In order to use the assay for such studies, it was necessary first to establish the immunological relevance of these fiber-bound cells. In particular, methods were developed for recovering viable cells from the fibers, and it was then shown that the recovered cells were highly enriched with respect to their antigen-binding specificity, and that they were normally responsive to a specific antigenic stimulus.

Recovery of viable cells. Removal of the fractionated cells from the fiber without damage depends on several factors. It has previously been noted that cells specifically bound to derivatized fibers cannot be

released by the addition of a competitive binding inhibitor (Edelman et al., 1971). It was suggested that, after being specifically bound, the cell membrane forms secondary attachments to the surface of the fiber, and therefore other physical means of removal were adopted. The physical methods using taut fibers required shearing of the cell-fiber bond, which can produce a lesion in the cell surface membrane. Damage to the cells is minimized by either (1) using a "soft" gelatin-coated fiber that presumably minimizes the shear required for release, or (2) avoiding shear forces altogether by melting the cells off a gelatin-coated fiber, and, in all cases, (3) using the appropriate medium and incubation conditions to allow repair of any lesions produced by the removal process. With these methods, fractionated cell populations with high viabilities can be obtained routinely from either derivatized or gelatin-coated fibers.

Rebinding of fractionated cells to antigen-coated fibers. A radical rearrangement or even removal of specific immunoglobulin receptors can result from the incubation of antigen-binding cells with soluble antigen or soluble anti-Ig (Taylor et al., 1971; Yahara and Edelman, 1972). It might be expected, therefore, that the specific binding of lymphocytes to antigen-derivatized fibers would cause similar effects. Previous studies (Rutishauser et al., 1972), however, showed that up to 50% of the cells bound to Dnp-fibers were capable of forming uniform rosettes in situ when incubated with cross-reacting 2,4,6-trinitrophenyl-derivatized erythrocytes. This suggested that, for these cells, the binding event did not induce a radical rearrangement of all the receptors. The failure of the remaining cells to form rosettes may reflect differences in the relative sensitivities of the rosette and fiber-binding assays, or in the antigen-binding properties of T and B cells.

Experiments based on the rebinding of fiber-fractionated cells to fibers of the same specificity circumvent many of the ambiguities encountered with the fiber-rosette assay. The results indicate that the isolated cells, even after plucking, were not grossly impaired in their ability to bind antigen. The conditions of the rebinding assays (incubation at 4^{o} for no more than 2 hr) do not allow time for a significant

amount of synthesis or rearrangement of receptors. These experiments indicate, then, that at least some receptors remain on or in the cells during fractionation.

Analysis of the enrichment obtained in the rebinding experiments indicates that most of the fractionated cells have hapten-specific receptors. Dnp-fractionated cells from both unimmunized and immunized animals were re-bound to antigen-coated fibers with equivalent efficiencies, consistent with the conclusion that in each case the population of cells recovered from the fibers is nearly a pure population of Dnp-specific cells.

Response of fractionated cells to antigenic stimulation. The stimulation by antigen of the fractionated antigen-binding B cells to differentiate into antibody-secreting cells suggests that their functional properties were not altered by the fractionation procedure. The data are consistent with the idea that the proportion of Dnp-sensitive B cells in the population of Dnp-binding cells is the same before and after fractionation. The magnitude of the response obtained with Dnp-fractionated cells is well correlated with the expected enrichment and with the results obtained in rebinding experiments.

The unusual exponential relationship between the number of cells transferred and the response of the recipient (Figure 9) remains unexplained. A number of phenomena could produce such an effect, such as competition for homing sites in the recipient spleen, or a toxic effect of the transferred cells on the recipient. Similarly, the apparent suppressive effects of one cell population on the response of another, as observed in the IgM production of unfractionated cells (Table VII), are as yet unexplained. These complexities of the transfer system make it difficult to analyze in detail the immune function of fiber-fractionated cells. Nevertheless, the results obtained in these experiments strongly support the conclusion that the cells bound to antigen-coated fibers are highly enriched not only with respect to their ability to bind the antigen, but also with respect to their ability to respond specifically to stimulation with the antigen.

Cross-reactivity among antigen-binding cells. As many as 2% of the spleen cells from an unimmunized adult mouse can bind specifically to the single haptenic determinant, Dnp, but only the small proportion of these cells having receptors with a relatively high affinity are stimulated by that antigen (Rutishauser et al., 1972). The lower affinity cells presumably either bind with higher affinity to other antigens, or fail to bind strongly to any antigen tested so far. A priori, it seems likely that the system contains both kinds of cells, because if there were no cells in the second category, the system would have exhausted its potential to recognize new antigens.

Studies of cross-reactivity at the level of the antigen-binding cell indicate that, in the non-immune adult, there is considerable cross-reaction among antigens such as sulfanilic acid and arsanilic acid or Dnp, which are considered to be entirely distinct at the level of the immune response (Landsteiner, 1945; page 168). There appears to be a striking gain in specificity, however, following immunization. In the non-immune animal soluble sulfanilate-BSA will inhibit the binding of cells to Dnp-derivatized fibers, although higher concentrations are required of this antigen than of Dnp. In immunized animals, however, the high-affinity cells are not inhibited except by Dnp. Thus, the high-affinity cells are also high-specificity cells, and the gain in specificity by clonal selection appears to occur first by binding, and then by triggering of only the high-affinity populations. This provides some fundamental insight into the relationship between the repertoire and the selection from the repertoire, in terms of amplification and its relation to specificity.

The overlapping arrangement of antigen-binding specificities would assure that at least some cells in the repertoire would be able to bind any antigen. At the same time, the selective triggering of only those cells with the highest affinity for the antigen would maximize the specificity of the immune response to any given antigen. Such a model emphasizes the functional relevance of all the antigen-binding cells detected by the fiber-binding assay. While only those cells with high affinity for the test antigen are likely to respond to it, the remaining cells

represent other, distinct antigen-binding specificities and are necessary for the completeness of the repertoire.

The Ontogeny of Specific Antigen-Binding Cells

These methods for the detection of cells functionally specific for each of a wide variety of antigens have been applied to the characterization of antigen-binding cells in the developing immune system. As mentioned above, the experiments in this thesis were designed to approach three questions concerning this development. 1) When and where in the course of development do specific antigen-binding cells appear? 2) How do the antigen-binding properties of cells from developing animals compare with those of cells from adults? 3) To what extent does the development of the repertoire of antigen-binding cells vary from individual to individual? It was hoped that on the basis of these data the processes responsible for the generation of antibody diversity and the commitment of each lymphocyte to the production of a single species of Ig could be described more precisely, and that some insight might be obtained into the mechanisms by which these processes are controlled.

Quantitation of antigen-binding cells as a function of age. In the developing Swiss-L mouse, cells specific for each of a variety of hapten and protein antigens appeared simultaneously, late in gestation. They were detected transiently in the liver, but the major site for their appearance was the spleen.

Antigen-specific cells could be detected in the liver between the 15th day of gestation and one day postpartum. Antigen-specific cells could first be detected in the spleen on the 16th day of gestation, and rapidly increased in number thereafter. It should be noted that the development of antigen-specific spleen cells measured here by the fiber assay exactly paralleled that previously reported for three antigens measured by the rosette assay (Spear et al., 1973), so that a consistent pattern of development has now been observed in studies of cells specific for each of 13 different antigens, using two independent assays for antigen-specific cells. The same pattern was observed for two antigens with Balb/c spleen cells.

These observations are in accord with the data that have been accumulated so far on lymphocyte development. Cells synthesizing Ig can be detected as early as the 12th day of gestation in the liver (Melchers et al., 1975). It should be noted, however, that cells with detectable Ig on their surfaces are not present until the 15th or 16th day of gestation, when they appear in the liver and spleen (Nossal and Pike, 1973; Spear et al., 1973). In addition, Melchers et al., (1975) have recently shown that cells reactive in vitro to B-cell mitogens such as bacterial lipopolysaccharide can be detected in the liver from the 18th day of gestation to about a week after birth. In the spleen, these reactive cells appear at the same time, but increase in number up to adulthood.

Hemopoietic activity, including the generation of lymphocytes, is confined to the liver, spleen, thymus, and bone marrow at this stage of development in the mouse (Metcalf and Moore, 1971; page 10). Inasmuch as no antigen-binding cells could be detected in the latter two tissues, all these data suggest that the sites for the initial appearance of specific antigen-binding cells are the fetal liver and spleen. In particular, it seems unlikely that these cells first appear elsewhere in the developing embryo and only secondarily migrate to the liver or spleen. The simultaneous appearance in these two organs of cells specific for each of the antigens tested argues strongly against a sequential generation of antigen-binding cells according to their antigenic specificities.

Relative avidities of fetal and adult antigen-binding cells. A key characteristic of mature antigen-binding cell populations is the presence within those populations of some cells with high avidity for the antigen, as discussed above. To assess the nature of the fetal antigen-binding cells in this respect, the distribution of avidities with which they bound antigen was compared to that for adult spleen cells. Fetal liver and spleen cells showed relative avidity distributions identical within experimental error to that of adult spleen cells for one antigen, and fetal and adult spleen cells showed relative avidity distributions identical within experimental error for three additional antigens. As

early as any antigen-binding cells can be detected in the fetus, they show the same relative avidity distributions as normal adult cells. In view of the large change in the relative avidity distribution caused by the deliberate immunization of adult animals, this similarity between fetuses and normal adults is most simply explained by two factors. First, it is consistent with the very restricted exposure to foreign antigens that these specific pathogen-free animals receive in the course of their post-natal development. Second, the effects of any exposure to foreign antigen which these animals might receive would be minimized because of the replenishment of the adult repertoire by the continuing generation of committed antigen-binding cells (Stocker at al., 1974; Nossal and Pike, 1975).

Possible mechanisms for the generation of antigen-binding cells. These results are in accord with the requirement of the clonal selection theory that the full repertoire of specific antigen-binding cells exist in the animal before exposure to foreign antigen. Within the limits of these experiments, fetal cell populations appear to express as wide a range of antigen-binding specificities as adult cell populations. Indeed, although only a small number of antigens has been tested here, the closely similar results obtained in all cases, and the parallel between these results and the appearance of Ig-bearing cells in the liver and spleen, suggest that the repertoire of these specificities is filled early and rapidly for a large number of antigens. These data, and those on the proportions of antigen-binding cells of different specificities as a function of age, also suggest that for the fetus there is no positive selection by foreign antigen after the appearance of specific antigen-binding cells.

These results are consistent, however, with certain forms of negative selection. Raff et al. (1975), and Nossal and Pike (1975), have proposed, for example, that an immature lymphocyte exposed to foreign antigen is immediately and irreversibly stripped of its surface Ig. Such a cell would not be detected either as an Ig-bearing cell or as an antigen-binding cell in experiments of the sort described here. If this negative selection were imposed by an invariant panel of self

antigens, such as the developing animal's own histocompatibility antigens (Jerne, 1971), its effect would be the complete absence of certain clones from the animal's repertoire at all stages of development. A constant negative selective force of this sort would cause no detectable maturation of the repertoire, either in terms of the range of antigen-binding specificities expressed, or in terms of the distribution of avidities with which any particular foreign antigen is bound.

To assess the role of negative selection more directly, it will be necessary to search for fine differences in the antigen-binding repertoires expressed by genetically distinct strains of mice, or to perturb the system by deliberately introducing foreign antigens into the fetus.

Obviously, general conclusions concerning the development of antigen-specific cells must be qualified by the fact that the number of antigens tested so far is still an extremely small fraction of the total number of antigens. These conclusions nevertheless suggest boundary conditions on possible mechanisms for the generation of antibody diversity. It would become necessary to postulate that a very large range of antibodies is already specified when the first cells bearing surface Ig appear in the fetus on the 15th day of gestation. Recent work indicates that the amount of DNA in the genome coding for antibody molecules is much smaller than the number of different antibodies that can be produced (Leder et al., 1975; Tonegawa, 1976), suggesting that the range of antibodies is generated by the somatic mutation (Cohn, 1968; Jerne, 1971) or recombination of a small number of genes (Gally and Edelman, 1970) in the precursors of the antigen-binding cells.

The generation of antibody diversity could therefore occur early in development with the expression of antibody specificities occuring at random within a short time interval later in development. Such a model is consistent both with the observed kinetics of appearance of antigen-binding cells, and with the degenerate set of antigen-binding specificities expressed by these cells in the mature animal. Clearly, as long as positive selection is not necessary, somatic mechanisms are sufficient for the rapid production of the full range of antigen-binding cell, and there is no reason a priori to favor germ-line models for

the generation of antibody diversity. This might be summarized by the statement that the only positive selection is clonal selection itself, in the mature animal.

Given that the young animal has the full range of antigen-binding cells, however, it remains necessary to explain its inability to respond to antigenic stimulation (Spear et al., 1973). Several factors probably contribute to this phenomenon. The spleen of the newborn Swiss mouse appears to contain both T and B antigen-binding cells in the same proportions as the adult mouse. However, Spear and Edelman (1974) have shown that the ratio of cells bearing the θ-antigen to cells bearing detectable surface Ig decreases approximately 50% between birth and adulthood, and that a subpopulation of cells fully responsive to T-cell mitogens, which seems to correspond to long-lived recirculating T lymphocytes, appears in the mouse only several weeks after birth, coincident with the onset of immune competence. Goidl and Siskind (1974) have shown that liver cells from fetal mice, on transfer to irradiated adults, give rise only to cells secreting low affinity antibodies after immunization. High affinity antibodies are produced, however, in similar experiments in which adult spleen cells are transferred or in which fetal cells are transferred together with bacterial lipopolysaccharide (Goidl and Siskind, 1975). These results indicate that the fetus possesses as wide a range of antigen-specific cells as the adult, but suggest that many of the fetal cells cannot respond to antigenic stimulation with the production of antibody. A possible explanation of this unresponsiveness of fetal B cells is that some of the receptors thought to be involved in T cell-B cell interaction appear only very late in development, well after the repertoire of antigen-binding specificities is established. Two such receptors are cell surface Ig with heavy chains of the δ-class (Vitetta et al., 1975) and the complement receptor (Gelfand et al., 1974). Both of these receptors can first be detected on B cells about 2 weeks after birth.

Before the appearance of these secondary receptors, the antigen-binding cells ought to be fully responsive to antigen only in the presence of substances such as lipopolysaccharide, which appear to

substitute for T cell-B cell collaboration in the induction of an immune response (Sjöberg et al., 1972). Further functional studies will be necessary to analyze in detail the last stages of the development of the immune system, in which antigen-binding cells already present in the neonatal animal develop the ability to respond to antigen.

Antigen-Binding Cells in Single Fetal Mice

All of the studies of specific antigen-binding cells in developing animals described so far were carried out on cells pooled from several animals. To determine the extent to which variation from animal to animal was being obscured by the pooling procedure, individual fetal spleens were assayed for antigen-binding cells. Because the fiber-binding assay is a sampling procedure, it cannot readily be used to characterize the small numbers of cells yielded by single fetal spleens. While the rosette assay is only capable of detecting cells specific for a very limited number of antigens, its high efficiency allows it to be used with very small cell populations. Both outbred Swiss-L and inbred CBA/J and Balb/c fetuses were tested. In the case of the Swiss mice, for each of the two antigens tested, SRBC and Tnp, the mean number of antigen-binding cells per spleen calculated from the individual measurements agreed with the number obtained from measurements of pooled cells. The distributions of the individual values about these means were unimodal, and the distribution of values obtained for any given litter of fetuses did not differ appreciably from that for the population as a whole. Similar distributions were obtained for the inbred Balb/c and CBA/J fetuses. These experiments suggest that the results obtained with pools of cells from several mice accurately reflect the immunological development of individuals.

It remained necessary, however, to determine more precisely the source of the substantial variation in numbers of RFC seen from individual to individual. The possible effect of variation in spleen size on the number of RFC per spleen was examined by determining RFC number as a function of size, using the numbers of nucleated cells in the individual spleens as an index of the latter. When outbred Swiss-L fetuses were examined in this way, no simple correlation could be found

between the number of RFC per spleen and the number of nucleated cells per spleen, for individuals from either the same or different litters.

Four possible sources of this variation in the frequency of RFC in individual fetal spleens seemed likely: 1) variability in the assay methods themselves; 2) variation in the development of individuals of the same genotype and parentage; 3) variation between individuals of the same genotype due to maternal effects; and 4) variation due to genetic differences between the individual fetuses. To distinguish among these possibilities, spleens from individual CBA/J and Balb/c fetuses were assayed for nucleated cells and for Tnp-specific or SRBC-specific RFC. For each of these strains of mice, the number of RFC per spleen specific for each antigen was a constant proportion of the number of nucleated cells per spleen. For each strain and each antigen, no more individual variation was seen either within or between litters than could be accounted for by sampling fluctuation alone. These results would appear to exclude variability due to the assay itself, to idiosyncratic variations in the development of fetuses of the same genotype, or to variations caused by maternal effects. These results suggest that the frequency of specific antigen-binding cells in the spleens of developing mice is subject to genetic controls. This tentative conclusion warrants intense and critical study because of its significance for the "unexplored" area of the clonal selection theory -- the generation of antibody diversity and the shaping of the repertoire.

It will be necessary in further studies to distinguish between controls affecting the repertoire itself and general controls on the development of the spleen or the immune system as a whole. In order to carry out such an analysis, it will be important to determine for individual fetuses the size of the total repertoire of antigen-binding cells within the nucleated cell population of the spleen. On the basis of these data it should be possible to characterize the antigen-binding repertoires of individual F_1 and backcross mice, as well as mice of other strains.

On a more general level, the fact that the relative proportions of Tnp-specific and SRBC-specific cells were the same in both strains of

mice, despite the higher absolute frequency of RFC observed in CBA/J fetuses, suggests that the composition of the repertoire in the fetus is independent of spleen size. Although only cells specific for two antigens have been examined so far, these studies support at the level of individual development the conclusion drawn from the studies of pooled cells that the repertoire fills early and uniformly.

Summary: the ontogeny of antigen-binding cells. The final product of the humoral immune system, the antibody-secreting cell, is generated by two distinct types of cell proliferation. One of these is the antigen-driven proliferation of a pre-existing and committed lymphocyte within the repertoire of the mature animal to form a clone of identical immunoglobulin producing cells. The other, which may be called the "hidden" aspect of clonal selection, has been emphasized in this work and consists of the initial generation of the repertoire of committed cells during ontogeny. This development has been studied here in terms of the appearance of specific antigen-binding cells, the avidity distributions of these cells in the fetus and the adult, and the extent and nature of individual variation in the developmental process.

Cells specific for each of the 11 antigens tested first appeared in the fetal mouse late in gestation, at which time they could be detected both in the liver and the spleen. In all cases, these cells disappeared from the liver within a day of birth but continued to increase in number in the spleen until adulthood. These data suggest that the range of antigens recognized by fetal and adult cells is the same, and hence that the repertoire of antigen-binding cells fills uniformly and early in development, rapidly reaching the proportions found in the adult.

The distributions of avidities with which fetal and nonimmune adult cells bound antigens were compared for 4 antigens and were found to be identical within experimental error in all cases. These data suggest that there is no positive selection by antigen following the appearance of specific antigen-binding cells, and hence that the generation of antibody diversity in the normal animal is not an antigen-driven phenomenon.

These findings at the level of the population of antigen-binding cells are in sharp contrast to the gradual maturation of the immune res-

ponse measured either in terms of the range of antigens capable of eliciting a response or in terms of the physico-chemical properties of the antibodies produced in the course of such a response. The results reported here suggest that the restricted ability of the neonatal animal to respond to antigenic stimulation is not due to the lack of antigen-specific cells, but rather to the absence of mature cells capable of the interactions needed for a full immune response.

Measurements of the numbers of cells specific for each of two antigens in the spleens of individual outbred Swiss-L fetal mice suggested that there were not subpopulations of individuals differing systematically from the fetal population as a whole. Significantly more variation among individuals was found, however, than would be expected if the actual number of spleen cells specific for a given antigen were constant from fetus to fetus. Similarly, the ratio of the number of antigen-binding cells to the number of nucleated cells in the spleen varied significantly from fetus to fetus in the outbred mice.

For the inbred strains of mice CBA/J and Balb/c, however, the ratio observed for each strain and each antigen was a constant within sampling error. In addition, the relative proportions of cells specific for the two antigens differed no more from CBA/J to Balb/c mice than would be expected in repeated samples of cells from the spleen of a single fetus. These results confirm at the level of the individual fetus the uniform pattern of development seen for the population of fetuses as a whole. In addition, they reveal a surprising precision in the proliferation of specific antigen-binding cell populations and suggest that the development of these cell populations may be subject to strong genetic controls both on the composition of the repertoire of antigen-binding specificities and on the process by which this repertoire is generated and expressed in the course of development.

Investigation of these problems is continuing. Whatever the outcome, it seems clear that the early events in the development of the immune system are of broad significance not only in immunology, but also as a general model for the control of growth and differentiation during embryonic development.

REFERENCES

Abney, E. R., I. R. Hunter, and R. M. E. Parkhouse (1976). Preparation and characterization of an antiserum to the mouse candidate for immunoglobulin D. Nature 259, 404.

Abney, E. R., and R. M. E. Parkhouse (1974). Candidate for immunoglobulin D present on murine B lymphocutes. Nature 252, 600.

Archer, O. K., D. E. R. Sutherland, and R. A. Good (1963). Appendix of the rabbit: a homologue of the bursa in the chicken. Nature 200, 337.

Basten, A., J. F. A. P. Miller, N. L. Warner, and J. Pye (1971). Specific inactivation of thymus-derived (T) and non-thymus-derived (B) lymphocytes by ^{125}I-labelled antigen. Nature New Biol. 231, 104.

Basten, A.,J. F. A. P. Miller, J. Sprent, and J. Pye (1972). A receptor for antibody on B lymphocytes. I. Method of detection and functional significance. J. Exp. Med. 135, 610.

Bianco, C., R. Patrick, and V. Nussenzweig (1970). A population of lymphocytes bearing a membrance receptor of antigen-antibody-complement complexes. I. Separation and characterization. J. Exp. Med. 132, 702.

Biozzi, G., C. Stiffel, D. Mouton, C. Liacopoulos-Briot, C. Decreusefond, and Y. Bouthillier (1966). Etude du phénomène de l'immunocyto-adhérence au course de l'immunisation. Ann. Inst. Pasteur 110, 7.

Burnet, F. M. (1959). The Clonal Selection Theory of Acquired Immunity, Cambridge University Press, New York.

Byrt, P., and G. L. Ada (1969). An in vitro reaction between labelled flagellin or haemocyanin and lymphocyte-like cells from normal animals. Immunology 17, 503.

Boyse, E. A., M. Miyazawa, T. Aoki, and L. J. Old (1968). Ly-A and Ly-B: Two systems of lymphocyte iso-antigens in the mouse. Proc. Roy. Soc. B 178, 175.

Boyse, E. A., K. Itakura, E. Stockert, C. A. Iritani, and M. Miura (1971). Ly-C: a third locus specifying alloantigens expressed only on thymocytes and lymphocytes. Transplantation 11, 351.

Boyse, E. A., and L. J. Old (1969). Some aspects of normal and abnormal cell surface genetics. Ann. Rev. Genet. 3, 269.

Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf (1970). Methods in Immunology, 2nd edition, W. A. Benjamin, New York.

Cantor, H., and E. A. Boyse (1975). Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses in a differentiation process independent of antigen. J. Exp. Med. 141, 1376.

Chanana, A. D., J. Schaedli, M. W. Hess, and H. Cottier (1973). Predominance of Theta-positive lymphocytes in gut-associated and peripheral lymphoid tissues of newborn mice. J. Immunol. 110, 283.

Click, R. E., L. Benck, and B. J. Alter (1972). Enhancement of antibody synthesis in vitro by mercaptoethanol. Cell. Immunol. 3, 156.

Cohn, M. (1968). The molecular biology of expectation, in Rutgers Symposium on Nucleic Acids in Immunology, O. J. Plescia and W. Braun, editors. Springer-Verlag New York Inc., New York. 671.

Cunningham, B. A., M. M. Pflumm, U. Rutishahuser, and G. M. Edelman (1969). Subgroups of amino acid sequences in the variable regions of immunoglobulin heavy chains. Proc. Natl. Acad. Sci. USA 64, 997.

Decker, J., J. Clarke, L. MacPherson, R. Weinstein, and E. E. Sercarz (1972). Early appearance of antigen-binding cells to two different antigens during fetal lymphoid development. Adv. Exp. Med. Biol. 29, 269.

Decker, J., J. Clarke, L. M. Bradley, A. Miller, and E. E. Sercarz (1974). Presence of antigen-binding cells for five diverse antigens at the onset of lymphoid development: lack of evidence for somatic diversification during ontogeny. J. Immunol. 113, 1823.

Diamantstein, T., W. Vogt, H. Rühl, and G. Bochert (1973). Stimulation of DNA synthesis in mouse lymphoid cells by polyanions in vitro. I. Target cells and possible mode of action. Eur. J. Immunol. 3, 448.

Dresser, D. W., and N. A. Mitchison (1968). The mechanism of immunological paralysis. Advan. Immunol. 8, 145.

Dwyer, J. M., N. L. Warner, and I. R. Mackay (1972). Specificity and nature of the antigen-combining sites on fetal and mature thymus lymphocytes. J. Immunol. 108, 1439.

Dwyer, J. M., and I. R. Mackay (1970). Antigen-binding lymphocytes in human fetal thymus. Lancet 1, 1199.

Dwyer, J. M., and I. R. Mackay (1972). The development of antigen-binding lymphocytes in foetal tissues. Immunology 23, 871.

Dwyer, J. M., and N. L. Warner (1971). Antigen binding cells in embryonic chicken bursa and thymus. Nature New Biol. 229, 210.

Edelman, G. M., U. Rutishauser, and C. F. Millette (1971). Cell fractionation and arrangement on fibers, beads and surfaces. Proc. Natl. Acad. Sci. USA 68, 2153.

Edelman, G. M., and W. E. Gall (1969). The antibody problem. Ann. Rev. Biochem. 38, 415.

Elliot, B. E., J. S. Haskill, and M. A. Axelrad (1973). Thymus-derived rosettes are not "helper" cells. J. Exp. Med. 138, 1133.

Elliot, B. E., and J. S. Haskill (1973). Characterization of thymus-derived and bone marrow-derived rosette-forming lymphocytes. Eur. J. Immunol. 3, 68.

Feldmann, M., P. C. L. Beverley, M. Dunkley, and S. Kontiainen (1975). Different Ly antigen phenotypes of in vitro induced helper and suppressor cells. Nature 258, 614.

Fleiss, J. L. (1973). Statistical Methods for Rates and Proportions, John Wiley and Sons, New York.

Gally, J. A., and G. M. Edelman (1970). Somatic translocation of antibody genes. Nature 227, 341.

Gally, J. A., and G. M. Edelman (1972). The genetic control of immunoglobulin synthesis. Ann. Rev. Genet. 6, 1.

Gelfand, M. C., G. J. Elfenbein, M. M. Frank, and W. E. Paul (1974). Ontogeny of B-lymphocytes. II. Relative rates of appearance of lymphocytes bearing surface immunoglobulin and complement receptors. J. Exp. Med. 139, 1125.

Glick, B., T. S. Chang, and R. G. Jaap (1956). The bursa of Fabricius and antibody production. Poultry Sci. 35, 224.

Goidl, E. A., and G. W. Siskind (1974). Ontogeny of B-lymphocyte function. I. Restricted heterogeneity of the antibody response of B lymphocytes from neonatal and fetal mice. J. Exp. Med. 140, 1285.

Goidl, E. A., and G. W. Siskind (1975). Ontogeny of the B-cell function in the mouse. Fed. Proc. 34, 966.

Greaves, M. F., J. J. T. Own, and M. C. Raff (1973). T and B Lymphocytes: Origins, Properties and Roles in Immune Responses. American Elsevier Publishing Co., Inc., New York.

Greaves, M. F., and G. Janossy (1972). Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev. 11, 87.

Gronowicz, E., A. Coutinho, and G. Möller (1974). Differentiation of B cells: sequential appearance of responsiveness to polyclonal activators. Scand. J. Immunol. 3, 413.

Haas, W. (1975). Separation of antigen-specific lymphocytes. II. Enrichment of hapten-specific antibody-forming cell precursors. J. Exp. Med. 141, 1015.

Haas, W., J. W. Schrader, and A.Szenberg (1974). A new, simple method for the preparation of lymphocytes bearing specific receptors. Eur. J. Immunol. 4, 565.

Haas, W., and J. E. Layton (1975). Separation of antigen-specific lymphocytes. I. Enrichment of antigen-binding cells. J. Exp. Med. 141, 1004.

Hayward, A. R., and Soothill, J. F. (1972). Reaction to antigen by human foetal thymus lymphocytes, in Ontogeny of Acquired Immunity (Ciba Foundation Symposium), American Elsevier Publishing Co., Inc., New York. 261.

Hilschmann, N., and L. C. Craig (1965). Amino acid sequence studies with Bence-Jones proteins. Proc. Natl. Acad. Sci. USA 53, 1403.

Hirst, J. A., P. C. L. Beverley, P. Kisielow, M. K. Hoffman, and H. F. Oettgen (1975). Ly antigens: markers of T cell function on mouse spleen cells. J. Immunol. 115, 1555.

Jerne, N. K. (1955). The natural selection theory of antibody formation. Proc. Natl. Acad. Sci. USA 41, 849.

Jerne, N. K. (1971). The somatic generation of immune recognition. Eur. J. Immunol. 1, 1.

Jerne, N. K., and A. A. Nordin (1963). Plaque formation in agar by single antibody producing cells. Science 140, 405.

Julius, M. H., and L. A. Herzenberg (1974). Isolation of antigen-binding cells from unprimed mice. Demonstration of antibody-forming cell precursor activity and correlation between precursor and secreted antibody avidities. J. Exp. Med. 140, 904.

Kunkel, H. G. (1954). Zone electrophoresis. Methods Biochem. Anal. 1, 141.

Landsteiner, K. (1945). The Specificity of Serological Reactions, 2nd edition, Harvard University Press, Cambridge.

Leder, P., T. Honjo, D. Swan, S. Packman, M. Nau, and B. Norman (1975). The organization and diversity of immunoglobulin κ and λ genes, in Molecular Approaches to Immunology, E. E. Smith, editor. Academic Press, New York. 173.

Loor, F. and B. M. Kindred (1973). Differentiation of T cell precursors in nude mice demonstrated by immunofluorescence of T cell membrane markers. J. Exp. Med. 138, 1044.

Marchalonis, J. J., and R. E. Cone (1973). Biochemical and biological characterization of lymphocyte surface immunoglobulin. Transplant. Rev. 14, 3.

McClain, D. A., J. L. Wang, and G. M. Edelman (1975). The effects of sodium metaperiodate on T. and B lymphocytes. Cell. Immunol. 15, 287.

McConnell, I., A. Munro, B. W. Gurner, and R. R. A. Coombs (1969). Studies on actively allergized cells. I. The cyto-dynamics and morphology of rosette-forming lymph node cells and inhibition of rosette-formation with antibody to mouse immunoglobulins. Int. Arch. Allergy Appl. Immunol. 35, 209.

Medawar, P. B., and E. Simpson (1975). Thymus-dependent lymphocytes. Nature 258, 106.

Melchers, F., H. von Boehmer, and R. A. Phillips (1975). B-lymphocyte interactions in the mouse. Organ distribution and ontogeny of immunoglobulin-synthesizing and of mitogen-sensitive cells. Transplant. Rev. 25, 26.

Metcalf, D., and M. A. S. Moore (1971). Haemopoietic Cells, American Elsevier Publishing Co., Inc., New York.

Metcalf, D., and R. Wakonig-Vaartaja (1964). Stem cell replacement in normal thymus grafts. Proc. Soc. Exp. Biol. Med. 115, 731.

Miller, J. F. A. P. (1961). Immunological function of the thymus. Lancet 2, 748.

Mishell, R. J., and R. W. Dutton (1967). Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. 126, 423.

Moore, M. A. S., and J. J. T. Owen (1966). Experimental studies on the development of the bursa of Fabricius. Develop. Biol. 14, 40.

Moore, M. A. S., and J. J. T. Owen (1967). Experimental studies on the development of the thymus. J. Exp. Med. 126, 715.

Mosier, D. E. (1974). Ontogeny of mouse lymphocyte function. I. Paradoxical elevation of reactivity ot allogeneic cells and phytohemagglutinin in Balb/c fetal thymocytes. J. Immunol. 112, 305.

Mosier, D. E., and B. M. Johnson (1975). Ontogeny of mouse lymphocyte function. II. Development of the ability to produce antibody is modulated by T lymphocytes. J. Exp. Med. 141, 216.

Nelson, J. B., and G. R. Collins (1961). The establishment and maintenance of a specific pathogen-free colony of Swiss mice. Proc. Anim. Care Panel 11, 65.

Nilsson, B. S., B. M. Sultzer, and W. W. Bullock (1973). Purified protein derivative of tuberculin induces immunoglobulin production in normal mouse spleen cells. J. Exp. Med. 137, 127.

Nossal, G. J. V., and B. Pike (1973). Studies on the differentiation of B lymphocytes in the mouse. Immunology 25, 33.

Nossal, G. J. V., and B. Pike (1975). Evidence for the clonal abortion theory of B-lymphocyte tolerance. J. Exp. Med. 141, 904.

Nowell, P. C. (1960). Phytohemagglutinin: an initiator of mitosis in cultures of human leukocytes. Cancer Res. 20, 462.

Osmond, D. G., and G. J. V. Nossal (1974a). Differentiation of lymphocytes in mouse bone marrow. I. Quantitative radioautographic studies of antiglobulin binding by lymphocytes in bone marrow and lymphoid tissues. Cell. Immunol. 13, 117.

Osmond, D. G., and G. J. V. Nossal (1974b). Differentiation of lymphocytes in mouse bone marrow. II. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labeling. Cell.

Immunol. 13, 132.

Osoba, D. (1970). Some physical and radiobiological properties of immunologically reactive mouse spleen cells. J. Exp. Med. 132, 368.

Owen, J. J. T. (1972). The origins and development of lymphocyte populations, in Ontogeny of Acquired Immunity (Ciba Foundation Symposium), American Elsevier Publishing Co., Inc., New York. 35.

Owen, J. J. T., M. D. Cooper, and M. C. Raff (1974). In vitro generation of B lymphocytes in mouse fetal liver, a mammalian bursal equivalent. Nature 249, 361.

Owen, J. J. T., M. C. Raff, and M. C. Cooper (1975). Studies on the generation of B lymphocytes in the mouse embryo. Eur. J. Immunol. 5, 468.

Owen, J. J. T., and M. C. Raff (1970). Studies on the differentiation of thymus-derived lymphocytes. J. Exp. Med. 132, 1216.

Owen, J. J. T., and M. A. Ritter (1969). Tissue interaction in the development of thymus lymphocytes. J. Exp. Med. 129, 431.

Pantelouris, E. M., and P. A. Flisch (1972). Responses of athymic (nude) mice to sheep red blood cells. Eur. J. Immunol. 2, 236.

Perey, D. Y. E., M. D. Cooper, and R. A. Good (1968). Lymphoepithelial tissue of the intestine and differentiation of antibody production. Science 161, 265.

Peterson, R. D., M. D. Cooper, and R. A. Good (1965). The pathogenesis of immunologic deficiency diseases. Am. J. Med. 38, 579.

Playfair, J. H. L. (1968). Strain differences in the immune response of mice. I. The neonatal response to sheep red cells. Immunology 15, 35.

Poljak, R. J. (1975). X-ray diffraction studies of immunoglobulins. Adv. Immunol. 21, 1.

Press, J. L., and N. R. Klinman (1974). Frequency of hapten-specific B cells in neonatal and adult murine spleens. Eur. J. Immunol. 4, 155.

Pritchard, H., and H. S. Micklem (1973). Haemopoietic stem cells and progenitors of functional T-lymphocytes in the bone marrow of 'nude' mice. Clin. Exp. Immunol. 14, 597.

Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue (1971). Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. J. Exp. Med. 133, 156.

Raff, M. C. (1970). Two distinct populations of peripheral lymphocytes in mice distinguishable by immunoflouresence. Immunology 19, 637.

Raff, M. C., J. J. T. Owen, M. D. Cooper, A. R. Lawton, M. Megson, and W. E. Gathings (1975). Differences in susceptibility of mature and immature mouse B-lymphocytes to anti-immunoglobulin-induced immunoglobulin suppression in vitro. J. Exp. Med., 142, 1052.

Raff, M. C., M. Megson, J. J. T. Owen, and M. D. Cooper (1976). Early production of intracellular IgM by B-lymphocyte precursors in mouse. Nature 259, 224.

Raff, M. C., and J. J. T. Owen (1971). Thymus-derived lymphocytes: their distribution and role in the development of peripheral lymphoid tissues of the mouse. Eur. J. Immunol. 1, 27.

Reif, A. E., and J. M. Allen (1965). The AKR thymic antigen and its distribution in leukemias and nervous tissues. J. Exp. Med. 120, 413.

Rittenberg, M. B., and K. L. Pratt (1969). Antitrinitrophenyl (TNP) plaque assay. Primary response of Balb/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. 132, 575.

Rowlands, D. T., D. Blakeslee, and E. Angala (1974). Acquired immunity in opossum (Didelphis virginiana) embryos. J. Immunol. 112, 2148.

Rutishauser, U., C. F. Millette, and G. M. Edelman (1972). Specific fractionation of immune cell populations. Proc. Natl. Acad. Sci. USA 69, 1596.

Rutishauser, U., P. D'Eustachio, and G. M. Edelman (1973). Immunological function of lymphocytes fractionated with antigen-derivatized fibers. Proc. Natl. Acad. Sci. USA 70, 3894.

Rutishauser, U., and G. M. Edelman (1972). Binding of thymus- and bone marrow-derived lymphoid cells to antigen-derivatized fibers. Proc. Natl. Acad. Sci. USA 69, 3774.

Seller, M. J. (1972). Bone marrow transplantation in a genetically determined anaemia in the mouse, in Ontogeny of Acquired Immunity (Ciba Foundation Symposium), American Elsevier Publishing Co.,

Inc., New York. 175.

Sherwin, W. K., and D. T. Rowlands (1974). Development of humoral immunity in lethally irradiated mice reconstituted with fetal liver. J. Immunol. 113, 1353.

Sherwin, W. K., and D. T. Rowlands (1975). Determinants of the hierarchy of humoral immune responsiveness during ontogeny. J. Immunol. 115, 1549.

Sigal, N. H., P. J. Gearhart, J. L. Press, and N. R. Klinman (1976). Late acquisition of a germ line antibody specificity. Nature 259, 51.

Silverstein, A. M., J. W. Uhr, K. L. Kraner, and R. J. Lukes (1963). Fetal response to antigenic stimulus. II. Antibody production by the fetal lamb. J. Exp. Med. 117, 799.

Sjöberg, O., J. Andersson, and G. Möller (1972). Lipopolysaccharides can substitute for helper cells in the antibody response in vitro. Eur. J. Immunol. 2, 326.

Snedecor, G. W., and W. G. Cochran (1967). Statistical Methods, 6th edition, Iowa State University Press, Ames.

Solomon, J. B. (1971). Foetal and Neonatal Immunology, American Elsevier Publishing Co., Inc., New York.

Spear, P. G., A.-L. Wang, U. Rutishauser, and G. M. Edelman (1973). Characterization of splenic lymphoid cells in fetal and newborn mice. J. Exp. Med. 138, 557.

Spear, P. G., and G. M. Edelman (1974). Maturation of the humoral immune response in mice. J. Exp. Med. 139, 249.

Stobo, J. D., and W. E. Paul (1972). Functional heterogeneity of murine lymphoid cells. II. Acquisition of mitogen responsiveness and of Θ antigen during the ontogeny of thymocytes and "T" lymphocytes. Cell Immunol. 4, 367.

Stocker, J. W., D. G. Osmond, and G. J. V. Nossal (1974). Differentiation of lymphocytes in the mouse bone marrow. III. The adoptive response of bone marrow cells to a thymus cell-independent antigen. Immunology 27, 795.

Taylor, R. B., W. P. H. Duffus, M. C. Raff, and S. DePetris (1971). Redistribution and pinocytosis of lymphocyte surface immunoglobulin

molecules induced by anti-immunoglobulin antibody. Nature New Biol. 233, 225.

Tonegawa, S. (1976). Reiteration frequency of immunoglobulin light chain genes: further evidence for somatic generation of antibody diversity. Proc. Natl. Acad. Sci. USA 73, 203.

Uhr, J. W., and G. Möller (1968). Regulatory effect of antibody on the immune response. Adv. Immunol. 8, 81.

Vitetta, E. S., U. Melcher, M. McWilliams, M. E. Lamm, J. M. Phillips-quagliata, and J. W. Uhr (1975). Cell surface immunoglobulin. XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. J. Exp. Med. 141, 206.

Westphal, O., O. Lüderitz, and R. Bister (1952). Über die Extraktion von Bakterien mit Phenol-Wassen. Z. Naturforsch. B 7b, 148.

Wigzell, H., and B. Andersson (1969). Cell separation on antigen-coated columns. J. Exp. Med. 129, 23.

Williams, C. A., and M. W. Chase, editors (1967). Methods in Immunology and Immunochemistry, Academic Press, New York. volume I.

Wu, A. M., J. E. Till, L. Siminovitch, and E. A. McCulloch (1967). A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J. Cell. Physiol. 69, 177.

Wu, A. M., J. E. Till, L. Siminovitch, and E. A. McCulloch (1968). Cytological evidence for a relationship between haemopoietic colony-forming cells and cells of the lymphoid system. J. Exp. Med. 127, 455.

Yahara, I., and G. M. Edelman (1972). Restriction of the mobility of lymphocyte immunoglobulin receptors by concanavalin A. Proc. Natl. Acad. Sci. USA 69, 608.

Zaalberg, O. B., V. A. van der Meul, and J. M. van Twisk (1966). Antibody production by single spleen cells: a comparative study of the cluster and agar plaque formation. Nature 210, 544.