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THE ONTOGENY OF ANTIGEN-BINDING CELLS

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Abbreviations used in this paper:

Ig = immunoglobulin
n.c. = nucleated cell
RFC = rosette-forming cell
SRBC = sheep red blood cell
TNP = 2,4,6-trinitrophenyl

When an animal is exposed to a foreign substance, or antigen, it responds by producing antibodies, protein molecules that bind 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, the responses are highly specific, distinguishing between closely related substances. Third, the immune system exhibits a specific "memory" in that later exposure to the same antigen causes an enhanced response. Finally, the system can distinguish structures present in the animal itself from foreign ones, and normally responds only to the latter.

Immune responses are now understood at the cellular and molecular levels in terms of the clonal selection theory (Jerne, 1955; Burnet, 1959). According to the theory, in the course of an animal's development, each cell in its immune system becomes committed to produce a single kind of antibody and to express copies of its antibody on its surface. An antigen interacts with the system by binding to those cells in this repertoire that are specific for it. Binding of antigen stimulates the cells to divide and differentiate, each stimulated cell giving rise to a clone of cells all making identical immunoglobulin (Ig) molecules.

The specifications of the theory concerning the cellular composition and behavior of the adult immune system have been corroborated by a wide variety of experimental evidence (reviewed, for example, by D'Eustachio *et al.* 1977). Beyond stipulating that the system arises in the absence of interactions with foreign antigens, however, the clonal selection theory makes no direct predictions concerning the ontogeny of the immune system, and this developmental aspect of the system has remained rather poorly understood. When and where do antigen-specific cells appear in the course of development? Does the process vary from one individual to the next? How is it regulated?

Our experiments were intended to approach this problem as directly as possible, by measuring the development of antigen-binding cells in fetal mice. Mice were used because they have a relatively short gestation period (19 to 20 days), and a wide variety of highly inbred, genetically homogeneous

strains is available. In the first part of this paper, we will describe the ontogeny of antigen-binding cells, focusing in particular on the questions of when and where antigen-binding cells appear, and how their antigen-binding properties compare with those of cells in the adult animal. In the second part of the paper, we will describe individual variation in the ontogeny of antigen-binding cells in order to analyze the genetic and physiological basis of controls affecting the generation of these cells. Together, the results of these experiments provide the basis for a preliminary description of the development of the repertoire of antigen-specific cells. In the final part of this paper, we will consider the results of these experiments together with data from other systems in order to define boundary conditions within which the molecular processes responsible for the generation of antibody diversity and the commitment of developing cells to the production of single species of Ig must operate.

Frequency and avidity of specific antigen-binding cells in developing mice.

Before describing our own experiments in detail, it is worthwhile to review somewhat more fully the properties of the cell types involved. A point not explicitly considered in the original statement of the clonal selection theory is that there are in fact two classes of antigen-specific lymphocytes (Figure 1). The first class, B lymphocytes, corresponds to the antibody-producing cells of the theory. The second class, T lymphocytes, does not produce detectable antibody, but carries out a variety of antigen-specific immune functions including a "helper" function necessary for the full stimulation of B cells. Interactions between cells of these two classes can greatly affect the course of an immune response, but they do not affect its original specificity, and we shall, therefore, be concerned here primarily with the repertoire of antigen-specific B cells.

Experiments carried out by several groups within the past few years indicate that the fetal liver and spleen are the sites of B-cell maturation at least in the mouse, and

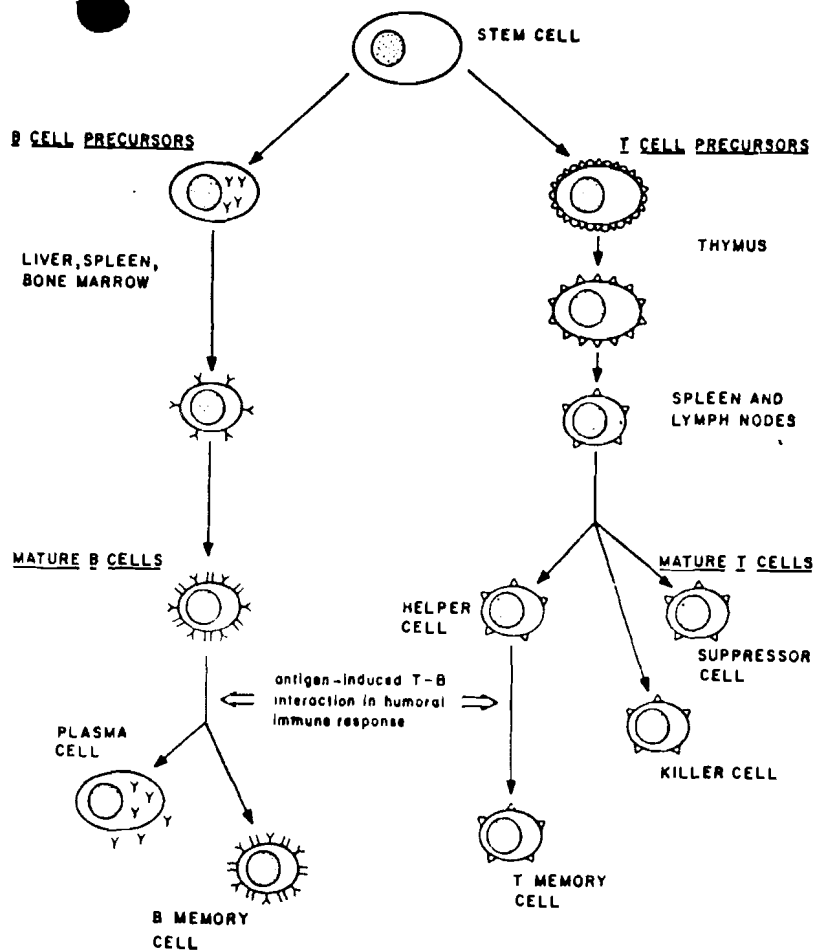


Figure 1. T and B lymphocytes. These two classes of cells are morphologically similar, but can be distinguished from each other and from their immature precursors on the basis of their location in the body, their antigenic markers, and a variety of functional properties (D'Eustachio *et al.*, 1977). B cells can be recognized and classified according to the amount and cellular distribution of Ig (Y) and of complement and Fc receptors (11). T cells can be classified according to the relative amounts of TL (O) and Thy-1 (Δ) antigens on their surfaces. In addition, the various functionally distinct subpopulations of mature T cells express different subsets of Ly antigens (not shown) and can be distinguished on that basis (Cantor and Boyse, 1976).

have provided a qualitative description of B-cell maturation in these tissues (Figure 1). Cells synthesizing Ig can be detected in the fetal liver as early as the 12th day of gestation (Melchers *et al.* 1975; Raff *et al.* 1976). If explants of 12-day fetal liver are placed in culture, lymphoid cells bearing Ig on their surfaces appear over a period of a week. Similar results are obtained with cultures of 14-day fetal spleen, but not with cultures of thymus or yolk sac at any stage of development. Because hemopoiesis, including lymphopoiesis, is confined to the liver, spleen, yolk sac, and thymus in the fetal mouse (Metcalf and Moore, 1971, p. 10), all of 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. In terms of clonal selection, the key problem in this lymphopoietic process is the generation of antigen-specific cells. More precisely, when and where in this process is the repertoire of antigen-binding specificities generated, what are the relative numbers of cells committed to each specificity, and how is the repertoire expressed in the course of development?

Several groups have approached this problem by analyzing the ontogeny of responsiveness to antigenic stimulation. The immune responses of fetal and neonatal animals have been characterized in terms of the range of antigens capable of eliciting a response (Klinman and Press, 1975; Rowlands *et al.* 1974; Silverstein *et al.* 1963), the antigen-binding affinities of the antibodies produced (Goldl and Siskind, 1974), and the idiotypic determinants expressed by these antibodies (Sigal *et al.* 1976, 1977). In every case, immune responsiveness matures in a well-defined and reproducible way in the course of development. The interpretation of these data is not straightforward, however. Two possible interpretations are that this maturation represents the sequential appearance of antigen-specific cells, or that it represents the maturation of additional cell functions required for efficient cell-antigen and cell-cell interaction following specific antigen binding. There is substantial evidence that such functional maturation does occur and that it is not complete in the mouse until at least three weeks after birth (Goldl *et al.* 1976; Mosier and Johnson, 1975; Spear and Edelman, 1974). In particular, this functional immaturity can include the inability of an antigen-specific B cell to respond to stimulation by antigen (Metcalf and Klinman, 1976; Nossal and Pike, 1975; Scott *et al.* 1977; Spear and Edelman, 1974;

Viratetta *et al.* 1977; Zitron *et al.* 1977). 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 appears to require that the appearance of antigen-binding cells be examined directly in the fetus and that the antigen-binding properties of these cells be compared with those of cells from adult animals.

Even direct binding between antigens and antibody on the cell surface can be misleading, however, unless the valence of the antigen is taken into account (Edelman, 1973). One of the most useful ways of controlling this parameter has been to analyze the binding of cell to antigenic determinants immobilized with equivalent densities on a solid matrix such as a nylon fiber (Rutishauser *et al.* 1972, 1973) or a red blood cells (McConnell *et al.* 1969). By using this approach, it has been possible to determine when and where in the course of development specific antigen-binding cells appear and to compare the antigen-binding properties of cells from developing animals with those of cells from adults. The two binding assays appear to give equivalent results, and the choice of a particular assay for any given experiment is largely a technical one. The rosette assay is extremely sensitive and can be adapted for use even with the very small number of cells obtained from single fetuses (D'Eustachio and Edelman, 1975). The fiber-binding assay, although somewhat less sensitive, is rapid and easily applied to comparative studies with different hapten and protein antigens (Rutishauser *et al.* 1972).

Spear *et al.* (1973) found that cells specific for each of three antigens, 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 frequency of Ig-bearing cells. In addition, fetal and adult spleen cell populations specific for the 2,4,6-trinitrophenyl (TNP) hapten bound the antigen with the same relative avidity distribution.

The numbers of cells capable of binding specifically to nylon fibers coated with each of 11 antigens have also

been determined as a function of age (D'Eustachio and Edelman, 1975). In the developing Swiss-L mouse, cells specific for each of these antigens first appeared late in gestation, in the liver and spleen (Figure 2). Antigen-specific cells could be detected in the liver between the 15th day of gestation and one day after parturition. Antigen-specific cells were first detected in the spleen on the 16th day of gestation and rapidly increased in number thereafter. In each organ, cells specific for all antigens tested appeared simultaneously. The development of antigen-specific spleen cells measured by the fiber assay exactly paralleled that measured by the rosette assay, 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. In all cases, the increase in number and frequency of antigen-binding cells paralleled the increase in Ig-bearing cells discussed above.

Inasmuch as no antigen-binding cells were detected in any fetal hemopoietic tissue except spleen and liver, all these data suggest that the initial sites of 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 and spleen.

A key characteristic of mature antigen-binding cell populations is the presence within these populations of some cells with a high avidity for the antigen (Rutishauser *et al.* 1972; Siskind and Benacerraf 1969). To assess the nature of fetal antigen-binding cells in this respect, the distribution of avidities with which they bound antigen was compared to that for adult spleen cells (Figure 3). Fetal liver and spleen cell populations showed relative avidity distribution 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 (D'Eustachio and Edelman, 1975). As early as any specific antigen-binding cells can be detected in the fetus, they show the same relative avidity distributions as normal adult cells. In view of the substantial change in the relative avidity distribution caused by the deliberate immunization of adult

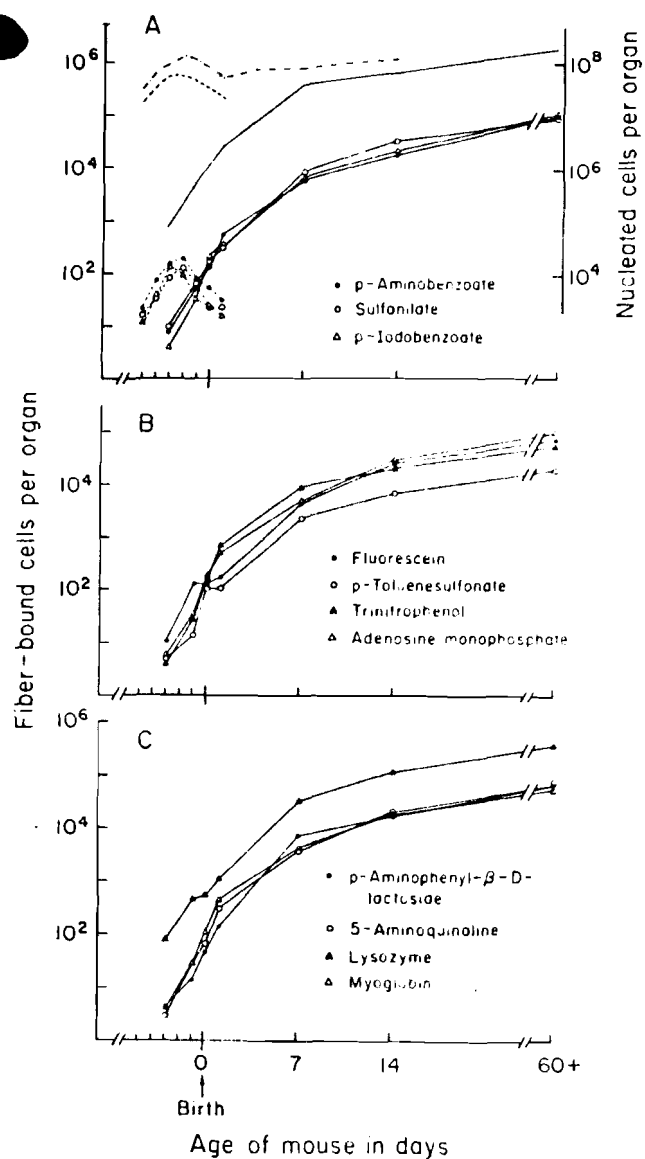


Figure 2. Numbers of cells per organ specific for each of 11 antigens in the spleens (—) and livers (---) of Swiss-L mice as a function of age. Antigen-specific cells were detected by means of the fiber-binding assay, and each point is the mean of two or more independent determinations. The numbers of lymphoid cells recovered per spleen and per liver as a function of age are shown in (A), together with the total number of nucleated cells per liver (-.-.-) (D'Eustachio and Edelman, 1975).

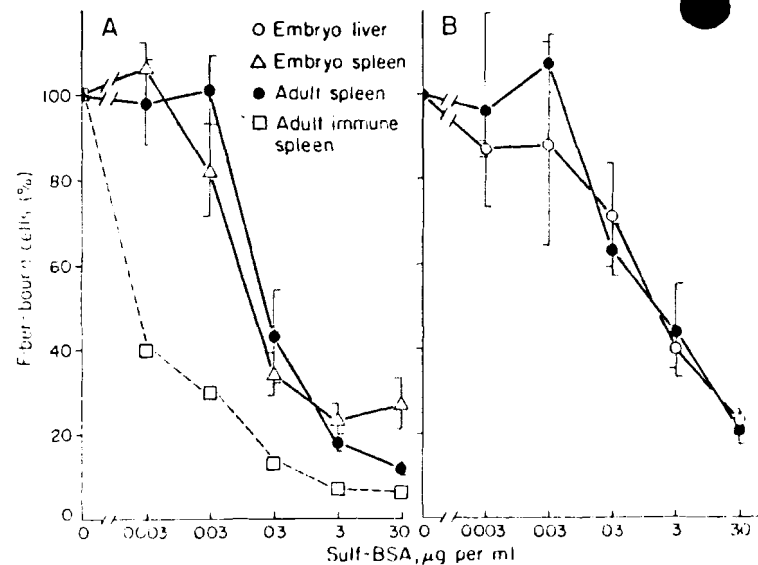


Figure 3. Numbers of cells bound to sulfanilate-coated fibers in the presence of soluble sulfanilate-BSA at several concentrations, expressed as a percentage of uninhibited control values. Cells from fetal spleen (A) and fetal liver (B) were compared with cells from adult spleen in parallel experiments. Liver cells were obtained from fetuses on the 17th day of gestation (0). Spleen cells were obtained from fetuses on the 18th day of gestation (Δ), or from adults (\bullet). Each value shown for these cells is the arithmetic mean of three or more independent determinations, plus or minus the standard error of the mean. Spleen cells from adult mice immunized against sulfanilate were assayed in the same way (\square). The results of a typical experiment are shown (D'Eustachio and Edelman, 1975).

animals (Figure 3), this similarity between fetuses and normal adults may seem surprising. It can probably be explained, however, by the continuing replenishment of the adult repertoire as a result of the generation of committed antigen-binding cells in the adult bone marrow (Stocker *et al.* 1974; Nossal and Pike, 1975). This constant turnover would tend to minimize the effects of any exposure to foreign

igen.

Variation in individual development.

Most of these measurements of antigen-binding cells in developing mice were carried out on pools of cells obtained from as many as 50 animals. To determine the extent to which variation from animal to animal was being obscured by the pooling procedure, spleens of individual 18-day Swiss-L fetuses were assayed for antigen-binding cells, using the rosette assay. For each of the two antigens tested, TNP and native sheep red blood cells (SRBC), the mean numbers of antigen-binding cells per spleen calculated from the individual measurements agreed with the numbers obtained from measurements of pooled cells. While the distributions of the individual values about these means were unimodal, they were quite broad, indicating that there is significantly more variation among individuals than would be expected from sampling fluctuation, assuming that the actual numbers of cells binding a specific antigen were constant, or nearly so, among fetuses. As much variation was observed among fetuses within a litter as in the fetal population as a whole (D'Eustachio and Edelman, 1975).

To determine the source of this fluctuation more precisely, the numbers of cells specific for each of four antigens in the spleens of individual randombred Swiss-L and inbred CBA/J and BALB/c fetal mice were determined as a function of the total numbers of nucleated spleen cells. All assays were carried out on fetuses at the 18th day of gestation (D'Eustachio *et al.* 1976; Cohen *et al.* 1977). For outbred Swiss-L fetuses (Figure 4), the ratio of antigen-binding cells to nucleated cells varied significantly more than could be explained 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 (Figure 5 and Table I).

These data suggest two surprising properties of fetal antigen-binding cell populations. First, they suggest the existence of two sets of controls that affect directly the generation of antigen-binding cells: one affecting the

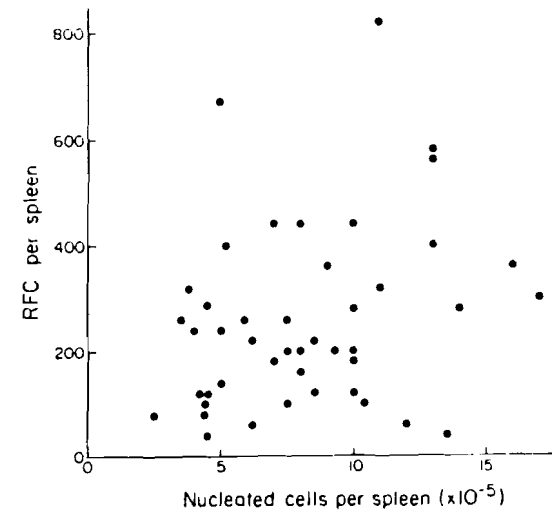


Figure 4. Numbers of TNP-specific rosette-forming cells (RFC's) in the spleens of Swiss-L fetal mice as a function of spleen size. RFC number per spleen was calculated from the number of RFC's observed in 25 μ l portions of the rosette assay mixture (equivalent to 1/20 spleen). The number of nucleated cells in the spleen was used as an index of its size. Each point represents the result obtained for one spleen. All fetuses were tested on the 18th day of gestation (D'Eustachio *et al.* 1976).

proportions of cells of different antigen-binding specificities within the population of committed lymphocytes (*i.e.*, the composition of the repertoire), and the other affecting the total size of that repertoire within the fetal spleen. Second, these data suggest that, at least for CBA/J and BALB/c mice, both controls appear to function independently of maternal or environmental effects, at least within the range of environmental variation in our breeding colonies. In particular, no more variation in the composition and size of the repertoire is seen from individual to individual within a strain than would be expected in replicate samples drawn from a homogeneous population of cells in a single individual. Thus, in fetuses

TABLE 1

*Frequencies and Ratios of RFC and Ig-Bearing Cells
in the Spleens of Inbred 18-Day Fetal Mice*

Mouse strain	TNP-SRBC RFC	Sheep 7 RBC RFC	Sheep 26 RBC RFC	Tosyl RFC	Ig-bearing Cells Per Spleen
(A) Positive cells per 10^5 nucleated cells (mean \pm s.d.)*					
CBA/J	32 ± 12	8 ± 7	12 ± 13	7 ± 5	$(7 \pm 2) \times 10^3$
BALB/c	8 ± 2	2 ± 0.4	6 ± 0.2	1 ± 0.3	$(2 \pm 1) \times 10^3$
Ratio ^{††}	3.8	4.0	1.6	7.0	4.0
(B) Positive cells per spleen**					
CBA/J	70 ± 54	12 ± 8	26 ± 22	12 ± 7	$(1.8 \pm 2.3^\dagger) \times 10^4$
BALB/c	58 ± 37	20 ± 5	28 ± 3	6 ± 4	$(1.2 \pm 1.3^\dagger) \times 10^4$

Footnotes to Table I

* Calculated from data for individual fetuses, weighing each fetus equally.

** Calculated from data for individual fetuses, except for Ig-bearing cells, which were calculated on the bases of $(2.6 \pm 2.6) \times 10^5$ nucleated cells per spleen for 41 CBA/J fetuses and $(7.3 \pm 3.2) \times 10^5$ nucleated cells per spleen for 24 BALB/c fetuses.

† Propagated standard deviation.

†† Based on pooled cell counts for each antigen and strain, CBA/J: BALB/c.

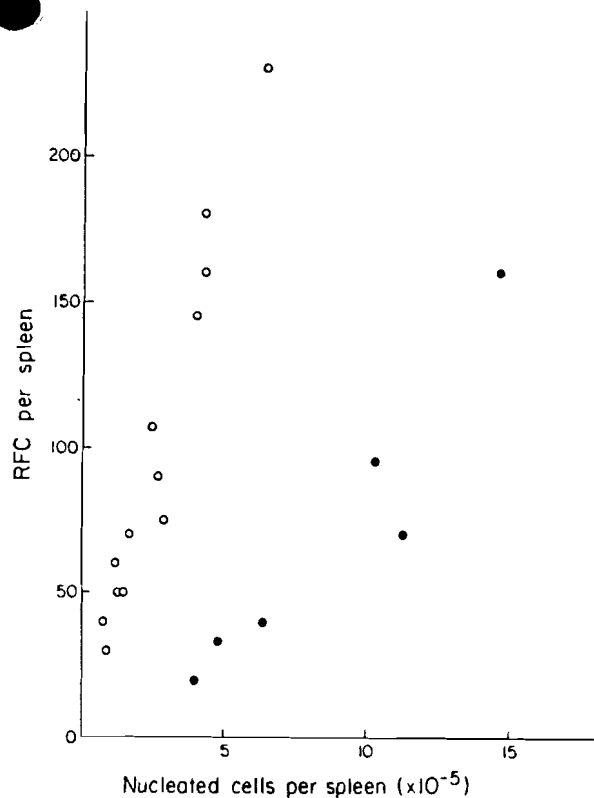


Figure 5. Number of TNP-specific RFC's in the spleens of individual CBA/J (O) and BALB/c (●) fetal mice as a function of spleen size. All fetuses were tested on the 18th day of gestation (D'Eustachio et al. 1976).

At 18 days of gestation, the number of nucleated spleen cells that bound the hapten antigens TNP and toluenesulfonyl and the surface antigens of native SRBC of different individual sheep was a constant proportion of the total number of nucleated spleen cells. The ratio of the proportion of spleen cells specific for a given antigen in CBA/J mice to the proportion in BALB/c mice of spleen cells specific for the same antigen equalled, within sampling fluctuation, the ratio of the proportion in CBA/J mice of nucleated spleen cells which had detectable surface Ig to the proportion of such

cells in BALB/c mice (Table I). Because the frequency of splenic T cells is low in these mice at this stage of development, this observation suggests that the nucleated cells of the fetal spleen may be viewed as falling into two subpopulations or compartments - those bearing detectable Ig on their surfaces (Ig+ cells) and those without detectable surface Ig. Within the compartment of Ig+ cells, the fraction of cells specific for a given antigen appears to be the same for both of the inbred strains examined, suggesting that the regulation of relative compartment size may be independent of the regulation of compartment composition.

This conclusion is supported by further analysis of antigen-binding cell populations in randombred Swiss-L fetal mice. While the absolute and relative frequencies of TNP-specific cells varied significantly from fetus to fetus (Figure 4), the ratio of the proportions of cells specific for two different antigens was constant within sampling fluctuation for all the fetuses tested (Table II). This observation suggests variation in repertoire size among Swiss-L fetuses while repertoire composition remained constant. At the same time the ratio of the proportions observed for the two antigens in Swiss-L fetuses differed from the ratio observed in the inbred strains. Hence the methods used for counting specific antigen-binding cell populations are sensitive enough to detect differences in the composition of the Ig+ compartment from strain to strain, and this result suggests that control of repertoire composition is not invariant for all strains of mice.

The observation that the proportion of antigen-specific cells among nucleated cells was invariant within a strain, but differed sharply between strains raised the possibility that this frequency was genetically determined. To test this possibility, we compared the proportions of TNP-specific cells in the spleens of F_1 hybrids (Figure 6) and of back-cross fetuses (Figure 7) to those observed in fetuses of the parental inbred strains (Figure 5). In the F_1 fetuses, the number of rosette-forming cells per spleen was proportional to the excess of the number of nucleated cells over a threshold of 2.5×10^5 . This threshold may indicate a delayed start in the expansion of the lymphoid compartment of the spleen relative to the nonlymphoid compartment. Nevertheless, although there was more fluctuation about a

TABLE II

Numbers of RFC for Two Different Antigens and Nucleated Cells
in the Spleens of Individual Swiss-L 18-Day Fetal Mice

Litter #	Fetus #	Observed Sample Counts				Calculated Estimates			
		TNP-SRBC RFC*	n.c.†	Sh.26 RBC RFC	n.c.	TNP PFC per Spleen	Sh.26 RFC per Spleen	10 ⁵ n.c. per Spleen	TNP RFC per Sh.26 RFC
1	1	153	412	8	318	765	40	9.1	19.1
	2	91	371	7	361	455	35	9.2	13.0
2	1	108	321	7	284	540	35	7.6	15.4
	2	58	316	5	418	290	25	9.2	11.6
	\bar{X}^f					513	34	8.8	14.8 ^{**}
	SD ††					198	6	0.8	3.3

Footnotes to Table II

* $G^2 = 21.8$ with 3 d.f. for homogeneity of TNP-SRBC RFC per nucleated cell (n.c.); $P < 10^{-4}$. Sheep 26 RBC RFC per n.c. $G^2 = 2.21$ with 3 d.f., $P = 0.53$. All experiments in this table were done blind with the equivalent of four aliquots for each antigen assay, using sheep 26 RBC as TNP carrier and as antigen.

** The probability that the difference between this ratio and the ratio observed for the same two antigens in BALB/c and CBA/J fetuses is due to sampling fluctuation is less than 10^{-9} .

† n.c. = nucleated cell.

†† SD = standard deviation.

$^f \bar{X}$ = arithmetic mean.

straight line than could be explained by sampling fluctuation alone, the constant of proportionality more closely resembled that for inbred CBA/J fetuses than that for inbred BALB/c fetuses. BALB/c x (BALB/c x CBA/J) F_1 backcross fetuses differed from F_1 fetuses and from fetuses of each inbred line (Figure 7). The backcross population appeared to fall into two distinct groups of approximately equal size, one with a relatively high proportion of rosette-forming cells to nucleated cells, and the other with a low proportion. These results are consistent with the control of the relative frequency of antigen-binding cells by a single autosomal Mendelian locus, with the allele at that locus expressed by CBA/J fetuses dominant to the allele expressed by BALB/c fetuses (Cohen *et al.*, 1977).

While genetic controls affecting the size of antigen-binding cell populations have not been reported previously, several controls affecting other quantitative aspects of splenic lymphoid cell populations have been observed. These other controls are consistent with our findings, and may reflect the same underlying regulatory mechanisms. At two weeks postpartum the frequency of splenic B cells expressing complement receptors varies significantly from strain to strain of inbred mouse. Within a strain, the amount of variation in this frequency is greatly reduced (Gelfand *et al.* 1974). The absolute number of these cells, however, appears to be constant even between strains (Ferreira and Nussenzweig 1976). Similarly, for at least one pair of strains, the frequency of splenic T cells differs significantly two weeks after birth, but the number of these cells is the same for both strains (Ferreira and Nussenzweig, 1976). These observations suggest that the sizes of the B- and T-cell populations in the spleen are tightly controlled in young inbred mice, and these controls appear to be due to the action of one or a few autosomal genes at least the predominant one of which maps near the major histocompatibility complex in the mouse (Gelfand *et al.* 1974; Ferreira and Nussenzweig, 1976).

What is the nonlymphoid cell population whose variation in number leads to these observed complementary variations in lymphoid cell frequency? A plausible candidate is the population of nucleated erythroid cell precursors, which make up a substantial part of the fetal spleen cell population of the mouse (Metcalf and Moore, 1971, pp. 214, 218), and whose

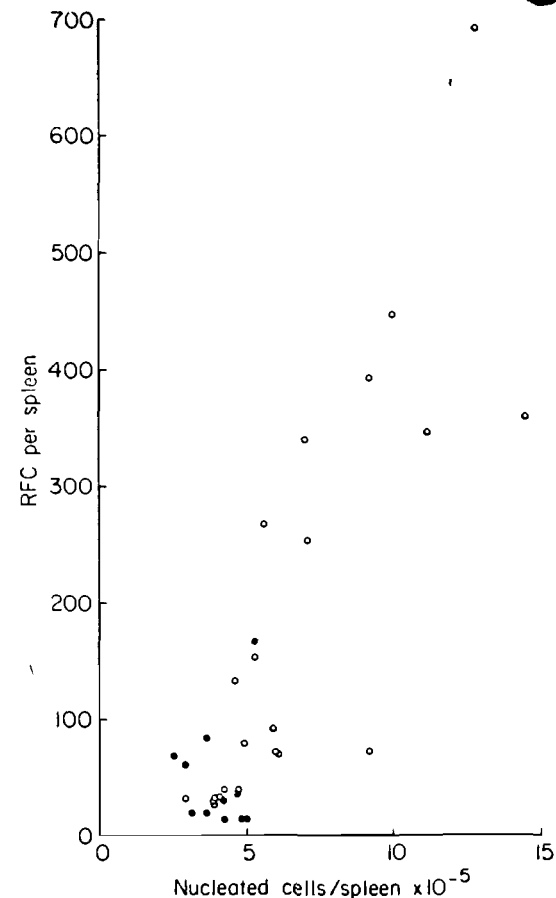


Figure 6. Numbers of TNP-specific RFC's in the spleens of BALB/c x CBA/J (O) and CBA/J x BALB/c (●) F_1 fetal mice as a function of spleen size. All fetuses were tested on the 18th day of gestation (Cohen *et al.* 1977).

maturation is controlled at least in part by genes linked to the major histocompatibility complex (Boubelik *et al.*, 1975). The exact numbers of regulatory genes and the level(s) at which they operate to affect cell populations in the developing spleen obviously remain to be worked out. Nevertheless, it appears to be possible for the first time to give a numerically precise account of the cellular

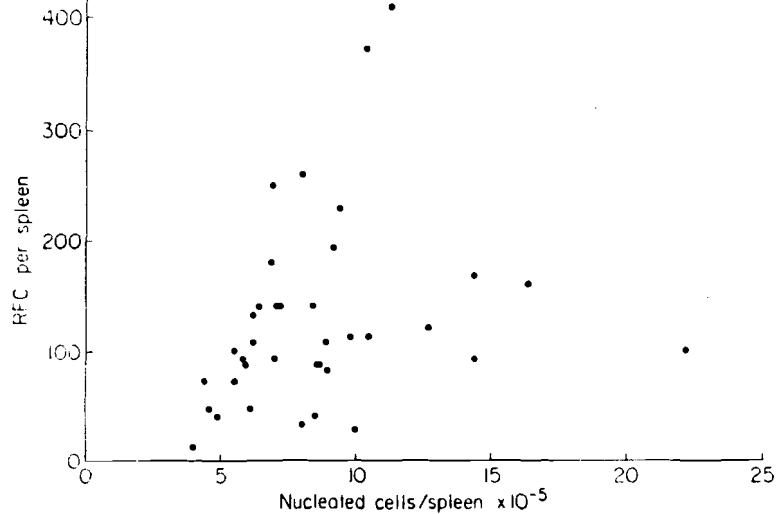


Figure 7. Numbers of TNP-specific RBC's in the spleens of BALB/c \times (BALB/c \times CBA/J) F_1 backcross fetal mice as a function of spleen size. All fetuses were tested on the 18th day of gestation (Cohen et al., 1977).

composition of a developing mammalian organ, to define the controls affecting that composition, and to relate both sets of parameters to the maturation of the organ's function.

Clonal selection and the origin of antibody diversity.

The results of our experiments can be summarized briefly. Cells specific for each of 11 antigens could be detected in the fetal mouse only in the last four days before birth, at which time they appeared in both the liver and the spleen. Although the numbers of antigen-binding cells present in fetuses were smaller than those in adults, no restriction could be detected in the variety of specificities expressed by the fetuses. Under these circumstances, the

hypothesis that cells of different antigenic specificities are generated in a well-defined temporal sequence during development (Klinman and Press, 1975; Silverstein et al., 1963) seems highly unlikely.

The distributions of relative avidities with which fetal and adult cells bound antigens were compared for four antigens, and were identical within experimental error in all cases. This result appears inconsistent with any maturation of antigen-specific cells at the level of antigen-binding itself (Cohn, 1968; Klinman and Press, 1975). It is wholly consistent with the wide range of observations suggesting that the functional consequences of this binding event may change as the cell and its environment in the animal both mature.

Measurements of the numbers of antigen-binding cells present in single fetal spleens showed no systematic deviation of individual fetuses from this pattern, suggesting that it is an accurate description of the immunological development of individual mice, and not merely the average of highly disparate populations. Furthermore, analysis of antigen-binding cell number in inbred and hybrid fetuses suggests that different genetic factors influence the fraction of fetal spleen cells which bear Ig on their surfaces, and the proportions among these Ig+ cells of cells specific for any given antigen. The most striking observation, however, is that the controls on the repertoire composition operate with such precision that by the time there are roughly 15,000 or 2^{14} , Ig+ cells in the fetal spleen, the proportions of Ig+ cells binding specifically to each of various antigens in different individual mice of a given genetic background fluctuate no more than could be explained by sampling fluctuation.

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. It nevertheless seems likely that the full range, if not the full density, of antigen-binding cells is already specified by the 18th day of gestation.

It becomes necessary to explain how such a wide repertoire is generated in the course of fetal development. Three factors might be supposed a priori to be directly involved; the genes coding for Ig variable regions inherited by the fetus in its germ-line genome; somatic mutational and recombinational mechanisms acting in the developing fetus to generate a larger set of genes from the germ-line sequences; and selective mechanisms, acting negatively or positively to alter the relative frequencies of antigen-binding specificities ultimately expressed in the repertoire.

A simple germ-line theory would suppose that each different antigen-specific cell expresses a different germ-line gene and that the proportions observed in the antigen-binding cell repertoire result from the regulated expression of these genes (Hood *et al.*, 1975). While this theory provides a ready qualitative explanation for the control of repertoire composition, recent experiments suggest that it is quantitatively inadequate to account for the diversity of specificities in the repertoire. In particular, the number of germ-line genes now appears to be as small as one per haploid genome per heavy or light chain subgroup in at least some cases (Leder *et al.*, 1975; Tonegawa, 1976).

It, therefore, appears that many, perhaps most, of the genes coding for the full repertoire of Ig variable regions are generated by somatic mutation or related alterations in germ-line DNA. Within this framework, our data indicate that the mechanism which generates the repertoire must yield reproducible proportions of antigen-binding specificities in the absence of selection by antigens other than those ordinarily present in the fetal mouse, and that this mechanism may function in as few as 14 generations of cell division. These data appear incompatible with any theory that positive selection by antigens outside of the individual fetus determines the composition of the repertoire of antigen binding specificities. In principle, however, this remarkably precise and early control of the repertoire could be explained by the action of negative selection on the repertoire generated by somatic mutation or recombination.

Nossal and Pike (1975) have proposed, for example, that an immature lymphocyte exposed to antigen is immediately and irreversibly stripped of its surface Ig. Such a cell

would not be detected either as an Ig+ cell or as an antigen-binding cell in experiments of the sort described in this paper. If this negative selection were imposed by an invariant panel of self-antigens, such as the developing animal's own histocompatibility antigens (Jerne, 1971), certain clones would be absent 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 the range of antigen-binding specificities expressed, or in the distribution of avidities with which any particular foreign antigen is bound. Such negative selection would be expected to lead to fine differences in the antigen-binding repertoires expressed by genetically distinct strains of mice. Within the limits of precision of the assays we have used, however, no differences have been detected in the composition of the BALB/c and CBA/J fetal repertoires in spite of differences between the two strains in a variety of genetic markers including histocompatibility antigens (Staats, 1976).

Our data suggest that the repertoire may be generated rapidly and somatically, and that it arises largely in the absence of positive or negative selection for cells of particular antigen-binding specificities. It is possible to construct models of somatic mutation compatible with these restrictions and with the observed constancy of the repertoire composition from individual to individual (J.E. Cohen, P. D'Eustachio and G.M. Edelman, unpublished observations). It will be of considerable interest to determine whether the frequency and distribution of Ig variable region sequences generated by such models accurately reflect the patterns seen in the sequences of actual Ig's, and to test the predictions of these models against further studies of fetal and adult lymphocytes and their precursors.

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DISCUSSION FOLLOWING PRESENTATION BY DR. D'EUSTACHIO

DR. STEELE: Based upon Dr. Mosier's work presented earlier, it appears that presenting an antigen shortly after birth permits one to modulate the size of the responding population. Have you attempted to influence this by presenting antigens early or by presenting antigens to the mother?

DR. D'EUSTACHIO: We haven't tried deliberately to do that. As a routine control for the rosette assays, I always assayed the spleen from the mother of the litter, and what we observed consistently in the inbred fetuses was that while rosette forming cell frequency in the maternal spleen varied significantly, rosette forming cell frequency in the inbred fetal spleens did not. At least this is some indirect evidence suggesting that the two compartments tend to behave somewhat independent. Whether I could force a change on the fetal compartment, I don't know.

DR. GOIDL: Have you attempted to use a different fiber in the assay?

DR. D'EUSTACHIO: I haven't. I should point out that when the assay was being developed a number of kinds of fibers were tested. Nylon appears to be entirely satisfactory in the sense that essentially all of the binding can be competitively inhibited by soluble antigen and essentially all binding can also be inhibited by antibody against mouse immunoglobulin. It is possible, in addition, to recover cells from the fiber and show that they have antigen specific immune function. Therefore, I don't think that the nylon has anything to do with what binds to the fiber.