FREQUENCY AND AVIDITY OF SPECIFIC ANTIGEN-BINDING CELLS IN DEVELOPING MICE*

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In the course of its development, the mouse acquires a large number of lymphoid cells, each committed to make a single type of antibody molecule. Through these molecules, the cells recognize an enormous number of different foreign antigens. Some aspects of the development of this system are fairly well understood. Cells capable of synthesizing immunoglobulin (Ig) can first be detected in the liver on the 12th day of a 19–20 day gestation period (1), but cells bearing surface Ig cannot be detected until the 15th day of gestation when they appear in the spleen and liver (2, 3). The appearance of these Ig-bearing cells in the liver is transient, and they disappear within a week of birth. In the spleen, however, their number continues to increase, reaching a plateau only in the adult animal.

The development of antigen-specific cells is less well understood. Although spleen cells specific for each of three antigens have been shown to appear and increase in number in parallel with the development of Ig-bearing cells (2), several investigators have demonstrated that the ability of developing animals to respond to various antigens is acquired in a well-defined sequence spread over a period of days or weeks (4–8). A more complete understanding of the functional development of the immune system clearly requires systematic information on the appearance and properties of cells specific for each of a wide variety of chemically diverse antigens. In particular, it seems important to determine, first, when and in which organs of the developing fetus specific antigen-binding cells first appear; second, how the numbers of these cells change during the animal's subsequent development; third, how this process varies from individual to individual; and fourth, how closely antigen-binding properties of fetal and adult cells resemble each other.

In the studies reported here, we have examined the antigen-binding properties of cells in the livers and spleens of mice as a function of age. 11 different hapten and protein antigens were studied. The fiber-binding assay (9) was used in many of these experiments, because it provides a simple and uniform method for studying the interactions of cells with any of a wide variety of antigens. In all cases, specific antigen-binding cells were first detected in the liver on the 15th day of a 19-day gestation period. The proportions of antigen-binding cells of different specificities were similar in fetal, neonatal, and adult tissues, and

* This work was supported by U.S. Public Health Service grants HD 07014 and AI 11378 from the National Institutes of Health, and by grant GB 37556 from the National Science Foundation.

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.

The numbers of antigen-binding cells measured in spleens from individual 18-day fetuses were unimodally distributed about the mean values for the population as a whole, and no systematic variation from these mean values could be detected, suggesting that the results obtained with pools of cells accurately reflect the development of individual mice. These results indicate that antigen-binding cells of various specificities arise relatively rapidly and in parallel during development, and therefore that strong antigenic selection is not likely to operate during ontogeny. This has several implications for theories on the origin of antibody diversity, and suggests that positive selection may not be required for somatic diversification to occur.

Materials and Methods

Mice. BALB/c mice bred in our laboratory from stock obtained from the Jackson Laboratories (Bar Harbor, Maine), and Swiss-L mice from the specific pathogen-free colony maintained at The Rockefeller University (10) were used in the present studies. Female mice (2- to 3-mo old) were housed with males of the same strain, and inspected daily for vaginal plugs. The day a plug was found was taken to be day 0 of gestation. Parturition occurred on day 19 of gestation in these mice. Breeding cages were checked daily for new births; the day of birth was taken to be the day the litter was found.

Antigens. Limulus hemocyanin was prepared by repeated centrifugation. Lysozyme (Worthington Biochemicals, Freehold, N. J.), myoglobin (Miles-Seravac, Maidenhead, Berks., England), and bovine serum albumin (BSA;[1] Pentex Chemicals Inc., Kankakee, Ill.), were obtained commercially and used without further purification. *p*-Aminobenzoic acid, sulfanilic acid, *p*-toluenesulfonic acid, *p*-iodobenzoic acid (all from Eastman Kodak, Rochester, N. Y.,), 5-aminoquinoline (Aldrich Chemical Co., Milwaukee, Wisc.), *p*-aminophenyl-β-D-lactoside (Cyclo, Los Angeles, Calif.), fluorescein isothiocyanate (Baltimore Biological Labs, Cockeysville, Md.), trinitrobenzenesulfonic acid (Pierce, Rockford, Ill.), and adenosine monophosphate (Calbiochem, San Diego, Calif.) were coupled to BSA or to *Limulus* hemocyanin by standard procedures (11).

Preparation of Antisera. Rabbit antiserum against mouse Ig was prepared as described previously (2). The immunoglobulin fraction of the antiserum was isolated by precipitation with ammonium sulfate at 35% saturation, followed by chromatography on DEAE-cellulose (DE52; W. & R. Balston, Maidstone, Kent, England). Antiserum against the θ(C3H) antigen was prepared by immunizing AKR/J mice with thymocytes from C3H/HeJ mice as described previously (12).

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, Md.) 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 number of cells estimated using hemacytometers. 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 (13).

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, Calif.) 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 through a syringe and 22 gauge needle. Each cell

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; MEM-DNase, Eagle's minimal essential medium supplemented with 20 μg/ml of deoxyribonuclease; RFC, rosette-forming cells; TNP, trinitrophenol.

suspension was transferred to a 12 × 75 mm disposable plastic tube (Falcon) and pelleted. The cells were resuspended in 0.8 ml of MEM supplemented with 1% heat-inactivated fetal bovine serum (Microbiological Associates).

Detection of Antigen-Binding Cells. Nylon fibers strung in circular polyethylene frames were coated with antigen, washed, and incubated with spleen cell suspensions as described previously (14). 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 nonspecific binding with no apparent effect on the number of lymphoid cells bound. All fiber-binding assays on liver cell suspensions were therefore carried out in the presence of 400 μg/ml of BSA. The number of cells bound to a given length of fiber was proportional to the number of antigen-specific cells in the incubation mixture (14, 15). Knowing the number of cells bound to a 25-cm length of fiber (the amount in one frame), 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. The numbers of B and T cells bound to antigen-coated fibers were estimated by determining the numbers of bound cells destroyed by treatment in situ with antiserum against mouse Ig or the θ(C3H) antigen and complement, as described previously (16).

To detect antigen-binding cells in single fetal spleens, the rosette assay was used because of its higher efficiency. The cell suspension prepared from a single spleen was divided into two 0.4-ml portions, each of which was mixed with 0.1 ml of a 1% suspension of red blood cells in MEM supplemented with 1% heat-inactivated fetal bovine serum, and assayed for rosette-forming cells (RFC) as described previously (2). Plain sheep red blood cells (SRBC; Microbiological Associates), or SRBC derivatized with the trinitrophenol hapten (TNP) (17) were used in these assays.

Results

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 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 I). If the assay was done in the presence of 400 μg/ml of rabbit antibodies against mouse Ig, 75–95% of the fiber binding was inhibited, whereas normal rabbit immunoglobulin caused less than 10% inhibition. 400 μg/ml of a particular soluble antigen-BSA or antigen-hemocyanin conjugate in the incubation mixture inhibited 50–95% of the binding to fibers coated with that antigen; 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 fiber-bound cells detected in the assay was linearly proportional to the number of cells in the incubation mixture, from 10^6 cells/dish to 10^8 cells/dish. Direct comparison of the numerical results obtained from the fiber assay with those of the rosette assay (2) 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 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 Fig. 1. 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

TABLE I

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 done 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 ± SE of the mean, based on three or more independent determinations.

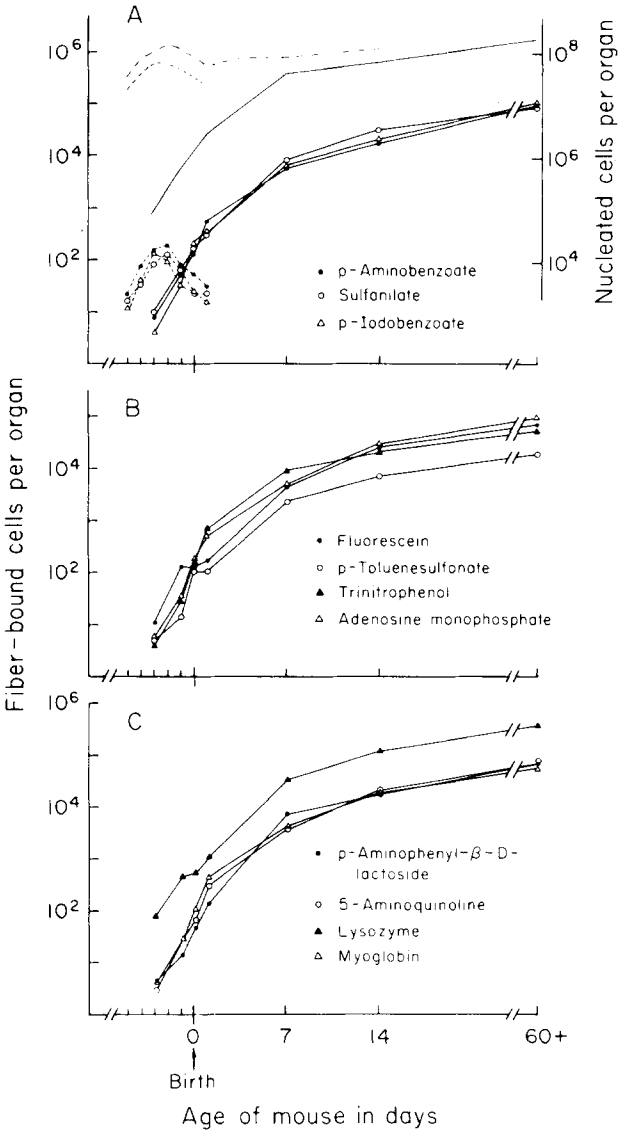

FIG. 1. Numbers of fiber-bound cells per organ specific for each of 11 antigens, in the spleens (——) and livers (-----) of Swiss-L mice as a function of age. These numbers were calculated from the numbers of cells bound per 25 cm of fiber and the numbers of nucleated cells per organ, as described in 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 hemacytometer counts of liver cell suspensions which had not been washed to remove damaged cells, and therefore includes both hemopoietic and parenchymal cells.

detected in the liver on the 15th day of gestation (Fig. 1 A). 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 (Fig. 1 A). In the spleen, specific antigen-binding cells were first detected on the 16th day of gestation (Fig. 1 A,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 I) and in the total number of cells in the spleen (Fig. 1 A). 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).

Antigen-Binding Cells in Single Fetal Mice. All of the experiments described above were performed with pools of 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 Swiss-L fetuses were tested for numbers of cells specific for each of two antigens, TNP and SRBC (Table II and Fig. 2). The rosette assay was used for these experiments because its very high efficiency (18) allowed accurate measurements of the numbers of antigen-specific cells in these small cell populations. For each antigen, the mean number of RFC per spleen, calculated from these measurements on individual fetal spleens (Table II) was in agreement with the corresponding numbers previously determined for pooled fetal spleens (2). For both antigens, the numbers of RFC measured in individual spleens was unimodally distributed about these mean values (Fig. 2).

To measure the variation in number of antigen-binding cells from fetus to fetus within any one litter, fetuses from several entire litters were assayed individually for TNP- or SRBC-specific RFC (Table II). Values of P calculated by a two-tailed Student's t test indicated that the results obtained for particular litters were not significantly different from those for the populations as a whole, nor were the two litters tested for TNP-specific RFC significantly different from each other.

In some of these experiments, the cell suspension from a single 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

TABLE II

Rosette-Forming Cells in Single Fetal Spleens

	Antigen	Mean no. RFC/ spleen	Standard deviation	Standard error of mean	No. of fetuses tested
All fetuses*	(*a*) SRBC	38	30	6	33
	(*b*) TNP	270	190	30	102
Individual litters*	(*c*) SRBC	27	18	7	6
	(*d*) TNP	200	120	40	10
	(*e*) TNP	260	200	70	9

* Two-tailed Student's t tests indicated no significant differences between individual litters and the populations of fetuses: comparing groups a and c, $0.3 < P < 0.4$; groups b and d, $0.2 < P < 0.3$; groups b and e, $0.95 < P < 0.98$; groups d and e, $0.3 < P < 0.4$.

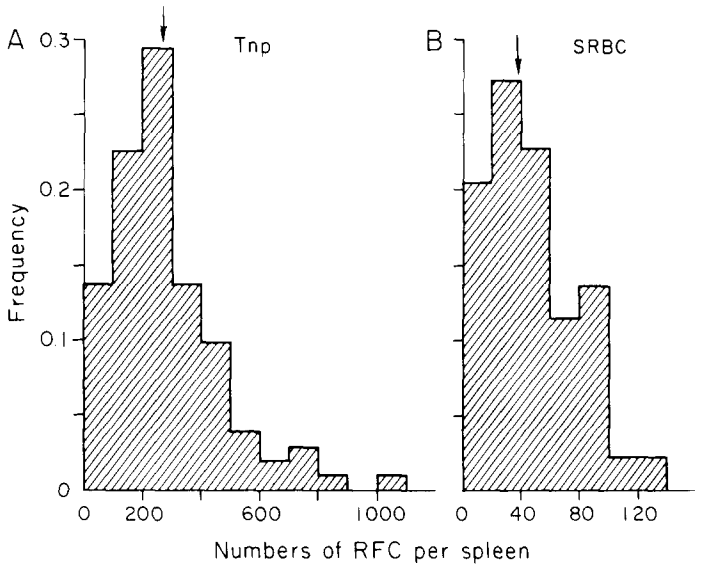

FIG. 2. Frequency distributions of the numbers of RFC in single fetal spleens. Spleens from individual 18-day fetuses were assayed for RFC, using TNP-coated SRBC (Fig. 2 A) or plain SRBC (Fig. 2 B) as the test antigens. The mean values observed for each antigen are shown by the arrows (see Table II).

that the relative scarcity of SRBC-specific cells in the fetal population as a whole was reflected in the individual fetuses. Spleens from a total of nine individual BALB/c fetuses gave a mean number of 74 TNP-specific RFC per spleen, and the individual values were unimodally distributed about this mean, in agreement with the results obtained with Swiss-L fetal spleens.

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. The relative avidity with which a lymphocyte binds to a soluble hapten-protein conjugate can be estimated from the concentra-

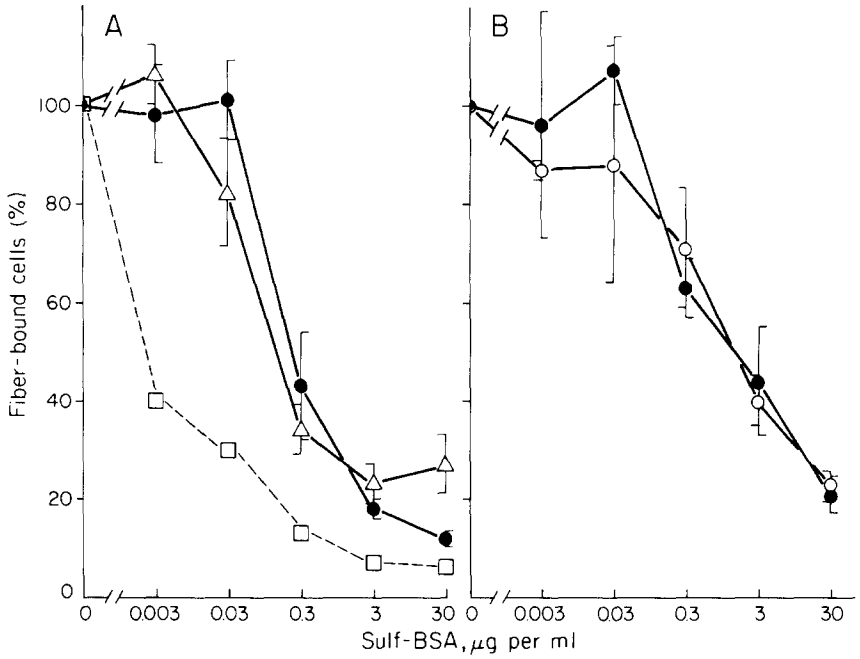

FIG. 3. Number of cells bound to sulfanilate-coated fibers in the presence of soluble sulfanilate-BSA at several concentrations, expressed as a percentage of uninhibited control values. Cells from fetal spleen (Fig. 3 A) and fetal liver (Fig. 3 B) were compared with cells from adult spleen in parallel 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 three or more independent determinations ± SEM. Spleen cells from adult mice immunized against sulfanilate (two intraperitoneal injections, one month apart, of sulfanilate-hemocyanin adsorbed on bentonite) were assayed in the same way (□). The results of a typical experiment are shown.

tion 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.

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 (Fig. 3). Both fetal spleen cells (Fig. 3 A) and fetal liver cells (Fig. 3 B) 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 (Fig. 3 A). The relative avidity distributions of spleen cells from fetuses and adults were compared for three additional antigens, *p*-aminobenzoate, TNP, and *p*-toluenesulfonate, and for each of these antigens the fetal and adult cells gave closely similar distributions.

Discussion

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. Fetal and adult antigen-binding cells were remarkably similar with respect to the relative numbers of cells specific for each of the antigens, and with respect to the range of avidities with which they bound any particular antigen.

The experiments described here indicate that the fiber-binding assay provides a general, rapid method for isolating and characterizing antigen-binding cells. 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 specific 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 fibers assay especially suitable for measuring the rates of appearance and properties of antigen-binding cells in the course of development.

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 (2), 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 (1). 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 (2, 3). In addition, Melchers et al. (1) 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 (19). 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.

Most of the present measurements 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 precedure, individual fetal spleens were assayed for antigen-binding cells. 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. Fetal BALB/c spleens were examined individually only for TNP-specific RFC, but again a unimodal distribution about the mean for the population was observed. These experiments suggest that the results obtained with pools of cells from several animals accurately reflect the immunological development of individual mice with respect to any one antigenic specificity. The possibility remains, however, that antigen-binding cells of different specificities appear sequentially in individual mice but that the sequence varies from animal to animal and therefore is not observed in experiments on pooled cells. If this were true, then at least some individual fetuses should contain SRBC-specific cells, but no, or very few, TNP-specific cells. Despite the large variation in the absolute numbers of antigen-specific cells per spleen observed in these experiments, no such fetuses were found. Although these data are limited, they do support the hypothesis that cells of different antigenic specificities are expressed with some variance beginning at the same point in development, but not in any fixed order and soon reach the proportions found in the adult animal.

A key characteristic of mature antigen-binding cell populations is the presence within those populations of some cells with high avidity for the antigen (15). 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 as an indication of the very restricted exposure to foreign antigens that these specific pathogen-free animals receive in the course of their postnatal development.

It is important to note that, while cells bound to an antigen-coated fiber are antigen-specific in the sense that they bind to only one or a few related antigens and do so via their cell-surface Ig receptors, only a relatively small proportion

of these cells bind that antigen with sufficient avidity to be triggered by it (15). In order to recognize a wide range of specificities in the immune response, the large proportion which bind a given antigen too weakly for triggering must therefore bind other antigens with a sufficient avidity to be stimulated (20). In both fetal and adult animals, the group of cells that binds TNP with low avidity includes, for example, some cells with a sufficiently high avidity for sulfanilate-BSA so that the latter compound blocks their binding to TNP-coated fibers (D'Eustachio, Rutishauser, and Edelman, unpublished observations).

The existence of cells with a wide range of avidities for a particular antigen therefore strongly suggests the existence of other triggering specificities, and although only a small number of antigens has been tested here, the closely similar results obtained in all cases suggest that the repertoire of these specificities is filled early and rapidly for a very 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 antigen after the appearance of specific antigen-binding cells. If this conclusion is correct, the generation of antibody diversity in the normal animal would appear not to be an antigen-driven phenomenon.

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 (21), suggesting that the range of antibodies is generated by the somatic mutation (22, 23) or recombination of a small number of genes (24) in the precursors of the antigen-binding cells, with no strong selection among the newly produced molecules according to their antigen-binding properties. The generation of antibody diversity could therefore occur early in development with the expression of antibody specificities occurring 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 cells, and there is no reason a priori to favor germ-line models for the generation of antibody diversity.

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 (2). Several factors probably contribute to this phenomenom. 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 (25) have shown that the ratio of cells being 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 (26) 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 (27). 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 (28) and the complement receptor (29). Both of these receptors can first be detected on B cells about 2 wk 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 (30). 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.

Summary

In order to analyze the development of antigen-specific cells we have compared the binding of diverse antigens by cells in the fetal, neonatal, and adult mouse. Although the numbers of antigen-binding cells present in fetuses and young animals were smaller than in adults, no restriction could be detected in the variety of specificities expressed in the fetuses, either with respect to the kinds of antigens bound, or to the range of avidities of binding.

Cells specific for each of the 11 antigens tested could be detected in the fetus only in the last 4 days before birth, at which time they appeared both in the liver and in 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. Measurements of the numbers of antigen-binding cells 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. 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. The proportions of T and B cells in neonatal and adult antigen-binding cell populations were also closely similar.

These data are consistent with models for the origin of antibody diversity in which the genes coding for the full repertoire of antibodies are generated somatically from a small number of germ line genes during the course of

development, in the absence of any strong selection with respect to antigenic specificity. They 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 needed for a full immune response.

The authors thank Dr. Urs Rutishauser for his advice and assistance with several aspects of this work, and Miss Mildred Decker for her valuable technical assistance.

Received for publication 15 July 1975.

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