CHARACTERIZATION OF SPLENIC LYMPHOID CELLS IN FETAL AND NEWBORN MICE*

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The capacity to respond to antigen by the production of humoral antibody arises in utero for some mammalian species but not until after birth for other species including mice (1). The development of this immunologic competence must depend in part upon the appearance and maturation of the cellular elements required for the immune response and in part upon the expression of a sufficiently wide range of antigen-binding specificities. In general, the production of antibody in the mature animal requires the interaction of two kinds of antigen-specific lymphocytes that differentiate along separate pathways, one mediated by the thymus and the other by the bursa in chickens or its equivalent in mammals (2–4). The thymus-derived cells (T cells) and bursa-derived or bone marrow-derived cells (B cells) have been most extensively studied in the mouse inasmuch as there are known cell surface markers in this species that allow the identification of both cell types (5). In contrast to T cells, B cells have sufficient immunoglobulin on their surfaces to be easily detected by a variety of immunological techniques (6); on the other hand, T cells but not B cells carry the membrane antigen specified by the θ-marker (7, 8).

Little is known about the expression of different antigen-binding specificities during development or about the rates of appearance and numbers of the two lymphocyte cell types in secondary lymphoid tissues during organogenesis. Nossal and Pike (9) have determined that B cells first appear in the spleens and blood of CBA/H/Wehi mice, on the 16th day of a 19–20 day gestation period. They found that the number of B cells increases rapidly until after birth and more slowly thereafter, reaching a plateau at about 5 wk of age. Quantitative estimates of the number of T cells in secondary lymphoid organs as a function of age have not been reported for mouse fetuses; in several studies of neonatal mice, however, the numbers of T cells in lymph nodes, Peyer's patches, and blood (10, 11), and in spleens (12) have been shown to increase with age. Although antigen-binding cells have been detected in fetal and neonatal tissues (13, 14), information on their rates of appearance, total numbers, and range of specificity is particularly sparse.

In the studies reported here, we have examined the lymphoid cells in the spleens of mice as a function of age in order to determine in a correlative

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fashion when T cells, B cells, and specific antigen-binding cells appear. We also wished to know whether cells that bind to different antigens appear sequentially or simultaneously and whether the onset of antibody synthesis in response to antigen is related to quantitative changes in lymphoid cell populations. The results indicate that T cells, B cells, and antigen-binding cells appear in the spleens of mice as early as the 15th day of a 19 day gestation period. Cells specific for each of three unrelated antigens were first detected during the same 24 h interval, from 15 to 16 days' gestation. The three kinds of antigen-binding cells were present in the same relative proportions in fetal, neonatal, and adult spleens; in addition, fetal and adult antigen-binding cells were similar in their ranges of avidities for a single antigen. B cells, T cells, and antigen-binding cells rapidly increased in number from the time of their appearance until about 7 days after birth. The numbers of all these cell types remained relatively constant from 1 to 2 wk after birth; nevertheless, consistent responses to antigen could not be detected until after 2 wk of age.

Our results suggest that although a rather large variety of antigen-binding specificities can be expressed even before birth, maturational changes required for the onset of response to antigen occur during the 2nd wk after birth. These changes may be qualitative in nature inasmuch as they are not evident from a mere consideration of the numbers of lymphoid cells present.

Materials and Methods

Mice.—Swiss-L mice from the specific pathogen-free colony maintained at The Rockefeller University (15) were used in the present studies. These colony-bred mice are the descendents of nine animals used to start the colony in 1926. Female mice (2–3 mo old) were housed with males and inspected daily for vaginal plugs. The day a plug was found was taken to be day 0 of gestation. Parturition regularly 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. Since most births occur at night, the mice could be as much as 12 h older than reported.

Immunization of Mice.—Mice of various ages were injected intraperitoneally with 10% sheep erythrocytes (SRBC)[1] (Microbiological Associates, Inc., Bethesda, Md.) in saline or with trinitrophenyl-hemocyanin (Tnp-Hcn) adsorbed onto bentonite (16) in phosphate-buffered saline ([PBS] 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The bentonite was autoclaved at a 1% (wt/vol) solution in H_2O before the adsorption. Amounts injected were 8 μl of 10% SRBC or 8 μg of Tnp-Hcn on bentonite per gram of body weight. Control animals received saline or bentonite in PBS, respectively.

Preparation of Antisera.—Anti-θ (C3H) serum was prepared in AKR/J mice by the method of Reif and Allen (7). Thymocytes from C3H/HeJ mice were used for immunization. The

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[1] *Abbreviations used in this paper:* fl-anti-Ig, fluorescein-labeled rabbit Ig directed against mouse Ig; Ig, immunoglobulin; MEM-1% FBS, Eagle's minimal essential medium supplemented with 1% fetal bovine serum; PBS, phosphate-buffered saline; PFC, plaque-forming cells; RFC, rosette-forming cells; RRBC, rabbit erythrocytes; SRBC, sheep erythrocytes; Tnp-Hcn, *Limulus* hemocyanin derivatized with 2, 4, 6-trinitrobenzenesulfonic acid; Tnp-SRBC, SRBC derivatized with 2, 4, 6-trinitrobenzenesulfonic acid.

serum obtained had a cytotoxic titer of 1:800 against thymocytes from CBA/J mice and 1:400 against thymocytes from Swiss-L mice. At optimal cytotoxic concentrations, greater than 95% of thymocytes and 25–35% of spleen cells from Swiss-L mice were killed in the presence of complement. 90% of the cytotoxic activity could be removed by two absorptions of the serum with equal volumes of CBA/J brain tissue. The mice used for the preparation and assay of anti-θ serum were obtained from Jackson Laboratories, Bar Harbor, Maine.

Antimouse immunoglobulin serum was prepared in rabbits by the injection of Swiss-L mouse immunoglobulin purified by starch block electrophoresis (17). The protein was emulsified with Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) and injected in the footpads at a dose of 1 mg per rabbit. Booster injections were given subcutaneously with incomplete Freund's adjuvant. Immunoglobulin was purified from the antiserum by precipitation with sodium sulfate at 18% saturation and chromatography on DEAE-cellulose. To couple fluorescein to the immunoglobulin, 10 mg of purified immunoglobulin and 0.4 mg of fluorescein isothiocyanate (Baltimore Biological Laboratories, Cockeysville, Md.) were dissolved in PBS containing 0.05 M sodium bicarbonate. The mixture was stirred at room temperature for 1 h and then passed over Sephadex G-25 equilibrated with PBS. Finally, the conjugated immunoglobulin was dialyzed against PBS for several hours.

Spleen Cell Suspensions.—The number of mice used to prepare each spleen cell suspension varied from 3 for adults and older animals to as many as 40 for fetuses at 15–16 days' gestation. The spleen capsules were punctured with needles and the cells gently forced out into Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) by pressing the organs against a wire mesh or squeezing with forceps. After gently pipetting the cell suspensions, clumps were removed by low-speed centrifugation and the cells were pelleted. The cells were washed with Hanks' solution and suspended in Eagle's minimal essential medium (Microbiological Associates) supplemented with 1% heat-inactivated fetal bovine serum (Microbiological Associates) (MEM-1% FBS). Cell counts were done using hemacytometers.

Cytotoxic Assays.—Cell suspensions at 3×10^7 nucleated cells per ml in MEM-1% FBS were mixed in 0.1-ml aliquots with equal volumes of anti-θ serum at the appropriate dilutions. The mixtures were kept at 0°C for 30 min and then diluted with 2 ml of MEM-1% FBS. The cells were pelleted by centrifugation, washed with 2 ml of MEM-1% FBS, and resuspended in 0.2 ml of guinea pig complement. The complement was obtained in lyophilized form (Grand Island Biological Co.), reconstituted as directed, diluted 1:10 with MEM-1% FBS, and absorbed with 1/20 vol of Swiss-L mouse spleen cells. The cells being tested were incubated with the diluted and absorbed complement for 45 min in a 37°C water bath. An equal volume (0.2 ml) of 0.16% trypan blue in saline was then added, and the mixtures were immediately examined microscopically to score for viable and nonviable cells. Control assays, in which the anti-θ serum was replaced by normal AKR/J mouse serum or by MEM-1% FBS, were always done in parallel with the tests for anti-θ cytotoxicity. Corrections for the fraction of cells dead in normal serum controls were applied to calculations of the percentage of cells killed by anti-θ serum.

Detection of Ig-Positive and θ-Positive Cells by Immunofluorescence.—The Ig-positive cells in the spleen were detected by their binding of fluorescein-labeled anti-immunoglobulin (fl-anti-Ig). Spleen cells (5×10^6) were suspended in 0.2 ml of MEM-1% FBS containing fl-anti-Ig at 1 mg/ml and 0.1 M sodium azide. After incubation of the mixture at 37°C for 15 min, the cells were washed and resuspended in 0.2 ml of MEM-1% FBS plus sodium azide for microscopic examination. For each determination, two aliquots were counted, scoring each for the fluorescent cells and for the total nucleated cells. About 200 cells were examined in each aliquot. The sum of θ-positive and Ig-positive cells in the spleen was detected by treating the cells first with anti-θ serum and then with fl-anti-Ig. Spleen cells (5×10^6) in 0.2 ml of MEM-1% FBS plus sodium azide were incubated with anti-θ serum at a final

dilution of 1:10 for 15 min at 37°C. The cells were washed with MEM-1% FBS + sodium azide; they were then incubated with fl-anti-Ig, washed, and examined as described for the detection of Ig-positive cells. The fluorescent cells included those stained directly with fl-anti-Ig (Ig-positive cells) and those stained indirectly due to the prior binding of mouse anti-θ antibodies (θ-positive cells). The number of θ-positive cells was estimated by subtracting the number stained with fl-anti-Ig alone from those stained by anti-θ plus fl-anti-Ig.

The microscopic examinations were done with a Zeiss universal microscope (Carl Zeiss, Inc., New York) alternating between phase-contrast and incident light fluorescence, using BG12 and BG38 exciting filters and a no. 53 barrier filter.

Detection of Antigen-Binding Cells.—Rosette assays were carried out by a slight modification of the method of McConnell et al. (18), using SRBC, Tnp-derivatized SRBC (16), or rabbit erythrocytes (RRBC) obtained from fresh blood. Erythrocytes at 10^8 per ml and spleen cells at 1–2 $\times$ 10^7 nucleated cells per ml were added in 0.1-ml aliquots to 0.3 ml of MEM-1% FBS in 12 $\times$ 75 plastic disposable tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). When Tnp-Hcn or anti-Ig was added to inhibit rosette formation, 0.1-ml aliquots of spleen cells were added to 0.3 ml of MEM-1% FBS containing the appropriate amount of inhibitor. The tubes were left at 0°C for 30 min before the addition of 0.1 ml-aliquots of erythrocytes. The inhibitor concentrations given refer to the final concentration after the addition of the erythrocytes. Controls for the inhibition experiments were similarly prepared although the number of rosettes obtained was the same whether the erythrocytes were added to the tubes 30 min after the spleen cells or at the same time. The rosette assay mixtures, containing a final concentration of 2–4 $\times$ 10^6 spleen cells per ml, were centrifuged for 10 min at 200 *g* and the pellets were left undisturbed for an additional 10 min. The entire procedure was carried out at 0°–4°C. The pellets were then gently resuspended and 10 or 25-μl aliquots placed between two glass slides separated by a spacer of tape. The suspensions were scanned at a magnification of 200 diameters. Lymphocytes with at least seven adherent erythrocytes were scored as rosette-forming cells (RFC). The bound red cells were usually uniformly distributed but sometimes formed rings or caps. For each determination at least two aliquots from duplicate tubes were scanned; if necessary, additional aliquots were scanned to bring the total count within the range of 10–100 RFC.

Detection of Antibody-Secreting Cells.—The Jerne plaque assay (19) was used to detect cells secreting antibodies against SRBC or against Tnp-SRBC (16).

RESULTS

Quantitation of Thymus-Derived and Bone Marrow-Derived Cells in the Spleen.—The percentages of nucleated spleen cells carrying easily detectable immunoglobulin receptors (B cells) or the θ-marker (T cells) were determined as a function of age by immunofluorescence techniques (Table I). The θ-positive cells were quantitated by subtracting the fraction of cells stained with fl-anti-Ig alone (Ig-positive cells) from the fraction stained with fl-anti-Ig after treatment of the cells with anti-θ serum (Ig-positive cells plus θ-positive cells). The specificity and reliability of this indirect procedure for estimating the number of θ-positive cells was ascertained by the following control experiments: (*a*) The number of spleen cells stained with fl-anti-Ig was the same before and after incubation with normal AKR/J mouse serum, which does not contain anti-θ antibodies. (*b*) More than 90% of thymocytes from CBA/J or Swiss-L mice were stained after incubation with anti-θ serum plus fl-anti-Ig but these cells, which are predominantly θ positive (10), were not stained after

TABLE I

Bone Marrow-Derived and Thymus-Derived Cells in the Spleens of Swiss-L Mice as a Function of Age

Age	Fluorescent cells as % of nucleated cells			Ratio of θ positive to Ig positive
	θ positive + Ig positive*	Ig positive‡	θ positive§	
days				
Gestation				
16	8.9 ± 0.4¶	1.1 ± 0.4¶	7.8 ± 0.6‖	7.1 ± 2.6‖
18	21.3 ± 2.3	4.0 ± 0.7	17.3 ± 2.4	4.3 ± 1.0
After birth				
0	41.5 ± 2.5	20.8 ± 2.7	20.7 ± 3.6	1.0 ± 0.2
1	50.0 ± 3.5	27.5 ± 1.8	22.5 ± 4.0	0.82 ± 0.16
7	55.0 ± 2.2	28.7 ± 2.2	26.3 ± 3.1	0.92 ± 0.13
14	61.3 ± 1.7	34.7 ± 1.0	26.6 ± 1.9	0.77 ± 0.06
21	60.2 ± 2.7	43.3 ± 3.1	16.9 ± 4.1	0.39 ± 0.10
28	67.0 ± 0.9	46.3 ± 1.9	20.7 ± 2.1	0.45 ± 0.05
60+	80.3 ± 2.4	53.0 ± 3.1	27.3 ± 3.9	0.52 ± 0.08

* Cells stained after treatment with anti-θ serum followed by fl-anti-Ig.

‡ Cells stained after treatment with fl-anti-Ig alone.

§ Calculated by subtracting percent Ig-positive cells from percent (θ-positive plus Ig-positive) cells.

¶ Mean ± standard error of mean, based on three independent determinations at each age.

‖ Calculated values ± propagated standard error.

treatment with fl-anti-Ig alone. (*c*) The antibodies in anti-θ serum that promoted the indirect staining of spleen cells or thymocytes by fl-anti-Ig could be removed by absorption of the serum with brain tissue, which also bears the θ-antigen (7), from CBA/J mice; two absorptions removed 85–90% of these antibodies, as detected by indirect immunofluorescence, whether the serum was tested against thymocytes, spleen cells from adults, or spleen cells from newborn mice. Cytotoxicity assays using anti-θ serum plus complement have confirmed the results obtained with indirect fluorescence. We preferred to use the fluorescence technique in most of our studies because, in our hands, it was more sensitive and reproducible than the cytotoxic assay.

The total numbers of nucleated cells, Ig-positive or B cells, and θ-positive or T cells in the spleens of mice at various ages are shown in Fig. 1. For analysis of the data, it is convenient to divide the developmental period of the mouse into three separate intervals, i.e., the first extending from 15 days' gestation to birth, the second extending from birth to 14 days of age, and the third from 14 days to adulthood. Both T cells and B cells could be detected in the spleen as early as 15 days' gestation. By the 16th day of gestation 1% of nucleated cells were Ig positive and almost 8% were θ positive (Table I). The B cells increased in number at a faster rate than the T cells and became

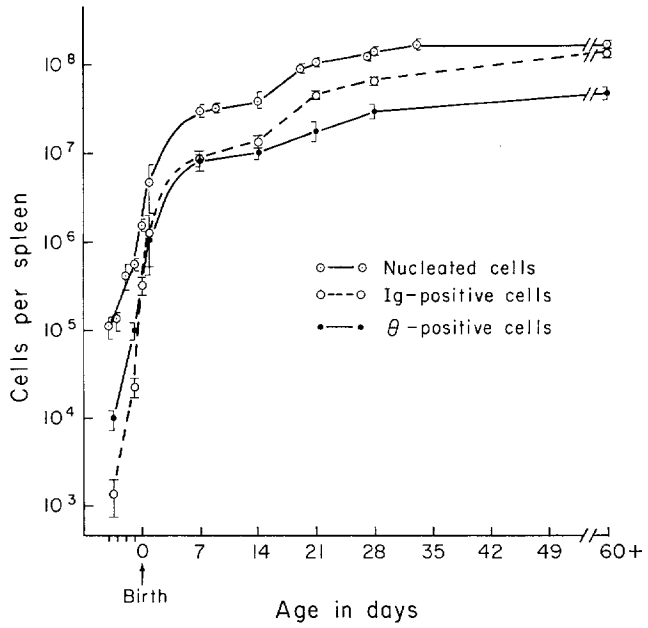

FIG. 1. Numbers of nucleated cells, Ig-positive cells, and θ-positive cells per spleen in Swiss-L mice as a function of age. Numbers of nucleated cells were determined directly by counting spleen cell suspensions in hemacytometers. The number of animals at the same age used to prepare each spleen cell suspension ranged from 3 for adults to as many as 40 (four litters) for fetuses. Each value shown is the arithmetic mean of four to seven independent determinations $\pm$ the standard error of the mean. Numbers of Ig-positive and θ-positive cells were calculated by multiplying the appropriate values presented in Table I by the mean number of nucleated cells per spleen at each age tested. These calculated values are shown with vertical bars representing the propagated standard error of the product of the means.

more numerous than T cells within 24 h after birth (Table I and Fig. 1). During this first interval, however, most of the cells in the spleen were neither T nor B cells, consistent with evidence that the spleen of the fetal mouse is predominantly erythropoietic and granulocytic (20). The process of birth itself did not immediately affect the rates of increase of T cells, B cells, or total nucleated cells.

The second interval was a period of little apparent activity. The rate of increase in the numbers of all cell types in the spleen decreased during the 1st wk after birth and reached a plateau during the 2nd week. Moreover, the ratio of T cells to B cells remained relatively constant during this interval (Table I). In contrast, the third interval was characterized by a significant increase in the number of spleen cells during the 3rd wk after birth due primarily to increases in the B cell population. This change was reflected in a decrease in the T cell to B cell ratio.

Quantitation of Antigen-Binding Cells.—Using the rosette assay, we have estimated the fraction of spleen cells capable of binding to each of three different antigens (Table II) and have calculated the numbers of specific antigen-binding cells per spleen (Fig. 2) as a function of age. Several control experiments demonstrated the specificity of this assay for the detection of antigen-binding cells: (*a*) Rabbit antimouse Ig at a concentration of 500 μg per ml inhibited 95% of the RFC detected in fetal or adult spleens, whereas normal

TABLE II

Antigen-Binding Cells in the Spleens of Swiss-L Mice as a Function of Age

Age	RFC per 10^6 nucleated cells*		
	Tnp-SRBC	SRBC	RRBC
days			
Gestation			
16	68 ± 7.6	5 ± 3.5	n.t.‡
18	216 ± 54	23 ± 2.7	31 ± 4.1
After birth			
0	843 ± 66	50 ± 9.4	40 ± 7.1
1	985 ± 89	n.t.	n.t.
7	1,210 ± 153	113 ± 19	98 ± 11
9	n.t.	108 ± 22	n.t.
14	2,010 ± 401	220 ± 14	159 ± 24
19	n.t.	271 ± 29	n.t.
21	2,560 ± 137	n.t.	150 ± 22
26	n.t.	243 ± 24	n.t.
28	2,980 ± 156	370 ± 41	223 ± 19
33	3,300 ± 247	285 ± 17	n.t.
60+	2,510 ± 227	266 ± 22	283 ± 32

* Data expressed as mean ± standard error of the mean, based on three to seven independent determinations at each age. The number of lymphocytes scanned for each determination ranged from 2×10^4 to 4×10^5 cells from each of two replicate tubes, depending on the number of RFC present.

‡ Not tested.

rabbit immunoglobulin inhibited at most 15% of the RFC. (*b*) Soluble Tnp-Hcn inhibited up to 90% of Tnp-RFC from adult and fetal spleens (Fig. 3) but only 15% of SRBC-RFC. (*c*) The number of RFC detected was linearly proportional to the number of spleen cells added in the assay for cell concentrations up to 4×10^6 nucleated cells per ml.

Cells capable of binding to each of three different antigens were first detected between 15 and 16 days' gestation in the spleens of animals pooled according to age. Their numbers increased rapidly and in parallel until about 1 wk after birth. Thus, at all ages tested, there were approximately 10 times as many Tnp-specific cells as cells specific for either erythrocyte type (Fig. 2). The cells that bound to the two kinds of underivatized erythrocytes were prob-

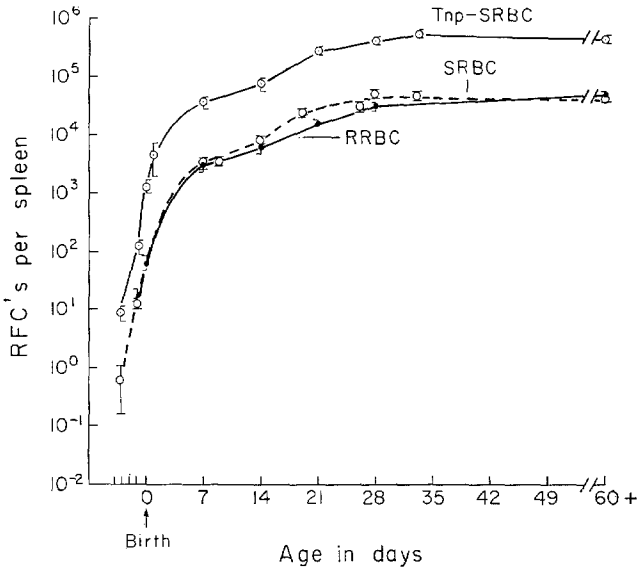

FIG. 2. Numbers of RFC specific for Tnp-SRBC, SRBC, and RRBC in the spleens of Swiss-L mice as a function of age. These numbers were calculated by multiplying the appropriate values in Table II (RFC per 10^6 nucleated cells) by the mean number of nucleated cells per spleen (Fig. 1) at each age tested. Vertical bars represent the propagated standard error of the product of the means.

ably partially overlapping populations. Nevertheless, a significant proportion of the lymphocytes that bound to SRBC did not bind to RRBC and vice versa inasmuch as rosette assays performed with a mixture of RRBC and SRBC yielded 50% more RFC than with either type of erythrocyte alone.

Antigen-binding cells and B cells were first detected in the spleen at about the same time and their numbers increased in parallel (Figs. 1 and 2). The ratio of RFC to Ig-positive cells remained relatively constant with age for all three antigens tested (values for Tnp-RFC shown in Table III). On the other hand, the ratio of RFC to θ-positive cells was small in the fetus but increased sharply with age (Table III).

Experiments were carried out to compare the range of avidities for the Tnp hapten in adult and fetal antigen-binding cells. The relative avidity with which a lymphocyte binds to a soluble Tnp-protein conjugate can be estimated from the concentration of conjugate required to inhibit rosette formation with Tnp-SRBC. The formation of rosettes by the higher avidity cells will be inhibited by the lower concentrations of the soluble competitor. It should be pointed out that differences in the avidity distributions of two cell populations can readily be demonstrated by using several concentrations of soluble competitor to inhibit rosette formation. For example, comparisons of

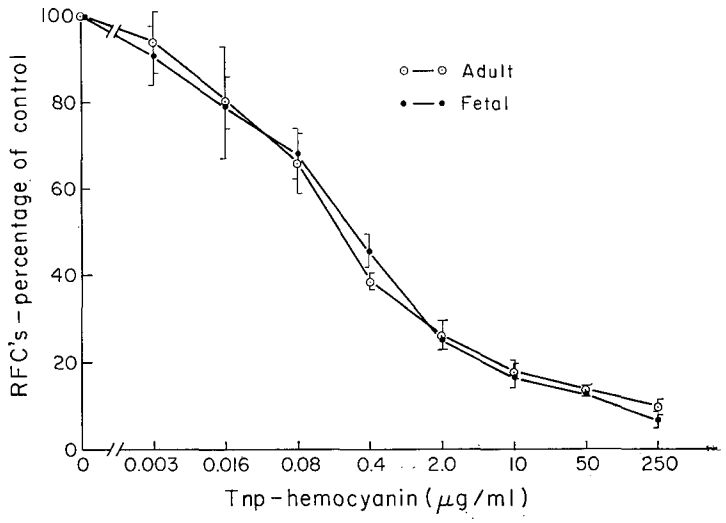

FIG. 3. Number of RFC formed by fetal or adult spleen cells with Tnp-SRBC in the presence of Tnp-Hcn at several concentrations, expressed as the percentage of uninhibited control values. The spleen cell suspensions were prepared from 50 fetuses (four litters) at 18 days' gestation and from 3 unimmunized adults. These suspensions were assayed for Tnp-RFC in the presence or absence of Tnp-Hcn as described in Materials and Methods. Each value shown is the mean of three independent determinations ± the standard error of the mean.

TABLE III

Ratios of Tnp-RFC to Ig-Positive Cells and to θ-Positive Cells in the Spleens of Swiss-L Mice as a Function of Age

	Age	$\frac{\text{Tnp-RFC}}{\text{Ig-positive cells}} \times 10^3$	$\frac{\text{Tnp-RFC}}{\theta\text{-positive cells}} \times 10^3$
	days		
Gestation			
	16	6.2 ± 2.4*	0.9 ± 0.1*
	18	5.4 ± 1.6	1.2 ± 0.4
After birth			
	0	4.1 ± 0.6	4.1 ± 0.8
	1	3.6 ± 0.4	4.4 ± 0.9
	7	4.2 ± 0.6	4.6 ± 0.8
	14	5.8 ± 1.2	7.6 ± 1.6
	21	5.9 ± 0.5	15.1 ± 3.8
	28	6.4 ± 0.4	14.4 ± 1.6
	60+	4.7 ± 0.5	9.2 ± 1.6

* Values calculated from data given in Tables I and II, ± the propagated standard error of the quotient of the means.

Tnp-RFC from normal adult mice and from mice immunized with Tnp-Hcn revealed a much higher proportion of high avidity cells in the RFC from the immunized animals. Specifically, 5 days after the secondary immunization of adult Swiss mice with Tnp-Hcn, 90% of the Tnp-RFC detected were inhibited by concentrations of Tnp-Hcn as low as 0.4 μg per ml, whereas in the unimmunized animals, concentrations of 250 μg per ml were required for the same degree of inhibition.

To characterize the distribution of avidities among adult and fetal Tnp-RFC, spleen cells pooled from several unimmunized adults as well as from a number of 18-day fetuses were mixed with various concentrations of Tnp-Hcn before centrifugation in the rosette assay. The results shown in Fig. 3 indicate that the degree of inhibition of fetal and adult Tnp-RFC at several Tnp-Hcn concentrations was not significantly different within the precision of the method. Thus, the fetal antigen-binding cells exhibited the same range of avidities for antigen as did the adult cells.

In Vivo Response to Antigen as a Function of Age.—The age at which the Swiss-L mice were able to respond to antigen in vivo was determined by assaying for antibody-secreting cells (plaque-forming cells or PFC) and for elevated numbers of RFC 5 days after the injection of SRBC or Tnp-Hcn. The results shown in Fig. 4 indicate that the mice were able to respond to either antigen within 5 days by the production of PFC when injected at 14 days of age or later but not when injected at 7 days or earlier. A response to antigen consisting of elevated numbers of RFC was detected in animals injected with SRBC at 14 days of age or later and in animals injected with Tnp-Hcn at 21 days or later. In a number of experiments we observed that the injection of Tnp-Hcn before 7 days of age resulted in slight decreases in the levels of RFC below control values. Although the PFC response to antigen reached adult levels by 4 wk of age, the RFC response at 4 wk was lower than adult levels.

DISCUSSION

B cells, T cells, and antigen-binding cells all appeared in the spleens of Swiss-L mice early in the development of this organ, at about 15–16 days' gestation. Their numbers increased rapidly during the interval before and immediately after birth and reached a plateau at 1 wk of age. Nevertheless, response to antigen could not be detected until after 2 wk of age. Fetal and adult antigen-binding cells were found to be remarkably similar with respect to the relative numbers of cells specific for each of three different antigens and with respect to the range of avidities with which Tnp-specific cells bound to antigen.

Our data show that lymphocytic infiltration of the spleen occurs earlier than had been previously suggested on the basis of conventional histologic techniques (21). The splenic anlage is first detectable in the mouse on day 13

of gestation (20). We found that both T lymphocytes and B lymphocytes could be detected in the spleen as early as day 15, although quantitation was difficult at this time because of the small numbers of cells. By day 16 of gestation, 1% of nucleated spleen cells had immunoglobulin receptors and almost

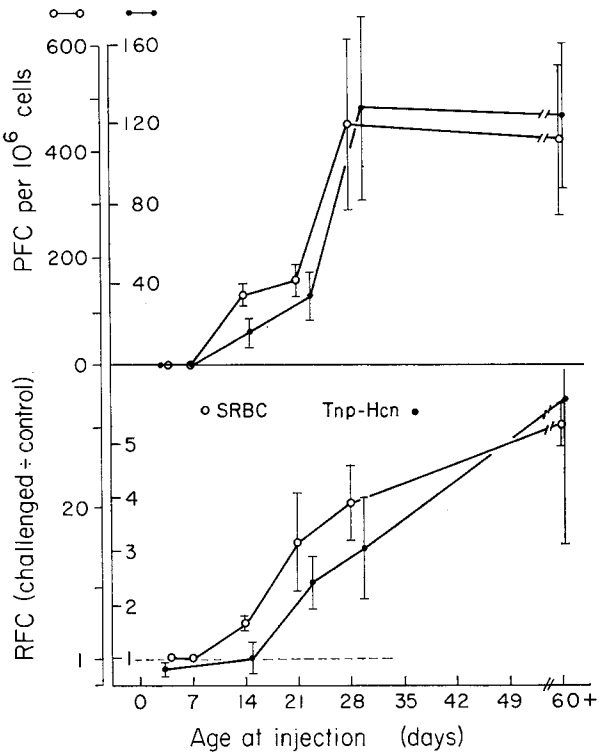

FIG. 4. Numbers of PFC and RFC produced in response to SRBC and to Tnp-Hcn as a function of age in Swiss-L mice. Several mice of the same age were injected with SRBC, Tnp-Hcn adsorbed onto bentonite, saline, or bentonite alone at the ages indicated and as described in Materials and Methods. The spleens from the mice in each experimental group (numbers in each group ranged from two for adults to five for the youngest mice tested) were pooled before the assays performed 5 days after injection. Mice injected with saline had less than 1 PFC per 10^6 spleen cells in assays with SRBC. Animals injected with bentonite had about 5 PFC per 10^6 spleen cells at every age tested when assayed against Tnp-SRBC. These background levels of PFC were subtracted from the values obtained for the numbers of PFC per 10^6 spleen cells in mice injected with the antigens. Each value shown in the top half of the figure represents the arithmetic mean of three independent determinations ± the standard error of the mean. The numbers of RFC per 10^6 spleen cells were determined for the experimental and control groups of animals in three separate series of experiments. The arithmetic means of the values obtained were used to calculate the ratios presented in the bottom half of this figure. The vertical bars indicate the propagated standard error of the quotient of the means.

8% expressed the θ-antigen (Table I). During this same 24 h interval from day 15 to day 16 of gestation, cells capable of binding to each of three different antigens were first detected. The appearance of lymphocytic cells in the spleen, and the rapid increases in their numbers before and immediately after birth, are probably not influenced by foreign antigens, for the fetus is largely protected from foreign substances (22). Furthermore, the exposure to antigens from the environment at birth apparently does not stimulate faster rates of increases for B cells, T cells, or antigen-binding cells (Figs. 1 and 2). We cannot, however, assess the role of endogenous antigens in the proliferation of lymphocytes and their migration to the spleen.

The site at which B cell precursors differentiate and become Ig-positive B cells remains to be determined for mammalian species. Tyan and Herzenberg (23) demonstrated, by the transfer of fetal tissues to irradiated adults, that the liver is the major source of B cell precursors from the 10th day of gestation until birth. By 2 or 3 days before birth, these cells can also be found in other organs including the spleen, bone marrow, and blood. Experiments reported by Nossal and Pike (9) appear to rule out the fetal liver and bone marrow as sites for the earliest detectable expression of Ig receptors. They found that Ig-positive cells appeared in these organs later during the development of CBA mice than in the spleen and blood. The percentage of B cells among nucleated cells was found to be slightly higher in blood than in the spleen before birth. Nevertheless, inasmuch as the total number of B cells was probably considerably larger in the spleen than in blood, the possibility exists that the commitment of B cell precursors to the production of one kind of immunoglobulin and to the expression of that immunoglobulin receptor occurs first in the spleen.

The T cells detected in the spleen presumably migrate from the thymus. If so, there is a substantial flow of cells from the thymus to the spleen at a time when the thymus is undergoing many changes during its own development. Our finding that the percentage of T cells present in the spleens of Swiss-L mice at birth is about $\frac{3}{4}$ of adult values (Table I) conflicts with results reported by Stobo and Paul (12) for BALB/c mice. Using a cytotoxic assay based on chromium release, they estimated the percentage of T cells present in the spleen at birth to be only about $\frac{1}{30}$ of adult values. On the other hand, at least two studies have shown that, in BALB/c mice (10) and Charles River albino/ICR mice (11), the percentage of T cells in blood, lymph nodes, and Peyer's patches at the time of birth is about $\frac{3}{4}$ of adult values. It seems likely therefore that many T cells would also have migrated to the spleen by this time.

Subsets of T cells have been described on the basis of their activities and density of cell surface markers (24–26). Although their functions and interrelationships have not yet been completely sorted out, at least some θ-positive cells in the peripheral lymphoid organs of adults can bind to antigens in the

rosette assay (27–29). Our finding that RFC remained in constant ratio to B cells at all ages tested, but that the RFC to T cell ratio increased sharply with age (Table III), suggests that many of the T cells detected in fetal and young animals do not bind to the antigens used. It remains to be determined whether these cells are immature precursors of antigen-binding cells or have some other function altogether.

Two changes in the splenic lymphoid cell populations were observed to occur between 2 and 3 wk of age, coinciding in time with the onset of response to antigen in Swiss-L mice: (*a*) an increase in total cell numbers, due primarily to increases in B cells (Fig. 1) and (*b*) a decrease in the T cell to B cell ratio (Table I). It is known that the response to antigen consists of the expansion of antigen-binding cell clones as well as the production of antibodies, and it therefore seems likely that the observed proliferation of cells is a result rather than a cause of immunologic competence. Furthermore, there was very little change in the numbers of B cells, T cells, or antigen-binding cells between 1 and 2 wk after birth (Fig. 1). Thus, the capacity of a Swiss-L mouse to respond to antigen at 2 wk but not at 1 wk of age may be due to a qualitative rather than a quantitative change in the spleen cell populations during this interval. We have some preliminary evidence from studies on the antigenic stimulation of spleen cells in vitro that T cell helper function may be a limiting factor in the development of immunologic competence, in spite of the large numbers of θ-positive cells present.

Cells capable of binding to each of three different antigens were first detected during the 24 h interval from 15 to 16 days' gestation. We were unable to demonstrate significant differences between fetal and adult antigen-binding cells in several properties: (*a*) the ratio of cells with one specificity to cells with another (Fig. 2); (*b*) the ratio of each kind of antigen-binding cell to B cells (Table III); and (*c*) the range of avidities with which Tnp-specific cells bound to antigen (Fig. 3). Based on the time of appearance of cells specific for the three antigens tested and on the observation that the proportions of cells with different specificities remained constant with age, we conclude that, for the antigens used here, the expression of one specificity does not lag more than 24 h behind the expression of another. Because the range of avidities is great, it seems likely that the variety of different specificities that can be expressed in the fetus is large. Of course, the number of particular antigen-binding specificities present in a single fetus will necessarily be limited by the number of lymphoid cells present.

If antigen-binding specificities are generated by the somatic mutation of a few germ line genes (30–32) with the subsequent selection of suitable mutations after the expression of antigen-binding receptors on cell surfaces, one would predict that the variety of specificities expressed early in the fetus would be quite limited. We were unable, for the few antigens tested so far, to detect any restriction in the range of specificities that can be expressed in the fetus.

It can be argued that this restriction might have been detectable in other organs or at earlier times. In this regard, it should be pointed out that the antigen-binding receptors of at least the B lymphocytes are expressed in the spleen earlier than they can be detected in other lymphoid organs (9) and that the rosette assay allowed us to detect antigen-binding cells about as early as we could detect B cells by immunofluorescence.

If the results obtained with the three antigens tested can be extended to other antigens, severe constraints would be placed on somatic mutation theories. It would become necessary to postulate that many suitable mutations occur before 16 days' gestation at a time when the total number of lymphoid cells is quite small. Furthermore, since we and others (9) have not detected immunoglobulin receptors before 15 days' gestation, it is not clear what mechanism could operate to afford selective advantage of the mutated genes over the germ line genes. Our results are more compatible with germ line (33) or somatic recombination (34) theories for the generation of antibody diversity. Since, according to these theories, the different antigen-binding specificities arise during evolution or are generated by recombinational events among evolutionarily selected genes, the expression of these specificities could occur at random within a short time interval and could therefore give rise to the observed kinetics of appearance of antigen-binding cells.

Several studies, predominantly with lamb fetuses (35) but also with neonatal mice (36), have revealed that the onset of response to different antigens occurs at different times. Furthermore, all individuals of the same strain follow the same temporal sequence in responding to a series of antigens. It has been suggested that this process may reflect the appearance or maturation of antigen-reactive clonal precursors at different times and rates (35). Since the mouse has a much shorter developmental period than the lamb, it is more difficult to demonstrate temporal sequences in the response to antigens and in the appearance of antigen-binding cells. In fact, our results show an almost simultaneous onset of response to at least two different antigens (Fig. 4) as well as the appearance of cells specific for these two antigens in the same 24 h interval during gestation (Fig. 2). The finding that the ratio of RFC to B cells remains constant with age for three different antigens suggests that other antigen-binding cells will also be found to arise in parallel and at the same time. It remains to be determined whether mice can be shown to respond at different times to two antigens for which antigen-binding cells arise almost simultaneously. In order to clarify these questions, we are continuing to investigate the kinetics of expression of antigen-binding specificities and the maturation of cell populations required for the immune response.

SUMMARY

In order to clarify the cellular events that precede the onset of immunological competence in the mouse, we have characterized and quantitated the lymph-

oid cells of the spleen as a function of age. Our results show that T cells and B cells both appeared in the spleens of Swiss-L mice as early as the 15th–16th day of gestation. Antigen-binding cells specific for each of three different antigens were also first detected during this same 24 h interval. The B cells and three varieties of antigen-binding cells increased in number rapidly and in parallel until about 1 wk after birth. The T cells, which were more numerous than B cells at first, increased in number somewhat more slowly.

Coincident with the onset of response to antigen, there was a further increase in B cell numbers and a decrease in the T cell to B cell ratio. The capacity to respond to antigen by cellular proliferation and synthesis of antibody did not arise until about 2 wk after birth although there were no quantitative changes in the total numbers of T cells, B cells, and antigen-binding cells between 1 and 2 wk of age. Some qualitative change, such as the functional maturation of an antigen-reactive cell, may be required during this interval for the onset of this immunological response. Although the numbers of antigen-binding cells present in fetuses and young animals were smaller than in adults, we have as yet been unable to detect any restriction in the variety of specificities that can be expressed in fetuses, either in the kinds of antigens bound or in the range of avidities with which a single antigen is bound.

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