ONTOGENY OF B-LYMPHOCYTE FUNCTION

I. Restricted Heterogeneity of the Antibody Response of B Lymphocytes from Neonatal and Fetal Mice*

By EDMOND A. GOIDL and GREGORY W. SISKIND‡

(From the Division of Allergy and Immunology, Department of Medicine, Cornell University Medical College, New York 10021)

The adult antibody response is generally characterized by a marked degree of heterogeneity of affinity of the antibody for the antigenic determinant (1, 2). With time after immunization there appears to be a preferential selection of high affinity antibody-producing B lymphocytes resulting in an increase in the average affinity of the serum antibody (2–19). It is usually assumed that individual B lymphocytes produce a homogeneous antibody product (20) and that the heterogeneity of serum antibody is a consequence of the stimulation, by antigen, of a number of different B lymphocytes (18). Most workers agree that some form of clonal selection theory (21) accounts for the specificity of the immune response. However, the mechanism which gives rise to the diversity of information required to synthesize the large array of different antibody molecules which can be formed by the normal adult animal is not known. Two alternative theories have been proposed: (*a*) that all information is coded in the germ line or (*b*) that limited information is coded in the germ line and that the available diversity is expanded by some process of somatic mutation or recombination.

Previous studies on the ontogeny of the immune response have suggested that there is an ordered progressive development of the ability to respond to different antigens (22–24). T-lymphocyte function has been shown to mature relatively late in development (25). B lymphocytes or their precursors can be detected relatively early in ontogeny (26–30). It is not clear whether the demonstrated limitations in the immune response of fetal or neonatal animals reflect a lack of B lymphocytes capable of synthesizing the antibody in question or a lack of development of necessary antigen "processing" or "localizing" mechanisms.

The present study was designed to examine the ontogeny of the functional capacity of B lymphocytes. In particular, we wished to study the maturation of the ability of B cells to generate an antibody response which is heterogeneous with respect to affinity. Since individual B lymphocytes produce homogeneous antibody (20), the heterogeneity of affinity can be assumed to reflect the degree of polyclonality of the antibody response. Immunization of immature animals

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was deemed inadequate to study the potential capacity of B lymphocytes since the response might be restricted as a consequence of immaturity of "antigen processing" mechanisms such as those involving T lymphocytes or macrophages. Therefore, a cell transfer system was employed in which the functional capacity of B cells from neonatal or fetal animals was assayed after transfer to adult, lethally irradiated recipients. Recipients also received excess adult thymus cells so that T-cell activity would not limit the response. It was found that cells from neonatal or fetal donors produced a response of highly restricted heterogeneity of affinity as compared with cells from adult donors. Maturation of the capacity to transfer a heterogeneous, adult type, immune response occurred between 1 and 2 wk after birth.

Materials and Methods

Animals. LAF_1 mice (Jackson Laboratories, Bar Harbor, Maine) were used. Fetal tissues were obtained by sacrifice of timed pregnant mice. Neonatal livers were taken from animals sacrificed within 18 h of birth. Germfree BALB/c mice were the generous gift of Dr. Richard Asofsky, National Institutes of Health, Bethesda, Md.

Antigens and Haptens. Dinitrophenyl (DNP) derivatives of bovine gamma globulin (BGG,[1] Miles-Yeda, Kankakee, Ill.) and ovalbumin (Ova, Miles-Yeda) were prepared by the reaction of 1-fluoro-2,4-dinitrobenzene (DNFB, Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) with the protein under alkaline conditions essentially as described by Eisen et al. (31). The derivatized proteins were purified by extensive dialysis against 0.001 M potassium phosphate buffer (pH 7.4) in the case of DNP-BGG and against 0.15 M NaCl in the case of DNP-Ova. The concentration of the product was determined from its "dry weight" and the degree of derivatization estimated from its absorbancy at 360 nm (ϵ for DNP-lysine was taken as 17,400). A single preparation of DNP-BGG was used which had an estimated 50 DNP groups per molecule of protein. The DNP-Ova preparations had between 7 and 11 DNP groups per Ova molecule.

DNP-ϵ-amino-N-caproic acid (DNP-EACA) was prepared by the reaction of DNFB with EACA (Sigma Chemical Co., St. Louis, Mo.) under alkaline conditions and was purified by repeated crystallization from hot water as described previously (5). The concentration was estimated spectrally assuming a molar absorbancy of 17,400 at 360 nm.

Immunization. Mice were immunized by the intraperitoneal injection of 500 μg DNP-BGG emulsified in complete Freund's adjuvant (CFA, containing 1 mg/ml *Mycobacteria butyricum*) so as to be in a final vol of 0.2 ml. Animals were sacrificed by cervical dislocation and their spleens assayed for plaque-forming cells 20 days after antigen injection. Previous studies had shown that the average affinity, and the degree of heterogeneity of affinity of the anti-DNP plaque-forming cells were maximal at this time.[2]

Cell Transfers. All studies were carried out by immunizing lethally (800 R) irradiated (Gammator M, Radiation Machinery Corp., Parsippany, N. J.) mice reconstituted with excess (1×10^8), adult, syngeneic, thymus cells plus B lymphocytes obtained from various sources. In this way the potential of the B-lymphocyte population to respond to DNP-BGG could be tested in an adult environment with excess normal adult thymus cells present in all cases. In all studies of ontogeny, B cells from a single donor were transferred into a single recipient. On the other hand, thymus cells were pooled from several donors so as to yield a relatively constant population of thymus cells. The

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[1] Abbreviations used in this paper: BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; DNFB, 1-fluoro-2,4-dinitrobenzene; EACA, ϵ-amino-N-caproic acid; HBSS, Hanks' basic salt solution; Ova, ovalbumin; PBS, phosphate-buffered saline; PFC, plaque-forming cells; V, variable.

[2] Goidl, E. A., J. J. Barondess, and G. W. Siskind. 1974. Studies on the control of antibody synthesis. VIII. Change in affinity of direct and indirect plaque-forming cells with time after immunization in the mouse. Loss of high affinity plaques late after immunization. Manuscript submitted for publication.

following sources of B lymphocytes were employed: (*a*) 17 day fetal liver (timed from appearance of vaginal plug); (*b*) neonatal liver obtained within 18 h after birth; (*c*) spleen and bone marrow from 7-day old mice; (*d*) spleen and bone marrow from 10-day old mice; and (*e*) spleen and bone marrow from 14-day old mice.

For cell transfer single cell suspensions were prepared by teasing the tissue in Hanks' basic salt solution (HBSS) (Grand Island Biological Co., Grand Island, N. Y.) containing 0.35 mg/ml Na_2HCO_3 and 0.02 mg/ml sodium heparin sulfate). The cells were filtered through a thin layer of cotton gauze to remove clumps, washed once, and resuspended in HBSS for injection. Recipients received thymus cells mixed with a source of B lymphocytes intravenously 2–4 h after lethal irradiation. Animals were injected with DNP-BGG in CFA intraperitoneally 24 h later.

Assay of Plaque-Forming Cells (PFC). Anti-DNP PFC in the spleen were assayed by the monolayer technique of Kennedy and Axelrad (32). Briefly, washed sheep red blood cells (SRBC), coated with DNP-Ova by a chromic chloride method (33), were adsorbed as a monolayer on to a poly-L-lysine (mol wt 30,000–70,000; Miles-Yeda) coated plastic petri dish. Cells were expressed from individual spleens by gentle manual disruption, were filtered through a thin layer of cotton gauze, were washed once with HBSS, and were resuspended in a known volume of HBSS for assay. Lyophilized guinea pig serum (GIBCO) dissolved in H_2O and diluted 1:10 in phosphate-buffered saline (PBS, 0.15 M NaCl, 0.01 M potassium phosphate buffer, pH 7.4) was used as a source of complement (C). Generally, each plate contained cells from $\frac{1}{30}$ of a spleen in a total vol of 2.0 ml C and 10 μl of rabbit antimouse globulin to develop indirect plaques. The plates were incubated at 37°C for 1 h and the plaques were counted using a dissecting microscope. The dilution of antimouse globulin antiserum used had been previously shown to bring about maximal development of indirect PFC and to cause approximately 75% inhibition of the number of direct PFC.

Assay of Avidity of Anti-DNP PFC. The avidity of the indirect anti-DNP PFC was assayed by inhibition of plaque formation by various concentrations of DNP-EACA essentially according to the method described by Andersson (34). Nine concentrations of DNP-EACA ranging from 1×10^{-9} to 1×10^{-5} M in half-log increments were used. The hapten dissolved in PBS was added to the incubation mixture. The inhibition of plaque formation is specific, as it had been previously shown that even the highest concentration of DNP-EACA had no effect on the number of anti-SRBC PFC detected in spleen cell suspensions from SRBC immunized mice. Plaque formation by high affinity antibody-forming cells in inhibited by low concentrations of hapten while plaque formation by low affinity antibody-forming cells requires a high concentration of hapten for inhibition. From the pattern of inhibition with various concentrations of hapten one can calculate the distribution of avidities of the PFC in the cell suspension. This method for measuring avidity has recently been analyzed mathematically by DeLisi and Goldstein (35) who were able to show that the technique could give a legitimate estimate of the relative affinity of the antibody produced by the PFC.

Results

Heterogeneity of Avidity of Adult Spleen Cells in a Cell Transfer Recipient. In Fig. 1 is illustrated the heterogeneity of avidity of anti-DNP PFC 20 days after DNP-BGG immunization of lethally irradiated mice reconstituted with syngeneic, adult thymus and adult spleen or adult bone marrow cells. For comparison, data illustrating the heterogeneity of affinity of the response of normal adult mice immunized in a similar manner is presented. As has been shown previously,[2] a marked degree of heterogeneity of avidity is seen in the antihapten PFC response. We have previously shown[2] that in this system maximum heterogeneity is present, in the primary response, at 20 days after antigen injection. It is apparent from the data (Fig. 1) that the degree of heterogeneity is similar in intact animals and in animals reconstituted with adult bone marrow or adult spleen cells as the source of B lymphocytes. Clearly, as has been previously shown in detail,[2] the cell transfer system does not appear to place any restriction on the heterogeneity of the PFC response with respect to avidity.

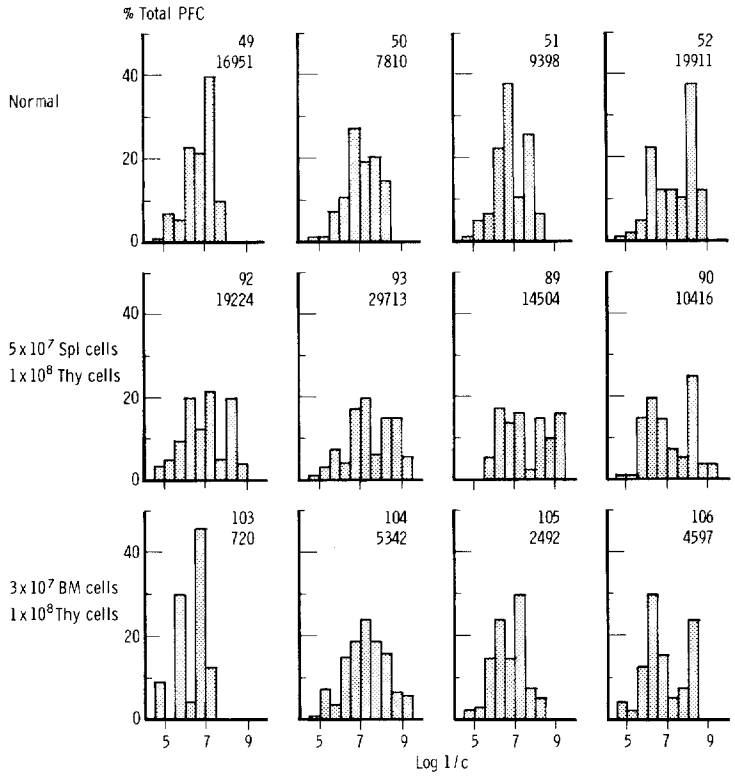

FIG. 1. Each histogram illustrates the distribution of indirect PFC with respect to avidity in a mouse spleen 20 days after immunization with 500 μg DNP-BGG in CFA. In the top row are data on normal animals. In the second row are data on lethally irradiated animals reconstituted with syngeneic adult spleen and thymus cells 1 day before immunization. In the bottom row are data on lethally irradiated mice reconstituted with adult syngeneic bone marrow and thymus cells 1 day before immunization. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Animal identification number (top) and the total PFC per spleen are given in the right upper corner of each histogram. Avidity increases to the right.

Furthermore, the degree of heterogeneity of avidity obtained when "central" lymphoid tissue (bone marrow) is used in reconstitution is indistinguishable from that seen when animals are reconstituted with "peripheral" lymphoid cells (spleen).

In Fig. 2 are illustrated the heterogeneity of avidity of anti-DNP PFC produced by 20 mice each reconstituted with spleen cells from one-third of an individual adult spleen. A highly heterogeneous response is obtained which is indistinguishable from what was seen above (Fig. 1) in mice reconstituted with pools of tissue from several donors. The number of spleen cells transferred was based on preliminary observations which suggested that when reconstitution was with one-third of an adult spleen, the total number of PFC per spleen obtained was comparable to what was seen in recipients of a single neonatal liver as the source of B cells. While all of the 20 recipients produced a distinctly heterogeneous

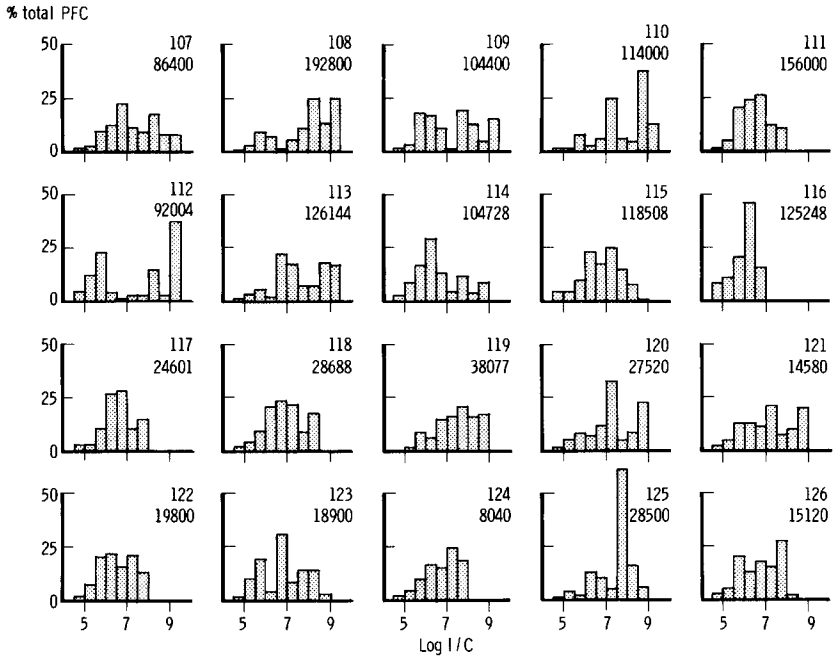

FIG. 2. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with cells from one-half of an individual adult syngeneic spleen and adult thymus cells 1 day before immunization with 500 μg DNP-BGG in CFA. Animals were sacrificed 20 days after immunization. Avidity increases to the right. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Animal identification number (top) and the total PFC per spleen are given in the right upper corner of each histogram.

response, four animals (nos. 111, 116, 117, and 124) showed what might be described as a slightly reduced degree of heterogeneity.

Ontogeny of the Ability of B Lymphocytes to Generate a Heterogeneous Response. In Figs. 3–7 are illustrated the heterogeneity of avidity of anti-DNP PFC from mice reconstituted with adult thymus cells and with 17 day fetal liver, neonatal liver, 7-day old spleens, 10-day old spleens, and 14-day old spleens, respectively, as the source of B lymphocytes. In all cases cells from a single donor were transferred to an individual recipient. The number of PFC per spleen is reasonably similar in all cases to what was seen in recipients of one-third of an adult spleen.

The effect on the immune response of omitting adult thymus cells and reconstituting solely with neonatal liver is indicated in Table I. It is apparent that DNP-BGG is a thymic-dependent antigen under these conditions, and that neonatal B lymphocytes can cooperate with adult T lymphocytes in the generation of an immune response.

From an examination of Figs. 3–7 it is clear that the B lymphocytes from young or fetal donors produce a response of restricted heterogeneity as compared with that of adult animals (Fig. 2). The ability to mount (in a cell transfer system) an adult type response is not fully developed until 2 wk of age.

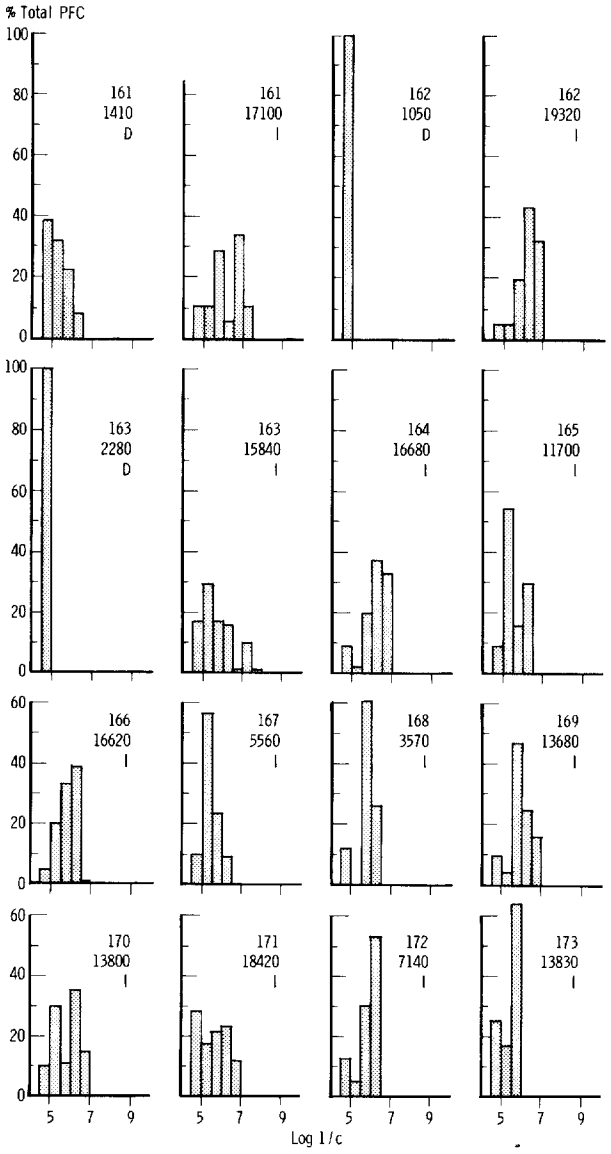

FIG. 3. Each histogram illustrates the distribution of PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with cells from the liver of an individual, 17 day fetal, syngeneic mouse and adult syngeneic thymus cells 1 day before immunization with 500 μg DNP-BGG in CFA. Animals were sacrificed 20 days after immunization. Avidity increases to the right. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Animal identification number (top) and the total PFC per spleen are given in the upper right corner of each histogram. D, direct PFC; I, indirect PFC.

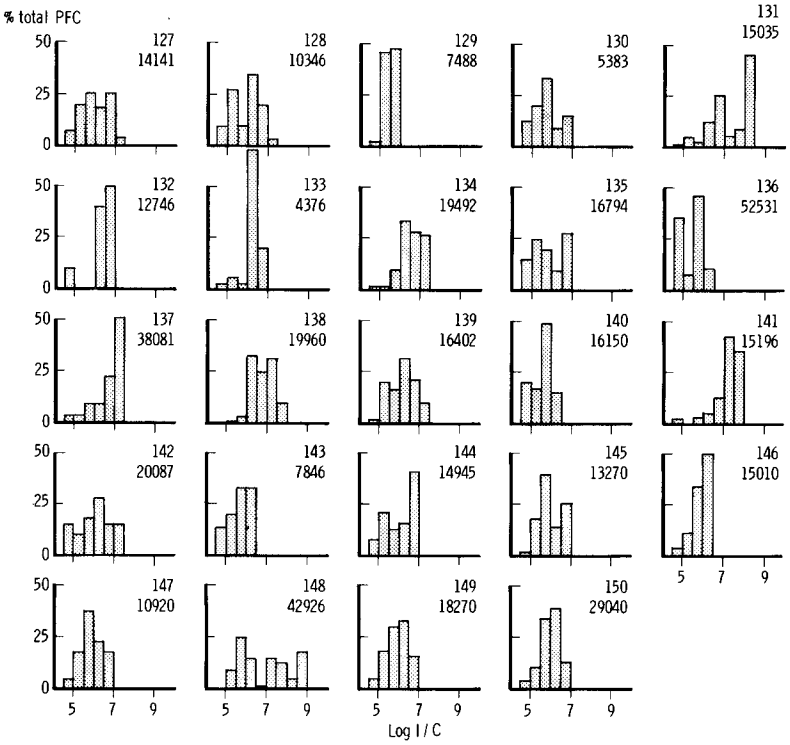

FIG. 4. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with cells from the liver of an individual syngeneic neonatal mouse and adult syngeneic thymus cells 1 day before immunization with 500 μg DNP-BGG in CFA. Animals were sacrificed 20 days after immunization. Avidity increases to the right. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Animal identification number (top) and the total PFC per spleen are given in the upper right corner of each histogram.

Mice reconstituted with liver from individual 17-day old fetal donors produce a response which is markedly restricted with respect to avidity (Fig. 3). The magnitude of the PFC response is comparable to that of mice reconstituted with one-third of an adult spleen. In most cases there were too few direct PFC present at 20 days after immunization to permit hapten inhibition studies to be performed accurately. Three recipients (nos. 161, 162, and 163) did have sufficient numbers of direct PFC for avidity measurements. In each case the direct PFC were even more highly restricted in avidity than were the indirect PFC in the same spleen.

The immune response of 24 animals each reconstituted with cells from an individual neonatal liver and pooled adult thymus is also clearly very restricted with respect to heterogeneity of avidity (Fig. 4). 22 of the animals could be described as showing a restriction in heterogeneity as compared with recipients of adult spleen cells. 9 of the 24 animals (e.g. 129, 132, and 146) are highly restricted in heterogeneity. Overall, the degree of restriction in heterogeneity may be

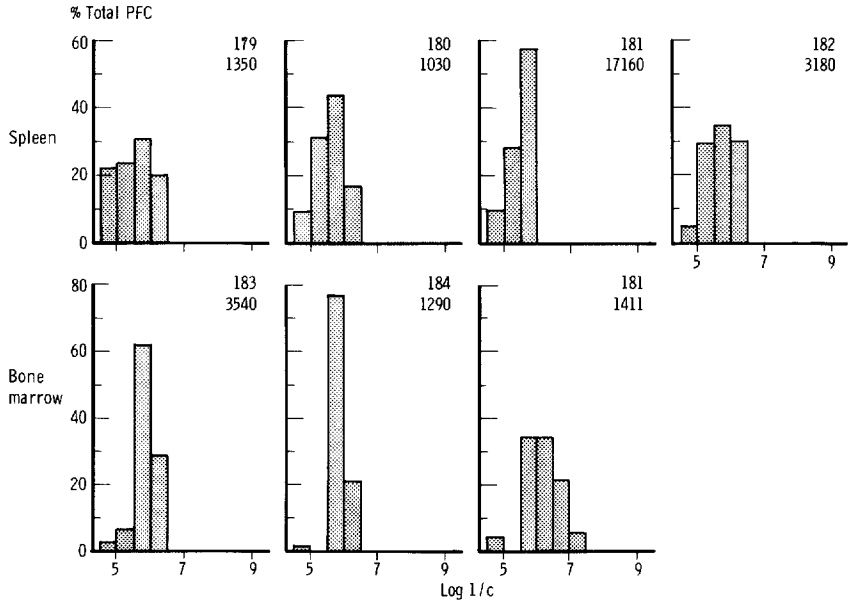

FIG. 5. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with cells from the spleen or bone marrow of an individual, 7-day old, syngeneic donor and adult syngeneic thymus cells 1 day before immunization with 500 μg DNP-BGG in CFA. The top row presents data on animals reconstituted with spleen cells and the bottom row presents data on animals reconstituted with bone marrow. Animals were sacrificed 20 days after immunization. Avidity increases to the right. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC precent in each subpopulation. Animal identification number (top) and the total PFC per spleen are given in the upper right corner of each histogram.

slightly less marked than in animals reconstituted with 17 day fetal liver. 21 of the animals showed an absence of high avidity PFC. However, 3 out of the 24 animals produced high avidity PFC; two of these being somewhat restricted in heterogeneity (nos. 131 and 141) and one being highly heterogeneous (no. 148). Thus, cells from fetal or neonatal tissues appear to be incapable of generating a normal adult immune response. The PFC response by these B cells, while of good magnitude, is restricted with respect to heterogeneity of avidity and, in most cases, is of low average avidity and lacking in high avidity subpopulations.

Animals reconstituted with spleen cells from individual 7-day old donors (Fig. 5) are very similar with respect to restriction in heterogeneity to mice reconstituted with neonatal liver. In contrast, spleen cells from 10-day old donors (Fig. 6) are capable of reconstituting irradiated mice to yield a response approaching that of adult mice. The responses of animals (Fig. 7) reconstituted with spleen cells from individual 2-wk old mice are indistinguishable in heterogeneity of avidity from the responses of animals reconstituted with spleen cells from adult donors. Thus, the ability of B lymphocytes to yield a heterogeneous response becomes adult in character by 2 wk after birth.

Comparison of the Heterogeneity of Response Using Bone Marrow or Spleen as the Source of B Lymphocytes. As was illustrated above (Fig. 1) adult bone

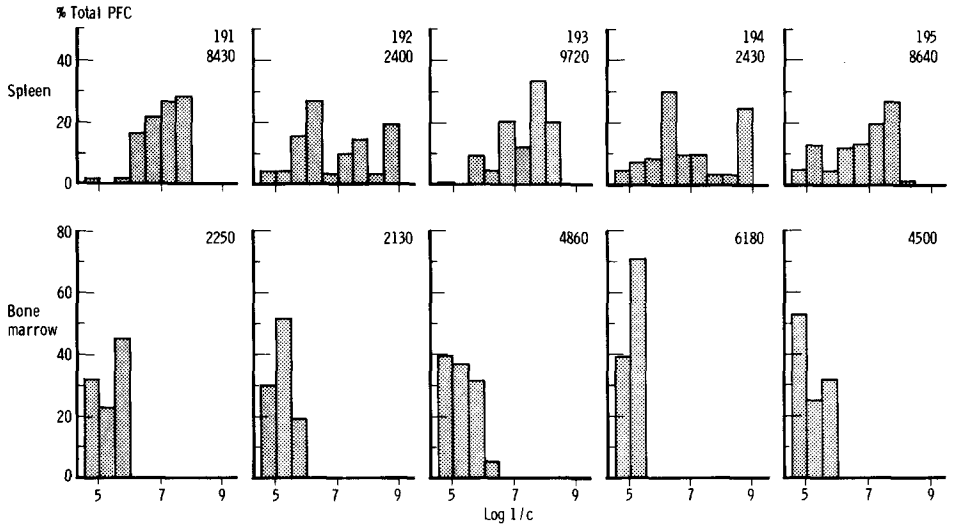

FIG. 6. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with cells from the spleen or bone marrow of an individual, 10-day old, syngeneic donor and adult syngeneic thymus cells 1 day before immunization with 500 μg DNP-BGG in CFA. The top row presents data on animals reconstituted with spleen cells and the bottom row data on animals reconstituted with bone marrow from the same individual donors. Animals were sacrificed 20 days after immunization. Avidity increases to the right. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Animal identification number (top) and the total PFC per spleen are given in the upper right corner of each histogram.

marrow and adult spleen behave similarly as a source of B cells in reconstituting irradiated, syngeneic mice. Using cells from 14-day old donors (Fig. 7) there appears to be a tendency, in about half of the comparisons (e.g. animals 153, 154, 155, 156, and 160), for mice reconstituted with bone marrow to be somewhat restricted with respect to heterogeneity of avidity as compared with animals reconstituted with spleen cells from the same individual donor. This tendency is even more apparent when 10-day old animals were used as donors. As can be seen in Fig. 6, mice reconstituted with bone marrow as the source of B lymphocytes yielded a response which is very restricted with respect to avidity as compared with animals reconstituted with spleen cells from the same donor. Thus, at 10 days of age, the potential of B cells from the spleen to reconstitute a heterogeneous immune response is approaching that of adult tissues. In contrast, the bone marrow behaves in a more immature manner. Animals reconstituted with bone marrow from 10-day old donors produce an anti-DNP response which is still highly restricted with respect to heterogeneity of avidity. With cells from 7-day old donors both bone marrow and spleen show marked restriction in heterogeneity (Fig. 5).

Heterogeneity of the Immune Response of Germfree Mice. The possibility existed that the restricted heterogeneity of the B-lymphocyte population in fetal and neonatal mice is the consequence of their lack of exposure to environmental antigens. The heterogeneity of the immune response of adult germfree mice to

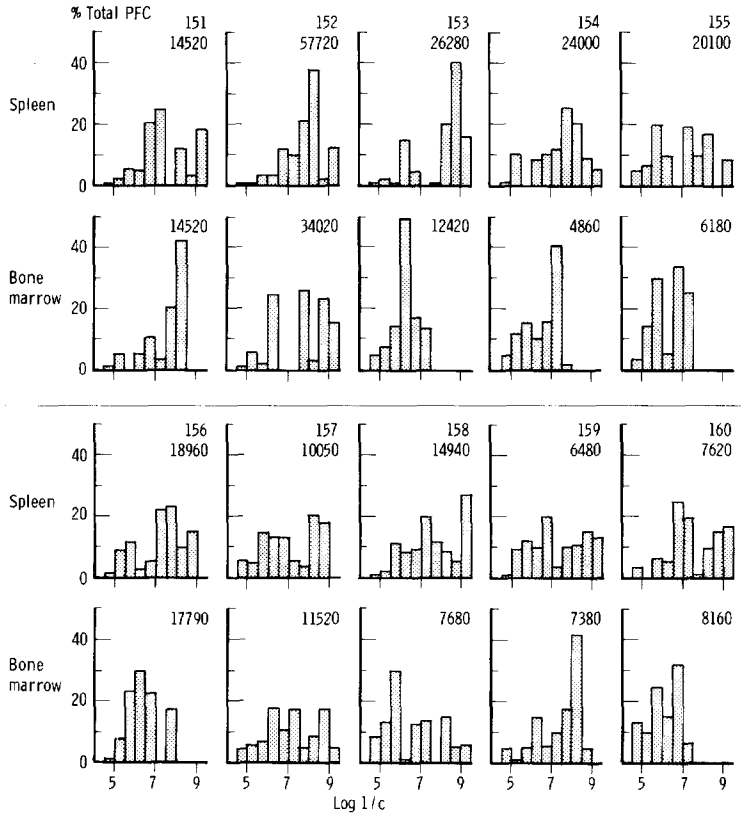

FIG. 7. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with cells from the spleen or bone marrow of an individual, 2-wk old, syngeneic donor and adult syngeneic thymus cells 1 day before immunization with 500 μg DNP-BGG in CFA. The top and third rows present data on animals reconstituted with spleen cells and the second and bottom rows data on animals reconstituted with bone marrow. In each case the same donor was used for bone marrow and for spleen. The data for the spleen recipient are presented directly above the data on the bone marrow recipient. Animals were sacrificed 20 days after immunization. Avidity increases to the right. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Animal identification number (top) and the total PFC per spleen are given in the upper right corner of each histogram.

DNP-BGG was therefore assayed to determine if the reduced exposure of germfree mice to environmental antigens would result in their also manifesting an immune response of restricted heterogeneity. As can be seen in Fig. 8, germfree mice produce an immune response indistinguishable from conventionally reared animals with respect to heterogeneity of avidity of PFC.

Statistical Analysis of Heterogeneity. Four different functions (see footnote to Table II) were used to describe the degree of heterogeneity of avidity of the PFC population from individual animals. The averages and standard deviations of these indices for the different groups of animals studied are presented in Table II. It is clear that the pattern of change in heterogeneity index of PFC avidity in animals reconstituted with B cells from different sources corresponds to what was

TABLE I

*Cooperation between B cells from Neonatal Liver and Adult T Cells in the Immune Response to DNP-BGG**

No thymus cells		1×10^8 thymus cells	
Direct PFC/spleen	Indirect PFC/spleen	Direct PFC/spleen	Indirect PFC/spleen
20	40	240	49,926
110	1,440	150	29,040
30	140	180	18,270
90	160	120	10,920

* Eight lethally irradiated mice were each reconstituted with cells from a single syngeneic neonatal liver with or without 1×10^8 adult syngeneic thymus cells. Animals were immunized with 500 μg DNP-BGG in CFA 1 day later, were sacrificed 20 days later, and were assayed for direct and indirect PFC. Background PFC per spleen in this system is 100–150 for direct plaques and 100–200 for indirect plaques.

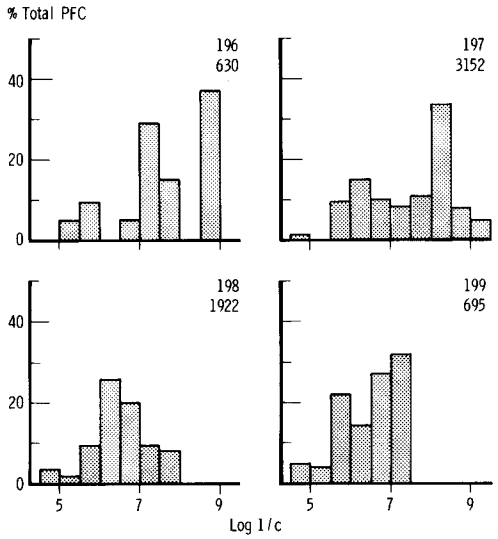

FIG. 8. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen 20 days after immunization of a "germfree" mouse with 500 μg DNP-BGG in CFA. Avidity increases to the right. The abscissa represents the log of the inverse of the free hapten concentration used in the plaque inhibition assay. The ordinate represents the percent of the total population of PFC present in each subpopulation. Animal identification number (top) and the total PFC per spleen are given in the upper right corner of each histogram.

intuitively apparent from a direct examination of the histograms. Animals reconstituted with 17 day fetal liver were of restricted heterogeneity and a progressive increase in heterogeneity was observed with increasing age of the donor mice. Animals reconstituted with 14-day old donor spleen or bone marrow were indistinguishable from recipients of adult tissues with respect to heterogeneity of PFC avidity.

TABLE II

*Heterogeneity of Avidity of PFC from Animals Reconstituted with Various Tissues as the Source of B Lymphocytes**

Source of B lymphocytes	Heterogeneity index			
	Simpson	McIntosh	Gini	Shannon
Day 17, fetal liver (9)‡	0.698 ± 0.093	0.444 ± 0.083	0.685 ± 0.094	1.898 ± 0.386
Neonatal liver (27)	0.724 ± 0.084	0.468 ± 0.076	0.711 ± 0.085	2.018 ± 0.366
Day 10, spleen (6)	0.794 ± 0.064	0.537 ± 0.066	0.782 ± 0.064	2.479 ± 0.315
Day 10, bone marrow (7)	0.529 ± 0.242	0.318 ± 0.156	0.515 ± 0.244	1.257 ± 0.629
Day 14, spleen (10)	0.842 ± 0.035	0.589 ± 0.041	0.830 ± 0.035	2.789 ± 0.212
Day 14, bone marrow (6)	0.799 ± 0.039	0.541 ± 0.045	0.787 ± 0.039	2.486 ± 0.244
Adult spleen (20)	0.816 ± 0.038	0.560 ± 0.051	0.804 ± 0.049	2.607 ± 0.240
Adult bone marrow (4)	0.785 ± 0.066	0.526 ± 0.070	0.772 ± 0.067	2.447 ± 0.367
Adult germfree spleen (4)	0.801 ± 0.038	0.542 ± 0.042	0.789 ± 0.039	2.485 ± 0.287

* Four different indices (44, 45) were employed to describe the heterogeneity of the PFC avidity distribution in individual animals. The data are presented as mean ± standard deviation of these heterogeneity indices. For each of the indices a higher number indicates greater heterogeneity. Distributions for the individual animals were presented in Figs. 1–4 and 6–8. Data for recipients of day 7 spleen or bone marrow were not included because too few animals were studied for statistical evaluation. The Shannon index is expressed as log base 2.

‡ Numbers in parentheses are the numbers of animals used.

With the data in this form it is possible to statistically analyze the differences in heterogeneity of the various groups of animals. The hypothesis of equality of variances is tested by the F-test in order to establish the applicability of the *t*-test. Groups were then compared by the *t*-test. If the hypothesis of equality of variances is rejected by the F-test, the groups were compared by a chi-square analysis. The results statistically confirmed the intuitive impressions described above: (*a*) Recipients of neonatal liver were statistically different from recipients of adult spleen, from day 14 spleen and bone marrow ($P < 0.01$) recipients, and from day 10 bone marrow and spleen ($0.02 < P < 0.05$) recipients. (*b*) Adult spleen recipients were statistically indistinguishable from adult bone marrow recipients, from adult germfree animals, and from day 14 spleen and bone marrow recipients ($P > 0.4$). Adult spleen recipients are significantly different from day 10 bone marrow recipients ($P < 0.02$) but indistinguishable from day 10 spleen recipients ($P > 0.4$). (*c*) Recipients of day 17 fetal liver were indistinguishable from recipients of neonatal liver ($P > 0.5$) and were significantly statistically different from recipients of adult spleen ($P < 0.01$).

Discussion

Data have been presented which show that B lymphocytes from fetal or neonatal donors are restricted with respect to their ability to yield a heterogeneous

antibody response such as is characteristic of adult animals. There occurs a progressive increase in heterogeneity of the B-cell population with time after birth. By 2 wk of age animals are indistinguishable from adults in regard to this parameter of immune function. It was also shown that the peripheral lymphoid tissue (spleen) seems to mature more rapidly than the central immune tissue (bone marrow) in regard to its ability to reconstitute an irradiated animal to produce an adult type heterogeneous immune response.

A number of previous workers have studied the ontogeny of the ability of fetal or neonatal animals to mount an immune response against various antigens (22–24). The data reported by these workers have suggested that there is an ordered sequential appearance of the capacity of animals to respond to various antigens. Montgomery and Williamson (36, 37) studied the heterogeneity of antibody produced by neonatal rabbits by isoelectric focusing and by equilibrium dialysis. Their results indicated that some of the neonatal animals produced antibody of restricted heterogeneity while others produced antibody which was indistinguishable from that of adults with respect to heterogeneity. Because these studies (22–24, 36, 37) were all carried out by immunizing neonatal or fetal animals it is not possible to determine if the restrictions on the immune response which were demonstrated were the consequence of immaturity of B-cell function, of T-cell function, of macrophage function, or of other antigen "processing" mechanisms. Data have been reported (38) which suggest that macrophage function in the immune response may be immature in neonatal animals. Studies have suggested that T-cell precursors (39) and some active T cells are present relatively early in development (40, 41). However, recent studies by Spear and Edelman (25) indicate that T lymphocytes are immature with respect to their response to mitogens until several weeks after birth.

It has been shown that B lymphocytes, or precursors of B lymphocytes, are present early in ontogeny (26–30) and that the major source of B-cell precursors in the fetal mouse, after the 10th day of gestation, is the liver (42). Spear et al. (26) showed an increase in the number of antigen-binding cells in the spleens of mice from day 15 of gestation to 1 wk after birth. The avidity of the antigen-binding cells from pooled neonatal or fetal tissues was found to be similar to the avidity of adult B cells. The present studies attempt to examine the ontogeny of the functional capabilities of B lymphocytes. In particular, we wished to examine the ontogeny of the catalogue of antibodies (B lymphocytes) available in the adult animal to respond to a haptenic determinant. Clearly, adult animals can produce a highly heterogeneous antibody response to a haptenic determinant (1, 2, 18, 19). The estimate of the number of different antibodies which an animal can potentially produce against a haptenic determinant is in the range of 8,000 (43, 46). If one accepts a clonal selection theory then this corresponds to an equivalent array of different B lymphocytes capable of responding to this antigenic determinant in the adult animal. It was hoped that by transferring neonatal cells into irradiated adult recipients one could examine the potential of B lymphocytes to respond to a haptenic determinant. Restrictions on the immune response of B lymphocytes as a result of immaturity of T cells, macrophages, or other features of the "neonatal environment" should be bypassed by the experimental design employed. The data indicate that B cells

from fetal and neonatal animals are restricted with respect to the catalogue of different anti-DNP antibodies which they can produce. In most cases they produce only low avidity PFC and lack the high avidity PFC normally present in adult animals. Occasional neonates (approximately 13%) have B cells capable of producing high avidity PFC. It should be kept in mind that before immunization, and early in the immune response, most of the antibody-producing cells or potential antibody-forming cells are of low affinity. The few high affinity antibody-producing B cells come to predominate in the total population of PFC only later in the immune response, after they have been selectively stimulated to proliferate.

The mechanism for the generation of diversity of antibody-producing cells is not known. Two general types of theories have been proposed: (*a*) Germ line theories hold that all information required to code for the entire catalogue of antibodies capable of being synthesized by an adult animal is inherited in the germ plasm. Individual adult B lymphocytes are thus genetically all identical and all possess the entire catalogue of information to make every antibody molecule. Presumably some type of randomly operating differentiation event results in individual B cells expressing only one (or perhaps a small number) gene for the variable (V) region of the light chain and of the heavy chain of the antibody molecule. Thus, individual B lymphocytes are restricted in function as a consequence of a differentiation event and not as a result of possessing limited genetic information. (*b*) On the other hand, somatic theories for the generation of diversity essentially postulate that the animal inherits a restricted catalogue of genetic information and that this catalogue is expanded by some random process of somatic mutation or recombination to yield the large catalogue available in the adult animal. According to such a theory, individual B cells in the adult animal differ with respect to the information they have coded in their DNA. B lymphocytes thus produce different antibodies as a consequence of differences in their individual genetic makeup.

What predictions might be made regarding the ontogeny of the heterogeneity of the antibody response based upon these two theories? Clearly, a somatic mutation theory would predict a random expansion of the capacity to produce different antibodies. One would thus expect that early in ontogeny the B-cell population would produce a response of restricted heterogeneity. It may be presumed that there are many more antibody molecules that can bind a particular antigenic determinant with low or medium affinity than can bind it with high affinity. That is, one would expect that there are far fewer chemical structures that will yield very high affinity interactions than could potentially yield weak interactions with a given antigenic determinant. Thus, one would predict that early in ontogeny there would be mainly low affinity antibody-producing cells present in most animals. Of course occasional animals might be found which by chance have high affinity antibody-forming cells present early in development. That is, one would predict on the basis of a somatic theory for generation of diversity, that early in ontogeny individual animals would differ one from the other as a consequence of the random nature of the process generating diversity. One would also predict that in most cases low affinity antibodies would be found early in ontogeny rather than high affinity antibodies.

Clearly, these predictions are fulfilled by the observations reported in the present paper.

Predictions based upon a germ line theory for the generation of diversity are more difficult to make. Since all cells contain all information it might be predicted that once the B lymphocytes have matured so that they are capable of responding and producing antibody they would yield a response which is heterogeneous like a normal adult animal. However, if one assumes that B cells must undergo some differentiation event which in effect restricts their ability to make different antibodies then it would be this differentiation event that controls the ontogeny of antibody heterogeneity and not the potential genetic capacity of the individual cells to make different antibodies. This differentiation event presumably must involve expressing certain V genes and irreversibly repressing all other V genes. If this differentiation event were to occur in a random manner in different cells (that is, the particular V genes turned on in any cell would be a chance event) then the operational properties of the system with respect to the ontogeny of heterogeneity of the immune response would be identical to those of a somatic theory for generation of diversity.

Thus, the data reported here on the ontogeny of the ability to produce a heterogeneous immune response could be consistent with either a germ line or a somatic theory for the generation of diversity. One might mention, however, that the occurrence of marked heterogeneity in the peripheral lymphoid cells of 10-day old animals while the bone marrow of these same animals is still restricted in heterogeneity suggests the operation of some type of differentiation mechanism to account for the increasing capacity to transfer a heterogeneous response. While the data certainly do not distinguish between different theories for generation of diversity they might appear slightly more compatible with a germ line theory.

Summary

The ontogeny of the ability of B lymphocytes to produce an antihapten response which is heterogeneous with respect to affinity for the antigenic determinant was studied in a cell transfer system. The heterogeneity of affinity of the immune response of lethally irradiated mice reconstituted with syngeneic, adult thymus cells and fetal or neonatal tissues as a source of B lymphocytes was studied. It was found that B cells from 17 day fetal liver or neonatal liver are highly restricted with respect to heterogeneity of affinity as compared with adult spleen or bone marrow. The B-cell population achieves an adult character with respect to heterogeneity of affinity by 2 wk of age. The peripheral lymphoid tissues (spleen) appear to mature in this respect more rapidly than do central lymphoid tissues (bone marrow). Spleens from 10-day old donors behave in an adult, heterogeneous manner while bone marrow from the same donors exhibit a marked restriction in heterogeneity of affinity. Germfree mice produce an immune response which is indistinguishable from conventionally reared adult animals with respect to heterogeneity of affinity.

The earlier appearance of the ability to transfer a heterogeneous immune response in spleen as compared with bone marrow suggests that the increasing

heterogeneity of the B-lymphocyte population which occurs between birth and 2 wk of age is the result of a differentiation event and not of a somatic mutation or recombination event.

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