ONTOGENY OF B-LYMPHOCYTE FUNCTION

II. Ability of Endotoxin to Increase
the Heterogeneity of Affinity of the Immune Response
of B Lymphocytes from Fetal Mice*

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We (1) have previously demonstrated that B lymphocytes from neonatal or late fetal mice produced an immune response which was highly restricted with respect to heterogeneity of avidity of the plaque-forming cells (PFC) as compared with the PFC response produced by B lymphocytes from adult donors. These studies were carried out by transferring the lymphoid population to be tested, together with excess adult thymus cells, into lethally irradiated, syngeneic mice. Under these conditions the functional capacity of the B lymphocytes from immature animals could be assayed in an adult environment in the presence of excess adult T lymphocytes. Thus, the restricted heterogeneity of the immune response observed with B lymphocytes from neonatal or 17-day fetal donors could be ascribed to a functional immaturity of the B lymphocyte itself. When evaluated in this system, it was found that B lymphocytes acquire adult functional capabilities between 7 and 10 days of age in LAF, mice. Our studies described above (1), together with work from a number of other laboratories (2–17), have thus demonstrated an ordered development of various functional and morphological characteristics of the immune system.

It was suggested (1) that the temporally abrupt maturation of the competence of B lymphocytes to generate an adult-type, heterogeneous, immune response implied a differentiation event rather than the accumulation of somatic mutations. If this hypothesis were correct, then the B-cell population present in a neonatal animal should have the genetic information required to synthesize the same heterogeneous population of antibody molecules as the adult is capable of producing. This prediction was tested in the present paper by determining whether neonatal or fetal B lymphocytes could be induced, in the transfer system described above, to produce a heterogeneous antibody response if given

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Abbreviations used in this paper: BGG, bovine gamma globulin; CFA, complete Freund’s adjuvant; DNP, dinitrophenyl; DsSO₄, dextran sulfate; EACA, -amino-N-caproic acid; LPS, lipopolysaccharide; PFC, plaque-forming cells; TNP, trinitrophenol.
either dextran sulfate (DxSO4) or endotoxin (lipopolysaccharide, LPS) two "polyclonal B-cell activators" (18) together with the antigen.

It was found that B lymphocytes from as early as day 14 of fetal life were capable of reconstituting irradiated, syngeneic recipients to give an antidi-nitrophenyl (DNP) response which was of markedly restricted heterogeneity of avidity. LPS or DxSO4 injected simultaneously with antigen could convert these responses into highly heterogeneous ones. In addition, secondary responses by irradiated animals reconstituted with day 14 or 16 fetal liver were highly heterogeneous and of high avidity, suggesting the selection of high affinity memory cells during the primary response despite the lack of detectable high avidity PFC. Both lines of evidence suggest that the information required to produce a heterogeneous immune response is already present in the day 14 fetal B-lymphocyte population.

Materials and Methods

Animals.  LAF1 mice (The Jackson Laboratory, Bar Harbor, Maine) were used. Fetal tissues were obtained by sacrifice of timed pregnant mice. Neonatal livers were taken from animals sacrificed within 12 h of birth. Age was designated taking the day of birth as "day 0" and the day of vaginal plug detection as "day 0 of fetal life."

Antigens and Haptens. The DNP derivative of bovine gamma globulin (BGG: Miles Laboratory, Kankakee, Ill.) was prepared by the reaction of 1-fluoro-2,4-dinitrobenzene (DNFB; Eastman Organic Chemicals Division, Eastman Kodak Co., Rochester, N. Y.) with BGG as described previously (19). The concentration of the product was determined by its "dry weight", and the degree of derivatization was estimated from its absorbancy at 360 nm (ε for DNP-lysine was taken as 17,400). Two preparations of DNP-BGG were used which had approximately 50 and 48 DNP groups per molecule of protein, respectively.

The DNP-α-amino-N-caproic acid (DNP-EACA) preparation used has been described previously (20). Escherichia coli endotoxin (LPS) was obtained from Difco Laboratories, Detroit, Mich. and DxSO4, mol wt 500,000, was obtained from Sigma Chemical Co., St. Louis, Mo.

Immunization.  Mice were immunized by the intraperitoneal injection of 500 μg DNP-BGG emulsified in complete Freund's adjuvant (CFA; containing 2 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 PFC 19-20 days after antigen injection. Previous studies (21) had shown that the average avidity and the degree of heterogeneity of avidity of the anti-DNP PFC were maximal at this time.

Cell Transfers. All studies were carried out by immunizing lethally (800 R) irradiated mice reconstituted with excess (1 × 10⁹) adult, syngeneic thymus cells plus B lymphocytes obtained from various sources. The source of fetal or neonatal B lymphocytes was the liver. This transfer system has been described in detail previously (1). In all studies of ontogeny, B cells from a single donor were transferred into a single recipient. On the other hand, adult thymus cells were pooled from several donors so as to yield a relatively constant population of thymus cells. Recipients were irradiated 2-4 h before cell transfer and were immunized 24 h after cell transfer. When indicated, 10 μg of endotoxin (LPS) or 100 μg of DxSO4 dissolved in saline were injected, intraperitoneally, at the time of immunization.

Assay of PFC. Anti-DNP PFC in the spleen were determined by the method of Jerne et al. (22), as modified for slide assay by Dresser and Greaves (23). Washed sheep red blood cells (SRBC) were conjugated by the Rittenberg and Pratt method (24) with 2,4,6-trinitrobenzene sulfonic acid (Sigma Chemical Co.). The slides were incubated for 1 h at 37°C. Lyophilized guinea pig serum (Grand Island Biological Co., Grand Island, N. Y.), dissolved in Millipore-filtered, de-ionized H₂O and diluted 1:7, was added as a source of complement, and the slides were incubated for an additional 45 min. Generally, each slide contained cells from one-twentieth to one-thirtieth of a spleen. Rabbit antimouse gamma globulin was used at the predetermined optimal dilution (1/300) to develop indirect plaques. Between 88 and 100% of the direct PFC were inhibited by the
addition of rabbit antimouse μ-chain antiserum (kindly provided by Dr. R. Asofsky, National Institutes of Health, Bethesda, Md.) to the assay plates. It should be noted that this was true even with animals reconstituted with day 14 or 16 fetal liver as the source of B lymphocytes.

Assay of Avidity of Anti-DNP PFC. The avidity distribution of the anti-DNP PFC was assayed by the inhibition of plaque formation using various concentrations of DNP-EACA, essentially according to the method of Andersson (25) as validated by previous work (26, 27). As previously described (1), concentrations of DNP-EACA ranging from $1 \times 10^{-9}$ to $1 \times 10^{-5}$ M, in half-log increments, were used.

Results

Heterogeneity of Avidity of the Anti-DNP Response of Irradiated Mice Reconstituted with B Lymphocytes from Neonatal Donors: Lack of Evidence for Suppressor T-Lymphocyte Activity Being Responsible for the Restricted Heterogeneity. We have previously shown (1) that when neonatal liver was used as a source of B lymphocytes for reconstituting irradiated recipients, the immune response was highly restricted with respect to heterogeneity of avidity. The restricted response presumably represents a small number of clones which, by chance, have matured earlier than the majority of B-lymphocyte clones. Therefore, it would be expected that different competent clones would be represented in different individual fetal mice. Such a hypothesis would lead one to expect that if a mixture of several neonatal livers were used to reconstitute irradiated adult mice, the recipients would produce a heterogeneous response, more closely approximating that produced by B lymphocytes from adult donors.

In Fig. 1 is illustrated the response of mice reconstituted with a mixture of five neonatal livers plus $1 \times 10^8$ adult thymus cells. The animals showed a heterogeneity of affinity approaching that of adult animals. If the restriction in heterogeneity were a consequence of suppressor T-lymphocyte activity (28) one would not have expected such an increased heterogeneity. Although not illustrated in the four examples shown in the top row of Fig. 1, more extensive data published previously (1) indicate that approximately one out of seven recipients of individual neonatal livers produce some high avidity PFC. Thus, it is not surprising to find, on a chance basis, that recipients of five neonatal livers have acquired the ability to produce very high avidity PFC. The number of PFC produced by animals receiving five neonatal livers appeared rather low compared with recipients of a single liver. This may reflect suppressor T-cell activity. However, since the two groups were not run simultaneously, a definitive comparison is not justifiable. For the purposes, of the present paper, the distributions of avidities are of more importance than the absolute magnitude of the response.

In a second series of experiments, irradiated animals were reconstituted with the equivalent of one-third of an adult spleen or with the equivalent of one-third of an adult spleen plus one neonatal liver. The animals were immunized with DNP-BGG, and the distribution of avidity of the anti-DNP PFC was determined 20 days later. The presence of neonatal liver did not result in any restriction in heterogeneity of avidity (Fig. 1). Grossly the magnitude of the direct and indirect PFC response was similar for animals receiving adult spleen alone (average: 1,695 direct and 5,618 indirect PFC) and animals receiving adult spleen plus neonatal liver (average: 1,746 and 4,767). The failure of the addition
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 (first) row are data on lethally irradiated mice reconstituted with syngeneic adult thymus cells and a single, syngeneic neonatal liver. In the second and third rows are data on lethally irradiated mice reconstituted with syngeneic adult thymus cells and one-third of a syngeneic adult spleen. The fourth row contains data on animals reconstituted with syngeneic adult thymus cells and five syngeneic neonatal livers. The bottom three rows contain data on lethally irradiated mice reconstituted with syngeneic adult thymus, one-third of an individual adult syngeneic spleen, plus one syngeneic neonatal liver. Animals were immunized 1 day after cell transfer. 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 total indirect PFC per spleen are given in the right upper corner of each histogram. Avidity increases to the right. The data in the first and second rows are representative data taken from previous work (1) and are offered only as a baseline for comparison.
of neonatal liver to increase the magnitude of the PFC response might be viewed as suggesting (but not proving) the presence of suppressor activity in the neonatal liver. The experiment does clearly demonstrate a failure of neonatal liver to alter the heterogeneity of the response by adult B lymphocytes. Thus, it appears unlikely that suppressor T-lymphocyte activity is responsible for the restriction in heterogeneity observed in animals reconstituted with neonatal or fetal B lymphocytes.

Secondary Response in Animals Reconstituted with B Lymphocytes from Fetal Donors. Irradiated mice reconstituted with adult thymus cells plus liver from day 14 or day 16 fetal mice as the source of B cells were immunized 1 day after cell transfer with DNP-BGG in CFA and were boosted 4 mo later with the same antigen in saline. Animals were sacrificed 5 days postboosting, and the distributions of avidities of splenic anti-DNP PFC were determined. The data presented in Fig. 2 clearly indicate that all animals produced a highly heterogeneous response of high average avidity and magnitude (compare with Fig. 1, 5, and 6). We have previously shown that 5 days after primary immunization LAF1 mice have only low avidity anti-DNP PFC (21). The animals reconstituted with day 14 or 16 fetal liver thus produced a secondary type response both in terms of the number of PFC, the predominance of indirect PFC, and the presence of large numbers of very high avidity PFC. The data suggest that selection for high affinity memory cells took place during the primary response even though no high avidity PFC were detectable during the primary response (Fig. 5 and 6). Such selection implies the presence of B lymphocytes bearing high affinity anti-DNP antibody in the 14- and 16-day fetal livers.

Effect of LPS on the Heterogeneity of the Anti-DNP PFC Response Produced by Irradiated Mice Reconstituted with B Lymphocytes from Fetal or Neonatal Donors. The data described above suggested that day 14 and 16 fetal mice already possessed the full catalogue of information required to synthesize the heterogeneous array of anti-DNP antibodies characteristic of the immune response of adult animals. The possibility that a "polyclonal B-cell activator" (17, 18) such as LPS might be capable of "turning on" B cells from neonatal or fetal donors so as to yield a heterogeneous response was investigated. Irradiated mice were reconstituted with adult thymus cells and neonatal liver as a source of B lymphocytes. The animals were immunized with DNP-BGG 1 day later and received 10 μg LPS intraperitoneally simultaneously. The distribution of avidities of the anti-DNP PFC in the spleens of these mice 20 days after immunization are compared in Fig. 3 with the response of representative animals reconstituted in the same manner but not receiving LPS. Clearly, the animals that received LPS made a highly heterogeneous response, comparable to that of adult animals.

Similarly designed experiments were carried out with irradiated mice reconstituted with fetal liver as the source of B lymphocytes. The results employing 18-, 16-, and 14-day fetal donors are illustrated in Fig. 4, 5, and 6, respectively. It is apparent from direct inspection of the figures that LPS was capable of converting a response of restricted heterogeneity into a highly heterogeneous one when liver from individual 18- or 16-day fetal donors was used to reconstitute irradiated mice. In contrast, (Fig. 6) LPS had no demonstrable effect on the response
Fig. 2. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with liver from an individual, day 14 (top two rows), or day 16 (bottom row) syngeneic fetal donor plus syngeneic adult thymus cells. Animals were immunized with 500 μg DNP-BGG in CFA 1 day after cell transfer and were boosted with 500 μg DNP-BGG in saline, intraperitoneally, 4 mo later. Splenic PFC were assayed 5 days after boosting. 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. The animal identification number (top) and total indirect PFC per spleen are given in the right upper corner of each histogram. Avidity increases to the right.

of animals reconstituted with 14-day fetal liver. While LPS markedly increased the heterogeneity of the response with B cells from day 16 or 18 fetal animals, it had no clear effect on the magnitude of the PFC response at 20 days after immunization. It can thus be concluded that B lymphocytes from 16-day fetal mice have the information necessary to synthesize the array of antibody molecules reflected in the heterogeneity of antibody avidity seen in the immune response of adult animals.

In the studies with neonatal donors (Fig. 3) littermate controls were not employed. The response in the absence of LPS, which had been extensively documented previously (1), was illustrated by four representative animals. This procedure was regarded as acceptable since the critical data in the present paper
Fig. 3. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with liver from an individual, syngeneic, neonatal donor plus syngeneic adult thymus cells. The animals were immunized with 500 μg DNP-BGG in CFA 1 day after cell transfer and were sacrificed for PFC assay 20 days later. The animals illustrated in the lowest two rows received 10 μg LPS intraperitoneally at the same time as the antigen injection. The animals in the top row are the same as those illustrated in Fig. 1 and are presented for comparison purposes. 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. The animal identification number (top) and the total indirect PFC per spleen are given in the upper right corner of each histogram. Avidity increases to the right.

are actually those obtained from studies with fetal donors where littermate controls were employed (see footnotes to Fig. 4, 5, and 6 for details). The apparent increased number of PFC in the control, as compared with the LPS-treated animals, in the studies with neonatal liver is probably merely due to the failure to use littermate controls. This effect was not seen in studies with fetal donors (Fig. 4, 5, and 6) where littermate controls were employed.

Effect of DMSO on the Heterogeneity of the anti-DNP PFC Response Produced by Irradiated Mice Reconstituted with B Lymphocytes from Day 14 Fetal Donors. The failure of mice reconstituted with day 14 fetal liver to produce a heterogeneous response upon immunization simultaneously with LPS administration could reflect either a lack of information to produce the entire array of anti-DNP antibodies or an inability of B cells from 14-day fetal mice to respond to LPS. The effect of a second "polyclonal B-cell activator," DMSO, (17), was therefore studied. Mice reconstituted with B cells from day 14 fetal donors produced a highly heterogeneous response, comparable to that of adult animals,
FIG. 4. Each histogram illustrates the distribution of indirect PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with liver from an individual, 18-day, syngeneic fetal donor plus syngeneic adult thymus cells. Animals were immunized with 500 μg DNP-BGG in CFA 1 day after cell transfer and were sacrificed for PFC assay 19 days later. The animals illustrated in the lowest two rows received 10 μg LPS intraperitoneally at the same time as the antigen injection. Groups of recipients which received livers from littermate donors were as follows: (a) 233, 234, 235, 242, 243, and 244; (b) 237, 238, and 241; (c) 236, 240, and 245. 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. The animal identification number (top) and the number of indirect PFC per spleen are given in the upper right corner of each histogram. Avidity increases to the right.

when immunized with DNP-BGG together with the injection of 100 μg DxsO₄ (Fig. 6). Thus, B lymphocytes as early as day 14 of fetal life possess the necessary information to produce a highly heterogeneous anti-DNP response.

**Ontogeny of the Capacity to Produce Indirect anti-DNP PFC.** As indicated in Table I animals reconstituted with B lymphocytes from day 18 fetal donors produce both direct and indirect PFC. In contrast, when the B-cell donors are day 14 or 16 fetal mice, few if any indirect PFC are observed. It should be noted that the values listed under "indirect PFC" in Table I are the total plaque counts in the presence of rabbit antimouse immunoglobulin antiserum. The number of
direct PFC has not been subtracted from the crude indirect plaque count. It is interesting that while DxsSO₄ and LPS are capable of increasing the heterogeneity (i.e., the polyclonality) of the PFC response with day 14 and 16 fetal donors, respectively, they do not bring about an increased production of indirect PFC.
Fig. 6. Each histogram illustrates the distribution of direct PFC with respect to avidity in the spleen of a lethally irradiated mouse reconstituted with liver from an individual, 14-day, syngeneic fetal donor plus syngeneic adult thymus cells. Animals were immunized with 500 μg DNP-BGG in CFA 1 day after cell transfer and were sacrificed for PFC assay 20 days later. The animals illustrated in the fourth and fifth rows down from the top received 10 μg LPS, intraperitoneally, at the same time as the antigen injection. The animals illustrated in the bottom row received 100 μg Dextran sulfate, intraperitoneally, at the same time as the antigen injection. Groups of recipients that received livers from littermate donors were as follows: (a) 262, 265, 276, 277, 278, 279, 280, 281, and 282. 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. The animal identification number (top) and the number of direct PFC per spleen are given in the upper right corner of each histogram. Avidity increases to the right.
TABLE I

Anti-DNP PFC Response in the Spleens of Irradiated Mice Reconstituted with Fetal B Lymphocytes and Immunized with DNP-BGG*

| Age of fetal liver donor days | Additional treatment‡ | Number of animals | Direct PFC /spleen | Indirect PFC /spleen |
|-----------------------------|------------------------|-------------------|-------------------|---------------------|
| 14                          | None                   | 15                | 1,113             | 575                 |
| 14                          | LPS                    | 10                | 930               | 686                 |
| 14                          | DxSO₄                  | 5                 | 661               | 654                 |
| 14                          | Boosted                | 7                 | 4,466             | 57,214              |
| 16                          | None                   | 4                 | 1,035             | 525                 |
| 16                          | LPS                    | 4                 | 1,155             | 762                 |
| 16                          | Boosted                | 3                 | 3,467             | 57,733              |
| 18                          | None                   | 7                 | 1,091             | 4,995               |
| 18                          | LPS                    | 6                 | 690               | 4,746               |

* Irradiated LAF mice were reconstituted with adult thymus cells and fetal liver as a source of B lymphocytes and were immunized with 0.5 mg DNP-BGG in CFA 1 day later. Animals were sacrificed 19 or 20 days after immunization and their splenic PFC assayed. Data are presented as arithmetic means. The animals are the same as those whose PFC avidity distributions were presented in Fig. 5, 6, and 7.

‡ Where indicated animals received 10 μg LPS or 100 μg DxSO₄ intraperitoneally at the same time as immunization. "Boosted" animals were assayed 5 days after boosting with 0.5 mg DNP-BGG in saline, intraperitoneally, 4 mo after primary immunization.

§ Indirect PFC were developed by addition of rabbit antimouse immunoglobulin antibody. The number of direct PFC was not subtracted from the value obtained in the presence of developing antiserum. Thus, the value for indirect PFC represents a maximum possible value.

Boosting of animals reconstituted with day 14 or 16 fetal liver resulted in a very marked response consisting mainly of indirect PFC.

Statistical Analysis of Data. The Shannon heterogeneity index (29) was used to describe the degree of heterogeneity of avidity of the PFC population of individual animals. The average value of this index for each of the experimental groups is presented graphically in Fig. 7. Both data reported in the present paper and data reported previously by Goidl and Siskind (1) are summarized in the figure. Clearly, the Shannon heterogeneity indices for responses of mice reconstituted with B cells from animals 7 days of age or younger are considerably lower (less heterogeneity) than when older donors are employed. Treatment with LPS (day 16 fetal or older donors), with DxSO₄ (day 14 fetal donors),

\[ I = -K \sum \frac{p_i}{\ln p_i} \]

where \( G \), total population; \( I \), calculated information content of the system; \( K \), a constant; \( M \), number of states ranging from 1,2, \ldots, j, \ldots, M \) which have respective probabilities of \( p_1, p_2, \ldots, p_M \). The maximal information content (maximum heterogeneity) of a system is represented by the equidistribution of information bits among all of the arbitrarily set states. The minimal information content (minimum heterogeneity) would require all bits of information to be contained in a single subpopulation.
FIG. 7. Scatter diagram illustrating the heterogeneity indices of anti-DNP PFC avidity distributions for irradiated mice immunized with 500 μg DNP-BGG in CFA 1 day after reconstitution with excess adult thymus cells plus the indicated tissue as the source of B lymphocytes. Distribution of avidities of splenic PFC were determined by inhibition with DNP-EACA at 19–21 days after immunization. Along the abscissa is indicated the age (in days) of the B lymphocyte donor. The Shannon heterogeneity index (29) is represented (log₂) on the ordinate. The larger the index, the greater the heterogeneity. The tissue source of B lymphocytes is designated as follows: BM, bone marrow; S, spleen; L, liver. Other treatments of recipient mice are indicated as follows: LPS, 10 μg endotoxin when immunized; DXSO₄, 100 μg DXSO₄ when immunized; 2°, data obtained 5 days after boosting at 4 mo after primary antigen injection. The data are a combination of that presented in this paper and that reported previously (1).

or boosting (day 14 or 16 fetal donors) led to responses that were comparable in heterogeneity index to those of irradiated mice reconstituted with adult lymphoid tissues.

The data from the histograms for each experimental group was assembled in matrix form. Matrices to be compared were then tested for equality of variances by the F test to establish the applicability of the t test. Matrices were then compared by the t test. If the hypothesis of equality of variances was rejected by the F test, the matrices were compared by a chi-square analysis. The results statistically confirmed the conclusions described above which were based upon direct inspection of the histograms. (a) LPS had no statistically significant effect on the heterogeneity of the response of animals reconstituted with 14-day fetal liver. (b) Recipients of day 14, 16, or 18 fetal liver were statistically significantly less heterogeneous than recipients of adult spleen (P values ranging from <0.01
to $<0.025$). (c) LPS treatment resulted in an increase in the heterogeneity of avidity of the anti-DNP response of animals reconstituted with day 16 or 18 fetal liver or with neonatal liver. The response of LPS-treated animals was statistically indistinguishable from that of animals reconstituted with adult spleen. (d) LPS-treated recipients of day 16 or 18 fetal or neonatal liver produced a response statistically significantly more heterogeneous than that of similarly reconstituted animals not treated with LPS ($P$ values ranging from $<0.01$ to $<0.025$). (e) LPS did not significantly increase heterogeneity of the response of animals reconstituted with 14-day fetal liver. (f) D$_{x}$SO$_{4}$ injection significantly increased the heterogeneity of the anti-DNP response by animals reconstituted with day 14 fetal liver ($P < 0.01$).

Discussion

We previously described and characterized a cell transfer system which facilitates studies on the functional capacity of B lymphocytes (1). Essentially, lethally irradiated mice are reconstituted with excess, adult, syngeneic thymus cells plus the B-lymphocyte population the functional capacity of which is to be evaluated. It was shown that adult T cells were capable of cooperating with fetal B lymphocytes in this system. In addition, it was established that the immune response to DNP-BGG in an irradiated mouse reconstituted with adult lymphoid tissue was similar in average affinity and heterogeneity of affinity to the response of intact animals (21).

Using this system the ontogeny of the functional capacity of B lymphocytes to produce a heterogeneous immune response has been studied. From data reported in the present paper and previously (1) the following conclusions can be made. (a) B lymphocytes from fetal or neonatal donors produce a response that is markedly restricted with respect to heterogeneity of affinity. (b) Based upon mixed cell transfer studies, the restriction in heterogeneity is probably not the consequence of suppressor cell activity. (c) Injection of "polyclonal B-cell activators" (LPS and D$_{x}$SO$_{4}$) will increase the heterogeneity of the anti-DNP response of mice reconstituted with fetal B cells. Thus, as early as 14 days of gestation, LAF$_{1}$ mice appear to have an adequate catalogue of information available so that, with appropriate stimulation, they can produce an anti-DNP response comparable to that of adult mice with regard to heterogeneity of affinity.

In the course of these studies, three distinct differentiation events affecting the functional capacity of B lymphocytes have been identified. (a) Between day 14 and 16 of fetal life B cells acquire the capacity to be "turned on" by LPS. That is, the ability of LPS to increase the heterogeneity of the response becomes apparent with cells from 16-day donors. (b) Between day 16 and 17 (see reference 1) of fetal life B lymphocytes acquire the capacity to reconstitute irradiated mice to give a normal indirect PFC response. Cells from 14- or 16-day fetal mice produce few if any indirect PFC even in the presence of excess adult thymus cells or treatment with LPS or D$_{x}$SO$_{4}$. (c) Between day 7 and 10 after birth, peripheral B lymphocytes acquire the capacity to produce an adult type heterogeneous response (1).

Klinman and Press (30) have reported that the B-cell repertoire specific for the DNP or TNP determinants is restricted in content in neonatal BALB/c mice.
Montgomery and Williamson (31) have reported that the antibody produced by
some, but not all, neonatal rabbits is of restricted heterogeneity. Kearney and
Lawton (32) demonstrated a maturation of B-cell response to LPS at day 17 of
fetal life, a timing that appears compatible with our results.

Since individual clones of B lymphocytes appear to synthesize homogeneous
antibody (33) it can be assumed that the heterogeneity of the immune response
reflects a polyclonality of the response (34, 35). Thus, studies on heterogeneity of
antibody affinity represent an effort to probe the degree of polyclonality of the
response. Based upon their anti-DNP response in the presence of DxSO₄ and
their secondary response, one can conclude that the B-lymphocyte population
from 14-day fetal mice has the genetic information required to produce an array
of anti-DNP antibodies indistinguishable (within the limitations of the assay
methods) from that produced by adult animals. In terms of theories of genera-
tion of antibody diversity, the ontogeny data reported here are consistent with
either a germ line theory or a somatic mutation theory in which a large fraction
of the mutations have already occurred relatively early in ontogeny (before day
14 of fetal life). It should be noted that Edelman's group (3, 36) observed that the
avidity of the antigen-binding cells in pools of fetal mouse tissue was similar to
that of normal adult mice. This observation appears consistent with the data
reported here.

The precise developmental event occurring between 7 and 10 days of age
which allows the expression of a heterogeneous B-lymphocyte response is not
known. Several lines of evidence suggest that this change represents a differen-
tiation event and not the accumulation of somatic mutations. (a) The acquisition
of an "adult-like" capacity to generate a heterogeneous response occurs in the
spleen relatively abruptly between 7 and 10 days of age. (b) Animals reconsti-
tuted with bone marrow from 10-day-old donors produce a "fetal-like," restricted
response, while animals reconstituted with spleen cells from the same donors
yield a heterogeneous response. (c) The B-lymphocyte population from day 14 of
fetal life appeared to contain all information needed to produce a heterogeneous
antibody response.

The finding that animals reconstituted with day 14 or 16 fetal liver give a
heterogeneous secondary response, late after priming, is worthy of some discus-
sion. Animals reconstituted with 14- or 16-day fetal B lymphocytes produce a
primary anti-DNP PFC response which is highly restricted with respect to
heterogeneity of avidity, is of low average avidity, is generally lacking in
detectable high avidity PFC, and appears to be lacking in indirect PFC. Despite
the restricted primary response, the secondary response 5 days after boosting is
highly heterogeneous, contains a large proportion of high avidity PFC, and
consists predominantly of indirect PFC. One possibility is that the heterogeneity
actually reflects a primary response by the host which has recovered from the
effects of irradiation and has repopulated its lymphoid tissues with host type
cells. Alternatively, it might represent a primary response by B lymphocytes of
fetal donor origin which have matured in the adult environment to be capable of
giving a normal heterogeneous response. These possibilities seem unlikely for
the following reasons. The normal primary anti-DNP PFC response 5 days after
antigen injection has been shown to be of low magnitude, to contain a large
percentage of direct PFC, to have an absence of detectable high avidity PFC, and to have a low average avidity (21). It is only later in the primary response (approximately 3 wk after antigen injection) that a predominance of high affinity, indirect PFC is observed. This general pattern was found to hold for both intact mice and irradiated recipients of adult lymphoid tissue (21). The pattern of PFC avidity and the number and class distribution of PFC observed in the secondary responses produced by animals reconstituted with day 14 or 16 fetal liver is indistinguishable from the secondary response to DNP-BGG by intact mice or by irradiated mice reconstituted with adult spleen (21). Thus, it appears that selection for high affinity memory cells actually took place during the primary response of irradiated mice reconstituted with day 14 or 16 fetal liver. This implies that the B-lymphocyte population present in the day 14 fetal liver contains cells bearing cell surface antibody molecules having the full range of avidities present in the normal adult. This population was capable of being selectively stimulated by antigen to proliferate. Thus, selection of a subpopulation of high avidity memory cells proceeded normally during the primary response despite the fact that no high avidity PFC were observed. One can speculate that the deficiency in the functional capacity of the B-cell population from fetal animals lies not in the absence of the specific clones of cells bearing specific antigen receptors, but rather in the ability of these clones of cells, after proliferating, to differentiate into antibody-producing cells. This implies the existence of two distinct signals or controls, one for proliferation and generation of memory cells, and a second for conversion to antibody-producing cells. It may be suggested that it is this latter capacity that matures between 7 and 10 days of age in LAF1 mice. The mechanisms controlling the differentiation process are not known. Kaplan and Goidl (37) have recently offered a possible hypothesis involving a type of in vivo "Hayflick phenomenon."

Summary

The ontogeny of the functional capacity of B lymphocytes to generate a heterogeneous response to a haptenic determinant was studied by cell transfer techniques in LAF1 mice. Fetal liver, as a source of B lymphocytes, was transferred into adult, syngeneic, irradiated animals. All recipients received excess adult thymus cells so that T-cell activity did not limit the response and were immunized with DNP-BGG. The heterogeneity of avidity of their anti-DNP PFC response was assayed by hapten inhibition of plaque formation.

Animals reconstituted with B lymphocytes from fetal donors produced a response that is highly restricted with respect to heterogeneity of affinity. Transfer studies using multiple fetal donors or mixtures of adult and neonatal cells for reconstitution suggest that the restriction in heterogeneity is not the consequence of suppressor T-lymphocyte activity. With animals reconstituted with B cells from day 16 or older fetal donors, injection of LPS together with antigen converted the response to a heterogeneous "adult-type" response. With animals reconstituted with B lymphocytes from day 14 fetal liver DxSO4, but not LPS, could convert the response to a highly heterogeneous one. Animals reconstituted with day 14 or 16 fetal liver as source of B lymphocytes were capable of producing a heterogeneous secondary response despite the fact that their pri-
mary response was of restricted heterogeneity. This implies the selection of high
affinity B-memory cells, in the absence of high affinity PFC, during the primary
response with fetal B lymphocytes. Animals reconstituted with day 14 or 16 fetal
liver produce only direct PFC, while animals reconstituted with day 18 fetal
liver produce both direct and indirect PFC.

Three differentiation events have therefore been defined in the functional
development of B lymphocytes: (a) between day 14 and day 16 of fetal life they
acquire responsiveness to LPS; (b) between day 16 and 18 of fetal development
they acquire the capacity to produce indirect PFC; (c) between day 7 and 10 after
birth they acquire the capacity to give a heterogeneous response after normal
immunization. In addition, it was shown that LAF1 mice already have all of the
information required to produce an "adult-type" heterogeneous anti-DNP re-
sponse at day 14 of fetal life.

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