ONTOGENY OF B-LYMPHOCYTE FUNCTION

V. Thymus Cell Involvement in the Functional Maturation of B-Lymphocytes from Fetal Mice Transferred into Adult Irradiated Hosts*

BY DAVID H. SHERR,† MYRON R. SZEWCZUK,§ AND GREGORY W. SISKIND

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

A number of investigators have demonstrated B lymphocytes, or their precursors, in fetal mice (1–17). However, the B-cell population does not appear to achieve adult characteristics until some time after birth (1–5). Factors involved in the regulation of B-lymphocyte maturation have not been identified. The purpose of this study is to demonstrate a role for thymus cells in promoting the functional maturation of B lymphocytes.

We have previously described the use of a cell transfer system to study the functional maturation of B lymphocytes. Lethally irradiated adult mice are reconstituted with neonatal or fetal tissues as a source of B cells plus adult thymus cells. In this system the functional capacity of B lymphocytes to respond to antigen can be assayed in an adult environment in the presence of adult T cells. It was found that lethally irradiated LAF1 mice reconstituted with adult spleen and thymus cells produce a normal plaque-forming cell (PFC) response to dinitrophenylated bovine gamma globulin (DNP-BGG) (4). As in the normal adult mouse, the anti-DNP response is highly heterogeneous with respect to antibody affinity. In contrast, irradiated mice reconstituted with fetal or neonatal liver cells plus adult thymus cells produce a response which is restricted with respect to heterogeneity of affinity and is of low average affinity. The B-cell population acquires the capacity to produce a normal heterogeneous adult-like response between 7 and 10 days after birth. It was further shown that mice reconstituted with day 14 or day 16 fetal liver as the source of B cells produce mainly, or only, direct PFC. The capacity to

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Abbreviations used in this paper: BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; DNP, 2,4-dinitrophenyl; EACA, e-amino-n-caproic acid; HBSS, Hanks' balanced salt solution; PFC, plaque-forming cell; SRBC, sheep erythrocytes.
produce indirect PFC matures between day 16 and 17 of fetal life (4). By delaying immunization for various times after cell transfer the kinetics of B-cell maturation in an adult environment can be studied. It was found that B cells from day 15 fetal mice and from neonatal mice both acquire the capacity to produce a heterogeneous response within 3 days after transfer into an irradiated syngeneic mouse (17). This suggests that the differentiation event normally occurring in the LAF, mouse between day 7 and 10 after birth is probably induced by some factor in the adult animal. In the present paper evidence is presented which indicates that this event in the functional maturation of B lymphocytes requires, or is facilitated by, adult thymus cells.

Materials and Methods

**Animals.** LAF, mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Adult recipients were 6-8 wk old. Fetal tissues were obtained from timed pregnancies (vaginal plug date taken as day 0 of fetal life). Neonatal livers were taken from animals sacrificed within 18 h of birth. Thymus cells were obtained from 3- to 4-wk-old weanling donors.

**Cell Transfers.** In all experiments, LAF, adult mice were lethally irradiated 2-4 h before cell transfer by exposure to 800 rads from a gamma source. Where indicated, recipients had been thymectomized 1-8 wk before irradiation. Fetal or neonatal livers were teased. The cells were passed through a thin layer of gauze; were washed once; and were injected intravenously together with or without 1 × 10⁸ pooled adult thymus cells, into lethally irradiated hosts as described previously (4). In all cases, tissues from a single B-cell donor were transferred into a single recipient. 6 or 28 days after transfer, all animals received 1 × 10⁸ thymus cells from weanling donors and were immunized on the following day. Thymus cell suspensions were prepared as pools from several donors.

**Antigens and Haptens.** DNP-BGG was prepared by the reaction of BGG (Miles Laboratories Inc., Miles Research Products, Kankakee, Ill.) with 2,4-dinitrobenzene sulfonic acid (Eastman Kodak Co., Rochester, N.Y.) under alkaline conditions essentially as described by Eisen et al. (18). The concentration of the conjugated protein was determined by dry weight analysis and the degree of derivitization estimated spectrophotometrically from its absorbance at 360 nm (ε for DNP-lysine = 17,400). The DNP-BGG used in these experiments had 55 DNP groups per BGG molecule. DNP-α-amino-n-caproic acid (DNP-EACA) was prepared by the reaction of 2,4-dinitrofluorobenzene (Eastman Chemical Co.) with EACA (Sigma Chemical Co., St. Louis, Mo.) as described previously (19).

**Immunizations.** Mice were immunized by the intraperitoneal injection of 500 μg DNP-BGG or 100 μg BGG emulsified in complete Freund’s adjuvant (CFA; containing 1.5 mg/ml Mycobacterium butyricum) in a final vol of 0.2 ml. Mice were sacrificed by cervical dislocation 19-21 days after immunization with DNP-BGG for assay of their splenic anti-DNP PFC. Mice immunized with BGG in CFA were boosted by the intraperitoneal injection of 500 μg BGG in saline 3 wk after priming and were sacrificed 13 days later for assay of their splenic anti-BGG PFC.

**Assay of Number and Affinity of PFC.** Anti-DNP PFC were assayed by the Dresser and Greaves (20) modification of the Jerne plaque assay (21) using 2,4,6-trinitrophenyl conjugated sheep erythrocytes (SRBC) prepared by the reaction of 2,4,6-trinitrobenzene sulfonic acid (Sigma Chemical Co.) with washed SRBC as described by Rittenberg and Pratt (22). Slides were incubated for 1 h at 37°C. Freshly frozen guinea pig serum (absorbed with 50% SRBC) was added, at a final dilution of 1:30, as a source of complement and the slides were incubated for an additional 45 min. The affinity distribution of anti-DNP PFC was determined by hapten inhibition of plaque formation as described by Andersson (23) and validated by previous work (24, 25). Concentrations of DNP-EACA ranging from 1 × 10⁻⁹ M to 1 × 10⁻³ M in half-log increments were used for inhibition.

Anti-BGG PFC were assayed as described previously (26) using BGG conjugated SRBC prepared by coupling the protein with 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide HCl (Sigma Chemical Co.) (27). Slides were incubated at 37°C for 1 h, complement was added, and incubation continued for an additional 2½ h. The affinity distribution of anti-BGG PFC was
assayed by plaque inhibition as described above using concentrations of BGG, absorbed with 50% SRBC, ranging from $1 \times 10^{-11}$ M to $1 \times 10^{-7}$ M in half-log increments.

Indirect PFC were developed by the addition of rabbit anti-mouse immunoglobulin antiserum at a final dilution of 1:300 which had been found to be optimal for plaque development.

**Results**

*Requirement for Thymus Cells to Promote Maturation of the Capacity of Fetal B Cells to Produce a Heterogeneous Anti-DNP Response.* Irradiated mice reconstituted with fetal or neonatal B cells and adult thymus cells have been shown to acquire an adult capacity to generate a heterogeneous response within 3 days of transfer (17). The role of thymus cells in the maturation of B lymphocytes was investigated in this cell transfer model. Irradiated adult mice were reconstituted with day 15 fetal liver together with or without $1 \times 10^8$ thymus cells from weanling donors. 6 days later all animals received $1 \times 10^8$ pooled weanling thymus cells and were immunized the following day. The affinity distributions of their splenic anti-DNP PFC are illustrated in Fig. 1 and their heterogeneity indices and ratios of indirect to direct PFC are presented in Table I. It is clear that mice receiving adult thymus cells together with the fetal liver produce an adult-like response with regard to heterogeneity of antibody affinity and have a preponderance of indirect PFC. In contrast, mice not given weanling thymus cells at the time of reconstitution produce a response of restricted heterogeneity of affinity. In addition, the immune response of mice reconstituted only with day 15 fetal liver consists mainly of direct PFC.

Thus, it appears that thymus cells are necessary if maturation of B-cell function is to take place in the reconstituted mice. It is interesting that the presence of a thymus in the irradiated animal does not alter the requirement for transferred thymus cells to promote B-cell maturation in these short-term experiments. It should be noted that all animals, both those which had and those which had not received thymus cells at the time of the original reconstitution, were given $1 \times 10^8$ weanling thymus cells 1 day before immunization. Since DNP-BGG is a thymic-dependent antigen, essentially no PFC are produced by irradiated mice which do not receive thymus cells (4). In addition, it should be mentioned that since anti-IgM was not added to inhibit direct plaques, the indirect plaque count undoubtedly includes some direct plaques and thus represents an overestimate of the true number of indirect (IgG) PFC. The developing antiserum which we use inhibits about 60% of direct plaques. However, in view of uncertainties in such estimates it was deemed preferable not to apply this correction to the data.

*Requirement for Thymus Cells to Promote Maturation of the Capacity of Fetal B Cells to Produce a Heterogeneous Anti-BGG Response.* The requirement for thymus cells for the maturation of the capacity of B cells to produce a heterogeneous response to the thymic-dependent protein antigen, BGG, was studied. Irradiated adult mice were reconstituted with neonatal or 14 day fetal liver as the source of B cells together with or without $1 \times 10^8$ weanling thymus cells. All animals received $1 \times 10^8$ pooled weanling thymus cells 6 or 28 days later and were immunized with BGG in CFA on the following day. The distributions of splenic anti-BGG PFC with respect to affinity are illustrated in
FIG. 1. Each histogram illustrates the distribution of indirect anti-DNP PFC with respect to affinity in the spleen of an LAF, mouse 19–21 days after immunization with 500 µg DNP-BGG in CFA. In the top row are data on lethally irradiated adult mice reconstituted with 10⁶ adult thymus cells together with a single day 15 fetal liver. In the second row are data on lethally irradiated, thymectomized adult mice reconstituted with day 15 fetal liver alone. The third row contains data on lethally irradiated adult mice reconstituted with day 15 fetal liver alone. The fourth row contains data on lethally irradiated thymectomized adult mice reconstituted with day 15 fetal liver together with 10⁶ adult thymus cells. All animals received 10⁶ adult thymus cells 6 days after fetal cell transfer and were immunized with DNP-BGG in CFA on the following day. In each case, data on five representative animals from groups of 6 to 18 mice are presented. 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), total indirect PFC per spleen and ratio of indirect to direct PFC are given in the right upper corner of each histogram. Affinity increases to the right.

Fig. 2 and the heterogeneity indices are presented in Table II. The results are similar to those described above using DNP-BGG. In both thymectomized and nonthymectomized recipients a maturation of immature B cells to produce a heterogeneous response with respect to antibody affinity is seen only if thymus cells are given. In the absence of thymus cells irradiated thymectomized mice reconstituted with neonatal liver give a response of restricted heterogeneity.
Thymus Cell Involvement in the Maturation of B Lymphocytes from Fetal Donors to Produce a Heterogeneous Indirect Anti-DNP PFC Response in Irradiated Adult Recipients*

| Recipient, reconstituted with | Time between reconstitution and immunization | Heterogeneity index | Number indirect PFC PFC/ spleen | Ratio of indirect to direct PFC |
|-----------------------------|---------------------------------------------|--------------------|----------------------------------|--------------------------------|
| Irradiated recipient, 10^7 thymus cell, adult spleen (10) | 1 | 2.79 ± 0.17 (N.S.) | 6,032 ± 1,313 | 2.6 ± 0.9 (N.S.) |
| Irradiated recipient, 10^6 thymus cells, day 15 fetal liver (6) | 7 | 2.57 ± 0.22 | 6,411 ± 1,007 | 2.1 ± 1.0 |
| Irradiated thymectomized recipient, 10^6 thymus cells, day 15 fetal liver (18) | 7 | 2.49 ± 0.27 (N.S.) | 19,203 ± 5,022 | 3.3 ± 1.6 (N.S.) |
| Irradiated recipient, day 15 fetal liver (10) | 7 | 1.54 ± 0.73 (P < 0.05) | 4,773 ± 3,411 | 1.3 ± 1.0 (P < 0.05) |
| Irradiated thymectomized recipient, day 15 fetal liver (11) | 7 | 1.54 ± 0.38 (P < 0.002) | 3,103 ± 1,789 | 1.0 ± 0.4 (P < 0.002) |

* Lethally irradiated mice were reconstituted with day 15 fetal liver or adult spleen with or without 10^6 syngeneic adult thymus cells as indicated in the first column (left). Recipients were thymectomized before irradiation as indicated. All animals received 10^6 adult thymus cells 6 days after reconstitution with fetal liver and were immunized with 500 µg DNP-BGG in CFA on the following day. Splenic anti-DNP PFC were assayed 19-21 days after immunization. Numbers in parenthesis indicate the number of animals studied. Data are presented as mean ± standard deviation.

† The Shannon heterogeneity index (4, 29) was used to describe the degree of heterogeneity of affinity of the indirect anti-DNP PFC population of individual animals. The average value ± standard deviation of this index for each experimental group is presented. The larger the index, the greater the heterogeneity. The significance of differences in heterogeneity indices and in the ratio of indirect to direct PFC were evaluated by the Mann-Whitney U test. All groups were compared with the irradiated recipients which received 10^7 thymus cells together with fetal liver cells (second row from top).

§ N.S. = not significant.

when immunized 29 days after cell transfer. The possibility existed that the presence of suppressor T cells in the fetal liver might be responsible for the restriction in heterogeneity of affinity. Treatment of day 14 fetal liver with anti-δ antisera and complement before cell transfer may have slightly reduced the degree of restriction in heterogeneity. However, mice reconstituted with anti-δ-treated fetal liver clearly produce a response of significantly restricted heterogeneity as compared with that of mice reconstituted with normal adult B lymphocytes.

Maturation, in the Absence of Thymus Cells, of the Capacity of Fetal B Cells to Produce Indirect Anti-DNP PFC. The kinetics of the maturation of fetal B cells to produce indirect PFC was followed in cell transfer recipients. Lethally irradiated thymectomized mice were reconstituted with day 15 fetal liver. At various times from 5 to 19 days later, the mice received 1 × 10^6 pooled weanling thymus cells and were immunized with DNP-BGG in CFA on the following day. The ratios of indirect to direct PFC, and the heterogeneity indices are presented in Table III. Mice immunized 6 days after cell transfer produce relatively few indirect plaques while mice immunized 13–20 days after cell transfer have a predominance of indirect PFC. Regardless of the time of
immunization, all animals have a response of restricted heterogeneity of affinity. Thus, while the presence of thymus cells appears to be necessary for B cells from immature donors to develop the capacity to generate an adult-like heterogeneous response, thymus cells are not required for B cells to develop the capacity to produce indirect PFC.
Table II

Thymus Cell Involvement in the Maturation of B Lymphocytes from Fetal Donors to Produce a Heterogeneous Anti-BGG Response in Irradiated Adult Recipients*

| Recipient, reconstituted with: | Time between reconstitution and immunization | Number indirect PFC | Heterogeneity index‡ |
|-------------------------------|------------------------------------------|---------------------|----------------------|
| Irradiated recipient, 10⁶ thymus cells, adult spleen (5) | 1 | 26,183 ± 5,304 | 2.78 ± 0.06 (NS)§ |
| Irradiated recipient, 10⁸ thymus cells, day 14 fetal liver (7) | 7 | 17,760 ± 7,554 | 2.39 ± 0.26 |
| Irradiated thymectomized recipient, 10⁶ thymus cells, day 14 fetal liver (9) | 7 | 21,662 ± 8,100 | 2.47 ± 0.23 (NS) |
| Irradiated recipient, day 14 fetal liver (7) | 7 | 18,471 ± 8,927 | 0.80 ± 0.73 (P < 0.006) |
| Irradiated recipient, anti-α-treated day 14 fetal liver (10) | 7 | 26,490 ± 17,169 | 1.54 ± 0.26 (P < 0.002) |
| Irradiated thymectomized recipient, neonatal liver (9) | 29 | 31,488 ± 6,669 | 1.98 ± 0.22 (P < 0.02) |

* Lethally irradiated mice were reconstituted with day 14 fetal liver or neonatal liver with or without 10⁶ syngeneic, adult thymus cells as indicated in the first column (left). Recipients were thymectomized before irradiation when indicated. Mice received 10⁶ weanling thymus cells 6 days after reconstitution with fetal liver and were immunized with 100 μg BGG in CFA 1 day later. Animals reconstituted with neonatal liver (row 6) received 10⁶ weanling thymus cells 28 days after neonatal cell transfer and were immunized with BGG 1 day later. All animals were boosted with 500 μg BGG in saline 21 days after primary immunization and their spleens were assayed 13 days after boosting for indirect anti-BGG PFC. The number of animals studied is indicated in parenthesis. Data are presented as mean ± standard deviation.

‡ The Shannon heterogeneity index (4, 29) was used to describe the degree of heterogeneity of affinity of the indirect anti-BGG PFC population of individual animals. The average value ± standard deviation of this index for each experimental group is presented. The larger the index, the greater the heterogeneity. The significance of difference in heterogeneity indices was evaluated by the Mann-Whitney U test. All groups were compared with irradiated recipients receiving 10⁶ thymus cells together with the fetal liver cells (second row from top).

§ NS = not significant.

Discussion

In the present studies data are presented which support the hypothesis that thymus cells are required to facilitate B-cell differentiation. In previous work we have shown that lethally irradiated adult mice reconstituted with syngeneic adult spleen and 1 × 10⁶ thymus cells produce an anti-DNP response which is
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**TABLE III**

| Time between reconstitution and immunization | Number of animals | Direct PFC | Indirect PFC | Ratio of indirect to direct PFC | Heterogeneity index† |
|---------------------------------------------|------------------|------------|--------------|-------------------------------|----------------------|
| days                                        |                  | PFC/spleen | PFC/spleen   |                               |                      |
| 6                                           | 11               | 2,600      | 2,590        | 1.0                           | 1.84 ± 0.38          |
| 13                                          | 9                | 4,250      | 18,210       | 4.3                           | 2.15 ± 0.19          |
| 20                                          | 4                | 1,990      | 5,730        | 2.9                           | 2.17 ± 0.26          |

* Lethally irradiated thymectomized mice were reconstituted with day 15 fetal liver. The animals were immunized with 500 μg DNP-BGG in CFA 6-20 days after cell transfer as indicated in the first column. All animals received 10⁶ syngeneic weanling thymus cells, intravenously 1 day before immunization. Splenic anti-DNP PFC were assayed 20 days after immunization. The results are expressed as geometric means.

† The Shannon heterogeneity index (4, 29) was used to describe the degree of heterogeneity of affinity of the indirect anti-DNP PFC population of individual animals. The average value ± standard deviation, of this index for each experimental group is presented. The larger the index, the greater the heterogeneity.

heterogeneous with regard to antibody affinity (4). In contrast, mice reconstituted with fetal or neonatal B cells and adult thymus cells produce a response of restricted heterogeneity. The B-lymphocyte population acquires the capacity to respond with an adult-like, heterogeneous response between 7 and 10 days after birth in LAF₁ mice (4). Mice reconstituted with B cells from day 14 to day 16 fetal donors plus adult thymus cells respond to immunization with DNP-BGG in CFA with mainly direct PFC. Between day 16 and 17 of fetal life the B-cell population acquires the capacity to produce a response consisting predominantly of indirect PFC. It appears that the B-cell population present in day 14 fetal mice already possesses the full catalogue of information required for generation of a heterogeneous response. This hypothesis is supported by the findings that: (a) mice reconstituted with day 14 fetal liver produce a highly heterogeneous response when given the polyclonal B-cell activator dextran sulfate at the time of immunization and; (b) mice reconstituted with B cells from day 14 fetal liver produce a highly heterogeneous, high affinity secondary response (15). The latter observations suggest that not only does the day 14 fetal B-cell population possess the full complement of genetic information necessary to synthesize the total array of anti-DNP antibodies but in addition probably expresses this information in the form of antigen receptors on the B-cell surface. Thus, one is able to selectively expand a population of high affinity anti-DNP memory B cells during the primary response despite the fact that high affinity antibody secreting cells are not detectable. Finally it was found that B cells from day 15 fetal donors or from neonatal donors would mature to be capable of generating a heterogeneous antibody response within 3 days in the lethally irradiated adult recipient (17). This suggested that the differentiation event leading to the acquisition of the capacity of the B-cell
population to produce a heterogeneous antibody response was "induced" by some "factor" in the internal environment of the adult animal.

In the present study it has been shown that, in the adult recipient, immature B cells acquire the capacity to give a heterogeneous response within 6 days of transfer only if adult thymus cells are given to the irradiated mice together with the immature B cells. It is important to note that all animals receive adult thymus cells 1 day before immunization. Thus, helper T cells are provided in adequate numbers (4, 28). The facilitation of B-cell maturation by thymus cells is clearly a function of thymic cells which is distinct from helper T-cell activity.

Maturation of the capacity of B cells to produce a response consisting of predominantly indirect PFC appears to be under different controls from the maturation to give a heterogeneous response. The two differentiation events have been shown to occur at different times during ontogeny (4). In addition, in the present studies, it was found that the capacity to produce indirect PFC matures within 2 wk in the cell transfer recipient even in the absence of thymus cells. That is, in the absence of thymus cells, the B-cell population matures in the sense of acquiring the capacity to produce indirect plaques but remains immature in the sense that it produces a response of restricted heterogeneity. It should be noted that in the presence of thymus cells, maturation of the B-cell population to give a predominantly indirect PFC response occurs rapidly (within 3 days). Furthermore, the B-cell population from day 15 fetal donors would have normally developed this capacity in approximately 2 days. Thus in the absence of thymus cells the maturation of B cells to produce indirect PFC is clearly delayed. Previous work has demonstrated that polyclonal B-cell mitogens can induce the production of a heterogeneous response by B cells from day 14 or day 16 fetal donors without significantly increasing the ratio of indirect to direct PFC (15). This also suggests that the two maturational events are under independent control.

The data presented here, using two different antigens, support the view that thymus cells facilitate, or are required for, the normal maturation of the B-cell population to be capable of producing a heterogeneous response. The apparent normal development of B-cell function in thymus-deprived animals suggests that the requirement for thymus cells is not absolute. It should be emphasized that the cell type in the thymus which is responsible for facilitation of B-cell maturation has not been identified.

Summary

Lethally irradiated mice reconstituted with adult thymus cells and neonatal or fetal liver cells produce an anti-2,4-dinitrophenyl or anti-bovine gamma globulin response of restricted heterogeneity of affinity in comparison with the response of mice reconstituted with B cells from adult donors. In addition, mice reconstituted with day 15 fetal B cells and adult thymus cells produce relatively few indirect plaque-forming cells (PFC). It was found that B cells acquire the capacity to produce a heterogeneous response, of predominantly indirect PFC, within 7 days of transfer only when thymus cells are transferred along with the B cells. B cells from fetal or neonatal donors transferred without young adult thymus cells develop the capacity to generate indirect PFC within 13
days after transfer to adult recipients, but continue to produce a response of restricted heterogeneity of affinity for up to 28 days after transfer. Thus, it has been shown that cells present in the thymus facilitate, or are necessary for the functional maturation of B lymphocytes. Furthermore, the data suggest that maturation of the B-cell population to produce a heterogeneous response is controlled independently of its maturation to be capable of producing indirect PFC.

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