

MATURATION OF B LYMPHOCYTES IN THE RAT*

I. MIGRATION PATTERN, TISSUE DISTRIBUTION, AND TURNOVER RATE OF UNPRIMED AND PRIMED B LYMPHOCYTES INVOLVED IN THE ADOPTIVE ANTIDINITROPHENYL RESPONSE

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(Received for publication 30 July 1973)

Studies of the adoptive antibody response of rats to horse spleen ferritin and diphtheria toxoid (DT)¹ indicate that the circulatory characteristics and life-span of thoracic duct B lymphocytes that initiate the primary response ("virgin" cells) and secondary response ("memory" cells) differ. Virgin B cells do not recirculate from the blood to the lymph and are short lived (1). Memory B cells continually recirculate and are relatively long lived (1). Similar studies of memory B cells in the spleen show that hapten (DNP)-primed cells recirculate and turn over slowly (2). Indeed, the recirculating cells can account for all the immunological memory transferred by spleen cells in the adoptive secondary anti-DNP response.

The object of the present investigation was to obtain further evidence for physiological changes in virgin and memory B lymphocytes involved in a well-defined antihapten response. Accordingly, the migratory pattern, tissue distribution, and turnover rate of thoracic duct, spleen, and bone marrow cells involved in the adoptive primary and secondary anti-DNP response to DNP-DT were studied. The results of the present study confirm our previous report that B cells involved in the adoptive primary response are relatively fixed, but B cells involved in the adoptive secondary response continually recirculate. The relative inability of bone marrow cells to carry immunological memory is related to the inability of recirculating cells to penetrate the marrow. However, studies of the rate of formation of B cells involved in the primary and secondary response to DNP show that both populations of cells turn over

* Supported by U.S. Public Health Service Grant AI-10293.

† Career Development Awardee AI-70018, U.S. Public Health Service.

¹ *Abbreviations used in this paper:* B, bursa equivalent; DNP, dinitrophenyl(ated); DNP-BSA, dinitrophenyl-bovine serum albumin; DNP-DT, dinitrophenyl-diphtheria toxoid; DT, diphtheria toxoid; Ig, immunoglobulin; M-199, tissue culture medium 199; M-199-FCS, tissue culture medium 199 with 5% fetal calf serum; ME, mercaptoethanol; NRS, normal rabbit serum; PBS, phosphate-buffered saline; RARBS, rabbit antirat B-cell serum; VBS-FCS, Veronal-buffered saline with 5% fetal calf serum.

slowly. These results differ from our previous findings, since unprimed B cells involved in the adoptive primary response to ferritin turn over rapidly. Differences in the turnover rates of unprimed cells using different antigens are discussed in the context of antigen-dependent B-cell maturation.

Materials and Methods

Animals.—Inbred Lewis rats were used in all experiments. Animals were purchased from Microbiological Associates, Inc., Bethesda, Md.

Preparation of Cell Suspensions.—The thoracic duct of adult male rats was cannulated by a modification of the technique of Bollman et al. (3). Rats were maintained in restraining cages and received a continuous intravenous infusion of Ringer's solution containing streptomycin (0.1 mg/ml) and heparin (1 U/ml) at 2 ml/h. Thoracic duct cells were collected at 4°C for 24 h in 5 ml of Ringer's solution with 100 U of heparin and 1 mg of streptomycin. Cells were harvested by centrifugation at 150 g for 10 min and resuspended in tissue culture medium 199 (M-199) (Grand Island Biological Co., Grand Island, N.Y.) before injection. Spleen and bone marrow cell suspensions were made in medium 199 by the method of Billingham (4).

X-Irradiation.—Rats received 500 R whole body X-irradiation from a single 250 kV (15 A) source. The source axis distance was 52 cm, and the dose rate was 105 R/min (0.25 mm of Cu plus 0.55 mm of Al filtration).

Immunization Procedures.—Rats were immunized to alum-adsorbed diphtheria toxoid (DT) (Parke, Davis and Co., Detroit, Mich.) by a single subcutaneous (7.5 Lf, 0.25 ml) and intraperitoneal (7.5 Lf, 0.25 ml) injection of 15 Lf toxoid. Fluid DT (Commonwealth of Massachusetts, Department of Health) was dinitrophenylated (DNP) by the procedure of Eisen et al. (5). The composition of the conjugate was DNP₁₁-DT. Immunization to DNP-DT was achieved by injecting 0.1 ml of an emulsion of equal volumes of DNP-DT in phosphate-buffered saline (PBS) and complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.) in each hind footpad to give a total dose of 0.4 mg of protein per animal.

Immunization to DNP-bovine serum albumin (DNP₃₅-BSA) was accomplished by injecting 0.1 ml of an emulsion of equal volumes of DNP-BSA in phosphate-buffered saline and complete Freund's adjuvant in each hind footpad to give a total dose of 0.4 mg of protein per rat. In studies of the adoptive secondary response, hosts were challenged with soluble DNP-DT by injecting 0.5 mg of protein intraperitoneally in 1 ml of phosphate-buffered saline.

Antibody Titrations.—Antibodies to DNP were measured by a previously described modification of the Farr assay (6). Antibody responses are expressed as the log₁₀ of that titer of antiserum that bound 33% of [³H]DNP-ethylaminocaproic acid (10⁻⁸ M). Mercaptoethanol (ME)-resistant antibody was measured by incubating equal volumes of a 1:2.5 dilution of whole antiserum and 2-ME (0.1 M in saline) for 30 min at room temperature before titration. All antibodies from day 14 were 2-ME resistant.

Rabbit Antirat B-Cell Serum (RARBS).—RARBS was kindly supplied by Dr. J. C. Howard, Cellular Immunology Research Unit, Sir William Dunn School of Pathology, Oxford University, Oxford, England, and Dr. D. W. Scott, Department of Immunology and Microbiology, Duke University, Durham, N. C. Rabbits were given intravenous injections of thoracic duct cells from August rats that were adult thymectomized, lethally irradiated, and bone marrow reconstituted (7). The antiserum was subsequently absorbed with thymocytes from Lewis rats. Details of immunizations and absorptions have been described elsewhere (2). The absorbed antiserum specifically kills B but not T cells in the thoracic duct lymph (2).

Cytotoxic Assay.—50 μl of RARBS and 25 μl of a suspension of thoracic duct, spleen, or bone marrow cells (5 × 10⁶ cells/ml) diluted in Veronal-buffered saline with 5% fetal calf

serum (VBS-FCS) was placed in 10×75 mm glass tubes, and the reaction mixture was allowed to incubate for 15 min at room temperature. 25 μ l of rabbit complement absorbed 1:1 (vol/vol) with rat thymocytes and diluted 1:4 in VBS-FCS was then added. Cells were harvested by centrifugation at 150 g 45 min later and resuspended in 50 μ l of M-199. 50 μ l of 0.2% trypan blue in M-199 was added just before counting the cells in a standard hemacytometer. Approximately 200 cells were counted from control tubes (containing normal rabbit serum (NRS) absorbed 1:1 (vol/vol) with rat thymocytes) for each point of the titration. When thymocytes were used as target cells, the diluent was 0.15 M fructose with 5% FCS. Fructose minimizes nonspecific killing of rat thymocytes by NRS, but does not alter specific killing by RARBS.

Results of the cytotoxic assay are expressed as the cytotoxic index:

$$\text{Cytotoxic index} = \frac{\% \text{ cells killed by antiserum} - \% \text{ cells killed by NRS}}{100 - \% \text{ cells killed by NRS}}$$

In Vitro Incubation of Thoracic Duct, Spleen, or Marrow Cells with RARBS.—Thoracic duct, spleen, or bone marrow cells were suspended in M-199-FCS at a concentration of 2×10^7 cells/ml. 20 μ l of RARBS was added to each milliliter of cell suspension. 15 min later absorbed rabbit complement diluted 1:4 in M-199-FCS was added to make a final dilution of 1:10 complement. The reaction mixture was allowed to incubate an additional 45 min at room temperature. Cells were harvested by centrifugation at 150 g for 10 min and resuspended at the desired cell concentration in M-199 before intravenous injection.

Preparation of Ficoll-Hypaque-Purified Bone Marrow Cells.—Bone marrow lymphocytes were separated from a suspension of bone marrow cells in M-199 by means of a Ficoll-Hypaque gradient (8). 15 parts of a 25% solution of Hypaque M 75% (Winthrop Laboratories, New York) were added to 24 parts of a 9% Ficoll solution (Pharmacia Fine Chemicals, Uppsala, Sweden) and stirred for 1 h at room temperature. 10 ml of the mixture was placed in a 40 ml conical centrifuge tube and carefully overlaid with 20 ml of bone marrow cells in M-199 ($10\text{--}15 \times 10^6$ cells/ml). Cells were spun at 20°C for 35 min at 400 g and then aspirated from the Ficoll-Hypaque middle layer. After two washes in M-199, the Ficoll-purified cells were used in cell transfer experiments or in cytotoxic assays for B lymphocytes. A mean of 66% small lymphocytes were present in these cell suspensions as compared with a mean of 7% in unfractionated bone marrow cells. The yield of purified cells was approximately 10% of bone marrow cells applied to the gradient.

Intravenous Injection of Cells and Bleeding Procedures.—Injection and bleeding techniques have been described elsewhere (1).

RESULTS

Adoptive Primary Anti-DNP Response Restored by DT-Primed Spleen Cells.—Lewis rats were given 500 R whole body X-irradiation. Graded numbers of DT-primed spleen cells were injected intravenously 2 h later. The adoptive hosts were challenged with DNP-DT in complete Freund's adjuvant 24 h after irradiation. Spleen cells were obtained from rats immunized to DT 4–8 wk before. Fig. 1 a shows the adoptive primary anti-DNP response restored by 2, 10, 50, and 100×10^6 spleen cells. Significant antibody titers were detected by days 12 or 14 for all cell doses above 2×10^6 .

Adoptive Primary Anti-DNP Response Restored by DT-Primed Spleen Cells Passaged through an Intermediate Host.—In order to determine whether B lymphocytes that restore the adoptive primary anti-DNP response are able

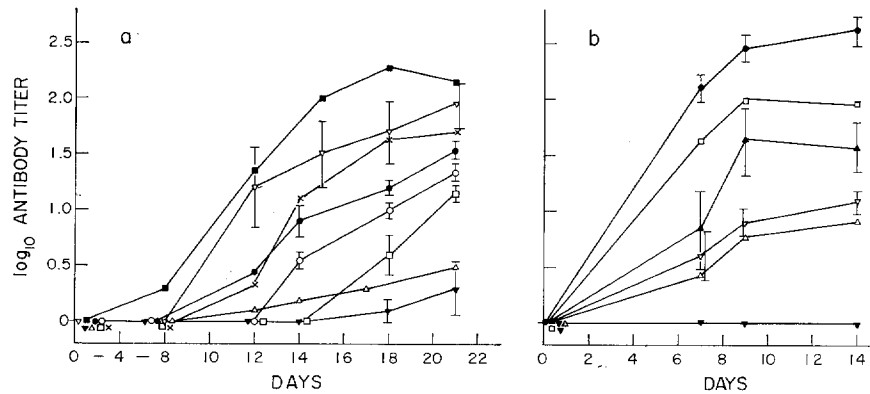


FIG. 1. Adoptive anti-DNP response of rats to DNP-DT. Spleen cells were obtained from donors immunized to DT or DNP-BSA 4-8 wk before. Cells were injected intravenously into X-irradiated hosts and DNP-DT was given 22 h later. Each point represents the mean response of four to nine rats with brackets showing the standard error of the mean. Responses are expressed as \log_{10} titer that bound 33% labeled antigen. (a) Adoptive primary response to 0.4 mg of DNP-DT in complete Freund's adjuvant. $\nabla-\nabla$, mean response of rats given 100×10^6 DT-primed spleen cells; $\bullet-\bullet$, mean response of rats given 50×10^6 cells; $\circ-\circ$, mean response of rats given 10×10^6 cells; $\blacktriangledown-\blacktriangledown$, mean response of rats given 2×10^6 cells; $\triangle-\triangle$, mean response with 30×10^6 passaged DT-primed spleen cells; $\blacksquare-\blacksquare$, mean response with 100×10^6 DT-primed spleen cells treated with [^3H]thymidine; $\times-\times$, mean response with 50×10^6 DT-primed spleen cells treated in vitro with NRS; $\square-\square$, mean response with 50×10^6 DT-primed spleen cells treated in vitro with RARBS. (b) Adoptive secondary response to 0.5 mg of DNP-DT in PBS. All rats were given 50×10^6 DT-primed spleen cells and graded numbers of DNP-primed cells. $\bullet-\bullet$, mean response of rats given 50×10^6 DNP-primed spleen cells; $\nabla-\nabla$, mean response of rats given 25×10^6 spleen cells; $\triangle-\triangle$, mean response of rats given 10×10^6 spleen cells; $\square-\square$, mean response with 50×10^6 DT-primed spleen cells treated in vitro with [^3H]thymidine; $\blacktriangle-\blacktriangle$, mean response with 10×10^6 passaged DNP-primed spleen cells; $\blacktriangledown-\blacktriangledown$, mean response with 50×10^6 DNP-primed spleen cells without antigen.

to recirculate from the blood to the lymph, DT-primed spleen cells were passaged through an intermediate host before cell transfer. Approximately 400×10^6 spleen cells were injected intravenously into intermediate hosts that had received 500 R whole body X-irradiation 2 h before. The thoracic duct of these animals was cannulated 24 h later and lymph was collected for 48 h. The passaged spleen cells were harvested from the lymph and injected intravenously into final hosts that had been irradiated 2 h previously. The reconstituted final hosts were challenged with DNP-DT 22 h later. Cells present in the lymph of the intermediate host are almost exclusively of donor origin (1, 2).

Fig. 1 *a* shows that 30×10^6 passaged DT-primed spleen cells restore an anti-DNP response that is similar in time-course and magnitude to that restored by 2×10^6 unpassaged cells. The decreased restorative action of passaged cells is related to the inability of splenic B cells to migrate from the

blood to the lymph, since the helper activity of passaged carrier-primed cells (T cells) is at least as great as that of unpassaged cells (2).

Adoptive Primary Anti-DNP Response Restored by DT-Primed Spleen Cells Treated with [³H]Thymidine.—The rate of formation (turnover rate) of DT-primed spleen cells that initiate the primary anti-DNP response was studied by treating rats immunized to DT with [³H]thymidine before cell transfer. DT-primed donors received intraperitoneal injections of [³H]thymidine (New England Nuclear, Boston, Mass.; specific activity 20 Ci/mmol) in aqueous solution every 8 h for a period of 48 h before removal of the spleen. Each injection contained 3.5 mCi in 3.5 ml of sterile water (24.5 mCi total dose). The interval between immunization and administration of [³H]thymidine was 4–6 wk. This dose of radioactive material produces a 20–100-fold decrease in the ability of rat thoracic cells to restore the adoptive primary response to ferritin (1), and a 10-fold decrease in colony-forming units in mouse bone marrow cells (9). The functional deficits are related to radiation damage after the uptake of radioactive material by rapidly dividing cells.

In order to minimize reutilization of [³H]thymidine in the adoptive hosts, 1 ml of a 10^{-4} M solution of cold thymidine was injected intraperitoneally into adoptive hosts after cell transfer, and thymidine (10^{-4} M) was added to the drinking water. Fig. 1 *a* shows that 100×10^6 [³H]thymidine-treated spleen cells restore an adoptive anti-DNP response that is at least as great as that of an equal number of untreated cells.

Adoptive Secondary Anti-DNP Response Restored by a Combination of Carrier (DT)- and Hapten (DNP)-Primed Spleen Cells.—In several experiments a constant number (50×10^6) of DT-primed spleen cells and a graded number of DNP-primed spleen cells were injected intravenously into irradiated hosts. The reconstituted hosts were challenged intraperitoneally with soluble DNP-DT 24 h after irradiation. Hapten (DNP)-primed cells were obtained from donors immunized to DNP-BSA 4–8 wk before.

Fig. 1 *b* shows the adoptive secondary response restored by 10, 25, and 50×10^6 DNP-primed spleen cells. Antibody titers were substantial by day 7 and a plateau was achieved by day 9. Although the response restored by 10 and 25×10^6 cells was similar, a more vigorous response was noted with 50×10^6 cells. A comparison of the adoptive primary and secondary responses shows that the adoptive secondary response was more rapid and required fewer cells to achieve similar antibody titers. Antibodies in both responses are resistant to treatment with mercaptoethanol.

Adoptive Secondary Anti-DNP Response Restored by Hapten-Primed Cells Passaged through an Intermediate Host.—The circulatory dynamics of hapten-primed spleen cells was studied by injecting 300×10^6 cells intravenously into an intermediate host and collecting donor cells in the thoracic duct lymph as before. The restorative action of the passaged cells was subsequently examined in irradiated hosts that received 50×10^6 unpassaged DT-primed spleen

cells and an intraperitoneal injection of DNP-DT. Fig. 1 *b* shows that 10×10^6 passaged cells restored a response that was greater than that restored by 25×10^6 unpassaged cells. A comparison of the adoptive primary and secondary responses shows that passaged cells were less efficient (~ 10 -fold) than unpassaged cells in the primary response and were more efficient (~ 3 -fold) than unpassaged cells in the secondary response.

Adoptive Secondary Anti-DNP Response Restored by Hapten-Primed Spleen Cells Treated with [^3H]Thymidine.— $[^3\text{H}]$ thymidine was administered to DNP-primed donors 4–6 wk after immunization. Spleen cells were obtained after the injection of 24.5 mCi as described before. Fig. 1 *b* shows that the anti-DNP response restored by 50×10^6 $[^3\text{H}]$ thymidine-treated cells was slightly below that restored by 50×10^6 untreated cells, but was considerably greater than that restored by 25×10^6 untreated cells.

Adoptive Primary Anti-DNP Response Restored by Passaged or [^3H]Thymidine-Treated Thoracic Duct Cells.—Fig. 2 *a* shows the adoptive primary anti-DNP

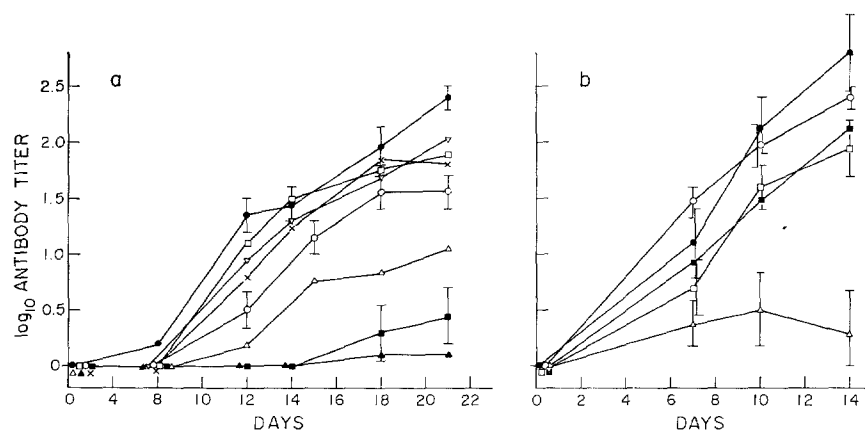


FIG. 2. Adoptive anti-DNP response of rats to DNP-DT. Thoracic duct cells were obtained from donors immunized to DT or DNP-BSA 4–8 wk before. Cells were injected intravenously into X-irradiated hosts and DNP-DT was given 22 h later. (a) Adoptive primary response to 0.4 mg of DNP-DT in complete Freund's adjuvant. ●—●, mean response of rats given 100×10^6 DT-primed thoracic duct cells; □—□, mean response of rats given 50×10^6 cells; ○—○, mean response of rats given 10×10^6 cells; ■—■, mean response of rats given 2×10^6 cells; ▽—▽, mean response of rats given 50×10^6 DT-primed thoracic duct cells treated with $[^3\text{H}]$ thymidine; △—△, mean response of rats given 50×10^6 passaged DT-primed thoracic duct cells; ×—×, mean response with 50×10^6 DT-primed thoracic duct cells treated in vitro with NRS; ▲—▲, mean response with 50×10^6 DT-primed thoracic duct cells treated in vitro with RARBS. (b) Adoptive secondary response to 0.5 mg of DNP-DT in PBS. All rats were given 50×10^6 DT-primed thoracic duct cells and graded numbers of DNP-primed cells. ●—●, mean response of rats given 25×10^6 DNP-primed thoracic duct cells; ○—○, mean response with 10×10^6 DNP-primed cells; △—△, mean response with 2×10^6 DNP-primed cells; ■—■, mean response with 10×10^6 DNP-primed cells treated with $[^3\text{H}]$ thymidine; □—□, mean response with 10×10^6 passaged DNP-primed cells.

response restored by 2, 10, 50, or 100×10^6 DT-primed thoracic duct cells in irradiated hosts challenged with DNP-DT in complete Freund's adjuvant. The adoptive response restored by 50×10^6 thoracic duct cells passaged through an irradiated intermediate host fell below that restored by 10×10^6 unpassaged cells (Fig. 2 *a*). Nevertheless, antibody titers were substantial by day 21 and well above those restored by 2×10^6 unpassaged cells. Treatment of DT-primed donors with [^3H]thymidine produced little effect on the anti-DNP response restored by 50×10^6 thoracic duct cells, since equal numbers of treated and untreated cells produced similar responses (Fig. 2 *a*).

Adoptive Secondary Anti-DNP Response Restored by Hapten-Primed Thoracic Duct Cells Treated with [^3H]Thymidine or Passaged through an Intermediate Host.—Fig. 2 *b* shows the adoptive secondary anti-DNP response restored by a constant number (50×10^6) of DT-primed thoracic duct cells and graded numbers of DNP-primed thoracic duct cells. Similar responses were produced by 10 and 25×10^6 DNP-primed cells, but a sharp fall-off was observed with 2×10^6 cells. Both passaged and [^3H]thymidine-treated cells (10×10^6) were slightly less efficient in restoring the anti-DNP response than unpassaged or untreated cells (Fig. 2 *b*).

Adoptive Primary Anti-DNP Response Restored by Passaged Bone Marrow Cells.—Fig. 3 *a* shows the adoptive primary anti-DNP response restored by unprimed bone marrow cells alone or in combination with 10×10^6 DT-primed spleen cells. The response restored by the combination of cells is greater than the sum of the responses restored by each cell inoculum independently.

In order to examine the migratory pattern of marrow cells involved in the adoptive anti-DNP response, 450×10^6 marrow cells were injected into each of four irradiated intermediate hosts, and cells were collected in the thoracic duct lymph as described before. A mean of 75×10^3 cells was collected in the intermediate host lymph during 48 h of thoracic duct drainage. This represents a yield of about 0.02% of the cells injected. Almost all of the cells were of donor origin, since fewer than 5×10^3 cells were obtained from uninjected irradiated rats. The mean yield of passaged thoracic duct cells in four similar experiments was 22%. The adoptive anti-DNP response restored by a combination of 50×10^3 passaged marrow cells and 10×10^6 DT-primed spleen cells was similar to that of 10×10^6 DT-primed spleen cells alone (Fig. 3 *a*).

Adoptive Secondary Anti-DNP Response Restored by Hapten-Primed Bone Marrow Cells.—Fig. 3 *b* shows the adoptive secondary anti-DNP response restored by a constant number (50×10^6) of DT-primed spleen cells and graded numbers of bone marrow, spleen, or thoracic duct cells from donors immunized to DNP-BSA 4–6 wk earlier. Although 10×10^6 hapten-primed thoracic duct cells produced a vigorous response by day 7, no detectable antibody was restored by 10×10^6 hapten-primed bone marrow cells by day 14. However, 100×10^6 hapten-primed marrow cells did restore a slowly rising response that was similar in time-course and amplitude to that of the adoptive

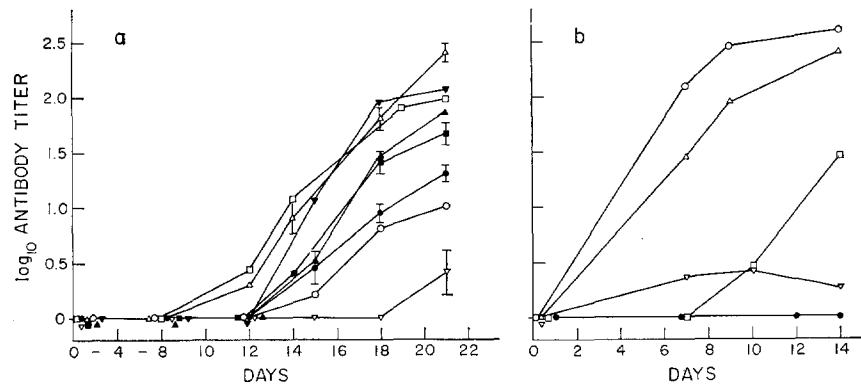


FIG. 3. Adoptive anti-DNP response of rats to DNP-DT. Bone marrow cells were obtained from unimmunized donors or from donors immunized to DNP-BSA 4-8 wk before. All spleen cells were obtained from DT-primed donors. Cells were injected intravenously into X-irradiated hosts and DNP-DT was given 22 h later. (a) Adoptive primary anti-DNP response to 0.4 mg of DNP-DT in complete Freund's adjuvant. Δ - Δ , mean response of rats given 10×10^6 DT-primed spleen cells and 100×10^6 unprimed bone marrow cells; \square - \square , mean response with 10×10^6 spleen cells and 50×10^6 bone marrow cells; \blacksquare - \blacksquare , mean response with 10×10^6 spleen cells and 10×10^6 bone marrow cells; \bullet - \bullet , mean response with 10×10^6 spleen cell alone; ∇ - ∇ , mean response with 100×10^6 bone marrow cells alone; \circ - \circ , mean response with 10×10^6 spleen cells and 50×10^6 passaged bone marrow cells; \blacktriangle - \blacktriangle , mean response with 10×10^6 spleen cells and 10×10^6 Ficoll-purified marrow cells treated in vitro with NRS; \blacktriangledown - \blacktriangledown , mean response with 10×10^6 spleen cells and 10×10^6 Ficoll-purified marrow cells treated in vitro with RARBS. (b) Adoptive secondary anti-DNP response to 0.5 mg of DNP-DT in PBS. All rats were given 50×10^6 DT-primed spleen cells and graded numbers of DNP-primed thoracic duct, spleen, or bone marrow cells. \circ - \circ , mean response of rats given 50×10^6 DNP-primed spleen cells; Δ - Δ , mean response of rats given 10×10^6 DNP-primed thoracic duct cells; ∇ - ∇ , mean response of rats given 2×10^6 DNP-primed thoracic duct cells; \bullet - \bullet , mean response of rats given 10×10^6 DNP-primed bone marrow cells; \square - \square , mean response of rats given 100×10^6 DNP-primed bone marrow cells.

primary response (Figs. 3 a and b). The response of 50×10^6 hapten-primed spleen cells is shown for comparison.

Cytotoxic Action of Rabbit Antirat B Cell Serum (RARBS) on Thoracic Duct, Spleen, Bone Marrow, and Thymus Cells.—The effect of RARBS on several rat lymphoid tissues was studied in an in vitro cytotoxicity assay. Fig. 4 shows representative titration curves with thoracic duct, spleen, unfractionated bone marrow, Ficoll-purified bone marrow, and thymus cells. The highest titers (titer at 50% kill) were observed with spleen target cells, and no killing of thymocytes was observed. An initial plateau up to a 1:40 dilution was noted with all target cells except for thymus cells. The cytotoxic index indicates the percent of lymphocytes killed in the case of thoracic duct cells, since almost all cells found in rat thoracic duct lymph are lymphocytes (10). However, the

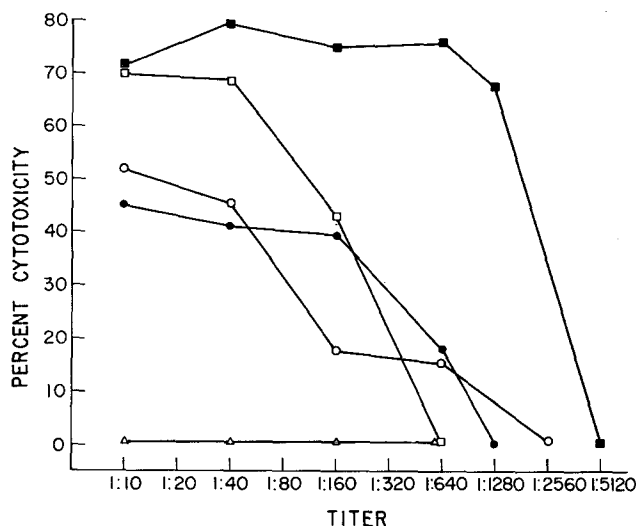


FIG. 4. Cytotoxic action of rabbit antirat B cell serum (RARBS) on rat lymphoid tissues.—●—●, thoracic duct cells; ■—■, spleen cells; □—□, unfractionated bone marrow cells; ○—○, Ficoll-purified bone marrow cells; △—△, thymus cells.

cytotoxic index in the case of spleen and bone marrow cells may include killing of cells other than lymphocytes.

Effect of RARBS on the Adoptive Primary Anti-DNP Response Restored by Spleen, Thoracic Duct, and Bone Marrow Cells.—The adoptive anti-DNP response restored by DT-primed thoracic duct cells treated in vitro with RARBS was studied. Fig. 2 *a* shows that the response restored by 50×10^6 thoracic duct cells incubated with RARBS was markedly reduced and was below that restored by 2×10^6 untreated cells at day 21. Treatment with RARBS also abolished the adoptive response restored by 50×10^6 DT-primed spleen cells on days 12 and 14, as compared with an equal number of untreated cells (Fig. 1 *a*). However, antibody titers were detectable by day 18 and were similar to those produced by 10×10^6 untreated cells by day 21. On the other hand, treatment with RARBS did not reduce the ability of bone marrow lymphocytes purified on a Ficoll-Hypaque gradient to restore the adoptive anti-DNP response (Fig. 3 *a*). It is of interest that the purified cells were no more efficient on a cell-per-cell basis in restoring the adoptive antibody response than unfractionated bone marrow cells, despite the 10-fold increase in the proportion of small lymphocytes in the former cell inocula.

DISCUSSION

Our previous studies of the adoptive antibody response of rats to horse spleen ferritin suggest that "virgin" B lymphocytes are nonrecirculating cells

that turn over rapidly (1). On the other hand, "memory" B cells continuously recirculate from the blood to the lymph and turn over slowly (1, 2). The object of the present investigation was to examine the migration pattern, rate of formation, and tissue distribution of B cells in a well-defined experimental system using a hapten-protein conjugate (DNP-DT) instead of ferritin as the antigen.

In order to determine the circulatory characteristics of unprimed splenic B cells involved in the adoptive primary anti-DNP response, DT-primed spleen cells were passaged (intravenous injection and subsequent collection in the thoracic duct lymph) through an irradiated intermediate host before transfer to the final host. The latter host was challenged with DNP-DT in complete Freund's adjuvant, since the primary antihapten response to soluble DNP-DT is barely detectable (Strober, S., unpublished observations). The restorative action of passaged and unpassaged cells was compared. Any reduction in the response restored by the former cells as compared with the latter would be related to the inability of unprimed B cells to migrate from the blood to the lymph, since the helper activity of passaged carrier-primed cells is at least as great as that of unpassaged cells (2). The experimental findings show that passaged cells were approximately 15-fold less efficient in restoring the adoptive primary anti-DNP response as compared with unpassaged cells. This indicates that the large majority of unprimed B cells in the spleen do not recirculate from the blood to the lymph.

On the other hand, studies of the adoptive secondary anti-DNP response to soluble DNP-DT show that hapten-primed spleen cells obtained from donors immunized to DNP-BSA continuously recirculate, since the restorative action of passaged hapten-primed cells was about three fold greater than that of unpassaged cells. The unprimed B cells in the spleen appear to change from non-recirculating to recirculating cells after exposure to antigen. It is unlikely that this change is due to the use of adjuvant in the primary response, since the same change has been noted when adjuvant was used to elicit both the adoptive primary and secondary responses (1).

The rate of formation of unprimed and DNP-primed spleen cells was investigated by administering a large dose (~ 25 mCi) of high specific activity [^3H]thymidine to the spleen cell donors for 48 h before cell transfer. This dose of radioactive material has been shown to reduce the number of colony-forming units in mouse bone marrow about 10-fold (9) and to reduce the adoptive primary antiferritin response restored by thoracic duct cells 20–100-fold (1). The extent of "thymidine suicide" is related to the turnover rate of the target cells. The experimental results show that the anti-DNP response restored by both unprimed and DNP-primed spleen cells was unaffected by treatment with [^3H]thymidine. This indicates that both populations of cells are turning over relatively slowly.

Similar studies of the circulatory characteristics and rate of formation of unprimed and primed thoracic duct cells involved in the adoptive anti-DNP

response were carried out. The results again show that the majority of unprimed B lymphocytes are unable to recirculate, since passaged DT-primed thoracic duct cells were about fivefold less efficient than unpassaged cells in restoring the adoptive primary anti-DNP response. However, it is of interest that a significant fraction (~20%) of unprimed thoracic duct cells are able to migrate from the blood to the lymph. Experiments with DNP-primed thoracic duct cells indicate that the large majority of hapten-primed cells recirculate. As in the case of spleen cells, both unprimed and DNP-primed thoracic duct cells turn over slowly, since the restorative action of both types of cells was not affected by treatment with [³H]thymidine.

Although the results of the migration studies of unprimed and DNP-primed spleen and thoracic duct cells confirm our previous findings using horse spleen ferritin as antigen, important differences were noted in studies of the rate of formation of unprimed and primed cells. Although treatment with [³H]thymidine produced a 20–100-fold decrease in the restorative action of unprimed thoracic duct cells when ferritin was used, no effect on the restorative action of unprimed cells was noted when DNP-DT was used. Differences in the turnover rate of unprimed cells to different antigens have been noted previously in studies using vinblastine instead of [³H]thymidine as the mitotic inhibitor (11).

Our initial interpretation of these results was that different populations of cells are involved in the adoptive antibody response to different antigens. However, in view of recent data (1, 2), it is more likely that differences in the turnover rate reflect differences in the extent of environmental priming of experimental animals to the antigen under investigation. For example, rats used in experiments with ferritin may have little or no previous exposure to antigens that cross-react with ferritin; so that unprimed ("true virgin" cells) and primed cells differ in both migratory behavior and turnover rate. Rats used in the present study may have some previous exposure to antigens that cross-react with DNP. This could account for the similar rate of formation of unprimed and intentionally primed B cells and for the significant fraction of unprimed thoracic duct cells that recirculate. Studies with sheep red blood cells suggest that Lewis rats have a considerable exposure to cross-reacting antigens, since both the circulatory characteristics and turnover rate of unprimed and primed cells are similar (Strober, S., unpublished observations). Experiments with germfree animals may provide definitive evidence for this hypothesis.

Studies of the adoptive primary anti-DNP response restored by a combination of unprimed bone marrow cells and DT-primed spleen cells show that both populations cooperate in initiating the primary response. In order to study the migration pattern of bone marrow cells involved in the response, we attempted to test the restorative action of passaged and unpassaged marrow cells. However, a mean of 75×10^3 cells (mainly small lymphocytes) was collected in the lymph of the intermediate hosts after injecting $400\text{--}500 \times 10^6$

cells intravenously (yield $\sim 0.02\%$). The yield of injected marrow small lymphocytes is also exceedingly low (0.1–0.2%) as compared with that of injected thoracic duct cells (mean of 22%). This indicates that only a minute fraction of bone marrow cells of Lewis rats are able to recirculate. Strain differences appear to play an important role in determining this fraction (12). Examination of the anti-DNP response restored by small numbers of passaged cells (50×10^6) showed no detectable activity.

A comparison of the restorative action of hapten-primed thoracic duct or bone marrow cells in the adoptive secondary anti-DNP response shows that 10×10^6 thoracic duct cells restore a vigorous response ($\sim \log_{10} 2$) at day 7, but no detectable response is restored by 100×10^6 marrow cells at that time. In addition, the time-course and magnitude of the anti-DNP response restored by equal numbers of primed and unprimed marrow cells is similar. The priming effect observed with thoracic duct or spleen cells (a more rapid response requiring fewer cells) was not demonstrated with marrow cells. This is consistent with previous findings that memory B cells recirculate, and recirculating cells do not easily penetrate the bone marrow.

The effect of rabbit antirat B cells serum (RARBS) on the ability of rat lymphoid cells to restore the adoptive primary anti-DNP response was studied. RARBS has been previously shown to kill B (hapten-primed), but not T (carrier-primed), lymphocytes (2). However, the specificity of the antiserum for cells other than lymphocytes has not been evaluated. The present findings show that RARBS abolished the restorative activity of DT-primed thoracic duct lymphocytes for a period of 21 days. It is likely that the effect was mediated by the killing of mature B lymphocytes. This indicates that residual B cells of the sublethally irradiated hosts do not play an important role in the adoptive primary response, since the injection of large numbers of helper cells does not restore a detectable anti-DNP response. RARBS only partially diminished the restorative activity of DT-primed spleen cells and had no effect on the activity of bone marrow cells purified on a Ficoll-Hypaque gradient. This suggests that the bone marrow, and the spleen to some extent, have the ability to generate mature B cells from cells that are not killed by RARBS (i.e., B-cell precursors). Thoracic duct cells appear to lack this regenerative capacity. Indeed, the slow rising response restored by bone marrow as compared with spleen or thoracic duct cells suggests that few mature B cells are involved in the adoptive primary response restored by the marrow.

Table I summarizes the tissue distribution and biological characteristics of different types of B cells or B-cell precursors identified in the present work. The actual derivation of one cell from the other along specific pathways of differentiation is likely but not proven. Since B-cell maturation may proceed by continuous rather than quantum changes, the different categories of B cells may refer to cells at given points in time rather than to stable populations of cells.

TABLE I
Stages of B-Cell Maturation

Cell type	Bone marrow	Spleen	Thoracic duct	Re-circulates	Rapid turnover	
Antigen-independent maturation	B-cell precursor	++	+	0	0	N.T.*
	Virgin B cell (B ₁)	±	+	+	0	+
Antigen-dependent maturation	Intermediate B cell	±	+	+	0	0
	Memory B cell (B ₂)	0	+	++	+	0

* Not tested.

Although antigen-dependent maturation has been shown in studies with ferritin and DNP, the extent of antigen-independent maturation remains to be elucidated. Presumably antigen-dependent maturation requires the presence of recognition sites on the surface of maturing cells and, therefore, would proceed after recognition sites are developed. It is unclear when maturing B cells acquire these sites, since other lymphocytes (T cells) without easily detectable surface Ig are able to bind antigen specifically (i.e., bear surface receptors) (13).

SUMMARY

The migration pattern, tissue distribution, and turnover rate of unprimed and primed B lymphocytes involved in the adoptive anti-DNP response was studied. The adoptive primary response restored by unprimed spleen or thoracic duct cells passaged through an intermediate host (intravenous injection and subsequent collection in the thoracic duct lymph) was markedly diminished as compared with that restored by unpassaged cells. On the other hand, the adoptive response restored by passaged spleen or thoracic duct cells from DNP-primed donors was greater than or the same as that restored with unpassaged cells, respectively. This suggests that unprimed B cells change from nonrecirculating to recirculating lymphocytes after exposure to antigen.

Studies of the adoptive anti-DNP response restored by unprimed or primed bone marrow cells showed little change in the time-course or amplitude of the response restored by either population of cells. The relative inability of marrow cells to carry immunological memory was related to the inability of recirculating memory cells to penetrate the marrow.

The turnover rate of unprimed and primed B cells was investigated by treating the cell donors with [³H]thymidine for 48 h before removal of thoracic duct or spleen cells. The adoptive anti-DNP response restored by unprimed or primed cells was not affected by [³H]thymidine treatment. This indicates that both populations of cells turn over slowly. However, our previous studies show that unprimed B cells involved in the adoptive antibody response to ferritin turn over rapidly. The different findings are discussed in the context of antigen-dependent B-cell maturation.

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