INITIATION OF ANTIBODY RESPONSES BY DIFFERENT CLASSES OF LYMPHOCYTES

V. FUNDAMENTAL CHANGES IN THE PHYSIOLOGICAL CHARACTERISTICS OF VIRGIN THYMUS-INDEPENDENT ("B") LYMPHOCYTES AND "B" MEMORY CELLS*

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Thymus-dependent, helper cells ("T" lymphocytes) and thymus-independent, antibody-forming precursor cells ("B" lymphocytes) cooperate in the initiation of the primary antibody response of rodents to a variety of antigens (1-3). Both cell lines also cooperate in the initiation of the secondary antibody response, and both carry immunological memory (4, 5). Recent cell transfer studies indicate that B memory cells determine the changes in the proportion of immunoglobulin classes and affinity of antibodies produced in the secondary response (6-8). These changes suggest that B memory cells are not derived by a simple expansion of unprimed B lymphocytes, but rather by heterogeneous expansion of subpopulations of unprimed B lymphocytes or by a qualitative change in immunological function.

On the other hand, the carriage of immunological memory by T lymphocytes could be explained by a homogeneous expansion of unprimed T cells. Miller et al. (5) and Mitchell et al. (4) have shown that both primed and unprimed T lymphocytes are able to cooperate with primed B lymphocytes in the adoptive secondary antibody response of mice to fowl gamma globulin and sheep red blood cells (SRBC), respectively. Approximately, ten times as many unprimed as primed T lymphocytes were required to restore responses of equal magnitude (4, 5). However, the adoptive secondary response to either antigen could not be restored by a combination of primed T lymphocytes and large numbers of unprimed B lymphocytes (4, 5). This suggests that a fundamental change in the immunological function of B but not T lymphocytes occurs after priming.

The present work was designed to determine whether priming changes the life-span and migratory behavior of B lymphocytes as well. Our previous studies

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1 Abbreviations used in this paper: BSA, bovine serum albumin; B lymphocytes, thymus-independent, antibody-forming precursor cells; DNP, dinitrophenyl; DNP-BSA, DNP-DT, dinitrophenyl-bovine serum albumin, dinitrophenyl-diphtheria toxoid, etc.; DT, diphtheria toxoid; HSF, horse spleen ferritin; ME, mercaptoethanol; SRBC, sheep red blood cells; T lymphocytes, thymus-dependent, helper cells.
provide indirect evidence for these changes, since unprimed and primed lymphocytes which restore the adoptive antibody response to diphtheria toxoid (DT) and horse spleen ferritin (HSF) in rats differ in their distribution in the lymphoid tissues (9), and in their sensitivity to the mitotic inhibitor, vinblastine (10). The present results provide direct evidence for physiological changes in unprimed and primed B cells. In particular, the findings indicate that unprimed B lymphocytes are short-lived (newly formed) cells which are unable to recirculate from the blood to the lymph, and that primed B lymphocytes (B memory cells) in the thoracic duct lymph are long-lived, recirculating cells.

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 procedure of Bollman et al. (11). Rats were maintained unaesthetized in restraining cages and received a continuous intravenous infusion of Ringer’s solution containing streptomycin (0.1 mg/ml) and heparin (1 unit/ml) at 2 ml/hr. Thoracic duct cells were collected at 4°C for 24 hr (unless otherwise indicated in the text) in 5 ml of Ringer’s solution with 100 units 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 (Grand Island Biological Co., Grand Island, N.Y.) before injection. Spleen cell suspensions were made in medium 199 according to the technique of Billingham (12).

X-Irradiation.—Rats were placed in Lucite containers and received 500 R whole body X-irradiation (except as noted in the text) from a single 250 kv (15 amp) source. The source axis distance was 52 cm, and the dose rate was 105 R/min (0.25 mm of Cu + 0.55 mm of Al filtration).

Neonatal Thymectomy.—Neonatal thymectomy was performed within 24 hr of birth by a modification of the technique of Miller (13). The mediastinum of thymectomized rats was examined for gross and microscopic evidence of thymus remnants at the end of each experimental protocol. Animals with remnants were discarded from the study.

Immunization Procedures.—Rats were immunized to alum-adsorbed DT (Parke, Davis & Co., Detroit, Mich.) by a single subcutaneous (7.5 Lf, 0.25 ml) and intraperitoneal injection (7.5 Lf, 0.25 ml) of 15 Lf toxoid. Bovine serum albumin (BSA) (Nutritional Biochemicals Corp., Cleveland, Ohio) and fluid DT (Commonwealth of Massachusetts Department of Health) were dinitrophenylated (DNP) by the procedure of Eisen et al. (14). The composition of the conjugates was DNP3~BSA and DNP13-DT. Rats immunized to DNP-BSA received 0.1 ml of an emulsion of equal volumes DNP-BSA in phosphate-buffered saline 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. Rats challenged with DNP-DT received a single intraperitoneal injection of 0.5 mg of protein in 1 ml of phosphate-buffered saline. Immunization to HSF (Pentex Biochemical, Kankakee, Ill.) was accomplished by the injection of 0.1 ml of emulsion of equal volumes HSF in saline and complete Freund’s adjuvant into each hind footpad. Each rat received a total dose of 2 mg of HSF.

Antibody Titration.—Antibodies to DT and HSF were measured in microtiter agglutination plates (Cooke Engineering Co., Alexandria, Va.) by a modification of the tanned red cell hemagglutination technique of Stavitsky (15). Fresh sheep cells were used in all assays, and tanning and sensitization were carried out a few hours before the titration procedure. Mecaptoethanol (ME)-resistant antibody was measured by incubating equal volumes of a 1:10 dilution of whole antiserum and 2-ME (0.1 ml in saline) for 30 min at room temperature before titration.
Antibodies to DNP were measured by a previously described (16) modification of the Farr assay (17). Serial fivefold dilutions of antiserum were made in 20% normal rat serum in saline. Diluted antisera were incubated with $10^{-8}$ M DNP-3H-ethylaminocaproic acid (kindly supplied by Dr. R. A. Asofsky, National Institute for Allergy and Infectious Diseases, Bethesda, Md.) for at least 1 hr at room temperature. Globulins were precipitated with 50% saturated ammonium sulfate for an additional 1 hr. Aliquots of the supernatant were counted in a scintillation counter, and antibody titers were recorded as the log$_{10}$ of that dilution of antiserum which bound 33% of the labeled ligand.

In Vitro Labeling of Thoracic Duct Cells with Uridine-3H.—Thoracic duct cells (10$^6$ cells/ml) were incubated in vitro in a culture medium containing medium 199 with 5% fetal calf serum and 1 $\mu$Ci/ml uridine-3H (New England Nuclear Corp., Boston, Mass.; specific activity 20 mCi/m mole). The incubation mixture was agitated slowly in a 37°C water bath for 45 min. The cells were washed twice in medium 199 before preparation of smears or intravenous injection.

Radioautography.—Radioautographs of uridine-3H-labeled cells were prepared with Kodak NTB emulsion (Eastman Kodak Co., Rochester, N. Y.). Slides were exposed for 17–21 days before developing and application of Giemsa stain.

In Vitro Incubation of Thoracic Duct Cells with Thymidine-3H.—Thoracic duct cells (1–2 $\times$ 10$^7$ cells/ml) were incubated in a tissue culture medium containing Ham's F12 medium without thymidine (Hyland Laboratories, Costa Mesa, Calif.), 10% (v/v) fetal calf serum, and thymidine-3H (New England Nuclear Corp.; specific activity 15 mCi/m mole) at a concentration of 10 $\mu$Ci/ml. The incubation mixture was placed in a 10 cm diameter plastic Petri dish (Falcon Plastics, Inc., Division of B-D Laboratories, Inc., Los Angeles, Calif.) which was maintained at 37°C for 24 hr in an atmosphere of 95% O$_2$ and 5% CO$_2$. Approximately 85–100% of cells were recovered and viability was greater than 90% as judged by trypan blue dye exclusion. Incubated cells were washed three times in medium 199 with 5% fetal calf serum before intravenous injection.

Intravenous Injection of Cells.—Cell suspensions were always made up in 0.5–1 ml of medium 199 and injected into the lateral tail vein of anesthetized rats.

Bleeding Procedures.—Anesthetized rats were bled from the tail vein on the days indicated in the figures. Approximately 0.2–0.5 ml of blood was allowed to clot at room temperature for 1 hr and then refrigerated for 12 hr. Serum was removed and stored at –20°C after centrifugation at 200 g for 10 min.

RESULTS

Inability of Unprimed Thoracic Duct Cells to Restore the Adoptive Antibody response to DT.—Adult male Lewis rats weighing 150–200 g received 500 R whole body X-irradiation and either spleen cells or thoracic duct cells from normal (unimmunized) donors 2 hr later. Each recipient was challenged with 15 Lf alum-adsorbed DT 24 hr after irradiation. Although 1 $\times$ 10$^6$ spleen cells restored a moderate anti-toxoid response, 1 $\times$ 10$^6$ thoracic duct cells did not restore a detectable response by day 20 (Fig. 1). Drainage of the thoracic duct of normal rats for 5 days before challenge with DT did not significantly decrease the anti-toxoid response as compared with intact controls (Fig. 1).

Restoration of the Adoptive Antibody Response to DT with DT-Primed Thoracic Duct Cells.—Adult male rats were primed with 15 Lf alum-adsorbed DT and thoracic duct cells were collected 4–8 wk later. Graded numbers of cells were transferred to sublethally irradiated (500 R) rats and each animal was challenged with DT as described in the previous section. As few as 1 $\times$ 10$^7$ cells
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restored a vigorous response by day 7 (Fig. 2). The log₁₀ cell dose vs. antibody titer plot was linear in this dose range (Fig. 2).

In order to determine whether residual radioresistant cells make a contribution to the adoptive antibody response after sublethal irradiation, the above experiments were repeated with groups of three animals each receiving 1000 R. Antibody titers in these groups at day 7 were almost identical with those shown in Fig. 2 for the three cell doses.

Restoration of the Adoptive Antibody Response to DT with Primed Thoracic Duct Cells Passaged through an Intermediate Host.—The migratory characteristics of DT-primed thoracic duct cells remained to be elucidated, since both recirculating and nonrecirculating cells are found in the thoracic duct lymph of the rat (18, 19). Accordingly, DT-primed cells were injected intravenously into normal rats and the thoracic duct of these intermediate hosts was cannulated 24 hr later. Thoracic duct cells were collected for 24 hr and then transferred to irradiated hosts which were subsequently challenged with DT.

In order to determine the yield of donor DT-primed cells in the lymph of the intermediate host, preliminary experiments were performed in which 5 × 10⁸ thoracic duct cells from normal donors were labeled in vitro for 45 min with uridine⁻³H and injected intravenously into a normal adult recipient. Thoracic duct cells were collected from 24 to 48 hr later and the per cent labeled cells in
the 24 hr lymph collection was examined by radioautography. Table I shows the average per cent labeled cells (200 cells counted) in donor (87%) and host (11%) lymph in two experiments. A considerable reduction in the proportion of labeled large and medium cells (>8 μm diameter) was noted in host as compared with donor lymph. These results were used to calculate the number of

\[ \text{Fig. 2. Adoptive antibody response of rats at day 7 to a single subcutaneous and intraperitoneal injection of 15 Lf alum-adsorbed DT. O, Mean response of rats given an intravenous injection of thoracic duct cells from DT-primed donors 2 hr after 500 R whole body X-irradiation. DT was injected 22 hr later. , Mean response of irradiated rats given thoracic duct cells passaged through a normal intermediate host. Brackets show the standard error of the mean. There were four to seven rats in each group.} \]

**TABLE I**

<table>
<thead>
<tr>
<th>Recovery of Uridine-( ^{3}H )-Labeled Cells in Rat Thoracic Duct Lymph</th>
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<tbody>
<tr>
<td>Per cent labeled cells</td>
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<td>Donor thoracic duct cells</td>
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<td>Host thoracic duct cells</td>
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Fig. 2 shows that the anti-DT response restored by primed, passaged cells at day 7 was slightly higher than that of primed, unpassaged cells at two different cell doses. However, differences between the two groups were not significant \((P > 0.05)\) as judged by the Student's \( t \) test.

**Restoration of the Adoptive Antibody Response to DNP-DT with Passaged Primed Cells.**—Although the results of the previous section show that DT-primed thoracic duct cells are able to recirculate from the blood to the lymph, the contribution of helper cell (T cell) and antibody-forming precursor cell (B
cell) activity was not assessed. A decrease in the precursor cell activity of passaged cells could have been masked by an increase in helper cell activity. In order to assess both helper and precursor cell activity, several experiments were performed in which a combination of carrier and hapten-primed cells were used to restore the adoptive antibody response to DNP-DT. We have previously shown that both cell types cooperate in the initiation of the anti-DNP response to this hapten-protein conjugate (20).

Fig. 3 shows the experimental scheme in which graded numbers of DT-primed thoracic duct cells were passaged through a normal intermediate host and injected together with \(25 \times 10^6\) DNP-primed cells into an X-irradiated host. DNP-DT was injected intraperitoneally 24 hr after irradiation. The adoptive anti-DT response was taken as a measure of B or precursor cell activity and the anti-DNP response was taken as a measure of T or helper cell activity (21, 22). Both \(10\) and \(25 \times 10^6\) unpassaged, DT-primed cells produced anti-DT and anti-DNP responses which were easily detectable by day 7 (Fig. 4). A greater difference was noted between the anti-DT response restored by the two cell doses as compared with the anti-DNP response. The anti-DT response restored by \(16 \times 10^6\) passaged, DT-primed cells fell between the responses restored by \(10\) and \(25 \times 10^6\) unpassaged cells. The anti-hapten response restored by the
passaged cells did not differ significantly ($P > 0.1$) at days 10 and 14 from the
responses restored by 10 or $25 \times 10^6$ unpassaged cells.

**Restoration of the Adoptive Antibody Response to DNP-DT with Primed Cells from Donors Treated with Thymidine-$^3$H.**—In further experiments the rate of
formation (turnover rate) of helper and precursor cells involved in the adoptive
secondary response to DNP-DT was studied. DT-primed donors weighing 125-

![Graph](image)

**Fig. 4.** Adoptive antibody response of rats to a single intraperitoneal injection of 0.5 mg
of DNP-DT. All rats were given $25 \times 10^6$ nonpassaged DNP-primed thoracic duct cells and
graded numbers of DT-primed thoracic duct cells intravenously 2 hr after 500 R whole body
X-irradiation. DNP-DT was given 22 hr later. (a) Mean anti-DT response of groups of five
to nine rats with brackets showing the standard error of the mean. (b) Mean anti-DNP re-
sponse expressed as log$_{10}$ titer which bound 33% of labeled antigen. $\bullet-\bullet$, Mean response
of rats given $25 \times 10^6$ nonpassaged DT-primed cells; $\bigcirc-\bigcirc$, mean response of rats given
$10 \times 10^6$ nonpassaged DT-primed cells; $\square-\square$, mean response of rats given $16 \times 10^6$ pas-
saged DT-primed cells. $\bigtriangleup-\bigtriangleup$, Mean response of rats given $25 \times 10^6$ DT-primed cells treated
in vivo with thymidine-$^3$H.

150 g received intraperitoneal injections of thymidine-$^3$H (New England Nu-
clear Corp.; specific activity 15 mCi/mmol) in aqueous solution every 8 hr for
a period of 48 hr before cannulation of the thoracic duct. Each dose of thymi-
dine-$^3$H contained 3.5 mCi in 3.5 ml of sterile water (24.5 mCi total dose).

Thoracic duct cells from thymidine-$^3$H-treated donors were collected for 24
hr and then injected together with DNP-primed cells into irradiated hosts as
described before. The thoracic duct cell output of the treated donors was about
equal to that of untreated rats. In order to minimize reutilization of the radio-
active label, each host received a single intraperitoneal injection of cold thymi-
dine (1 ml, 10^{-4} \text{ M thymidine in water}) within a few hours after cell transfer. Cold thymidine was also added to the drinking water (10^{-4} \text{ M}) for the remainder of the experiment. DNP-DT was administered to each host 24 hr after irradiation.

Fig. 4 shows that the anti-DT and anti-DNP responses restored by a combination of 25 \times 10^6 treated, DT-primed cells and 25 \times 10^6 untreated, DNP-primed cells was similar to that restored by 10 \times 10^6 DT- and 25 \times 10^6 DNP-primed cells from untreated donors.

**Inability of Passaged Thoracic Duct Cells from Normal Donors to Restore the Adoptive Primary Antibody Response to HSF.**—Although thoracic duct cells from normal rats do not restore the adoptive primary response to alum-adsorbed DT, these cells do restore the primary response to HSF (10). In order to determine the migratory characteristics of the active cells, passaged and unpassaged thoracic duct cells were tested for their ability to restore the adoptive anti-HSF response of irradiated hosts. Accordingly, 5 \times 10^6 thoracic duct cells from normal donors were injected intravenously into syngeneic intermediate hosts which had received 500 R 24 hr before. The thoracic duct of the intermediate hosts was cannulated 24 hr after the injection, and cells were collected for a period of 48 hr. The passaged cells were subsequently injected intravenously into irradiated hosts challenged with HSF. Equal numbers of passaged cells were injected 2 and 24 hr after irradiation, and antigen was administered simultaneously with the second injection of cells.

The fraction of donor cells in the intermediate host lymph was determined by incubating 5 \times 10^6 cells from normal donors with uridine-^{3}H in vitro for 45 min. The cells were subsequently injected into an intermediate host and collected as described above. Radioautographs of donor and intermediate host lymph obtained in two experiments showed that an average of 84% of cells in the donor lymph and 87% of cells in the host lymph (200 cells counted) were labeled. These results were used to calculate the number of passaged cells transferred to recipients challenged with HSF.

Fig. 5 shows the adoptive primary response to HSF restored by 2, 10, 50, and 180 \times 10^6 unpassaged thoracic duct cells from normal donors. The responses restored by 50 and 180 \times 10^6 cells were almost identical. Moderate titers were observed at day 12 and a plateau was noted at day 18. A less vigorous response was restored by 10 \times 10^6 cells, and that restored by 2 \times 10^6 cells was barely detectable by day 20. Almost all the antibody produced after day 14 was resistant to 2-ME (Fig. 5 b).

The adoptive response produced by 180 \times 10^6 passaged thoracic duct cells is also shown in Fig. 5. Although antibody was detectable by day 12, titers did not rise thereafter. Titers at days 14, 18, and 20 fell between those restored by 2 and 10 \times 10^6 unpassaged cells.

**Inability of Thoracic Duct Cells from Thymidine-{^{3}H}-Treated Donors to Restore the Adoptive Primary Antibody Response to HSF.**—In several experiments,
thoracic duct cells from unimmunized donors treated with thymidine-³H were tested for their ability to restore the adoptive primary response to HSF. Each normal donor received 24.5 mCi of the radioactive nucleotide for 48 hr before cannulation of the thoracic duct as described previously. Cells from treated donors were transferred to syngeneic hosts 2 hr after whole body X-irradiation and HSF was injected 22 hr later. Cold thymidine (10⁻⁴ M, 1 ml) was injected intraperitoneally immediately after antigenic challenge and thymidine was added to the drinking water (10⁻⁴ M).

Fig. 5 shows the total and 2-ME-resistant antibody response restored by 180 × 10⁶ thymidine-³H-treated thoracic duct cells. Antibody was not detectable at day 18 and one out of five rats showed a small but detectable titer at day 20. This response was similar to that produced by 2 × 10⁶ untreated thoracic duct cells (Fig. 5).

Cooperation between Thymidine-³H-Treated or Passaged Thoracic Duct Cells
from Normal Donors and Spleen Cells from Neonatally Thymectomized Donors.—Fig. 6 shows that the anti-HSF response of neonatally thymectomized rats at day 12 is similar to that of age controlled normal rats. However, the adoptive response to HSF restored by 50 × 10⁶ thoracic duct or spleen cells from neonatally thymectomized rats is markedly reduced as compared with that restored by an equal number of cells from normal donors (Fig. 7).

In order to test the hypothesis that passaged or thymidine-³H-treated thoracic duct cells from normal donors lack B lymphocytes, a combination of 50 × 10⁶ spleen cells from thymectomized donors (a source of B lymphocytes), and 50 × 10⁶ passaged or thymidine-³H-treated thoracic duct cells from normal donors was injected into irradiated hosts. The reconstituted rats were challenged with HSF as described before. Fig. 7 shows that none of the individual cell inocula restored a detectable anti-HSF response until day 18. How-

ever, a combination of the spleen cells and passaged thoracic duct cells or spleen cells and thymidine-³H-treated thoracic duct cells produced a detectable response by day 14. Thereafter, the response rose rapidly and was similar to that restored by 50 × 10⁶ cells from normal donors by day 20 (Fig. 7). In each case, the day 20 titer produced by the combination of cells was significantly greater (P < 0.01) than the sum of the titers restored by either cell type alone.

Restoration of the Adoptive Primary Response to HSF with Thoracic Duct Cells Incubated In Vitro with Thymidine-³H.—Our previous studies have shown that in vitro incubation of normal thoracic duct cells in tissue culture medium 199 for 24 hr markedly reduces their ability to restore the adoptive anti-HSF response (10). However, in vitro incubation of thoracic duct cells in Ham’s F12 tissue culture medium with 10% fetal calf serum for 24 hr (see Materials
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and Methods for details) does not affect the restorative activity. Fig. 8 shows
that $1 \times 10^6$ incubated thoracic duct cells produced an adoptive anti-HSF
response which was almost identical with that produced by $180 \times 10^6$ unincu-
bated cells. The addition of thymidine-$3\text{H}$ (specific activity 15 mCi/mmole) at
a concentration of 10 $\mu$Ci/ml to the above culture medium (with cold thymi-
dine removed) did not alter the restorative action of the incubated cells (Fig. 8).

Fig. 7. Adoptive antibody response of rats to a single subcutaneous injection of HSF in
complete Freund’s adjuvant. Hosts were given an intravenous injection of lymphoid cells
from unimmunized syngeneic donors 2 hr after 500 R whole body X-irradiation. HSF was
given 22 hr later. (a) Total antibody response. (b) 2-ME-resistant antibody response. □—□,
mean response of rats given $50 \times 10^6$ untreated spleen cells from intact donors; ○—○,
mean response of rats given $50 \times 10^6$ untreated thoracic duct cells from intact donors; ■—■,
mean response of rats given $50 \times 10^6$ untreated spleen cells from thymectomized donors;
●—●, mean response of rats given $50 \times 10^6$ untreated thoracic duct cells from thymectomized donors;
△—△, mean response of rats given $50 \times 10^6$ thoracic duct cells from thymectomized donors;
▌—▌, mean response of rats given $50 \times 10^6$ thoracic duct cells treated in vivo with thymidine-$3\text{H}$;
▌—▌, mean response of rats given $50 \times 10^6$ thoracic duct cells passed through an irradi-
ated intermediate host; ▲—▲, mean response of rats given a combination of $50 \times 10^6$ tho-
racic duct cells treated in vivo with thymidine-$3\text{H}$ and $50 \times 10^6$ spleen cells from thymec-
tomized donors; ▼—▼, mean response of rats given a combination of $50 \times 10^6$ thoracic
duct cells passed through an irradiated intermediate host and $50 \times 10^6$ spleen cells from a
thymectomized donor. Brackets show the standard error of the mean. There were five to
seven rats in each group.

Restoration of the Adoptive Secondary Response to HSF with Passaged or
Thymidine-$3\text{H}$-Treated Thoracic Duct Cells from Immunized Donors.—Fig. 9
shows the adoptive anti-HSF response restored by 2, 10, 20, and $50 \times 10^6$
thoracic duct cells from donors immunized to HSF 4-8 wk before. The re-
sponse restored by 20 and $50 \times 10^6$ cells rose rapidly after day 8, but that
restored by $2 \times 10^6$ cells was not detected until day 14. Differences in the
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pre- and postboost titers of intact rats immunized to HSF are shown for comparison (Fig. 9).

In several experiments HSF-primed thoracic duct cells were passaged through X-irradiated intermediate hosts and tested for their ability to restore the adoptive antibody response to HSF. Fig. 9 shows that \(20 \times 10^6\) passaged cells produced a response which was not significantly different in time-course and magnitude from that produced by \(20 \times 10^6\) unpassaged cells.

Treatment of HSF-primed donors with thymidine-\(^3\)H produced a slight reduction in the restorative activity of the primed cells. The response restored by \(20 \times 10^6\) treated cells was similar to that restored by \(10 \times 10^6\) untreated cells, but was considerably greater than that restored by \(2 \times 10^6\) untreated cells (Fig. 9).

**DISCUSSION**

The migratory behavior and turnover rate of lymphocytes which initiate the primary and secondary antibody response of rats to protein antigens was investigated. Studies of the primary response of Lewis rats to DT show that drainage of the thoracic duct of normal rats for 5 days before antigenic challenge does not alter the magnitude of the anti-DT response. Thoracic duct
cells ($1 \times 10^9$) from these donors do not restore the adoptive antibody response of sublethally irradiated (500 R) hosts. However, spleen cells ($1 \times 10^9$) from normal donors restore a vigorous response. These results indicate that a population of lymphocytes involved in the primary response to DT do not recirculate from the blood to the lymph, and are, instead, associated with the solid lymphoid tissues.

On the other hand, as few as $1 \times 10^7$ thoracic duct cells from rats immunized to DT 4–8 wk earlier restore a vigorous adoptive secondary antibody response. It is unlikely that cells from the sublethally irradiated hosts are involved in the response since similar responses are observed in lethally irradiated (1000 R) recipients. In addition, Ellis et al. (23) have shown that thoracic duct cells from rats primed to tetanus toxoid are the precursors of the antibody-forming cells in the adoptive anti-toxoid response of lethally irradiated rats. Antibody-forming cells of donor origin were identified by the cytotoxic action of antisera.

**Fig. 9.** Adoptive antibody response of rats to a single subcutaneous injection of HSF in complete Freund’s adjuvant. Hosts were given an intravenous injection of thoracic duct cells from HSF-primed donors 2 hr after 500 R whole body X-irradiation. HSF was given 22 hr later. $\bigcirc$ $\sim$ $\bigcirc$, mean response of rats given $50 \times 10^6$ untreated cells; $\triangledown$ $\sim$ $\triangledown$, mean response of rats given $20 \times 10^6$ untreated cells; $\bullet$ $\sim$ $\bullet$, mean response of rats given $10 \times 10^6$ untreated cells; $\Delta$ $\sim$ $\Delta$, mean response of rats given $2 \times 10^6$ untreated cells; $\square$ $\sim$ $\square $, mean response of rats given $20 \times 10^6$ cells passaged through an irradiated intermediate host; $\bigcirc$ $\sim$ $\bigcirc$, mean response of rats given $20 \times 10^6$ cells treated in vivo with thymidine-$^3$H; $\blacktriangle$ $\sim$ $\blacktriangle$, mean difference in pre- and postboost titers of intact rats primed to HSF 4–8 wk before is shown for comparison. Brackets show the standard error of the mean. There were five to seven rats in each group.
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directed against donor histocompatibility antigens on the surface of specifically immunofluorescent cells.

In order to determine whether memory cells are able to recirculate from the blood to the lymph, DT-primed thoracic duct cells were injected intravenously into normal intermediate hosts and subsequently collected in the thoracic duct lymph. The passaged cells were compared with unpassaged cells for their ability to restore the adoptive secondary response to DT. The yield of DT-primed cells in the lymph of the intermediate hosts was determined by labeling donor cells with uridine-3H in vitro before cell transfer. The per cent of labeled cells in donor and host lymph was used to calculate the number of DT-primed cells injected into each irradiated recipient. A comparison of the cell dose vs. antibody response plots of passaged and unpassaged cells shows that passaged cells completely maintain their restorative activity.

Although these findings show that memory cells in the thoracic duct lymph are able to recirculate from the blood to the lymph, the contribution of helper cell (T cell) and antibody-forming precursor cell (B cell) activity was not assessed. A decrease in the precursor cell activity of passaged cells could have been masked by an increase in helper cell activity. In order to assess both activities, a combination of carrier and hapten-primed cells were used to restore the adoptive antibody response to DNP-DT. We have previously shown that both cell types cooperate in the initiation of the anti-DNP response to this hapten-protein conjugate (20).

Graded numbers of passaged or unpassaged DT-primed thoracic duct cells with a constant number of DNP-primed cells were transferred to irradiated hosts challenged with DNP-DT. The adoptive anti-DNP response was taken as a measure of helper cell activity and the anti-DT response was taken as a measure of precursor cell activity (21, 22). The experimental results show that the DT-primed, passaged and unpassaged cells restore quite similar anti-DNP and anti-DT responses. This shows that the majority of both primed, helper and precursor cells are able to recirculate from the blood to the lymph. Our recent report that hapten-primed thoracic duct cells are able to recirculate provides further evidence for this point (24). In addition, Howard has shown that the majority of uridine-3H-labeled B lymphocytes from the thoracic duct of thymectomized, lethally irradiated rats restored with syngeneic bone marrow are able to migrate from the blood to the lymph (25). However, the migratory pathways of the B lymphocytes differ from those of T lymphocytes (26).

A comparison of the studies of the adoptive primary and secondary responses to DT suggests that the migratory patterns of unprimed and primed lymphocytes differ. However, one could argue that active recirculating lymphocytes are found both in the thoracic duct lymph and spleens of unimmunized rats. The spleen may have an additional activity not present in the lymph which allows for the full expression of the immunological function of the recirculating cells. In order to show conclusively that the migratory behavior of unprimed
and primed lymphocytes differ, we studied the adoptive primary and secondary response of rats to another protein, HSF. Both unprimed and primed thoracic duct cells are able to restore the antibody response of irradiated hosts to this antigen (10). The mechanisms responsible for the differences in the restorative action of unprimed thoracic duct cells to HSF and DT have not been elucidated.

Thoracic duct cells from unimmunized donors were passaged through irradiated intermediate hosts and subsequently tested for their ability to restore the adoptive response to HSF. In vitro labeling of donor cells with uridine-3H showed that almost all the cells in the intermediate host lymph are of donor type. The response restored by passaged cells is reduced 20- to 100-fold as compared with that produced by equal numbers of unpassaged cells. This indicates that a population of thoracic duct cells involved in the adoptive primary response to HSF is unable to recirculate from the blood to the lymph. In addition, the findings suggest that nonrecirculating lymphocytes play a role in the primary response of rats to protein antigens such as DT and HSF whether or not the active cells are found in the thoracic duct lymph.

The turnover rate of thoracic duct cells involved in the adoptive primary response to HSF was investigated by administering a large dose (~25 mCi) of thymidine-3H (specific activity 15 mCi/m mole) to unimmunized donors for 48 hr before cannulation of the thoracic duct. A 24 hr pulse of a similar dose of thymidine-3H in vivo has been reported to reduce the number of colony-forming units in mouse bone marrow approximately fivefold (27). The extent of “thymidine suicide” depends upon the proliferative rate of the cell population under investigation. Cells from thymidine-3H-treated donors were transferred to irradiated hosts challenged with HSF. Cold thymidine was administered to the cell recipients to minimize reutilization of the radioactive label. The adoptive primary response restored by the treated cells is approximately 100-fold less than that restored by an equal number of untreated cells. The results show that a population of thoracic duct cells involved in the adoptive response is newly formed at least every 48 hr. These findings are consistent with the marked reduction in the restorative action of unprimed thoracic duct cells after in vivo treatment with the mitotic inhibitor, vinblastine (10). The newly formed cells are almost certainly short lived, since the rate of disappearance of lymphocytes labeled in vivo with thymidine-3H is directly related to the rate of appearance of the labeled cells (28, 29). The short life-span implies either early cell death, or, more probably, continued cell division.

Approximately 5-10% of rat thoracic duct cells are large (>8 μ diameter) lymphocytes which rapidly incorporate thymidine-3H in vitro (30). In order to determine whether these are the short-lived cells involved in the adoptive primary response to HSF, thoracic duct cells from normal donors were incubated in vitro with thymidine-3H at a concentration of 10 μCi/ml for 24 hr
before cell transfer. This concentration of radioactive thymidine is 100 times that which heavily labels 25–35% of thoracic duct large lymphocytes in vitro in 1 hr (30), and abolishes the in vitro antibody response of mouse spleen cells to SRBC (31). Our previous experiments showed that in vitro incubation of thoracic duct cells in culture medium 199 for 24 hr in the absence of radioactive materials markedly reduces their ability to restore the adoptive primary response to HSF (10). However, the tissue culture conditions described in the present study almost completely maintain the restorative action and viability of thoracic duct cells during the 24 hr period of incubation without radioactive thymidine. In this way the effects of specific thymidine-3H killing of dividing cells could be tested. The addition of thymidine-3H to the tissue culture medium did not alter the restorative action of these cells, and suggests that large lymphocytes which divide in the lymph in vitro do not play a role in the adoptive primary response. The experimental results also suggest that the active newly formed thoracic duct cells enter the lymph from a distant generative compartment and do not continue to divide while they are in the lymph.

Experiments with neonatally thymectomized rats suggest that the primary antibody response to HSF is independent of T lymphocytes, since the response of intact and thymectomized animals is not significantly different 12 days after the injection of HSF. However, thoracic duct and spleen cells from thymectomized donors show a marked decrease in their ability to restore the adoptive anti-HSF response as compared with equal numbers of cells from intact donors. These findings are not surprising in view of the fact that only a small fraction of the total number of T lymphocytes in a normal rodent is required to completely restore the primary response of thymectomized hosts (16, 32). A comparison of the restorative action of limited numbers of cells from normal and thymectomized donors is, therefore, a more sensitive indicator of T cell dependence than a comparison of the responses of the donors themselves to the same antigen.

In several experiments, combinations of passaged or thymidine-3H-treated thoracic duct cells from normal donors and spleen cells from thymectomized donors were tested for their ability to restore the adoptive primary response to HSF. The response restored by either combination of cells was considerably greater than the sum of the responses restored by each cell type alone. These findings strongly suggest that the population of nonrecirculating, short-lived (newly formed) cells present in the thoracic duct lymph of unimmunized rats are B lymphocytes, since the restorative activity of passaged or thymidine-3H-treated thoracic duct cells can be reconstituted with B cells. In addition, the results suggest that unprimed T lymphocytes are recirculating cells which are turning over slowly (long lived). Definitive evidence for this point is lacking, since specific T or B cell markers are not presently available in the rat.

Although passaged thoracic duct cells from unimmunized donors are unable
to restore the adoptive primary response to HSF, passaged cells from immunized donors restore a vigorous secondary response which is similar to that produced by an equal number of unpassaged cells. This indicates that there is a fundamental change in the migratory behavior of thoracic duct lymphocytes after exposure to antigen. Primed (memory) thoracic duct cells are able to recirculate from the blood to the lymph; whereas, unprimed cells are not. These results are in agreement with those obtained using DT as antigen, and serve to explain the differences in the lymphoid tissue distribution of unprimed and primed lymphocytes involved in the adoptive antibody response to DT (9). The altered migratory properties of primed and unprimed cells may be related to changes in the cell membrane which occur after priming, since changes in the homing patterns of thoracic duct cells have been reported to be related to changes in the constituents of the surface membrane (33, 34).

<table>
<thead>
<tr>
<th>TABLE II</th>
<th>Thoracic Duct Cells Involved in the Primary and Secondary Antibody Response</th>
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<tbody>
<tr>
<td>1° Antibody response</td>
<td>2° Antibody response</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>Recirculating</td>
<td>Recirculating</td>
</tr>
<tr>
<td>Long lived</td>
<td>Long lived</td>
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<tr>
<td>B1 lymphocytes</td>
<td>B2 lymphocytes</td>
</tr>
<tr>
<td>Nonrecirculating</td>
<td>Recirculating</td>
</tr>
<tr>
<td>Short lived (newly formed)</td>
<td>Long lived</td>
</tr>
</tbody>
</table>

Treatment of HSF-primed donors with thymidine-3H for 48 hr before cannulation of the thoracic duct produced a slight (~2-fold) reduction in the adoptive secondary response restored by the treated cells. On the other hand, similar treatment of unimmunized donors produced at least a 100-fold reduction in the adoptive primary response. These experimental results indicate that the turnover rate of a population of unprimed lymphocytes is much greater than that of primed (memory) lymphocytes. The findings are consistent with our previous report of the markedly increased sensitivity of unprimed thoracic duct cells to the mitotic inhibitor, vindblastine, as compared with primed cells (10). Experiments with DNP-DT show that the majority of both primed helper (T) and precursor (B) cells are relatively long lived (slowly turning over). The slight reduction in the restorative activity of thymidine-3H-treated, primed cells is difficult to interpret, since the reutilization of the radioactive label by donor cells was not assessed. Our previous experiments suggest that reutilization could account for the depressed responses, since in vivo treatment of primed cells with a nonreutilizable mitotic inhibitor, vinblastine, resulted in augmented adoptive secondary responses to HSF (10).
Table II lists the physiological characteristics of primed and unprimed T and B lymphocytes suggested by the present study. Both unprimed and primed T cells found in the thoracic duct lymph are long-lived, recirculating lymphocytes. On the other hand, unprimed B cells are nonrecirculating, short-lived lymphocytes (B₁ cells) which undergo a fundamental biological change to long-lived, recirculating lymphocytes (B₂ cells) found in the thoracic duct lymph after priming. Changes in the physiological functions of unprimed and primed B cells may parallel fundamental changes in immunological functions recently suggested by several investigators (4, 5).

The rapid turnover of unprimed B lymphocytes may play an important role in generating the considerable diversity of antibody combining sites ("V" regions) of rats by means of continuous somatic mutation or recombination (35, 36) of either B cell precursors or B cells themselves. On the other hand, the long-lived memory B lymphocytes form more stable clones of cells which may not divide until they are stimulated by antigen. Fig. 10 shows two possible models of "clone stabilization." In the first, all B lymphocytes derived from a given stem cell are committed to the expression of a single "V" region. Interaction between antigen and the mature B cell gives rise to a clone of long-lived memory cells which also express the identical V region. On the right, somatic mutation or recombination occurs continuously among rapidly dividing B cells or B cell precursors, such that a given B cell may express a V region which differs from that of its ancestors. Interaction between antigen and a transient mutant or recombinant B cell gives rise to a clone of memory cells which express the V region of the unprimed cell during their long life-span.

Fig. 10. "Clone stabilization." Two models are shown. On the left, "B" lymphocytes derived from a given stem cell are committed to the expression of a single "V" region. Interaction between antigen and the mature B cell gives rise to a clone of long-lived memory cells which also express the identical V region. On the right, somatic mutation or recombination occurs continuously among rapidly dividing B cells or B cell precursors, such that a given B cell may express a V region which differs from that of its ancestors. Interaction between antigen and a transient mutant or recombinant B cell gives rise to a clone of memory cells which express the V region of the unprimed cell during their long life-span.
cell division proceeds. Interaction between antigen and a transient mutant or recombinant B cell gives rise to a clone of memory cells which continue to express the V region of the unprimed cell during their long life-span. In this way, antigen may stabilize the expression of transient mutant or recombinant V regions which would otherwise be short lived.

SUMMARY

The life-span and migratory characteristics of rat thoracic duct cells which initiate the adoptive primary and secondary antibody response to diphtheria toxoid (DT) and horse spleen ferritin (HSF) were investigated. The experimental results show that thoracic duct lymphocytes from normal (unimmunized) donors are able to restore the adoptive response of irradiated hosts to HSF. Thoracic duct cells passaged through an intermediate host (intravenous injection and subsequent collection in the thoracic duct lymph) showed a marked reduction in their restorative action as compared with unpassaged cells. In addition, the restorative action of cells from donors treated with thymidine-\(^{3}H\) for 48 hr before cannulation of the thoracic duct was markedly decreased. This indicates that a population of lymphocytes involved in the adoptive primary response is unable to recirculate from the blood to the lymph and is turning over rapidly (short lived). The nonrecirculating, short-lived lymphocytes are probably "B" cells, since a combination of spleen cells from neonatally thymectomized rats and passaged or thymidine-\(^{3}H\)-treated cells restores a vigorous response to HSF.

On the other hand, passaged or thymidine-\(^{3}H\)-treated thoracic duct cells from donors immunized to DT or HSF are able to restore a vigorous adoptive secondary antibody response. Experiments with the hapten-protein conjugate, DNP-DT, show that the majority of both helper ("T") and precursor ("B") cells are able to recirculate and are slowly turning over (long lived). The findings suggest that T lymphocytes involved in both the primary and secondary antibody response are recirculating, long-lived cells. However, B lymphocytes involved in the primary response are nonrecirculating, short-lived cells ("B\(_{1}\)" cells) which undergo a fundamental physiological change to recirculating, long-lived cells ("B\(_{2}\)" cells) involved in the secondary antibody response.

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REFERENCES


