BIOLOGICAL CHARACTERISTICS OF T AND B MEMORY LYMPHOCYTES IN THE RAT*

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Both thymus-derived (T) and bursa-equivalent (B) lymphocytes carry immunological memory and cooperate in the induction of the secondary antibody response to a variety of antigens in rodents (1–4). Mitchison (3, 4) and Raff (5) have shown that cooperation between carrier- and hapten-primed cells in the adoptive secondary antibody response to a hapten-protein conjugate in mice is an example of T and B memory cell cooperation.

We have recently studied the biological characteristics of carrier-and hapten-primed cells present in the thoracic duct lymph of the rat (6, 7). Our findings show that both types of cells are relatively long lived and recirculate from the blood to the lymph. These studies did not investigate the biological characteristics of memory cells in the solid lymphoid tissues such as the spleen. It is possible that a second population of memory cells that is turning over rapidly and does not recirculate is found in the spleen, but not in the thoracic duct lymph. This possibility was suggested by the early work of Gowans and Uhr (8). These investigators showed that thoracic duct small lymphocytes carry immunological memory to bacteriophage $\phi\chi174$, but prolonged thoracic duct drainage of immunized rats does not reduce the anamnestic response of the drained rats to a subsequent challenge with the bacteriophage (8).

This report investigates the hypothesis that two populations of memory cells exist in the rat: a recirculating population found in the thoracic duct lymph and a sessile population found in the solid lymphoid tissues such as the spleen. The experimental findings presented here do not agree with this hypothesis, and instead indicate that recirculating, nondividing lymphocytes can account for both T and B cell memory in the thoracic duct lymph and the solid lymphoid tissues. The contribution of fixed or rapidly turning over lymphocytes to immunological memory is small or negligible as compared with that of the recirculating, relatively long-lived lymphocytes.

Materials and Methods

Animals.—Inbred Lewis rats were used in all experiments. Animals were purchased from Simonsen Laboratories, Gilroy, Calif.

Preparation of Cell Suspensions.—The thoracic duct of adult male rats weighing 150–200 g

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was cannulated by a modification of the procedure of Bollman et al. (9). Rats were maintained unanesthetized in restraining cages and received a continuous intravenous infusion of Ringer's solution containing 0.1 mg/ml streptomycin and 1 U/ml heparin at 2 ml/h. Thoracic duct cells were collected in sterile flasks containing 5 ml of Ringer's solution with 100 U of heparin and 1 mg of streptomycin. Cells were collected at 4°C for 24 h (unless otherwise noted in the text) and then harvested by centrifugation at 150 g for 10 min. Spleen cell suspensions were made in tissue culture medium 199 (M-199)[1] according to the technique of Billingham (10).

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

Neonatal Thymectomy.—Neonatal thymectomy was performed within 24 h of birth by a modification of the technique of Miller (11).

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. 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. (12). The composition of the conjugates was DNP_{35}-BSA and DNP_{13}-DT. Rats were immunized to DNP-BSA by injecting 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 were challenged with 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 (13). Antibody responses are expressed as the $\log_{10}$ of that dilution of antiserum that bound 33% of [^{3}H]DNP-ethylaminocaproic acid (10^{-8} M).

In Vitro Labeling of Thoracic Duct Cells with [^{3}H]Uridine.—Thoracic duct cells (10^7 cells/ml) were incubated in vitro in tissue culture medium 199 with 5% fetal calf serum (M-199-FCS) and mCi/ml [^{3}H]uridine (New England Nuclear, Boston, Mass.; specific activity 20 Ci/mmol). The incubation mixture was agitated slowly in a 37°C water bath for 45 min. Cells were harvested by centrifugation and washed twice with M-199 before use.

Autoradiography.—Autoradiographs of [^{3}H]uridine-labeled cells were prepared with Kodak NTB emulsion (Eastman Kodak Co., Rochester, N. Y.). Slides were exposed for 17–28 days before developing and application of Giemsa stain.

Intravenous Injection of Cells and Bleeding Procedures.—Injection and bleeding techniques have been described in detail previously (7).

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. Rabbits were given an intravenous injection of approximately 180 × 10^6 thoracic duct cells from August rats that were adult thymectomized, lethally irradiated, and bone marrow reconstituted (14). Two intravenous booster injections containing 100–190 × 10^6 thoracic duct cells were administered 3 and 8 wk later. Serum obtained 7 days after the last immunization was absorbed four times with Lewis thymocytes. The absorbed serum specifically identifies B lymphocytes in Lewis rats (J. C. Howard, manuscript in preparation).

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[1] *Abbreviations used in this paper*: ALS, antilymphocyte serum; DNP-BSA, dinitrophenyl-bovine serum albumin; DNP-DT, dinitrophenyl-diphtheria toxoid; DT, diphtheria toxoid; M-199, tissue culture medium 199; M-199-FCS, M-199 with 5% fetal calf serum; NRS, normal rabbit serum; RARBS, rabbit antirat B cell serum; VBS-FCS, Veronal-buffered saline with 5% FCS.

Cytotoxic Assay.—100 μl of RARBS diluted in Veronal-buffered saline with 5% FCS (VBS-FCS) was placed in 10 × 75 mm glass culture tubes. 50 μl of a suspension of thoracic duct cells in VBS-FCS ($5 × 10^6$ cells/ml) was added, and the reaction mixture was allowed to incubate for 15 min at room temperature. Thereafter, 100 μl of rabbit complement diluted 1:4 in VBS-FCS was added, and cells were harvested by centrifugation at 150 *g* 45 min later. The pellets were resuspended in 100 μl of M-199. 100 μl 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]) for each point of the titration. The cytotoxic assay was altered when thymocytes were used as target cells, in that the cells were suspended in 0.15 M fructose with 5% FCS instead of VBS-FCS. Fructose minimizes the nonspecific killing of rat thymocytes by NRS (J. C. Howard personal communication), but does not alter specific killing by RARBS.

Results of the cytotoxic assay were 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 Hapten- or Carrier-Primed Thoracic Duct Cells with RARBS.— Thoracic duct cells obtained 4–8 wk after immunization were suspended in M-199–FCS at a concentration of 10^7 cells/ml. 0.2 ml of undiluted RARBS was added to 9.8 ml of the cell suspension, and the mixture remained at room temperature for 15 min. Thereafter, 6.6 ml of rabbit complement diluted 1:4 in M-199–FCS was added, and the cells were allowed to incubate for an additional 45 min. The reaction mixture was spun down at 150 *g* for 10 min, and the pellet was washed once in M-199–FCS. The washed cells were resuspended in M-199 at a concentration of $25 × 10^6$ cells/ml, and 1-ml aliquots were transferred intravenously to cell recipients.

Preparation of Antilymphocyte Serum (ALS).—ALS was prepared by a modification of the technique of Levey and Medawar (15). Approximately $1 × 10^9$ rat thymocytes were suspended in 1 ml of M-199 and injected intravenously into an adult male New Zealand white rabbit. An intravenous booster injection of $1 × 10^9$ thymocytes was administered 2 wk later. Serum was obtained 8 days after the second injection and stored at −40°C.

Aliquots of ALS were inactivated at 56°C for 30 min and then absorbed four times with equal volumes of packed rat red blood cells before use. Absorptions were carried out at room temperature for 20 min. The cytotoxic titer (50% kill) of the absorbed ALS run against rat thoracic duct cells was approximately 1:4,000. A single intraperitoneal injection of 1 ml of ALS into each of four normal rats reduced the mean peripheral blood small lymphocyte count from 10,900 to 3,200/mm³ after 24 h.

RESULTS

Cooperation Between Hapten- and Carrier-Primed Spleen Cells in the Adoptive Antibody Response to DNP-DT.—Adult male Lewis rats were immunized to alum-adsorbed DT or to DNP-BSA, and spleen cells were removed 4–8 wk later. Inocula of $50 × 10^6$ DT or DNP-primed cells were injected separately or together into the lateral tail vein of rats that received 500 R whole body X-irradiation 2 h earlier. The cell recipients were challenged with DNP-DT 24 h after irradiation. Anti-DNP titers were measured for 14 days thereafter. Table I shows that the anti-DNP response restored by either DT or DNP-primed spleen cells alone was minimal, but the response restored by a combination of cells was quite vigorous by day 7. The sum of the responses restored by the separate cell inocula was approximately 100-fold less than that restored by the combination of cells at days 7, 9, and 14.

Restoration of the Anti-DNP Response with Hapten-Primed Cells Passaged Through an Intermediate Host.—In order to determine whether hapten-primed spleen cells are able to recirculate from the blood to the lymph, several experiments were performed in which 300×10^6 spleen cells from donors primed to DNP-BSA were injected intravenously into an intermediate host that had been irradiated 24 h before (Fig. 1). The thoracic duct of the irradiated host was cannulated 24 h later, and thoracic duct cells were collected for 48 h. The passaged cells were injected together with unpassaged carrier-primed cells into the final hosts that received 500 R 2 h earlier. Cell recipients were challenged with DNP-DT 22 h later.

The contribution of donor and host cells in the thoracic duct lymph of the intermediate host was assessed by injecting 300×10^6 normal spleen cells

TABLE I

Adoptive Anti-DNP Response to DNP-DT Restored by Primed Spleen Cells

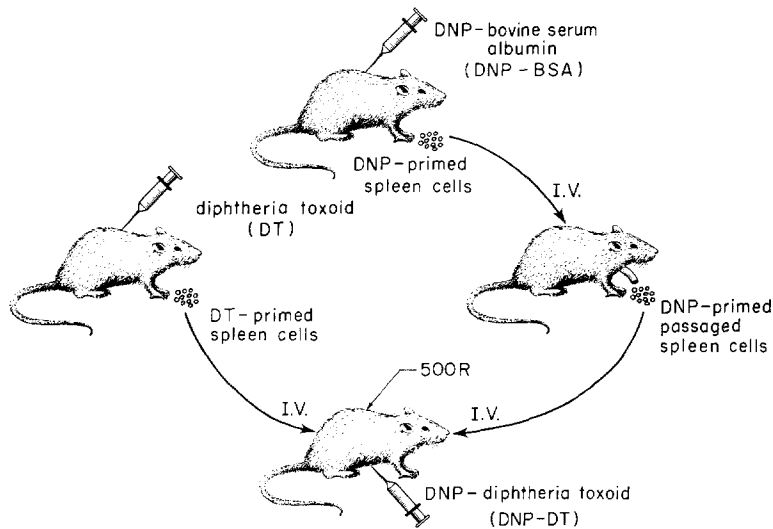| Cells primed to DT	Cells primed to DNP-BSA	Log$_{10}$ antibody titer		
		Day 7	Day 9	Day 14
50×10^6	–	0.06 ± 0.01	0.14 ± 0.02	0.05 ± 0.02
–	50×10^6	0.00	0.35 ± 0.22	0.45 ± 0.19
50×10^6	50×10^6	2.20 ± 0.12	2.47 ± 0.11	2.66 ± 0.14

Fig. 1. Experimental scheme. Lewis rats are immunized with DNP-BSA or DT. DNP-primed spleen cells are injected intravenously into an irradiated intermediate host and recovered in the thoracic duct lymph. The passaged DNP-primed cells and nonpassaged DT-primed cells are injected into an irradiated final host. Cell recipients are subsequently challenged with DNP-DT.

labeled in vitro with [³H]uridine into two irradiated rats. Thoracic duct cells were collected from the intermediate hosts as described above, and the percent labeled small lymphocytes in the donor spleen cell inocula and the percent labeled small lymphocytes in the pooled host thoracic duct cells were determined by counting 200–300 cells. An average of 59% labeled donor cells and 62% labeled host cells was observed in two experiments. This indicates that the contribution of host lymphocytes to the thoracic duct cells is negligible, providing that the donor small lymphocyte populations are labeled randomly (i.e., that the labeled and unlabeled cells do not have different migratory patterns).

In order to compare the relative activity of passaged hapten-primed cells to unpassaged cells, the anti-DNP response restored by graded numbers of unpassaged DNP-primed cells injected together with a constant number of DT-primed cells (50×10^6) was studied. Fig. 2 shows that there was a rapid decline in the anti-DNP response when the number of hapten-primed cells was decreased from 50 to 25×10^6 cells. However, there was little change in the response on further reduction of the cell number from 25 to 10×10^6 cells. A comparison of the responses restored by passaged and unpassaged DNP-primed cells (Fig. 2) shows that the former cells are at least two to three times more active than the latter, since the anti-DNP titers restored by 10×10^6 passaged cells at days 9 and 14 were significantly greater (as judged by the Student's t test, $P < 0.01$) than those restored by 25×10^6 unpassaged cells. Hapten-primed thoracic duct cells were also several fold more active than an equal number of unpassaged hapten-primed spleen cells (Fig. 2). The responses restored by 10×10^6 passaged spleen cells and 10×10^6 thoracic duct cells were similar at day 9, but the latter cells were superior at day 14.

Estimation of the Percent Recirculating Lymphocytes Present in the Spleen.—Approximately 1×10^9 thoracic duct cells from normal donors were labeled in vitro with [³H]uridine. The labeled cells were washed twice in M-199 and injected intravenously into a rat that had been irradiated 24 h before. The thoracic duct of the irradiated intermediate host was cannulated 24 h later, and cells were collected for 48 h. Each 12 hourly collection of cells was harvested by centrifugation and injected intravenously into a normal rat. The thoracic duct of the normal recipient was cannulated 72 h after the last injection of cells, and lymph was collected for 12 h. Immediately thereafter, the spleen was removed, and both the thoracic duct cells and spleen cells were harvested and smeared for autoradiography. After exposure for 28 days, the percentage of labeled nucleated cells was determined (2,000 cells counted). Cells containing four or more grains were considered positive. The percent labeled cells in the thoracic duct lymph was 2.1% and the percent labeled cells in the spleen was 1.3%. If the thoracic duct lymph is assumed to contain only recirculating cells, then the estimate of recirculating cells in the spleen is about 60% (1.3/2.1).
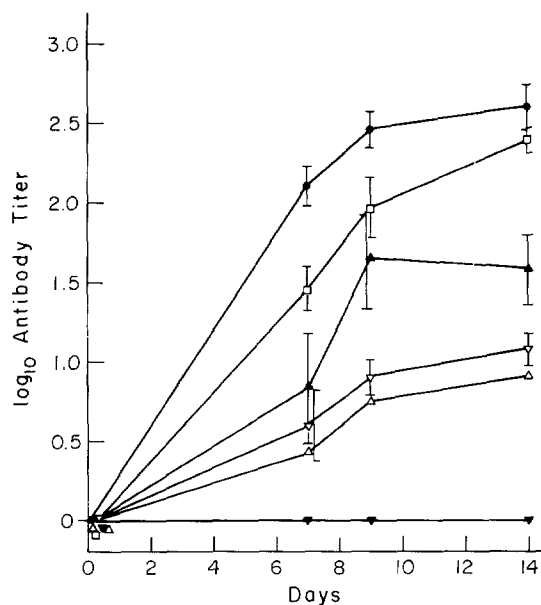
Fig. 2. Adoptive anti-DNP response of rats to a single intraperitoneal injection of 0.5 mg of DNP-DT. All rats were given 50×10^6 nonpassaged DT-primed spleen cells and graded numbers of DNP-primed spleen or thoracic duct cells intravenously 2 h after 500 R whole body X-irradiation. DNP-DT was given 22 h later. Each point represents the mean response of groups of from four to seven rats with brackets showing the standard error of the mean. Responses are expressed as $\log_{10}$ titer that bound 33% of labeled antigen. ●—●, mean response of rats given 50×10^6 nonpassaged DNP-primed spleen cells; ▽—▽, mean response of rats given 25×10^6 nonpassaged spleen cells; △—△, mean response of rats given 10×10^6 nonpassaged spleen cells; ▲—▲, mean response of rats given 10×10^6 passaged spleen cells; □—□, mean respons of rats given 10×10^6 nonpassaged thoracic duct cells; ▼—▼, mean response of rats given 50×10^6 nonpassaged spleen cells with no subsequent challenge with DNP-DT.

Effect of Prolonged Thoracic Duct Drainage on the Activity of Hapten-Primed Spleen Cells.—The thoracic duct of two rats immunized to DNP-BSA 6 wk earlier was cannulated and the lymph was allowed to drain for 5 days. The thoracic duct cannula was removed on the 5th day and the animals were returned to their cages. Spleen cells were collected from the drained rats after a 48 h rest and injected together with DT-primed cells into irradiated rats. The cell recipients were challenged with DNP-DT as in previous experiments. Fig. 3 shows that thoracic duct drainage produced at least a 25-fold decrease in the anti-DNP response restored by DNP-primed spleen cells, since 2×10^6 cells from untreated donors produced a more vigorous response than 50×10^6 cells from drained donors.

Effect of [³H]Thymidine on the Activity of Hapten-Primed Spleen Cells.—The rate of formation (turnover rate) of memory cells in the spleen was studied by treating rats immunized to DNP-BSA with [³H]thymidine before cell trans-
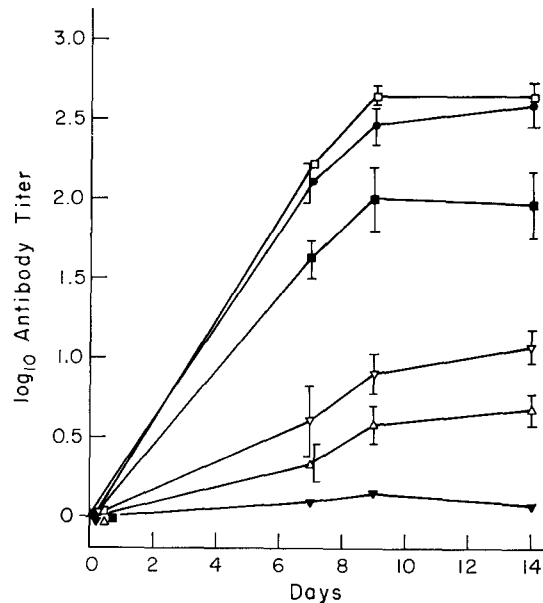
Fig. 3. Adoptive anti-DNP response of rats to a single intraperitoneal injection of 0.5 mg of DNP-DT. All rats were given 50×10^6 untreated DT-primed spleen cells and graded numbers of DNP-primed cells intravenously 2 h after 500 R whole body X-irradiation. DNP-DT was given 22 h later. Each point represents the mean response of groups of four to seven rats and brackets show the standard of the mean. ●—●, mean response of rats given 50×10^6 untreated DNP-primed spleen cells; ▽—▽, mean response of rats given 25×10^6 untreated cells; △—△, mean response of rats given 2×10^6 untreated cells; ▼—▼, mean response of rats given 50×10^6 cells obtained after 5 days of thoracic duct drainage; □—□, mean response of rats given 50×10^6 cells from donors treated with ALS; ■—■, mean response of rats given 50×10^6 cells from donors treated with [³H]thymidine.

fer. Approximately 6 wk after immunization, 125–150-g donors were given intraperitoneal injections of [³H]thymidine (New England Nuclear; specific activity 20 Ci/mmol) in aqueous solution every 8 h for a period of 48 h before removal of the spleen. Each dose of [³H]thymidine contained 3.5 mCi in 3.5 ml of sterile water (24.5 mCi total dose).

An inoculum of 50×10^6 spleen cells from the treated donor was injected together with 50×10^6 untreated DT-primed spleen cells into irradiated hosts as described before. In order to minimize reutilization of the radioactive label, each host received a single intraperitoneal injection of cold thymidine (1 ml, 10^{-4} M thymidine in water) within a few hours after cell transfer. Cold thymidine was also added to the drinking water (10^{-4} M) for the remainder of the experiment. DNP-DT was administered to each host 24 h after irradiation. Fig. 3 shows that the response restored by 50×10^6 [³H]thymidine-treated spleen cells was decreased as compared with that restored by an equal number of

untreated cells. However, the decrease was less than twofold, since the treated cells produced a significantly greater ($P < 0.01$) response than that produced by 25×10^6 untreated DNP-primed spleen cells.

Restoration of the Anti-DNP Response with Passaged or [³H]Thymidine-Treated Carrier-Primed Cells.—The migratory properties and rate of formation of carrier-primed cells were studied by comparing the restorative activity of passaged or [³H]thymidine-treated cells with that of untreated cells from DT-primed donors. Fig. 4 shows the anti-DNP response restored by graded numbers of untreated DT-primed spleen cells injected together with a constant number of DNP-primed cells. The response produced by 10×10^6 DT-primed cells was not significantly different ($P > 0.05$) from that produced by 50×10^6 cells. However, a sharp decline in the response was observed when the number of carrier-primed cells was reduced to 2×10^6 (Fig. 4).
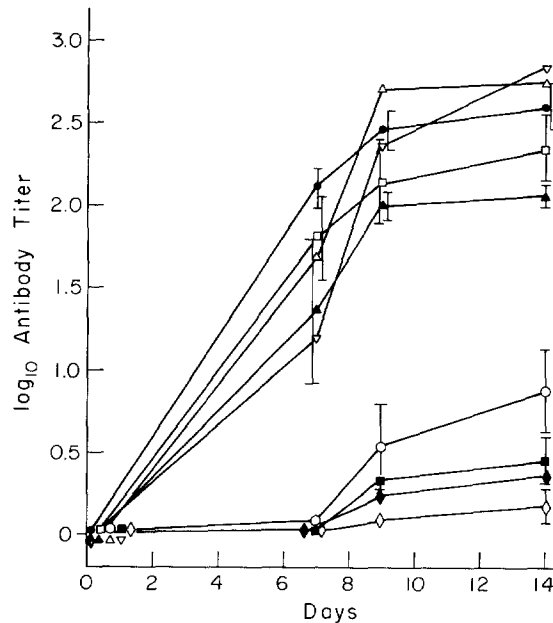

Fig. 4. Adoptive anti-DNP response of rats to a single intraperitoneal injection of 0.5 mg of DNP-DT. All rats were given 50×10^6 untreated DNP-primed spleen cells and graded numbers of DT-primed spleen cells intravenously 2 h after 500 R whole body X-irradiation. DNP-DT was given 22 h later. Each point represents the mean response of groups of four to seven rats and brackets show the standard error of the mean. ●--●, mean response of rats given 50×10^6 untreated DT-primed spleen cells; □—□, mean response of rats given 10×10^6 untreated cells; O--O, mean response of rats given 2×10^6 untreated cells; ■—■, mean response of rats given no DT-primed cells; △—△, mean response of rats given 10×10^6 passaged DT-primed spleen cells; ▲—▲, mean response of rats given 50×10^6 spleen cells from donors treated with ALS; ◆—◆, mean response of rats given 10×10^6 spleen cells from donors treated with ALS; ◇—◇, mean response of rats given 50×10^6 cells obtained after 5 days of thoracic duct drainage; ▽—▽, mean response of rats given 10×10^6 cells from donors treated with [³H]thymidine.

In several experiments, spleen cells from DT-primed donors were passaged through irradiated intermediate hosts using the protocol described for the passage of cells from hapten-primed donors. Although the mean anti-DNP response produced by 10×10^6 passaged cells was greater than that produced by 50×10^6 unpassaged cells at days 9 and 14, the differences were not significant ($P > 0.05$) (Fig. 4). The response restored by DT-primed spleen cells from donors drained of thoracic duct cells for 5 days was decreased at least 25-fold, since 50×10^6 DT-primed cells from drained donors restored a response that was below that restored by 2×10^6 DT-primed cells from untreated donors (Fig. 4).

The effect of treatment of DT-primed donors with [^{3}H]thymidine (24.5 mCi) for a period of 48 h before removal of the spleen is also shown in Fig. 4. The response restored by 10×10^6 treated spleen cells did not differ significantly ($P > 0.05$) from that restored by an equal number of untreated cells.

Differential Effect of ALS on Carrier- and Hapten-Primed Spleen Cells.—In several experiments, 1 ml of ALS was injected intraperitoneally into rats immunized to DT or DNP-BSA 4–8 wk earlier. Spleen cells from the carrier- or hapten-primed donors were removed 24 h later and injected together with untreated hapten- or carrier-primed cells respectively into irradiated hosts. Cell recipients were subsequently challenged with DNP-DT. Fig. 3 shows that 50×10^6 treated and 50×10^6 untreated DNP-primed spleen cells restored almost identical anti-DNP responses when injected with 50×10^6 untreated DT-primed cells. On the other hand, the response produced by a combination of 50×10^6 untreated DNP-primed cells and 50×10^6 treated DT-primed cells was significantly decreased ($P < 0.01$) as compared with that produced when 50×10^6 untreated DT-primed cells were used (Fig. 4). The mean antibody titers restored by the former cell combination fell below that restored by 10×10^6 untreated DT-primed cells at days 7, 9, and 14 (Fig. 4). A more striking difference was observed when the responses restored by 10×10^6 ALS-treated DT-primed cells were compared to those restored by 10×10^6 untreated DT-primed cells (Fig. 4).

Cytotoxic Action of Rabbit Antirat B Cell Serum (RARBS) on Thoracic Duct Cells and Thymocytes.—The cytotoxic action of RARBS was tested in an in vitro assay using thoracic duct cells and thymocytes from normal rats, and thoracic duct cells from rats that were thymectomized within 24 h of birth as target cells. Fig. 5 shows that the antiserum killed 80–90% of thoracic duct cells from neonatally thymectomized rats up to a dilution of 1:640. Thereafter, a sharp decline in the percent cytotoxicity was observed. Thymocytes were not killed by the antiserum. A plateau (approximately 40% cytotoxicity) was observed at dilutions 1:10 to 1:160 when the antiserum was titered against normal thoracic duct cells.

Effect of RARBS on Carrier- and Hapten-Primed Thoracic Duct Cells.—We have previously shown that carrier- and hapten-primed thoracic duct cells cooperate in the restoration of the anti-DNP response to DNP-DT (6, 7). In
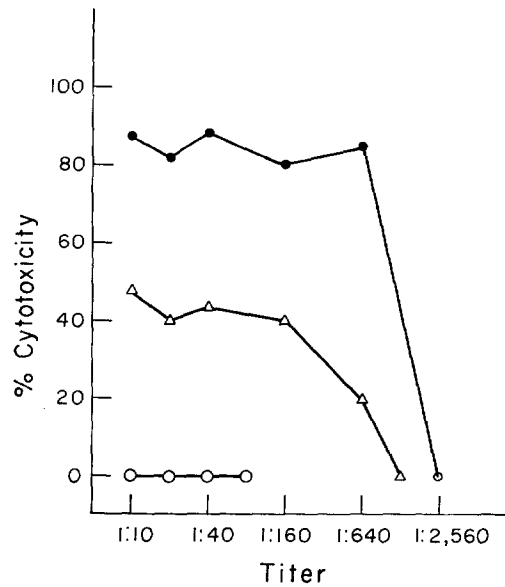
Fig. 5. Cytotoxic action of rabbit antirat B cell serum (RARBS) on thoracic duct cells and thymocytes. ●—●, thoracic duct cells obtained from neonatally thymectomized rats; △—△, thoracic duct cells obtained from normal rats; ○—○, thymocytes obtained from normal rats.

order to determine whether the former cells are T lymphocytes and the latter cells are B lymphocytes, we tested the sensitivity of hapten- and carrier-primed thoracic duct cells to the cytotoxic action of RARBS. Fig. 6 a shows the anti-DNP response restored by 50×10^6 untreated DT-primed thoracic duct cells injected together with 25, 10, or 2×10^6 DNP-primed thoracic duct cells treated in vitro with NRS. Although no significant difference was observed in the response restored by 50 and 10×10^6 DNP-primed cells, a marked decrease in the response was noted when the number of DNP-primed cells was reduced to 2×10^6. The anti-DNP response restored by 50×10^6 untreated DT-primed cells and 25×10^6 DNP-primed cells treated in vitro with RARBS is also shown in Fig. 6 a. No detectable antibody titers were observed at days 7, 9, and 14.

Fig. 6 b shows the antibody response restored by 25 or 10×10^6 DT-primed cells treated in vitro with NRS combined with 25×10^6 untreated DNP-primed cells. The titers produced by 10×10^6 DT-primed cells were significantly different from those produced by 25×10^6 DT-primed cells at days 7 and 10. Treatment of the DT-primed spleen cells with RARBS produced a slight reduction in the anti-DNP response at day 7, but by day 14 there was no significant difference between the titers produced by the treated and untreated cells.
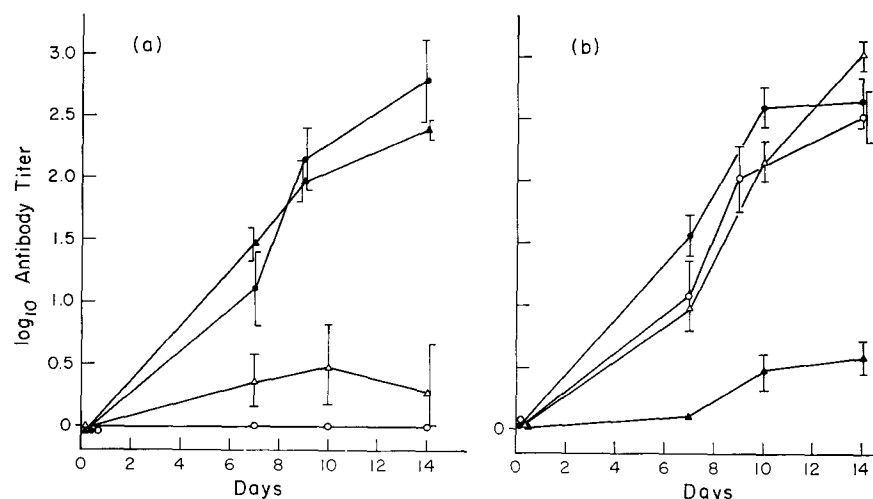
FIG. 6. Adoptive anti-DNP response of rats given an intraperitoneal injection of 0.5 mg of DNP-DT. All rats were given a combination of DT and DNP-primed thoracic duct cells 2 h after 500 R whole body X-irradiation. DNP-DT was given 22 h later. Each point represents the mean of groups of four to seven rats with brackets showing the standard error of the mean. (a) Effect of RARBS on hapten-primed cells. ●—●, mean response of rats given 50 × 10⁶ untreated DT-primed cells and 25 × 10⁶ DNP-primed cells treated in vitro with NRS; ▲—▲, mean response of rats given 50 × 10⁶ untreated DT-primed cells and 10 × 10⁶ DNP-primed cells treated in vitro with NRS; △—△, mean response of rats given 50 × 10⁶ untreated DT-primed cells and 2 × 10⁶ DNP-primed cells treated in vitro with NRS; ○—○, mean response of rats given 50 × 10⁶ untreated DT-primed cells and 25 × 10⁶ DNP-primed cells treated in vitro with RARBS. (b) Effect of RARBS on carrier-primed cells. ●—●, mean response of rats given 25 × 10⁶ DT-primed cells treated in vitro with NRS and 25 × 10⁶ untreated DNP-primed cells; △—△, mean response of rats given 10 × 10⁶ DT-primed cells treated in vitro with NRS and 25 × 10⁶ untreated DNP-primed cells; ▲—▲, mean response of rats given 25 × 10⁶ untreated DNP-primed cells; ○—○, mean response of rats given 25 × 10⁶ DT-primed cells treated in vitro with RARBS and 25 × 10⁶ untreated DNP-primed cells.

DISCUSSION

Gowans and Uhr (8) showed that small lymphocytes found in the thoracic duct lymph of the rat carry immunological memory. These investigators suggested that a second population of memory cells that is relatively fixed and does not recirculate from the blood to the lymph is present in the solid lymphoid tissues such as the spleen. We have recently examined the origin, lifespan, and migratory characteristics of thoracic duct lymphocytes that carry immunological memory to the hapten-protein conjugate DNP-DT (6, 7). Our findings indicate that both T and B memory cells present in the thoracic duct lymph of the rat are long-lived, recirculating lymphocytes. The object of the present study was to determine whether a population of nonrecirculating, short-lived, memory cells is present in the spleen.

The adoptive secondary antibody response to DNP-DT was used to investigate the nature of splenic memory cells in the rat. The experimental findings presented here show that hapten (DNP-BSA) and carrier (DT)-primed spleen cells obtained from donors 4–8 wk after immunization act synergistically in the restoration of the anti-DNP response of sublethally irradiated (500 R) rats to DNP-DT. The mean antibody titers restored by a combination of both types of cells were approximately 100-fold greater than the sum of the mean titers restored by either type of cell independently. Cooperation between hapten- and carrier-primed spleen cells in the adoptive antibody response of mice to 4-hydroxy-5-iodo-3-nitrophenacetyl-ovalbumin (NIP-OA) has been reported previously by Mitchison (3, 4).

The migratory characteristics of hapten-primed spleen cells were examined by injecting DNP-primed cells intravenously into a sublethally irradiated intermediate host and collecting the thoracic duct cells 24 h later. The passaged cells (i.e. those that migrated from the blood to the lymph) were injected together with large numbers of nonpassaged DT-primed cells into irradiated final hosts that were challenged with DNP-DT. The anti-DNP response restored by this combination of cells was compared with that restored by graded numbers of nonpassaged DNP-primed spleen cells injected together with a large number of DT-primed spleen cells. A similar experimental protocol was used to examine the migratory properties of carrier-primed cells.

An examination of the responses restored by graded numbers of nonpassaged hapten- and carrier-primed cells shows that there is a superabundance of the latter cells relative to the former cells in the rat spleen. A sharp decline in the adoptive anti-DNP response was observed when the number of hapten-primed cells was reduced from 50 to 25×10^6. On the other hand, a reduction in the number of carrier-primed cells from 50 to 10×10^6 did not significantly decrease the anti-DNP response. However, a significant decline in the response was noted when the number of carrier-primed cells was reduced to 2×10^6.

The anti-DNP response restored by passaged hapten-primed spleen cells was at least 2.5-fold greater than that restored by an equal number of nonpassaged cells in the limited range of cell doses tested. This shows that at least some spleen cells that carry immunological memory to the hapten are able to recirculate from the blood to the lymph. Further experiments showed that hapten-primed thoracic duct cells as well as the passaged hapten-primed spleen cells are more efficient in restoring the anti-DNP response than nonpassaged spleen cells. Recirculating cells, therefore, contain a greater proportion of hapten-primed (B) memory cells than nonrecirculating cells.

Could the recirculating memory cells account for all the memory cells in the spleen? This point was investigated by calculating the percentage of recirculating cells in the rat spleen. Thoracic duct cells were labeled in vitro with [³H]uridine, passaged through an irradiated intermediate host, and injected into a normal rat. The percentage of labeled cells in the thoracic duct lymph

and the spleen of the normal host was examined 3 days later. The ratio of labeled cells in the lymph to labeled cells in the spleen was 2.1:1.3. If one assumes that the thoracic duct cells of the normal host are made up entirely of recirculating cells, then the estimate of recirculating cells in the spleen is about 60%. This figure is in reasonable agreement with that of 57–71% calculated by Ford (16) in his studies of the kinetics of lymphocyte migration in the isolated perfused rat spleen. These findings suggest that recirculating cells could account for the anti-DNP response restored by hapten-primed spleen cells, since the recirculating cells are two to three times more efficient than the unfractionated cells and make up over half of the spleen cell population.

If, in fact, all splenic memory cells are recirculating lymphocytes, then one should be able to withdraw hapten-primed memory cells from the spleen by prolonged thoracic duct drainage. Accordingly, several experiments were performed in which the thoracic duct of rats immunized to DNP-BSA was cannulated and the lymph was allowed to drain for 5 days. Spleen cells from the drained donor were injected together with DT-primed cells into irradiated hosts that were challenged with DNP-DT. The anti-DNP response restored by cells from the drained donors was reduced at least 25-fold as compared with that restored by an equal number of hapten-primed cells from untreated donors. This suggests that the large majority of hapten-primed memory cells in the spleen can be mobilized into the thoracic duct lymph within 5 days. The contribution of "fixed" memory cells to the adoptive anti-DNP response is, therefore, quite small as compared with that of the recirculating cells.

Similar studies of the migratory patterns of carrier-primed spleen cells show that passaged DT-primed cells are as efficient as unpassaged cells in restoring the antibody response to DNP-DT. In addition, the response restored by carrier-primed cells obtained from rats after prolonged thoracic duct drainage is reduced at least 25-fold as compared with that restored by cells from untreated donors. This indicates that the large majority, if not all, T and B memory cells in the spleen are recirculating lymphocytes.

These results are not inconsistent with the findings of Gowans and Uhr (8). The present report examined the effect of thoracic duct drainage on the ability of a limited number of primed spleen cells to restore the adoptive anti-DNP response. Comparisons were made on a cell dose vs. response basis. The report of Gowans and Uhr examined the effect of thoracic duct drainage on the anamnestic response of the intact rat to bacteriophage ϕx174. The pool of recirculating lymphocytes in an intact adult rat contains approximately 1.75×10^9 cells (17). If 95% of the recirculating pool were withdrawn from the phage-immunized rat, then approximately 90×10^6 recirculating cells would remain. The residual cells would be capable of producing a vigorous secondary response to ϕx174, since 20×10^6 thoracic duct cells from immunized donors restore a vigorous adoptive response in irradiated hosts (8).

Recent studies by Gowans and his colleagues (18) show that spleen cells

obtained from rats immunized to tetanus toxoid and depleted of thoracic duct cells by prolonged thoracic duct drainage are able to restore the adoptive secondary response of irradiated hosts. These investigators raised the possibility that the active cells are residual recirculating small lymphocytes, since the restorative activity of the splenic small lymphocytes purified by velocity sedimentation was equal to that of the unfractionated spleen cells. Further evidence for this point was obtained from experiments in which the adoptive secondary response of irradiated rats reconstituted with toxoid-primed thoracic duct cells was unaffected by chronic thoracic duct drainage (19). The findings of Gowans and his colleagues are, therefore, consistent with the present finding that re- circulating memory cells can account for the large majority, if not all, of the memory cells in the spleen.

The vigorous anamnestic response of thoracic duct cell-depleted rats can also be explained by the presence of antigen depots in the regional nodes draining the site of primary immunization. Persistent antigen may recruit and trap recirculating memory cells in these nodes and slowly stimulate the formation of new memory cells. Studies of the regional nodes draining the site of *Salmonella* flagellin injections in rats by Miller and Koskimies (20) provide some evidence for the latter point. The present report did not study the memory cells in the regional nodes in order to avoid the complicating factors of cell trapping and stimulation by antigen depots. The present results are, therefore, pertinent to those solid lymphoid tissues that do not drain the sites of primary immunization.

In order to determine the effect of ALS on hapten- and carrier-primed spleen cells, DNP-BSA or DT-primed donors received a single injection of 1 ml of ALS 24 h before cell transfer. Untreated hapten- or carrier-primed cells were injected together with treated DT or DNP-primed cells respectively into irradiated hosts that were challenged with DNP-DT. Although ALS has no effect on the anti-DNP response retored by hapten-primed cells, a fivefold reduction in the restorative action of carrier-primed cells was observed after ALS treatment. Mitchison reported a similar differential effect of ALS on hapten- and carrier-primed cells in the mouse (3). These findings indicate that ALS is able to specifically eliminate T but not B recirculating lymphocytes. The different migratory pathways (21, 22) and kinetics of recirculation (23) of T and B memory cells probably contribute to the different sensitivities of the two cell populations to the action of ALS in vivo.

The turnover rate (rate of formation) of hapten- and carrier-primed spleen cells was investigated by injecting DNP-BSA and DT-primed donors with 3.5 mCi of [³H]thymidine (specific activity 20 Ci/mmol) every 8 h for 48 h before removal of the spleen (~25 mCi, total dose). [³H]Thymidine was used to "suicide" rapidly dividing cells. We have previously shown that this dose of [³H]thymidine produces a 20–100-fold decrease in the ability of thoracic duct

cells from unimmunized rats to restore the adoptive primary response to horse spleen ferritin in irradiated hosts (7). However, only a slight reduction (<2-fold) was noted in the anti-DNP response restored by [³H]thymidine-treated hapten-primed cells in the present study, and no significant reduction was noted in the response restored by treated carrier-primed cells. The slight reduction observed with the former cells may have been due to reutilization of the radioactive label, since we have obtained enhanced adoptive secondary responses to ferritin when donors were treated in vivo with vinblastine, a nonreutilizable mitotic inhibitor (24). These findings indicate that the majority of T and B memory cells or memory cell precursors do not divide within 48 h and are turning over at a considerably slower rate than B lymphocytes involved in the adoptive primary response (7).

Although we have assumed that hapten-primed cells are B lymphocytes and that carrier-primed cells are T lymphocytes, we have not formally proven this point. The availability of rabbit antirat B cell serum (RARBS) (kindly supplied by Dr. J. C. Howard, Cellular Immunology Unit, Sir William Dunn School of Pathology) allowed us to investigate the lineage of DT and DNP-primed cells. The specificity of RARBS was first tested in an in vitro cytotoxicity assay using thymocytes and thoracic duct cells from normal rats, and thoracic duct cells from neonatally thymectomized rats as target cells. The antiserum killed 0% of thymocytes, approximately 40% of normal thoracic duct cells, and 80–90% of thoracic duct cells from thymectomized donors. This suggests that about 40% of normal thoracic duct cells are B lymphocytes. This figure is somewhat higher than that observed in mice (25), but was similar to the percentage of immuno-globulin-bearing cells (35%) detected in normal thoracic duct lymph by an indirect immunofluorescent technique (S. Strober, unpublished observations).

The effect of RARBS on hapten- and carrier-primed cells was determined by incubating DT or DNP-primed thoracic duct cells in vitro with RARBS before injection into irradiated hosts. Untreated DNP or DT-primed cells were transferred together with treated cells and the recipients were challenged with DNP-DT. The anti-DNP response restored by treated hapten-primed cells was at least 10-fold less than that restored by untreated cells. On the other hand, no significant effect was observed on the adoptive response restored by treated carrier-primed cells. This suggests that in rats as well as mice (5), hapten-primed cells are B lymphocytes and carrier-primed cells are T lymphocytes.

Although the genesis of T and B memory cells was not examined in this report, the studies of Wakefield and Thorbecke (26, 27) provide evidence that some of these cells may be generated in the white pulp of the spleen within the 1st 2 wk after priming. These investigators observed the adoptive antibody response to sheep red blood cells restored by white pulp cells in irradiated mice. Cells obtained from donors 9–14 days after immunization to SRBC produced

an adoptive response that increased as the lag time between cell transfer and antigenic challenge increased. No increase was observed when a single pulse of the mitotic inhibitor, bromodeoxyuridine, was given to recipients immediately after cell transfer, but before antigenic challenge. These results strongly suggest that new memory cells were generated in the irradiated host by rapidly dividing donor cells. Similar studies using white pulp cells obtained from donors 4 wk after immunization showed no increase in the adoptive antibody response with increasing lag time and no effect of bromodeoxyuridine on the adoptive response. This indicates that the rapidly dividing generative compartment in the white pulp of the spleen is short lived, and that the large majority of memory cells obtained 4 wk after immunization are turning over very slowly (long lived).

The experimental findings presented herein indicate that both T and B memory cells are relatively long-lived, recirculating lymphocytes; however, our previous report indicates that virgin B lymphocytes are nonrecirculating cells that are turning over at least once every 48 h (7). This suggests that the interaction with antigen changes the virgin B lymphocyte from a short-lived, nonrecirculating cell to a long-lived, recirculating memory B cell. The relevance of these changes to theories of the generation of diversity of antibody molecules and selection of clones of memory cells by antigen has been discussed elsewhere (7).

SUMMARY

The adoptive secondary antibody response of rats to the hapten-protein conjugate dinitrophenyl-diphtheria toxoid (DNP-DT) was used to investigate the migratory properties and rate of formation of T and B memory cells in the spleen. The experimental findings show that hapten (DNP-BSA)- and carrier (DT)-primed spleen cells act synergistically in the restoration of the adoptive anti-DNP response. Passage of both hapten- and carrier-primed spleen cells through an intermediate host (intravenous injection and subsequent collection in the thoracic duct lymph) showed that both cell types are able to recirculate from the blood to the lymph. In addition, memory to the hapten or carrier could be withdrawn from the spleen by prolonged thoracic duct drainage.

The rate of formation of hapten- and carrier-primed spleen cells was studied by treating donors with [^{3}H]thymidine for 48 h before cell transfer in an attempt to "suicide" rapidly dividing cells. Only a slight reduction in the adoptive response to the hapten or carrier was noted upon transfer of treated cells to irradiated hosts. In further experiments, the cell lineage of hapten- and carrier-primed cells was determined by treating each cell type in vitro with rabbit antirat B cell serum (RARBS) and complement. Although treatment with RARBS did not affect the adoptive response restored by carrier-primed cells, the same treatment abolished the response restored by hapten-primed cells.

These findings indicate that T and B memory cells in the spleen of the rat are relatively long-lived, recirculating lymphocytes. The contribution of fixed

or rapidly turning over cells to immunological memory is small or negligible as compared with the latter cells.

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