IDENTIFICATION OF MARROW-DERIVED AND THYMUS-DERIVED SMALL LYMPHOCYTES IN THE LYMPHOID TISSUE AND THORACIC DUCT LYMPH OF NORMAL RATS

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All the specific radiosensitive cells required for the induction and expression of the primary antibody response to sheep erythrocytes in the rat are normally present among the small lymphocytes in thoracic duct lymph (1, 2). Experiments with radiation chimeras have now made it clear that the activity of thoracic duct cells in this response is due to the presence in rat lymph of two populations of small lymphocytes, one derived from the thymus (T lymphocytes)[1] and the other derived from the bone marrow without thymic influence (B lymphocytes).[2] Thymus-marrow collaboration in the rat, first reported by Johnston and Wilson (3), is similar to that originally described in mice by Mitchell and Miller (4).

The present work extends the earlier findings on radiation chimeras by demonstrating that thymus- and marrow-derived small lymphocytes are present in the thoracic duct lymph of normal rats, and establishes three criteria by which the two populations may be distinguished. These are: (*a*) a marked deficiency of uridine incorporation in vitro by B lymphocytes relative to T lymphocytes; (*b*) a small difference in the rate of sedimentation; (*c*) a physiological segregation after transfusion into the blood, into clearly distinct zones within the spleen, lymph nodes, and Peyer's patches, which define the areas through which the two cell populations recirculate.

Materials and Methods

Animals.—Young adult male and female rats of the inbred HO and AO strains were used in this study.

Collection from the Thoracic Duct of Pure Marrow-Derived Lymphocytes and Artificial Mixtures of Marrow-Derived and T Lymphocytes.—Adult rats were thymectomized, irradiated with 1000 rads from a ^{60}Co source, and then injected intravenously with 10^7 bone marrow cells from

[1] *Abbreviations used in this paper:* B lymphocytes, peripheral bone marrow-derived small lymphocytes; DAB, Dulbecco's A + B (buffered salt solution); D/E/FCS, fetal calf serum in Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; T lymphocytes, peripheral thymus-derived small lymphocytes.

[2] Scott, D. W., and J. C. Howard. Collaboration between thymus-derived and marrow-derived thoracic duct lymphocytes in the hemolysin response of rat. *Cell Immunol.* In press.

lymphocyte-depleted, syngeneic donors (5). Pure marrow-derived lymphocytes were obtained from the thoracic duct of these B rats 1–3 months after irradiation and marrow reconstitution. The small lymphocytes, which constituted about 75% of the total population of these marrow-derived lymphocytes will be referred to throughout this paper as B lymphocytes. Fuller details of these procedures are given in the previous paper (5). Artificial mixtures of marrow-derived and T lymphocytes were obtained from lymph by first preparing B rats, injecting them intravenously 14–28 days later with 10^9 dissociated thymocytes, and cannulating their thoracic ducts 1–4 wk later still.[2]

Fractionation of Lymphocytes by 1 g Sedimentation.—This technique, in which cells are allowed to sediment through a shallow density gradient for a few hours, separates cells primarily on the basis of differences in size and to some extent on differences in density (6). 10–15 ml of washed thoracic duct lymphocytes, suspended at a concentration of 10^7/ml in buffered salt solution (DAB, Dulbecco's A + B, Oxoid Ltd., London) containing 100 μg/ml streptomycin, 200 units/ml penicillin, and 0.2% (w/v) bovine serum albumin, were allowed to sediment at 4°C for 8–9 hr through a "buffered step" ⅓–2% gradient of bovine serum albumin in DAB. 15-ml fractions were collected by upward displacement. Details and a discussion of the method have been published elsewhere (7).

In experiments where cells were labeled with radioactive uridine after sedimentation, 30, 5, and 3% (v/v) fetal calf serum (FCS) replaced the 2, ⅓, and 0.2% bovine serum albumin, respectively, and all dilutions were made in Dulbecco's (D) modified Eagle's (E) medium instead of DAB. 1.0-ml aliquots of the fractions in this D/E/FCS could then be labeled directly without further treatment.

Radioactive Labeling of Lymphocytes In Vitro.—

Preparative labeling: Lymphocyte suspensions for intravenous transfusion or for separation by 1 *g* sedimentation were labeled in vitro with uridine-5-^{3}H or uridine-^{14}C (U) (450 μCi/mM) (Radiochemical Centre, Amersham, England) as described in the previous paper (5).

In one experiment thoracic duct lymphocytes were labeled both with uridine-^{14}C and sodium chromate-^{51}Cr. The suspension containing 2.5×10^7 cells/ml was labeled for 30 min with uridine-^{14}C at 0.5 μCi/ml, after which sodium chromate-^{51}Cr was added to a final concentration of 25 μCi/ml and incubation continued for a further 60 min. An aliquot of cells from the same suspension labeled continuously with uridine-^{14}C for 90 min showed that sodium chromate did not interfere with uridine uptake.

Analytical labeling: In one experiment it was necessary to compare the uptake of radioactive uridine in large numbers of cell fractions obtained after 1 *g* sedimentation. In these cases, cells were already suspended in D/E/FCS (see 1 *g* sedimentation) at concentrations varying between 10^5 and 1.5×10^6/ml. 1.0-ml aliquots of cell suspension were dispensed in triplicate into 10-ml disposable plastic round-bottomed centrifuge tubes. 0.01 ml of uridine-^{3}H was added to each tube from a Hamilton syringe to give a final concentration of 5 μCi/ml. Tubes were incubated under 5% CO_2 in air for 60 min with shaking every 15 min. Cell suspensions from each tube were then deposited on Millipore membrane filters (Millipore Corp., Bedford, Mass.) and washed exhaustively with phosphate-buffered saline (PBS, Dulbecco A, Oxoid Ltd.). The volume of the wash was that required to reduce the membrane-associated counts in the absence of cells to a constant level of about 200 cpm.

Scintillation Counting of Lymphocyte Suspensions.—Lymphocyte suspensions from sedimentation experiments were digested by adding 0.1 ml 10 N NaOH to 1 ml of a suspension containing a known number of cells and incubating for 15 min at 80°C. The pH was adjusted with 0.2 ml 10 N HCl and 15 ml of Triton X-100-toluene scintillant (Rohm and Haas Co., Philadelphia, Pa.) added to each sample. Other labeled samples were prepared for counting as described in the accompanying paper (5). ^{14}C and ^{51}Cr were counted simultaneously in a liquid scintillation spectrometer with appropriate spillover corrections.

Radioautography.—Lymphoid tissues for radioautography were fixed in formol-alcohol for 12 hr. Paraffin sections at 4 μ were dipped in Ilford K5 emulsion (Ilford Ltd., Ilford, Essex, England), exposed for 2–8 wk, and stained through the emulsion with Unna-Pappenheim. Dark-ground illumination was used for most of the photomicrographs since it gives, optically, a strikingly increased yield of radioautographic grains.

Counting of Labeled Cells in Histological Sections.—To analyze the distribution of radioactively labeled cells in the spleen, well-marked regions of white pulp showing both periarteriolar and follicular lymphocyte aggregations were chosen at low magnification by transmitted light. Two photographs were taken of each region of white pulp at higher magnification, one by transmitted light, the other by dark-ground illumination. After coding the photographs, periarteriolar and follicular areas were marked out on transmitted light exposures in which labeled cells were not visible, and traced on to the corresponding coded dark-ground exposure where labeled cells could be seen clearly. Labeled cells were then counted in the periarteriolar and follicular regions by two observers independently and the individual values were extracted from the paired scores. To allow for variation in the relative areas of periarteriolar and follicular tissue in successive fields, the marked regions were cut out, weighed, and the concentration of labeled cells expressed as a density per unit area of follicular or periarteriolar tissue.

RESULTS

Distribution of Uridine-^{3}H–Labeled Lymphocytes after Intravenous Transfusion: Definition of Two Subpopulations.—The route of recirculation of rat lymphocytes was defined by Gowans and Knight (8) by transfusing thoracic duct cells which had been labeled in vitro with adenosine-^{3}H. A wide variation was observed radioautographically in the amount of tritium incorporated into the RNA of individual small lymphocytes. After transfusion, heavily labeled small lymphocytes accumulated rapidly in the periarteriolar lymphocyte sheaths of the spleen and in the deep cortex of lymph nodes and this characteristic distribution was maintained for a long period.

In the present study the distribution of thoracic duct lymphocytes after transfusion was studied by first labeling their RNA in vitro with uridine-^{3}H. A radioautographic examination of the tissues of normal rats 48 hr after receiving 10^9 labeled thoracic duct lymphocytes from normal syngeneic donors showed intensely labeled cells in the traffic areas originally defined with the adenosine label but, in addition, areas of lymphoid tissue, previously unlabeled, showed numbers of lightly labeled cells (Figs. 1–5). These lightly labeled cells defined the so-called "thymus-independent" areas of lymphoid tissue which are not depleted by chronic thoracic duct drainage in the rat and which are unaffected in neonatally thymectomized animals (9, 10). They will be described in this study as the "follicular" areas of the splenic white pulp and lymph nodes. A follicular area normally consists of small lymphocytes arranged in an arc around the germinal center, and should not be confused with the "marginal" zone which completely encloses individual lymphatic nodules in the rat spleen and is rich in reticuloendothelial elements. Sometimes the germinal center may not be clearly defined, in which case the

follicular zone consists of an accumulation of small lymphocytes without a well-marked central area.

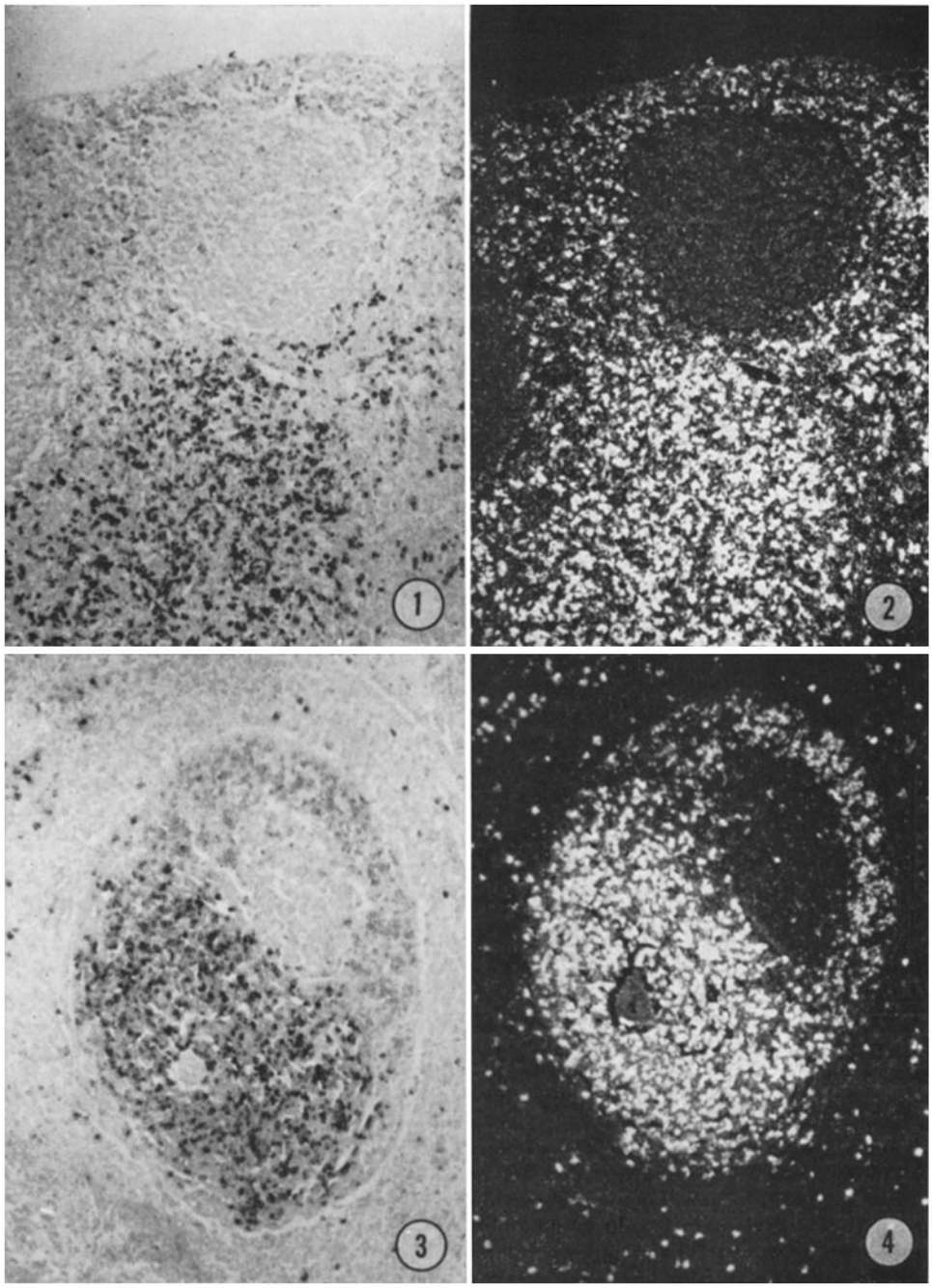
In the lymph nodes and spleen, the germinal centers themselves appear as unlabeled islands enclosed by lightly labeled cells (Figs. 1–4); in the nodes the area of light labeling often extends laterally from the follicle, beneath the marginal sinus. In Peyer's patches (Fig. 5) the germinal centers show a massive conical cap of lightly labeled cells pointed towards the mucosal surface, and are partly surrounded by a narrower strip of lightly labeled cells. Between the follicles there are restricted areas of heavily labeled cells, confirming the original observation that Peyer's patches are on the route of recirculation of lymphocytes as defined by adenosine labeling (8).

A careful reexamination of the original radioautographs of lymphoid tissue after transfusion of adenosine-^{3}H–labeled lymphocytes prepared in the experiments of Gowans and Knight (8) has revealed that there are indeed areas of lightly labeled cells corresponding to those illustrated in Figs. 1–5, but the cells are few in number and too weakly labeled to give a clear picture of their distribution.

These findings suggested that small lymphocytes from the thoracic duct are normally a mixture of two populations, one labeling heavily with RNA precursors, the other labeling lightly, and segregating into distinct areas of lymphoid tissue. The subsequent experiments aimed to show that the heavily and lightly labeled small lymphocytes in lymph correspond to thymus-derived and marrow-derived cells, respectively.

Uptake of Uridine by Normal Thoracic Duct Lymphocytes, by Marrow-Derived Lymphocytes, and by Artificial Mixtures of Marrow-Derived and T Lymphocytes.—Suspensions of pure marrow-derived thoracic duct lymphocytes and artificial mixtures of marrow-derived and T lymphocytes were labeled with uridine-^{3}H in vitro at the same time and under the same conditions as suspensions of thoracic duct lymphocytes from normal rats. Table I shows that the uptake of uridine-^{3}H by marrow-derived lymphocytes is consistently only 20–40% of the normal despite the presence of high proportions of large lymphocytes which incorporate large quantities of uridine. It can be seen in Fig. 13 that this high incorporation by large lymphocytes increases the radioactivity of the whole marrow-derived population to 2.5 times that of B small lymphocytes alone. The uridine uptake by artificial mixtures of marrow-derived and T lymphocytes was intermediate between marrow-derived and normal lymphocytes (Table I). Thus B small lymphocytes incorporate low quantities of uridine relative to lymphocytes from normal donors, and the intentional addition of T lymphocytes raises the level towards normal.

Distribution in Lymphoid Tissue of Uridine-^{3}H–Labeled B Lymphocytes and of Artificial Mixtures of B and T Lymphocytes.—Thoracic duct lymphocytes from B rats were labeled in vitro with uridine-^{3}H or uridine-^{14}C and transfused intravenously in doses of $1–2 \times 10^{8}$ into normal syngeneic re-



FIGS. 1–5. Radioautographs (RA) of tissues from a rat 48 hr after an intravenous injection of 10^9 normal thoracic duct lymphocytes which have been labeled in vitro with uridine-^{3}H. Heavily labeled small lymphocytes have localized in the areas already familiar as traffic zones for recirculation (8). In addition, lightly labeled small lymphocytes have migrated into follicular areas around germinal centers and are clearly seen in the dark ground views. (RA exposure: 42 days)

FIGS. 1 and 2. Cervical lymph node. Bright- and dark-field views of the same area. Heavily labeled cells in deep cortex; lightly labeled cells around germinal center. × 75.

FIGS. 3 and 4. Spleen. Bright- and dark-field views of the same area of white pulp. Heavily labeled cells in the periarteriolar region; lightly labeled cells around the germinal center. × 120.

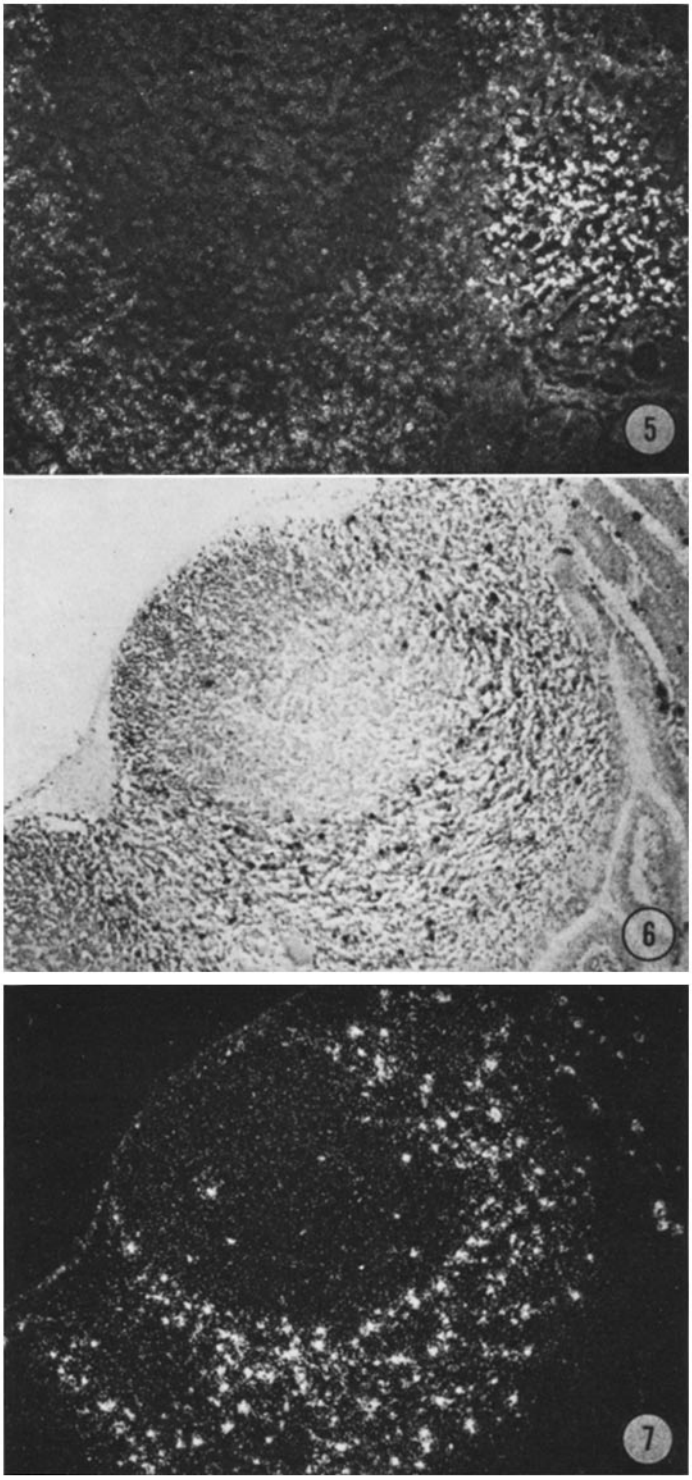
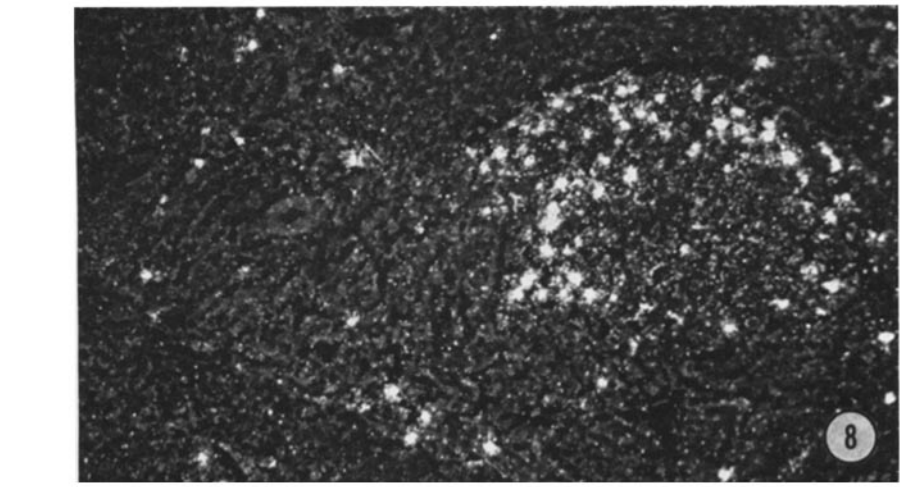
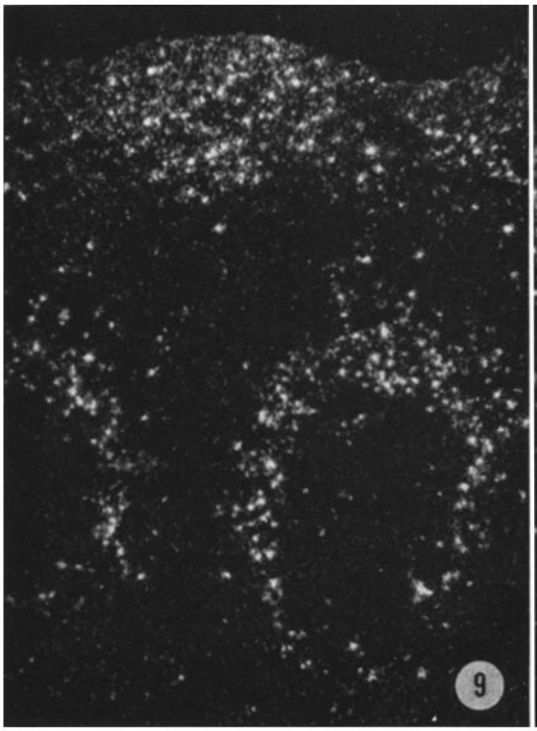
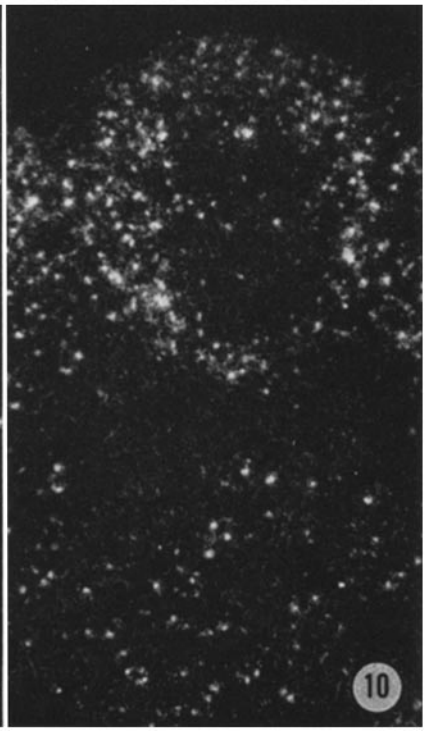


FIG. 5. Peyer's patch from lower ileum. Dark-ground view with mucosal surface below. A wide band of lightly labeled cells caps the germinal center. A small interfollicular zone is occupied by heavily labeled cells. × 120.

Legend to Figs. 6–7 on following page.







FIGS. 6–10. Radioautographs of tissues from a rat 24 hr after an intravenous injection of 2×10^8 marrow-derived lymphocytes labeled in vitro with uridine-^{14}C (lymphocytes from thoracic duct of irradiated, thymectomized rats restored with syngeneic marrow). The location of lightly labeled small lymphocytes is best seen in the dark-ground photographs.

FIGS. 6 and 7. Peyer's patch from lower ileum. Bright- and dark-fields of same area with mucosal surface at lower right. Labeled cells restricted to follicular zone around germinal center. The interfollicular area (*lower left*) lacks the concentration of heavily labeled cells seen in Fig. 5. (RA exposure: 103 days) × 75.

FIG. 8. Splenic white pulp. An arteriole at the center of the unlabeled periarteriolar sheath is clearly seen on the left. Labeled cells are concentrated in the follicular zone on the right. Contrast with Fig. 4 in which the periarteriolar region is packed with heavily labeled cells. (RA exposure: 64 days) × 120.

FIGS. 9 and 10. Cervical lymph nodes. Labeled cells can be seen in the dark-ground views around germinal centers in the deep (Fig. 9) and superficial (Fig. 10) cortical areas. The concentration of labeled cells below the marginal sinus in Fig. 9 is probably also follicular in location but the labeling extends laterally from it on either side in a band beneath the sinus. Contrast with Fig. 2 in which the deep cortex is packed with heavily labeled cells. (RA exposure: 103 days) × 75.

cipients which were killed for radioautographic study 24 hr later. The distribution of labeled B small lymphocytes in the spleen, nodes, and Peyer's patches (Figs. 6–10) contrasted strikingly with that obtained previously with transfusions of normal lymphocytes; the labeled cells were concentrated almost exclusively around germinal centers in precisely the follicular regions defined by the lightly labeled cells from normal lymph. Owing to the low uptake by B lymphocytes, and the difficulty of obtaining them in large num-

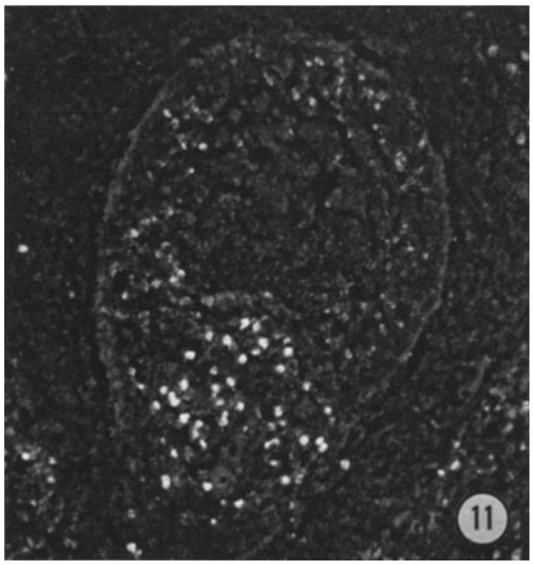


FIG. 11. Radioautograph of spleen from a normal rat given an artificial mixture of B and T small lymphocytes labeled in vitro with uridine-^{14}C (lymphocytes from thoracic duct of B rat given intravenous injection of thymus cells). Heavily labeled cells in periarteriolar region of white pulp (*below*); lightly labeled cells in arc around germinal center (*above*). The exclusively follicular localization seen in Fig. 8 has now been restored qualitatively to normal. (RA exposure: 28 days) × 120.

bers (5), the distribution of labeled cells was not easy to demonstrate photographically. Satisfactory results were obtained when B lymphocytes were labeled with uridine-^{14}C and photographed under dark-ground illumination. When artificial mixtures of marrow-derived and T lymphocytes were collected from the thoracic duct, labeled, and transfused into normal syngeneic recipients, as well as lightly labeled cells in follicular areas, heavily labeled cells were once again seen in the thymus-dependent traffic areas (Fig. 11). The experimental addition of T cells to the B cell population thus restored the pattern of distribution of transfused lymphocytes to normal, although quantitatively a relative deficiency of T cells persisted.

Relative Separation of Thoracic Duct Lymphocytes into B and T Populations

by Velocity Sedimentation.—The separation of lymphoid cells by sedimentation at 1 *g*, described by Miller and Phillips (6), was used in the present experiments to resolve two subpopulations of small lymphocytes in rat thoracic duct lymph, distinguished quantitatively by their uridine uptake in vitro and qualitatively by their distribution in vivo.

TABLE I

Radioactivity in Thoracic Duct Lymphocytes after Incubation with Uridine-^{3}H In Vitro for 75 Min

Experiment No.	Radioactivity (cpm/10^6 cells) in lymphocytes from thoracic duct*		
	Normal	Marrow-derived‡	Marrow-derived + T§
1	15,908	4724	
	16,180		
2	6874	5123	
	11,816	3122	
	12,676		
	10,864		
3	10,445	4033	
	8657		
4	12,802	5466	7684 (1)
			8345
5	15,044	2654	5591 (2)
	10,637		5946
	Overall % large lymphocytes (mean and range)		
	4.7	19.0	13.7
	(2.0–9.5)	(11.7–26.3)	(8.8–15.9)

* Each value is measurement on lymphocytes from one rat.

‡ Thoracic duct lymphocytes from thymectomized, irradiated, and marrow-reconstituted donors (B donors).

§ Thoracic duct lymphocytes from B donors injected with 10^9 thymocytes 2 wk (1) or 4 wk (2) before cannulation.

Thoracic duct lymphocytes from normal rats were labeled in vitro with uridine-^{3}H and sedimented for 8–9 hr at 1 *g*. The specific activity in cpm/10^6 cells was calculated for each fraction from the sedimentation chamber as a ratio relative to the specific activity of the unfractionated cells. The results of six such experiments are shown in Fig. 12 in which the specific activity data (*upper*) can be superimposed on the frequency distribution of sedimentation velocities (*lower*).

The specific activity profile plotted against sedimentation velocity shows

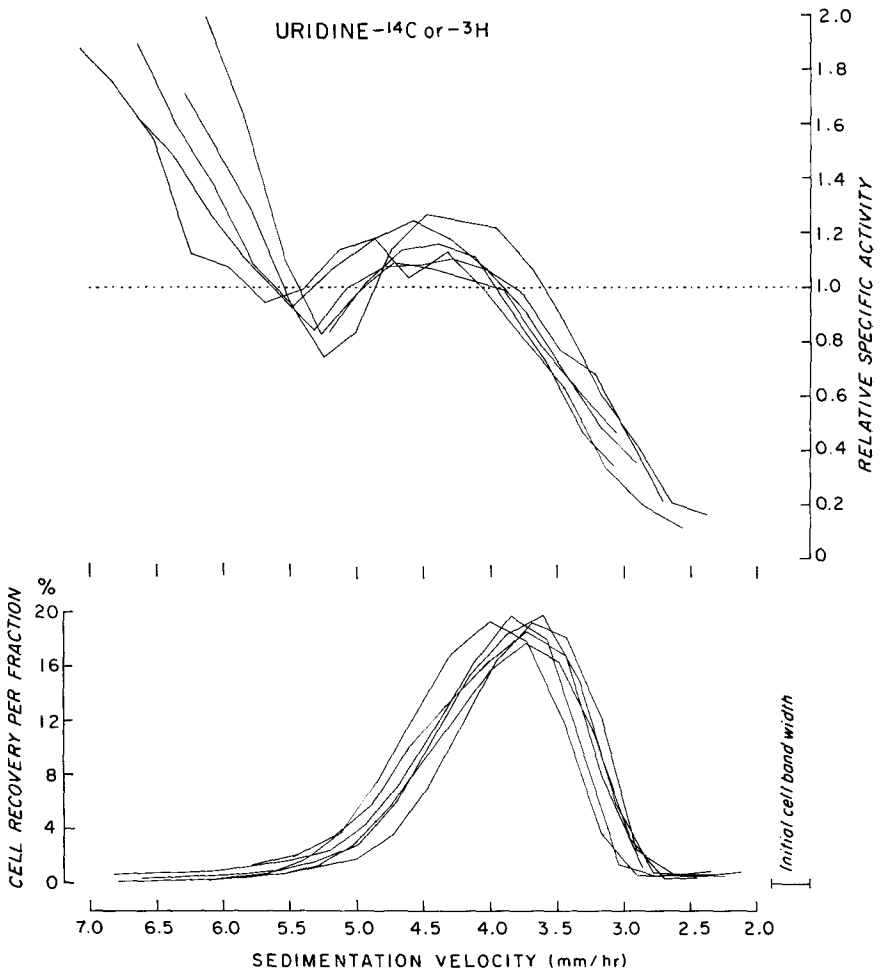


FIG. 12. Sedimentation of uridine-^{3}H– and uridine-^{14}C–labeled thoracic duct lymphocytes from normal donors.

Ordinates.—(*Lower graph*): "Cell Recovery per Fraction" represents the number of cells in a given 15-ml fraction as a percentage of the total cells recovered. Total recovered cells ranged from 107 to 157 million, representing a mean recovery of 90% of the number of cells loaded. (*Upper graph*): "Relative Specific Activity" represents the ratio: specific activity of given fraction (cpm/10^6 cells)/specific activity of initial suspension (cpm/10^6 cells). The value 1.0 therefore corresponds to the initial specific activities, which were 8155, 4443, 5519, 13464, 10808 (^{3}H), and 3239 (^{14}C) cpm/10^6 cells in the six experiments.

"Initial Cell Band Width" is the range of velocities covered by the loaded band of cells at the start of sedimentation. Velocities are calculated relative to the *top* of the band (0 mm/hr).

several characteristic features. Under the small lymphocyte mode the specific activity rises steeply with increasing sedimentation velocity to a maximum beyond the peak of small lymphocytes. The subsequent trough and steep rise in specific activity at still higher sedimentation velocities mark regions rich in non–S-phase and S-phase large lymphocytes, respectively (7).

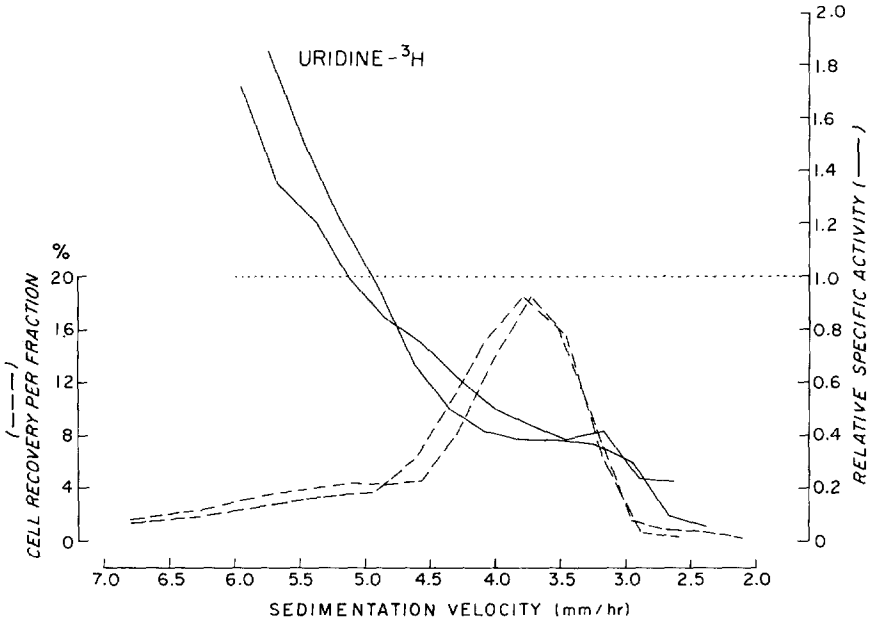


FIG. 13. Sedimentation of uridine-^{3}H–labeled thoracic duct lymphocytes from thymectomized, irradiated marrow-reconstituted rats. Each experiment represents the sedimentation of lymphocytes pooled from two donors. Ordinates as in Fig. 12. Total recovered cells: 132 and 104 million. Initial specific activities: 3979 and 3777 cpm/10^6 cells.

Note the homogeneous low specific activity of B small lymphocytes compared with the small lymphocytes from normal donors (Fig. 12).

Curves identical to those shown in Fig. 12 were obtained when samples from each fraction of cells were labeled individually with uridine-^{3}H after sedimentation and when samples of lymphocytes had been collected from the thoracic duct for either long or short periods before labeling and sedimentation. These two results suggested that the marked correlation between sedimentation velocity and uridine uptake among small lymphocytes was not an experimental artefact. Experiments recorded in the next two sections showed that this correlation was in fact due to the partial separation of two distinct small lymphocyte populations, one of relatively low sedimentation velocity and low uridine uptake corresponding to B lymphocytes, the other of relatively high

sedimentation velocity and high uridine uptake corresponding to T lymphocytes.

Sedimentation of Uridine-^{3}H–Labeled Marrow-Derived Lymphocytes and of Artificial Mixtures of Marrow-Derived and T Lymphocytes.—When populations of pure marrow-derived lymphocytes from B donors were labeled with uridine-^{3}H and sedimented, the normal correlation between sedimentation velocity

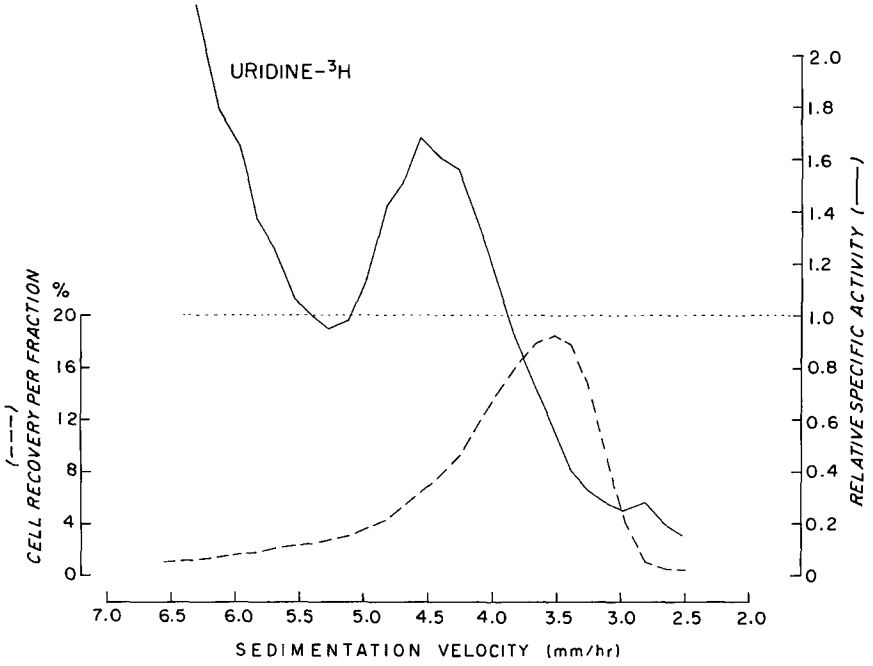


FIG. 14. Sedimentation of uridine-^{3}H–labeled thoracic duct lymphocytes pooled from two thymectomized, irradiated, marrow-reconstituted donors given 10^9 thymocytes 14 days before thoracic duct cannulation. Ordinates as in Fig. 12. Total recovered cells: 135 million. Initial specific activity: 6307 cpm/10^6 cells.

Note the reconstitution by thymocytes of the normal specific activity profile (Fig. 12) compared with the specific activity profile of pure marrow-derived lymphocytes (Fig. 13).

and uridine uptake among small lymphocytes was abolished (Fig. 13). B small lymphocytes were nearly homogeneous in uridine uptake, with a specific activity of only about 0.4 that of the unfractionated population, emphasizing the deficient uridine uptake among B lymphocytes relative to cells from normal donors (see also Table I).

The high frequency of large lymphocytes in suspensions of marrow-derived thoracic duct cells (5) was evident in the cell distribution profiles especially at sedimentation velocities greater than 5.5 mm/hr and their high uridine uptake intruded on the small lymphocyte specific activity plateau at still lower velocities.

The cell distribution profiles of pure marrow-derived cells showed a deficiency of rapidly sedimenting small lymphocytes relative to the profiles of cells from normal donors. This deficiency would be expected if the rapidly

TABLE II

The Histological Distribution in the Spleen of Slow and Fast Sedimenting Small Lymphocytes after Intravenous Transfusion

Label	Uridine-^{3}H		Uridine-^{14}C + sodium chromate-^{51}Cr	
Sedimentation fractions	Slow	Fast	Slow	Fast
Donor inocula				
Velocity of pooled fractions (mm/hr)	3.1–3.6	4.1–5.3	2.6–3.4	4.2–4.9
No. of cells injected ($\times 10^{6}$)	20.8	30.4	19.4	31.4
Specific activity (uridine) cpm/10^{6} cells	6109	11,856	1625	3428
Labeled cells %*	62	84	98	99
Recipient spleens				
Periarteriolar area				
No. of labeled cells‡	102	176	see Fig. 17	see Fig. 16
Area units	12.7	7.3		
Cells/unit area	8.37	24.19		
Follicular area				
No. of labeled cells‡	64	44		
Area units	10.4	7.3		
Cells/unit area	6.01	6.02		
Cells/unit area, total§	7.0	15.0	—	—

Normal thoracic duct lymphocytes were labeled in vitro with uridine-^{3}H or with both uridine-^{14}C and sodium chromate-^{51}Cr before sedimentation. Pools of slow and fast small lymphocyte fractions were injected intravenously into normal recipients and radioautographs of spleens prepared 24 hr later.

* Frequency of labeled cells counted on radioautographs prepared from cell pools before injection.

‡ See Materials and Methods for technique of counting.

§ The ratio of these two values (0.47) is lower than the ratio of injected cell doses (0.67) suggesting that a lower proportion of slow than fast cells was detected in radioautographs, presumably as a consequence of the lower uptake of uridine.

sedimenting, high uridine uptake small lymphocytes from normal lymph were T cells, but too few experiments have been performed to assess its statistical significance.

When artificial mixtures of uridine-labeled marrow-derived and T lymphocytes were sedimented; the normal specific activity profile was reconstituted

(Fig. 14). The high specific activity mode in rapidly sedimenting small lymphocytes is clearly marked. Thus the characteristic specific activity profiles of normal thoracic duct cells after sedimentation can be explained by a relative separation of their constituent B and T lymphocytes; the experimental addition of T lymphocytes to the thoracic duct lymph of B rats restores a rapidly sedimenting, high uridine uptake population of small lymphocytes.

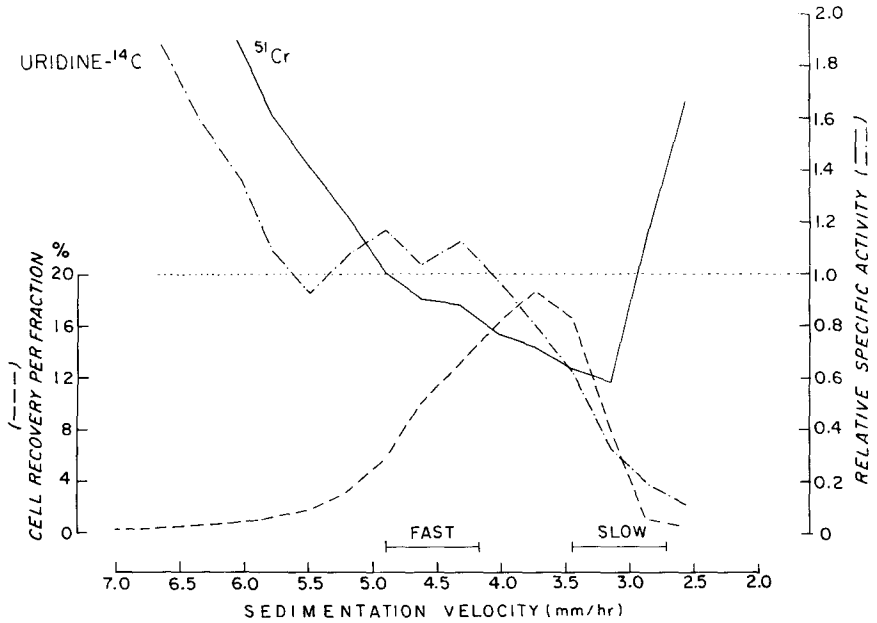


FIG. 15. Sedimentation of thoracic duct lymphocytes from a normal donor after labeling with both uridine-^{14}C and sodium chromate-^{51}Cr, before transfusion into normal recipients. Ordinates as in Fig. 12. Total recovered cells: 133 million. Initial specific activities: 3239 (^{14}C), 9013 (^{51}Cr) cpm/10^6 cells. Slow and fast represent pooled fractions transfused into normal recipients (see Table II and Figs. 16, 17.)

Note that the labeling of small lymphocytes with ^{51}Cr is relatively homogeneous.

The Distribution In Vivo of Normal Small Lymphocytes of High and Low Sedimentation Velocity.—It cannot be assumed that the populations of normal small lymphocytes showing high and low incorporation of uridine in vitro in sedimentation experiments (Fig. 12) necessarily correspond to those showing heavy and light labeling respectively in radioautographs of lymphoid tissue 24 hr after transfusion (Figs. 1–5). The population labeling lightly in vivo could have had two other origins: (*a*) from an undetected population of lightly labeled cells sedimenting in vitro at the same rate as the heavily labeled group; (*b*) from a subpopulation of cells labeling heavily in vitro but losing label in

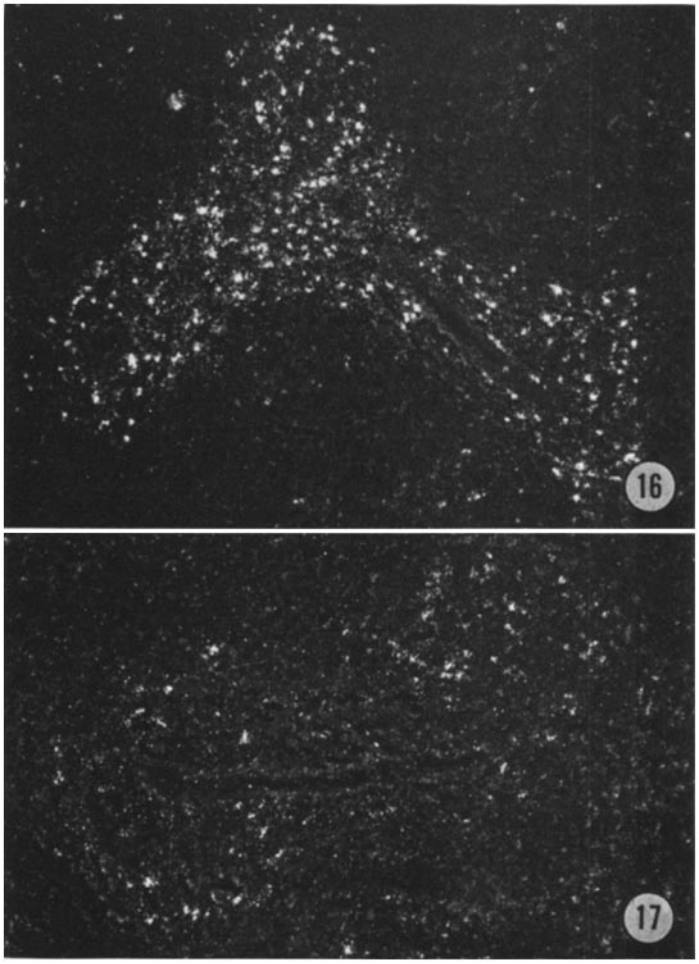
vivo by turnover of intracellular radioactivity. The second possibility arises because the radioactivity initially in the cells was measured by scintillation counting and was located partly in trichloroacetic acid–soluble intracellular pools, while labeling in vivo was detected by radioautography and was almost exclusively in RNA. Consequently, a considerable loss of label might have occurred by turnover of intracellular pools and of RNA.

These objections were answered by two experiments which established that when slowly sedimenting cells with low uridine uptake in vitro were separated from normal lymph, they did, in fact, localize in the areas of the spleen which showed light labeling after the transfusion of unfractionated cells; and conversely that the fast sedimenting, high uridine uptake fractions were depleted of cells migrating into these areas but rich in cells localizing in the heavily labeled zone.

In the first experiment pools of "slow" and "fast" small lymphocytes prepared by sedimenting ^{3}H-labeled uridine normal thoracic duct cells were injected intravenously into normal rats from which spleens were taken and prepared for radioautography 24 hr later. Table II records the number of labeled cells counted in periarteriolar and follicular areas of the splenic white pulp (see Materials and Methods). It can be seen that the proportion of cells migrating to the periarteriolar regions was strikingly greater after transfusions of fast, high uridine uptake fractions. The fate of the slow fractions was more difficult to determine in the radioautographs because a lower proportion of the cells showed detectable labeling initially (slow:fast; 62%:84%) and the individual cells were more lightly labeled. This difficulty was reduced in the second experiment.

Slow and fast fractions were prepared by sedimenting normal thoracic duct lymphocytes which had been labeled both with uridine-^{14}C and sodium chromate-^{51}Cr. Fig. 15 plots sedimentation velocity against the specific activity of the two isotopes and shows that small lymphocytes of different sedimentation velocity label more uniformly with ^{51}Cr than with uridine. There is a rapid rise of ^{51}Cr specific activity at high sedimentation velocities associated with large lymphocytes, and high activities at very low velocities due to the few contaminating erythrocytes; in between, small lymphocytes are labeled relatively evenly. In this experiment uridine-^{14}C at a low concentration was used simply to monitor the enrichment of high uridine-uptake cells in fast fractions; ^{51}Cr, which labeled almost 100% of cells in both fast and slow fractions (Table II), was responsible for most of the radioautographic image.

Radioautographs were prepared from the spleens of recipients of slow and fast fractions 28 hr after injection and were photographed under dark-ground illumination. A sufficiently high concentration of labeled cells was present to demonstrate directly the entrance of slow cells into the follicular areas of white pulp and of fast cells into the periarteriolar areas (Figs. 16, 17).



FIGS. 16 and 17. Radioautographs of spleen from rats given small lymphocytes from the thoracic duct of normal donors. Thoracic duct lymphocytes had been labeled in vitro with both uridine-^{14}C and sodium chromate-^{51}Cr and separated into slow and fast fractions by velocity sedimentation (see text and Table II). 2–3 $\times$ 10^7 cells were given intravenously to normal recipients. (RA exposure: 28 days) $\times$ 120.

FIG. 16. Labeled cells from the fast fractions (T lymphocytes) are localized in the periarteriolar region around arterioles which are cut both transversely (*lower left*) and longitudinally (*center to lower right*). Follicular areas, seen in vague outline at lower center and right center, are virtually unlabeled.

FIG. 17. Labeled cells from slow fractions (B lymphocytes) restricted to two follicular zones (*upper right, lower left*) in splenic white pulp. An arteriole, cut longtitudinally, runs across the center of the white pulp but, in contrast to Fig. 16, labeled cells have not accumulated around it.

DISCUSSION

The present experiments have established that the thoracic duct lymph of rats normally contains two distinct populations of small lymphocytes which migrate from the blood into separate well-demarcated areas of the spleen, lymph nodes, and Peyer's patches; and that these two populations correspond to the marrow- (B) and thymus- (T) derived lymphocytes, the existence of which was first inferred from immunological experiments on mice (11).

The point of departure for these studies was the observation that small lymphocytes from the thoracic duct, which showed a wide variation in the intensity of labeling after incubation in vitro with uridine-^{3}H, appeared to segregate after intravenous injection so that distinct areas in lymphoid tissue contained either lightly or heavily labeled cells. Heavily labeled small lymphocytes occupied the areas already identified as traffic zones for lymphocyte recirculation (8), namely the periarteriolar sheaths of the splenic white pulp, the deep cortex of the lymph nodes, and the interfollicular zones in Peyer's patches. In contrast, lightly labeled small lymphocytes localized in areas which either surrounded or capped the germinal centers; this "follicular" localization was particularly striking in the Peyer's patches.

The key evidence that cells which labeled lightly or heavily in vitro migrated into the areas showing light or heavy labeling, respectively, after transfusion came from fractionating labeled thoracic duct lymphocytes by the technique of velocity sedimentation (6); fast and slow fractions separated by this technique were enriched in heavily and lightly labeled small lymphocytes, respectively, and these localized in the areas of corresponding label intensity after transfusion. Thus two populations had been distinguished by differential localization in lymphoid tissue, differential uptake of uridine in vitro, and by a differential distribution of sedimentation velocities. The range of sedimentation velocities involved is sufficiently small for the difference between the two populations to be explained in terms of differences either in cell size or cell density. It remained to correlate this list of distinguishing features with marrow and thymus derivation.

Lymphocytes obtained from the thoracic duct of thymectomized, irradiated, bone marrow–restored rats (B rats) are derived from the marrow (5). Studies on these marrow-derived lymphocytes and on lymphocyte populations from B rats given injections of thymus cells provided strong evidence that the two populations of small lymphocytes found in normal thoracic duct lymph are derived separately from the thymus and the marrow. Thus, B lymphocytes were shown to be a lightly labeled class of small lymphocyte which localized in follicular areas after intravenous transfusion; and injections of thymus cells restored to the lymph of B rats a class of heavily labeled, more rapidly sedimenting small lymphocytes which homed into their characteristic zones in lymphoid tissue.

The follicular location of marrow-derived small lymphocytes in normal

lymphoid tissue, described in the present work, corresponds closely to the distribution of thymus-independent lymphoid cells inferred from histological studies on thymus-deprived animals (9, 10, 12) and from studying the fate, after intravenous injection, of lymph node and spleen cells from neonatally thymectomized donors (13). Evidence that the lymphocytes occupying follicular areas in mice are a distinct population was obtained recently by mapping the distribution of cells bearing receptors for the C'3 component of complement (14). This population does not carry the θ antigen (15). However, the derivation of θ-negative cells in mice is uncertain (16, 17) and the present study provides positive evidence that follicular small lymphocytes are, in fact, marrow derived.

Wide variation in intensity of labeling in the RNA of thoracic duct small lymphocytes exposed in vitro to adenosine-^{3}H (8), and deficiency of uridine and cytidine uptake among small lymphocytes from the thoracic duct of neonatally thymectomized rats (18) were observed before the existence of two separate populations of lymphocytes had been postulated by Mitchell and Miller (11). More recently, Austin (19) transfused rat lymph node cells labeled with uridine-^{3}H and noted lightly labeled cells in the follicular areas, but the light labeling was attributed to a high rate of RNA breakdown. The functional significance of the difference in uridine uptake between marrow- and thymus-derived cells, found in the present study, is not known.

A point which requires emphasis is that marrow-derived small lymphocytes, which localize in follicular areas of lymphoid tissue, are normally present in the thoracic duct lymph; indeed, the first indication of their existence, in the present work, came from studying the fate of labeled thoracic duct cells after syngeneic transfer. Since marrow-derived small lymphocytes from the thoracic duct reenter lymph after transfusion into the blood (5), the follicular areas must be regarded as regions through which B lymphocytes normally recirculate, in the same way as the periarteriolar and deep cortical zones are the traffic areas for the recirculation of T lymphocytes. It is not clear how the segregation of recirculating B and T lymphocytes into different areas of lymphoid tissue can be reconciled with the contiguity implied by collaboration between cell types in antibody responses.

SUMMARY

These experiments show that small lymphocytes from the thoracic duct of rats are normally a mixture of thymus-derived and marrow-derived cells, and define the traffic areas in lymphoid tissues through which the two populations recirculate.

Thoracic duct lymphocytes were labeled in vitro with uridine-^{3}H and their histological distribution in the lymphoid tissues of normal recipients was demonstrated by radioautography. Labeled lymphocytes occupied two adjacent areas distinguished by a marked difference in the intensity of labeling;

heavily labeled cells were found in thymus-dependent traffic areas of lymphocyte recirculation, while lightly labeled cells localized in the thymus-independent follicular areas around germinal centers.

A corresponding heterogeneity of uridine uptake among small lymphocytes from normal donors was demonstrated by sedimentation at 1 *g*; slowly sedimenting cells incorporated little uridine and localized in follicular areas after transfusion while rapidly sedimenting cells incorporated more uridine and localized in thymus-dependent areas after transfusion.

Experimentally prepared marrow-derived small lymphocytes behaved in sedimentation studies and after transfusion like a pure population of the lightly labeled small lymphocytes in normal lymph. Artificially reconstituted mixtures of marrow-derived and thymus-derived lymphocytes were qualitatively indistinguishable from normal lymphocyte populations.

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BIBLIOGRAPHY

1. Ellis, S. T., J. L. Gowans, and J. C. Howard. 1969. The origin of antibody forming cells from lymphocytes. *Antibiot. Chemother.* **15:**40.
2. Howard, J. C., and J. L. Gowans. The role of lymphocytes in antibody formation. III. The origin from small lymphocytes of cells forming direct and indirect haemolytic plaques to sheep erythrocytes in the rat. *Proc. Roy. Soc. Ser. B Biol. Sci.* In press.
3. Johnston, J. M., and D. B. Wilson. 1970. Origin of immunoreactive lymphocytes in rats. *Cell. Immunol.* **1:**430.
4. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic duct lymphocytes. *Proc. Nat. Acad. Sci. U.S.A.* **59:**266.
5. Howard, J. C. 1972. The life-span and recirculation of marrow-derived small lymphocytes from the rat thoracic duct. *J. Exp. Med.* **135:**185.
6. Miller, R. G., and R. A. Phillips. 1969. Separation of cells by velocity sedimentation. *J. Cell. Physiol.* **73:**191.
7. Hunt. S. V., S. T. Ellis, and J. L. Gowans. The role of lymphocytes in antibody formation. IV. Carriage of immunological memory to tetanus toxoid by lymphocyte fractions prepared by velocity sedimentation. *Proc. Roy. Soc. Ser. B Biol. Sci.* In press.
8. Gowans, J. L., and J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. Roy. Soc. Ser. B Biol. Sci.* **159:**257.
9. Waksman, B. H., B. G. Arnason, and B. D. Janković. 1962. Role of the thymus in immune reactions in rats. III. Changes in the lymphoid organs of thymectomized rats. *J. Exp. Med.* **116:**187.
10. Parrott, D. M. V., M. A. B. de Sousa, and J. East. 1966. Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. Exp. Med.* **123:** 191.
11. Mitchell, G. F., and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune

response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128:**821.

12. Keuning, F. J., and A. A. van den Broek. 1968. Role of marginal zone cells of lymphoid follicles in the immune response of rabbits. *Exp. Hematol.* **17:**4.
13. Parrott, D. M. V., and M. A. B. de Sousa. 1971. Thymus-dependent and thymus-independent populations: origin, migratory patterns and lifespan. *Clin. Exp. Immunol.* **8:**663.
14. Dukor, P., C. Bianco, and V. Nussenzweig. 1970. Tissue localization of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. *Proc. Nat. Acad. Sci. U.S.A.* **67:**991.
15. Bianco, C., and V. Nussenzweig. 1971. Theta-bearing and complement-receptor lymphocytes are distinct populations of cells *Science (Washington).* **173:**154.
16. Miller, J. F. A. P., and J. Sprent. 1971. Thymus-derived cells in mouse thoracic duct lymph. *Nature (New Biol.) (London).* **230:**267.
17. Schlesinger, M., and I. Yron. 1970. Serologic demonstration of a thymus-dependent population of lymph-node cells. *J. Immunol.* **104:**798.
18. Rieke, W. O. 1966. Lymphocytes from thymectomized rats: immunologic, proliferative, and metabolic properties. *Science (Washington).* **152:**535.
19. Austin, C. M. 1968. Patterns of migration of lymphoid cells. *Aust. J. Exp. Biol. Med. Sci.* **46:**581.