

ANATOMICAL DISTRIBUTION OF T AND B LYMPHOCYTES IN THE RAT

DEVELOPMENT OF LYMPHOCYTE-SPECIFIC ANTISERA*

BY IRVING GOLDSCHNEIDER AND D. D. MCGREGOR†

(From the Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut 06032, and The Trudeau Institute, Saranac Lake, New York 12983)

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Two major classes of lymphocytes have been shown to exist in the rat as they do in the mouse: thymus-derived (T)¹ lymphocytes and bone marrow-derived (B) lymphocytes. Rats thymectomized at birth show a selective deficiency of T lymphocytes in peripheral lymphoid tissue, blood, and lymph (1, 2), and an associated defect in cell-mediated immunity as evidenced by failure of the residual lymphocytes to initiate delayed-type hypersensitivity reactions (3) and to respond vigorously to foreign histocompatibility antigens in vivo (3) or in vitro (4). Similar cellular and functional defects have been described in adult rats after either thymectomy and irradiation (5, 6) or chronic drainage of lymph and cells from a thoracic duct fistula (2, 7).

Neonatal thymectomy and chronic lymph drainage have little effect upon B lymphocytes in the rat (1, 2), although antibody responses that depend upon the collaboration of T and B lymphocytes are often severely depressed (5-9). B lymphocytes have been distinguished from T lymphocytes by their buoyant density, decreased rate of incorporation of RNA precursors, and propensity to migrate to the "thymus-independent" areas of peripheral lymphoid tissue (10, 11).

In the present study, antilymphocyte sera (ALS) of restricted specificity have been used to further delineate the tissue distribution of T and B lymphocytes in the rat.² The results indicate that antisera raised in rabbits against rat

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† Research Associate, American Cancer Society.

¹ *Abbreviations used in this paper:* ALS, antilymphocyte serum; ALS_B, B-lymphocyte-specific ALS; ALS_T, T-lymphocyte-specific ALS; ALS_{T+B}, lymphocyte-specific ALS; AMS, antimacrophage serum; ATS, antithymocyte serum; B, bone marrow-derived; BSS, balanced salt solution; Ig, immunoglobulin; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; T, thymus-derived; TDL, thoracic duct lymphocytes; TxBM, thymectomized, irradiated, and bone marrow-reconstituted.

² A preliminary report of this study has been presented (Goldschneider, I., and D. D. McGregor. 1972. Specific antisera against rat T and B lymphocytes. *Fed. Proc.* **31**:776).

thoracic duct lymphocytes (TDL) and absorbed with red blood cells (RBC), peritoneal exudate cells (PEC), and either thymocytes or lymphoid cells from T-cell-deficient donors can be made specific for subpopulations of lymphocytes that correspond or closely overlap with B and T lymphocytes, respectively.

Materials and Methods

Animals.—1-3-mo old male and female Lewis rats and members of the (Lewis × DA)F₁ hybrid cross were used throughout.

Operative Procedures.—

Incannulation of thoracic duct: The thoracic duct was incannulated in its short intra-abdominal course as described previously (2).

Thymectomy, irradiation, and reconstitution of rats: 4-6-wk old Lewis rats were thymectomized and 7 days later were exposed to 800 rads of whole body X- and γ -irradiation (12). Immediately after irradiation, the animals were reconstituted with 2×10^6 syngeneic bone marrow cells injected intravenously. Spleen, bone marrow, PEC, and TDL were obtained from these rats 6 wk after irradiation.

Cell suspensions.—All cells were washed twice in cold Tyrode's solution, centrifuged at 100 g for 10 min, and resuspended in Dulbecco's phosphate-buffered saline (PBS) (pH 7.4) at a concentration of 2×10^7 cells per ml.

TDL: Lymph from donor rats was collected into 5 ml of Tyrode's solution containing 100 U of heparin and 500 μ g of streptomycin.

Thymocytes, lymph node cells, and spleen cells: Etherized rats were exsanguinated from the aorta or by cardiac puncture. The thymus, mesenteric lymph node, and spleen were then removed, and the cells expressed into Tyrode's solution. The thymus was freed of adherent lymph nodes before dissociation.

Bone marrow cells: Plugs of marrow were expressed from the femurs and tibiae of exsanguinated rats by perfusing the exposed marrow cavities with Alsever's solution. Clumps of cells were dispersed by gentle aspiration using a fire-polished Pasteur pipette.

Blood lymphocytes: Freshly collected rat blood was defibrinated by swirling in a glass flask for 5 min. Two parts of the defibrinated blood were added with gentle mixing to one part of 3% gelatin (Gelatin and Glue Research Association, Birmingham, England) in Hanks' balanced salt solution (BSS). The cells were allowed to sediment at 37°C for 30 min in a 50-ml conical centrifuge tube before the upper two-thirds of the buffy coat (approximately 95% lymphocytes) was removed.

RBC: Heart or aortic blood was collected from donor rats which had been exposed to 1,000 rads of whole body irradiation 24 h before exsanguination. The blood was collected into equal volumes of Alsever's solution and the buffy coat discarded after centrifugation.

PEC: Rats were injected intraperitoneally with 3 ml of 12% sodium caseinate per 100 g body weight (Nutritional Biochemicals Corporation, Cleveland, Ohio). 48 h later the exudate cells were harvested by flooding the peritoneal cavity with 20 ml PBS.

Antisera.—

Preparation: Several batches of ALS were raised in rabbits given two intravenous injection of approximately 10^9 Lewis TDL, spaced 10 days apart. The TDL donors were either normal rats or thymectomized rats which had been irradiated and reconstituted with syngeneic bone marrow cells 6 wk before incannulation. Antithymocyte serum (ATS) and an anti-macrophage serum (AMS) were prepared in a similar manner by injecting rabbits with approximately 10^9 Lewis thymocytes and 5×10^8 Lewis PEC, respectively. In each case, the animals were bled 7 days after the second injection of cells. Sera obtained before and after immunization were heat inactivated (56°C for 30 min), passed through a Millipore filter (Millipore Corp., Bedford, Mass.) and stored at 4°C.

A rabbit IgG fraction against rat gamma globulin (rabbit antirat Ig), a fluorescein-

conjugated rabbit IgG fraction against rat gamma globulin (FITC-rabbit antirat Ig), and a fluorescein-conjugated goat IgG fraction against rabbit IgG (FITC-goat antirabbit IgG) were obtained commercially (Cappel Laboratories, Downingtown, Pa). All were checked for specificity by immunoelectrophoresis against whole rat or rabbit serum using purified rat gamma globulin or rabbit IgG as standards.

Absorption: Sera, usually diluted 1:5 with PBS, were mixed with equal volumes of packed cells and absorbed at 4°C for 30 min. The cells were removed by centrifugation and the sera resterilized by passage through a Millipore membrane. Multiple absorptions (2-8) were often necessary to completely remove reactivity for any cell type, as judged by indirect immunofluorescence. Absorptions of a single serum with cells from more than one source were done sequentially.

Certain antisera were absorbed with normal rat serum. This was done by mixing aliquots of antiserum and rat serum in varying proportions before testing the reactivity of the antiserum against cells or tissues. In experiments using cell suspensions, the effect of absorption was quantified by the change in antibody titer; in other experiments using frozen sections of rat tissues the effect was measured qualitatively, by the intensity or change in pattern of fluorescence.

Elution: Antisera specific for lymphocyte surface antigens were prepared from aliquots of ALS which had been absorbed with RBC and PEC. Two ml of a 1:5 dilution of ALS was added to 2×10^9 viable TDL. The cells were incubated at 4°C for 30 min, then washed three times in cold PBS to remove unabsorbed antibody. After washing, the cells were resuspended in 10 ml of 0.1 M citric acid and incubated at 4°C for 20 min with gentle mixing (13). This elution process was repeated three times, and the pooled eluates were concentrated by vacuum dialysis against PBS to the original volume and to neutral pH. The concentrated eluates were "clarified" by centrifugation ($1000 g \times 15$ min), passed through a $0.45 \mu\text{m}$ Millipore membrane, dispensed into ampoules and stored at -20°C. Approximately 50% of the antibodies to lymphocyte surface antigens were recovered in the eluates as judged by antibody titers against rat TDL.

Enumeration of Lymphocytes.—For the immunofluorescence assay of TDL or cell suspensions prepared from thymus or lymph nodes, the number of specifically fluorescing lymphocytes was expressed as a percentage of the total number of nucleated cells per microscopic field. When a suspension of spleen cells or bone marrow cells was analyzed, however, the percentage of fluorescing lymphocytes was computed from the differential lymphocyte count performed on a sample of the same cell suspension prepared as a smear and stained with Wright's stain.

Immunofluorescence.—

Cell suspensions: Suspensions of living cells were examined by indirect immunofluorescence as described previously (14). Briefly, 10^6 washed cells suspended in 0.05 ml PBS were mixed with 0.05 ml serially diluted rabbit antiserum in a U-well microtiter tray, and incubated for 20 min at 4°C. The cells were washed in the tray three times with cold PBS, then incubated at 4°C for 20 min in 0.025 ml of a 1:20 dilution of FITC-goat antirabbit IgG. At this dilution, the fluorescein-conjugated antiserum did not react detectably with any of the rat cells in suspension. After rewashing three times in cold PBS, the microtiter plate was placed on ice, and samples were withdrawn and examined by darkfield-fluorescence microscopy. Normal rabbit sera, obtained before immunization of each rabbit, were used as controls in each experiment.

Frozen sections: Tissues from 8-12-wk old Lewis or (Lewis \times DA) F₁ hybrid rats were frozen in O.C.T. compound (Scientific Products, Inc. Waltham, Mass.) at -20°C; $4\text{-}\mu\text{m}$ sections were prepared in a cryostat and dried onto chrome alum "subbed" slides. To test for immunofluorescence, the sections were exposed to either normal rabbit serum or immune rabbit serum (usually diluted 1:10 or 1:20, depending on the titer) for 20 min at 4°C. The sections were then washed three times in PBS, incubated for 20 min with FITC-goat antirabbit IgG (1:20 dilution), rewashed, and mounted in buffered glycerin.

Photomicrographs of frozen sections under darkfield-fluorescence illumination were taken using Polaroid type 57 B & W film (ASA 3000) (Polaroid Corporation, Cambridge, Mass.). In order to positively identify the structure or area being photographed, coordinates were taken and the same field was rephotographed after the section was fixed with alcohol-formalin-acetic acid and stained with hematoxylin and eosin.

RESULTS

Absorption of ALS.—The antisera raised in rabbits immunized with rat TDL reacted with many cell types in addition to lymphocytes. These included rat RBC, macrophages, granulocytes, bone marrow cells, hepatocytes, myocardial cells, and fibroblasts. An antiserum specific for lymphocytes as judged by indirect immunofluorescence was prepared by absorbing the crude ALS sequentially with RBC and PEC from lymphocyte-deficient TxBM rats. Antibodies to lymphocyte plasma membrane constituents subsequently were obtained by reacting the absorbed ALS and TDL and eluting the attached antibodies with 0.1 M citric acid. Further absorption of the concentrated eluate, designated ALS_{T+B}, with spleen or bone marrow cells from TxBM rats resulted in antiserum (ALS_T) which reacted with thymocytes and a subpopulation of peripheral lymphocytes. An antiserum with an entirely different specificity was prepared by absorbing ALS_{T+B} with rat thymocytes. This antiserum (ALS_B) reacted with a second subpopulation of peripheral lymphocytes. The procedure followed in preparing these various reagents is schematized in Fig. 1.

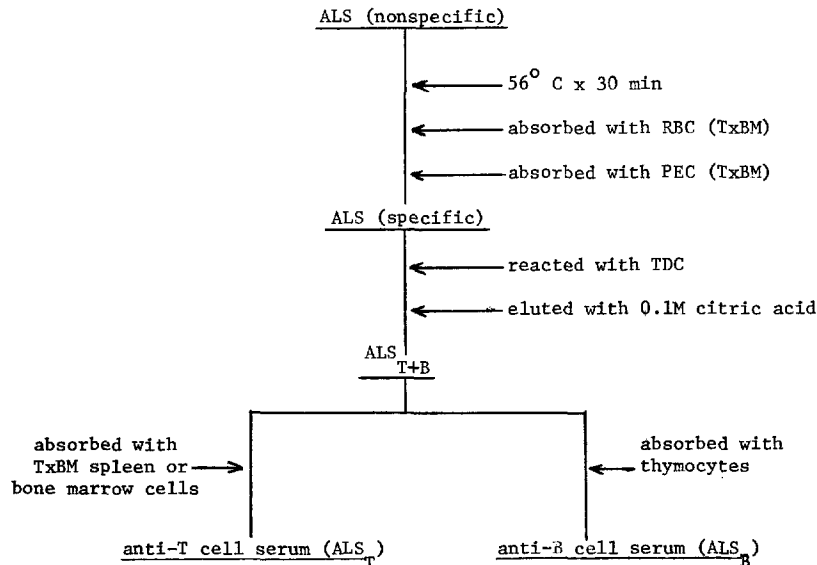


FIG. 1. Scheme of absorption of antilymphocyte serum. See Materials and Methods for details of absorption and elution procedures. In some experiments ALS_B was absorbed further with normal rat serum to remove antibodies to gamma globulins.

Specificity of ALS.—Frozen sections of normal rat lymph node, spleen, and thymus were exposed to either normal rabbit serum or differentially absorbed ALS raised in the same animals. The normal rabbit serum was heat-inactivated and absorbed with rat RBC and PEC, but was not further purified prior to use. In each case, tissue sections were examined for fluorescence after development with FITC-goat antirabbit IgG. Reproducible results were obtained in each of several experiments using 2–4 different batches of ALS. The anatomical locations of cells binding the various antisera are illustrated schematically in Fig. 2. Photomicrographs of representative tissue sections are shown in Figs. 3–11.

ALS prepared against TDL from normal rats: ALS_{T+B} reacted with plasma membrane constituents of all lymphocytes in lymph node and spleen except germinal center cells. The antiserum reacted weakly with the cytoplasm of plasma cells, but not detectably with their surface membranes. Erythropoietic

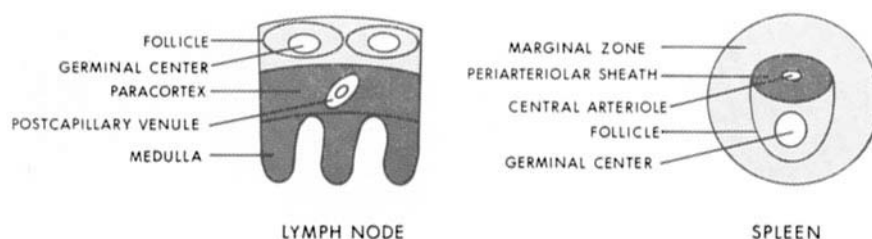
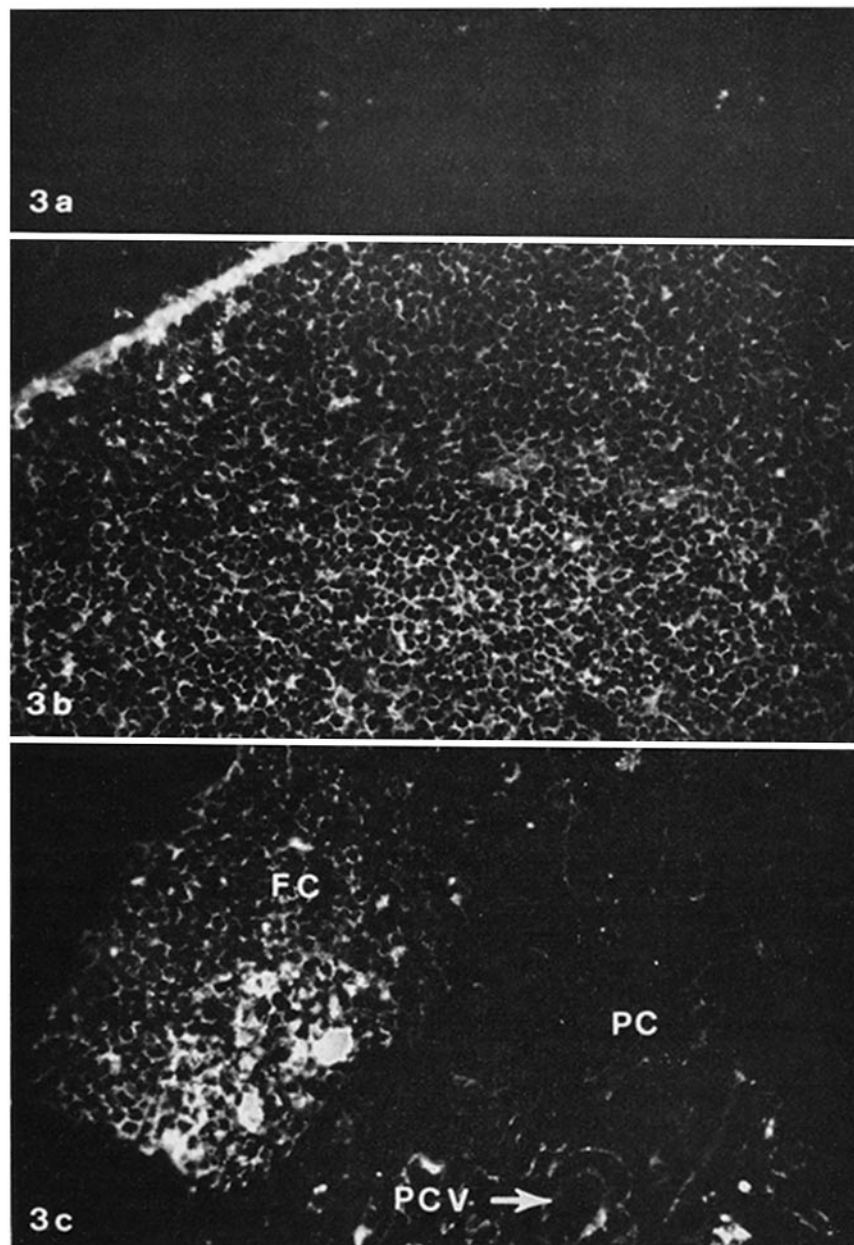


FIG. 2. Schematized diagram of rat lymph node and spleen (white pulp) showing anatomical locations of lymphocytes whose plasma membranes react with ALS_B (light grey area) or ALS_T (dark grey area). Germinal center lymphocytes do not react with either antiserum (white area). The cytoplasm, but not the surface membranes, of plasma cells in medullary cords reacts weakly with ALS_B . Macrophages do not react with either antiserum.

and myelopoietic cells in spleen and endothelial cells of postcapillary venules in lymph node were not stained. Macrophages often exhibited an intense yellow-brown autofluorescence, but did not stain with ALS_{T+B} .

ALS_B reacted selectively with lymphocytes in the follicular cortex of lymph node (Fig. 3 *a-c*). Plasma cells in the medulla showed weak cytoplasmic fluorescence. Lymphocyte-like cells within germinal centers did not fluoresce (Fig. 4 *a, b*), although dendritic cells, which have receptors on their surfaces for cytophilic antibodies (15, 16), were often stained (Fig. 4 *c-e*). ALS_T reacted with lymphocytes in the paracortex, medullary cords, and lymph sinuses of lymph node (Fig. 5 *a, b*). Follicular lymphocytes were not stained (Fig. 5 *c*), nor were plasma cells.

In the spleen, ALS_B reacted strongly with lymphocytes in the lymphocyte corona (mantle) of lymphoid follicles and in the marginal zone of the white pulp (Figs. 6 *a, b* and 8 *b*). Staining was limited to the surface membranes of follicular lymphocytes, whereas those in the marginal zone appeared to exhibit both membrane and cytoplasmic fluorescence. Lymphocyte-like cells within



FIGS. 3-5. Frozen sections of lymph nodes exposed to either normal rabbit serum or ALS were washed and reacted with FITC-goat antirabbit IgG. *FC*, follicular cortex; *PC*, paracortex; *MC*, medullary cord; *LS*, lymph sinus; *Fol*, follicle; *GC*, germinal center; *PCV*, postcapillary venule.

FIG. 3. Serial sections of mesenteric lymph node cortex. X 320. (a) Normal rabbit serum: Scattered macrophages in follicular cortex and paracortex show bright yellow autofluorescence. Lymphocytes are not stained. (b) ALS_{B+T} : The surfaces of all lymphocytes in the follicular cortex and paracortex are stained with equal intensity. There is no cytoplasmic or nuclear fluorescence. (c) ALS_B : Lymphocytes in the follicular cortex fluoresce, whereas those in the paracortex do not. Endothelial cells lining a postcapillary venule are also negative. The large bright spots are autofluorescent macrophages.

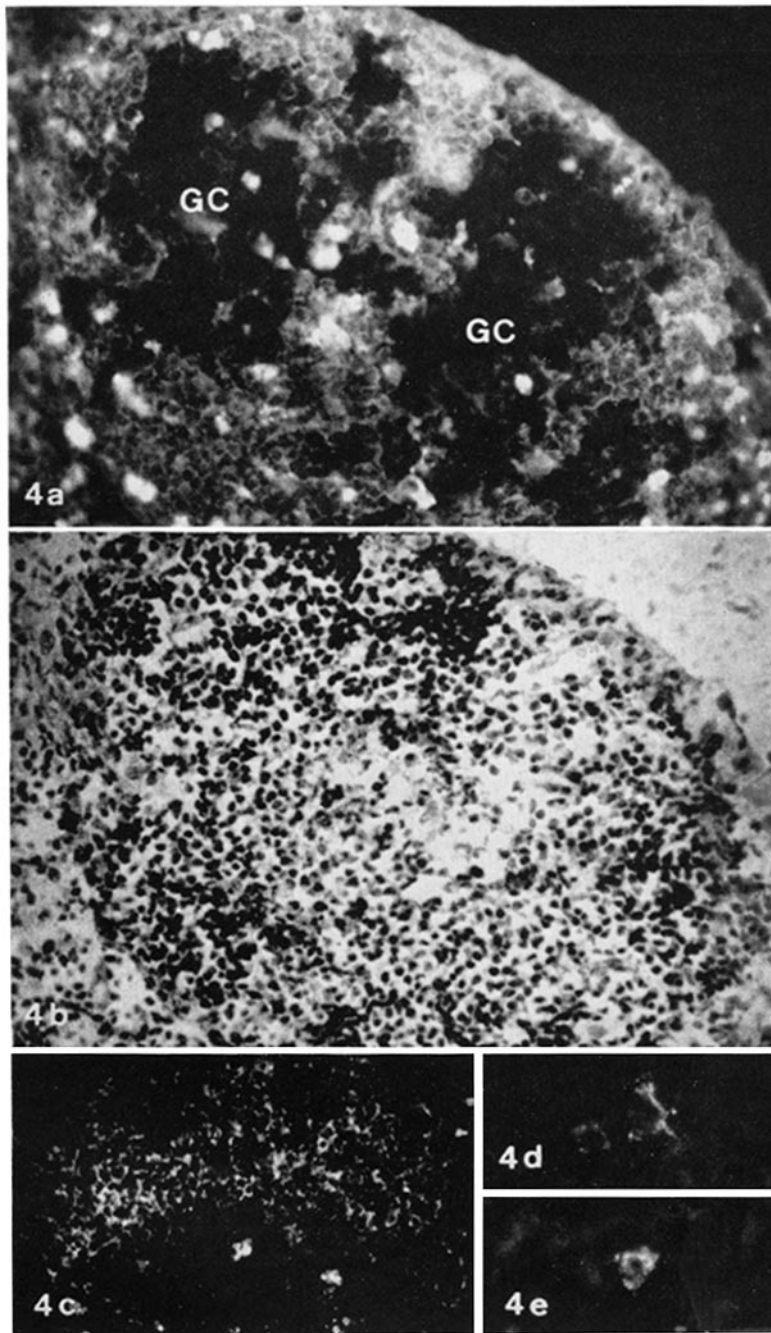


FIG. 4. Follicular cortex of mesenteric and cervical lymph nodes exposed to ALS_B. (a) Lymphocytes in follicle show bright surface membrane fluorescence. Germinal center cells do not fluoresce. The follicle is outlined by autofluorescent macrophages. $\times 320$. (b) Same field as (a) stained with hematoxylin and eosin. Follicular lymphocytes have densely staining nuclei; germinal center lymphocytes have pale-staining nuclei. Dendritic cells (not well visualized in photomicrograph) have large, vesicular nuclei and long cytoplasmic processes which extend between lymphocytes. (c) Positive fluorescence of dendritic cells in germinal center. Note irregular stippled appearance of cell processes. Dendritic cells are concentrated within the outer (capsular) hemisphere of the germinal center and tend to obscure the non-fluorescing germinal center lymphocytes in this region. $\times 200$. (d) and (e) Fluorescing dendritic cells in germinal centers. Note stellate shape of cell bodies, elongated cell processes, and stippled pattern of surface fluorescence. Germinal center lymphocytes do not fluoresce. $\times 500$.

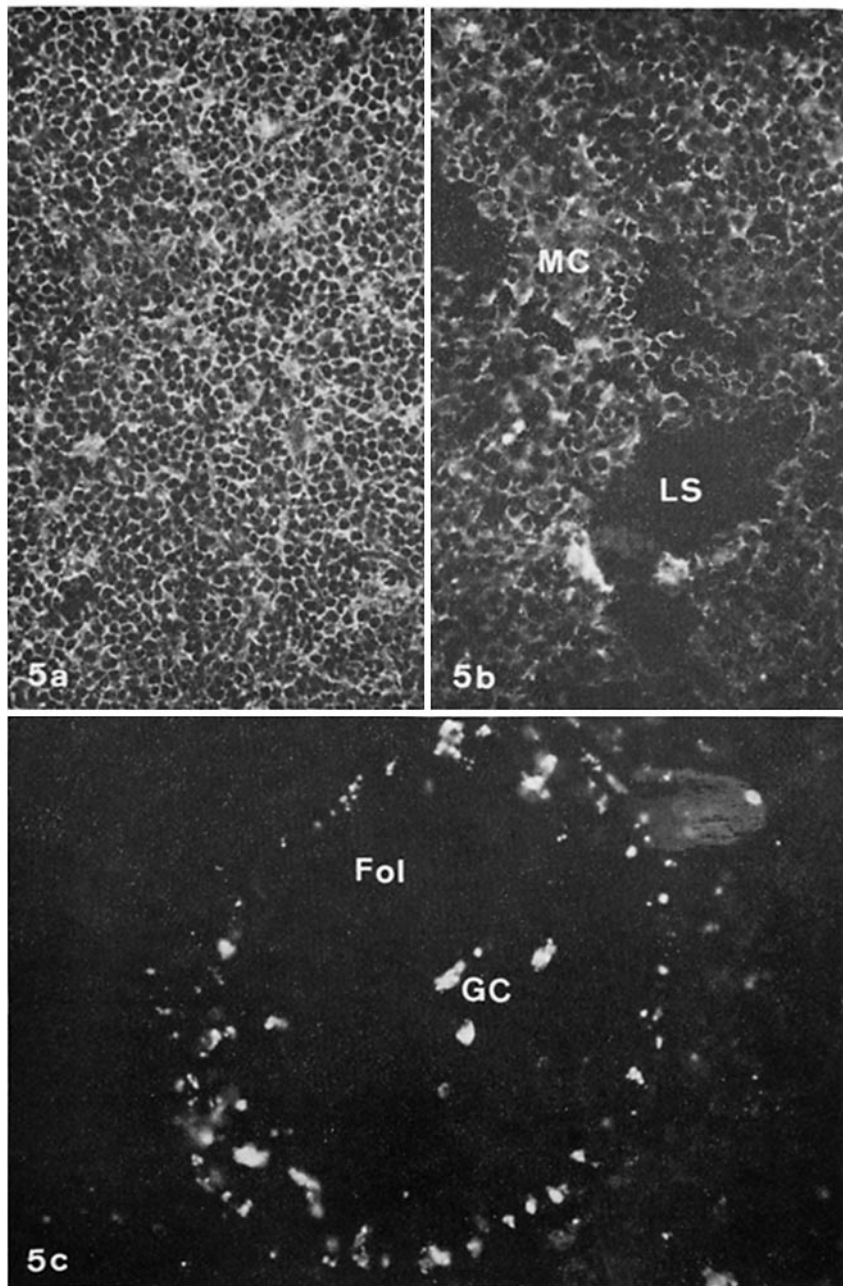
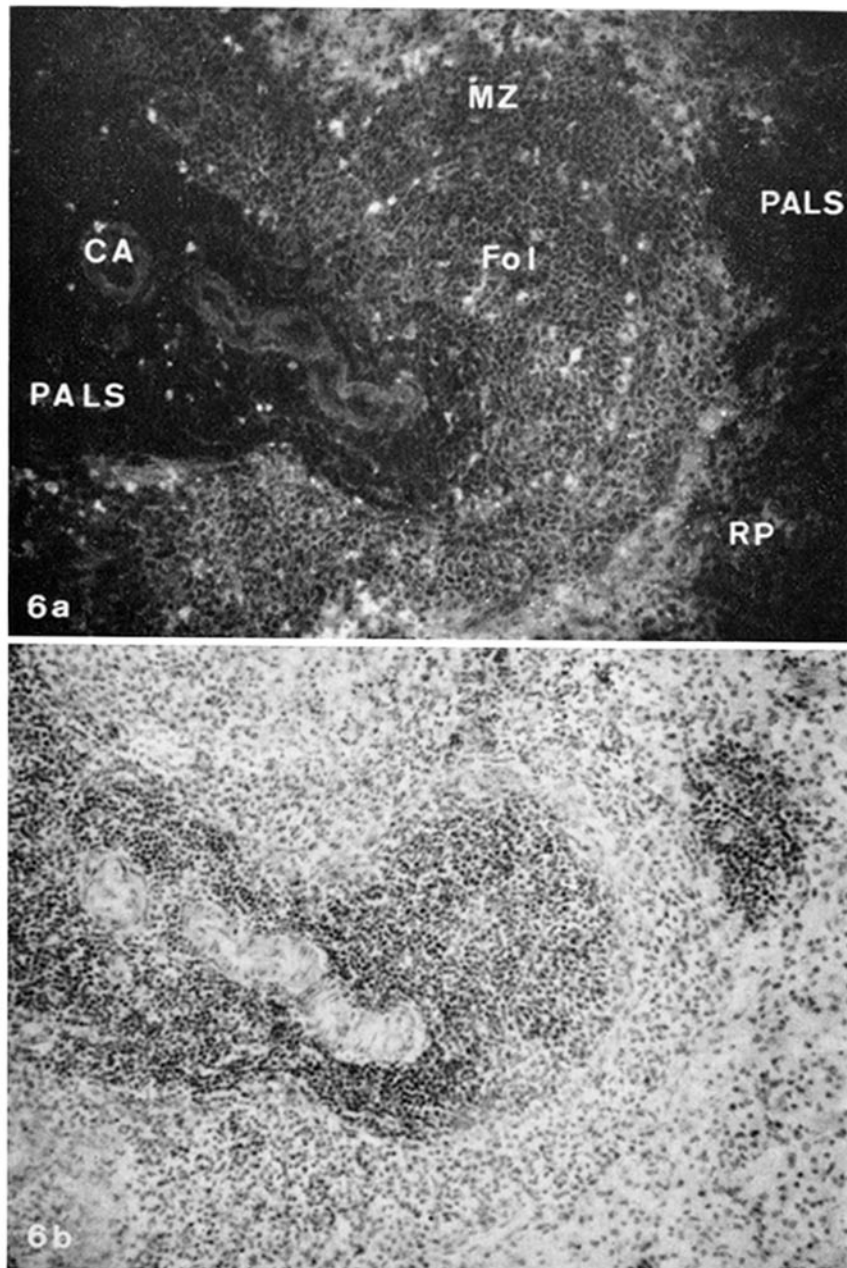


FIG. 5. Mesenteric lymph node exposed to ALS_T . Single section, 3 different fields. $\times 320$. (a) Paracortex: All lymphocytes show intense membrane fluorescence. Endothelial cells of postcapillary venules (not shown) do not fluoresce. (b) Medulla: The surfaces of small lymphocytes in medullary cords and lymph sinuses fluoresce intensely. Reticuloendothelial cells and plasma cells have dull autofluorescent cytoplasm and indistinct cell borders. (c) Follicular cortex: Macrophages alone exhibit intense autofluorescence; lymphocytes in follicle and germinal center are not stained.



FIGS. 6-8. Frozen sections of spleen exposed to either normal rabbit serum or ALS were washed and reacted with FITC-goat antirabbit IgG. *CA*, central arteriole; *PALS*, periarteriolar lymphoid sheath; *Fol*, follicle; *GC* germinal center; *MZ*, marginal zone; *RP*, red pulp.

FIG. 6. Spleen exposed to ALS_B. (a) Lymphocytes in follicle and marginal zone of white pulp show bright surface membrane fluorescence, whereas those in periarteriolar sheath (longitudinal section, left; cross-section, right) do not. Germinal center cells (not shown) do not fluoresce. Macrophages in red pulp exhibit yellow-brown cytoplasmic autofluorescence. $\times 200$. (b) Same field as *a* stained with hematoxylin and eosin.

germinal centers were not stained, although dendritic cells in this area were frequently positive. The cytoplasm of some plasma cells in the red pulp showed weak fluorescence. ALS_T , on the other hand, stained the surfaces of lymphocytes within the periarteriolar lymphocyte sheath of white pulp of spleen (Figs. 7 *a-d* and 8 *a*). A few cells in the marginal zone were also stained, but the great majority of lymphocytes within the marginal zone and virtually all follicular lymphocytes were negative.

The specificity of ALS_T and ALS_B for T and B cells, respectively, was also revealed in the binding of the antisera to frozen sections of thymus. ALS_T reacted strongly with the surface membranes of thymocytes (Fig. 9 *a*), whereas ALS_B did not react at all (Fig. 9 *b*).

ALS prepared against TDL from T-cell-deficient rats: An attempt was made to raise a potent and relatively specific antiserum against rat B lymphocytes by immunizing rabbits with TDL from donors which had been thymectomized, irradiated, and reconstituted with syngeneic bone marrow cells. However, the resulting antisera had antibodies against both T and B lymphocytes in titers comparable to those found in ALS produced against TDL from normal rats.

ATS: Rabbits were injected with thymocytes from normal rats, and the resultant ATS were differentially absorbed by the standard procedure (Fig. 1). After absorption the ATS were found to have antibodies only against T lymphocytes; but the antibody titers were approximately 10-fold less than the titers of similarly absorbed antisera raised against TDL.

Antiserum to rat gamma globulin: Rabbit antirat Ig was reacted with frozen sections of rat lymph nodes, spleen, and thymus. The anatomical and cytological localization of fluorescence staining was identical to that seen with ALS_B (compare Fig. 3 *c* with 10 *a*; and Figs. 6 *a* and 8 *b* with 10 *b*). All activity of the anti-Ig serum and approximately 85% of the activity of ALS_B was removed by absorption with normal rat serum. In addition, the staining of lymph node and spleen sections by FITC-rabbit antirat Ig was blocked by pre-treating the sections with ALS_B . It would seem therefore that the activity of ALS_B is vested mainly in antibodies against rat gamma globulin. This was substantiated by immunodiffusion studies which revealed a single line of identity between several lots of ALS_B , all of which cross-reacted with lines formed by the antirat gamma globulin and normal rat serum (Fig. 12).

ALS_T and ATS, on the other hand, could not be demonstrated to react with rat gamma globulin either by blocking experiments or immunodiffusion.

AMS: Antisera were prepared by immunizing rabbits with rat PEC. After absorption with RBC, thymocytes, and TDL, the antisera were incubated with frozen sections of spleen and thymus. AMS reacted with the surface of macrophages throughout the spleen (Fig. 11 *a, b*) and with scattered macrophages in thymus, but not with lymphocytes. Fibrous connective tissue was intensely stained by AMS, a phenomenon that was not observed in experiments using ALS_B or ALS_T .

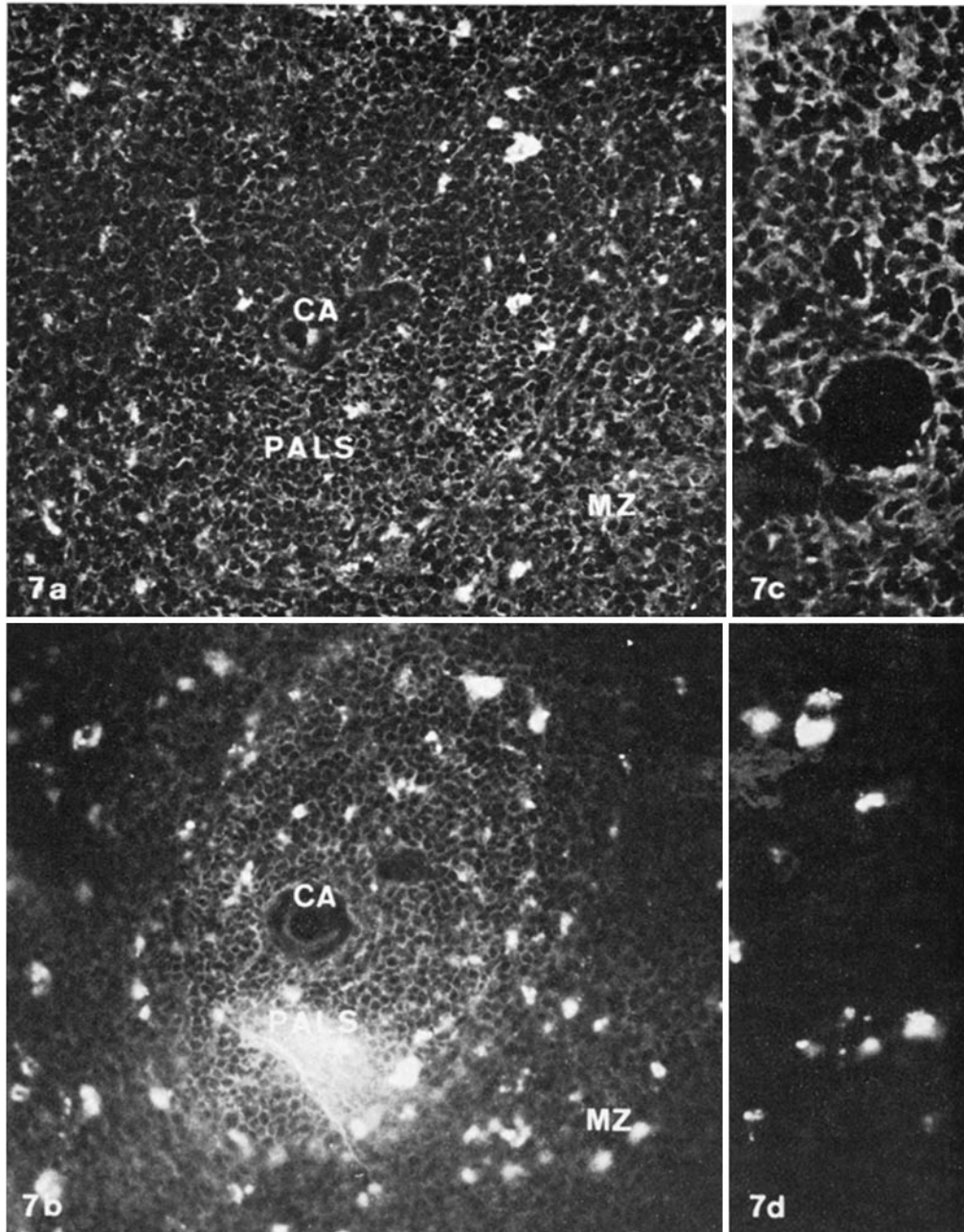


FIG. 7. Serial sections of spleen. (a) White pulp exposed to ALS (RBC absorbed): Cells in periarteriolar sheath and marginal zone fluoresce. A lymphoid follicle is not present in this field. $\times 320$. (b) White pulp exposed to ALS_T: Lymphocytes in periarteriolar sheath alone show intense membrane fluorescence. In other fields, similar cells were occasionally seen in the marginal zone. Larger uniformly stained cells are autofluorescent macrophages. $\times 320$. (c) Red pulp exposed to ALS (RBC absorbed): All cells in splenic cords fluoresce. Sinusoids are empty. $\times 500$. (d) Red pulp exposed to ALS_T: There is no specific fluorescence. Macrophages show intense autofluorescence.

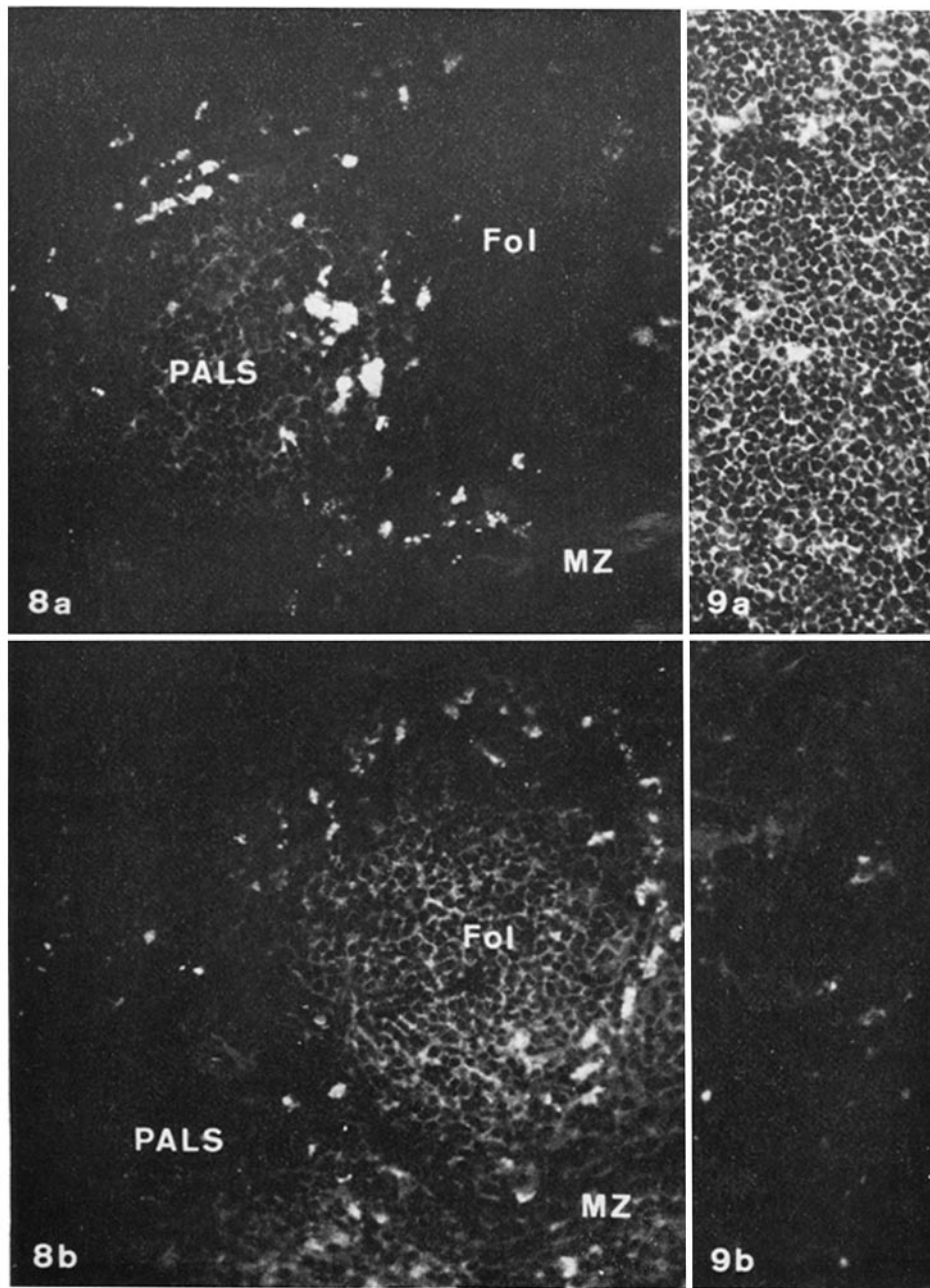


FIG. 8. Serial sections of splenic white pulp. $\times 320$. (a) ALS_T : Specific fluorescence of cells in periarteriolar sheath only. (b) Adjacent section exposed to ALS_B : Specific fluorescence of cells in follicle and marginal zone; lymphocytes in periarteriolar sheath do not stain.

FIG. 9. Serial sections of thymus. $\times 320$. (a) ALS_T : The surface membranes of all thymocytes are stained. (b) Adjacent section exposed to ALS_B : Thymocytes are unstained.

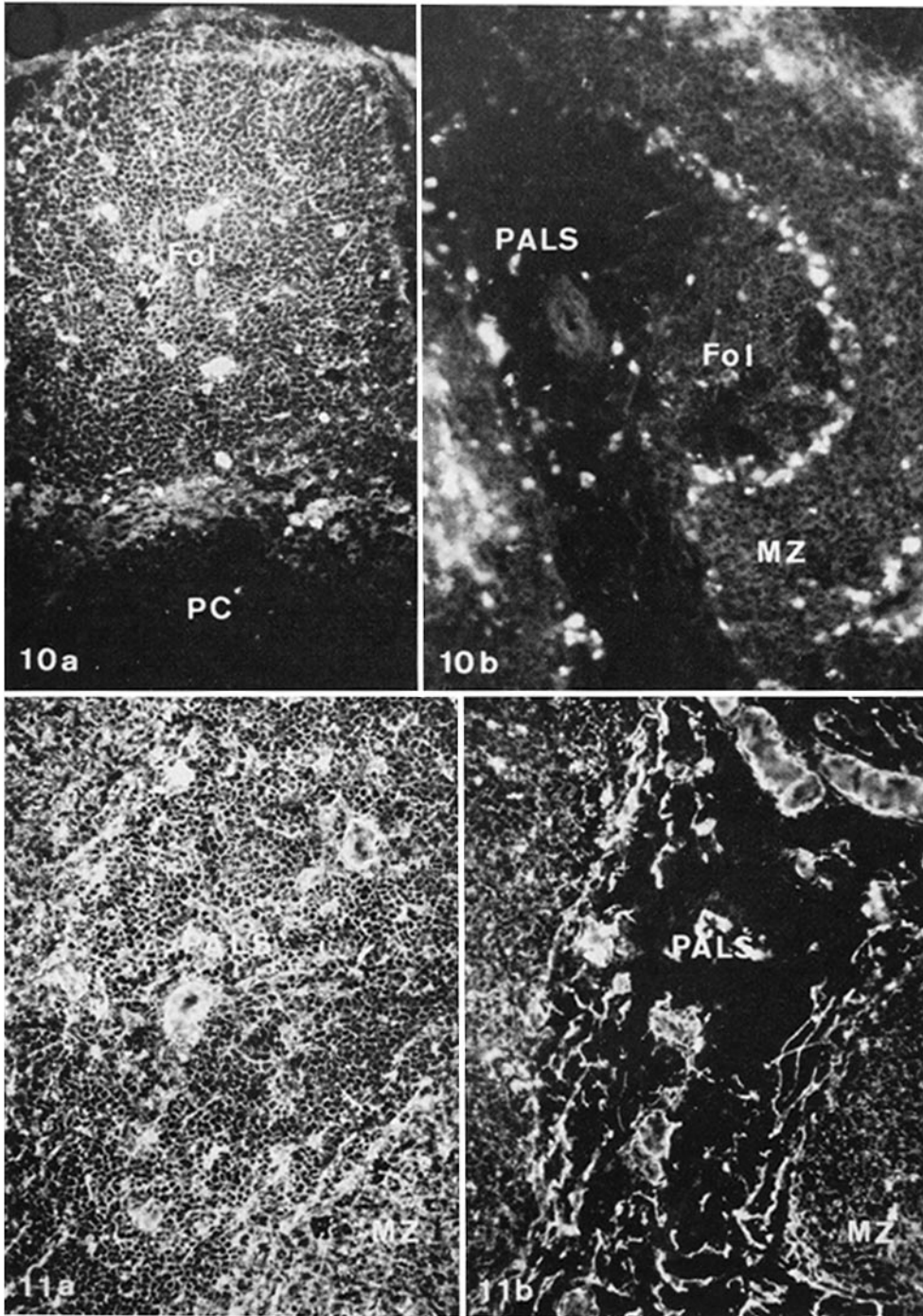
Distribution of B and T Lymphocytes in Suspensions of Cells from Normal Tissues.—Suspensions of living cells from lymphoid tissues, blood, thoracic duct lymph, and the bone marrow of normal rats were exposed to ALS_{T+B} , ALS_T , ALS_B , and antirat Ig. In each case, the cells were examined for fluorescence after development with FITC-goat antirabbit IgG. Normal rabbit serum controls were negative.

Table I indicates that nearly all lymphocytes from the thymus, thoracic duct, lymph node, and spleen could be identified as either T or B lymphocytes as judged by their serum-binding properties. Thymocyte suspensions contained the highest proportion of T cells (>99%) and spleen the lowest (50%). Approximately the same proportion of lymphocytes reacted with anti-Ig serum as with ALS_B ; and pretreatment of the cells with ALS_B inhibited their reaction with FITC-anti-Ig. Similarly, the titers of ALS_B against lymphocytes was reduced eightfold by absorbing the antiserum with normal rat serum before its addition to target cells. In contrast, the titers of ALS_T were reduced only slightly by this procedure, a result that could be explained by dilution alone.

Approximately 15% of cells in the marrow suspensions were scored cytologically as "small lymphocytes", yet only 59% of these reacted with ALS_{T+B} . This implies that the remaining 41% either were not lymphocytes or were lymphocytes that lacked either T- or B-cell surface antigens. A third possibility, namely that a portion of T or B cells were not detected due to problems of counting efficiency was excluded by an experiment in which lymph node cells were added to a suspension of bone marrow cells in numbers equal to or 10 times less than that of the lymphocytes already present in the marrow. Samples of the resultant cell mixtures were reacted with ALS_{T+B} , ALS_T , ALS_B , and the anti-Ig serum. In each case, the efficiency of detecting the added T and B lymphocytes was greater than 90%.

Origin of T Lymphocytes in Bone Marrow.—As shown in Table I, approximately 13% of lymphocyte-like cells in the bone marrow, or 1% of the total nucleated cells, stained with ALS_T . It was of interest to determine whether T lymphocytes in the bone marrow belong to a mobilizable pool of recirculating cells. Accordingly, lymph and cells were drained from a thoracic duct fistula for 8 days. By the end of this period, blood lymphocyte levels had decreased by more than 90%, yet there was no obvious effect upon the proportion of lymphocyte-like cells in the bone marrow. The marrow of both normal and lymphocyte-depleted rats contained cells capable of binding ALS_B , but cells with antigenic determinants for ALS_T were not found in the marrow of the incannulated animals.

Makeup of Lymphocyte Populations in TxBM Rats.—Thymectomized, irradiated, and bone marrow-reconstituted rats have been designated "B" rats, because their lymphoid tissue, blood, and lymph are presumed to be populated by B lymphocytes almost exclusively (17). An effort was made to substantiate this contention by measuring the binding of ALS_T and ALS_B to TDL obtained



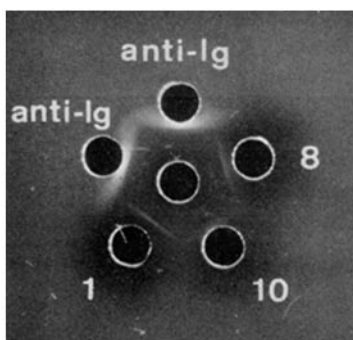


FIG. 12. Immunoprecipitin patterns formed by rabbit antisera and normal rat serum. Anti-Ig, rabbit antirat gamma globulin; arabic numerals, rabbit antirat B-cell sera (ALS_B) prepared from ALS raised in rabbits no. 1, 8, and 10, respectively; center well, normal rat serum. There is complete fusion of the lines formed by ALS_B , 1, 8, and 10; and partial fusion of the lines formed by ALS_B 1 and anti-Ig serum, and by ALS_B 8 and anti-Ig serum.

from freshly incannulated TxBM donors. Approximately 14% of the cells issuing from the thoracic duct of these animals were stained by ALS_T whereas 84% were stained by ALS_B . These values compare with 87% stained by ALS_T and 11% stained by ALS_B in the thoracic duct of freshly incannulated normal donors (Table I). Assuming that the absolute number of cells binding ALS_B in the lymph of the TxBM rats was normal (18), the results suggest that the cell population binding ALS_T had been reduced to approximately 2% of the normal value.

Simplified Protocol for Preparing Antisera Specific for Rat T and B Lymphocytes.—In the foregoing experiments an effort was made to prepare antisera that were specific for antigenic determinants on the plasma membranes of the two major lymphocyte populations in the rat, namely T and B cells. To this end, antibodies eluted from the surfaces of living lymphocytes were absorbed

FIG. 10. Frozen sections of lymph node and spleen exposed to rabbit antirat Ig were washed and reacted with FITC-goat antirabbit IgG. (a) Mesenteric lymph node. Surface membranes of follicular lymphocytes are intensely stained, whereas lymphocytes in paracortex are not. Macrophages in junctional area between follicle and paracortex show yellow-brown cytoplasmic autofluorescence. Compare with Fig. 3 c. $\times 200$. (b) White pulp of spleen. Cells in follicle and marginal zone show specific fluorescence. Lymphocytes in periarteriolar sheath do not fluoresce. Compare with Fig. 6 a. $\times 200$.

FIG. 11. Serial sections of white pulp of spleen. $\times 200$. (a) Exposed to AMS (RBC absorbed): All cells in periarteriolar sheath, follicle (not shown), and marginal zone fluoresce. (b) Exposed to AMS (RBC, THY, and TDC absorbed): Only cells in marginal zone fluoresce. The pattern of fluorescence is irregular and reticulate due to the elongated processes of many cells, and perhaps to staining of intercellular fibers. Macrophages in sinuses and cords of red pulp also exhibited intense surface staining (not shown). Note fluorescence of fibrous connective tissue in periarteriolar sheath, and absence of fluorescence of lymphocytes in this region. Lymphocytes in follicles and germinal centers were also negative.

TABLE I

Distribution of T and B Lymphocytes in Suspensions of Cells from Lewis and (Lewis × DA)_{F1} Hybrid Rats

Specificity of antiserum	Percentage of lymphocytes positive by indirect immunofluorescence*					
	Thymus	Thoracic duct lymph	Lymph node	Spleen	Bone marrow	Blood†
ALS _{T+B}	>99	>99	>99	>95	59 (50-69)	>99
ALS _T	>99	87 (85-91)	81 (79-85)	~50‡	13 (8-15)	61 (58-65)
ALS _B	0.1 (0-0.5)	11 (8-14)	18 (14-21)	~50	38 (33-40)	40 (37-45)
Anti-Ig	0.1 (0-0.5)	9 (7-12)	15 (11-18)	~50	40 (27-45)	33 (27-41)

* Mean of 4-6 experiments. Range given in parentheses.

† Lymphocyte-rich fraction from buffy coat (see methods).

‡ Approximate values. Clumping of spleen cells precluded exact quantitation.

with purified T cells obtained from thymus or with purified B cells obtained from the spleen or bone marrow of T-cell-deficient (TxBM) donors. In retrospect these cumbersome precautions were unnecessary, for it had been our experience in this and other studies (14) that antisera raised against viable cells have little detectable antibody activity against cytoplasmic or nuclear constituents. Furthermore, we have found that erythrocytes, bone marrow cells, and peritoneal exudate cells from normal rats can be used to remove unwanted antibodies from ALS without seriously decreasing the titer against T cells.

A modified version of the original protocol for preparing ALS_T and ALS_B is presented in Fig. 13. Antisera absorbed in this manner have binding properties comparable to those prepared by the more rigorous methods employed in this investigation.

DISCUSSION

A congeries of evidence from studies in the chicken (19, 20), mouse (21-23), rat (1-4, 24), and other mammalian species including man (25) has established the existence of at least two developmentally and functionally distinct populations of lymphocytes. T lymphocytes are produced under the influence of the thymus, whereas B lymphocytes arise from stem cells in the bone marrow and are formed in both normal and thymectomized subjects. Antisera directed toward lymphocyte surface antigens have been exploited in studies of the tissue distribution and function of T and B lymphocytes in the mouse (26-29) and chicken (30). Relatively little is known about the antigenic differences between ontogenetically distinct lymphocyte populations in other species. Anti-Ig sera have generally been used to identify B cells, because B cells have readily detectable Ig receptors at the cell surface (27, 28, 31). T-cell-specific antigens have only recently been described in guinea pig and man (32-34). In the rat, thymus-specific antigens have been known for some time (35-37); however,

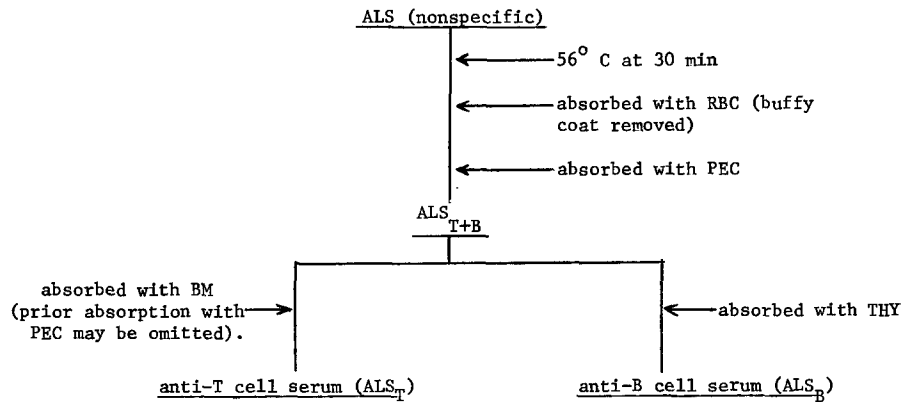


FIG. 13. Modified scheme for absorption of antilymphocyte serum. ALS_B may be further absorbed with normal rat serum to remove antibodies to immunoglobulins.

relatively little is known about the antigenic makeup of peripheral T lymphocytes. Iverson (38) presented indirect evidence that an antiserum raised against rat TDL contained antibodies specific for T lymphocytes. More recently, an antigen similar to murine Thy-1 (θ) antigen has been described on thymus-dependent lymphocytes in the rat (39-41).

In the present experiments, antisera raised in rabbits against thoracic duct lymphocytes were found after differential absorption to contain antibodies specific for antigens on the surface of rat T and B lymphocytes. Using these antisera, the anatomical distribution and quantitative representation of T and B cells in lymphocyte-rich tissues and body fluids of the rat were determined by indirect immunofluorescence. Our findings are in basic agreement with those reported in the mouse (27-29, 42, 43). Thus, approximately 99% of thymocytes, 90% of TDL, and progressively smaller portions of lymphocytes obtained from lymph nodes, blood, spleen, and bone marrow were identified as being T cells in the rat. In frozen sections of lymph node and spleen, T and B cells were located in the thymus-dependent and thymus-independent regions described previously (1, 2). However, unlike the results of Gutman and Weissman (42) who used anti-Thy-1 and anti-Ig sera as markers for T and B lymphocytes in the mouse, we are unable to detect the presence of small numbers of T cells in germinal centers or the presence of Ig molecules on the surface of germinal center lymphocytes.

Results of the current investigation indicate that rat B cells, like mouse B cells, have Ig receptors at high concentration at the cell surface, whereas T cells do not. Rabbit antirat Ig sera reacted selectively with lymphocytes in the thymus-independent areas of lymph node and spleen. Moreover, all antisera made "specific" for B lymphocytes reacted strongly with rat gamma globulin in immunodiffusion and immunoelectrophoresis assays (D. Stetchschulte,

personal communication); and most of the antibody activity of ALS_B could be neutralized by normal rat serum.

In contrast, none of the antisera made specific for T cells had detectable anti-immunoglobulin activity as judged by immunodiffusion and immunoelectrophoresis assays, or by inhibition studies using normal rat serum. It would seem therefore that if Ig molecules are present on T cells, they are represented in low concentration or are sterically hindered from binding rabbit anti-Ig serum. The latter possibility cannot be discounted. Recent studies in the rat (44) have shown that antigen-stimulated T cells have readily detectable surface Ig molecules, but that unstimulated T cells or T cells that have been stimulated by plant mitogens do not.

The ontogenetic relationship of germinal center lymphocytes, follicular lymphocytes, and plasma cells has not been definitively established, although there is evidence that they may be lineal descendants (45). In the present experiments, we were unable to establish an antigenic relationship between these three cell types using anti-B-cell sera and anti-Ig sera. Follicular lymphocytes reacted strongly with both antisera, whereas germinal center lymphocytes and plasma cells reacted with neither antiserum. Similarly, Takahashi et al. (27) could not demonstrate Ig receptors on the surface of most γ -G plaque-forming cells and cells from several mouse myelomas. In addition, Takahashi et al. (46) have described an alloantigen (PC.1) on plasma cells that is absent from lymphocytes which are not engaged in antibody production. These observations suggest either that thymus-independent lymphocytes comprise more than one cell line or that germinal center lymphocytes and follicular lymphocytes undergo marked antigenic changes during the course of differentiation to antibody-forming cells. The finding of Raff et al. (29) of an antigen (MBLA) common to the surfaces of plaque-forming cells, MOPC-315 myeloma cells and follicular lymphocytes in the mouse, but not to T cells, supports the latter interpretation.

The results of the current investigation revealed an unexpected antigenic relationship between follicular lymphocytes and a population of cells in the marginal zone of splenic white pulp. Both were stained by ALS_B and by anti-Ig serum. Despite the widely canvassed view that the marginal zone is populated almost entirely by reticuloendothelial cells, careful morphological studies have shown that medium-size "lymphocytes" are the major cell component in this area (47, 48). It is probable, therefore, that the Ig-bearing cells in the marginal zone belong to the B-cell series. The origin of these marginal zone lymphocytes is not known, but there is evidence for two possible sources. Howard et al. (11) and Sprent (18) have shown that B lymphocytes continuously recirculate from the blood to the follicular region of splenic white pulp. The route followed has not yet been determined; it is conceivable, however, that it lies along a pathway that includes blood sinuses in the marginal zone. Keuning and Bos (49), on the other hand, have suggested that the marginal zone contains antigen-activated

B lymphocytes which are descendants of follicular lymphocytes. It may be significant therefore that the majority of Ig-bearing cells in this region are larger than follicular lymphocytes and appear to have Igs not only at the cell surface, but also in the cytoplasm. This raises the fascinating possibility that some "lymphocytes" in the marginal zone represent an intermediate state of B-cell differentiation between antigen-recognition cells and antibody-forming cells.

There is great interest at the moment in the type(s) of lymphocytes present in the bone marrow. This interest stems in part from the use of bone marrow as a source of hematopoietic and lymphoid cell precursors in both clinical and experimental situations. Inocula prepared from parental strain bone marrow have been shown to be 50-100 times less potent than TDL in causing lethal graft-versus-host reactions in F_1 hybrid recipients (50, 51), suggesting that about 1% of nucleated bone marrow cells or 10% of bone marrow lymphocytes, are T cells. This estimate was substantiated in the present investigation by the finding that 13% of bone marrow lymphocytes have receptors for ALS_T . It also was shown that these lymphocytes could be withdrawn from the marrow by chronic thoracic duct drainage, a procedure which also depletes the marrow of histocompatibility antigen-reactive cells (52) and of cells capable of collaborating with B lymphocytes in the hemolysin response to sheep erythrocytes (53, 54).

Although rats can be severely depleted of T lymphocytes by thymectomy and irradiation, such animals retain a limited ability to initiate cell-mediated immune responses. For example, they can shed first-set skin allografts from distantly related donors (unpublished observations) and can defend themselves against the intracellular bacterial parasite, *Listeria monocytogenes* (12). The results of the current study provide a plausible explanation for these findings, for they indicate that TxBM rats are not completely divested of T lymphocytes. Approximately 14% of cells issuing from the thoracic duct of freshly incannulated TxBM donors had receptors for ALS_T , although the total pool of these cells had been reduced 98%. It is logical to postulate that some T lymphocytes derive from the bone marrow with which the irradiated animals were reconstituted. But there is also evidence that some host T cells may have escaped the lethal effects of irradiation (12). Regardless of their origin, it is evident that the presence of T cells in the lymph of TxBM rats would in itself explain our failure to raise specific anti-B-cell sera by immunizing rabbits with TDL obtained from such donors.

The precursors of peripheral T cells have been shown to belong to a line of dividing cells in bone marrow which cannot be withdrawn by chronic drainage from the thoracic duct (51, 52), and which lack antigens that are present in high concentration on thymocytes (55). These precursor cells have not yet been identified morphologically, but it is plausible to think that they, and possibly also the precursors of B lymphocytes, are part of the lymphocyte-like cell population in the bone marrow that fails to stain with either ALS_T or ALS_B .

SUMMARY

A method is described whereby antisera raised in rabbits to rat thoracic duct lymphocytes were made specific for the plasma membrane antigens of T and B lymphocytes. These lymphocyte-specific antisera were used in immunofluorescence assays to study the distribution of B and T cells in lymphocyte containing tissues and body fluids of the rat.

Rabbit antirat B-cell serum (ALS_B) reacted selectively with the surfaces of lymphocytes in the lymphoid follicles of lymph node cortex and in the follicles and marginal zones of splenic white pulp, but not with the surfaces of germinal center cells or plasma cells. An identical pattern of fluorescent staining was obtained with rabbit antirat Ig serum. It was shown by blocking, absorption, and immunoprecipitation studies that ALS_B was composed in large part of antibodies to rat Ig, but that it contained antibodies to other B-cell antigens as well.

Rabbit antirat T-cell serum (ALS_T) reacted selectively with the surfaces of lymphocytes in the paracortex of lymph node and in the periarteriolar sheath of spleen, and with thymocytes. ALS_T did not display anti-Ig activity.

ALS_T reacted with approximately 100% thymocytes and with 90% thoracic duct, 80% lymph node, 60% blood, 50% spleen, and 10% bone marrow lymphocytes in suspensions of cells from these sources. ALS_B reacted with the remainder of the lymphocytes in the suspensions, except for bone marrow in which only 59% of lymphocytes had detectable B- or T-cell surface antigens.

The population of T lymphocytes in rat bone marrow was depleted by drainage of lymphocytes from a thoracic duct fistula, thereby establishing their membership in the pool of recirculating T cells. Approximately 14% of lymphocytes issuing from the thoracic duct of TxBM donors reacted with ALS_T. The presence in these animals of a small number of T cells, calculated to be approximately 2% of the normal value, may account for the limited capacity of TxBM rats to respond to antigens that induce a cell-mediated immune response.

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