

## FUNCTIONAL HETEROGENEITY OF MURINE LYMPHOID CELLS

### V. LYMPHOCYTES LACKING DETECTABLE SURFACE $\theta$ OR IMMUNOGLOBULIN DETERMINANTS

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Lymphocyte populations of several species have been divided into two broad categories on the basis of ontogenetic and functional studies, as well as by differences in cell surface antigens (1-3). Although all lymphocytes appear to derive originally from stem cells in hematopoietic tissue (4), the subpopulations can be characterized on the basis of the organ in which their final differentiation is believed to occur. Thus, one population is thymus derived or thymus dependent (T lymphocytes), is involved in cellular immune responses (5, 6), and exhibits "helper" function in the antibody response to certain antigens (7). The lymphocytes of the other major population participate in the humoral immune response as precursors of antibody-secreting cells (2) and also mediate the destruction of antibody-coated target cells (8, 9). These cells are referred to as B lymphocytes and undergo final differentiation, in birds, in the bursa of Fabricius (1, 10). The site of differentiation of this cell type in mammals is not definitely established and consequently, in mammals, these cells are termed bone marrow-derived lymphocytes. In mice, specific markers have been described that allow cells of these two populations to be distinguished from each other (3). The isoantigenic marker  $\theta$  is found on thymocytes and thymus-derived lymphocytes; T lymphocytes contain insufficient immunoglobulin (Ig) on their surface to be detected by fluorescent or radioactive anti-immunoglobulin, at least by conventional procedures. On the other hand, bone marrow-derived lymphocytes possess easily demonstrable amounts of Ig on their surface while bearing no detectable  $\theta$ -determinants (11). In addition, this latter group of cells has receptors for the third component of complement (12) and for aggregated immunoglobulin (13, 14), allowing them to bind both antigen-antibody-complement and antigen-antibody complexes.

In the present communication we describe the existence of a population of murine lymphoid cells that, by conventional techniques, do not bear detectable  $\theta$  or Ig determinants on their surface and do not have receptors for the third component of complement. Suspensions of lymphocytes containing 55-78% of such  $\theta^-$ , Ig $^-$  cells have been obtained by purification procedures, and electron micrographs of these cells show them indeed to resemble lymphocytes. These

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$\theta^-$ , Ig $^-$  cells constitute 3–13% of splenic lymphocytes and 0–4% of lymph node lymphocytes in normal mouse strains. In NZ mice, however, such cells constitute more than 20% of splenic lymphocytes (15).

### Materials and Methods

*Animals.*—BALB/c, C3H, AKR, NZB, NZW, and (NZB  $\times$  NZW) $F_1$  (B/W) mice were obtained from the Division of Research Services of the National Institutes of Health. CBA/J and C57BL/6 mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

*Cell Suspensions.*—Single-cell suspensions were prepared from the spleen and from inguinal and mesenteric lymph nodes by gentle teasing of the lymphoid organs with forceps in chilled RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.).

*Antisera.*—Anti- $\theta$ -C3H, anti- $\theta$ -AKR, and burro antimouse thymocyte antisera (ATS)<sup>1</sup> were prepared as previously described (16). The ATS was absorbed with BALB/c erythrocytes before use. In the presence of complement, it was capable of lysing essentially all mouse lymphocytes. Rabbit antimouse  $\kappa$ -chain antiserum, prepared by immunization of a panel of rabbits with a mixture of several  $\kappa$ -type Bence Jones proteins, was a gift of Doctors R. Mage and K. R. McIntire. Purified anti- $\kappa$ -antibody was prepared from this antiserum by absorption to and elution from agarose affinity columns to which mouse  $\kappa$ -type myeloma proteins of the  $\gamma_1$  and  $\gamma_2$  classes had been coupled. The purified antibody was capable of precipitating mouse immunoglobulins of the  $\kappa$ -class as well as  $\kappa$ -type Bence Jones protein. Absorption of the antibody with a  $\kappa$ -type  $\gamma$ A myeloma protein (HOPC 8; a gift of Dr. M. Potter) coupled to agarose removed all the antibody activity. Rabbit antimouse  $\lambda$ -chain antiserum, raised by the immunization of rabbits with the Bence Jones protein from mice bearing the tumor RP-20, was a gift of Doctors R. and M. Mage. Rabbit antiserum directed against MOPC 315, a myeloma protein with  $\lambda$ -chains with different antigenic determinants from RP-20, was kindly provided by Dr. H. Eisen. Antiserum to BALB/c and C57BL/6 histocompatibility determinants were prepared by the immunization of C57BL/6 mice with BALB/c spleen cells and the immunization of BALB/c mice with C57BL/6 spleen cells, respectively. 100 million cells were given, intraperitoneally, weekly for 4–5 wk and sera harvested 1 wk after the last injection. We thank Dr. R. Sharon for the gift of these sera.

*Quantitation of Ig- and of  $\theta$ -Bearing Cells.*—

*Cytotoxicity assay:* A  $^{51}\text{Cr}$  release cytotoxicity assay, which has previously been described in detail (16), was employed. Briefly, cell suspensions from which the red cells had been removed by hypotonic lysis with Tris-NH $_4$ Cl buffer were chromated and washed. The cells were brought to a concentration of  $1 \times 10^6$  per ml; 50  $\mu\text{l}$  of cells was incubated with 50  $\mu\text{l}$  of antisera at room temperature for 15 min. 50  $\mu\text{l}$  of a 1:4 dilution of guinea pig complement (C; Baltimore Biological Laboratories, Cockeysville, Md.) was added and the mixture incubated for 30 min at 37°C. After sedimentation of the cells by centrifugation, 50  $\mu\text{l}$  of supernatant was removed and the radioactivity of this sample measured in a  $\gamma$ -ray spectrometer. Specific lysis caused by a given antiserum was calculated as follows:

$$\% \text{ Specific lysis} = \frac{\text{Radioactivity released by antiserum} + \text{C} - \text{Radioactivity released by normal serum} + \text{C}}{\text{Radioactivity released by freeze-thawing} - \text{Radioactivity released by normal serum} + \text{C}}$$

Frequency of lymphocytes bearing a given marker was calculated by dividing maximal specific lysis caused by antiserum to that marker by the specific lysis caused by absorbed ATS.

<sup>1</sup> *Abbreviations used in this paper:* A, antibody; ATS, antimouse thymocyte antiserum; BSA, bovine serum albumin; C, complement; CRL, complement receptor lymphocytes; E, erythrocytes; SRBC, sheep red blood cells.

*Immunofluorescence:* To  $5 \times 10^6$  lymphoid cells suspended in 0.5 ml of medium containing 1% bovine serum albumin (BSA; Miles Laboratories, Inc., Kankakee, Ill.), 0.1 ml of anti- $\theta$ -antiserum or of normal mouse serum was added. The cells were incubated at 4°C for 30 min and then washed twice in 5% BSA in medium. The washed cells were resuspended in 0.5 ml of cold 20% normal rabbit serum and 250  $\mu\text{g}$  of anti- $\kappa$ -antibody was added to all tubes. After a 30 min incubation at 4°C, the cell pellet was washed by centrifugation through 5% BSA. Fluoresceinated goat antirabbit immunoglobulin (0.1 ml; a gift of Dr. Ira Green) was added to all tubes and a further 30 min incubation at 4°C carried out. After three final washes through 5% BSA, the cells were examined in the living state under dark-field illumination. Small round cells were initially located with a tungsten light source and fluorescence was then determined by switching to an ultraviolet light source. The frequency of  $\kappa$ -bearing cells was determined from the fraction of fluorescent cells observed when the cells were initially incubated in normal mouse serum. The combined frequency of  $\kappa$ -bearing and of  $\theta$ -bearing cells was determined from the fraction of fluorescent cells observed when the cells were initially incubated in anti- $\theta$ -serum and the frequency of cells lacking both  $\theta$  and Ig ( $\theta^-$ , Ig $^-$  cells) was equivalent to that of the nonfluorescent small round cells after such treatment. Finally, the frequency of  $\theta$ -bearing cells was taken as the frequency of fluorescent cells when anti- $\theta$  had been used minus the frequency of fluorescent cells when normal mouse serum had been used.

*Autoradiography:* The frequency of lymphocytes bearing either  $\theta$ - or  $\kappa$ -determinants was also quantitated by using an autoradiographic technique similar to that described for immunofluorescence.  $5\text{--}10 \times 10^6$  lymphoid cells were suspended in 1% BSA and either anti- $\theta$ -antiserum or normal mouse serum was added to all tubes. After a 30 min incubation at 4°C, the cells were washed twice in 5% BSA and resuspended in 0.5 ml of 20% normal rabbit serum; 0.125  $\mu\text{g}$  of [ $^{125}\text{I}$ ]anti- $\kappa$ -antibody (25–36  $\mu\text{Ci}/\mu\text{g}$ ; radioiodinated by the chloramine T method) was added to all tubes. The cells were incubated again at 4°C for 30 min and then washed three times by centrifugation through 5% BSA. The cell pellet was smeared on glass slides, dipped in nuclear track emulsion (NTB-2; Eastman Kodak Co., Rochester, N.Y.), and exposed for 2–10 days. The slides were developed and then stained with methyl green-pyronin. Small, round, nonpyroninophilic cells were scored for grains. The frequency of  $\theta^+$  cells, of  $\kappa^+$  cells, and of  $\theta^-$ ,  $\kappa^-$  cells was determined as described under immunofluorescence. Increasing the amount of anti- $\kappa$ -antibody to 0.25  $\mu\text{g}$  or increasing the exposure time to 21 days did not result in an increase in the number of positive cells.

*Removal of complement receptor lymphocytes:* Many B lymphocytes possess a receptor for C3 and can form rosettes with erythrocytes (E) coated with antibody (A) and complement (C). The procedures for forming such rosettes have been reported previously (12, 17). Sheep red blood cells (SRBC) were sensitized with 19S rabbit anti-SRBC antibody and with diluted normal mouse serum as a source of C (EAC). As a control, SRBC were sensitized in a similar manner except that C was omitted (EA). After washing,  $1 \times 10^8$  EAC or EA were mixed with  $2 \times 10^6$  spleen cells at 37°C, for 30 min in the presence of 0.01 M EDTA. The cells were then layered onto a two-step gradient (10%/35%) of BSA (Pathocyte no. 5; Miles Labs.). The rosettes were pelleted by centrifugation at 30,000 g for 30 min in a Spinco model L ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.). Cells banding at the interface were collected and evaluated for surface  $\theta$  and  $\kappa$  using the immunofluorescent technique described above.

*Electron Microscopy.*—Cell suspensions enriched in  $\theta^-$ ,  $\kappa^-$  spleen cells were fixed for a total of 45 min at room temperature in phosphate-buffered glutaraldehyde (1%, pH 7.2). Immediately after addition of the fixative, the spleen cells were centrifuged at 1800 g and the pellet was minced in  $\sim 2\text{-mm}$  cubes. The specimens were postfixed in 1% osmic acid, dehydrated through graded ethanol and propylene oxide solutions, and embedded in Maraglas (Polysciences, Inc., Warrington, Pa.). Thin sections were prepared, stained with lead citrate-uranyl acetate, and examined in a Philips EM-300 electron microscope (Philips Electronic Instruments, Mount Vernon, N.Y.).

## RESULTS

*Frequency of  $\theta$ -Bearing and of  $\kappa$ -Bearing Cells in Splenic Lymphocyte Populations.*—In our initial studies on the frequency of  $\theta$ -bearing and of  $\kappa$ -bearing spleen cells by a quantitative cytotoxic reaction, we routinely observed that the sum of the maximum number of cells that could be lysed with anti- $\theta$  and C and with anti- $\kappa$  and C was substantially less than the number that were lysed with an absorbed ATS and C. The ATS appeared capable of lysing most or all lymphocytes. Thus, ATS lysed 82–88% of spleen cells; we have tentatively assumed that this defines a lower estimate for the number of lymphocytes in the population. In normal strains, between 13 and 24% of the spleen cells that could be lysed by ATS were not lysed by either anti- $\theta$  or anti- $\kappa$  (Table I).

TABLE I  
*Frequency of  $\theta$ -Bearing,  $\kappa$ -Bearing, and  $\theta^-$ ,  $\kappa^-$  Lymphocytes in Splenic Lymphocyte Populations*

Strain	N	% $\theta$ -Bearing cells			% $\kappa$ -Bearing cells			% $\theta^-$ , $\kappa^-$ Cells		
		$^{51}\text{Cr}$	Fl	$^{125}\text{I}$	$^{51}\text{Cr}$	Fl	$^{125}\text{I}$	$^{51}\text{Cr}$	Fl	$^{125}\text{I}$
BALB/c	3	43 $\pm$ 2	42 $\pm$ 5	39 $\pm$ 1	40 $\pm$ 1	46 $\pm$ 1	49 $\pm$ 1	17 $\pm$ 3	10 $\pm$ 5	12 $\pm$ 1
C3H	3	39 $\pm$ 2	51 $\pm$ 2	43 $\pm$ 1	37 $\pm$ 1	44 $\pm$ 3	51 $\pm$ 1	24 $\pm$ 2	5 $\pm$ 1	7 $\pm$ 1
C57BL/6	3	43 $\pm$ 1	50 $\pm$ 2	44 $\pm$ 1	43 $\pm$ 3	47 $\pm$ 2	45 $\pm$ 1	14 $\pm$ 2	3 $\pm$ 0	11 $\pm$ 1
CBA/J	3	41 $\pm$ 2	50 $\pm$ 2	45 $\pm$ 1	46 $\pm$ 1	47 $\pm$ 2	46 $\pm$ 2	13 $\pm$ 2	3 $\pm$ 2	9 $\pm$ 3
AKR	2	37	39	40	44	48	49	19	14	12
NZB	3	34 $\pm$ 1	49 $\pm$ 4	46 $\pm$ 1	24 $\pm$ 1	24 $\pm$ 2	31 $\pm$ 2	42 $\pm$ 1	28 $\pm$ 2	22 $\pm$ 2
NZW	3	44 $\pm$ 1	49 $\pm$ 1	43 $\pm$ 0.3	20 $\pm$ 1	24 $\pm$ 1	29 $\pm$ 1	36 $\pm$ 3	27 $\pm$ 0.3	28 $\pm$ 1
B/W	3	23 $\pm$ 3	43 $\pm$ 1	39 $\pm$ 3	17 $\pm$ 2	22 $\pm$ 2	34 $\pm$ 0.3	60 $\pm$ 4	33 $\pm$ 5	27 $\pm$ 2

Frequency of cells bearing indicated markers was determined by a  $^{51}\text{Cr}$  release cytotoxicity assay ( $^{51}\text{Cr}$ ), fluorescence after incubation with fluoresceinated antisera (Fl), or autoradiographic detection after incubation with  $^{125}\text{I}$ -labeled antibody ( $^{125}\text{I}$ ). N refers to the number of individual animals in a group. Values are means  $\pm$  standard error of the mean.

Although it is possible that some of these cells were not lymphocytes but were still lysed by the absorbed ATS, this seemed unlikely as it would require a non-lymphocyte population of more than 12–18% of the spleen cells. Alternatively, it was possible that a substantial number of these cells did indeed bear  $\theta$ - or  $\kappa$ -determinants but escaped lysis by our reagents. This is clearly a possibility, despite the fact that values for both  $\theta$ - and  $\kappa$ -bearing cells were plateau values and increasing the concentration of antiserum did not increase the percentage of cells lysed by either serum.

To further investigate these possibilities, the frequency of  $\theta$ -bearing,  $\kappa$ -bearing, and  $\theta^-$ ,  $\kappa^-$  lymphocytes was determined using both an indirect immunofluorescence technique and autoradiography. These procedures tended to yield slightly higher values for  $\theta$ -bearing cells and for  $\kappa$ -bearing cells and somewhat lower values for  $\theta^-$ ,  $\kappa^-$  cells. Nonetheless, the frequency of  $\theta^-$ ,  $\kappa^-$  cells from immunofluorescence data ranged from 3 to 14% in the "normal" strains studied.

Using autoradiographic procedures we found the frequency of these cells varied from 7 to 12% (Table I). The latter procedure is probably the most reliable as it combines sensitivity with positive identification of the  $\theta^-$ ,  $\kappa^-$  cells as lymphocytes by standard light microscope criteria (Fig. 1).

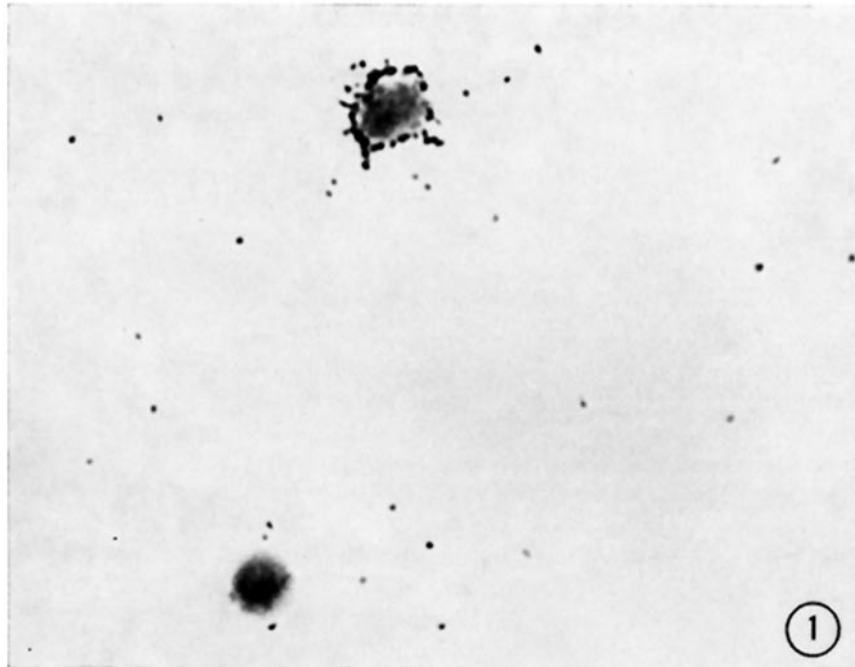


FIG. 1. Autoradiograph of a cell preparation incubated with anti- $\theta$  and  $^{125}\text{I}$ anti- $\kappa$ . One heavily labeled lymphocyte and one unlabeled lymphocyte are shown. The latter cell is classifiable as a  $\theta^-$ ,  $\kappa^-$  cell.

Furthermore, as we have previously reported (15), NZ mice demonstrate a striking increase in the frequency of  $\theta^-$ ,  $\kappa^-$  cells. Spleens of 4-6-wk old NZB, NZW, and (NZB  $\times$  NZW) $F_1$  mice have from 27 to 33%  $\theta^-$ ,  $\kappa^-$  cells by fluorescence assay, and by autoradiography, 22 to 28%  $\theta^-$ ,  $\kappa^-$  cells. These  $\theta^-$ ,  $\kappa^-$  cells are not simply Ig-bearing cells with surface  $\lambda$ -determinants. Thus, evaluation of the frequency of  $\lambda$ -bearing cells in spleens of BALB/c and of NZB mice by indirect immunofluorescence with anti- $\lambda$ -antiserum reveals that only 3 and 5% of the cells, respectively, bear such determinants. Moreover, evaluation of the frequency of  $\theta^-$ ,  $\kappa^-$  cells in the spleens of BALB/c mice by the use of anti- $\kappa$ - and anti- $\lambda$ -reagents simultaneously yields a frequency of  $\theta^-$ ,  $\kappa^-$  cells of 2-10%. Consequently, we will subsequently refer to  $\theta^-$ ,  $\kappa^-$  cells as  $\theta^-$ , Ig $^-$  cells.

The population of  $\theta^-$ , Ig $^-$  cells might be contributed to by the following cell types: (a) T lymphocytes lacking sufficient  $\theta$  to be detected by our procedures; (b) B lymphocytes lacking sufficient surface immunoglobulin to be detected by our procedures or possessing surface immunoglobulin of a type that our reagents do not detect or in a configuration not available to

our reagents; (c) nonlymphoid cells that resemble lymphocytes; (d) lymphocytes that are not classical B or T cells.

A series of experiments aimed at dealing with some of these possibilities and at describing other characteristics of these cells constitutes the remainder of this communication.

*Are  $\theta^-$ , Ig $^-$  Cells Thymus-Derived Lymphocytes Bearing Too Little  $\theta$  to Be Detected by Our Reagents?*—If  $\theta^-$ , Ig $^-$  cells are  $\theta^-$  T lymphocytes, procedures that diminish the frequency of T cells should also diminish the frequency of  $\theta^-$ , Ig $^-$  cells. The two procedures that we have utilized to decrease the frequency of T cells are (a) in vivo treatment with ATS and (b) thymectomy, irradiation, and reconstitution with bone marrow cells.

CBA/J mice received 1.0 ml of ATS intraperitoneally; 48 h later their spleens were removed and the number of  $\theta^+$ ,  $\kappa^+$  and  $\theta^-$ , Ig $^-$  cells evaluated by both immunofluorescence and autoradiography. Spleen cell populations from ATS-treated animals had 6–7%  $\theta^+$  cells as compared with 39–41% in spleen cell suspensions from normal animals (Table II). The  $\theta^-$ , Ig $^-$  cells, on the other hand, constitute 23–26% of the splenic lymphocytes of ATS-treated animals, but only 12–13% of the spleen cells of normal animals. Similarly, spleens from thymectomized, irradiated (850 R) mice reconstituted only with bone marrow have a marked reduction in their content of  $\theta$ -bearing cells and an increase in the frequency of  $\theta^-$ , Ig $^-$  cells (Table II). Both these findings strongly suggest that  $\theta^-$ , Ig $^-$  cells are not  $\theta^-$  T cells.

Moreover, there is an increase in the frequency of  $\theta^-$ ,  $\kappa^-$  lymphocytes in the peripheral and mesenteric lymph nodes of BALB/c mice that have been thymectomized as adults, irradiated (750 R), and reconstituted with anti- $\theta$ -treated bone marrow. When such animals are evaluated 6 wk after reconstitution, 13.4 and 12.2% of peripheral and mesenteric lymph node cells fail to be labeled by

TABLE II  
*Effect of ATS and of Thymus Deprivation on Frequency of  $\theta^-$ , Ig $^-$  Splenic Lymphocytes*

Treatment	% $\theta$ -Bearing cells		% $\kappa$ -Bearing cells		% $\theta^-$ , Ig $^-$ cells	
	F1*	$^{125}\text{I}$ *	F1	$^{125}\text{I}$	F1	$^{125}\text{I}$
None‡	41	39	46	49	13	12
ATS§	7	6	70	68	23	26
Tx; X ray	4	5	66	68	30	27

\* F1 refers to the use of fluoresceinated antisera to determine the percent of lymphocytes of the various types.  $^{125}\text{I}$  refers to the use of [ $^{125}\text{I}$ ]antibody for the same purpose.

‡ Normal CBA mice.

§ CBA mice received 1.0 ml of ATS intraperitoneally. 48 h later, spleen cell suspensions were prepared and the frequency of the various cell types was determined.

|| CBA mice in this group were thymectomized (Tx) at 6 wk of age. 1 wk later they were irradiated with 850 R and reconstituted with  $5 \times 10^6$  syngeneic bone marrow cells. Spleen cells were examined 6–10 wk later.

anti- $\theta$  plus [ $^{125}$ I]anti- $\kappa$ , whereas the frequency of unlabeled lymphocytes in normal BALB/c mice was 10.3 and 6.4%, respectively (Table III). This confirms the finding obtained with spleen cells that thymus deprivation does not diminish the frequency of  $\theta^-$ , Ig $^-$  cells. In addition, the occurrence of  $\theta^-$ , Ig $^-$  cells in lymph nodes strengthens the contention that these cells are lymphocytes, as the frequency of nonlymphoid cells in lymph node is considerably less than that in spleen.

In order to more rigorously test the possibility that  $\theta^-$ , Ig $^-$  cells might be  $\theta^-$  T cells, we prepared chimeric mice in which the T cells could be identified by a histocompatibility marker.

Adult C57BL/6 mice were thymectomized, irradiated with 850 R, and reconstituted with  $5 \times 10^6$  C57BL/6 bone marrow cells and with  $80 \times 10^6$  (C57BL/6  $\times$  BALB/c)F $_1$  thymocytes. In such mice, therefore, histocompatibility antigens of the BALB/c should be restricted to T lymphocytes and antisera to BALB/c antigens should only identify T cells. 8 wk after reconstitution, spleens were removed and evaluated for  $\theta$ -bearing cells, cells bearing C57BL/6 antigens, cells bearing BALB/c antigens, and cells bearing  $\kappa$ -chains.

TABLE III

$\theta^-$ ,  $\kappa^-$  Lymphocytes in Peripheral and Mesenteric Lymph Nodes of Normal and Thymus-Deprived BALB/c Mice

Animal	Nodes	Anti- $\theta$ + anti- $\kappa$ +/total	NMS + anti- $\kappa$ +/total	$\kappa^+$	$\theta^+$	$\theta^-$ , $\kappa^-$ *
				%	%	%
Normal	Peripheral	556/620 (89.7%)	128/327 (39.1%)	39.1	50.6	10.3
ATXBM	Peripheral	567/655 (86.5%)	300/400 (75%)	75.0	11.6	13.4
Normal	Mesenteric	383/409 (93.6%)	50/200 (25%)	25.0	68.6	6.4
ATXBM	Mesenteric	383/436 (87.8%)	426/479 (88.9%)	88.9	0	12.2 (11.1)‡

Mesenteric and peripheral lymph node cells from normal BALB/c mice and BALB/c mice thymectomized as adults, irradiated (750 R), and reconstituted with anti- $\theta$ -treated bone marrow (ATXBM) were incubated with either anti- $\theta$  or normal mouse serum (NMS) and [ $^{125}$ I]anti- $\kappa$ , and the frequency of  $\theta$ -bearing,  $\kappa$ -bearing, and  $\theta^-$ ,  $\kappa^-$  lymphocytes was determined by autoradiography.

\* The percent of  $\theta^-$ ,  $\kappa^-$  cells is 100 — the percent of cells stained with anti- $\theta$  + anti- $\kappa$ .

‡ The figures in parentheses represent 100 — the frequency of  $\kappa$ -bearing cells.

Two separate experiments were performed. In one instance indirect immunofluorescence was used for identification of the various cell types; in the other case, the cells were identified autoradiographically (Table IV). The frequency of T lymphocytes was essentially the same when determined using either anti- $\theta$  alone, anti-BALB/c alone, or anti- $\theta$  plus anti-BALB/c. Thus, our anti- $\theta$ -reagent appears to be capable of detecting essentially all the T cells and, in turn,  $\theta^-$  T cells cannot make any substantial contribution to the population of  $\theta^-$ , Ig $^-$  cells.

TABLE IV  
 $\theta^-$ ,  $Ig^-$  Cells Are not  $\theta^-$  T cells

Reagents	Positive lymphocytes	Conclusion†
	%	
Exp. A Immunofluorescence*		
Anti- $\kappa$	45	45% $\kappa$ -bearing cells
Anti- $\theta$ + anti- $\kappa$	80	35% $\theta$ -bearing cells; 20% $\theta^-$ , $\kappa^-$ cells
Anti-BALB/c + anti- $\kappa$	82	37% BALB/c-bearing cells; 18% BALB/c <sup>-</sup> , $\kappa^-$ cells
Anti- $\theta$ + anti-BALB/c + anti- $\kappa$	84	39% BALB/c-bearing or $\theta$ -bearing cells; 16% $\theta^-$ , BALB/c <sup>-</sup> , $\kappa^-$ cells
Anti-C57BL/6 + anti- $\kappa$	99	Control
Anti- $\theta$ + anti-C57BL/6 + anti- $\kappa$	100	Control
Exp. B Autoradiography		
$[^{125}I]$ Anti- $\kappa$	58	58% $\kappa$ -bearing cells
Anti- $\theta$ + $[^{125}I]$ anti- $\kappa$	78	20% $\theta$ -bearing cells; 22% $\theta^-$ , $\kappa^-$ cells
Anti-BALB/c + $[^{125}I]$ anti- $\kappa$	74	16% BALB/c-bearing cells; 26% BALB/c <sup>-</sup> , $\kappa^-$ cells
Anti- $\theta$ + anti-BALB/c + $[^{125}I]$ anti- $\kappa$	72	14% BALB/c-bearing or $\theta$ -bearing cells; 28% $\theta^-$ , BALB/c <sup>-</sup> , $\kappa^-$ cells
Anti-C57BL/6 + $[^{125}I]$ anti- $\kappa$	97	Control
Anti- $\theta$ + anti-C57BL/6 + $[^{125}I]$ anti- $\kappa$	99	Control

C57BL/6 mice were thymectomized at 6 wk of age and were irradiated with 850 R 2 wk later. At that time, they were given  $5 \times 10^6$  C57BL/6 bone marrow cells and  $80 \times 10^6$  (C57BL/6  $\times$  BALB/c) $F_1$  thymus cells. 8 wk later, spleen cell suspensions were prepared and evaluated by immunofluorescence (exp. A) or autoradiography (exp. B).

\* In the immunofluorescence study, the cells were exposed to fluorescent goat antirabbit  $\gamma$ -globulin after exposure to the reagents cited in the table.

† The percent of  $\kappa$ -bearing cells is the percent of positive cells when anti- $\kappa$  alone is used. The percent of  $\theta$ -bearing and/or BALB/c-bearing cells is the percent of positive cells observed when anti- $\theta$  and/or anti-BALB/c followed by anti- $\kappa$  are used minus the percent of positive cells when anti- $\kappa$  alone is used. The percent of  $\theta^-$ ,  $\kappa^-$ ; BALB/c<sup>-</sup>,  $\kappa^-$ ; and  $\theta^-$ , BALB/c<sup>-</sup>,  $\kappa^-$  cells is the percent of negative cells observed when anti- $\theta$  and/or anti-BALB/c followed by anti- $\kappa$  are used. Finally, anti-C57BL/6 + anti- $\kappa$  and anti- $\theta$  + anti-C57BL/6 + anti- $\kappa$  are control combinations of sera in which all cells should be positive.

#### *Are $\theta^-$ , $Ig^-$ Cells B Lymphocytes Bearing Too Little Surface Ig for Detection?*

This possibility is considerably more difficult to rule out than the previous one, as a pure source organ for B lymphocytes has not been identified in mammals. Advantage may be taken of the fact that many B lymphocytes have been demonstrated to bear an  $Mg^{++}$ -independent receptor for the third component of C and can form rosettes with erythrocytes sensitized with antibody and C. Moreover, if 19S antibody is used and the rosettes are formed in the presence of 0.01 M EDTA, neither monocytes or polymorphonuclear leukocytes form rosettes, and under these conditions rosette-forming cells are exclusively B lymphocytes. Such cells are referred to as complement receptor lymphocytes (CRL).

In order to determine if  $\theta^-$ ,  $Ig^-$  cells bear an  $Mg^{++}$ -independent receptor for C3, spleen cells from mice were incubated with erythrocytes coated with 19S antibody and complement (EAC) in the presence of 0.01 M EDTA. After the rosettes had formed, they were separated from spleen cells that had not formed rosettes by layering the cells on a two-step BSA gradient (10%/35%) and centrifuging them at 30,000  $g$  in a Spinco model L ultracentrifuge. Under these conditions, the rosettes, which are more dense than lymphocytes, are pelleted while the



remaining cells band at the 10%/35% interface. The frequency of  $\theta$ -bearing cells,  $\kappa$ -bearing cells, and  $\theta^-$ ,  $Ig^-$  cells in the population recovered from the interface was evaluated by immunofluorescence and compared with a control population that had been incubated with erythrocytes coated with antibody without complement (EA) and then treated in a similar manner to the cells incubated with EAC. In our hands, this EA reagent fails to form rosettes with lymphocytes.

In three experiments, removal of CRL diminished the frequency of  $\kappa$ -bearing lymphocytes, although significant numbers of such cells still remained. The frequency of  $\theta$ -bearing cells and of  $\theta^-$ ,  $Ig^-$  lymphocytes was increased as a result of the removal of CRL and the two cells types increased in frequency to essentially the same degree (Table V). The latter point is demonstrated by the fact

TABLE V  
*Removal of Complement Receptor Lymphocytes and Enrichment of  $\theta^-$ ,  $Ig^-$  Lymphocytes*

Exp. no.		$\kappa$ -Bearing cells %	$\theta$ -Bearing cells		$\theta^-$ , $Ig^-$ cells	
			%	(% of $\kappa^-$ cells)	%	(% of $\kappa^-$ cells)
1	EA	37	46	(73)	17	(27)
	EAC	17	55	(66)	28	(34)
2	EA	48	41	(79)	11	(21)
	EAC	14	66	(77)	20	(23)
3	EA	48	32	(61.5)	20	(38.5)
	EAC	23	47	(61)	30	(39)

Spleen cells were incubated with sheep erythrocytes coated with antibody (EA) or antibody and complement (EAC). Rosettes were removed by centrifugation on a 10%/35% BSA gradient. Cells banding at the interface were evaluated for surface- $\theta$  and  $\kappa$  by immunofluorescence.

that little or no change in the percent of  $\kappa^-$  cells constituted by the  $\theta^+$  and by the  $\theta^-$ ,  $Ig^-$  cells occurred as a consequence of the removal of CRL. This provides strong evidence that  $\theta^-$ ,  $Ig^-$  cells lack the C receptor. Nonetheless, it does not rule out the possibility that  $\theta^-$ ,  $Ig^-$  cells are B lymphocytes at a stage in their differentiative history in which they lack detectable Ig and the complement receptor.

Finally, the removal of CRL from a spleen cell population results in some enrichment in the frequency of  $\theta^-$ ,  $Ig^-$  cells and suggests a potential strategy for the partial purification of these cells.

*Are  $\theta^-$ ,  $Ig^-$  Cells Nonlymphoid Cells That Resemble Lymphocytes by Light Microscopy?*—Although the quantitation of  $\theta^-$ ,  $Ig^-$  cells by autoradiography allows these cells to be identified, by light microscopy, as resembling lymphocytes, one cannot be certain that they are not cells of another lineage that might be confused with lymphocytes. To investigate this point, we have prepared populations of cells enriched in  $\theta^-$ ,  $Ig^-$  cells to study their morphologic and functional properties.

The principal procedure we have employed to partially purify  $\theta^-$ ,  $Ig^-$  cells is as follows:

Spleen cell populations were passed over glass bead columns to remove adherent cells as described in Materials and Methods. The effluent cells were collected and  $50 \times 10^6$  cells were treated with 0.5 ml of anti- $\theta$ -antiserum for 30 min at 4°C. These cells were washed with medium and resuspended in 0.5 ml of medium containing 300  $\mu$ g of anti- $\kappa$ -antibody; 0.5 ml of C was then added. The mixture was incubated at 37°C for 45 min and the dead cells were removed by centrifugation on a two-step BSA gradient (10%/35%). The remaining cells were evaluated for surface  $\theta$ ,  $\kappa$ , and rabbit immunoglobulin.

The results of three such preparative experiments are shown in Table VI. By this procedure, from 50 to 75% of the recovered cells are  $\theta^-$ ,  $Ig^-$  and not coated with rabbit Ig.

TABLE VI  
*Partial Purification of  $\theta^-$ ,  $Ig^-$  Cells*

Exp. no.	Reagents	Fluorescent lymphocytes	$\theta^-$ , $Ig^-$ cells
		%	%
1	Fl anti-rabbit Ig	40	
	Anti- $\kappa$ + Fl anti-rabbit Ig	45	
	Anti- $\theta$ + anti- $\kappa$ + Fl anti-rabbit Ig	50	50
2	Fl anti-rabbit Ig	18	
	Anti- $\kappa$ + Fl anti-rabbit Ig	20	
	Anti- $\theta$ + anti- $\kappa$ + Fl anti-rabbit Ig	35	65
3	Fl anti-rabbit Ig	10	
	Anti- $\kappa$ + Fl anti-rabbit Ig	15	
	Anti- $\theta$ + anti- $\kappa$ + Fl anti-rabbit Ig	25	75

Electron micrographs of such enriched preparations reveal a predominant cell with a high nuclear to cytoplasmic ratio, prominent segregated nucleoli, occasional large mitochondria, and small amounts of endoplasmic reticulum (Fig. 2). One striking feature is the great similarity of these cells to each other (Fig. 3). Their morphologic appearance is clearly consistent with classification as lymphocytes.

Such cell preparations were evaluated for phagocytic activity and for their capacity to be stained by 3,3'-diaminobenzidine. Incubation of these preparations with polystyrene beads failed to demonstrate any cells that ingest such beads indicating that the great majority of  $\theta^-$ ,  $Ig^-$  cells are not actively phagocytic. In addition, passage of spleen cell populations over glass bead columns tended to increase the relative frequency of  $\theta^-$ ,  $Ig^-$  cells, indicating that such cells do not adhere to glass surfaces.

Exposure of preparations enriched for  $\theta^-$ ,  $Ig^-$  cells to 3,3'-diaminobenzidine

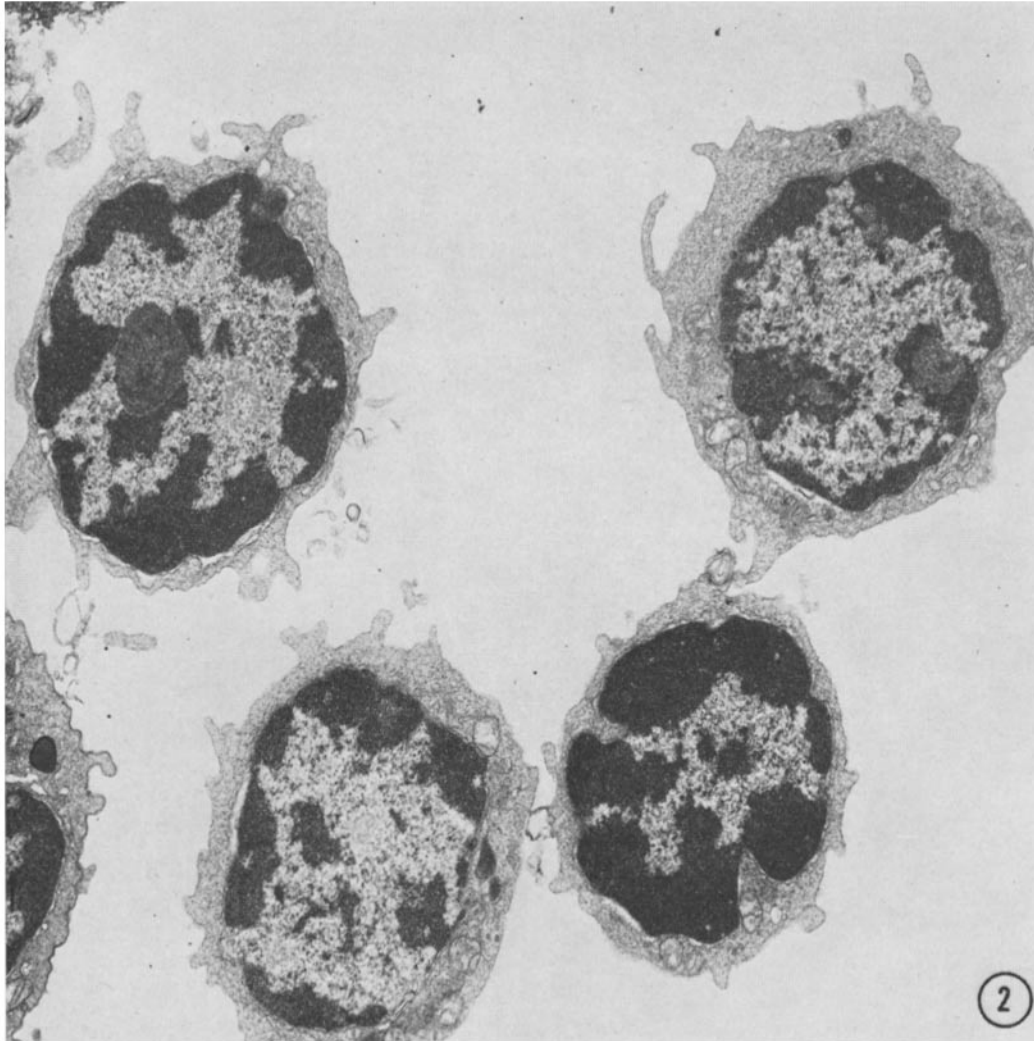


FIG. 2. Electron micrograph of an enriched preparation of  $\theta^-$ ,  $Ig^-$  cells.  $\times 10,000$ .

and  $H_2O_2$  causes no development of reaction product visible by electron microscope examination. This indicates that the cells do not contain significant amounts of ribosomal hemoglobin and are, therefore, not red cell precursors sufficiently differentiated to synthesize hemoglobin (18). Secondly, the cells do not possess subcellular organelles or granules that contain 3,3'-diaminobenzidine reaction product. The development of reaction product in this instance would indicate the presence of peroxidase activity. Such activity is known to be present

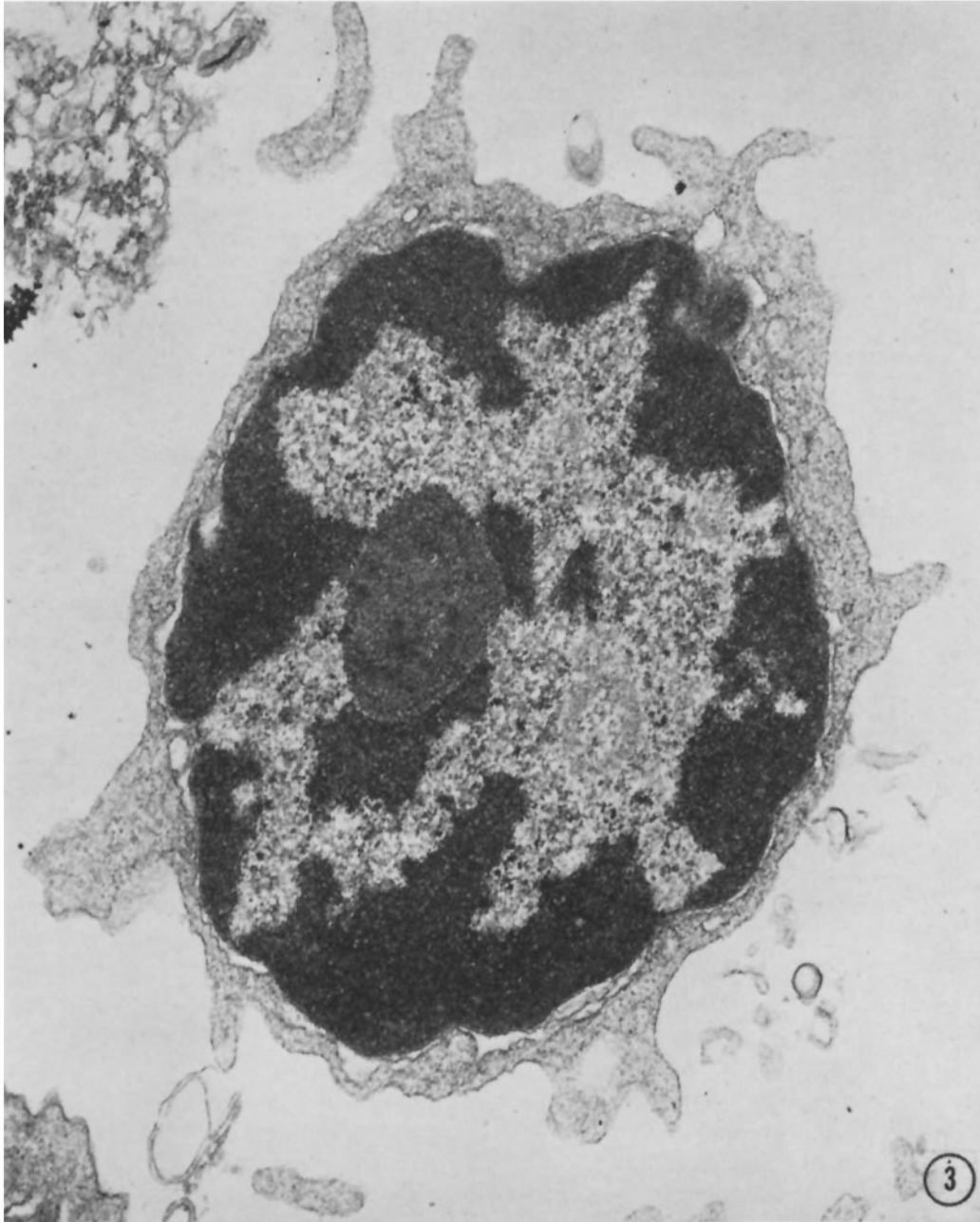


FIG. 3. Higher magnification photograph of one of the cells shown in the previous figure.  
× 24,000.

in the granules of early granulocyte precursors (19) and in the endoplasmic reticulum of monocyte precursors (20). The absence of peroxidase activity in  $\theta^-$ ,  $Ig^-$  cells suggests that they are not identifiable as erythroid, myeloid, or monocytoïd precursors.

Finally, we have previously reported (15) that enrichment for  $\theta^-$ ,  $Ig^-$  cells in lymphoid populations obtained from NZB spleens does not lead to a parallel enrichment in cells capable of forming colonies in the spleens of lethally irradiated syngeneic recipients. This further supports the thesis that  $\theta^-$ ,  $Ig^-$  cells are not myeloid or erythroid precursors.

Although selective cytolysis with antisera directed at the  $\theta$ - and  $\kappa$ -determinants coupled with removal of adherent cells is the most convenient method of obtaining enriched  $\theta^-$ ,  $Ig^-$  cell populations, the theoretical objection may be raised that some of these cells are either  $\theta^+$  or  $Ig^+$  cells in which the formation of surface antigen-antibody complexes has led to "cap" formation and subsequent endocytosis of the specific marker (21). We regard this as unlikely as these cells should have been lysed due to the presence of C. Nonetheless, we felt that an alternate procedure to obtain enriched populations of  $\theta^-$ ,  $Ig^-$  cells would be useful to establish the morphologic characteristics of such cells. We thus used the procedure of equilibrium centrifugation on a discontinuous BSA gradient.

A preparation of  $100-200 \times 10^6$  nucleated spleen cells was suspended in 1 ml of 33% BSA and placed on the bottom of a cellulose nitrate tube. This was overlaid with 1 ml each of 29, 26, 23, and 10% BSA and centrifuged at 15,000 *g* for 30 min. Cells were collected from each interface and from the pellet, chromated, and evaluated for  $\theta^-$ ,  $Ig^-$  cells by specific cytolysis. The highest percent of such cells (55%) was found at the 10%/23% interface. These cells were passed over a glass bead column to remove adherent cells and examined under the electron microscope.

Such cells were identical in appearance to those illustrated in Figs. 2 and 3.

*Other Characteristics of  $\theta^-$ ,  $Ig^-$  Cells.*— $\theta^-$ ,  $Ig^-$  cells are found principally in the spleen in normal mouse strains. As we have previously reported (15), the frequency of  $\theta^-$ ,  $Ig^-$  cells in lymph nodes of BALB/c, C3H, C57BL/6, and CBA mice ranges from 0 to 6% and few, if any,  $\theta^-$ ,  $Ig^-$  cells are found in the thymus. The existence of  $\theta^-$ ,  $Ig^-$  lymphocytes in the bone marrow is difficult to quantify because of the great variety of cell types in that organ. In young NZB, NZW, and B/W F<sub>1</sub> mice,  $\theta^-$ ,  $Ig^-$  cells occur in lymph nodes in a considerable frequency (up to 20%) and, as noted above, there is a small increase in the frequency of  $\theta^-$ ,  $Ig^-$  cells in the mesenteric lymph node of thymus-deprived mice.

The final characteristic of  $\theta^-$ ,  $Ig^-$  lymphocytes that we have evaluated is whether they are a rapidly or slowly dividing group of cells. To do this CBA/J mice were injected with tritiated thymidine according to protocols designed to label rapidly or slowly dividing cells (22), and the fraction of  $\theta^-$ ,  $\kappa^-$  cells labeled

with tritiated thymidine was evaluated by a combined autoradiographic-immunofluorescence procedure (23).

To evaluate rapidly dividing cells, 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]TdR (6.7 Ci/mM) per gram of weight was injected every 6 h for 5 days and the mice were then sacrificed. To label slowly dividing cells, 0.75  $\mu\text{Ci}$  of [ $^3\text{H}$ ]TdR per gram of weight was administered daily for 17 days. 14 days later the animals were sacrificed.

As seen in Table VII, only 18% of the  $\theta^-$ , Ig $^-$  cells labeled under the protocol designed to label rapidly dividing cells, while 36% of the remaining lymphocytes labeled under these conditions. However, 80% of the  $\theta^-$ , Ig $^-$  were labeled when the protocol for labeling slowly dividing cells was used, while only 23% of the remaining cells labeled under this protocol. Thus,  $\theta^-$ , Ig $^-$  cells or their immediate precursors are, in general, relatively slowly dividing cells.

TABLE VII  
[ $^3\text{H}$ ]TdR Labeling Characteristics of  $\theta^-$ , Ig $^-$  Cells

	Indicated cells labeled
	%
Protocol for rapidly dividing cells	
$\theta^+$ cells + $\kappa^+$ cells	36
$\theta^-$ , Ig $^-$ cells	18
Protocol for slowly dividing cells	
$\theta^+$ cells + $\kappa^+$ cells	23
$\theta^-$ , Ig $^-$ cells	80

Cells bearing either the  $\theta$ - or  $\kappa$ -determinants were identified by immunofluorescence in which anti- $\theta$ , anti- $\kappa$ , and fluorescent goat antirabbit Ig were used.  $\theta^-$ , Ig $^-$  cells are those lymphocytes not fluorescent after such treatment. Cells containing grains were identified by examining the same preparation under dark-field illumination with a tungsten light source.

#### DISCUSSION

In this communication, we have described the existence of a group of lymphocyte-like cells on which neither the  $\theta$ -antigen nor detectable amounts of immunoglobulin can be found. The principal evidence for the lack of surface immunoglobulin and of  $\theta$  on these cells is their failure to be lysed by either anti- $\theta$  or anti- $\kappa$  and complement, and their lack of labeling in immunofluorescence and autoradiographic studies with these reagents and with anti- $\lambda$ -antibody as well. It is obvious that all detection procedures have a lower limit of sensitivity and we cannot be certain that the cells classified as  $\theta^-$ , Ig $^-$  do not bear small amounts of either marker. Nonetheless, it is very unlikely that  $\theta^-$ , Ig $^-$  cells bear limited amounts of  $\theta$  as these cells increase in relative frequency after thymus deprivation. With regard to surface Ig, it is likely that a considerable heterogeneity exists in the density of such molecules on lymphocytes. Indeed, it has been

reported that virtually all lymphocytes, including T cells, possess limited amounts of Ig (24). Our procedures would not detect the amounts of Ig said to be present on T cells and might not label B lymphocytes bearing limited amounts of surface Ig. Thus, it is possible that  $\theta^-$ , Ig $^-$  cells (as defined here) might possess small amounts of surface Ig.

The  $\theta^-$ , Ig $^-$  cells have the following additional characteristics: (a) They lack the C3 receptor present on many B lymphocytes. (b) They do not adhere to glass or plastic surfaces nor are they actively phagocytic. (c) They lack endogenous peroxidases and hemoglobin. (d) They or their immediate precursors are relatively slowly dividing cells. (e) They are found predominantly within the spleen.

One can with some confidence exclude  $\theta^-$ , Ig $^-$  cells from the T cell line, primarily on the basis of the chimera experiment reported in the Results section. In a sense, then, if the  $\theta^-$ , Ig $^-$  cell is a lymphocyte it must be a B lymphocyte since such a cell is generally considered to be any lymphocyte, derived from a bone marrow precursor, which differentiates independently of the thymus. Indeed, Lamelin et al. have recently described a population of Ig $^-$  cells bearing mouse B lymphocyte antigens (25).

The B lymphocyte class contains the precursors of antibody-forming cells. Such cells generally possess easily detectable surface Ig but there may well be stages in their differentiation when the surface Ig is not detectable. For example, during differentiation from a nonimmunoglobulin-bearing stem cell, a lymphocyte-like Ig $^-$  cell might be an intermediate on the path to the fully differentiated cell. Similarly, the descendants of Ig-bearing precursors of antibody-forming cells are plasma cells many of which have little detectable surface Ig (26). Again, an intermediate Ig $^-$  lymphocyte might exist. Our studies thus far cannot rule out either possibility definitively and, indeed, it seems likely that representatives of both cell types may contribute to the  $\theta^-$ , Ig $^-$  cell pool.

Another alternative that must be seriously considered is that  $\theta^-$ , Ig $^-$  cells, although "bone marrow-derived," are lymphocytes of quite a different lineage than the cells that act as precursors of antibody-secreting cells. Although several possibilities might be mentioned, including a cell acting as a macrophage precursor or one acting as the precursor of a cell capable of destroying antibody-coated target cells, we have no evidence to implicate any role for  $\theta^-$ , Ig $^-$  cells thus far. A determination of the nature of these cells will require their purification, labeling, and transfer to a syngeneic recipient in which their fate can be followed. In preliminary experiments, in which only a partial purification of  $\theta^-$ , Ig $^-$  cells has been achieved, such cells were labeled with [ $^3$ H]uridine and injected into syngeneic recipients. 24 h later, all labeled cells were  $\theta^-$ , Ig $^-$  lymphocyte-like cells. These experiments obviously require extension and refinement in an effort to follow  $\theta^-$ , Ig $^-$  cells.

It is most provocative, however, that the frequency of  $\theta^-$ , Ig $^-$  cells is markedly elevated in NZB, NZW, and (NZB  $\times$  NZW) $F_1$  mice. These mice are subject

to a variety of autoimmune disorders, particularly late in life.  $\theta^-$ , Ig $^-$  cells are increased in number relatively early in life in the NZ mice, and thus these cells may have pathogenetic significance rather than merely representing one of a wide variety of abnormalities present in these animals after the onset of disease.

## SUMMARY

An appreciable percent (3–14%) of the lymphocyte-like cells of the mouse spleen lack both the  $\theta$ -isoantigen and sufficient surface immunoglobulin to be detected by conventional immunofluorescence or autoradiographic procedures. These  $\theta^-$ , Ig $^-$  cells are increased in frequency after treatment of mice with antithymocyte serum or in mice that have been thymectomized, irradiated (850 R), and reconstituted with bone marrow cells. Moreover, in chimeric C57BL/6 mice in which the T cells are derived from (BALB/c  $\times$  C57BL/6)F<sub>1</sub> donors,  $\theta^-$ , Ig $^-$  cells also lack BALB/c histocompatibility antigens. These experiments indicate that  $\theta^-$ , Ig $^-$  cells are not  $\theta^-$  T lymphocytes.

Removal of complement receptor lymphocytes from spleen cell populations increases the frequency of  $\theta^-$ , Ig $^-$  cells, indicating that such cells lack the complement receptor. Partially purified populations of  $\theta^-$ , Ig $^-$  cells have been obtained by cytolysis by anti- $\theta$ - and anti- $\kappa$ -antibody and complement and by density gradient ultracentrifugation. These cells closely resemble lymphocytes in morphology. The only exceptional feature is the existence of prominent nucleoli. The  $\theta^-$ , Ig $^-$  cells lack hemoglobin and endogenous peroxidases, are not actively phagocytic, and do not adhere to glass. This suggests they are not of the erythroid, myeloid, or monocytoid lines. [<sup>3</sup>H]Thymidine labeling studies indicate that  $\theta^-$ , Ig $^-$  cells are members of a relatively slowly dividing cell pool. Whether  $\theta^-$ , Ig $^-$  cells are members of the "classical" B lymphocyte line or belong to another, as yet undescribed, lineage is not yet certain.

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