

DIFFERENTIATION ANTIGENS IDENTIFY SUBPOPULATIONS OF RABBIT T AND B LYMPHOCYTES

Definition by Flow Cytometry

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Although it is known that rabbit lymphocytes, like those of other species, may be divided into the broad categories of T and B cells (see references 1 and 2 for review), no studies have provided further characterization of these populations. In man and mouse a number of cell surface antigens characteristic of T cells or T cell subsets (3–6), as well as B cell subpopulations (7–10), have been described. Cell function is highly correlated with these differentiation antigens. For example, Lyt-2 in the mouse and Leu-2 in man identify the suppressor-cytotoxic effector T cell subset, and the human Leu-3 antigen (which has no murine homologue) defines the helper-inducer T cell subset.

Investigations of the lymphoid cell populations of the rabbit have been hindered by the lack of immunologic reagents specific for differentiation antigens on rabbit lymphocytes. Although several heterologous anti-rabbit T cell antisera (11–16) and a monoclonal antibody that recognizes what may be the rabbit homologue of Thy-1 (17) have been described, reagents to identify subsets of rabbit lymphocytes within the two general populations of T and B cells have not been reported.

A panel of 24 monoclonal antibodies directed against lectin-purified cell surface glycoproteins of the rabbit T cell line RL-5 was recently prepared; a number of these antibodies precipitated glycoproteins from detergent lysates of both the cell line and normal rabbit spleen cells.¹ This report describes the results of flow cytometry (FC)² experiments in which six of these monoclonal antibodies were used to define subpopulations of rabbit splenocytes and thymocytes. Functional and immunofluorescence criteria indicate that one of the antibodies is specific for a surface determinant present exclusively on all rabbit T cells. The panel of reagents defines four thymocyte subsets and in the spleen delineates two T cell subsets, two B cell subsets, and a null cell subset.

Materials and Methods

Animals. The rabbits used in these experiments were randomly selected outbred animals that were bred and maintained in the colony of the Laboratory of Immunogenetics, National

¹ Wilkinson, J. M., J. A. Sogn, D. L. Wetterskog, and T. J. Kindt. Manuscript submitted for publication.

² *Abbreviations used in this paper:* Con A, concanavalin A; F/P ratio, molar fluorescein/protein ratio; FACS, fluorescence-activated cell sorter; FC, flow cytometry; FITC, fluorescein isothiocyanate; MLR, one-way mixed lymphocyte reaction; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; TRITC, tetramethylrhodamine isothiocyanate.

Institutes of Health.

Reagent Preparation. Monoclonal antibodies were derived from BALB/c mice immunized with cell surface glycoproteins of the rabbit T cell line RL-5, isolated by detergent lysis and subsequent chromatography on lectin-affinity columns. Single-cell preparations of spleens from these mice were fused with the HAT-sensitive myeloma line P3x63-Ag8-U1, and the resulting hybridomas were assayed by an enzyme-linked immunosorbent assay system using polystyrene plates coated with whole RL-5 cells or suitable negative controls. Complete details of the preparation and screening of the monoclonal reagents will be published separately.¹ The 9AE10 monoclonal, an IgM antibody reported to be specific for a rabbit T cell determinant with a molecular weight of 25,000 (17), was the generous gift of Dr. K. L. Knight, University of Illinois Medical Center, Chicago, IL.

Mouse IgG was isolated from ascites fluid by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose and labeled with fluorescein isothiocyanate (FITC) (18) or biotin (19). Table I summarizes the monoclonal antibodies described, the molar fluorescein/protein (F/P) ratio of the FITC-antibody conjugates, and the specificities of these antibodies. Avidin (Vega Biochemicals, Tuscon, AZ) was conjugated with sulforhodamine 101 phosphoryl chloride (Molecular Probes, Plano, TX) under conditions identical to those used for fluorescein (18).

Sheep anti-rabbit μ conjugated to FITC and to tetramethylrhodamine isothiocyanate (TRITC), as well as TRITC- and FITC-conjugated goat anti-mouse Ig and anti-mouse μ were purchased from N. L. Cappel Laboratories, Cochranville, PA. All reagents were passed through appropriate immunoabsorbents to remove any crossreactivity with noncognate immunoglobulins. The specificity of anti-rabbit μ was tested by cytoplasmic double immunofluorescence with FITC- and TRITC-labeled antibodies of different specificities (e.g., anti-rabbit IgA or anti-rabbit IgG) on rabbit spleen cell suspensions; doubly labeled cells were never observed. Anti-mouse Ig and anti-mouse μ did not bind to rabbit lymphoid cells, as determined by FC.

Cell Staining and FC. Single-cell suspensions of lymphoid tissues were prepared in RPMI 1640 medium containing 0.1% bovine serum albumin and 3 mM sodium azide. In a 12 x 75 polystyrene tube, 10⁶ cells were pelleted, stained with saturating amounts of conjugated antibody for 30 min at 0°C, and washed twice in the same medium. When biotinylated antibody was used, a second incubation with sulforhodamine-101-conjugated avidin was performed in the same manner. The unlabeled 9AE10 monoclonal was facilitated by a second incubation with FITC-conjugated anti-mouse μ .

FC was performed with a FACS-II (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) equipped with an argon ion laser producing 200 MW at 488 nm and a krypton ion laser producing 150 MW at 568 nm. Data were acquired with and analyzed by a PDP 11/34 computer (Digital Equipment Corporation, Maynard, MA) using programs written by T. Chused and Willis Chung. Except where otherwise noted, "light scatter" refers to narrow (15°) angle forward light scatter. In analytical studies data from 50,000 to 200,000 light scatter-gated viable cells were collected. Logarithmic amplification was used in the fluorescence channels. To simplify presentation, populations with increasing fluorescence are called dull, intermediate, and bright.

TABLE I
Properties of FITC-Monoclonal Antibody Conjugates

Antibody	F:P ratio	Specificity*	
		Thymus	Spleen
L11/135	2.3	Th1a, Th1b, Th2, Th3	T1, T2
L12/27	2.4	Th1a, Th1b, Th2	T1, T2, B1, B2, null
L12/201	2.5	Th1a, Th1b, Th2, Th3	T1, T2, B1, B2, null
L13/64	3.3	Th2, Th3	T1, T2, B2, null
W4/28	4.2	Th1a, Th1b, Th2, Th3	T1, T2, B2, null
W4/86	2.5	Th1a, Th1b, Th2, Th3	T1, T2, B2

* Subpopulations as defined in Tables II and VI.

For preparative sorting, cells were stained at 0°C in the absence of azide, and culture medium was used as sheath fluid. Gates were set such that 5% of the cells between the positive and negative peaks were discarded.

"Panning" of Cells on L11/135 Plates. IgG was isolated from ascites fluid of mice carrying the L11/135 hybridoma. The antibody was diluted into phosphate-buffered saline (PBS) at a concentration of 25 µg/ml, and 5 ml of this solution was placed into 100-mm petri plates. After overnight incubation at 4°C, the antibody solution was removed, and the plates were washed extensively with PBS. A suspension of rabbit spleen cells (1.7×10^7 cells in 5 ml of medium) was placed on the plates and left at room temperature for 1 h. Nonadherent cells were then aspirated for further analysis. This procedure caused neither a significant decrease in viability nor resulted in macrophage depletion.

Mitogen Stimulation of Lymphoid Cells. Cells were cultured in round-bottom microtiter plates (Linbro Chemical Co., Hamden, CT) in RPMI 1640 medium containing 10% fetal bovine serum, 4 mM glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), gentamycin (10 µg/ml), and 5×10^{-5} M 2-mercaptoethanol. Each well contained 1×10^5 cells in a volume of 100 µl. Mitogens used were concanavalin A (Con A, Sigma Chemical Co., St. Louis, MO) at a concentration of 10 µg/ml, and phytohemagglutinin (PHA) (Burroughs-Wellcome Ltd., Beckenham, England) at a concentration of 0.04 µg/ml (0.005 mU/ml). Cultures were incubated for 72 h at 37°C in 7% CO₂ in air, then pulsed for 18 h with 1 µCi [³H]thymidine (specific activity, 6.7 Ci/mmol). Cells then were harvested with a PHD Cell Harvesting System (Cambridge Technology, Cambridge, MA), and the incorporated radioactivity was measured in a scintillation counter (Beckman Instruments Inc., Fullerton, CA).

One-Way Mixed Lymphocyte Reaction (MLR). Cells were cultured, pulsed, and harvested as described for the mitogen response assays, except that flat-bottomed microtiter plates (Costar, Data Packaging, Cambridge, MA) were used. Stimulator cells were irradiated (2,500 rad) and equal numbers (3×10^5 cells/well) of stimulator and responder cells were added to each well.

Results

The panel of 24 monoclonal antibodies was screened by FC on the RL-5 cell line, rabbit thymus, and spleen cells, as described elsewhere.¹ These reagents gave six staining patterns. A representative example was selected from each group as the basis of this report. Each of these six antibodies, the 9AE10 monoclonal, and anti-rabbit µ were used alone and in two-color FC analyses with each of the other reagents to characterize reactive populations from thymus and spleen. Because it is not possible to include all the data, illustrative examples and tabular summaries are provided.

Thymocyte Subpopulations. The staining of thymocytes with the selected antibodies and with 9AE10, an antibody previously reported to be T cell specific (17), is shown in Fig. 1. L11/135 and W4/86 produced a single peak with a slight shoulder on the right side. W4/28 and L13/64 gave one peak with a more pronounced shoulder to the right. L12/201 gave two closely spaced peaks. Comparison with the unstained control shows that these five antibodies, L11/135, W4/86, W4/28, L13/64, and L12/201, labeled all thymocytes. L12/27 delineated three subpopulations, the dullest of which was only slightly above the unstained level.

Antibody-mediated aggregation of cells, particularly thymocytes, is an occasional problem in FC. It does not correlate with staining intensity. Such aggregated cells are lost from the FC analysis. L12/27 and L12/201 caused variable aggregation of thymocytes, which fortuitously helped demonstrate three thymocyte subpopulations that differed in cell size and in the differentiation antigens they expressed. These subpopulations are designated as Th1, Th2, and Th3, in order of increasing size. L12/27 markedly aggregated the cells it stained and removed them from the analysis, thereby increasing the proportion of large, L12/27-dull, Th3 cells, as shown in Fig. 2.

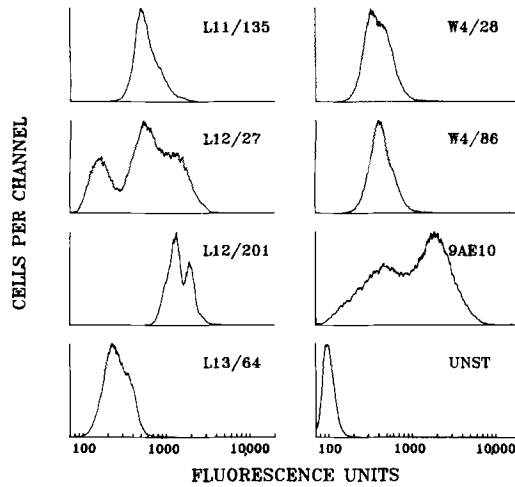


FIG. 1. Flow cytometry analysis of rabbit thymocyte staining by the panel of monoclonal antibodies and 9AE10. UNST, unstained control.

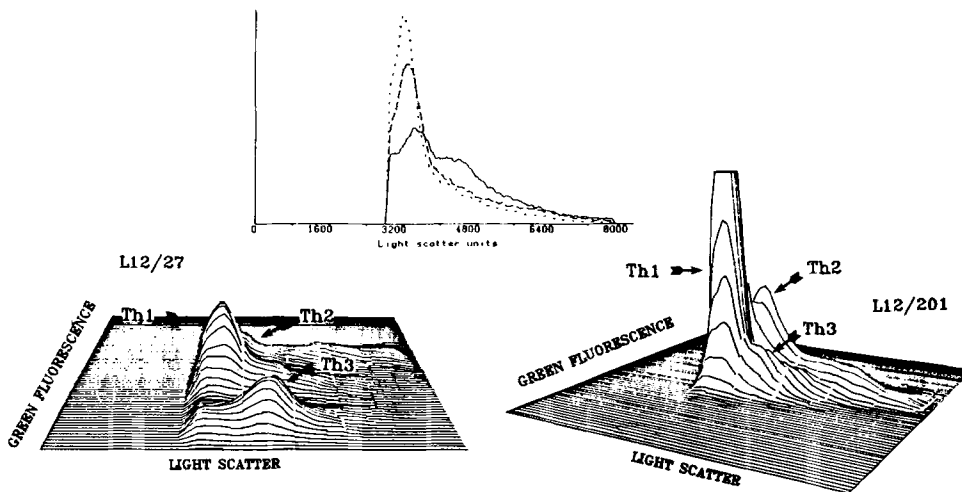


FIG. 2. Rabbit thymocyte staining by L12/27 (left) and L12/201 (right) as a function of light scatter. The fluorescence scale is logarithmic and the light scatter scale linear. L12/27 removes a large fraction of the positive Th1 and Th2 cells by aggregation, which enhances the visibility of the L12/27-negative, light scatter-large Th3 subset. L12/201 aggregates fewer cells and reveals the L12/201-bright, light scatter-intermediate Th2 peak. In both displays, aggregated cells are present in the very high light scatter and fluorescence region. The inset shows the total light scatter distribution for thymocytes stained with L12/27 (—) or L12/201 (---) and for the unstained control (.....).

L12/201, which does not cause significant aggregation, reveals the light scatter-intermediate Th2 cells as a separate peak from the major Th1 population (Fig. 2).

The 9AE10 monoclonal has been shown to be specific for a rabbit T cell marker with a molecular weight of 25,000, and it was thought that this determinant might be the rabbit homologue of Thy-1 (17). In our experiments this antibody gave a broad bimodal distribution of thymocyte staining (Fig. 1). In contrast to the other reagents

used, significant differences in 9AE10 staining intensity that were not correlated with light scatter were observed. Whereas the Th3 subpopulation of large cells was bright for 9AE10, the Th1 group stained bimodally with this antibody, indicating that it is, in fact, composed of two subsets, which are designated Th1a and Th1b. Because the Th2 cells are only slightly larger than the Th1 cells, the 9AE10 distribution of the Th2 subpopulation could not be accurately determined. The complete phenotypes of the three thymocyte subsets are given in Table II.

Splenocyte Staining. The results of staining spleen cells with this panel of reagents is shown in Fig. 3. L12/27, which gave a trimodal distribution in thymus, stained all spleen cells with equal intensity. L13/64, W4/28, and W4/86 gave similar distributions with ~25% of the cells in a negative or dull peak and the remainder in a positive peak. Anti- μ produced a trimodal distribution with 55% in the two positive peaks. As described below, the brighter peak consisted of cells much larger than those in the intermediate peak. L11/135 stained 40% of the spleen cells. The bimodal distribution of the L11/135-positive cells seen in Fig. 3 was not observed in all samples tested; in some, a single peak was seen (cf. Fig. 4). The L11/135-negative cells gave two peaks.

TABLE II
Rabbit Thymus Cell Subsets Determined by Flow Cytometry

Subpopulation:	Th1a	Th1b	Th2	Th3
Percent of cells:	25	50	15	10
Light scatter:	+	+	++	+++
L11/135	+	+	++	++
L12/27	+	+	++	tr*
L12/201	++	++	+++	+
L13/64	tr	tr	+	+
W4/28	+	+	++	+
W4/86	+	+	++	+
9AE10	+	++	++	+++

* Trace staining, not considered significant.

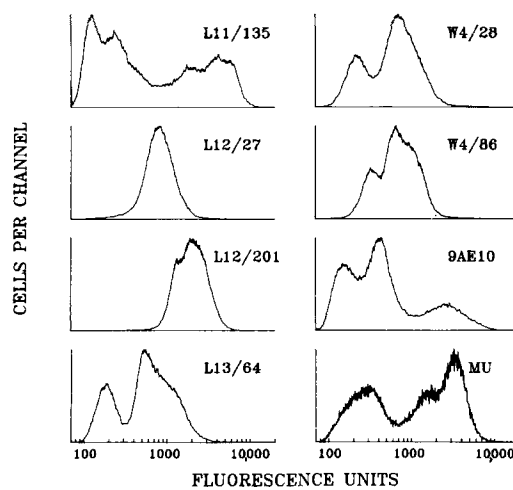


FIG. 3. Flow cytometry analysis of rabbit splenocyte staining by the panel of monoclonal antibodies 9AE10 and anti- μ .

The one on the right contained the larger B cells, which also exhibited more staining by L13/64, W4/28, and W4/86. This subpopulation may possess greater autofluorescence and/or bind small quantities of the antibody via Fc receptors (20). However, such staining was not inhibited by normal mouse serum or by preformed immune complexes (data not shown). 9AE10 yielded a positive peak containing 20% of the cells, a negative peak containing 30%, and an intermediate peak with 50%.

L11/135 Identifies All Peripheral T Cells. The accounting of the subpopulations described above suggested that L11/135 might be staining the subpopulation complementary to that stained by anti- μ . This was confirmed by two-color FC, which revealed five subsets of rabbit splenic lymphocytes, as shown in Fig. 4. Most of the μ -negative cells were positive for L11/135 (box 1 of Fig. 4), indicating that this monoclonal reagent identified a major subpopulation, presumably T lymphocytes, distinct from the surface μ -positive B cells.

To investigate the functional capabilities of cells recognized by L11/135, the following experiment was performed: rabbit splenocytes were stained with the antibody, and the positive cells were separated from the negatives by preparative FC. The two populations of cells (i.e., L11/135-negative and L11/135-positive) were then placed in culture with the mitogens Con A or PHA, and their proliferative responses were assayed. Results of this experiment are shown in Table III. The response of spleen cells stained with L11/135 before separation is shown as a baseline control. It is evident that the response of rabbit splenocytes to the T cell mitogens Con A and PHA resides in the L11/135-positive cells.

Similarly, when L11/135-positive cells were removed from a suspension of rabbit splenocytes by "panning," the fraction of the nonadherent cells stained by L11/135 was decreased from 63% to 1%, the fraction stained by anti-rabbit μ was increased from 38% to 78%, and the ability of these cells to respond in MLR (Table IV) or to

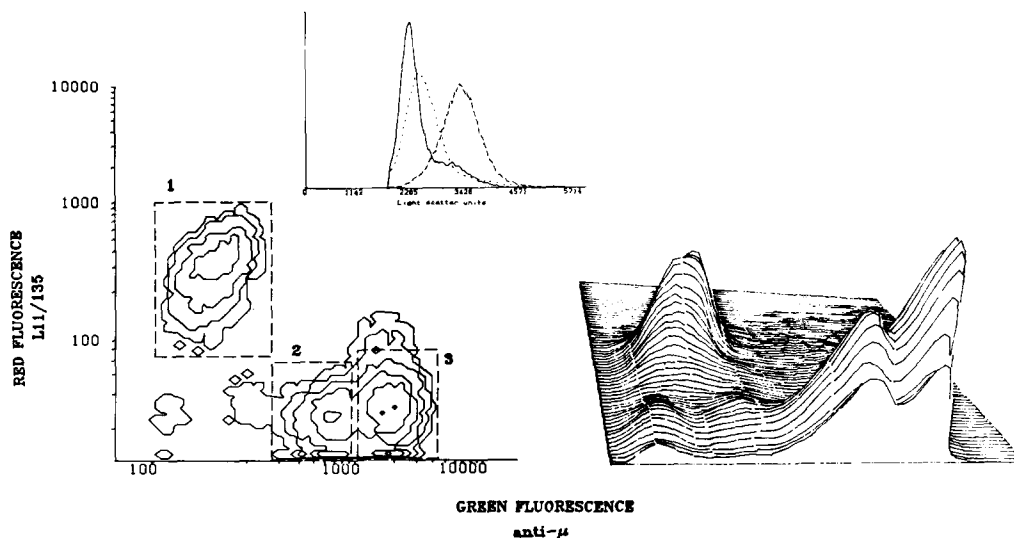


FIG. 4. Staining of rabbit spleen by FITC anti- μ and biotinylated L11/135 with sulforhodamine-avidin. The μ -negative, L11/135-positive cells (box 1) have intermediate light scatter (.....) (inset). The μ -bright cells (box 3) have high light scatter (---) and the μ -intermediate cells (box 2) have low light scatter (—).

TABLE III
Mitogen Responses of Rabbit Spleen Cells Separated by Preparative Flow Cytometry after Staining with T Lymphocyte-specific Monoclonal Antibodies

Responding cells	Mitogen added		
	None	Con A	PHA
Experiment 1			
L11/135 positive	15,042 ± 3,819*	522,485 ± 24,638	48,807 ± 7,500
L11/135 negative	4,298 ± 381	42,287 ± 2,227	7,441 ± 372
Unseparated	9,517 ± 185	422,682 ± 21,870	37,384 ± 3,906
Experiment 2			
9AE10 positive	1,105 ± 209	437,834 ± 35,598	ND‡
9AE10 intermediate	7,811 ± 812	146,183 ± 17,242	ND
9AE10 negative	4,736 ± 670	16,174 ± 3,584	3,969 ± 1,371
Unseparated	12,562 ± 1,098	476,965 ± 27,435	31,275 ± 4,964

* Results expressed as the mean counts per minute of quintuplicate cultures ± 1 SD.

‡ Not done.

TABLE IV
One-Way Mixed Lymphocyte Responses of Unseparated and L11/135-negative Splenocytes

Responding cells	Stimulator cells	
	Autologous	Allogeneic
Unseparated spleen	4,678 ± 880*	31,561 ± 3,740
Splenocytes not adherent to L11/135 plate	1,624 ± 578	3,726 ± 592

* Results are expressed as mean counts per minute of triplicate cultures ± 1 SD.

TABLE V
Con A Responses of Unseparated Spleen Cells and Splenocytes Depleted of L11/135-positive Cells by Plate Adherence

Responding cells	Mitogen added	
	None	Con A
Unseparated spleen	4,332 ± 542*	64,106 ± 12,329
Splenocytes not adherent to L11/135 plate	1,995 ± 385	981 ± 151

* Results are expressed as mean counts per minute of quintuplicate cultures ± 1 SD.

Con A (Table V) was lost. These data, taken together with the surface staining patterns of this monoclonal in comparison with anti-rabbit μ lead to the conclusion that L11/135 is specific for a rabbit T cell surface determinant.

The 9AE10 antibody gave two positive peaks when spleen cells were tested (Fig. 3). Because the bright peak contained only 26% of the cells and L11/135 stained ~40%, 9AE10 appeared not to react equally with all peripheral T cells. To clarify the relationship between L11/135 and 9AE10, these monoclonals were compared in the two-color study shown in Fig. 5. Slightly more than half of the L11/135-positive cells stained brightly with 9AE10 (box 4, Fig. 5), while half stained weakly (box 3, Fig. 5). Furthermore, isolation by preparative FC of the dull, intermediate, and bright 9AE10 populations seen in Fig. 3 showed that both the intermediate and bright groups contained significant numbers of PHA- and Con A-responsive cells (Table III). The

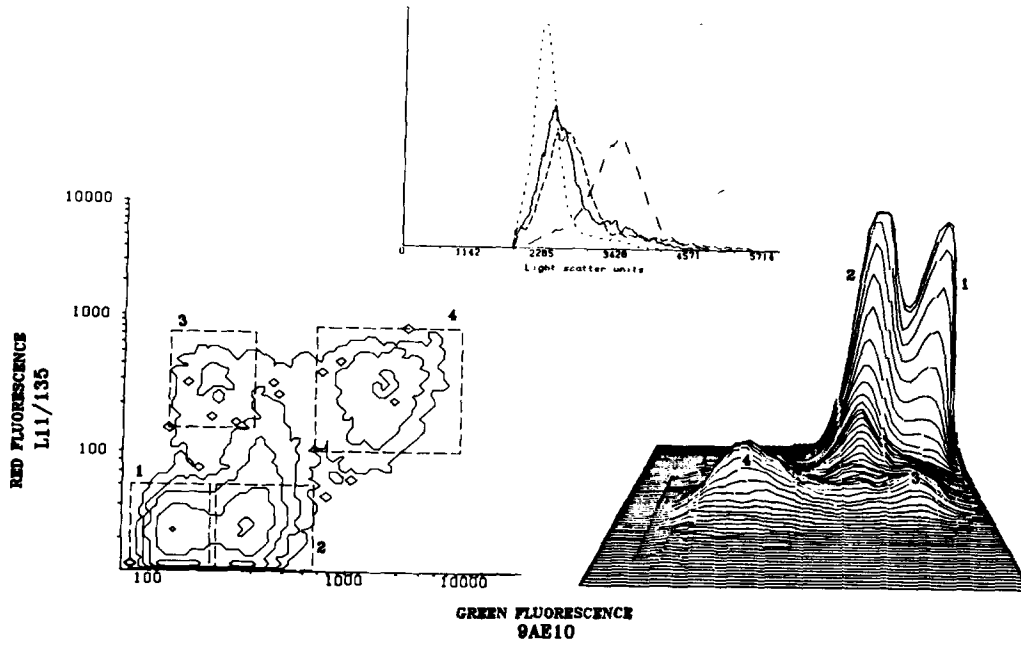


FIG. 5. Staining of rabbit spleen cells by 9AE10-FITC-conjugated anti-mouse μ and biotinylated L11/135 with sulforhodamine-avidin. The L11/135 positive cells include both 9AE10-bright (box 4) and 9AE10-dull cells (box 3). The 9AE10-dull cells (box 3), nevertheless, stain more intensely than the L11/135-negative, low light scatter B cells (box 1). The high light scatter B cells (box 2) have apparent 9AE10 staining levels slightly greater than the 9AE10-dull T cells (box 3), presumably due to Fc-mediated binding of anti-mouse μ . Note that the isometric view on the right has been rotated 180°. The light scatter profiles of the cells in each box are shown in the inset: box 1,; box 2, -----; box 3, ———; and box 4, ----.

TABLE VI
Rabbit Spleen Cell Subsets Determined by Flow Cytometry

Subpopulation:	T1	T2	B1	B2	Null
Percent of cells:	14	26	24	31	5
Light scatter:	++	+++	+	++++	++
L11/135	++	++	-	-	-
L12/27	+	+	+	+	+
L12/201	++	++	+	+	+
L13/64	+	++	-	+	-
W4/28	+	++	-	+	+
W4/86	+	+	-	+	?‡
9AE10	tr*	++	-	-	?
μ	-	-	+	++	-

* Trace staining.

‡ Staining properties could not be determined.

population that gave intermediate 9AE10 staining contained both the T cells of box 3 and the large B cells of box 2 (Fig. 5). The nature of this apparent staining of the large B cells was not clear. Further extensive two-color comparisons (*vide infra* and data not shown) provided additional evidence that rabbit peripheral T cells can be divided into two groups, as indicated in Table VI.

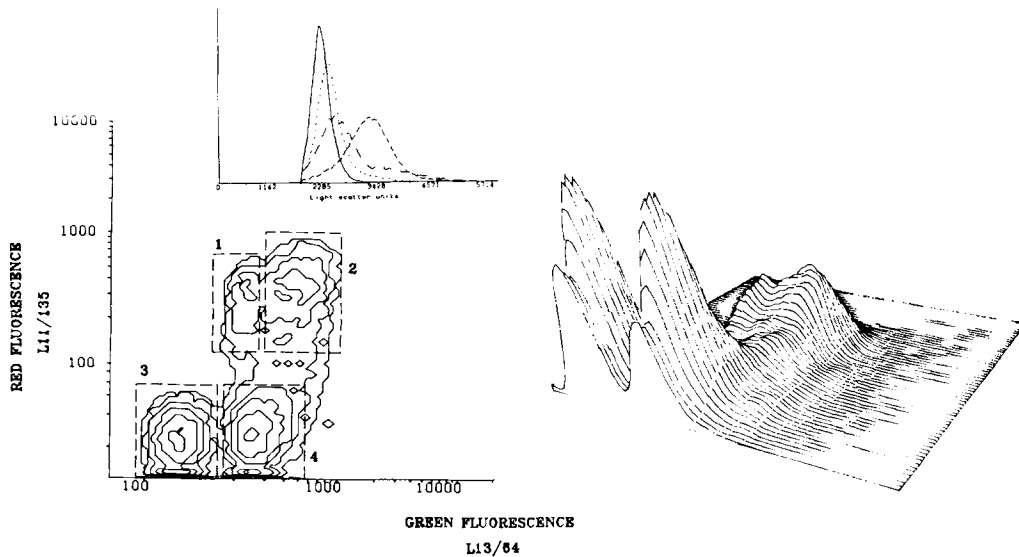


FIG. 6. Staining of rabbit spleen cells by FITC L13/64 and biotinylated L11/135 with sulforhodamine-avidin. The L11/135-positive cells have a bimodal distribution of L13/64 staining. The L13/64-intermediate cells (box 1) have lower light scatter (.....) (inset) than the L13/64-bright cells (box 2, — — —). The L11/135-negative B cells are also bimodal for L13/64. The L13/64-negative cells (box 3) have low light scatter (— — —), and the L13/64-positive cells (box 4) have high light scatter (-----).

Rabbit Splenic B Cells Contain Two Subpopulations. When stained with anti- μ , as shown in Fig. 4, the B cells formed two groups: (a) intermediate-staining cells (box 2, Fig. 4) with lower light scatter than the μ -negative cells and (b) bright-staining cells that were very large by light scatter (box 3, Fig. 4). Approximately 5% of the cells near the origin were stained by neither L11/135 nor anti- μ and thus appear "null." Anti- μ stained 55%, and the T cell-specific monoclonal L11/135 stained 40%, accounting for 95% of the spleen cells. Because L13/64, W4/28, and W4/86 stained ~75% of the spleen cells, they must define one or more additional subsets. This was investigated in a series of two-color studies, an example of which is shown in Fig. 6. L13/64 stained all of the L11/135-positive T cells and about half of the L11/135-negative cells. Multiparameter analysis revealed that the L13/64-positive, L11/135-negative cells (box 4, Fig. 6) were very large as judged by light scatter and thus correspond to the μ -bright, L13/64-positive B cells. Ultracentrifugation of the directly labeled reagents did not affect the result. Similar results were obtained with the other members of the panel, which raised the possibility of binding via Fc receptors as discussed below. These experiments appeared to define two B cell subpopulations whose characteristics are given in Table VI. Note that the B cell subset with high narrow angle forward light scatter did not have the increased 90° light scatter characteristic of monocytes and neutrophils.

In addition, L13/64 produced a bimodal distribution of the L11/135-bearing T cells. The cells in box 1 of Fig. 6 are smaller and stain less intensely with L13/64 than those in box 2, an additional demonstration of peripheral T cell subsets. The extensive multiparameter FC data summarized in Table VI gave clear evidence for subpopu-

lations of splenic T and B lymphocytes when staining patterns and light scatter profiles were analyzed.

Discussion

The panel of monoclonal anti-rabbit lymphocyte antibodies used in this study defines multiple lymphocyte subpopulations in rabbit thymus and spleen and invites comparison with our knowledge of man and mouse, the other species in which lymphoid cell populations have been extensively investigated.

The thymus experiments indicate at least four distinct subpopulations (Fig. 1, Table II). Staining with L12/27 and L12/201 and size as determined by light scatter define the Th1, Th2, and Th3 subsets. Th3 cells, which are large by light scatter, stain weakly with L12/27 and L12/201 but brightly with 9AE10 and closely resemble the rapidly proliferating subcortical cells in the mouse, which are bright for Thy-1 and are likely to be the least mature subpopulation. The behavior of L12/27, which stains all peripheral lymphocytes but not Th3, is consistent with this interpretation. The bulk of thymocytes, Th1, are small by light scatter and are stained in an intermediate fashion by L12/27 and L12/201. By analogy with the mouse, Th3 cells probably are immature cortical cells. Th2 cells, of intermediate size, have the same level of L12/27 and L12/201 staining as peripheral cells. Again, by analogy with the mouse, Th2 cells likely are mature medullary cells, and this subset likely includes those thymocytes with immunologic function. Such a developmental pathway, Th3 \rightarrow Th1 \rightarrow Th2, would imply that rabbit thymocytes acquire the differentiation antigens recognized by L12/27 and L12/201 as they mature.

The 9AE10 antibody distinguishes thymocyte subsets that are not correlated with size. Th1 cells have a bimodal distribution of 9AE10 staining, which further subdivides them into Th1a and Th1b. This difference was also observed in the peripheral T cell subsets T1 and T2. If Th2 cells are the most mature thymocytes, as suggested above, they would also be expected to exhibit a bimodal 9AE10 distribution. Because the light scatter of the Th2 cells is fairly close to that of the Th1 cells, it was not possible to ascertain the 9AE10 distribution of this group. Other methods of isolating the most mature thymocytes may allow this question to be answered. The data suggest, however, that the 9AE10 intermediate and bright thymocyte subpopulations may be the precursors of the corresponding peripheral T cell subsets.

Both human and mouse splenic T populations contain two antigenically and functionally distinct subsets: helper-inducer and suppressor-cytotoxic, which are specifically recognized by a series of monoclonal antibodies (3-6). In terms of percentages of cells stained and staining profiles, none of the anti-rabbit monoclonals closely resembled the subset-specific anti-mouse or anti-human reagents.

The L11/135 antibody is particularly noteworthy because it appears to be specific for a differentiation antigen present on all rabbit T cells. This was shown by the two-color immunofluorescence analysis of L11/135 and anti-rabbit μ , in which the two reagents clearly reacted with distinct populations. Further, it was demonstrated that the ability of rabbit spleen cells to respond to Con A, PHA, or to allogeneic splenocytes resided in the population recognized by L11/135. The 9AE10 monoclonal has also been reported to recognize all peripheral T cells (17). However, in a two-color comparison of L11/135 and 9AE10 it was observed that cells brightly positive for 9AE10 comprise a subset of the L11/135-positive cells (Fig. 5). This apparent

discrepancy is probably explained by the use of an IgM antibody in complement-mediated cytotoxicity, which would be expected to remove cells bearing low densities of the 9AE10 antigen, to select subpopulations for functional studies (21). These results, taken together with the other extensive two-color FC analyses, provide evidence for the existence of at least two T cell subsets in rabbit spleen.

A further use of the L11/135 antibody is its adaptability to simple preparative methodology for the purification of T cell-free lymphoid populations by panning techniques. Direct plating methods for removing T cells in other species have not yet been described. The ease with which this reagent may be used to remove T cells can likely be attributed to the high density of its recognized antigen on rabbit T cells. This determinant is a glycoprotein with a molecular weight of 120,000. A similar pan-T antigen of human cells was recently reported (22 and personal communication from S. C. Meuer). Further structural characterization of the L11/135 antigen and the glycoproteins detected by the other antibodies described in this report will be published separately.¹

The B cell subsets, B1 and B2, differ strikingly in cell size as measured by light scatter. B2, which has much greater light scatter than B1, was stained by L13/64 (Fig. 6), W4/28, and W4/86 as intensely as were peripheral T cells. This staining occurred with directly labeled antibody and was not affected by ultracentrifugation before staining. Even after division of the fluorescence intensity by light scatter intensity, which is closely proportional to total cell surface area, the B2 subset was brighter for L13/64, W4/28, and W4/86, as well as μ (data not shown). One possible explanation for these results is that the B2 subset has a greatly increased capacity for Fc-mediated binding (20). However, attempts to inhibit the binding of these antibodies with mouse serum and preformed immune complexes were unsuccessful, implying that L13/64, W4/28, and W4/86 stain T cells specifically but at levels that overlap the nonspecific uptake by the B2 subset. T cell staining by L11/135, on the other hand, is much brighter than the nonspecific B cell uptake. The rabbit B cell subsets resemble those of the mouse, in which the μ -bright subpopulation is large and gives intermediate staining for the T cell antigen Lyt-1 (23). The ontogenic relationship of B1 to B2 is not yet known, nor are their functional capacities.

A tabulation of the percents and phenotypes of the two T cell, two B cell, and null cell subsets identified in rabbit spleen during this study is given in Table VI. Identification of these populations will allow detailed analysis of the functional properties related to them. The possibility of using the available monoclonals to carry out physical separations of cell populations will greatly facilitate these investigations.

Summary

A panel of six monoclonal antibodies produced against cell surface glycoproteins of a rabbit T lymphocyte line was used with flow cytometry to define rabbit lymphocyte subpopulations. Four thymocyte populations were characterized by size and expression of cell surface antigens and appear to represent stages in thymocyte differentiation. Rabbit spleen contained five subpopulations: two of T lineage, two of B, and a null cell subset. Bimodal distribution of staining of thymocytes and peripheral T cells was observed using an antibody (9AE10) directed against a Thy-1 analogue in the rabbit, suggesting two separate T cell lineages. One of the monoclonal reagents, L11/135, reacted strongly with peripheral rabbit T cells as shown by two-color immuno-

fluorescence. In functional studies, only the L11/135-bearing cells responded to the T cell mitogens concanavalin A and phytohemagglutinin and to allogeneic splenocytes. The thymocyte subpopulations and the peripheral T and B cell subsets differ from those described in mouse and man.

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