

T CELL SUBSETS DEFINED BY EXPRESSION OF
Lyt-1,2,3 AND Thy-1 ANTIGENS
Two-Parameter Immunofluorescence and Cytotoxicity Analysis with
Monoclonal Antibodies Modifies Current Views*

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Antibodies to mouse T cell antigens such as Thy-1, Lyt-1, Lyt-2, and Lyt-3 have been used to characterize T cell subpopulations and to determine precursor-progeny relationships (1). Although the original Lyt-1⁺2⁺, Lyt-1⁺2⁻, and Lyt-1⁻2⁺ classifications have been very useful, several recent findings appear to be inconsistent with the model proposed by Cantor and Boyse, i.e., that Lyt-1⁺2⁺ immature T cells give rise to Lyt-1⁺2⁻ helper cells and Lyt-1⁻2⁺ cytotoxic and suppressor cells (1). For example, cytotoxic T cells originally classified as Lyt-1⁻ (2) have in some cases been depleted by anti-Lyt-1 and complement (3-5). Furthermore immunofluorescence studies in thymus (6) and peripheral lymphoid tissue (7, 8) have indicated that Lyt-1 is expressed on a larger proportion of cells than would be expected from the cytotoxic depletion studies (2). The immunofluorescence studies (5, 7) also showed that Lyt-1⁺2⁻ cells comprise ~50% of the cortisone-resistant thymocytes, indicating that T cells are already divided into separate lineages in the thymus.

Quantitative examination of the question of Lyt-1, Lyt-2, and Lyt-3 expression has recently been facilitated by the production of monoclonal rat antibodies to Thy-1 and Lyt antigens (7). These antibodies can be purified to a single molecular species and thus can be used at high antibody/cell ratios in cytotoxic assays. They also can be directly conjugated with fluorochromes and be used with very low backgrounds in quantitative single-cell fluorescence assays for measuring determinant expression by flow cytometry with the fluorescence-activated cell sorter (FACS) ¹

We show here that although subpopulations can be clearly distinguished on the basis of quantitative phenotypes for these antigens, the qualitative designations Lyt-1⁻2⁺ and Lyt-1⁺2⁺ need to be modified. Lyt-1⁺2⁻ cells express higher levels of Lyt-1 than do Lyt-1⁺2⁺ cells, and are also more easily killed by anti-Lyt-1 plus complement. We also show that although all Thy-1⁺ cells also bear Lyt-1, there is a clear negative correlation in the surface density of these two antigens. There is a small subpopulation

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¹ Abbreviations used in this paper: Ars, arsanilic acid, C, complement, FACS, fluorescence-activated cell sorter, FE, fluorescein equivalent, F/P, fluorescein protein

of lymphocytes that have a high density of Lyt-1 but no detectable Thy-1. Many of these latter cells are located in B cell areas (primary follicles) and germinal centers of spleen and lymph nodes. We compare the densities of Thy-1, Lyt-1, Lyt-2, and Lyt-3 antigens on thymocytes and peripheral (spleen and lymph node) T lymphocytes. Assuming a developmental sequence of cortical thymocytes → medullary (hydrocortisone resistant) thymocytes and T lymphocytes (9, 10), we infer from these data several cell surface changes which occur during maturation.

Materials and Methods

Monoclonal Antibodies. The production and characterization of monoclonal rat anti-mouse antibody-producing hybridoma cell lines has been described (7). Briefly, LOU/Ws1/M rats were immunized with mouse (SJL/J or C3H/HeJ) spleen cells or thymocyte membranes. Spleen cells from these rats were fused with the mouse myeloma NS-1 (11) and hybridomas selected essentially as described (7). Hybrid culture supernates were screened by immunofluorescence and analysis on the FACS. After cloning, the target antigens of the antibodies produced by several of the hybridoma cell lines were identified by comparative immunoprecipitation studies with conventional alloantisera.

Conventional Alloantisera. Alloantisera by Lyt-1.1, Lyt-1.2, Lyt-2.1, Lyt-2.2, Lyt-3.1, and Lyt-3.2 were generously provided by Dr. R. C. Nowinski (Fred Hutchinson Cancer Research Center, Seattle, Wash.) and Dr. F.-W. Shen (Sloan-Kettering Institute for Cancer Research, New York). These antisera were prepared as described (12).

Immunofluorescence of Cell Suspensions. Monoclonal antibodies used for fluorescence were 30-H12 (anti-Thy-1.2), 53-2.1 (anti-Thy-1.2), 53-6.7 (anti-Lyt-2), 53-7.3 (anti-Lyt-1), and 53-5.1 (anti-Lyt-3). Immunofluorescence staining of lymphoid cells was performed using three independent systems, including (a) direct staining with fluorescein-conjugated monoclonal antibodies; (b) indirect staining with biotin-conjugated antibody followed by fluorescein or rhodamine-conjugated avidin (Vector General Inc., Woodland Hills, Calif.); (c) indirect staining with a second step of fluorescein-conjugated mouse (SJL/J) anti-rat Ig. For direct conjugations with biotin or fluorescein, monoclonal antibodies were first purified from supernate on goat anti-rat Ig affinity columns. (The goat anti-rat Ig serum was kindly provided by Dr. R. Wilsnack, Huntingdon Research Center, Brooklandville, Md.) Fluorescein conjugations were with fluorescein isothiocyanate according to Goding (13). Biotin conjugations were with 120 µg biotin succinimide (Vector General Inc.) per mg of purified monoclonal antibody. In each of these three fluorescence labeling systems, all reagents were centrifuged at 100,000 g before use, and 10⁶ target lymphoid cells/well were stained in microtiter plates using saturation levels of first- and second-step reagents. Cells were stained on ice in the presence of 0.1% NaN₃ as previously described (14).

Quantitative fluorescence measurements were made on a modified FACS (Becton Dickinson FACS Systems, Mountain View, Calif.) fitted with a logarithmic amplifier that showed the fluorescence intensity distribution over a 1-10⁴ range. The geometric mean fluorescence of particular cell populations was calculated. After calibration of the FACS system with free fluorescein (by Dr. D. R. Parks, Stanford University, Stanford, Calif.), the mean fluorescence obtained with directly fluorescein-conjugated purified monoclonal antibodies was converted to fluorescein equivalents (FE). In this way mean numbers of antibody molecules bound to positive cells in thymus, spleen, and lymph node were determined by the following calculation: [mean FE (positive cells) - mean FE (unstained cells)]/fluorescein:protein (F/P) ratio of purified antibody.

The mean autofluorescence of unstained cells was equivalent to ~10⁴ fluorescein molecules. F/P ratios of the antibodies were (a) anti-Thy-1.2 (53-2.1), 2.45; (b) anti-Lyt-1 (53-7.3), 2.86; (c) anti-Lyt-2 (53-6.7), 3.60; and (d) anti-Lyt-3 (53-5.1), 3.12. Normally, 10,000 cells were analyzed for single-color and 100,000 for two-color fluorescence.

Immunofluorescence of Tissue Sections. Spleens and lymph nodes (brachial and axillary) were removed from either normal mice or from mice immunized 10 d previously with 20 µl of a 1% solution of sheep erythrocytes injected into the front footpads. The tissues were frozen, cut, fixed in acetone, and stained as described (15). All first- and second-stage antibodies were

titered and used at optimal levels. For single-color staining, the first-stage antibody was followed by rhodamine- or fluorescein-conjugated rabbit anti-rat Ig (absorbed with mouse Ig, kindly provided by Dr. R. Scollay, Stanford University)

Mice. BALB/cNHx, BALB/cGa, C57BL/6J, C57BL/6.H-2^k, AKR/J, and SJL/J mice were bred at Stanford University. The C57BL/6 Ly congenics (C57BL/6 *Lyt*-1.1, C57BL/6.*Lyt*-2.1) were bred at Stanford from stock provided by Dr. E. A. Boyse (Sloan-Kettering Institute for Cancer Research). All mice were male or female animals of 8–12 wk of age.

Cytotoxicity. Cytotoxicity studies were performed in microtiter plates (Dynatech Laboratories, Div. of Dynatech Corp., Alexandria, Va.) with 2×10^6 cells (nylon-enriched splenic T cells [16] or lymph node cells) in 100 μ l of antibody at the indicated dilution. After 45 min on ice, cells were pelleted and resuspended in 100 μ l of a 1/12 diluted rabbit complement for a 30 min incubation at 37°C. The percentage of dead cells were determined by staining an aliquot with a mixture of the nucleic acid-binding dyes ethidium bromide and acridine orange (17). The cells at each titration point were then divided into three equal portions for immunofluorescence staining with directly fluorescein-conjugated monoclonal anti-*Thy*-1.2, *Lyt*-1, *Lyt*-2, and *Lyt*-3. In each experiment, blocking controls were included, with complement replaced by medium and the cells at each antibody titration point stained as above

Results

Specificities of the Monoclonal Antibodies Used for FACS and Cytotoxic Analyses. The demonstration that the rat monoclonal antibodies (53-7.3, 53-6.7, and 53-5.1) react with the *Lyt*-1, *Lyt*-2, and *Lyt*-3 antigens, respectively, was not straightforward because these antibodies react with cells from all mouse strains. First, these antibodies detect framework (species) determinants rather than allodeterminants. Second, the frequencies of cells in thymus and spleen that express the antigens recognized by these antibodies (see below) were not consistent with the frequencies of *Lyt*-1-, *Lyt*-2-, and *Lyt*-3-bearing cells reported by Cantor and Boyse (2) and many others based upon cytotoxicity with conventional alloantisera. Therefore, in addition to using two-dimensional gel analysis to show that the same molecules are precipitated by the monoclonal antibodies and their corresponding conventional antisera (7), we used a variety of alloantibodies to block binding of the fluorescent-labeled monoclonal antibodies to viable cells to verify that the monoclonal antibodies react with the cell surface molecules carrying *Lyt*-1 or *Lyt*-2 and *Lyt*-3 allodeterminants.

The results of the blocking studies (J. A. Ledbetter and L. A. Herzenberg. Unpublished observations.) show that the binding of the anti-*Lyt*-2 antibody (53-6.7) is completely blocked by *Lyt*-2 alloantibodies that react with the allelic *Lyt*-2 determinant expressed on the target cells but not by antibodies to the other *Lyt*-2 allele or by *Lyt*-1 or *Lyt*-3 alloantibodies. Similarly, the anti-*Lyt*-1 monoclonal antibody (53-7.3) is blocked (partially) only by anti-*Lyt*-1 alloantibodies reactive with the appropriate allele. A third monoclonal antibody, 53-5.1, which immunoprecipitates the same molecular species as anti-*Lyt*-2, is blocked by *Lyt*-3 alloantibodies but not by *Lyt*-2 alloantibodies.

This latter antibody (53-5.1) appears to distinguish between allelic products at the *Lyt*-3 locus because it reacts with a >200-fold higher affinity to *Lyt*-3.2-bearing cells than to *Lyt*-3.1-bearing cells. Thus, the biochemical and blocking data identify the target antigens of these antibodies as *Lyt*-1, *Lyt*-2, and *Lyt*-3. We now go on to examine in detail the presence of these antigens on lymphoid cells of normal mice.

*Quantitative Levels of *Thy*-1 and *Lyt* Antigens Vary between Tissues.* The directly fluorescein-conjugated anti-*Lyt*-1, anti-*Lyt*-2, anti-*Lyt*-3, and anti-*Thy*-1.2 monoclonal antibodies were each used alone and in combination with the others in (FACS)

staining experiments. Table I and Fig. 1 show the numbers of cells bearing each antigen and the distribution of antigen densities on these cells. Thymus, spleen, and lymph node lymphocytes were studied. The changes in expression of the Lyt determinants are discussed in detail below.

Lyt-1 Is on All T Cells and Increases during Maturation. All thymocytes and splenic (Thy-1-bearing) T cells express Lyt-1 antigen; however, there is a clear bimodal distribution of antigen densities that correlates with maturation (Fig. 1a). In thymus, approximately 15–20% of the cells stain brightly for Lyt-1. These form a shoulder on the brighter side of the main population. The splenic or lymph node Lyt-1⁺ cells are

TABLE I
Quantitative Cellular Expression and Density of T Cell Antigens by FACS Analysis*

Antibody	Thymus		Spleen		Lymph node	
	Positive cells	Antigen density [‡]	Positive cells	Antigen density [‡]	Positive cells	Antigen density [‡]
	%		%		%	
Anti-Thy-1 2 (53-2 1)	100	85	30	37	57	39
Anti-Lyt-1 (53-7 3)	>95	1.5	34	3.8	59	4.4
Anti-Lyt-2 (53-6 7)	82	5.7	13	6.5	21	6.7
Anti-Lyt-3 (53-5 1)	82	6.0	11	6.8	20	7.2

* C57BL/6 Lyt-2 1 cells were stained with directly fluorescein-conjugated antibodies and analyzed on a FACS-II

[‡] Antigen densities of positive cells are expressed as mean number of antibody molecules bound/cell ($\times 10^4$), calculated as described in Materials and Methods

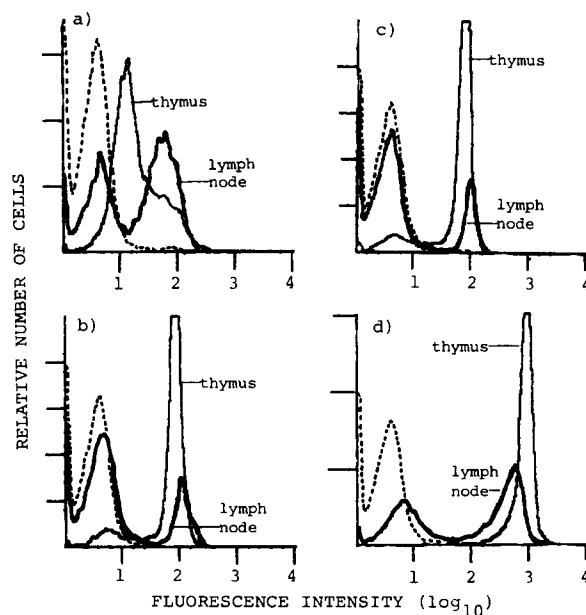


FIG 1 Immunofluorescence staining of C57BL/6J thymocytes and lymph node cells with (a) anti-Lyt-1 (53-7 3), (b) anti-Lyt-2 (53-6 7), (c) anti-Lyt-3 (53-5 1), and (d) anti-Thy-1 2 (53-2 1). Cells were stained with directly fluorescein-conjugated antibodies and analyzed on a FACS-II with a logarithmic amplifier. The dotted line shows the autofluorescence of unstained lymph node cells.

about four times brighter than the main thymocyte population and correspond in brightness to the smaller number of bright thymus cells. Because this latter is the cortisone-resistant thymocyte population (6), these data indicate that levels of Lyt-1 antigen increase during T cell maturation (Table I).

Lyt-2 and Lyt-3 Are Lost from Most T Cells as Thymocytes Mature and Migrate to the Periphery. Lyt-2- and Lyt-3-bearing cells comprise between 80 and 90% of thymocytes. The fluorescence distribution patterns are identical for Lyt-2 and Lyt-3 (Fig. 1). The antigen densities of Lyt-2 and Lyt-3 are almost identical (Table I), and these two antigens are expressed on the same cells ([18]; and J. A. Ledbetter, R. V. Rouse, H. S. Micklem, and L. A. Herzenberg. Unpublished two-color immunofluorescence data.). The variation in frequency of Lyt-2⁺3⁺ cells in thymus that is seen between individual mice is not a result of estimation errors, which are at most 1 or 2%.

The frequency of Lyt-2⁺3⁺ cells in spleen and lymph node of normal mice is usually only 27–40% of the Thy-1 cells. Again, the variation is between individual mice and not caused by estimation problems. These data indicate that Lyt-2 and Lyt-3 are lost from the majority of T cells during maturation, and that Lyt-1⁺2⁻3⁻ cells already exist in the thymus. Other data (H. S. Micklem, J. A. Ledbetter, and L. A. Herzenberg. Manuscript in preparation.) indicate that these cells are all in the hydrocortisone-resistant subpopulation of thymocytes.

The average Lyt-2 and Lyt-3 antigen densities are slightly higher in spleen and lymph node than in thymus (Table I). However, the peripheral (spleen or lymph node) Lyt-2⁺3⁺ cells correspond in brightness to the brightest thymocytes, indicating that these antigens do not change substantially in amounts on positive cells during maturation.

Thy-1.2 Decreases in Amount during Maturation. For Thy-1.2, we found the immunofluorescence of thymocytes and peripheral T cells closely agreed with previous reports using conventional reagents (9, 19). Thy-1.2-stained thymocytes give a very bright peak with a duller shoulder (Fig. 1 d). The latter corresponds with the hydrocortisone-resistant subpopulation (H. S. Micklem, J. A. Ledbetter, and L. A. Herzenberg. Manuscript in preparation.). In spleen and lymph node the brighter Thy-1.2-bearing cells were equivalent in brightness to the duller cells in the thymus.

In summary, the results of the quantitative staining analyses for Thy-1.2, Lyt-1, Lyt-2, and Lyt-3 antigens in thymus, lymph node, and spleen imply the following changes during T cell maturation: (a) Lyt-1 increases substantially; (b) Thy-1 decreases substantially; and (c) Lyt-2 and Lyt-3 are lost from most cells.

Thus, by comparing thymus, spleen, and lymph node cells on a population basis, Lyt-1 and Thy-1 antigen densities appear to change in opposite directions during T cell development but frequencies remain essentially the same. In contrast, Lyt-2 and Lyt-3 change in frequencies of positive T cells but only slightly in density on positive cells.

Cells with High Densities of Antigen Are More Easily Depleted by Complement-dependent Cytotoxicity. Our FACS results are inconsistent with reported frequencies for Lyt-1-bearing cells as determined by cytotoxic analyses. These inconsistencies, we show here, are mainly caused by inefficient killing of cells that express low levels of surface antigens. That is, cells with higher amounts of antigen are more easily killed than cells with low amounts of antigen. When cells are killed with increasing amounts of antibody, the residual surviving cells become progressively duller; however, even

under optimal killing conditions, some low-antigen-density cells often remain, especially when anti-Lyt-1 is used as the cytotoxic reagent. In addition, a small subpopulation of Lyt-1 bright cells is resistant to Lyt-1 cytotoxicity. This point is demonstrated in studies, presented below, of Thy-1 and Lyt expression on T cell populations that survive after cytotoxic depletion of cells with higher antigen densities.

For example, lymph node cells surviving Thy-1.2 cytotoxicity (distinguished from dead cells by forward-angle light scatter) were analyzed by immunofluorescence staining and quantitative FACS analysis for Thy-1.2, Lyt-1, and Lyt-2 antigens (Table II). The fluorescence determinations were done at each point of a Thy-1.2 titration curve that gave progressive depletion of the Thy-1.2⁺ cells. Staining was performed with directly fluorescein-conjugated antibodies. Controls showing no blocking by the cytotoxic antibody of the fluorescein-conjugated antibody were included at each antibody dilution. To avoid blocking, we used a cytotoxic IgM monoclonal anti-Thy-1.2 alloantibody (F7D5, generously provided by Doctors Ed Clark and Phil Lake, International Can Res (ICRF), University College, London) that did not affect the binding of the anti-Thy-1.2 monoclonal antibody (30-H12) used for immunofluorescence. (This is caused by a large affinity difference: 30-H12 prevents the binding of F7D5; data not shown.) The anti-Thy-1.2 cytotoxic antibody (F7D5) did not block the binding of anti-Lyt-1 or anti-Lyt-2 used for immunofluorescence.

The results (Table II) show that the brightest Thy-1.2 staining (highest antigen density) cells are killed by anti-Thy-1.2 at lower concentrations than the dull Thy-1.2 cells. Virtually all the Thy-1.2 cells were eliminated by higher concentrations of anti-Thy-1.2 plus complement (C).

Bright Thy-1 Cells Have Dull Lyt-1. Table II shows that killing progressively more cells with anti-Thy-1.2 causes the average Lyt-1 density of surviving cells to increase, demonstrating that the high density Thy-1.2 cells have low levels of Lyt-1. This negative correlation of Thy-1 and Lyt-1 on lymph node cells is also seen on splenic T

TABLE II
*Relationship between Cytotoxicity with Anti-Thy-1.2 and Fluorescence Intensity of Surviving Cells with Anti-Thy-1.2, Anti-Lyt-1, and Anti-Lyt-2**

Anti-Thy-1.2 dilution‡	Dead	Immunofluorescence of surviving cells§					
		Thy-1.2		Lyt-1		Lyt-2	
		Positive	Mean of positive	Positive	Mean of positive	Positive	Mean of positive
	%	%		%		%	
C alone	8.1	67	162	71	74	28	120
1/50,000	15	52	123	50	83	18	115
1/10,000	45	26	76	25	98	8.4	110
1/5,000	57	8.0	63	14	107	3.0	107
1/1,000	70	1.7	55	7.2	126	1.4	100

* Cytotoxicity performed on BALB/cNHx lymph node cells.

‡ The anti-Thy-1.2 cytotoxicity was with an IgM monoclonal antibody (F7D5) kindly provided by Doctors E. A. Clark and Phil Lake, University College, London. This antibody does not block the binding of the anti-Thy-1.2 monoclonal antibody (30-H12) used for fluorescence.

§ Surviving cells were distinguished by forward-angle light scatter (14) on the FACS. Immunofluorescence was with directly fluorescein-conjugated monoclonal anti-Thy-1.2 (30-H12), anti-Lyt-1 (53-7.3), and anti-Lyt-2 (53-6.7).

cells and is similar to the negative correlation seen in the thymus where, compared with peripheral T cells, higher levels of Thy-1, and lower levels of Lyt-1 per cell were found (Table I).

Some Lyt-1-bearing Cells Do not Express Detectable Amounts of Thy-1. A small population consisting of the brightest Lyt-1 cells remained resistant to Thy-1.2 killing even at high concentrations of antibody (Table II). The presence of a Lyt-1 bright, Thy-1.2⁻ population was confirmed by two-color immunofluorescence (see below). In contrast with Lyt-1, the Lyt-2-bearing cells were depleted in proportion to the Thy-1.2 antibody concentration and were almost completely depleted under conditions of total Thy-1.2 kill (Table II). Therefore by coupled cytotoxicity and fluorescence analysis virtually all Lyt-2⁺ cells also have Thy-1.

Some Lyt-1-bearing Cells Are not Susceptible to Depletion by Anti-Lyt-1 Plus C. Similar experiments were done obtaining cytotoxic titration curves with Lyt-1 on lymph node cells followed by immunofluorescence staining for Thy-1, Lyt-1, and Lyt-2 on surviving cells (Table III). To minimize blocking, we used an anti-Lyt-1.1 monoclonal antibody (W3/L1, a kind gift of Dr. U. Hämmerling) for cytotoxicity that gave a maximum of 15% blocking of the anti-Lyt-1 (53-7.3) used for fluorescence. Again, blocking controls were included at each antibody concentration. The anti-Lyt-1.1 (W3/L1) gave no blocking of anti-Lyt-2 and anti-Thy-1.2 antibodies used for fluorescence. The results (Table III) were consistent with the anti-Thy-1.2 cytotoxic titration and were as follows. (a) Nearly all the bright Lyt-1 cells were more easily killed by lower amounts of anti-Lyt-1 than the dull Lyt-1 cells. Even at the highest antibody concentrations, a population of very dull Lyt-1 cells (Fig. 2) survived. Interestingly, a

TABLE III
Relationship between Cytotoxicity with Anti-Lyt-1.1 and Fluorescence Intensity of Surviving Cells with Anti-Thy-1.2, Anti-Lyt-1, and Anti-Lyt-2*

α-Lyt-1.1 Dilution‡	Dead	Immunofluorescence of surviving cells§					
		Lyt-1		Lyt-2		Thy-1.2	
		Positive cells	Intensity (mean of positive)	Positive cells	Intensity (mean of positive)	Positive cells	Intensity (mean of positive)
C alone	10	55	72	24	186	53	589
1/100,000	15	46	62	28	182	42	661
1/50,000	24	41	59	28	182	38	692
1/10,000	28	33	51	20	178	29	708
1/5,000	32	30	51	18	174	27	589
1/1,000	41	23	52	14	166	25	550
1/100	50	19	58	11	162	18	537

* Cytotoxicity performed on C57BL/6.Lyt-1.1 lymph node cells.

‡ The anti-Lyt-1.1 cytotoxicity was with an IgM monoclonal antibody (clone W3/L1), generously provided by Dr. U. Hämmerling, Memorial Sloan-Kettering Cancer Center, New York. This antibody gave partial (~15%) blocking of the anti-Lyt-1 antibody (53-7.3) used for fluorescence. Blocking controls were included at each antibody dilution, and the Lyt-1 staining data were corrected for blocking.

§ Surviving cells were distinguished by forward-angle light scatter on the FACS (14). Immunofluorescence was with directly fluorescein-conjugated anti-Lyt-1 (53-7.3), anti-Lyt-2 (53-6.7), and anti-Thy-1.2 (53-2.1).

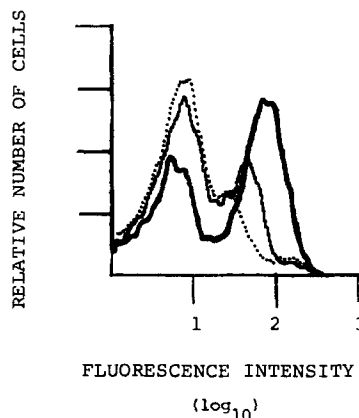


Fig. 2. Relationship between Lyt-1 cytotoxicity and Lyt-1 fluorescence intensity of surviving C57BL/6 Lyt-1.1 lymph node cells. Three Lyt-1 fluorescence distribution curves are shown, after treatment with complement alone (—); after a partial kill with 1/10,000 dilution of anti-Lyt-1.1 (W3/L1) plus complement (---); and after a more extensive kill with 1/100 dilution of anti-Lyt-1.1 (W3/L1) plus complement (· · ·). For additional details of cells surviving partial anti-Lyt-1 depletion, compare Table III

small population of the brightest Lyt-1 cells also survived. This represents ~2% of the original lymph node T cells. (b) At lower antibody concentrations, anti-Lyt-1 selectively killed dull Thy-1.2 cells, but at higher concentrations both bright and dull Thy-1.2 cells were killed. A sizable fraction of the Thy-1.2 cells, 34% in this experiment, were not depleted by anti-Lyt-1. In other experiments we found the C source to affect critically the percentage kill of Thy-1.2 cells by anti-Lyt-1 (data not shown). In general, rabbit C gave higher plateau level kills than guinea pig C. However, a sizable population of Thy-1.2 cells (range: 17–38%) was resistant to Lyt-1 cytotoxicity in each of these experiments. (c) At lower concentrations of Lyt-1, the Lyt-2 cells were enriched in the surviving population (Table III). At high concentrations of Lyt-1, the Lyt-2 cells were depleted by only 54% in this experiment (Table III). This is generally consistent with the finding of many others that after Lyt-1 cytotoxic depletion there are functional Lyt-2⁺ cells remaining. Using a sandwich technique for Lyt-1 killing with arsanilic acid (Ars)-conjugated antibody and rabbit anti-ARS plus rabbit C, we have depleted the Lyt-2 population by a maximum of 70% (data not shown). In these experiments, the Igh-1b allotype suppressors (Ig) were as much as 40% depleted even though these cells were the prototype Lyt-1⁻2⁺3⁺ suppressor T cell population (20).

Virtually All Lyt-2⁺3⁺ Cells Express At Least Low Levels of Lyt-1. We examined in more detail the overlap of Lyt-1 and Lyt-2 cells by additive fluorescence staining, i.e., anti-Lyt-1 alone compared with anti-Lyt-1 plus anti-Lyt-2 and Lyt-3. With either spleen or lymph node cells, the number of cells that stained with a mixture of all three antibodies was only 2% more than the number of cells that stained with anti-Lyt-1 alone. Because these stains gave well-defined minimums between peaks of positive and negative cells, the size of an Lyt-1⁻2⁺3⁺ population if it exists is at most 3% of total Thy-1.2 cells (Fig. 3). Furthermore, by comparing the fluorescence distribution patterns, it was apparent that the Lyt-1⁺2⁺3⁺ cells express lower amounts of Lyt-1 than the Lyt-1⁺2⁻3⁻ cells, i.e., the Lyt-2 and Lyt-3 antibodies stained a

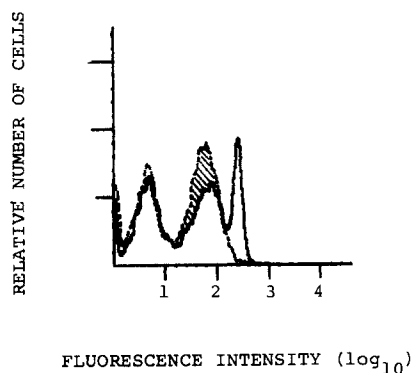


FIG 3 Immunofluorescence staining of C57BL/6 lymph node cells with anti-Lyt-1 alone (---) compared with anti-Lyt-1 plus anti-Lyt-2 and anti-Lyt-3 (—) Cells were stained with directly fluorescein-conjugated antibodies and analyzed on a FACS-II with a logarithmic amplifier The hatched area represents the Lyt-1 fluorescence intensity of the Lyt-1⁺2⁺3⁺ cells.

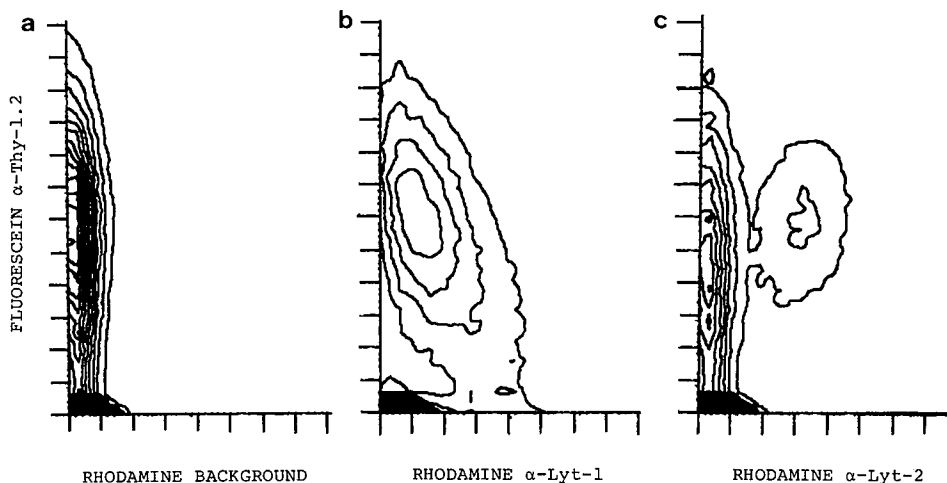


FIG. 4 Two-color immunofluorescence analysis of BALB/cNHx nylon-enriched splenic T cells on a FACS-II with standard linear amplifiers Staining for Thy-1.2 in each panel was with directly fluorescein-conjugated anti-Thy-1.2 (30-H12) (α -Thy-1.2) Staining for Lyt-1 or Lyt-2 was with biotin-conjugated antibodies (53-7.3 or 53-6.7) followed by rhodamine-conjugated avidin. The first panel shows the fluorescein anti-Thy-1.2 staining and the rhodamine background (rhodamine-avidin alone) The contour lines represent 75 cells/channel intervals

population of cells predominantly among the dull Lyt-1 cells (Fig. 3, shaded area). Higher-density Lyt-1 cells were primarily Lyt-2⁻3⁻.

Two-Color Cell Suspension Immunofluorescence for Thy-1.2, Lyt-1, and Lyt-2. The correlations of staining with the monoclonal T antibodies are readily examined by FACS simultaneous detection of fluorescein and rhodamine on individual cells (21). Nylon-purified splenic T cells were used. Fig. 4 shows the negative correlation between Thy-1 and Lyt-1 antigen densities using fluorescein-conjugated anti-Thy-1.2 in combination with biotin-anti-Lyt-1 followed by rhodamine-conjugated avidin (panel b). In panel c, the Lyt-2 cells are seen as a discrete population with intermediate to bright levels of Thy-1. The contour plots from these data graphically illustrate the correla-

tions of Thy-1 with the Lyt antigens and agree well with the cytotoxicity results presented above.

Similar two-color staining experiments for Lyt-1 and Lyt-2 depicted on contour plots (Fig. 5) show that the brightest Lyt-1 cells are Lyt-2 negative. Conversely, Lyt-2-positive cells stain dully for Lyt-1. Again quantitative staining analysis rationalizes how at certain antibody concentrations, selective cytotoxic depletion of the Lyt-1 subset or of the Lyt-2 subset can occur. These data also rationalize the failures to get total depletion of the Lyt-2 cells using anti-Lyt-1 and C because most of the Lyt-2 cells express low-density Lyt-1.

Section Staining For Thy-1, Lyt-1, and Lyt-2. To examine further the distribution of cells bearing Thy-1, Lyt-1, and Lyt-2 antigens, we stained frozen sections of BALB/cGa and C57BL/6.H-2^k spleen and lymph node. The distribution of positive staining with all three antibodies (Fig. 6) was similar to that seen with conventional rabbit anti-mouse T serum (22). In addition to confluent T zone staining (lymph

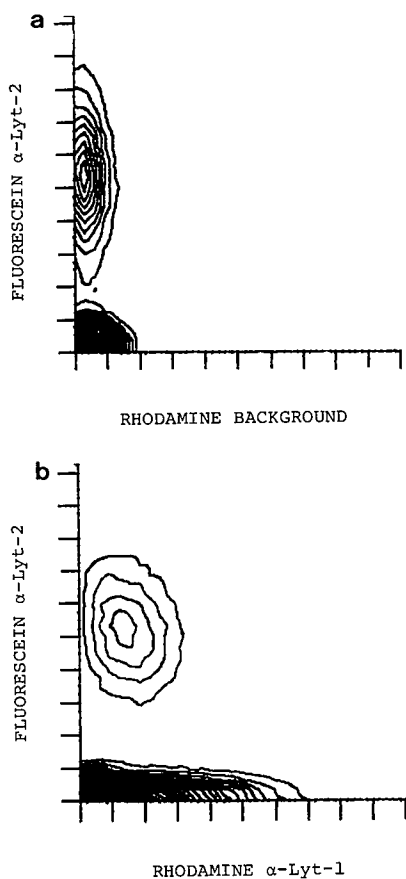


FIG. 5. Two-color immunofluorescence analysis of BALB/cNHx nylon-enriched splenic T cells on a FACS-II with standard linear amplifiers. Directly fluorescein-conjugated anti-Lyt-2 (53-6.7) (α -Lyt-2) and biotin-conjugated anti-Lyt-1 (53-7.3) followed by rhodamine-conjugated avidin were used (b) (a) shows the fluorescein anti-Lyt-2 staining and the rhodamine background (rhodamine-avidin alone). The contour lines represent 75 cells/channel intervals

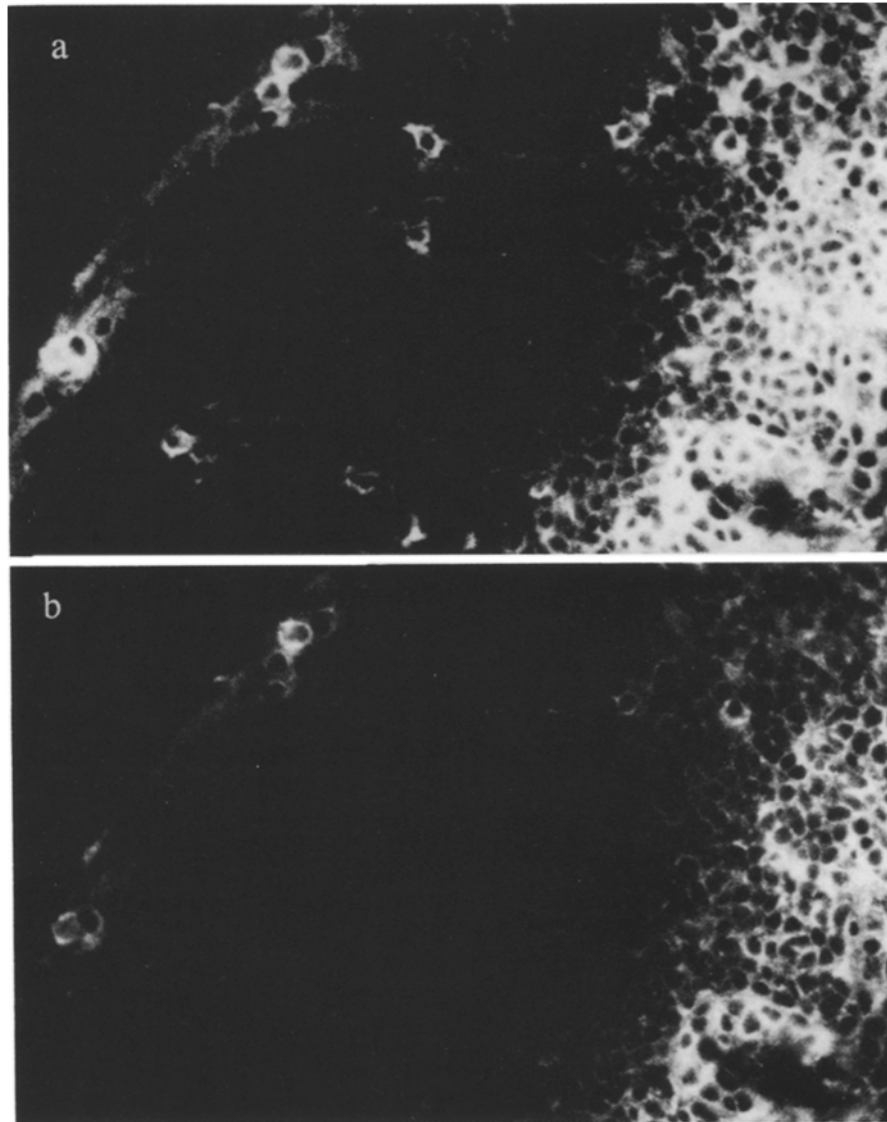


FIG 6 Two-color immunofluorescent staining of frozen section of a germinal center from a BALB/cGa immunized lymph node with mixed anti-Lyt-1 (53-7.3) and biotin-conjugated anti-Thy-1.2 (30-H12) followed by mixed fluorescein-conjugated avidin and rhodamine-conjugated rabbit anti-rat Ig. The same field is viewed with (a) rhodamine filters, and (b) fluorescein filters. The paracortex (right side) is stained and the germinal center is largely unstained in both, but four Lyt-1⁺Thy-1⁻ cells are present within the germinal center seen in (a) but not (b).

node paracortex and spleen periarteriolar sheath), occasional discrete cells were identified within B zones (primary follicles) and germinal centers. These cells were examined using two-color immunofluorescence to determine if Lyt-1⁺Thy-1⁻ cells could be visualized in these locations.

Using a mixture of anti-Lyt-1 (53-7.3) and biotin-conjugated anti-Thy-1.2 (30-H12) followed by a second-stage mixture of fluorescein-conjugated avidin and rhodamine-conjugated rabbit anti-rat Ig on the same section, Lyt-1⁺ and/or Thy-1⁺ cells stained red and Thy-1⁺ cells stained green. Thus, by simply changing filters while examining a microscopic field, Lyt-1⁺Thy-1⁻ cells would stain red but not green, whereas Lyt-1⁺Thy-1⁺ cells would stain with both colors. Using this technique, Lyt-1⁺Thy-1⁻ cells (stained red but not green) were identified within primary follicles and germinal centers (Fig. 6).

Discussion

Using monoclonal antibodies and quantitative immunofluorescence with a FACS, we show that all peripheral T cells have some Lyt-1 antigen. In general, the density of Lyt-1 is low (the mean number of antibody molecules bound per positive spleen cell is only 3.8×10^4 compared with 37×10^4 for Thy-1.2); however, the Lyt-1 antigen density is quite heterogeneous, because the brightest Lyt-1⁺ spleen cells have ~14 times as much antigen as the dullest Lyt-1⁺ cells. This large variation in Lyt-1 antigen density is nonrandom in that Lyt-1 appears to increase as T cells mature. Thus the immature (cortisone-sensitive) thymocytes have the lowest levels of Lyt-1, whereas the mature (cortisone-resistant) thymocytes (6) and peripheral T cells have about four times more Lyt-1. The demonstration that Lyt-1 increases as T cells mature is consistent with the negative correlation between Lyt-1 and Thy-1 antigen densities of T cells, because Thy-1 expression has been shown to decrease progressively as cells mature in the thymus and migrate to the periphery (9). The dramatic increase in Lyt-1 expression during T cell maturation is accompanied by a loss of Lyt-2 and Lyt-3 because the cells with highest levels of Lyt-1 are Lyt-2⁻3⁻.

Our finding that Lyt-1 is on all T cells might seem to conflict with earlier cytotoxic depletion studies in which cytotoxic effector T cells and suppressor cells were classified as Lyt-1⁻. The failure to detect Lyt-1 on these Lyt-2⁺3⁺ cells, however, can be explained by the difficulty in killing with antibody and C cells that carry small numbers of Lyt-1 determinants. The Lyt-1⁺2⁺3⁺ cells (including suppressor and cytotoxic effector T cells) have lower levels of Lyt-1 than the Lyt-1⁺2⁻3⁻ cells, and a proportion of the Lyt-1 cells are very difficult to kill with anti-Lyt-1, even with monoclonal antibodies and the best lots of rabbit C. Lyt-1⁺ cells that escape cytotoxicity are predominantly dull Lyt-1, Lyt-2⁺3⁺ (although a small subpopulation of bright Lyt-1 cells also escapes cytotoxicity)

The differential expression of Lyt-1 on T cell subpopulations is particularly important in view of the previous distinction between Lyt-1⁺2⁺3⁺ precursor T cells and Lyt-1⁻2⁺3⁺ cytotoxic effector T cells (23, 24). By Lyt-1 cytotoxicity (but not by fluorescence) we also see a subdivision of the Lyt-2⁺ set because many Lyt-2⁺ cells are not killed by anti-Lyt-1. These show slightly duller than average Lyt-2 staining, indicating that cytotoxicity with anti-Lyt-1 selectively subdivides the Lyt-2 population. On the other hand, two-color immunofluorescence and additive staining show the Lyt-2⁺ population as being rather uniformly dull Lyt-1, giving no indication of two phenotypically distinct Lyt-2⁺ subpopulations. There is ample evidence that Lyt-1 cytotoxicity varies in different laboratories. For example, of the T cells originally called Lyt-1⁻ (cytotoxic T cells and suppressor T cells) the cytotoxic effector cells have in several cases been depleted by anti-Lyt-1 and C (3-5). Furthermore, Igh-1b

allotype suppressor cells are also substantially depleted using a sandwich technique for Lyt-1 cytotoxicity with Ars-conjugated antibody and rabbit anti-Ars plus C (T. Tokuhisa and Lee Herzenberg. Personal communication.). Therefore, although Lyt-1 cytotoxicity may be useful for functional distinctions, the extent of Lyt-1 cytotoxicity is primarily dependent upon the source of C and the antibody concentration. We recommend that, as originally suggested by Shiku et al. (3), Lyt-1 phenotypes of T cell subpopulations be represented by Lyt-1 \uparrow (high) vs. Lyt-1 \downarrow (low) for cells that differ in their ease of depletion in cytotoxicity. Therefore, phenotyping of functional subpopulations needs to be based upon quantitative measurement of Lyt-1 expression.

The relationship of antigen density to susceptibility to C-dependent cytotoxicity is important for the interpretation of functional studies of T cell subpopulations using cytotoxic depletion. We show here that average surface density for these antigens is well correlated with susceptibility to cytotoxicity because high antigen-density cells are more easily killed than low antigen-density cells. We show this to be the case for Thy-1.2 and, in general, Lyt-1 cytotoxicity. There is, however, a small subpopulation of Lyt-1 bright cells which are resistant to even the highest concentrations of anti-Lyt-1. It is possible that these cells represent a specific functional subpopulation.

There is another example in which the relationship of antigen density to cytotoxicity is more complex. Anti-T200 detects an antigen expressed in equal amounts on both T and B cells but kills only T cells (25) even at very high concentrations. Because this is true for two different monoclonal anti-T200 antibodies, it does not appear to be a result of the nature of the antibody. Rather, subtle structural differences or presentation of an antigen on the cell surface may also influence susceptibility to cytotoxicity. B cells have a form of T200 with a lower molecular weight (180,000) than do T cells (200,000) (26). Thus, in the case of Lyt-1, the inability to kill a small subpopulation of bright cells may be because these have a slightly different molecular form or presentation of Lyt-1.

The numbers of cells that have Lyt-1 or Thy-1 in spleen, lymph node, and peripheral blood are approximately equal, although in many animals there are some 2-5% more Lyt-1 cells than Thy-1 cells. Quantitative two-color immunofluorescence shows that these two antigens are negatively correlated in surface density and represent two partially nonoverlapping populations. This analysis revealed a small population of Lyt-1 bright cells which had undetectable levels of Thy-1. At our current levels of sensitivity with the FACS, cells that have 10^4 fluorescein molecules are readily detectable above background. This corresponds to approximately 3×10^3 bound antibody molecules at our normal fluorescein conjugation ratios for monoclonal antibodies. Therefore more sensitive techniques may in the future detect very low levels of Thy-1 on these cells. We also found by examination of monoclonal antibody-stained frozen sections of spleen and lymph node that there are some cells which are Thy-1 negative and strongly positive for Lyt-1 in B cell areas such as primary follicles and germinal centers. It is likely that these are the same cells seen in cell suspensions by FACS analysis. Such differential expression by T cells may also occur within T zones of lymph node and spleen, but fail to be observed as a result of the confluence of positive cells in those areas.

The presence of an Lyt-1 $^+$ Thy-1 $^-$ cell population raises again the problem of defining murine T lymphocytes by possession of Thy-1. Because during T cell maturation, levels of Thy-1 decrease while levels of Lyt-1 increase, and cell-surface

densities of the two antigens are negatively correlated, the bright Lyt-1⁺, Thy-1⁻ cells may be the most mature T cells. Alternatively, because the function of these cells is unknown, it is possible that they are not thymus dependent.

The proportion of T cells that express surface Lyt-2 reported here, and in a previous publication (8), are unexpectedly low in comparison with the figures found by Cantor and Boyse (2) on the basis of cytotoxicity. The Lyt-1⁺2⁺3⁺ and Lyt-1⁻2⁺3⁺ categories of these authors accounted between them for some 65% of total T (Thy-1⁺) cells—about twice what we usually find. However, it is to be expected that the frequency will vary according to the immunological status of the animal, and we have seen considerable variation ourselves with occasional animals in the 60–70% range.

Summary

Using monoclonal antibodies and multiparameter fluorescence analyses, we show that the expression of Lyt-1, Lyt-2, and Lyt-3 on T cell subpopulations is more complex than was originally recognized by the cytotoxic depletion studies with conventional reagents that defined the Lyt-1⁺2⁺3⁺, Lyt-1⁺2⁻3⁻, and Lyt-1⁻2⁺3⁺ populations. We detect at least some Lyt-1 on all T (Thy-1-bearing) lymphocytes; however, in agreement with previous studies, we find that Lyt-2⁺3⁺ cells are more difficult to deplete with anti-Lyt-1 than Lyt-1⁺2⁻3⁻ cells.

Surprisingly, we found a small subpopulation of cells carrying relatively large amounts of Lyt-1 and no Thy-1 detectable by fluorescence-activated cell sorter analysis. We also detect cells with this phenotype histologically in B cell zones (primary follicles) and germinal centers in spleen and lymph nodes. In general, the Lyt-1 only population represents ~2% of spleen cells.

The relative quantitative expression of Thy-1, Lyt-1, Lyt-2, and Lyt-3 changes systematically during T cell maturation. Among Lyt-1⁺2⁺3⁺ cells in the thymus, Thy-1 and Lyt-2 are high, whereas Lyt-1 is low. Among splenic T cells, in contrast, Thy-1 is low, Lyt-1 is high, and Lyt-2 and Lyt-3 are a little higher than in thymus. In general, Thy-1 expression is negatively correlated with Lyt-1. Thus, even among splenic and lymph node T cells subpopulations exist that tend to be either high Thy-1 and low Lyt-1 or vice versa. Lyt-2⁺3⁺ cells represent ~85% of thymocytes but only ~35% of splenic or lymph node T cells. The Lyt-2⁺3⁺ cells are found predominantly in the low Lyt-1, high Thy-1 subpopulation.

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