CELLULAR IMMUNOLOGY 15, 180–196 (1975)

Characterization of Subpopulations of T Lymphocytes

I. Separation and Functional Studies of Peripheral T-Cells Binding Different Amounts of Fluorescent Anti-Thy 1.2 (Theta) Antibody Using a Fluorescence-Activated Cell Sorter (FACS)[1]

HARVEY CANTOR,[2] ELIZABETH SIMPSON,[3] VICKI L. SATO, C. GARRISON FATHMAN,[4] AND LEONARD A. HERZENBERG

Department of Genetics, Stanford University School of Medicine, Stanford, California 94305

Received May 4, 1974

Peripheral T lymphocytes in mice can be distinguished by the presence of the Thy 1.2 (theta) cell surface antigen. The fluorescence-activated cell sorter (FACS) was used to analyze and separate T-cells from peripheral lymphoid cell suspensions after incubation with fluorescein-labeled anti-Thy 1.2 (F anti-Thy 1.2). Stained cells were markedly reduced in nu/nu mice, in mice carrying the Thy 1.1 allele (theta-AKR), and were not seen after incubation with anti-Thy 1.2 that had been absorbed with CBA brain. According to these criteria, the stained cells were termed "T lymphocytes."

Among the T lymphocytes, there was considerable heterogeneity of fluorescent staining. The FACS was used to separate T-cells from other cells and further to separate T-cells with high intensity F anti-Thy 1.2 fluorescence (bright T-cells) from those with less F anti-Thy 1.2 fluorescence (dull T-cells). Separated bright T and dull T lymphocytes were shown to have several different functional properties. Dull T-cells appeared more sensitive to small doses of ALS *in vivo*, homed to lymph node in higher proportions than did bright T-cells, and were not affected by the short-term effects of thymectomy in adult life. Bright T lymphocytes, by contrast, were resistant to the *in vivo* effects of ALS, homed preferentially to spleen rather than lymph node in irradiated hosts, and were reduced shortly after adult thymectomy. Separated populations of bright and dull T-cells showed reduced ability to produce cytotoxic activity after *in vitro* sensitization, while mixtures of these two subpopulations of T-cells produced synergistic cytototoxic responses. The ontogenic and functional implications of these findings are discussed.

INTRODUCTION

In rodents, cells migrate from the thymus to peripheral lymphoid tissues to maintain a population of thymus-derived (T) cells (1). Many studies have suggested that this population is composed of recirculating, functionally long-lived lymphocytes that have been seeded from the thymus during neonatal life (2, 3). For example, while thymectomy in neonatal life results in a depletion of lymphocytes in the recirculating pool as well as severe immunological defects (3, 4) thymectomy in

[1] Support by NIH Grants GM 17367 and AI 08917 and Fellowships GM 52049–02 and IC 69.

[2] Present address: Department of Medicine, Harvard Medical School, Children's Cancer Research Center, 35 Binney Street, Boston, MA 02115.

[3] Present address: Clinical Research Centre, Harrow, Middlesex, UK.

[4] Present address: Immunology Branch, NCI, NIH, Bethesda, MD 20014.

be accompanied by progressive loss of Thy 1.2 antigen as detected by fluorescent antisera.

The higher proportion of bright splenic T cells neonatally, followed by the progressive increase in proportion of dull spleen T cells with age, are in agreement with the findings of Ollsson and Claësson (17) and are consistent with the idea that T_2 cells develop with time from T_1 in peripheral lymphoid tissue, but do not exclude the possibility that they are different cell lines, increasing in the periphery at different rates.

Cooperative interactions between subpopulations of T lymphocytes have been shown to occur during the production of graft versus host reactions in mice (8–10). Recently, similar interactions have been observed in the production of a cytotoxic response to alloantigens (11–13). Synergy was observed after sensitization of peripheral lymph node T lymphocytes and thymocytes (11, 12) and also was obtained using peripheral lymph node T cells and T cells from the spleens of animals given small doses of ALS (13). The present findings extend these observations, insofar as our findings indicate that separated subpopulations of splenic T cells interact synergistically in the *in vitro* generation of cytotoxicity. The latter finding, that synergy occurs between subpopulations of T cells obtained from the same peripheral lymphoid tissue, suggests that this T–T interaction may well be a physiologic one, occurring between T-cell subpopulations located within the same tissue *in vivo* that are distinguished by different amounts of Thy 1.2 antigen.

ACKNOWLEDGMENTS

Skillful technical assistance was provided by Messrs. R. Stovel, R. T. Merrill, T. Knaak, D. Hewgill. C57BL/6J mice were kindly provided by Dr. Irving Weissman.

REFERENCES

1. Weissman, I., *J. Exp. Med.* **126**, 291, 1967.
2. Miller, J. F. A. P., and Mitchell, G. F., *Transplant. Rev.* **1**, 3, 1969.
3. Miller, J. F. A. P., *Proc. Roy. Soc. London B* **156**, 415, 1962.
4. Good, R. A., Dalmasso, A. P., Martinez, C., Archer, O. K., Pierce, J. C., and Papermaster, B. W., *J. Exp. Med.* **116**, 773, 1962.
5. Taylor, R. B., *Nature (London)* **208**, 1334, 1965.
6. Metcalf, D., *Nature (London)* **208**, 1336, 1965.
7. Miller, J. F. A. P., *Nature (London)* **208**, 1337, 1965.
8. Cantor, H., and Asofsky, R., *J. Exp. Med.* **131**, 235, 1970.
9. Cantor, H., and Asofsky, R., *J. Exp. Med.* **135**, 764, 1972.
10. Tigelaar, R. T., and Asofsky, R., *J. Exp. Med.* **137**, 239, 1973.
11. Wagner, H., *J. Exp. Med.*, **138**, 1397, 1973.
12. Tigelaar, R. T., and Feldman, M., *Transplantation Proc.* in press, 1973.
13. Cantor, H., and Simpson, E., submitted for publication.
14. Bach, J. F., and Dardenne, M., *Immunology* **6**, 394, 1973.
15. Greaves, M. F. and Hogg, N. M. *In* "Progress in Immunology (B. Amos, Ed.), Academic Press, New York, 1971.
16. Stobo, J. B., and Paul, W. F., *J. Immunol.* **110**, 362, 1973.
17. Ollson, L., and Claësson, M. H., *Nature (London) New Biol.* **244**, 750, 1973.
18. Stobo, J. B., and Paul, W. E., and Henney, C. S., *J. Immunol.* **110**, 652, 1973.
19. Hulett, H. R., and Bonner, W. A., Sweet, R. G., and Herzenberg, L. A., *Clin. Chem.* **19**, 813, 1973.
20. Levey, R. H., and Medawar, P. B., *Ann. N.Y. Acad. Sci.* **129**, 164, 1966.
21. Reif, A. E., and Allen, J. M. V., *Nature (London)* **209**, 521, 1966.

adult life has been reported to result in little detectable effect upon the levels of recirculating lymphocytes and the primary immune response for at least 8–12 mo after surgery (5–7).

Recent studies of T lymphocytes that participate in the production of graft-versus-host (GVH[5]) responses (8–10), cytotoxic responses (11–13), antigen binding (14, 15), and response to mitogens (16) have suggested a functional heterogeneity within the T-cell class based on different homing properties and sensitivity to antilymphocyte serum (ALS) and adult thymectomy. For example, studies of the cells responsible for the production of GVH and cytotoxic responses directed at alloantigens have indicated that these cellular responses are mediated by an interaction between two T-cell subpopulations. One T-cell subpopulation between two T-cell subpopulations. One T-cell subpopulation (T_1) appears to be primarily restricted to thymus and spleen, is resistant to small doses of ALS, and preferentially homes to spleen in irradiated hosts. A second type of T cell (T_2), found mainly in lymph node, lymph, and blood, is quite sensitive to ALS and homes preferentially to lymph nodes in irradiated hosts.

Two apparently conflicting observations on the amount of Thy 1.2 antigens on peripheral T cells have been made (17, 18), although these studies are in agreement that there is a decrease from the thymus to the periphery. The reason for the discrepant observations on two peripheral subpopulations are not clear, but different methods have been used, and it is clear that two subpopulations have been defined by each of the methods employed. Based on the notion that fluorescein-labeled anti-Thy 1.2 serum (F anti-Thy 1.2) would stain those peripheral T cells bearing more Thy 1.2 determinants, more brightly than peripheral T cells with fewer such determinants, the brightness of individual lymphoid cells directly stained with F anti-Thy 1.2 was determined in a fluorescence-activated cell sorter (FACS) (19). Fluorescence profiles, i.e., frequency distributions of cells according to fluorescence brightness, were used to estimate the relative proportions of bright T and dull T cells in lymph nodes and spleen and to compare these with the brightness of thymus- and cortisone-resistant thymus-cell populations similarly stained. The effect of various doses of ALS, and of adult thymectomy on the relative proportion of bright T and dull T cells in the spleen was also examined.

The ability of the FACS to sort cells according to brightness was exploited to separate splenic T cells into bright and dull populations and perform several types of functional studies on them, alone, or in combination. These studies included migration patterns and the ability to make cytotoxic responses to alloantigens. Clear indications of synergy of the bright T- and dull T-cell populations in development of cytotoxicity were observed, supporting previous suggestions of a cooperative interaction between subpopulations of T cells. The relationship of these two populations to each other in ontogeny is further explored and discussed.

MATERIALS AND METHODS

Animals

BALB/cN and C57BL/6J mice were obtained from the Departments of Genetics and Pathology, Stanford University, Stanford, CA.

[5] Abbreviations. ALS, antilymphocyte serum; ATx, adult thymectomy; B, bone marrow derived; FACS, fluorescence-activated cell sorter; F anti-Thy 1.2, fluorescein labeled anti-Thy 1.2 globulin; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; F/P, molar fluorescein: protein ratio of the conjugate; GVH, graft versus host; PBS, Dulbecco's phosphate-buffered saline; PBS/FCS, PBS with 5% FCS.

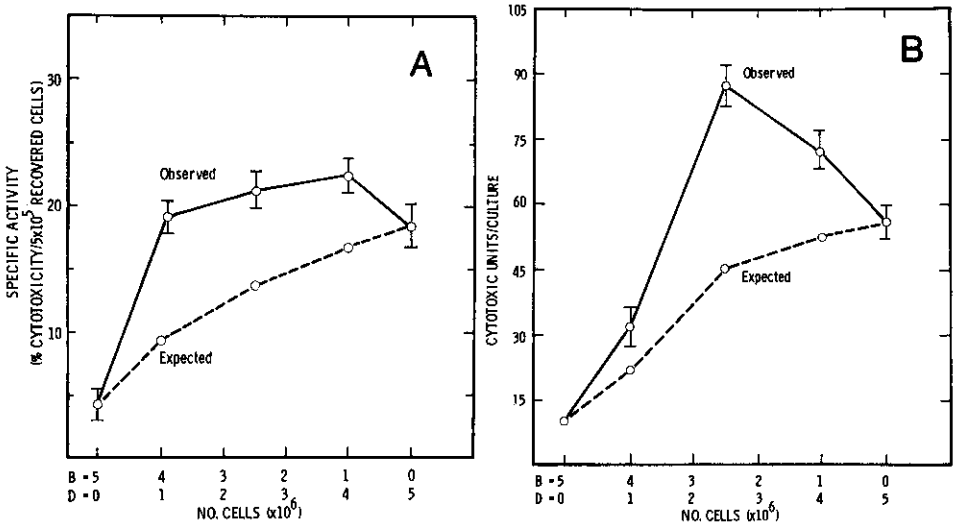

FIG. 13. A and B. Specific and total lytic activity after sensitization of bright T and dull T cells alone as compared with that obtained after sensitization of mixtures of bright T and dull T cells in different proportions. Observed lytic activity is compared with the lytic activity expected from the sum of the activities of the separated and dull components.

dull and bright T cells. These slopes were used to predict the activity of mixtures of these cells (the "expected" curves in Figs. 13A and 13B).

The observed activity of actual mixtures of bright and dull T cells is also plotted in Figs. 13A and 13B. It can be seen that the observed activity of cell mixtures is higher than the expected values, and that in all cases mixtures of bright T and dull T cells give higher activity than either alone. The highest activity per culture was seen with mixtures of bright T and dull T cells at ratios of 1 : 1, which is the ratio in unseparated populations.

DISCUSSION

We have separated two populations of splenic T cells by intensity of staining with F anti-Thy 1.2. The specificity of staining for this alloantigen was established by complete absorption of the staining activity with thymus (data not presented) or brain of mice carrying the *Thy-1*^{b} allele, therefore expressing Thy 1.2 antigen, and by lack of staining of AKR (*Thy-1*^{a} allele, therefore Thy 1.2 negative) thymus or spleen. Furthermore, we showed that staining for Thy 1.2 occurred exclusively on T cells in lymphoid organs. Since the nature of the method used, FACS fluorescence analysis, permits cells stained at background levels, as well as those stained above these levels, to be seen in fluorescence profiles, for simplicity we speak of stained cells as those which have a fluorescence intensity greater than a level objectively chosen to discriminate between background and specific staining. For example, in the staining of AKR or nu/nu spleen cells with F anti-Thy 1.2 serum, and of BALB spleen cells with this reagent after brain absorption, only the background stained cells were seen, and this background was the same as unstained cell suspensions.

The brightness of fluorescence per cell is presumably a direct function of the amount of F anti-Thy 1.2 and thus of the amount of Thy 1.2 antigen per cell, at

A typical fluorescence profile is shown in Fig. 1. The area under each profile is proportional to the numbers of cells analyzed. The abscissa is relative fluorescence intensity per cell while the ordinate is the relative number of cells with each level of fluorescence. Identical fluorescence profiles of different populations of stained lymphocytes can be obtained only if the cells are incubated under identical conditions with the same fluorescent reagent. Profiles plotted on the same figure or for a given experiment are from cells stained in parallel on the same day with the same reagents. Minor differences between profiles on different days are observed. The same scales are used throughout, except for Fig. 3A, by using appropriate scaling factors.

Cell Migration

Suspensions of lymphoid cells were treated with hemolytic Gey's solution and washed with PBS/FCS to remove red cells, and labeled with ^{51}Cr by incubating 10^7 cells per ml with 50 μCi/ml ^{51}Cr for 30 min at 37°C (26). In experiments involving separation, cells were ^{51}Cr labeled after nylon column purifications and FACS separation. Appropriate numbers of these labeled lymphocytes were injected intravenously into syngeneic recipients that had been irradiated (700 R) 24 hr previously. Approximately 20 hr after injection, the irradiated recipients were sacrificed and radioactive counts in liver, spleen, mesenteric, and peripheral (femoral, axillary, and brachial) lymph nodes were determined.

In Vitro Sensitization and Cytotoxic Assay

Sensitization of splenic T cells against allogeneic cells was carried out according to the method of Wunderlich and Canty (27). One-milliliter aliquots of 5×10^6 BALB/cN splenic T (responder) cells in fortified Eagle's medium were incubated in 16 × 10-mm petri dishes (Linbro) together with 5×10^5 irradiated (2500 R) C57BL/6 (H-2b) spleen (stimulator) cells on a rocker platform for 5 days in an atmosphere of 7% CO_2, 10% O_2, and 83% N_2. In these experiments, since the cells to be sensitized represented almost 100% Thy 1.2-bearing lymphocytes after fluorescence separation, induction of cytotoxic activity was not seen unless 2.5×10^5 cells obtained from peritoneal washings were added to the cultures as a source of macrophages.

In wells in which fewer than 5×10^6 responder T cells were used, the number of BALB/cN cells was made up to 5×10^6 by the addition of non-T cells prepared by treating BALB/cN spleen cells with anti-Thy 1.2 serum plus guinea pig complement.

The cultures were fed daily with a nutrient medium (28), and harvested after 5 days. The yield after 5 days ranged from 20% to 50% and viability determinations with trypan blue showed that 95%–99% of the remaining cells excluded the dye. The cytotoxic activity of these cells was assayed according to a modification of the method of Canty and Wunderlich (29). Viable sensitized cells (5×10^5) in 1 ml Eagle's Minimal Essential medium with 10% FCS were incubated with 1×10^5 ^{51}Cr-labeled EL-4(H-2b) target cells for 4 hr at 37°C. At the end of this time, the cultures were quenched with 1 ml of cold PBS/FCS and centrifuged at 2000 rpm for 10 min. Then 1-ml aliquots of the supernatant of each culture were counted for ^{51}Cr activity. The cytotoxic activity of each sensitized cell population

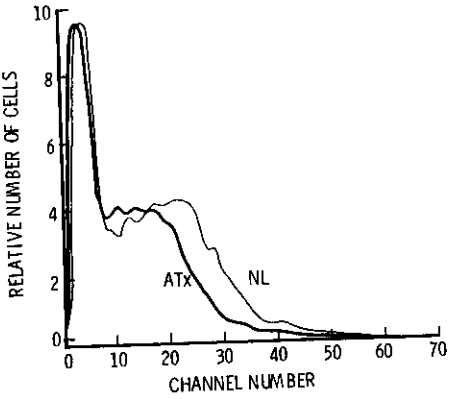

FIG. 10. Fluorescence profiles of spleen cells from adult-thymectomized (ATx) and sham-thymectomized (NL) donors after staining with F anti-Thy 1.2 (see text for details of protocol).

The Ontogeny of Bright and Dull T Cells in the Spleen

The foregoing experiments suggested that the two subpopulations of T cells exhibited different migratory properties, tissue distribution, and sensitivity to ALS and adult thymectomy. The ontogenic relationship between T_1 (bright T, ALS resistant, adult thymectomy sensitive) and T_2 (dull T, lymph node seeking, rapidly recirculating, ATx resistant) is not clear. They could be two developmental stages of the same cell type or they could arise independently. To explore these possibilities we looked at the proportion and number of bright T and dull T cells in spleen as the animal aged.

Spleen cell suspensions obtained from mice at various intervals after birth were stained with F anti-Thy 1.2 and the fluorescence profile of each cell population was examined. The proportion of cells in the T region increased from 15% in newborn mice to 35%–40% by 10 wk of age, and remained constant to 30 wk (Fig. 11). When a FACS fluorescent threshold was set to register only the brightest third of cells within the T region of adult spleen, it was found that the proportion of total cells brighter than this threshold was relatively constant in spleen cells ob-

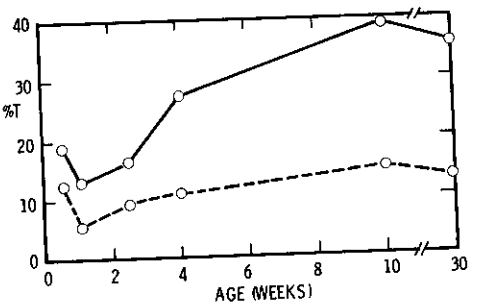

FIG. 11. Total T lymphocytes (——) and bright T lymphocytes (– –) at different intervals after birth. The proportion of T lymphocytes increased from 15%–20% in newborn mice to 35%–40% by 10 wk of age, while bright T cells, corresponding to approximately the brightest third of all adult T cells, remained relatively constant throughout the interval examined. These results represent the mean values of three experiments.

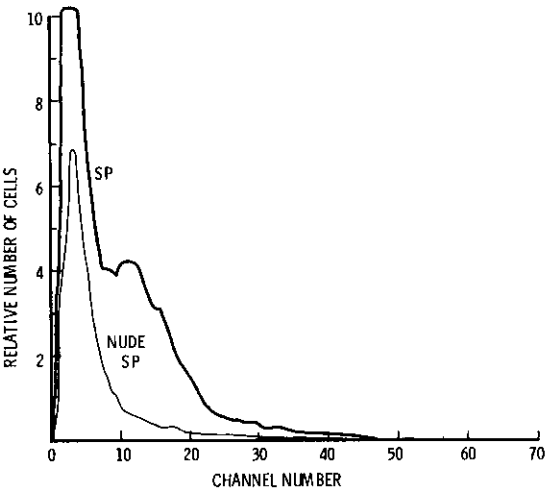

FIG. 2. Fluorescence profile of spleen cells (——) and spleen cells from nu/nu, mice (——) after incubation with F anti-Thy 1.2.

nu/nu mice (Fig. 2) or Thy 1.2-negative spleen cells from AKR mice [data not shown]. Two other controls for specificity of anti-Thy 1.2 staining were done: the first is shown in Fig. 3A; the reagent absorbed with CBA brain (30) did not stain any BALB/cN thymus cells above background. The second showed that the F anti-Thy 1.2 did not stain Thy 1.1, AKR thymus cells (Fig. 3B). The sensitivity of the FACS is great enough to see every cell, whether fluorescent or not. The "less-fluorescent" peak obtained with spleen and lymph node in Figs. 1A, 2, 3B, 4, 5, and 6 represents unstained, non-T, cells. In fact (data not given here), cells not exposed to a fluorescent reagent have an apparent "fluorescence profile" like the "less-fluorescent" peak in these figures. The conclusion that cells within the brighter peak were T lymphocytes is also supported by the observation that the number of cells in this peak was invariably 35%–45% of the total numbers of spleen lymphocytes, similar to the proportions killed after treatment with anti-Thy 1.2 plus guinea

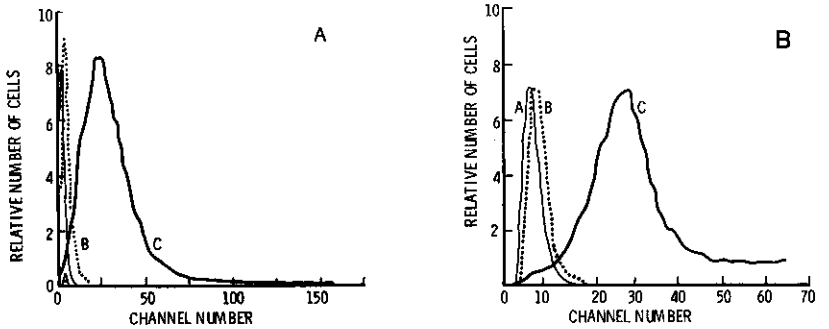

FIG. 3. A. Fluorescence profile of thymus cells from BALB/c mice after incubation with F anti-Thy 1.2 (C), anti-Thy 1.2 sera that had been absorbed × 1 with CBA brain (B), or after incubation with fluorescent rabbit anti-mouse immunoglobulin (A). Note the difference in abscissa scale from other figures. B. Fluorescence profiles of AKR thymus cells (B) and BALB/c thymus cells (C) after parallel incubation with F anti-Thy 1.2, compared with fluorescence pattern of BALB/c thymus cells incubated with fluorescent rabbit anti-MIg (A).

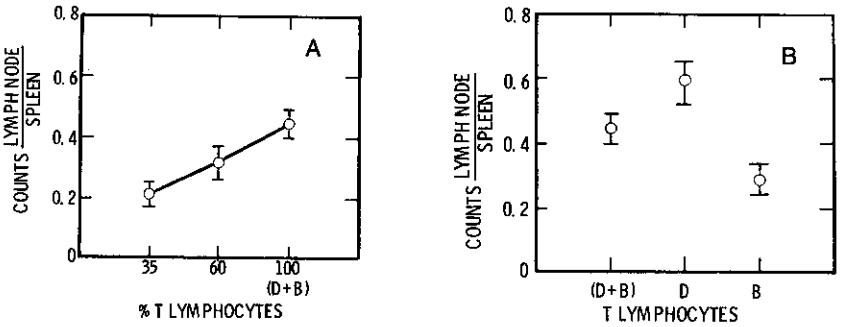

FIG. 8. ^{51}Cr-Labeled cells, containing increasing proportions of Thy 1.2-bearing (T) lymphocytes, were injected intravenously into the lethally irradiated syngeneic recipients. The ^{51}Cr counts in lymph nodes (axillary, brachial, femoral and mesenteric), and spleen were determined 18 hr later. Increasing proportions of T-cells in the inoculum were reflected in increasing lymph node/spleen ratios of the counts recovered (8A). The F anti-Thy 1.2-stained dull T-cell fraction (D) was enriched for lymph node-seeking lymphocytes, while the bright T fraction (B) showed a relative depletion of lymph node-seeking cells (8B).

after incubation with anti-Thy 1.2 at a 1 : 100 dilution, and demonstrates a marked enrichment for each cell type, although there was a small but significant contamination of the separated bright T cells with dull T cells and vice versa. The bright T and dull T cells were then labeled with ^{51}Cr and injected into syngeneic irradiated hosts and the homing patterns of these separated splenic T-cell subpopulations were compared with those of splenic T cells enriched by nylon wool passage and with equal mixtures of bright T and dull T cells (Figs. 8A and 8B). In the same experiment, a further group of irradiated mice was injected with ^{51}Cr-labeled spleen cells which had not been enriched for T cells by nylon-column passage: this population contained approximately 35% T cells, and a comparison of the migratory properties of this population was made with those of the nylon column, T-cell enriched population (60% T cells in this experiment) and a pure T-cell population (equal numbers of bright T and dull T cells). Figure 8B shows that the proportion of cells migrating to lymph nodes as compared with spleen increased directly with the concentration of T lymphocytes in the inoculum. Figure 8A shows that migration to lymph nodes was most characteristic of dull T cells; the brights by contrast appeared to be depleted of lymph node-seeking T cells. This finding could not be attributed to the amount of antibody absorbed on the cell surface after incubation with anti-Thy 1.2 (1 : 100) changing the migratory patterns of T cells, since incubation of cells with increasing concentrations of anti-Thy 1.2 serum prior to migration did not affect homing properties, unless very high concentrations of antiserum (1 : 20) were used (Table 2). Moreover, these results could not be attributed to a higher proportion of dead cells in the bright population since scatter gating was used (see Methods) to eliminate dead cells prior to injection and the viability of both populations after separation, as judged by trypan blue dye exclusion, was greater than 90%.

The Effect of Adult Thymectomy on Thy 1.2-Bearing Cells in Spleen and Lymph Nodes

Previous studies have shown that adult thymectomy (ATx) has little or no effect upon recirculating T lymphocytes found mainly in the thoracic duct within

TABLE 1

EFFECT OF ALS UPON THE PERCENTAGE OF THY 1.2-BEARING LYMPHOCYTES IN SPLEEN AND LYMPH NODE

	ALS (dose)	Percent residual Thy 1.2-bearing cells[a]	
		Spleen	Lymph node
Lot 14	0.15 ml	93	52
	0.25 ml	53	19
Lot 15	0.25 ml	62	31
	0.50 ml	47	25

[a] As assessed from cytotoxic effects of anti-Thy 1.2 serum (1:10) plus guinea pig complement, 24 hr after subcutaneous injection of ALS or normal rabbit serum.

We then compared the fluorescence profile of cells from different lymphoid tissues stained with fluorescent anti-Thy 1.2. Thymocytes presented the brightest fluorescent profile, while the cortisone-resistant subpopulation of thymocytes was substantially less fluorescent, showing a pattern very similar to that of splenic T cells (Fig. 4). Some splenic T cells were slightly more fluorescent than those of lymph node (Fig. 5). Also shown in Fig. 5 is the profile of nu/nu spleen cells which serves as a marker of non-T cells, although a small proportion of cells from these spleens consistently showed "T region" fluorescence.

The Effect of ALS Upon Thy 1.2-Bearing Cells in Spleen and Lymph Node

A cytotoxic assay was used to determine the proportion of residual T cells in spleen and lymph node 24 hr after various doses of ALS. Table 1 shows the results. Residual T cells were determined by treatment with anti-Thy 1.2 serum and guinea pig complement. Cells taking up trypan blue above background levels after this treatment were taken to be lymphocytes bearing Thy 1.2 determinants. A single dose of 0.15 ml ALS resulted in depletion of approximately 50% of Thy 1.2-bearing cells in lymph node and only a 7% depletion in spleen. Although increasing doses of ALS produced greater depletion of both spleen and lymph node T cells, the depletion of lymph node T cells was consistently greater than the reduction seen in spleen.

The results of this experiment can be compared with a FACS analysis of the fluorescence profile of spleen cells after depletion of recirculating T lymphocytes 24 hr after the subcutaneous administration of different doses of ALS (Fig. 6). The bulk of the cells removed after the administration of the small dose (0.2 ml) of ALS appeared to reside in the dullest fraction within the splenic T-cell region. Administration of larger doses of ALS (0.5 ml) removed equal proportions of bright and dull cells within the T region.

Migratory Properties of T Lymphocytes Bearing Different Concentrations of Thy 1.2 Antigen

In order to further test the idea that a decreased amount of surface Thy 1.2 was a marker for rapidly recirculating T lymphocytes, a comparison was made of the migratory properties of bright T and dull T cells separated from nylon-purified splenic T cells. Figure 7 shows the profiles of these two separated populations