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Analysis of Ly-6.2-Bearing Murine Lymphocyte Subpopulations in Relation to the T-Lymphocyte Markers, Thy-1, Lyt-1, and Lyt-2[1]

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Using immunofluorescence with a monoclonal anti-Ly-6.2 antibody and FACS analysis we have confirmed that the Ly-6.2 antigen is present on approximately 70% of mature T cells and B cells but on few immature lymphocytes. There is a wide range of antigen density among the Ly-6.2$^+$ populations, with the mean density higher on T cells than B cells. Following Con A activation of splenocytes there was a sixfold increase in Ly-6.2 antigen density though approximately 20% of the activated lymphocytes were Ly-6.2$^-$. The increase in Ly-6.2 density was specific since similar density increases did not occur for the closely linked antigens ThB and H 9/25. By panning a predominantly T-cell population for Lyt-2-bearing cells, it was found that Lyt-2$^+$ lymphocytes were either negative or dully staining for Ly-6.2. However, activated cells bearing the Lyt-2 antigen were all Ly-6.2 positive. Double-staining experiments showed that T cells which had high Ly-6.2 antigen densities also had high Thy-1 antigen densities. Corticosteroid-resistant thymocytes were highly enriched for Ly-6.2-bearing cells compared to untreated thymocytes and had staining profiles for Ly-6.2 which were similar to peripheral T cells, supporting the idea that steroid treatment selects for a phenotypically mature thymic population.

INTRODUCTION

Functional subpopulations of T lymphocytes have been mainly characterized in recent years by their expression of the Lyt-1 and Lyt-2, 3 markers, the phenotype Lyt-1$^+$, 2$^-$, 3$^-$ representing helper and Lyt-1$^-$, 2$^+$, 3$^+$ representing both killer and suppressor cells (1-3). Deviations from this model, were noted, however, when in certain instances cytotoxic cells were depleted by anti-Lyt-1 and complement treatment (4, 5) suggesting that Lyt-1 may be more widely distributed than previously demonstrated. In fact flow cytometry analyses revealed the presence of Lyt-1 on all thymocytes (6, 7). This finding was confirmed with monoclonal antibodies and extended to demonstrate a broad spectrum of expression of Lyt-1 on all lymphocytes, with killer cells (Lyt-2$^+$, 3$^+$) expressing lower levels of Lyt-1 than helper cells (Lyt-2$^-$, 3$^-$). Furthermore, Lyt-1 expression was negatively correlated with Thy-1 expression (8).

The spectrum of Lyt-1 and Thy-1 expression on mature lymphocytes is matched

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by the heterogeneity among helper and killer or suppressor subpopulations (9–12); the further definition of these functional subpopulations, however, is not possible based on the Lyt-1, 2, 3, and Thy-1 antigens alone. Several other lymphocyte markers are currently under investigation. One of these, Ly-6, has been described in two allelic forms Ly-6.2 and Ly-6.1 (13, 14). A particularly interesting feature of this antigen is that it belongs to a newly discovered group of determinants which appear to be coded for or regulated by the same genetic region. This region codes for Ly-6, H 9/25 (15), and controls the expression of ThB on B cells although not on thymocytes (16). It also codes for the antigens Ala-1 (17), Dag (18), and Ly-8 (19); these last three may well be identical with Ly-6 (20). To date all strains tested which carry the Ly-6.2 allele are H 9/25 positive and have high levels of B-cell surface ThB. This multiplicity of determinants has hampered investigation on the nature and distribution of the antigens since conventional alloantisera may be active against any or all of these specificities.

Recently, a monoclonal antibody was described by Kimura *et al.* (21) which was reactive in cytotoxicity studies against 70% of lymph node cells, 60% of spleen cells and which was shown by absorption studies to be present on thymus, bone marrow, liver, brain, and kidney cells. We now report a quantitative FACS analysis of the antigen density of Ly-6.2 on lymphoid cells using the same monoclonal antibody. Further, we correlate the density of this antigen to the density of the T-cell markers, Thy-1, Lyt-1, and Lyt-2 on various lymphoid cells.

MATERIALS AND METHODS

Mice. Eight- to twelve-week-old female mice of the inbred strains SJL/J, C57BL/6J, and BALB/cN bred in the Genetics Department, Stanford University, were used.

Reagents for fluorescence staining. Monoclonal anti-Ly-6.2 (S8.106) was kindly provided by Dr. U. Hammerling of the Sloan Kettering Cancer Institute. It was used in conjunction with a fluorescein-conjugated monoclonal anti-Igh-1a (21-74.4) (22), the second-step reagent. Monoclonal antibodies against Thy-1, Lyt-1, Lyt-2, Lyt-3, and ThB were produced by the following rat spleen derived hybridoma clones and were kindly provided by Dr. J. Ledbetter; 59 Ad-2.2, 53-7.3, 53-6.7, 53-5.1, and 49-h4, respectively, (23). All of these antibodies were used as direct fluorescein conjugates.

Fluorescence staining with anti-Ly-6.2. Lymphoid cell suspensions in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 1% fetal calf serum and 0.1% NaN_3 were incubated for 30 min on ice at a concentration of 10^6 cells per well in microtiter plates with saturating levels of anti-Ly-6.2. They were washed and incubated for a further 30 min with fluorescein-conjugated anti-allotype monoclonal reagent anti-Igh-1a (IgG_{2a}).

Additive staining. The cell suspensions were first incubated with anti-Ly-6.2 for 30 min, washed, and then stained for 30 min with saturating levels of fluorescein-conjugated anti-Igh-1a and fluorescein-conjugated anti-Thy-1, anti-Lyt-1, or anti-Lyt-2. Prior to additive staining we confirmed that there was no interaction between the anti-Igh-1a and the additional rat antibody stains.

Two-color staining. The cells were first incubated with anti-Ly-6.2 and biotin conjugated (24) anti-Thy-1 (Becton-Dickinson, Mountain View, Calif.), washed,

and further incubated with fluorescein-conjugated anti-Igh-1a and rhodamine-conjugated avidin (Vector Laboratories, Burlingame, Calif.). Controls showed no interaction between Ly-6.2 and Thy-1 staining.

Cell separations. Spleen lymphocytes were separated into T and B subpopulations using nylon wool. (25). B cells were recovered from the gently washed nylon wool by agitation in 20 ml of medium in a beaker. Contaminating cell levels were examined routinely by staining with fluorescein-conjugated anti-Thy-1 or anti-Fab and FACS analysis; they did not exceed 7%.

Preparation of blast cells. Spleen cells were cultured at 4×10^6/ml in RPMI medium containing 15% fetal calf serum and 4 μg/ml Con A or 10 μg/ml LPS for 3 days at 37° in a 5% CO_2 atmosphere.

Positive selection (Panning) of Lyt-2+ lymphocytes. A method modified from Wysocki and Sato (26) was used.

Bacteriological grade plastic plates (Fisher Scientific Co., Canada) were coated with affinity-purified goat anti-rat or monoclonal IgG 2b anti-arsanilic acid (clone 31-64 kindly donated by Malcolm Gefter, MIT) antibodies by incubating overnight at 4°C with 5 ml phophate-buffered saline (PBS) containing 100 μg/ml of the reagent. The medium containing antibody was harvested and any remaining unbound sites on the plate were blocked using 5 ml of 5% bovine serum albumin in PBS for 1 hr. The plates were then washed once with RPMI 1640 containing 1% new born calf serum, 25 m*M* Hepes, and 0.1% NaN_3. Lymphocytes obtained from mesenteric lymph nodes were suspended in similar RPMI 1640 medium at 10^7 cells/ml and were incubated for 40 min on ice with a saturating concentration of monoclonal anti-Lyt-2 (53-6.7) or the arsanilate conjugate (Becton-Dickinson, Mountain View, Calif.). The cells were washed once and 30×10^6 in 5 ml were overlayed onto appropriately coated plates and incubated for 1 hr in the cold. Unbound cells were gently harvested, the plates were washed to remove nonadherent cells with 5 ml cold medium and rinsed with a further 5 ml. The plates were then overlayed with 5 ml fresh medium and incubated at 37°C for 30 min. The bound cells were harvested by vigorous pipetting. They were stained with fluorescein-conjugated monoclonal anti-Lyt-3 (5.1) (8) to determine the percentage of Lyt-2^+, 3^+ lymphocytes. The anti-Ly-3 used for staining is not blocked by the anti-Lyt-2 used for cell selection.

Cortisone treatment. The mice were injected intraperitoneally with 125 mg/kg body wt of hydrocortisone acetate 48 hr prior to testing.

FACS analysis. A modified FACS II (Becton Dickinson FACS systems, Mountain View, Calif.) fitted with a logarithmic amplifier covering a 10^4-fold range of fluorescence intensity was used. The FACS was calibrated with free fluorescein (by Dr. D. R. Parks, Stanford University, Stanford, Calif.). The geometric mean fluorescence for each population was then used to determine the fluorescein equivalent (FE) from which the mean number of second step antibody molecules bound to positive cells was calculated using

$$\frac{\text{mean FE (positive cells)} - \text{mean FE (unstained cells)}}{\text{F/P ratio of purified antibody}}.$$

Since theoretically under saturating conditions a maximum of two anti-allotype second-step monoclonal antibodies can bind to each first-step antibody molecule and the first-step antibody can bind to a maximum of two antigen molecules on

TABLE 1

The Distribution and Relative Mean Density of Ly-6.2 Antigens on Lymphocytes from Lymphoid Organs of SJL/J Mice

Lymphoid organ	Positive cells (% ± SD)	Relative density[a] of Ly-6.2 ± SD[b]
Spleen	63.4 ± 2.8	8,970 ± 1383
Lymph node	73.5 ± 3.4	8,048 ± 1257
Peripheral blood	84.4 ± 4.2	12,450 ± 5161
Thymus	11.4 ± 7.8	4,600 ± 1860
Bone marrow	8.7 ± 4.0	n.c.[c]

[a] Relative density is equal to number of second step antibodies bound per cell (see Materials and Methods).

[b] The standard deviation represents real biological variation since the FACS analysis is reproducible to within 1%.

[c] Not calculated due to the relatively high ratio of nonspecific to specific binding of antibody on bone marrow cells.

the cell surface, the number of second-step antibodies should give a reasonable approximation of the total number of antigenic sites per cell.

RESULTS

Distribution of Ly-6.2 on Lymphocyte Populations in Lymphoid Organs

The proportion of Ly-6.2-bearing lymphocytes in the spleen, lymph nodes, peripheral blood, bone marrow, and thymus in SJL mice are shown in Table 1 along with the relative mean fluorescence of the total populations; representative curves are shown in Fig. 1. Similar results were obtained with C57BL/6 mice. It can be seen from Fig. 1 that in mature lymphocyte populations (peripheral blood, lymph node, and spleen) most of the cells are Ly-6.2+ whereas in the immature (bone marrow and thymus) few cells bear Ly-6.2. In the thymus the positive population is presumably derived from the mature medullary lymphocytes since it was greatly enriched by corticosteroid administration; however, some mature (approximately 30%) thymocytes are Ly-6.2 low or negative (Fig. 1). It can also be seen from Fig. 1 that lymphocytes have a wide range of Ly-6.2 antigen density with no clear demarcation between stained and unstained populations. However, experiments in which cells were stained with both anti-Thy-1 and anti-Ly-6.2 indicated that the T cells accounted for most of the unstained population (figure not shown). Two-color fluorescence staining with rhodamine-labeled anti-Thy-1 and fluorescein-labeled anti-Igh-1a second-step reagent for anti-Ly-6.2 suggested that the brightest anti-Ly-6.2 staining population were mainly Thy-1 bright cells but that some T cells, ranging from Thy-1 bright to dull, were dull staining or negative for Ly-6.2.

Relative Distribution of Ly-6.2, Thy-1, Lyt-1, and Lyt-2 Antigens on T Cells

Spleen lymphocytes were separated on nylon-wool columns; the nonadherent T-cell population contained 5–7% of B cells and gave a staining pattern with anti-Ly-6.2 similar to that for whole spleen (Fig. 2). Additive staining with anti-Ly-6.2 and anti-Thy-1 or anti-Ly-6.2 and anti-Lyt-1 indicated that the cells which re-

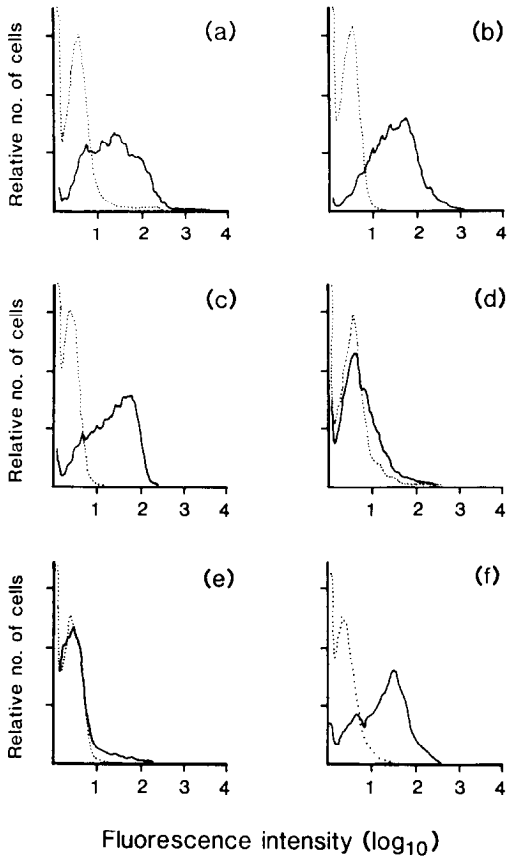

FIG. 1. Immunofluorescent staining of SJL/J lymphocytes (—) from (a) spleen, (b) lymph node, (c) peripheral blood, (d) bone marrow, (e) thymocytes (f) corticosteroid-treated thymocytes. Cells were incubated with monoclonal anti-Ly-6.2 and then with fluorescein-conjugated monoclonal anti-Igh-1a as the second step. They were analyzed on a FACS II with a logarithmic amplifier. The dotted line shows the fluorescence of cells stained with the second step alone.

mained unstained with anti-Ly-6.2 were indeed mostly T cells (Fig. 2a); it can therefore be concluded that whereas most T cells are Ly-6.2$^+$ a proportion (approximately 30%) are Ly-6.2$^-$.

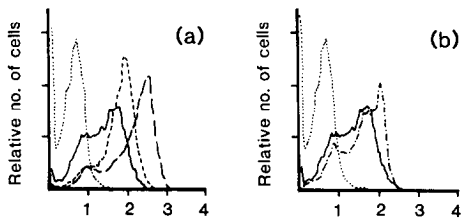

FIG. 2. Immunofluorescence staining of nylon-wool-enriched SJL/J splenic T cells with (a) anti-Ly-6.2 followed by fluorescent-conjugated anti-Igh-1a alone (——) or with additive staining with either fluorescein-conjugated anti-Lyt-1 (- - -) or fluorescein-conjugated anti-Thy-1 (— —): (b) additive staining with anti-Ly-6.2 and fluorescein-conjugated anti-Igh-1a and fluorescein-conjugated anti-Lyt-2 (-·-). The dotted line shows the fluorescence of cells stained with fluorescein-conjugated anti-Igh-1a alone. Cells were analyzed on a FACS II fitted with a logarithmic amplifier.

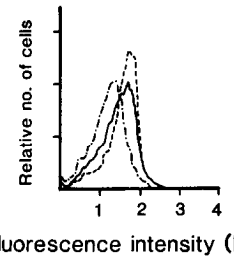

FIG. 3. Immunofluorescence staining of positively selected (panned) Lyt-2+, 3+ SJL/J lymph node lymphocytes (-·-), Lyt-2+, 3+-depleted lymphocytes (- - -) and control treated lymphocytes (——) stained with anti-Ly-6.2 and fluorescein-conjugated anti-Igh-1a. Cells were analyzed on a FACS II with a logarithmic amplifier.

We also double stained with anti-Ly-6.2 and anti-Lyt-2 (Fig. 2b). Some of the Ly-6.2$^-$ cells were Lyt-2$^+$ though most were Lyt-2$^-$. However, it also appeared that the brightest Ly-6.2$^+$ cells were Lyt-2$^-$. To further test this negative correlation between Ly-6.2 and Lyt-2 we selected Lyt-2+3+ cells from lymph node cells (chosen because they represent a predominantly T-cell population) by panning (see Materials and Methods). The cells were coated with either an arsanilate-conjugated anti-Lyt-2 monoclonal antibody or with an unconjugated rat anti-Lyt-2 monoclonal antibody; the plates were coated with anti-ars or anti-rat antibodies, respectively. In each case the unbound population was significantly depleted of Lyt-2+ cells (contaminating Lyt-2+, 3+ cells comprised 0.4 to 2.3% of the total population), whereas the bound population was highly enriched (over 80% Lyt-2+, 3+). The two populations and a control population obtained from uncoated plates were then stained for Ly-6.2 and it was indeed found that the Lyt-2-depleted population had a mean fluorescence which was at least twice that of the Lyt-2-enriched population (Fig. 3).

Ly-6.2 on B Cells

The adherent B-cell population contained 5–7% T-cell contamination but was mainly Ly-6.2$^+$ (Fig. 4). It can be seen that the mean brightness is less than for T cells and thus most B cells have a lower density of Ly-6.2 antigen. To test whether the B cells acquired antigen in the form of complexes shed from T cells in a way

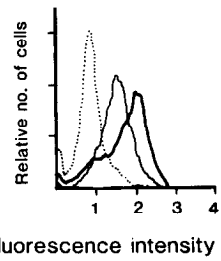

FIG. 4. Immunofluorescence staining of nylon-wool-separated T-cell-enriched (——) and B cell enriched (——) SJL/J splenic lymphocytes stained with anti-Ly-6.2 and fluorescein-conjugated rabbit anti-mouse IgG_{2a}. Cells were analyzed on a FACS II with a logarithmic amplifier. The dotted line represents the fluorescence of B cells stained with fluorescein-conjugated anti-IgG_{2a} alone. The T-cell background was less than that of the B cells.

analogous to what happens with indirect staining for the Thy-1 antigen (Ledbetter *et al.*, unpublished) we incubated a population of Ly-6.2$^-$ B cells from BALB/c with SJL/J T cells prior to staining. The subsequent staining pattern (figure not shown) indicated two clearly defined populations, a negative one (corresponding to BALB/c B cells) and a positive one (corresponding to SJL/J T cells) suggesting that shedding of Ly-6.2 by T cells and acquisition by B cells do not occur. Furthermore, incubating spleen cells for 2½ hr in the cold or at 37°C in medium with or without sodium azide did not alter the staining profiles of the populations. Thus, it is likely that the B-cell Ly-6.2 antigen is inherent to the cell rather than absorbed onto it.

Ly-6.2 on Activated Lymphocytes

We examined the relative increase in Ly-6.2 expression when spleen cell populations were activated by Con A and LPS. In 3-day cultures of Con A-activated cells the increase in mean fluorescence due to staining of activated cells was six times greater than that found for a normal population of spleen cells. Lymphocytes activated with LPS, however, showed a 3-fold increase in Ly-6.2 antigen density and only a 1.6-fold increase in the ThB density. With the Con A-activated cells two distinct populations could be distinguished, a Ly-6.2$^-$ (approximately 20%) and a brightly staining Ly-6.2$^+$ population (approximately 80%). Double-staining experiments again indicated that Ly-6.2$^-$ cells were Thy-1$^+$ and Lyt-1$^+$. They were, however, Lyt-2$^-$ (Fig. 5).

Relative Distribution of Ly-6.2, Thy-1, Lyt-1, and Lyt-2 Antigens on Cortisone-Resistant Thymocytes

Using cortisone treated animals we investigated the relationship between Ly-6.2, Thy-1, Lyt-1, and Lyt-2 in cortisone-resistant thymocytes. As with separated T cells most of the cortisone-resistant thymocytes stained with Ly-6.2 while about 30%, like splenic T cells did not. These were, however, stained with anti-Lyt-1 and anti-Thy-1. The Ly-6.2$^-$ population contained both Lyt-2$^+$ and Lyt-2$^-$ cells, again similar to the splenic T-cell picture (Fig. 2), and the two-color staining profile showed that the brightest Ly-6.2$^+$ cells are Lyt-2$^-$.

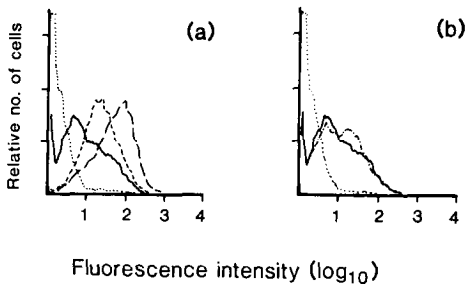

FIG. 5. Immunofluorescence profiles of 3-day Con A-activated C57BL/6 spleen cells stained with anti-Ly-6.2 and fluorescein-conjugated anti-Igh-1a alone (——) or additively in (a) with fluorescein-conjugated anti-Lyt-1 (- - -) or fluorescein-conjugated anti-Thy-1 (— —); in (b) with fluorescein-conjugated anti-Lyt-2 (-·-). Cells were analyzed on a FACS II with a logarithmic amplifier. The fluorescence detector was adjusted in this experiment to collect light between wavelengths 525 and 580. The apparent brightness is less in this figure because of the altered calibration.

DISCUSSION

In this report we have confirmed the presence of the Ly-6.2 antigen on most mature T lymphocytes but have demonstrated that there is marked heterogeneity of antigen density within the Ly-6.2 positive population (Fig. 1). We were unable to correlate Ly-6.2 density with Thy-1, Lyt-1 or Lyt-2 antigen density but using additive staining techniques we showed that Lyt-2^+ cells were confined to the duller or negative Ly-6.2 staining cells (Fig. 2). This was confirmed by positive selection (panning) experiments in which Lyt-2 cells were separated from the total population and stained for Ly-6.2. Approximately 30% of the bound cells (which were 80% Lyt-2^+) were negative for Ly-6.2. The remaining Ly-6.2^+ cells were almost exclusively in the dull range (Fig. 3).

Although Con A activation greatly increased the antigenic density of Ly-6.2 on spleen cells, additive staining suggested that Lyt-2 cells were again found among the duller staining Ly-6.2^+ blast cells (Fig. 5b). There was still a distinct negative population which was shown by additive staining to consist mainly of T cells. In contrast to the unstimulated population, however, the Ly-6.2^- cells were Lyt-2 negative. Thus the Ly-6.2^-, Lyt-2^+ cell of the normal T-cell population is either lost upon Con A activation, or alternatively, becomes Ly-6.2 positive. The latter seems more likely since previous workers reported that in an allogeneic system the precursors of killer T cells which are Lyt-2^+ were Ly-6.2^- whereas the effector cells were Ly-6.2^+ (27–29).

One possible role suggested for Ly-6.2 is that it may be a signal for T-lymphocyte migration from the thymus to the periphery (30). The basis of this suggestion was the finding that Ly-6.2 was only present on a small population of normal thymocytes which was corticosteroid resistant and thus represented the mature population. Our results make this suggestion improbable since we find a consistent Ly-6.2-negative population in normal peripheral cells and steroid-treated thymocytes. We favor the interpretation that cortisone-resistant Ly-6.2^- cells from the thymus are the source of Ly-6.2^- T cells in the peripheral organs, especially as we also find similarity of antigen presentation of Thy-1, Lyt-1, and Lyt-2, 3 determinants between mature thymocytes and peripheral T cells (Matossian-Rogers and Rogers, unpublished observations); this suggests that the phenotype of peripheral cells is established in the thymus before migration. A similar conclusion was obtained (H. S. Micklem, J. A. Ledbetter, and L. A. Herzenberg—manuscript in preparation)based on the phenotypic similarity of cortisone-resistant thymocytes with peripheral T cells and by Scollay and Weissman (31), who found the thymocyte subclasses Lyt-1^+2^- and Lyt-1^+2^+ represented in all thymic subpopulations including subcapsular lymphoblasts.

The marked increase in Ly-6.2 antigen density on mitogen stimulation is not a general phenomenon for other antigens coded for in the same genetic region since Con A and LPS activation only marginally increased the expression of ThB and H 9/25 (figures not shown). Nor is there any apparent correlation between the cellular distribution of these three antigens despite their close genetic linkage. It is well documented that ThB is confined to peripheral B cells and approximately 50% of immature thymocytes. By contrast, Ly-6 and H 9/25 are absent from immature thymocytes but present on mature thymocytes and peripheral T cells.

However, whereas Ly-6 is present on approximately 70% of these cells, H 9/25 is confined to approximately 20% of mature T cells (Matossian-Rogers and Rogers, unpublished observations; also (15)). Thus our analysis confirms the distinctions between these antigens. Ala-1, however, is known to increase markedly on activated cells (17) supporting the idea that the same antigen is defined by the monoclonal anti-Ly-6.2 antibody. The significance of the increase in Ly-6 on activation and its relationship to the function of the antigen remains to be elucidated.

Although Ly-6.2 is a well established T-cell marker, there has been controversy concerning its expression on B cells which is important in evaluating its relationship with the other genetically linked markers Ly-8, Ala-1, and Dag. Ly-6 was initially described as a T-cell marker (13) but was later shown to be also present on activated B cells (32); Ly-8 was originally described on both B and T lymphocytes in resting spleen. Sachs *et al.* (18) reported that Dag was present on 90% of spleen cells and although Ala-1 was first thought to be confined to mitogen activated B and T lymphocytes, the activity of the antiserum detecting this determinant was later shown to be absorbed by normal lymphocytes.

We have now conclusively shown that Ly-6.2 is also present on B cells albeit at a lower mean antigen density than on T cells. This confirms and extends the findings of Kimura *et al.* (21), who detected the antigen on 60% of B cells using the same monoclonal antibody. Their cytotoxicity and absorption data did not allow them to distinguish between the possibilities of a qualitative or quantitative difference in the relative distribution of Ly-6.2 on B and T cells. Our findings of a quantitative difference in antigen density may clarify the discrepancies of previous work since on this basis it would be more difficult to characterise B cells as bearing the antigen compared to T cells.

Current evidence strongly suggests identity of the Ly-6, Ala-1, Ly-8, and Dag antigens. Differences between the antisera detecting them have been found but these would be expected due to the nature of conventional antisera or to affinity differences of the antibodies. Even so, at present it would be premature to rule out nonidentity of these markers. Despite their close genetic linkage it is still possible that the antigens may differ; for example, Ly-6.2 is genetically linked but clearly distinguishable in terms of tissue distribution from H9/25 (15) and ThB (16). Differences in tissue distribution between Ly-6 and other antigens may be more subtle. Immunoprecipitation studies which are now under way should enable us to clarify this problem. However, a biological role of the Ly-6.2 antigen is still to be found.

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