# PHENOTYPIC AND FUNCTIONAL PROPERTIES OF MURINE THYMOCYTES

## I. Precursors of Cytolytic T Lymphocytes and Interleukin 2-producing Cells Are All Contained within a Subpopulation of "Mature" Thymocytes as Analyzed by Monoclonal Antibodies and Flow Microfluorometry

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The thymus is generally considered the major anatomical site for the maturation of immunocompetent T lymphocytes (1). However, when compared with T cells from peripheral lymphoid tissues, the frequency of immunocompetent cells in the thymus is relatively low (2–4). A variety of methods have been used to identify and enrich for immunocompetent cells in the thymus. From such studies (5, 6), it was concluded that immunocompetent thymocytes belonged to a subpopulation of ~10% of all thymus cells that were characterized by their resistance to administration of hydrocortisone in vivo. Such cortisone-resistant thymocytes (CRT)[1] were medium-sized cells that stained relatively weakly with anti-theta antisera and strongly with anti-H-2 antisera (reviewed in ref. 7, 8). Furthermore, these cells were not agglutinated by peanut lectin (9). The phenotype of this subpopulation of thymocytes, which is indistinguishable from that of peripheral T cells, is generally referred to as "mature." By comparison, the bulk of ("immature") thymocytes that were characterized by strong staining with anti-theta antisera and weak staining with anti-H-2 antisera were considered to be immunoincompetent.

In contrast to these earlier experiments, it has recently been reported (10) that thymocytes with an immature phenotype gave rise to cytolytic T lymphocytes (CTL) when stimulated with H-2 alloantigens or haptenated syngeneic cells in the presence of T cell growth factor (TCGF, also known as interleukin 2 or IL-2) under bulk culture conditions. These results were interpreted as showing that immature thymocytes differentiated into mature cells in the presence of IL-2. Furthermore, it was

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[1] *Abbreviations used in this paper:* C, rabbit complement; CRT, cortisone-resistant thymocytes; CTL, cytolytic T lymphocyte; CTL-P, precursor of CTL; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FLS, forward light scatter; FMF, flow microfluorometry; IL-2, interleukin 2 (also known as T cell growth factor, or TCGF); IL-$2_{50}$, dose of responder cells required for 50% maximum IL-2 production; MLC, mixed leukocyte cultures; 2° MLC SN, supernatant from secondary mixed leukocyte cultures; PNA, peanut agglutinin.

proposed that thymocyte maturation in vivo was controlled by intrathymic IL-2 production. However, in such studies it is difficult to exclude the possibility that maturation can be accounted for by the preferential survival and/or proliferation of a small number of contaminating mature cells during the culture period.

Flow microfluorometry (FMF) and monoclonal antibodies to cell surface determinants are two recent advances that have helped considerably in our understanding of thymocyte subpopulations (11). In addition, culture systems have recently been described that allow the responsiveness of T lymphocytes to be accurately quantitated. Mixed leukocyte microculture (micro-MLC) systems initially containing optimum concentrations of IL-2 allow a minimum estimate to be made of the frequencies of precursors of CTL (CTL-P) in thymocyte suspensions (4). In addition, the ability of cell populations to produce IL-2 can be quantitated by measuring their ability to support the proliferation of an IL-2-dependent CTL clone (12).

The purpose of the experiments described herein was to combine FMF with these quantitative functional assays to test whether functional activity could be generated from immature thymocytes. We will show that all CTL-P and IL-2-producing (helper) activity resides in a subpopulation of thymocytes having an immunologically mature phenotype, namely medium size, cortisone-resistant cells expressing low levels of Thy-1 and high levels of H-2K. We will further show that these mature cells can be subdivided into two distinct subpopulations based on staining with Lyt-2, with all CTL-P being Lyt-2$^+$ and all IL-2-producing cells Lyt-2$^-$.

## Materials and Methods

Adult mice of strains C57BL/6, CBA/JT6, AKR/J, DBA/2, and (C57BL/6 × DBA/2)F$_1$ were obtained from the mouse colony at the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland. The original breeding pairs were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Single cell suspensions were prepared in Dulbecco's modified Eagle's medium (DMEM) containing 5% (vol/vol) fetal bovine serum (FBS) and 10 mM Hepes buffer. For use as responder cells in micro-MLC, cells were kept at 4°C before culture. For the preparation of thymocytes, adult mice 4–6 wk of age were killed by ether anesthesia and the thymuses removed free of parathymic lymph nodes. Cortisone-resistant thymocytes were removed 48 h after a single intraperitoneal injection of 4 mg hydrocortisone acetate (Hydrocortifor, Vifar, Geneva). The recovery of hydrocortisone-resistant thymocytes (CRT) was generally 3–5% of that obtained from saline-injected control mice.

*Antibodies.*   Monoclonal rat IgG antibodies directed against nonpolymorphic determinants of the Lyt-2 molecule (53-6.7) were kindly provided by Dr. J. Ledbetter. Monoclonal rat IgM antibodies directed against Thy-1.2 (AT83) and against nonpolymorphic determinants of Lyt-2 (3.168.8.1) were the gift of Dr. F. Fitch. Monoclonal mouse IgG antibodies directed against H-2K$^b$ (B8-24-3) were donated by Dr. G. Kohler. The properties of all of these reagents have been described in detail elsewhere (13–15).

Fluorescein isothiocyanate (FITC)-coupled rabbit anti-rat immunoglobulin was obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands. FITC-coupled rabbit anti-mouse immunoglobulin was prepared as described elsewhere (16).

For negative selection experiments with anti-Lyt-2 antibodies, normal thymocytes or CRT (50 × 10$^6$ cells/ml) were incubated with a 1:10 dilution of anti-Lyt-2 hybridoma supernatant fluid (3.168.8.1) together with a 1:20 final concentration of rabbit complement (C) (Cedarlane Laboratories, Hornsby, Canada) for 45 min at 4°C. Treated cells were passed over Ficoll-Hypaque (d = 1.077) and washed twice before phenotypic or functional analysis.

*Fluorescence Staining and Fluorescence-activated Cell Sorter (FACS) Analysis.*   Indirect immunofluorescence was used in all instances, and all incubations were carried out at 4°C. For analytical purposes, samples of 1 × 10$^6$ thymocytes in 0.1 ml DMEM were incubated for 20–30 min with

2 $\mu$g of monoclonal antibodies to Lyt-2 (53-6.7), H-2K$^b$, or Thy-1.2. After washing twice in medium and centrifuging through FBS, the pellets were resuspended in 0.1 ml of medium supplemented with an appropriate concentration (~50 $\mu$g) of the relevant FITC-coupled anti-immunoglobulin reagent for an additional 20–30 min. The concentrations of monoclonal Ab and FITC-coupled anti-immunoglobulin used were determined by titration of both reagents independently. Control samples were incubated with the FITC-coupled reagent only. Finally, all samples were centrifuged over a layer of FBS and analyzed on a flow cytometer (FACS II; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, Calif.). For sorting purposes, aliquots of $10 \times 10^6$ cells in 1 ml were stained with the same final concentrations of first- and second-step reagents described above.

Flow cytometric analysis was performed using a Spectra-Physics laser (Spectra-Physics Inc., Mountain View, Calif.; 800 milliwatt output at 488 nm) and a conventional FACS II electronics system modified to allow 3-parameter sorting (17). Viable cells were routinely gated by a combination of narrow-angle forward light scatter (FLS) and perpendicular light scatter, as described by Van den Engh et al. (17). FITC-fluorescence was measured on the third channel using a cut-on filter (KV 550, Schott, Mainz, Germany) to eliminate laser light and a green interference filter (K-55, Balzers, Liechtenstein). For each determination, 10,000–40,000 gated events were accumulated. In some instances, additional gating conditions were used to obtain FLS distributions of a subpopulation of fluorescent cells.

Results of experimental and control cell populations are presented as fluorescence histograms, with the number of cells on the y-axis and the fluorescence intensity on the x-axis. Both parameters are expressed in arbitrary linear units. The percentage of positive cells in each histogram is calculated only when the distribution is clearly bi- or triphasic. In each case, the percentage of cells under each peak will be reduced by the percentage of control cells in the same interval of fluorescence to yield a net value for percent positivity.

*FACS Sorting.* Before sorting cells, the sample tubing of the FACS was flushed sequentially with detergent, sterile distilled water, and 96% ethanol. Then, the tubing was rinsed for a minimum of 30 min with a solution of gentamycin (50 $\mu$g/ml) in PBS, which was also used throughout the sorting procedure. The fluorescence profiles of thymocytes stained with monoclonal antibodies were determined as described above. Because the staining profiles obtained using the Lyt-2 and H-2K$^b$ reagents were clearly biphasic (see results), gating conditions were established such that positively stained cells were always deflected to the right and unstained cells to the left. The few percent (3–5% of all cells) of cells whose fluorescence intensity coincided with the inflection point of the curve were discarded. The removal of these cells did not significantly alter the ratios of positive to negative cells in the sorted population. However, with the Thy-1.2 reagent, the distinction between weakly staining and brightly staining cells was more arbitrary (see results). For the one experiment in which thymocytes were stained and sorted according to Thy-1.2 fluorescence intensity, a greater proportion of cells was discarded.

Positively or negatively selected cells were collected in sterile plastic tubes, whose inside surfaces had been coated with sterile FCS. The flow rate of viable cells being sorted was generally $1 \times 10^3$ to $2 \times 10^3$ gated events per second, and, depending on the sample being collected, between $10^5$ and $1.5 \times 10^6$ cells were collected.

Once collected, cells were immediately diluted in culture medium. The cell counts provided by the FACS were used to determine the cell concentration in this cell suspension, and dilutions were made accordingly. These cell suspensions provided responder cells for micro-MLC and for the helper cell assay. For CTL-P frequency determinations, responder cells were added to wells already containing irradiated stimulator cells and secondary MLC 2° (MLC SN).

*Micro-MLC.* Micro-MLC were prepared as previously described (18) in DMEM containing additional amino acids (19), 10 mM Hepes, $5 \times 10^{-5}$ M 2-mercaptoethanol, 10% (vol/vol) FBS, and 10–25% (vol/vol) supernatant from 2° MLC SN (20) as a source of IL-2. Microcultures were set up in round-bottomed microtiter plates (Greiner, Nürtingen, West Germany) and incubated at 37°C in a humidified incubator containing 5% $CO_2$ in air. To each well was added 100 $\mu$l medium containing 2° MLC SN, followed by 50 $\mu$l containing $10^6$ irradiated (1,000 rad) DBA/2 stimulator spleen cells. Finally, the required number of responder cells in 50 $\mu$l was added, and the microtiter plates were wrapped in aluminium foil and placed in the incubator. Generally 24–32 replicates at each of several responder cell doses were established.

*Assay of Cytolytic Activity.* The cytolytic activity of individual micro-MLC was determined by replacing 100 μl of culture supernatant from 7–8-d cultures with 100 μl containing 3 × 10³ ^{51}Cr-labeled P-815 target cells. The cell pellets and target cells were then mixed thoroughly and incubated for 4 h at 37°C. The specific ^{51}Cr release of each microculture was then determined by removing 100 μl of culture supernatant and counting in a γ-counter. Spontaneous release from ^{51}Cr-labeled target cells was determined by incubating labeled cells in wells containing irradiated stimulator cells alone. Maximum release was determined by lysing ^{51}Cr-labeled cells in 0.1 N HCl. Cytolytically positive microcultures were defined as those showing specific ^{51}Cr release values exceeding the mean spontaneous release value by >3 SD (the minimum positive ^{51}Cr release value). Minimal estimates of CTL-P frequencies were calculated by the statistical method of log likelihood maximization, as described by Taswell (21).

*Assay for IL-2 Production by Thymocytes.* A CTL clone that is dependent on the presence of IL-2 for proliferation was used to evaluate IL-2 production by different thymocyte populations. L3 is a CTL clone of C57BL/6 origin that is specific for H-2D$^d$ and causes lysis of 10,000 P-815 target cells in a 3½ h assay at an effector-to-target cell ratio of 0.3 (22). Because L3 cells express a constant amount of cytolytic activity per cell, proliferation of L3, measured in terms of cytolytic activity, is directly proportional to the amount of IL-2 produced in each culture. Further details of this assay will be described elsewhere.[2] Briefly, serial 3.2-fold dilutions of thymocyte responder cells were prepared, and 50 μl of each dilution was mixed with 100 μl (7.5 × 10⁵) irradiated (2,000 rad) allogeneic or semisyngeneic spleen cells in triplicate round-bottomed microtiter wells (Greiner). For control cultures, responder thymocytes were replaced by either 50 μl medium (negative control) or 50 μl of a standard source of IL-2 (positive control). 2° MLC SN was used as the source of IL-2 and routinely gave 50% maximum stimulation of L3 cells at a dilution of 1:32. Where noted, spleen stimulator cells were depleted of T cells by treatment with monoclonal anti-Thy-1.2 and C before culture. Cultures were then incubated for 24 h at 37°C in a 5% CO₂ incubator, after which they were exposed to gamma radiation (2,000 rad), and 50 μl medium containing 500 L3 cells were added per well. After incubation at 37°C for an additional 6 d, the cytolytic activity of each well was determined. The cells in each well were resuspended, and 100 μl was mixed with 100 μl containing 2 × 10³ ^{51}Cr-labeled P-815 tumor target cells in round-bottomed microtiter wells. After an incubation of 3½ h at 37°C, the plates were centrifuged, and 100 μl were removed for counting in a well-type scintillation counter. Data were normalized to the control culture values by the formula:

$$\text{relative IL-2 production} = \frac{\text{cpm(experimental)} - \text{cpm(medium)}}{\text{cpm(IL-2 standard)} - \text{cpm(medium)}} \times 100$$

Little normalization of data was in fact required because the percent specific ^{51}Cr release averaged 0–10% for medium control cultures and 95–100% for IL-2 control cultures.

## Results

*Definition of Four Thymocyte Subpopulations Using Monoclonal Antibodies and FMF.* Before undertaking functional studies, normal adult thymocytes were stained with monoclonal antibodies directed against Lyt-2, H-2, and Thy-1 antigens, followed by appropriate FITC-conjugated anti-Ig reagents. When analyzed by FMF (Fig. 1), staining with anti-Lyt-2 antibodies resolved a discrete subpopulation of Lyt-2⁻ thymocytes, as observed previously by Mathieson et al. (11). In a series of 18 experiments, a mean of 82% of C57BL/6 or CBA thymocytes were positively stained by this nonpolymorphic reagent (Table I).

When the monoclonal anti-H-2 antibodies were used to analyze thymocytes, an interesting dissociation was observed between reagents detecting H-2K and H-2D

[2] Glasebrook, A. L., and H. R. MacDonald. Quantitative analysis of Interleukin 2 (IL-2) production by murine lymphoid cells. I. Tissue distribution and surface phenotype of IL-2 producing cells in normal and primed populations. Manuscript in preparation.
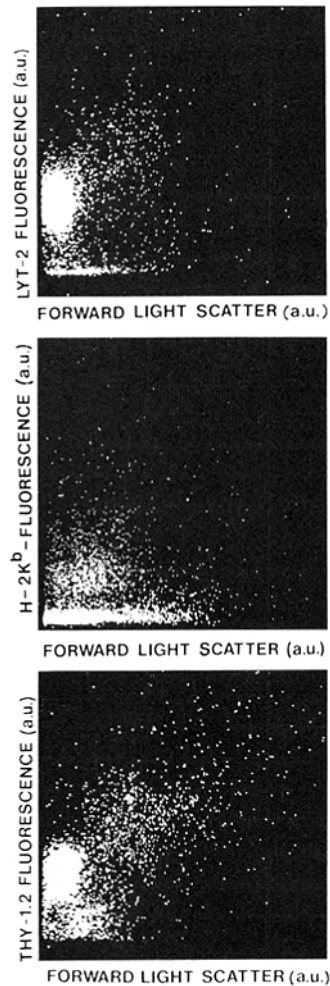
FORWARD LIGHT SCATTER (a.u.)

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Fig. 1.   FMF analysis of murine thymocytes stained with monoclonal antibodies. Normal adult C57BL/6 thymocytes were stained with optimum concentrations of monoclonal antibodies directed against Lyt-2, H-2K$^b$, or Thy-1.2, followed by appropriate fluoresceinated anti-Ig reagents. Samples were run on an FACS II, gated to exclude nonviable cells. Each photograph correlates the fluorescence intensity and forward light scatter (a cell size-related parameter) of 10,000 individual cells.

specificities. Whereas anti-H-2D$^b$ monoclonal antibodies reacted relatively uniformly with all C57BL/6 thymocytes (data not shown), anti-H-2K$^b$ monoclonal antibodies reacted preferentially with a subpopulation comprising ∼15% of the total cells (Fig. 1 and Table I). This differential staining was not restricted to the H-2$^b$ haplotype because a similar proportion of AKR/J thymocytes was clearly stained with a monoclonal anti-H-2K$^k$ antibody (data not shown).

A more complex staining pattern was obtained when C57BL/6 or CBA thymocytes were stained with a monoclonal anti-Thy-1.2 reagent (Fig. 1). In this instance, a triphasic fluorescence pattern was reproducibly observed, with 15%, 60%, and 23% of the cells staining with low, intermediate, and high intensity, respectively (Table I).

TABLE I

*Thymocyte Subpopulations in C57BL/6 Mice, Defined by Monoclonal Antibodies and FMF\**

| Antibody (number of experiments) | Subpopulation | Percent thymocytes |
|---|---|---|
| | | mean ± SD |
| Anti-Lyt-2 (18) | Positive | 82 ± 7 |
| | Negative | 18 ± 7 |
| Anti-H-2K$^b$ (8) | Positive | 17 ± 6 |
| | Negative | 83 ± 6 |
| Anti-Thy-1.2 (11)‡ | Low | 15 ± 4 |
| | Intermediate | 60 ± 8 |
| | High | 23 ± 4 |

\* Normal adult (4–6 wk old) thymocytes stained by indirect immunofluorescence and analyzed by FMF, as shown in Fig. 1.
‡ 2 ± 2% of cells in thymocyte suspensions were not stained by this reagent.

This triphasic staining pattern, which was previously observed with conventional anti-Thy-1.2 antibodies (23), was again not unique to the monoclonal anti-Thy-1 antibody used. A similar pattern was obtained with a monoclonal anti-Thy-1.1 antibody and a heterologous rabbit antiserum raised against purified Thy-1 (data not shown).

Because CRT are known to be significantly enriched for functional T cells defined in a variety of assays (5–8), we therefore compared the surface phenotype of these cells to that of normal thymocytes. As shown in Fig. 2, CRT from C57BL/6 mice contained fewer Lyt-2$^+$ cells than were obtained for normal thymus (37 ± 4% in four experiments). When stained with anti-H-2K$^b$ or anti-Thy-1.2 monoclonal antibodies, CRT exhibited a homogeneous phenotype (Fig. 2). In contrast to normal thymocytes, all CRT stained positively with the anti-H-2K$^b$ reagent and only weakly with the anti-Thy-1.2 reagent. This staining pattern, which was observed previously for CRT using conventional antisera (24), is similar to that of peripheral T cells and thus has been termed a mature phenotype.

Additional information relevant to the identification of thymocyte subpopulations can be obtained from analysis of FLS. As shown by van den Engh et al. (17), FLS measurements in populations of regularly shaped cells are roughly proportional to the square of the mean cellular radius. By using such analysis on subpopulations of thymocytes stained with a particular monoclonal antibody and electronically "gated" according to their fluorescence intensity (cf. Fig. 1), relative size distributions of phenotypically defined subpopulations could be obtained. Fig. 3 represents a comparison of the FLS distribution of CRT with those obtained for subpopulations of normal C57BL/6 thymocytes that have been gated according to Lyt-2, H-2K$^b$, or Thy-1.2 fluorescence intensity. In each panel, the FLS distribution of the total thymocyte population has been included to provide an internal size standard. It can readily be seen that CRT represent a relatively homogeneous population of medium-sized cells. Interestingly, strikingly similar FLS distributions were obtained by analyzing the subpopulations of normal thymocytes that were negative for Lyt-2, positive for H-2K$^b$, or weakly staining with Thy-1.2 (Fig. 3). In each case, the gated subpop-
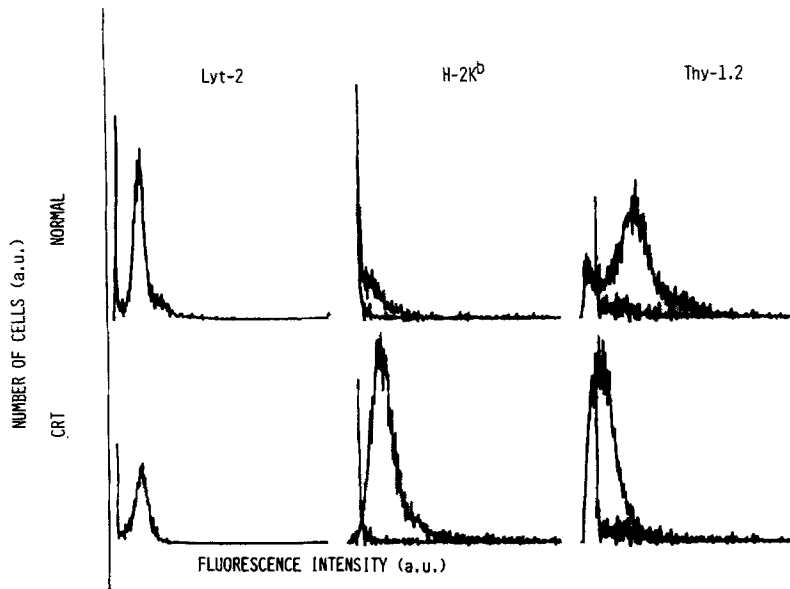
Fig. 2. Surface phenotype of CRT as measured by FMF. Normal adult C57BL/6 thymocytes or CRT (obtained 48 h after administration of hydrocortisone acetate in vivo) were stained as described in Fig. 1 and analyzed on an FACS II. Fluorescence histograms for each reagent are normalized to peak height.
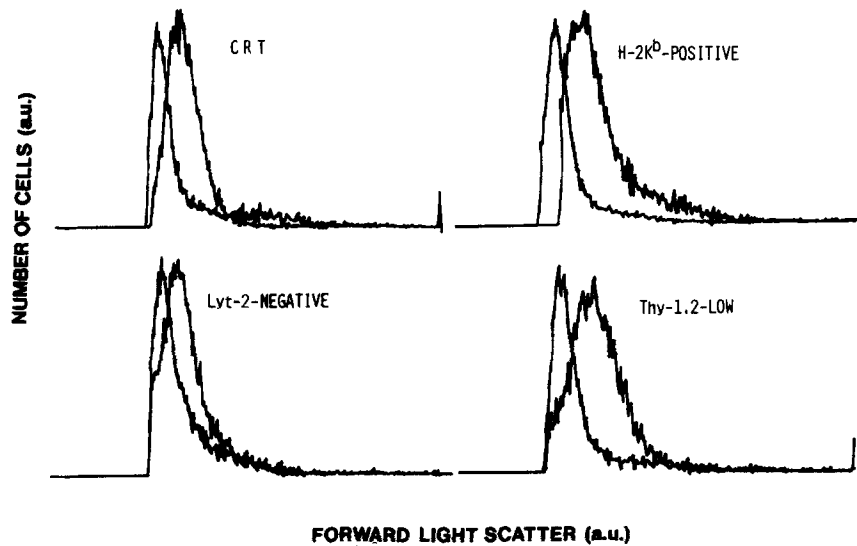


Fig. 3. FLS analysis of thymocyte subpopulations. Normal adult C57BL/6 thymocytes were stained and analyzed as described in Figs. 1 and 2. In each panel, the FLS distribution of all thymocytes (left curve) is compared to that of a particular subpopulation that has been gated according to its staining pattern with the indicated monoclonal antibody. The FLS distribution of unstained CRT is included for comparison. All curves have been normalized to the same peak height.

ulation analyzed comprised ~15–20% of total thymocytes and corresponded in size to CRT. It should be noted, however, that CRT also contain a subpopulation of Lyt-2$^+$ cells (Fig. 2). When similar light scatter analysis was performed on CRT stained with anti-Lyt-2 antibodies, the Lyt-2$^+$ cells were found to be slightly larger (in FLS) than the negative population (data not shown).

FLS analysis of thymocytes stained with anti-Thy-1.2 further showed that the three subpopulations of cells with low, intermediate, and high fluorescence intensity were clearly distinct in size (see Fig. 1). Thus the cells with intermediate fluorescence, comprising 60% of thymocytes, were relatively small cells, whereas the 15% dull staining cells were medium-sized, and the 23% bright cells were large.

By combining the results of the FLS and surface phenotype studies, it is possible to conclude that the normal adult thymus contains at least four clearly separable subpopulations of lymphocytes, as summarized in Table II. Populations A and B, which are characteristic of the mature or CRT phenotype, both represent medium-sized lymphocytes that homogeneously express high levels of H-2K and low levels of Thy-1. These two subpopulations, which together account for 15% of thymocytes, can be clearly differentiated on the basis of Lyt-2 expression, with population A being Lyt-2$^+$ and B being Lyt-2$^-$. Although a direct determination of the relative proportion of these two populations in normal thymus has not been carried out (such a comparison would require double labeling methods), results obtained with CRT

TABLE II

Summary of Subpopulations of Normal Thymocytes Detected by Monoclonal Antibodies and FMF

| Subpop-ulation | Percent total cells | Size (FLS) | Surface phenotype | | | Classification |
|---|---|---|---|---|---|---|
| | | | Lyt-2 | H-2K | Thy-1 | |
| A | 5* | Medium‡ | + | + | Low | Mature |
| B | 10* | Medium‡ | – | + | Low | Mature |
| C | 60 | Small | + | – | Intermed. | Immature |
| D | 23 | Large | + | – | High | Immature |

* Relative proportion of cells in these subpopulations estimated from data obtained with cortisone-resistant thymocytes.
‡ Cells in population A are slightly larger than those in B (see text).

TABLE III

Elimination of CTL-P in Normal and CRT Preparations by Treatment with Monoclonal Anti-Lyt-2 Antibodies plus C*

| Thymocyte preparation | Treatment | Reduc-tion in cell number‡ | CTL-P frequency | CTL-P per 10$^6$ cells treated§ |
|---|---|---|---|---|
| Normal | C | — | 1/1322 | 756 (—) |
| Normal | Anti-Lyt-2 +C | 90 | 1/45119 | 2.2 (99.7) |
| CRT | C | — | 1/164 | 6,098 (—) |
| CRT | Anti-Lyt-2 + C | 40 | 1/4521 | 133 (98) |

* Normal C57BL/6 thymocytes or CRT (obtained 48 h after injection of 4 mg hydrocortisone acetate i.p.) were treated with monoclonal anti-Lyt-2 antibodies and/or C. Treated cells were then assayed for their frequency of CTL-P directed against H-2$^d$ alloantigens, as described in detail in Materials and Methods.
‡ Expressed relative to C control.
§ Percent reduction is in parentheses.

would suggest that Lyt-2$^-$ cells outnumber Lyt-2$^+$ cells by a ratio of ~2:1 (Fig. 2). A third cell population (C in Table II), which accounts for the majority of normal thymocytes, is characterized by its small size, weak expression of H-2K antigens, and intermediate expression of Thy-1. A fourth population (D) is comprised of very large cells that stain brightly with Thy-1 and weakly with H-2K reagents. The latter two subpopulations, which are Lyt-2$^+$, together account for 85% of normal thymocytes and correspond to cells with an immature surface phenotype.

*CTL-P Are Lyt-2 Positive.* Using limiting dilution analysis of negatively selected cells, alloreactive CTL-P from peripheral lymphoid tissues have been previously shown to be Lyt-2$^+$ (25, 26). Similarly, CTL-P in normal thymus or CRT populations could be eliminated by treatment with monoclonal anti-Lyt-2 antibody plus C (Table III). Because 90% of normal thymus cells were eliminated by the antibody treatment, any enrichment for CTL-P in a positively selected population would be difficult to demonstrate. Thus CRT that were enriched for CTL-P (ref. 27 and Table III) and that contained a mean of 37% Lyt-2$^+$ cells were used to positively select CTL-P using this reagent.

CRT stained with anti-Lyt-2 antibodies in indirect immunofluorescence were sorted on an FACS II, gated to exclude nonviable cells. Positive and negative cells, defined as in Fig. 2, were used as responder cells in limiting dilution microcultures containing irradiated allogeneic DBA/2 splenic stimulator cells and 2°MLC SN as a source of IL-2. Control responder populations were stained with anti-Lyt-2 and passed through the FACS II but not sorted according to fluorescence intensity. Unstained and unsorted normal thymocytes served as an additional control responder population.

The cytotoxicity results of groups of 24 individual microcultures from a representative experiment are shown in Fig. 4. Whereas all cultures containing 500 Lyt-2$^+$ CRT were cytotoxic, only 17/24 unsorted and 1/24 Lyt-2$^-$ CRT were positive at this cell dose. The data of Fig. 4 were also plotted as the number of responder cells cultured vs. the log of the proportion of negative (i.e., noncytolytic) cultures (Fig. 5). For all groups of responder cells, it can be seen that there was a linear relationship between these two parameters, thus indicating that a single cell type, namely a CTL-P, was limiting the response.

Using the statistical method of log likelihood maximization (21), the frequencies of CTL-P in the four responder cell populations shown in Fig. 5 were calculated to be 1/3,934 for normal thymocytes, 1/421 for unsorted CRT, 1/148 for Lyt-2$^+$ CRT, and 1/16,954 for Lyt-2$^-$ CRT. These frequencies, combined with the proportion of total cells in each subpopulation, may be used to quantitate the recovery of CTL-P. Thus, in the experiment shown in Figs. 4 and 5, the 36% of sorted cells that were Lyt-2$^+$ contained 98.5% of all recovered CTL-P (experiment 1, Table IV). In two additional experiments (Table IV), the difference in frequency between Lyt-2$^+$ and Lyt-2$^-$ CRT was 280-fold and 388-fold, respectively. Thus, Lyt-2$^+$ CRT contained between 98.5 and 99.5% of recovered CTL-P.

*CTL-P Are H-2K Positive.* The H-2K phenotype of CTL-P in the normal C57BL/6 thymus was then determined. Thymocytes stained with monoclonal anti-H-2K$^b$ antibody were sorted into positive and negative subpopulations (cf. Fig. 1) and assayed for CTL-P content using a similar protocol to that described above. As shown in Fig. 6, CTL-P were enriched 4.4-fold in H-2K$^b$-positive thymocytes as compared with the control unsorted population. More importantly, H-2K$^b$-negative
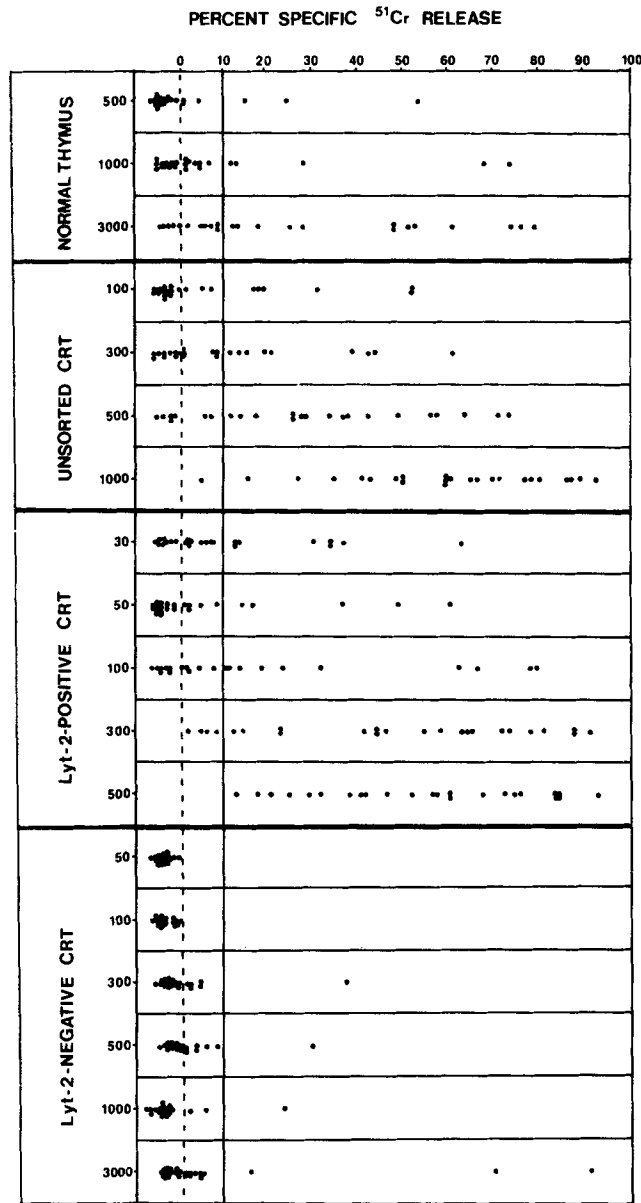
PERCENT SPECIFIC $^{51}$Cr RELEASE



Fig. 4. Cytolytic activity of individual micro-MLC, prepared using as responder cells normal C57BL/6 thymocytes or CRT sorted according to Lyt-2 phenotype. CRT obtained 48 h after in vivo hydrocortisone administration were stained by indirect immunofluorescence with monoclonal anti-Lyt-2-antibody, sorted on an FACS II into Lyt-2-positive and Lyt-2-negative subpopulations, as shown in Fig. 2, and cultured in micro-MLC for 7 d with irradiated DBA/2 spleen stimulator cells and IL-2. Normal thymocytes were stained with anti-Lyt-2, passed through the FACS II, and collected unsorted. Each point represents the specific $^{51}$Cr release of individual microcultures initially containing the indicated number of responder cells. The solid line represents 3 SD above the mean (dotted line) $^{51}$Cr release from cultures containing irradiated stimulator spleen cells alone and defines the minimum positive $^{51}$Cr release value.
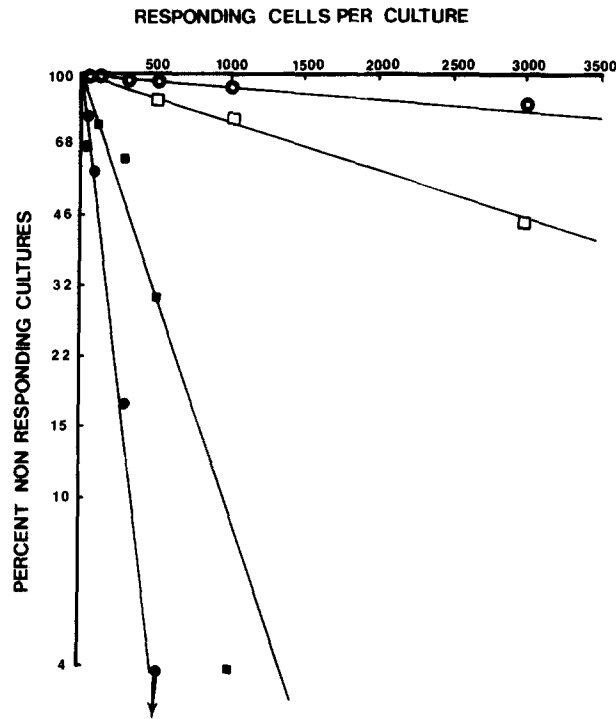
RESPONDING CELLS PER CULTURE



Fig. 5. Minimal estimates of CTL-P frequencies in normal thymocytes and CRT sorted according to Lyt-2 phenotype. The cytotoxicity data shown in Fig. 4 are plotted semilogarithmically as the percent nonresponding (i.e., noncytolytic) cultures vs. the dose of responder cells. CTL-P frequencies determined by the method of maximum log likelihood were 1/3,934 for normal thymus ($\square$), 1/421 for unsorted CRT ($\blacksquare$), 1/148 for Lyt-2-positive CRT ($\bullet$), and 1/16,954 for Lyt-2-negative CRT ($\bigcirc$).

TABLE IV

Quantitation of CTL-P in Subpopulations of CRT Sorted according to Lyt-2 Fluorescence*

| CRT population | Percent of total cells | | | Reciprocal of CTL-P frequency | | | CTL-P per 10⁶ cells sorted‡ | | | Percent of total CTL-P recovered | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experiment 1§ | Experiment 2 | Experiment 3 | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 1 | Experiment 2 | Experiment 3 | Experiment 1 | Experiment 2 | Experiment 3 |
| Control | 100 | 100 | 100 | 421 | 383 | 417 | 2,375 | 2,611 | 2,398 | — | — | — |
| Lyt-2⁺ | 36 | 35 | 36 | 148 | 124 | 112 | 2,432 | 2,823 | 3,214 | 98.5 | 99.3 | 99.5 |
| Lyt-2⁻ | 64 | 65 | 64 | 16,954 | 34,749 | 43,488 | 38 | 19 | 15 | 1.5 | 0.7 | 0.5 |

* CRT from C57BL/6 mice were sorted according to Lyt-2 fluorescence intensity (see Fig. 2) and assayed for their content of CTL-P directed against H-2ᵈ alloantigens, as described in detail in Material and Methods.
‡ Product of CTL-P frequency × fraction of sorted cells.
§ Data from Figs. 4 and 5. The CTL-P frequency of normal C57BL/6 thymocytes in this experiment was 1/3,934.

thymocytes, comprising 81% of all sorted cells, were depleted by 30-fold in CTL-P as compared with the control population. Similar results were obtained in two other experiments where the difference in frequencies between H-2K$^b$-positive and negative thymocytes was 277-fold and 47-fold, respectively (Table V). Thus, in terms of absolute numbers, the H-2K$^b$-positive subpopulation accounted for between 91 and 98% of the total recovered CTL-P (Table V).

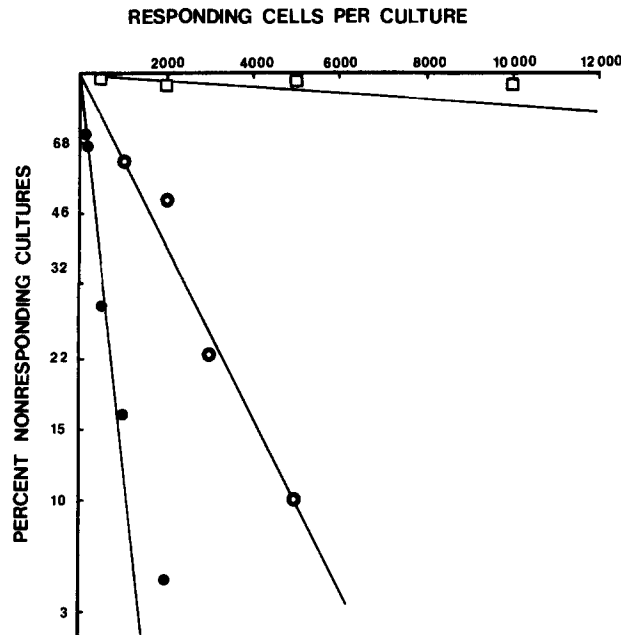*Most CTL-P Are Weakly Thy-1 Positive.*   Because the fluoresence profiles of the Thy-

Fig. 6. Minimum estimates of CTL-P frequencies in subpopulations of normal adult C57BL/6 thymocytes sorted according to H-2K$^b$ phenotype. Normal thymocytes were stained by indirect immunofluorescence with monoclonal anti-H-2K$^b$ antibody and sorted on an FACS II, as shown in Figs. 1 and 2. The cytotoxicity data of a typical experiment in which stained unsorted, H-2K$^b$-positive, and H-2K$^b$-negative subpopulations were used as responder cells in micro-MLC are shown plotted semilogarithmically. CTL-P frequencies determined by the method of maximum log likelihood were 1/2,149 for unsorted thymocytes (O), 1/486 for H-2K$^b$-positive thymocytes (●), and 1/65,962 for H-2K$^b$-negative thymocytes (□).

1.2-stained subpopulations of normal thymocytes tended to overlap (Fig. 2), the distinction between low, intermediate, and highly staining cells was often arbitrary. Compared with the Lyt-2 staining of CRT and the H-2K$^b$ staining of normal thymocytes, the Thy-1.2 reagent was the least interesting for sorting purposes. However, in the one experiment carried out with this reagent, 83% of CTL-P were found in the Thy-1.2 dull, medium-sized subpopulation that comprised 25% of sorted cells (data not shown).

*IL-2-producing T Cells Are Enriched among CRT.* The previous data demonstrated that CTL-P were enriched in CRT relative to normal thymocytes (Table III). We therefore examined whether helper or IL-2-producing T cells were also enriched among CRT. Varying numbers of responder C57BL/6 normal thymocytes or CRT were cultured with irradiated allogeneic DBA/2 spleen cells, as described in Materials and Methods. After culture for 24 h, the plates were irradiated, and 500 IL-2-dependent CTL clone cells (L3) specific for H-2D$^d$ (22) were added to each well. The plates were then cultured for an additional 6 d, and each well was assayed for cytolytic activity using $^{51}$Cr-labeled P-815 (H-2$^d$) tumor target cells. Control experiments established that irradiated thymocytes did not generate any detectable cytolytic activity (data not shown). Thus, the magnitude of specific $^{51}$Cr release observed per well was directly proportional to the proliferation of the IL-2-dependent cytolytic clone (22). Data were normalized to control values obtained from wells in which L3
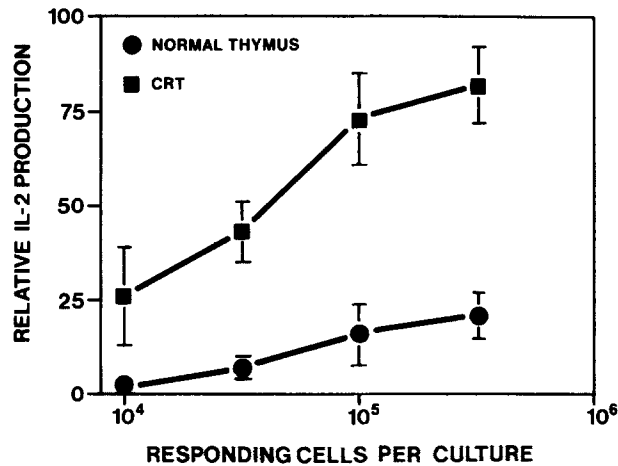
Fig. 7. Relative IL-2 production by normal or cortisone-resistant thymocytes (CRT). The indicated number of normal C57BL/6 thymocytes or CRT were cultured for 24 h with $7.5 \times 10^5$ irradiated DBA/2 spleen stimulator cells. After this initial culture, the plates were irradiated (2,000 rad), and 500 cells from an IL-2-dependent CTL clone (L3) specific for $H-2D^d$ were added to each well. After a further 6 d, the cytolytic activity of L3 cells in each well was assayed by adding $2 \times 10^3$ $^{51}$Cr-labeled P-815 tumor target cells. Each point represents the mean ± SD relative IL-2 production (calculated from the cytotoxicity values as described in Materials and Methods) of triplicate wells. The dose of responder cells required for 50% maximum IL-2 production (IL-2$_{50}$ value) was $4.2 \times 10^4$ for CRT (■) and $3 \times 10^5$ for normal thymocytes (●).
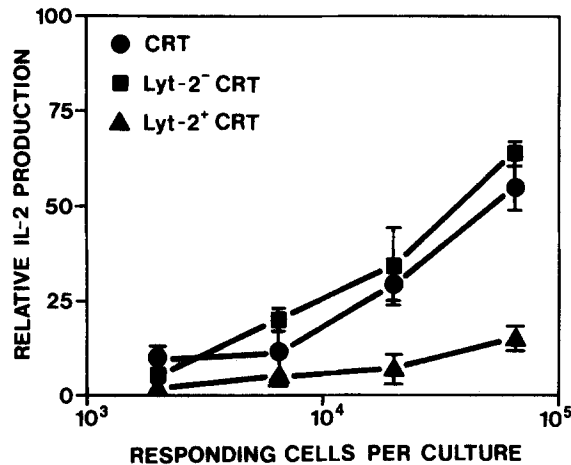


Fig. 8. Relative IL-2 production by C57BL/6 CRT sorted according to Lyt-2 phenotype. CRT, obtained 48 h after hydrocortisone administration and stained by indirect immunofluorescence with monoclonal anti-Lyt-2 antibodies, were sorted on an FACS II, and the relative IL-2 production of each subpopulation was determined as described in the legend to Fig. 7. The IL-2$_{50}$ value was 5.2 $\times 10^4$ for CRT (●), $3.8 \times 10^4$ for Lyt-2$^-$ CRT (■), and $>10^5$ for Lyt-2$^+$ CRT (▲).

cells were cultured with stimulator spleen cells in the presence or absence of a source of IL-2 (2° MLC SN) and are expressed as relative IL-2 production.[2]

Results obtained in a representative experiment are shown in Fig. 7. CRT stimulated with alloantigen were much more efficient in promoting the proliferation of IL-2-dependent CTL clone cells compared with normal thymocytes. The dose of re-

sponder thymocytes required for 50% maximum IL-2 production was used for comparisons of different responder cell populations and is arbitrarily defined as IL-$2_{50}$. In Fig. 7, the IL-$2_{50}$ value for CRT was $4.2 \times 10^4$ cells, whereas the value for normal thymocytes could not be determined but was at least $>3 \times 10^5$ cells. Results obtained in three independent experiments indicated an IL-$2_{50}$ value for CRT of 4.8 $\pm 0.6 \times 10^4$ (mean $\pm$ SD) and a value for normal thymocytes of $>5 \times 10^5$.

*CRT Producing IL-2 Are Lyt-$2^-$.* CRT of C57BL/6 origin stained with rat monoclonal anti-Lyt-2 antibodies and FITC-conjugated rabbit anti-rat Ig were sorted into Lyt-$2^-$ and Lyt-$2^+$ populations on the FACS as described above. The sorted CRT populations as well as stained unsorted control cells were then tested for IL-2 production and CTL-P frequency. The results shown in Fig. 8 demonstrate that essentially all of the IL-2-producing activity present in unsorted CRT was found in the Lyt-$2^-$ subpopulation. Because the majority of CRT were Lyt-$2^-$ (64% in this particular experiment), IL-2-producing activity was only slightly enriched for in the Lyt-$2^-$ CRT subpopulation (IL-$2_{50}$ = $3.8 \times 10^4$) compared with unsorted CRT (IL-$2_{50}$ = $5.2 \times 10^4$). The Lyt-$2^-$ CRT subpopulation, however, contained <1% of the total recovered CTL-P in this experiment (experiment 3, Table IV). Conversely, the Lyt-$2^+$ CRT subpopulation, which demonstrated little detectable IL-2-producing activity (IL-$2_{50}$ $>10^5$), contained >99% of CTL-P (experiment 3, Table IV). These results demonstrate that for CRT, essentially all IL-2-producing cells are Lyt-$2^-$, whereas CTL-P are Lyt-$2^+$.

*IL-2 Production in a Normal Thymus Is Mediated by Lyt-$2^-$ Cells.* The IL-2-producing activity of normal thymus is relatively low when compared with CRT (Fig. 7). Because normal thymus contains only 15% Lyt-$2^-$ cells, we therefore examined whether Lyt-$2^-$ normal thymus cells were enriched in IL-2-producing activity. C57BL/6 normal thymocytes were therefore treated with rat monoclonal anti-Lyt-2 antibody and C. CTL-P were >99% depleted by this treatment (Table III). Varying numbers of C-treated or anti-Lyt-2 plus C-treated responder thymocytes were assayed
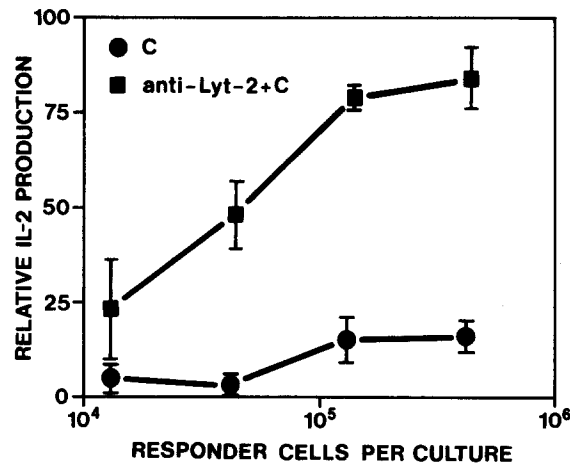


Fig. 9. Relative IL-2 production by Lyt-2-depleted normal C57BL/6 thymocytes. Normal adult thymocytes were treated with C alone or with monoclonal anti-Lyt-2 antibody plus C. Dead cells were removed by passage over Ficoll-Hypaque, and the relative IL-2 production of C-treated and anti-Lyt-2 plus C-treated cells was then determined as described in the legend to Fig. 7. The IL-$2_{50}$ value was $4.8 \times 10^4$ for Lyt-$2^-$ thymocytes (■) and $>4 \times 10^5$ for C-treated thymocytes (●).
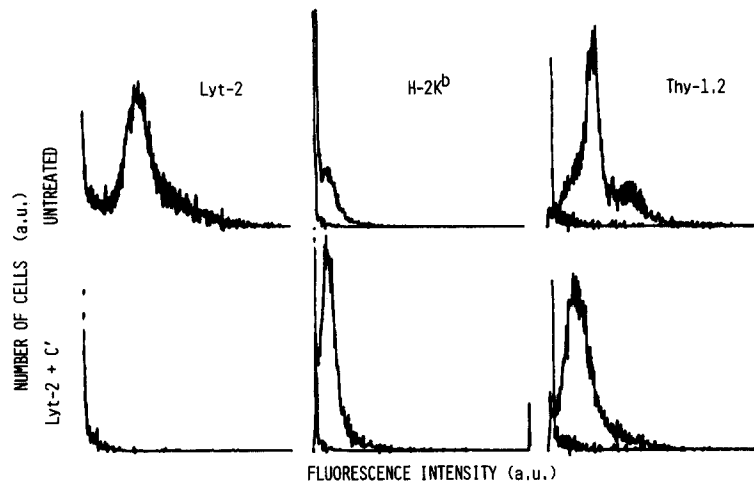
FIG. 10. Surface phenotype of Lyt-2⁻ thymocytes. C57BL/6 thymocytes treated with a monoclonal anti-Lyt-2 antibody plus C were passed over Ficoll-Hypaque (to remove dead cells) and subsequently stained with monoclonal antibodies against Lyt-2, H-2K$^b$, and Thy-1.2 (cf. Figs. 1 and 2). Fluorescence histograms for each reagent were normalized to peak height.

for relative IL-2 production. In this particular experiment, the stimulator spleen cells were depleted of T cells by incubation with rat monoclonal anti-Thy1.2 antibodies and C to exclude the possibility of IL-2 production by these cells.

The results obtained are shown in Fig. 9. Whereas C-treated normal thymocytes demonstrated little IL-2-producing activity (IL-2$_{50}$ >4 × 10$^5$), anti-Lyt-2 and C-treated thymocytes demonstrated a level of IL-2-producing activity (IL-2$_{50}$ = 4.8 × 10$^4$) similar to that observed with CRT responders (Fig. 7). These data were confirmed in a second experiment where IL-2$_{50}$ values for control and Lyt-2-depleted thymocytes were 2 × 10$^5$ and 3.8 × 10$^4$, respectively.

*Surface Phenotype of Lyt-2-negative Normal Thymocytes.* Because the Lyt-2-negative fraction of CRT and normal thymocytes contained the bulk of IL-2-producing activity, it was important to investigate, using the monoclonal anti-H-2K$^b$ and anti-Thy-1.2 antibodies, the phenotype of Lyt-2⁻ thymocytes. When normal thymocytes were treated with anti-Lyt-2 antibody plus C, the resultant Lyt-2⁻ cells were all homogeneously H-2K$^b$-positive and Thy-1.2-low (Fig. 10). This result indicated that no further enrichment for IL-2-producing activity could be obtained by positively selecting subpopulations of Lyt-2⁻ cells stained with anti-H-2K$^b$ or anti-Thy-1.2 antibodies. Furthermore, it demonstrated that Lyt-2⁻ thymocytes were phenotypically indistinguishable from mature thymocytes both in terms of FLS profile (see Fig. 3) and H-2K$^b$ or Thy-1.2 fluorescence (Fig. 10).

## Discussion

This report is the first in which monoclonal antibodies to Lyt-2, H-2K$^b$, and Thy-1.2 antigens have been combined with FMF and quantitative functional microassays to identify subpopulations of cells in normal as well as cortisone-resistant thymocyte suspensions. Positively selected subpopulations were assayed for their CTL-P content and their ability to produce IL-2 when stimulated with H-2 alloantigens. Both CTL-P and IL-2-producing cells were present in a thymocyte subpopulation with a mature

phenotype, i.e. medium size, H-2K$^b$-positive and Thy-1.2-low. However, these two cell types could be clearly distinguished on the basis of Lyt-2 fluorescence, with all CTL-P being Lyt-2$^+$ and all IL-2-producing cells Lyt-2$^-$.

In agreement with the previous report of Mathieson et al. (11), we found a clearly distinct subpopulation of Lyt-2$^-$ cells comprising ~15% of all cells in a normal thymus. After administration of hydrocortisone acetate, the proportion of Lyt-2$^-$ thymocytes increased to 63% of CRT. Whereas Mathieson et al. (11) found that the Lyt-2$^-$ subpopulation in normal thymus or CRT was larger (as measured by FLS) than the majority of cells, we only found this to be the case with normal thymocytes. Among CRT, the Lyt-2$^-$ and Lyt-2$^+$ cells were both medium-sized, with the Lyt-2$^+$ cells being slightly larger than the Lyt-2$^-$ cells. This result suggests that in a normal thymus, all Lyt-2$^-$ cells are of medium size, whereas the Lyt-2$^+$ cells are found in the small, medium, and large subpopulations (see Table II). The presence of a small number of medium-sized Lyt-2$^+$ cells in a normal thymocyte suspension is difficult to demonstrate due to the relative excess of small and large Lyt-2$^+$ cells. However, after hydrocortisone administration, which depletes the thymus of both small and large lymphocytes, this subpopulation of medium-sized Lyt-2$^+$ cells is clearly seen.

With a monoclonal anti-H-2K$^b$ antibody, a distinct subpopulation (17%) of medium-sized, brightly-fluorescent normal C57BL/6 thymocytes was identified. This result is in agreement with a recent study of Scollay et al. using a monoclonal anti-H-2K$^k$ antibody to stain H-2$^k$ thymocytes (30). Of some interest was the finding that a monoclonal anti-H-2D$^b$ reagent stained all thymocytes homogeneously when used at an optimum concentration; however, at lower antibody concentrations a medium-sized subpopulation was preferentially stained (unpublished data). Differential rates of expression (31) and turnover (32) of K vs. D antigens by lymphocytes have been described. The latter property varied between H-2 haplotypes, and genetic analysis revealed an Ir gene association; however, peripheral lymphocytes from H-2$^b$ mice were found to synthesize K$^b$ and D$^b$ at equal rates (32). It is thus unclear why the expression of detectable levels of the H-2K gene product should be confined to a subpopulation of thymocytes.

The phenotypic data reported here using a monoclonal anti-Thy-1.2 antibody are similar to those reported by Fathman et al. (23) using conventional anti-theta reagents. Thus, normal thymocytes could be subdivided into three subpopulations of Thy-1.2$^+$ cells, with 15% of cells having low, 60% having intermediate, and 23% having bright fluorescence. Shortman et al. (33) recently reported that thymocytes stained by monoclonal or conventional anti-Thy-1 antibodies gave different FMF profiles. In their study, the proportion of cells with low Thy-1 fluorescence was greater with monoclonal antibodies than with conventional alloantisera. In contrast,we have obtained the same fluorescence profiles with monoclonal anti-Thy-1 antibodies or with xenogeneic antisera to purified mouse Thy-1. The reasons for this discrepancy are not clear; however, in certain experiments, an unusually high proportion of weakly staining small thymocytes was observed with both the monoclonal and conventional reagents, regardless of whether direct or indirect immunofluorescent staining was used (unpublished data). Similar problems with analysis of Thy-1 staining by FMF have been encountered by others (R. Scollay, personal communication, and reference 34).

The phenotypic data using all three monoclonal reagents were summarized in Table II. Here, four major subpopulations of thymocytes were defined according to

their staining profiles. Thus, medium-sized cells that stained weakly with Thy-1.2 and brightly with H-2K$^b$ were either Lyt-2$^+$ (A) or Lyt-2$^-$ (B). The other two subpopulations, C and D, stained brightly with Thy-1.2 and Lyt-2 but weakly with H-2K$^b$. Such cells were either small (C) or large (D).

Having characterized thymocyte subpopulations on the basis of surface phenotype, the functional activity of positively and negatively-selected subpopulations was then quantitatively determined. Thus, alloreactive (anti-H-2$^d$) CTL-P in normal C57BL/6 thymus were recoverd in a subpopulation that was medium-sized Lyt-2$^+$, H-2K$^{b+}$ and Thy-1.2 low. In this regard, thymic CTL-P were phenotypically indistinguishable from CTL-P in peripheral lymphoid tissues (25, 26). Furthermore, the characterization of thymic CTL-P as medium-sized lymphocytes (as assessed by FLS) confirms previous bulk culture studies in which CTL precursor activity was confined to a subpopulation of medium-sized thymocytes or splenic T cells as defined by velocity sedimentation (35).

It should be noted that for CTL-P frequency determinations using separated subpopulations, the control population was stained with antibody and passed through the FACS. Because the plating efficiency of CTL-P in the culture system used is not known, use of such a control is essential in allowing comparisons to be made of the CTL-P content of different subpopulations. As shown in Tables IV and V, the total recovery of CTL-P in positively and negatively-selected subpopulations compared favorably with the control population, thus demonstrating that the functional activity of CTL-P in all sorted subpopulations was not affected either by staining with antibody or passage through the FACS.

Previous attempts to demonstrate IL-2 production by thymocytes have met with only limited success (reviewed in 36). However, using a recently developed microassay system[2] to quantitate IL-2 production by lymphocytes stimulated by H-2 alloantigens, comparable enrichment for such activity was found in the Lyt-2$^-$ subpopulation of either normal or cortisone-resistant thymocytes. This subpopulation, which was also found to be medium-sized, H-2K$^{b+}$ and Thy-1 low, hence corresponds to population B (Table II). Although the assay system as currently used does not allow the frequency of IL-2-producing cells to be directly determined, the degree of enrichment for IL-2 production in the Lyt-2$^-$ subpopulation together with the absence of detectble activity among the Lyt-2$^+$ cells suggests that most (if not all) alloantigen-induced IL-2 production attributable to thymocytes is mediated by Lyt-2$^-$ cells. This does not

TABLE V

*Quantitation of CTL-P in Normal Thymus Sorted according to H-2K$^b$ Fluorescence* *

| Popula-tion | Percent of total cells | | | Reciprocal of CTL-P frequency | | | CTL-P per 10$^6$ cells sorted‡ | | | Percent of total CTL-P recovered | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Experi-ment 1 | Experi-ment 2 | Experi-ment 3 | Experi-ment 1 | Experi-ment 2 | Experi-ment 3 | Experi-ment 1 | Experi-ment 2 | Experi-ment 3 | Experi-ment 1 | Experi-ment 2 | Experi-ment 3 |
| Control | 100 | 100 | 100 | 2,149 | 5,045 | 2,703 | 465 | 198 | 370 | — | — | — |
| H-2K$^b$ positive | 19 | 15 | 18 | 486 | 911 | 542 | 391 | 165 | 332 | 97.0 | 98.2 | 91.0 |
| H-2K$^b$ negative | 81 | 85 | 82 | 66,962 | 252,983 | 25,445 | 12 | 3 | 33 | 3.0 | 1.8 | 9.0 |

* Normal thymocytes from C57BL/6 mice sorted according to H-2K$^b$ fluorescence intensity (Fig. 2) were assayed for their content of CTL-P directed against H-2$^d$ alloantigens, as described in detail in Materials and Methods.
‡ Product of CTL-P frequency × fraction of sorted cells.

exclude the possibility that the Lyt-2$^+$ subpopulation of thymocytes may be capable of IL-2 production when stimulated by mitogens or other antigens. However, the surface phenotype of IL-2-producing cells in MLC-primed populations was found to be Lyt-2$^-$ (37), thus suggesting that peripheral T cells and thymocytes do not differ qualitatively in this regard. In quantitative terms, it should be noted that the relative IL-2 production by Lyt-2$^-$ thymocytes[2] or CRT (38) is much lower ($\sim$10-fold) than by Lyt-2$^-$ peripheral (spleen or lymph node) T cells.

It has been argued that the generation of CTL from thymic CTL-P is dependent upon interaction with an antigen-specific helper cell (39). Because the cytotoxicity data presented in this report using positively-selected Lyt-2$^+$ responder cells in the presence of optimum concentrations of IL-2 showed single-hit kinetics, i.e., one cell limiting the response, it may be concluded that such a postulated helper cell is not obligatory for the activation of thymic CTL-P. However, as is the case for CTL-P from peripheral lymphoid organs, such activation does require the presence of IL-2. Thus, in addition to the phenotypic similarities presented above, this further suggests that the activation requirements of thymic and extrathymic CTL-P are indistinguishable. Much of the confusion regarding apparent differences between these two populations of CTL-P may have arisen from the use of suboptimum culture systems, combined with the relatively low frequency of CTL-P and IL-2-producing cells in unselected thymus cell suspensions.

The thymus is generally implicated as the site of maturation of functional T cells (1). In this context, recent reports from several laboratories (10, 40, 41) have suggested that immature thymocytes can mature into functional T cells, including CTL-P, in the presence of IL-2. The results presented here are in direct contrast to these studies. Using limit dilution analysis in optimum concentrations of IL-2 to quantitate CTL-P recovery from thymocyte subpopulations stained by monoclonal antibodies and separated by FMF, no evidence for the differentiation of CTL-P from an immature thymocyte subpopulation of cells was found. Thus, all CTL-P were quantitatively recovered in a subpopulation of thymocytes with a mature phenotype, i.e., medium-sized, H-2K$^+$, Thy-1-low, and Lyt-2$^+$.

Several reasons for the discrepancy between the present results and previous data may be suggested. First, Wagner et al. (10), Irle et al. (40), and Kruisbeek et al. (41) identified and separated immature thymocytes according to their binding to the lectin peanut agglutinin (PNA). Although such a technique clearly enriches for cells with an immature phenotype (9, 40, 42), recent studies using FMF indicate that all thymocytes are stained to some extent by fluorescent PNA (43). Hence, it cannot be excluded that cell populations separated according to PNA binding may be contaminated with a small number of cells with a mature phenotype. In contrast to PNA, the fluorescence of thymocytes stained with H-2K$^b$ is clearly biphasic (Fig. 1), thus allowing a clear separation of phenotypically mature and immature cells.

A second important difference between the present studies and previous reports is the use of limiting numbers of responding cells to quantitate the functional activity of thymocyte subpopulations. When mass culture conditions are used, as was the case for studies with PNA-agglutinated thymocytes (10, 41), it is difficult to exclude the possibility that selective survival (24) and/or proliferation of a small number of contaminating cells having a mature phenotype could account for the activities measured after several days of culture, particularly when a source of IL-2 has been

added. Indeed, recent studies of Chen, Scollay, and Shortman (personal communication) indicate that PNA$^+$ thymocytes obtained after two cycles of cell sorting are devoid of functional activity when analyzed under limiting dilution conditions.

In conclusion, the functional data presented here argue in favor of a unique subpopulation of immunologically competent cells within the normal thymus. These cells are medium-sized, H-2K$^+$, and Thy-1-low. A distinction in functional activity of these cells may be made according to their Lyt-2 phenotype, however, with IL-2-producing cells being Lyt-2$^-$ and CTL-P being Lyt-2$^+$. The fact that either of these functional subpopulations can be positively selected from the normal thymus by using monoclonal antibodies combined with FMF technology should allow a more precise analysis of the specificity repertoire of immunocompetent precursor cells that have not yet migrated out of the thymus.

## Summary

The correlation between surface phenotype and function in subpopulations of murine thymocytes has been investigated using flow microfluorometry (FMF). C57BL/6 thymocytes stained with monoclonal antibodies directed against Lyt-2, H-2K$^b$, and Thy-1.2 and passed on an FACS II flow cytometer could be resolved into at least four distinct subpopulations on the basis of fluorescence and forward light scatter (FLS) measurements. (a) Medium-sized Lyt-2$^+$ cells that stained strongly with H-2K$^b$ and weakly with Thy-1.2 (5% of total cells); (b) medium-sized Lyt-2$^-$ cells with other properties as in (a) (10%); (c) small Lyt-2$^+$ cells that stained weakly with H-2K$^b$ and strongly with Thy-1.2 (60%); and (d) large Lyt-2$^+$ cells that stained weakly with H-2K$^b$ and very strongly with Thy-1.2 (23%). Cortisone-resistant thymocytes (CRT) were found to correspond phenotypically to populations (a) and (b).

The distribution of cytolytic T lymphocyte precursors (CTL-P) directed against H-2$^d$ alloantigens in subpopulations of C57BL/6 thymocytes that had been sorted according to the phenotypic criteria described above was then investigated. CTL-P in sorted and control populations were quantitated by limiting dilution analysis of mixed leukocyte microcultures established in an excess of interleukin 2 (IL-2). These studies established that all thymus CTL-P could be quantitatively recovered in a subpopulation of cells that was cortisone-resistant, medium-sized, Lyt-2$^+$, H-2K$^{b+}$, and weakly stained with Thy-1.2.

In parallel studies, the production of IL-2 by subpopulations of C57BL/6 thymocytes was quantitatively assessed using a recently developed sensitive microassay system. Graded numbers of sorted or control thymocytes were stimulated with irradiated T cell-depleted allogeneic cells and assayed for their ability to support the growth of an IL-2-dependent cytolytic T lymphocyte clone. Using this method, IL-2 production was found to reside entirely in a subpopulation of cortisone-resistant, medium-sized Lyt-2$^-$ thymocytes. Further phenotypic analysis of this subpopulation of cells indicated that it was homogeneously H-2K$^{b+}$ and weakly staining with Thy-1.2. Taken together with the CTL-P results, these data directly demonstrate that a subpopulation of thymocytes with a mature phenotype (i.e., cortisone-resistant, medium-sized, H-2K$^{b+}$, and weakly staining with Thy-1.2) accounts for all the functional activity in the thymus. Reasons for the apparent discrepancy between these results and other recent studies will be discussed.

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