A SPECIFIC BIOSYNTHETIC MARKER FOR IMMATURE THYMIC LYMPHOBLASTS

Active Synthesis of Thymus-Leukemia Antigen Restricted to Proliferating Cells*

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The thymus is the site of considerable cell proliferation and turnover (1–3). Virtually all lymphoid cells in the organ can be labeled after 3–4 d of exposure to tritiated thymidine. It is striking, therefore, that the small cells forming the dominant population are themselves inactive in DNA synthesis. Proliferation takes place only in a minority class of large and medium-sized lymphoblasts, which traverse a full cell cycle in as little as 8 h and give rise to the small, postmitotic cells by asymmetric division (4–6). It has been estimated that the period of rapid division lasts only 1–1.5 d, after which the accumulated small cells remain in the thymus for 3–4 d before death or export (5, 6).

The lymphoblasts thus represent the immediate precursors of most other thymocytes and, very likely, the ultimate precursors of T cells in the periphery. Although a great deal has been learned in recent years about the signals that trigger proliferation in mature, functional T cells (7–10), those that drive the intense mitotic activity of thymic lymphoblasts are completely obscure. It is not known, for example, whether any part of the thymocyte antigen receptor must be engaged. For mature lymphocytes, the linkage between antigen recognition and mitogenesis is a central feature of immune specificity, and, to the extent that they have been stimulated successfully in vitro, small thymocytes also seem to require contact with antigen or a corresponding lectin (11–13). If, in contrast, proliferation signals for thymic blast cells bypass their antigen receptors, then the processes converting these blasts into small lymphocytes take on profound functional significance. On the other hand, if interaction with stromal histocompatibility antigens turns out to be required, it may be possible to formulate mechanisms in precise terms to explain the apparent influence of the thymic stroma on the antigens that T cells later recognize as "self" (14, 15).

Before the triggering requirements of thymic blast cells can be compared with those of small thymocytes and peripheral T cells, the blasts must be identifiable by some characteristics other than size and mitotic activity. The studies reported here were designed to search for patterns of gene expression that distinguish the proliferating cells in the thymus from the majority populations in both cortex and medulla. To this end, populations highly enriched in cycling cells have been isolated by centrifugal elutriation and characterized by biosynthetic labeling. This has permitted a direct

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comparison between the products of the small, peanut agglutinin-binding cells of the thymic cortex and those of their rapidly dividing precursors. The results show a clear and unexpected difference between them in *de novo* synthesis of the surface glycoprotein, thymus-leukemia antigen (TL).[1]

Materials and Methods

Animals. C57BL/6-*Tla*^{a} (B6-TL$^+$), C57BL/6 (B6), and A/J mice were bred in the animal facility at The Salk Intitute. Age- and sex-matched animals were used in individual experiments, usually between 4 and 6 wk of age.

Antisera. Lyt-2, Lyt-3 glycoprotein complex (Lyt-2/3) was detected with a monoclonal antibody specific for the Lyt-2.2 allele (αLyt-2) (16). This was generously provided by Ulrich Hammerling (Memorial Sloan-Kettering Cancer Center, New York), as was the monoclonal antibody 18/20 (17) against the TL.3 determinant. The latter monoclonal was used for immune precipitation of TL because it was free of reactivity with Qa-1 (18). For fluorescent staining of surface TL$^+$ cells, a different monoclonal TL antibody (10-79.4) was used. This was kindly donated by Michael Chorney and Fung-Win Shen (Memorial Sloan-Kettering Cancer Center) as a high-titer ascites, and showed the same specificity in immune precipitation as 18/20 (E. Rothenberg and M. Chorney, unpublished observations). Fluorescein-isothiocyanate-conjugated goat anti-mouse IgG was obtained through the Research Resources program of the Division of Cancer Cause and Prevention, Biological Carcinogenesis Branch of the National Cancer Institute, Bethesda, Md., courtesy of Dr. John Cole.

Cell Fractionation by Centrifugal Elutriation. Single-cell suspensions of thymocytes were prepared as described previously (19–21) or by pressing thymus fragments gently through a 200-mesh stainless steel screen. The suspension medium was HEPES-buffered Dulbecco's modified Eagle's medium (DME) with 5% heat-inactivated fetal bovine serum (FCS; Hy-Clone, Sterile Systems, Inc., Logan, Utah). The cells were washed and resuspended at up to 10^8 cells/ml in the same medium or in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution supplemented with 5 mM Na azide and 2.5 mg/ml of bovine serum albumin (BSS/BSA). A sample of 5 ml (up to 5×10^8 cells) was loaded into a spinning JE-6 elutriator rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 18–22°C and elutriated with a continuous flow of BSS/BSA. The flow rate was controlled by a Cole-Parmer Masterflex pump equipped with a ten-turn Helipot potentiometer (Beckman Instruments, Inc.). The first 150–200 ml was collected as flowthrough; subsequently, small, medium, and large cell fractions of the same volume were harvested by sequential increases in the pump speed. The elutriated cells were collected in centrifuge bottles with 1.5 ml FCS and kept on ice thereafter.

Various settings of rotor and pump speeds were used throughout this study. For many experiments, a rotor speed of 1,950 rpm was used. In this case, flowthrough cells were collected at ~8 ml/min, the small-cell peak fraction at 11.5 ml/min, medium cells at 15 ml/min, and large cells at 21–23 ml/min. An equivalent but more rapid fractionation was achieved with the rotor speed set at 2,850 rpm, loading the sample at 16.5 ml/min, and collecting small cells at 21.5 ml/min, medium cells at 28 ml/min, and large cells at 37–45 ml/min. Settings were chosen to separate most of the small cells from the less viable cells in the flowthrough; thus, all fractions except the flowthrough were >95% viable. The flowthrough was also occasionally contaminated with unfractionated cells if back pressure developed in the rotor during sample loading. Overall recovery of cells depended on the viability of the initial cell suspension but frequently exceeded 90%. In the worst cases, small thymocytes contaminated the medium cell fraction to an extent of 50% and the large cell fraction to 20–30%.

After fractionation, the cells were recovered by centrifugation at 2,000 rpm for 10 min at 4°C. They were washed once in isotonic buffered saline solution without azide before incubation

[1] *Abbreviations used in this paper:* BSS/BSA, Hanks' balanced salt solution without Ca^{2+} or Mg^{2+}, containing 5 mM Na azide and 2.5 mg bovine serum albumin per milliliter; C, haploid DNA content; DME, Dulbecco's modified Eagle's medium; FCS, heat-inactivated fetal bovine serum; Lyt-2/3, Lyt-2, Lyt-3 glycoprotein complex as detected by αLyt-2; PBS, Dulbecco's phosphate-buffered saline (with Ca^{2+} and Mg^{2+} unless stated); PBS/1%, Dulbecco's PBS with 1% (vol/vol) fetal bovine serum; PNA, peanut agglutinin; TL, thymus-leukemia antigen.

with [^{35}S]methionine. In control experiments, exposure of thymocytes to azide followed by washing had no effect on the subsequent synthesis of any of the gene products studied here, whether monitored immediately or after an additional 18 h in tissue culture (data not shown).

Fractionation Based on Peanut Agglutinin (PNA) Binding. Cells were fractionated by a modification of the "panning" technique described by Wysocki and Sato (22). Petri dishes (no. 1005, Falcon Labware, Becton, Dickinson & Co., Oxnard, Calif.) were coated at room temperature with 4 ml of a 20 μg/ml solution of PNA (Vector Laboratories, Inc., Burlingame, Calif.) in 0.15 M NaCl, 0.05 M Tris-HCl, pH 9.5. The concentration of 20–25 μg/ml was chosen to bind the maximum percentage of cells without blocking or redistributing the PNA receptors as subsequently assayed by staining with fluorescein-conjugated PNA. After 1–2 h excess PNA was removed, and the plates were washed twice with Dulbecco's phosphate-buffered saline (PBS) and once with chilled PBS supplemented with 1% FCS (PBS/1%). Suspensions of no more than 3.5×10^7 cells in PBS with 5% FCS and 1 mM Na azide were gently applied to the plates (10^7 cells/ml) and incubated at 4°C for 75–90 min. Unbound PNA$^-$ cells were then collected with two washes of PBS/1%. The adherent PNA$^+$ cells were eluted by incubating the plates for 5–10 min at room temperature with 4–5 ml of 0.2 M D-galactose in PBS/1%; they were harvested by thoroughly flushing the plates with a pasteur pipette. The plates were washed once or twice more with galactose-containing medium to complete the elution. Before further processing, the PNA$^-$ cells were also exposed to galactose to remove any trace of surface-bound PNA. Overall recovery from this procedure was consistently ~70%; it was not improved by reducing the time that the cells were allowed to adhere to the plates.

Determination of DNA Content. Cells were fixed in 70% ethanol and stained with mithramycin (23) using the method described by Hyman and Stallings (24). Mithramycin was generously donated by Pfizer, Inc. (New York). The cells were then analyzed by flow microfluorometry in a Los Alamos-type fluorescence-activated cell sorter, using an excitation wavelength of 457.9 nm. Data were recorded as linear fluorescence intensity histograms from a 1024-channel pulse height analyzer (Fig. 1, left panels) or from a 256 × 256-channel dual parameter display of fluorescence vs. light scatter (Fig. 1, right panels). The fraction of cells in G_0/G_1 was calculated from the number of cells in the left-hand half of the 2C DNA content peak, divided by the overall number of cells in the distribution (objects with ≥6C DNA content excluded).

Fluorescent Staining of Surface TL. Cells were pelleted in 3-ml glass conical tubes and resuspended (4×10^6 cells in 40 μl) in BSS/BSA or a 1:10 dilution of monoclonal anti-TL antibody in BSS/BSA. They were incubated for 20 min on ice, diluted with 1 ml of BSS/BSA, and centrifuged through a 0.4-ml cushion of FCS. The pellets were then resuspended in 40 μl of fluorescein-conjugated goat anti-mouse IgG, diluted 1:20 in BSS/BSA. After 30 min on ice, the cells were diluted and centrifuged as before and finally resuspended in Ca^{2+}- and Mg^{2+}-free PBS with 1% (vol/vol) formaldehyde. Before analysis on the cell sorter, the suspensions were filtered through nylon mesh (Nytex; TETKO, Inc., Elmsford, N. Y.).

For flow microfluorometry, an excitation wavelength of 488 nm was used, and the cell distribution histogram was displayed on a 256 × 256-channel dual-parameter matrix plotting the logarithm of fluorescence intensity vs. forward angle light scatter (linear scale). The logarithmic amplifier was set to give a three-decade full scale in the fluorescence dimension. Fractions of TL-positive cells (log fluorescence channels 72-256) were calculated for populations gated according to light scatter to exclude dead cells (in light scatter channels 1–40). The small cells in this analysis (light scatter channels 40–140) included 84% of the total viable cells, and the large cells (channels 141–256) correspondingly included 16% of the total. Genetically TL-negative thymocytes stained similarly to the B6-TL$^+$ thymocytes exposed to second-stage reagent alone (Fig. 2 B).

Cytotoxic Elimination with Anti-TL.3 Antibody. Individual thymocyte samples were suspended at 2×10^7 cells/ml in a 1:200 dilution of monoclonal anti-TL.3 (18/20) in HEPES-buffered DME, or in HEPES-buffered DME alone. After incubation for 30 min on ice, the cells were diluted with 5 ml of HEPES-buffered DME that contained 2.5 mg/ml of bovine serum albumin, then centrifuged and resuspended at the original cell concentration in rabbit complement. A pretitered batch of rabbit complement, kindly provided by Robert Hyman of The Salk Institute, was used at a final dilution of 1:9 in HEPES-buffered DME. The cells were incubated 45 min at 37°C with intermittent agitation. They were then diluted and washed as

before and finally resuspended in [^{35}S]methionine-containing medium for biosynthetic labeling, as described below. After 30 min at 37°C, the cells were lysed and incorporation was determined by precipitation of aliquots of lysate with trichloroacetic acid.

Metabolic Labeling of Cells. Cells were biosynthetically labeled with [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) in methionine-deficient DME medium with 2% dialyzed FCS, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. Incubations were for 30–50 min at 37°C in a humidified atmosphere of 10–13% CO_2. Concentrations of isotope were typically 500 μCi/ml to prepare lysates for immune precipitation or 135 μCi/ml for analytical purposes (e.g., Table III). The incorporation of label by the large lymphoblasts was reduced as much as threefold in incubations at $>3 \times 10^7$ cells/ml, although the small lymphocytes seemed unaffected by high cell density. Consequently, labeling of thymocytes before elutriation tended to underestimate the differences between large and small thymocytes in methionine uptake (Table I, experiment 2) unless the incubation was carried out in larger volumes than those routinely used here. Labeling was terminated as described previously (19).

Immune Precipitation and Gel Electrophoresis. These conditions were as described previously (19, 20). To resolve intracellular TL from background actin, the concentration of sodium dodecyl sulfate (Bio-Rad Laboratories, Richmond, Calif.) in the running buffer was raised to 0.2%, as discussed previously (25). The gels were processed for fluorography (26) and exposed for 7 d at −70°C.

Results

Enrichment of Blast Cells by Elutriation. The experiments described here were designed to compare small cortical thymocytes with presumptive cortical blast cells in their synthesis of specialized, tissue-specific gene products. Because protein synthesis cannot be analyzed in individual cells, it was necessary to separate the dividing precursor cells physically from the postmitotic cells. Fractionation by size has been reported to give excellent resolution between these populations when unit gravity sedimentation was used (2, 27). For this study, to reduce fractionation time and preserve biosynthetic activity, large and small cells were separated instead by centrifugal elutriation (28, 29). Typical distributions of B6-TL$^+$ thymocytes after a single round of elutriation are shown in Table I. Similar results were obtained with thymocytes from B6 mice.

To assay for enrichment of cells in different growth states, the DNA-binding fluorescent dye mithramycin was used to stain elutriated thymocytes. DNA content per cell was then estimated by flow microfluorometry, as shown in Fig. 1. Typically, at least 75–85% of unfractionated thymocytes were in G_0/G_1 (Fig. 1, top panels) as judged by their 2C (diploid) DNA content, with the remainder in S (2C–4C), G_2 +

TABLE I

Fractionation of Thymocytes by Elutriation

Cell sample	Experiment 1		Experiment 2		Experiment 3		
	Fraction of cells	cpm/cell*	Fraction of cells	cpm/cell	Fraction of cells	PNA$^+$ cpm/cell	PNA$^-$ cpm/cell
Total	—	1.1	—	0.83	—	1.46	
Flowthrough	36%	0.32	18%	0.59	16%	0.33	0.18
Small	58%	0.70	65%	0.62	73%	0.22	0.38
Medium	6%	3.1	15%	1.5	8.5%	1.2	1.6
Large	0.6%	6.7	2%	3.9	2.5%	5.7	5.8
Labeled before or after elutriation:	After		Before		After		
Age of mice	4 wk		5 wk		6 wk		

* Labeling for 45 min with [^{35}S]methionine.

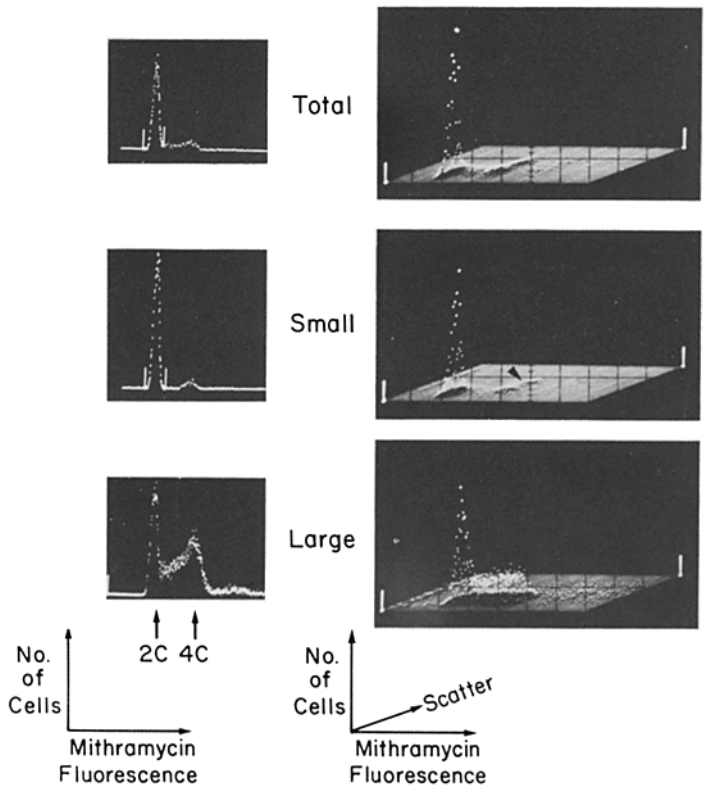


FIG. 1. DNA content of elutriated thymocytes. Left- and right-hand panels show results from two independent experiments. Unfractionated cells, cells from the peak small-cell fraction, and cells from the largest cell fraction were stained with mithramycin and analyzed by flow microfluorometry. Mithramycin fluorescence intensity is proportional to DNA content. 2C denotes the DNA content of cells in G_0 or G_1 phase; 4C is the DNA content of cells after one full round of DNA replication (G_2 + M phase). In the experiment shown in the right-hand panels, the DNA content histogram is displayed with a third dimension recording the forward angle light scatter, a measure of the size of the fluorescent objects. In the unfractionated and small cells, a significant proportion of the objects with 4C DNA content scatter distinctly more light than the majority of the cells (arrow in middle right panel). These are probably doublets of cells that have only 2C DNA content individually.

M (4C), or G_0/G_1 doublets ("4C" DNA content). Elutriated "small" thymocytes were relatively depleted in S and G_2 + M cells (Fig. 1, middle panels). In contrast, elutriated "large" thymocytes were highly enriched for actively cycling cells (Fig. 1, bottom panels). Here, <40% of the cells were in G_0/G_1, with 35–50% in G_2 + M, and the remainder in S phase. The medium fraction also included elevated percentages of cells with partially or completely replicated DNA (data not shown). Since the most primitive thymocytes apparently traverse a full cell cycle in 6–10 h (4–6), they are likely to be concentrated in the medium- and large-cell fractions.

Large and small elutriated thymocytes also showed dramatic differences in their rates of [^{35}S]methionine incorporation in vitro (Table I). The amount of label incorporated per cell in a 30–50-min incubation was routinely 5–20-fold higher in the large cells than in the cells from the peak small cell fraction, although the viabilities were equally high. This difference was not an artifact of stress during the fractionation procedure, for it was seen whether the cells were labeled before or after elutriation

(Table I, compare experiments 1 and 2). The large cells thus represent the enrichment of a thymocyte population that is highly atypical with respect to overall protein synthesis as well as with respect to DNA synthesis.

The extent of this enrichment by elutriation alone depended upon the age and strain of the mice. In B6 and B6-TL$^+$ mice >7 wk old, a simple decrease in the lymphoblast population could be inferred from both a reduced yield of cells in the large fraction and a lower level of [^{35}S]methionine incorporated per large cell. In A/J mice, on the other hand, the number of cells recoverable in the large fraction was dramatically increased to >15% of the total; however, these also incorporated <1/3 as much label per cell as the large B6 thymocytes (data not shown). Similar results were obtained with animals from 2.5 to 6.5 wk of age. The difference in thymocyte fractionation patterns may be related to the known differences between A/J and B6-background mice in various aspects of thymus physiology (30).

Enrichment of Cortical Thymocytes by PNA Binding. There is some evidence that most cortical and medullary thymocytes develop from separate pools of dividing precursor cells (31–34). To relate phenotypic differences between cells to maturation, it was important to be able to compare small thymocytes with lymphoblasts within the same lineage. Therefore, cortical and medullary thymocytes were fractionated physically on the basis of their binding by the lectin PNA (35, 36). PNA binds to ~90% of thymocytes (PNA$^+$), and these display characteristic markers of cortical cells. The unbound cells, in surface phenotype, functional competence, and in vivo steroid resistance, correspond closely to medullary cells and peripheral T cells (36–41). The first cells repopulating the thymus after irradiation include both PNA$^+$ and PNA$^-$ cells (42), so it is likely that the distinction is present in the most primitive cells of each lineage. To isolate cortical lymphoblasts for comparison with their postmitotic descendants, the size fractionation and PNA fractionation were combined.

Primitive lymphoblasts were first separated from small resting cells by elutriation; then small, medium, and large cells were fractionated by binding to PNA-coated petri

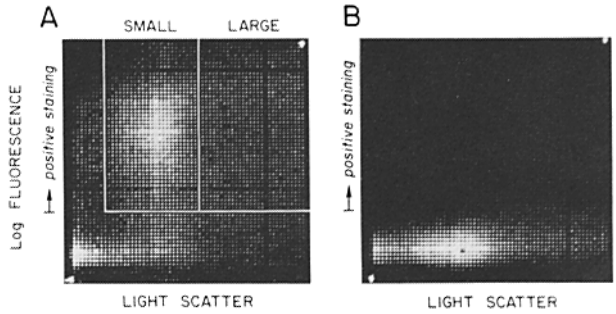
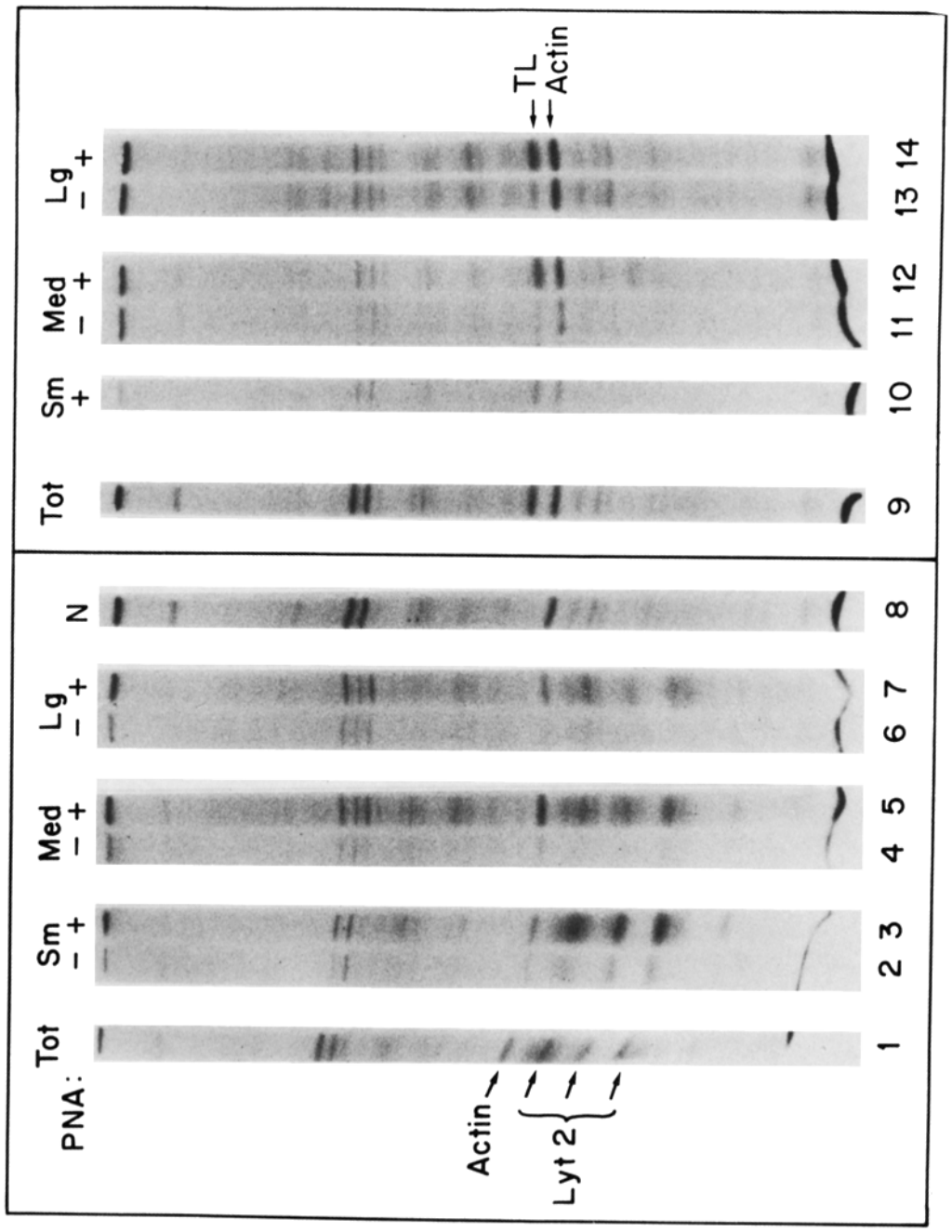


FIG. 2. Surface expression of TL on B6-TL$^+$ thymocytes. Thymocytes were stained with monoclonal antibody against TL (10-79.4) and fluoresceinated goat anti-mouse IgG. They were analyzed for forward-angle light scatter and fluorescence intensity on a flow microfluorometer equipped with a log amplifier for fluorescence detection. The full scale (channels 1-256) of log fluorescence covers three decades. Light scatter was detected with linear amplification. (A) Thymocytes stained with anti-TL. The dual-parameter display shows the analysis of 35,000 events. Light scatter channels 1-40 contained dead cells or debris. White outlines indicate the boundaries of the distributions of small cells (scatter channels 41-140) and large cells (scatter channels 141-256) with positive fluorescence (fluorescence channels 72-256). The percentages of positive cells are given in the text. (B) Control staining with second stage alone. Using the same scale and gain settings as in A, 5.5% of the cells were positive.


PNA:
Tot
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TL
Actin
Actin
Lyt 2
1
2 3
4 5
6 7
8
9
10
11 12
13 14

TABLE II

Relative Levels of TL Synthesis in Different Populations of Thymocytes

Ratio between cell samples	A		B	
	TL synthesis per 2×10^6 cpm*		TL labeled per cell‡	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Unfractionated/unfractionated	(1.0)	(1.0)	(1.0)	(1.0)
Small PNA^+/unfractionated	0.26	0.34	0.05	0.05
Medium PNA^+/unfractionated	1.9	1.4	1.5	1.2
Large PNA^+/unfractionated	2.3	1.8	11.2	7.0
Small, medium PNA^-/unfractionated	<0.03	<0.03	<0.01	<0.01
Large PNA^-/unfractionated	0.39	0.19	1.9	0.75
Large PNA^+/small PNA^+	8.8	5.2	209	135

* Anti-TL immune precipitates were prepared from inputs of 2×10^6 cpm of [^{35}S]methionine-labeled protein and analyzed by sodium dodecyl sulfate gel electrophoresis and fluorography. Radioactivity incorporated into the TL band was quantitated by scanning the fluorograms with a Zeineh microdensitometer (LKB Instruments, Inc., Rockville, Md.) and integrating the areas under the peaks with a planimeter (Hewlett-Packard Co., Palo Alto, Calif.). The value shown is the ratio between these areas in the two samples specified.

‡ The value in A was converted to the ratio of the amounts of newly labeled TL per cell by multiplying it by the ratio of counts per minute incorporated per cell in the two samples. This calculation compares the different amounts of TL synthesized per cell, if the specific activities of the methionine pools are the same in the two populations of cells.

dishes ("panning"; 22). In B6-TL^+ thymocytes, PNA^+ cells formed the majority of each size fraction, comprising up to 90% of the small cells, 85% of the medium cells, and 80% of the largest cells. The PNA^- fraction of the large cells was at least as enriched as the PNA^+ fraction in cells with >2C DNA content (data not shown), however, in agreement with other evidence for a PNA^- lymphoblast population (37, 42). As shown in Table I, experiment 3, the rate of [^{35}S]methionine incorporation per cell was similar for PNA^+ and PNA^- cells in any one size fraction. Therefore, in both classes of cells, any developmental transition from large to small must be accompanied by a sharp decline in methionine uptake and/or protein synthesis per cell.

Distinctions between the six pools of thymocytes yielded by this fractionation scheme could be revealed by analysis of their patterns of ongoing protein synthesis, using immune precipitation from metabolically labeled cell lysates. To compare different samples, the radioactive inputs were normalized to total [^{35}S]methionine

FIG. 3. Synthesis of Lyt-2/3 and TL in fractionated thymocytes. Cells were elutriated into small, medium, and large size classes. They were then incubated with [^{35}S]methionine, washed, and separated into PNA^+ and PNA^- fractions. Labeling was for 45 min, and the amounts of isotope incorporated per cell are given in Table I (experiment 3). For lanes 1–7, 1×10^6 cpm of labeled protein were analyzed by immune precipitation with anti-Lyt-2.2 monoclonal antibody. None of the three bands indicated as Lyt-2/3 could be precipitated from lysates of congenic thymocytes bearing the alternative Lyt-2 allele (B6-Lyt-2.1:3.1). For lanes 9–14, 2×10^6 cpm of labeled protein from the indicated lysates were subjected to immune precipitation with anti-TL.3 monoclonal antibody. Lysates from small PNA^- thymocytes (not shown) contained at least twofold less labeled TL than those from small PNA^+ cells. Lane 8 was a normal mouse serum precipitate from 2×10^6 cpm of the unfractionated thymocyte lysate (N). Tot, unfractionated thymocytes; Sm, "small" fraction; Med, "medium" fraction; Lg, "large" fraction; PNA^-, unbound by PNA-coated plates; PNA^+, bound to and eluted from PNA-coated plates.

incorporation rather than to cell number. Thus, *de novo* synthesis of each product was measured as a fraction of the overall protein synthesis in a cell population. This in effect corrected for differences in cell volume, and for the replacement of large-cell components otherwise diluted by rapid division.

Restriction of TL Synthesis to Blast Cells. The TL glycoprotein (19, 43) is known to be expressed on the surfaces of 70–80% of all thymocytes in B6-TL$^+$ mice, specifically marking the cortical cells (43–45). When thymocytes were analyzed by indirect immunofluorescence with monoclonal antibody to TL (Fig. 2), ~80% of the cells were positive, with good distinction between TL$^+$ and TL$^-$ cells. Most of the positive cells were small, as defined by light scatter (Fig. 2). The percentages scored as TL$^+$ were 78% in the small size class and 82% in the large class, although the latter stained more heterogeneously than the small TL$^+$ cells.

When fractionated thymocytes were assayed for ongoing TL synthesis, however, a very different result emerged. Compared with unfractionated cells, synthesis of the TL glycoprotein was enhanced in PNA$^+$ medium and large thymocytes (Fig. 3, cf. lanes 9, 12, and 14; Table II). On the other hand, in small thymocytes TL synthesis was sharply reduced, even in relation to their low overall protein synthesis (Fig. 3, lane 10; Table II). The contrast was more dramatic when considered in terms of TL synthesis per cell, since corresponding amounts of radiolabel were incorporated by >10 times as many small cells as large cells. In other experiments, the same pattern of TL synthesis was observed whether labeling was carried out before or after elutriation (data not shown), ruling out selective damage to the small cells during centrifugation. These results indicate that the majority of thymocytes that express surface TL are not actively engaged in the synthesis of this glycoprotein to a significant extent. The TL on their surfaces must therefore be a relic of prior synthesis in their lymphoblast precursors.

Selective Regulation of TL Synthesis. Biosynthetic labeling cannot indicate what fraction of the cells in a heterogeneous population makes a particular product, or indeed what fraction contributes to ongoing protein synthesis at all. The low activity of small cells in making TL could be due either to selective regulation of TL synthesis during the transition to the resting state or to elimination of surface TL$^+$ cells from the metabolically active pool. The question is not trivial because of the high rate of death, in vivo and in vitro, among small cortical thymocytes (27, 31, 46, 47). Thus it was important to establish whether cells expressing surface TL were responsible for any of the protein synthesis in the small-cell fraction.

To assay the level of overall protein synthesis in cells bearing surface TL, size-fractionated thymocytes were treated with monoclonal anti-TL antibody and complement, or with complement alone, and then labeled with [^{35}S]methionine. The resulting levels of methionine incorporation per input cell are summarized in Table III. Cytotoxic elimination of surface TL$^+$ cells reduced protein synthesis in unfractionated thymocytes by 66%; a similar fraction of synthesis was also vulnerable to elimination in both large and small thymocyte populations. The elimination was specific for expression of surface TL, because incorporation was not reduced with respect to the complement control when the thymocytes were obtained from congenic, TL$^-$ B6/J mice (data not shown). Therefore, cells bearing TL were still responsible for the majority of observed protein synthesis, in small cells as well as in large cells.

TABLE III

Biosynthetic Activity in Thymocytes Bearing Surface TL

Sample	Fraction of cells	C'* alone	αTL + C'	Fraction TL^+‡
		cpm/input cell	*cpm/input cell*	
Unfractionated	—	0.024	0.008	66%
Small	82%	0.019	0.006	67%
Medium	7.3%	0.088	0.043	51%
Large	0.8%	0.27	0.09	67%

* Complement.

‡ Percent reduction in protein synthesis after specific cytotoxic elimination, calculated from values given.

This means that the cessation of *de novo* TL synthesis must represent a regulatory event affecting the expression of this gene product specifically.

Maintenance of Lyt-2/3 Synthesis by Small Cortical Thymocytes. An example of a tissue-specific gene product that continued to be made in postmitotic thymocytes was Lyt-2/3. The Lyt-2/3 antigen marks the surfaces of certain functional subclasses of peripheral T cells (48–51) and virtually all cortical or PNA^+ thymocytes (32, 40–42). In the thymus, therefore, there is extensive overlap between the cells expressing surface Lyt-2/3 and those expressing surface TL (52, 53). Unlike TL, however, the three glycoprotein chains of the Lyt-2/3 complex ("Lyt-2"; 54, 55) were strongly labeled in PNA^+ cells of all size classes (Fig. 3, lanes 3, 5, and 7). In agreement with their steady-state pattern of membrane expression (40, 41), synthesis of these molecules was low or undetectable in the corresponding PNA^- populations (Fig. 3, lanes 2, 4, and 6). Therefore the ongoing production of Lyt-2/3 by small cells was not due to rare Lyt-$2/3^+$ medullary thymocytes; it could be firmly attributed to the cortical population that was so poor in TL synthesis.

Discussion

In the work presented here, a rapid and gentle cell fractionation procedure has been used to study the primitive, dividing lymphoblasts in thymus glands from normal B6-TL^+ mice. This protocol is not claimed to yield homogeneous pools of cells. What it does, instead, is to enrich physiologically important minor populations to the point where features of their phenotypes can be discerned. Although a given pool of cells may not be pure, characteristics unique to it can be identified by the way they partition among the cell fractions. This is a vital point for assigning characteristics, such as immune responsiveness or biosynthesis of specific products, that are difficult to assay at a single-cell level. In the case of dividing lymphoblasts, it is impossible to conclude what percentage of the cell population is synthesizing a particular marker; however, if a marker that is made at an enhanced rate in the lymphoblast fraction is made poorly in the small-cell fraction, then the high rate of synthesis cannot be attributed to contamination by the small cells.

A result of this analysis has been the identification of a minor population of thymocytes that appears to be responsible for most of the organ's active synthesis of TL glycoprotein. Although most of the cells in the thymus, especially small cortical cells, exhibit surface TL, *de novo* synthesis of this molecule is virtually confined to cell fractions enriched in dividing lymphoblasts. PNA-binding lymphoblasts appear to be

most active, though the data in Fig. 3 and Table II do not rule out some TL synthesis by the largest PNA^- cells. A considerable body of literature indicates that small cortical thymocytes are all postmitotic descendants of large- and medium-sized cells (4–6, 27). The present results confirm the precursor-product relationship between those large cells that actively make TL and the surface TL-positive majority population of thymocytes. The continued display of TL by the latter cells may simply reflect their lack of membrane turnover.

The interest of this finding lies in its specificity. In spite of a generalized reduction in [^{35}S]methionine incorporation per cell, small thymocytes do not shut off production of other surface glycoproteins to nearly the same extent as TL. Synthesis of Lyt-2/3 accounts for at least as high a fraction of total protein synthesis in small PNA^+ cells as in large ones. This contrasts sharply with the considerable reduction in TL synthesis even in relation to the diminished overall incorporation of label by these cells. Because virtually all small PNA^+ cells display both Lyt-2/3 and TL on their surfaces (52, 53), it is likely that cells continuing to make Lyt-2/3 but not making TL were derived from precursors that actively produced both. Moreover, the difference between the ways TL and Lyt-2/3 are regulated does not simply reflect the difference between a marker of strictly intrathymic cells and a marker of mature, peripheral T cells. Synthesis of another product specific for cortical thymocytes, terminal deoxynucleotidyl transferase (18, 20, 56–58), is maintained in the small cells like Lyt-2/3, not aborted like TL (data not shown). Consistent with this behavior in vivo, TL synthesis shuts off when unfractionated thymocytes are incubated in short-term tissue culture, whereas synthesis of terminal transferase, H-2, and Lyt-2/3 continues (D. Triglia and E. Rothenberg, unpublished observations) (18, 21). This means that *de novo* synthesis of TL is not a common feature of all metabolically healthy cortical cells, but rather a property specific to the large cortical lymphoblasts.

In the medium and large cell elutriator fractions, the enhancement of TL synthesis increases monotonically with the increased proportion of cycling cells. It seems highly probable, then, that TL is only made by thymocytes during their early period of rapid proliferation. This could mean either that TL synthesis is cell cycle dependent or that TL synthesis marks a unique stage in the thymic developmental progression. To test these possibilities critically, postmitotic cells of a rigorously purified TL^+ lineage must be restimulated to cycle and assayed for renewed synthesis of the glycoprotein. In preliminary studies, enriched PNA^+ populations have shown no signs of increased TL production with mitogenesis (E. Rothenberg and D. Triglia, unpublished observations). If confirmed, this would indicate that TL synthesis is not linked to proliferation generally but to a unique phase of proliferation. TL shutoff, in other words, appears to be irreversible.

The central purpose of this investigation has been to identify patterns of biosynthesis that distinguish the stem cell-like thymic lymphoblasts from their cortical and medullary descendants. Maintenance of these patterns could then be monitored in thymocytes responding to experimental manipulation, to indicate any change in their developmental state. The results presented here indicate that Lyt-2/3 and TL may represent valuable parts of such a pattern. In cells derived from the thymic cortex, the maintenance of Lyt-2/3 synthesis—possibly extending into functional maturity—allows this product to be used as a rough index of cell viability. Conversely, because ongoing synthesis of TL is even more limited in developmental time than its surface

expression, it may prove to be an exceptionally sensitive indicator of developmental transitions. If TL synthesis were not actively restored, then stimulation of small cortical thymocytes would necessarily give rise to TL^- progeny cells. No additional "maturation" events need occur (11, 59, 60); the TL initially present on the cell membranes would simply be diluted out. On the other hand, stimulation of thymocytes in such a way as to maintain TL synthesis, as in TL^+ lymphomas, could indicate that some blast cell population was also being maintained in a distinct "primitive" state. TL synthesis may thus provide an assay for the unique proliferation requirements of an elusive but important population of early lymphoid cells.

Summary

Large cortical thymocytes from C57BL/6-*Tla*^{a} mice have been prepared rapidly and in high yield by a combination of centrifugal elutriation and differential binding to peanut agglutinin (PNA)-coated plates. The cells in these lymphoblast-rich fractions were clearly distinct from the majority of thymocytes, with up to 70% in the S or G_2 + M phases of the cell cycle and an average rate of [^{35}S]methionine incorporation per cell up to 20 times higher than that of the majority population.

The populations of cells resolved in this fractionation were characterized by monitoring their rates of synthesis of specific glycoproteins, thymus-leukemia antigen (TL) and the Lyt-2, Lyt-3 complex (Lyt-2/3), relative to their total protein synthesis. Cells that bound to PNA synthesized high levels of Lyt-2/3, consistent with their identification as cortical thymocytes. Those that failed to bind made little or no Lyt-2/3, as expected for medullary cells. The fraction of dividing lymphoblasts that bound to PNA was enriched in cortical thymocyte precursors, including all the large cells detectably active in synthesizing Lyt-2. It differed sharply from the small cortical cells, however, in the synthesis of TL. Although both populations displayed abundant surface TL, the TL glycoprotein was produced actively in fractions containing dividing cells but made at a drastically reduced rate by the nondividing majority of cortical thymocytes. Thus, TL seems to be made at a narrowly circumscribed stage of early thymocyte development that is correlated with rapid proliferation. In most of the descendants of such blast cells, the TL glycoprotein is presumably retained on the cell surface as long as no substantial membrane turnover takes place. Ongoing TL synthesis may therefore serve as a marker for a unique developmental state which terminates rapidly in normal differentiation but may be extended by agents that give rise to TL^+ thymic lymphomas.

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