PROLIFERATION OF THYMIC STEM CELLS WITH AND WITHOUT RECEPTORS FOR INTERLEUKIN 2

Implications for Intrathymic Antigen Recognition

By J. P. LUGO, S. N. KRISHNAN, R. D. SAILOR, P. KOEN, T. MALEK,* AND E. ROTHENBERG

*From the Division of Biology, California Institute of Technology, Pasadena, California 91125 and the *Laboratory of Immunology, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland 20205*

The thymus plays a vital role in the ontogeny of T lymphocytes. Within this organ, T cell precursors acquire the phenotypic markers and the functional reactivities characteristic of mature T cells (1). In addition, nonlymphoid components of the thymus influence the antigen recognition repertoire of developing pre-T cells such that the mature T cells that derive from them recognize foreign antigens in the context of "self" major histocompatibility antigens (2, 3). The molecular and cellular events involved are still largely obscure. A conspicuous aspect of this developmental process is the rapid cell division that is induced in primitive lymphoblasts by contact with the thymic stroma (4–6). Among the descendants of these lymphoblasts are mature peripheral T cells (7, 8), although the vast majority of their progeny appear to die in situ (6, 9). Until now, it has been unknown whether a single mechanism drives thymic mitogenesis or whether different subpopulations of lymphoblasts respond to different signals, perhaps resulting in different fates.

For mature T lymphocytes, growth is strictly linked to antigen recognition. This proliferation is mediated by the polypeptide growth hormone interleukin 2 (IL-2)[1] (reviewed in ref. 10). To respond to IL-2, both helper and non-helper T cells must express receptors for IL-2 (IL-2-R), which occurs only upon stimulation with antigen or a mitogenic lectin (11). Thus, antigen binding per se is not mitogenic for mature T cells, but rather an inducer of hormone responsiveness. The interaction of IL-2 with its receptor is the only signal known to drive mature T cells to divide.

We wished to determine whether intrathymic pre–T cell proliferation is also regulated by IL-2. It has been proposed (12–14) that thymocyte division is triggered by contact with self antigens. Regardless of the ultimate fate of the cells, any clonal proliferation that is driven by antigen is likely to involve induction of IL-2-R on the proliferating cells. On the other hand, proliferation that is not

This research was supported by grants AI 19752 and CA 32911 from the National Institutes of Health, U.S. Public Health Service. J. Lugo was supported by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research. S. Krishnan was supported in part by a Summer Undergraduate Research Fellowship from the California Institute of Technology.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; FITC, fluorescein isothiocyanate; IL-2, interleukin 2; IL-2-R, IL-2 receptor; mAb, monoclonal antibody; PNA, peanut agglutinin.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/85/5/1048/15 $1.00
Volume 161 May 1985 1048–1062

antigen-induced may or may not depend on IL-2 and its receptor. Thus, a thymocyte population expressing IL-2-R may include cells undergoing selection on the basis of antigen specificity. Thymocytes that proliferate without IL-2-R must instead be responding to a novel mitogenic mechanism.

Antibodies against IL-2-R are now available (15–20), but this question has not been investigated before. Although >95% of thymocytes lack detectable IL-2-R expression (11, 15–18), the dividing thymocytes that we would expect to express this molecule constitute a small minority, and are themselves heterogeneous in phenotype (1, 21), functional reactivity (22), and anatomical location within the thymus (1). In this report, we have isolated thymocytes that are in cycle, and we present biochemical and flow-cytometric evidence that some of the lymphoblasts do express IL-2-R at levels sufficient to drive mitogenesis. However, these cells comprise only a small minority of the dividing cells. Virtually all the lymphoblasts of "cortical" phenotype and most of "medullary" phenotype lack detectable amounts of IL-2-R. Thus, most intrathymic proliferation appears to be IL-2–independent.

Materials and Methods

Animals and Cells. C57BL/6 (B6) mice and the B6 congenic lines, C57BL/6-Tla^a (B6-TL$^+$), and C57BL/6–Lyt-2.1,3.1 (B6–Lyt-2.1,3.1) were bred and maintained in the animal facility at the California Institute of Technology. Mice 4–6 wk of age were used in all experiments unless otherwise indicated. MTL2.8.2 cells (23) were cultured as described (22).

Preparation of Thymocyte Subpopulations. Thymocytes were separated according to their size and their peanut agglutinin (PNA) binding phenotype using the techniques of centrifugal elutriation and PNA "panning" in petri dishes, as previously described (22). Overall cell recovery ranged from 65 to 98%. In some experiments, mice were injected intraperitoneally with India ink 30–90 min before thymus removal, and the thymus lobes were checked to exclude the presence of intensely stained lymph nodes before they were used. The results obtained in these cases were the same as those shown.

Antisera and Monoclonal Antibodies (mAb). Mouse IL-2-R was detected by the rat IgM mAb 7D4 and 3C7 (15, 16). The mouse mAb AD4.15 (24) was produced by the corresponding hybridoma, grown as an ascites tumor in BALB/c × B6 F_1 female mice. IgM from the ascites was purified by S300 gel filtration chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) to >90% purity, based on electrophoretic analysis. The mAb AD4.15 was used as a negative control because its affinity for Lyt-2.2, its target antigen, is too low for immune precipitation, and, like 7D4, it requires a second antibody to bind to Staphylococcal protein A. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rat 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. J. Cole. A high-titer goat anti–rat IgG antiserum was kindly provided by Drs. B. Omary and I. S. Trowbridge (Salk Institute, La Jolla, CA).

Radiolabeling of Lymphocytes and Immune Precipitations. Cell surface iodinations were performed essentially as described by Siadak and Nowinski (25). Pulse-chase metabolic labeling with [^{35}S]methionine has also been described (26, 27). After the radiolabeling step, cells were lysed in detergent lysis buffer (26) at cell densities ranging from 2×10^7 to 10^8 cells/ml. Cellular debris was removed by centrifugation, and the clarified supernatants were precleared overnight with normal rabbit serum and fixed Staphylococcus aureus (Pansorbin; Calbiochem-Behring Corp. San Diego, CA) as previously described (26). Detergent lysates of [^{35}S]methionine-labeled thymocytes were precleared a second time with goat anti–rat IgG and protein A–Sepharose 4 B beads (Pharmacia Fine Chemicals). To precipitate mouse IL-2-R, mixtures of 3–5 μl of 7D4 culture supernatant and radiolabeled cell extracts were incubated overnight at 4°C, followed by the addition

of goat anti-rat IgG, and Pansorbin or protein A–Sepharose 4 B 60 min later. After a final 15–30 min incubation, the fixed bacteria or protein A–coated beads were washed with lysis buffer, and the receptors were eluted by boiling in electrophoresis sample buffer. For immune precipitation from ^{35}S-labeled thymocyte lysates, the protein A–Sepharose 4 B beads were washed in a high-salt detergent buffer (15). Slab gel electrophoresis and autoradiography were carried out as previously described (27). The intensity of autoradiographic signals was quantitated by using a densitometer (E-C Apparatus Corp., St. Petersburg, FL) to scan films on which the signal was not overexposed. When data had to be taken from two exposures, the results were standardized using signals that were within linear range in both exposures.

Flow Cytometry. For flow microfluorometry, an Ortho Cytofluorograph 50H cell sorter (Ortho Diagnostic Systems Inc., Raritan, NJ) was used, with an argon laser excitation wavelength of 488 nm. Fluorescence staining was performed as previously described (21). Cell populations were gated according to light scatter to exclude most dead and aggregated cells. Relative cell size was estimated by forward light scatter.

For determination of DNA content, ethanol-fixed cells were stained with propidium iodide (5 or 40 μg/ml; Calbiochem-Behring Corp.) in 1 ml phosphate-buffered saline containing 100 μg/ml heat-treated RNase. The relative number of cells in each phase of the cell cycle (G_0/G_1, S, and G_2 + M) was estimated by a cell cycle analysis algorithm (Joseph Trotter, Salk Institute).

Results

Centrifugal Elutriation and PNA Panning Enrich for Dividing Cortical and Medullary Thymocytes. Proliferating thymic lymphoblasts are among the largest cells found in the thymus (21). We therefore enriched for these cells on the basis of size using centrifugal elutriation. Three distinct size classes (small, medium, and large) were resolved (Fig. 1, *A*, *D*, and *G*), with the lymphoblasts concentrated in the medium and large fractions (22). Although the lymphoblast and postmitotic cell populations are clearly distinct, each size class is heterogeneous, including both cortical and medullary cells (21, 22). These cells were further fractionated by exploiting differences in their abilities to bind the lectin PNA. Overall, cortical thymocytes (~85% of all thymic lymphocytes) bind much more PNA (PNA^+) than do medullary thymocytes (PNA^-) (1). Large, dividing thymic lymphoblast cells are also separable into PNA^+ or PNA^- cell populations (21, 22, 28), though a correlation with anatomical location has yet to be established for these cells. Nevertheless, the combination of size fractionation and differential PNA binding collects actively dividing thymocytes into the large and medium size classes, away from the majority of small resting cells, and further subdivides the cells in these populations according to their "cortical" or "medullary" PNA-binding phenotype.

Analysis of the DNA content of cells in the resulting populations confirmed the high enrichment of cycling cells in the medium and large size fractions (Fig. 1). In the large size fraction, 60% of the cells were in S phase, with another 15% in G_2 + M (Fig. 1, *B* and *C*). In contrast, only 10% of the unfractionated thymocytes were found in S and G_2 + M together (data not shown). Medium-sized thymocytes also contained a high proportion of dividing cells (>25% in S and G_2 + M) (Fig. 1, *E* and *F*), though less of them than the large fraction. Small cells, on the other hand, were relatively depleted of cycling cells (Fig. 1, *H* and *I*), but comprised the bulk of elutriated cells (Table I). Within any given size class, PNA^+ and PNA^- cells had indistinguishable cell cycle distributions (Fig. 1). Therefore, any difference in phenotype between PNA^+ and PNA^- thymocytes

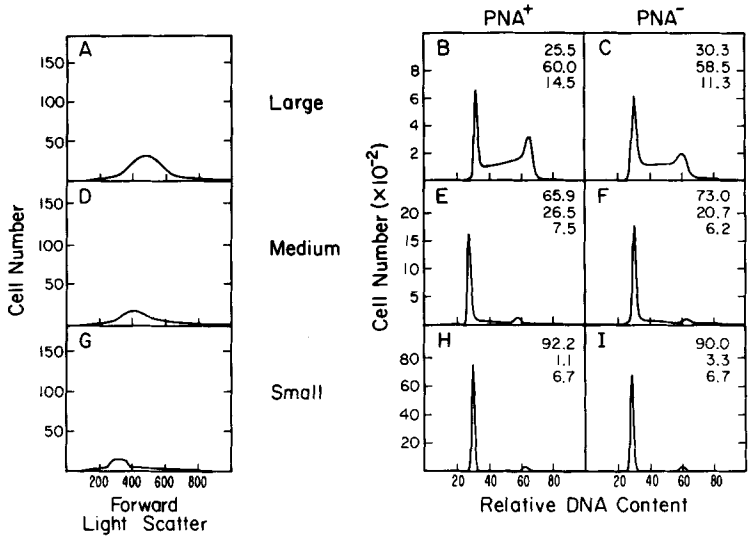


FIGURE 1. Flow-cytometric measurements of cell sizes and DNA contents in fractionated thymocyte populations. Forward light scatter intensity (abscissa) was used to measure relative cell size (*A*, *D*, and *G*). Elutriated thymocytes were fixed in 1% formaldehyde in isotonic phosphate-buffered saline for light scatter analysis. To measure DNA content, cells fractionated according to both size and PNA binding were fixed in ethanol and then stained with the DNA-binding chromophore propidium iodide, as described in Materials and Methods. Propidium iodide fluorescence intensity (abscissa in *B*, *C*, *E*, *F*, *H*, and *I*) is directly proportional to DNA content. The DNA content of G_0 resting cells and G_1 diploid cells corresponds to a fluorescence intensity of about 28 arbitrary units. The sets of three numbers in *B*, *C*, *E*, *F*, *H*, and *I*, from top to bottom, denote the percentages of cells in G_0/G_1, S, and G_2 + M, respectively.

of the same size class most likely reflects differences in their patterns of gene expression that are not simply due to differences in their proliferative state or their rate of protein synthesis (21).

Selective Expression of IL-2-R by PNA⁻ Lymphoblasts. To determine whether any of the six populations of thymocytes described above contain cells that express IL-2-R, we surface labeled each population with ^{125}I, lysed the cells in detergent, then tested the lysates for molecules precipitable with the rat mAb 7D4. This mAb inhibits IL-2-driven murine T cell proliferation by binding to the mouse IL-2-R (15, 16). From lysates of surface-labeled MTL.2.8.2 cells, an IL-2-dependent T cell clone (23), 7D4 precipitated a broadly migrating glycoprotein of 53–63 kilodaltons (kD) apparent molecular mass (p56) (Fig. 2, *12* and *13*). Some thymocyte populations contained cells that also expressed molecules similar to p56 IL-2-R. Lysates of medium and large PNA⁻ cells yielded 1/5 the amount of putative receptor that was precipitated from a corresponding number of MTL2.8.2 cells (Fig. 2, cf. *8*, *10*, *12*, and *13*).

To confirm that these molecules were IL-2-R, we analyzed their intracellular precursors by biosynthetic pulse labeling with [^{35}S]methionine. From extracts of pulse-labeled MTL.2.8.2 cells, 7D4 specifically precipitated two glycoproteins with apparent masses of 36 kD (p36) and 38 kD (p38) (Fig. 3*A*, *2*). After a 1 h chase with cold methionine, these 7D4-binding molecules disappeared and were replaced by a heterogeneous 54–62 kD protein matching the apparent mass of

TABLE I

Cells Expressing IL-2-R and Cells in S Phase in Various Thymocyte Fractions

Fraction	PNA Phenotype	Fraction of total thymocytes*	IL-2-R$^+$ cells‡	Cells in S Phase§
			%	
Small	−	18.7 ± 0.0	0.7 ± 0.5	3.3
	+	63.8 ± 1.3	0.6 ± 0.2	1.1
Medium	−	5.2 ± 0.8	11.2 ± 2.7	20.7
	+	8.3 ± 0.0	2.9 ± 0.6	26.5
Large	−	1.4 ± 0.2	11.0 ± 0.3	58.5
	+	2.5 ± 0.3	1.9 ± 0.5	60.0

* Values were those obtained in two experiments, normalized for recovery after panning.

‡ Positives as defined in Fig. 4. Values were determined in two experiments as: (percent cells stained with mAb 7D4 and FITC–goat anti–rat IgG) − (percent cells stained only with FITC–goat anti–rat IgG). These values agree well with those obtained for thymocytes subjected to size fractionation alone (three experiments). 1–3% of unfractionated thymocytes stained for IL-2-R (data not shown).

§ Estimated by flow cytometry. These values are more accurate than estimates of the total proliferative fraction because G_0 cells can not be distinguished from G_1 (cycling) cells, and some G_0/G_1 doublets may be counted as G_2 + M cells (4C DNA content).

the p56 surface-iodinated molecules (Fig. 3*A*, *3–7*). Thus, the 36 and 38 kD intracellular molecules, and the 53–63 kD cell surface protein detected by 7D4 were taken to be diagnostic forms of the IL-2-R.

As shown in Fig. 3*B* (*10–13*), medium and large thymocytes synthesized two 7D4-reactive molecules that were indistinguishable from the p36 and p38 molecules precipitated from MTL2.8.2 cell lysates (Fig 3*A*, *2*). A 1 h chase with cold methionine also converted these thymocyte molecules into a heterogeneous p56 glycoprotein (data not shown). We conclude that the molecules detected by 7D4 in PNA$^-$ lymphoblast lysates are, in fact, IL-2-R. Posttranslational variations in glycosylation or other modifications could account for the slight differences in mobility we detected when comparing thymocyte and MTL2.8.2 IL-2-R (Fig. 2*A*, *10*, *12*; see also Fig. 5). Such differences have been noted before (29, 30) in comparisons of IL-2-R derived from different human T cell lines.

In contrast to the medium and large PNA$^-$ cells, PNA$^+$ lymphoblasts expressed few cell surface IL-2-R (Fig. 2, *3–6*). Densitometric scans revealed that medium and large PNA$^+$ cells contained only 3–6% of the receptor material found in their PNA$^-$ counterparts. Furthermore, biosynthetic labeling experiments indicated that de novo synthesis of IL-2-R was also restricted to medium and large PNA$^-$ cells (data not shown). These results rule out the possibility that IL-2-R are lost from the surface of PNA$^+$ thymocytes because of rapid exfoliation or internalization in response to IL-2. Instead, they indicate a major distinction between these two classes of proliferating cells.

The small cells in the thymus also failed to express any IL-2-R detectable by immune precipitation (Figs. 2 and 3). This was not surprising in view of their G_0

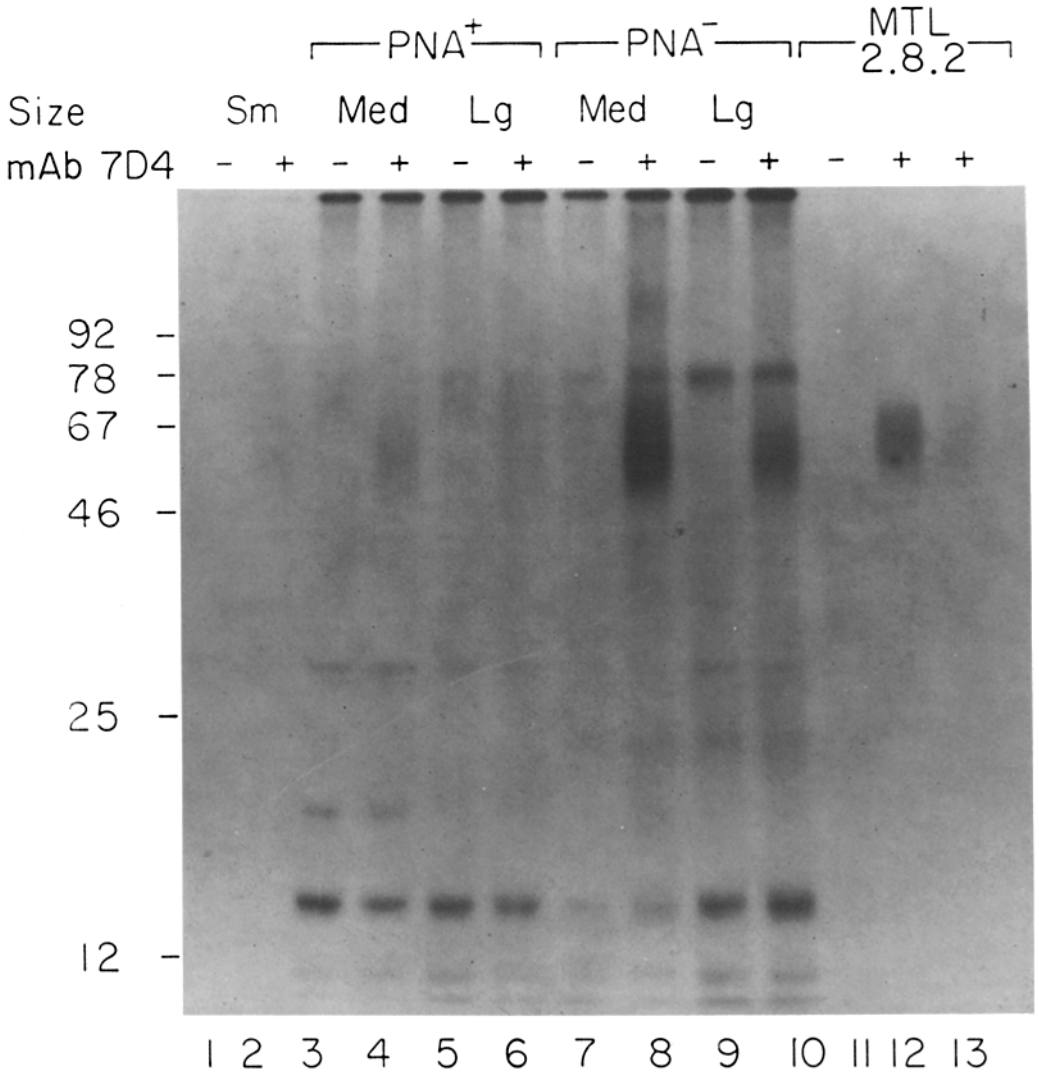
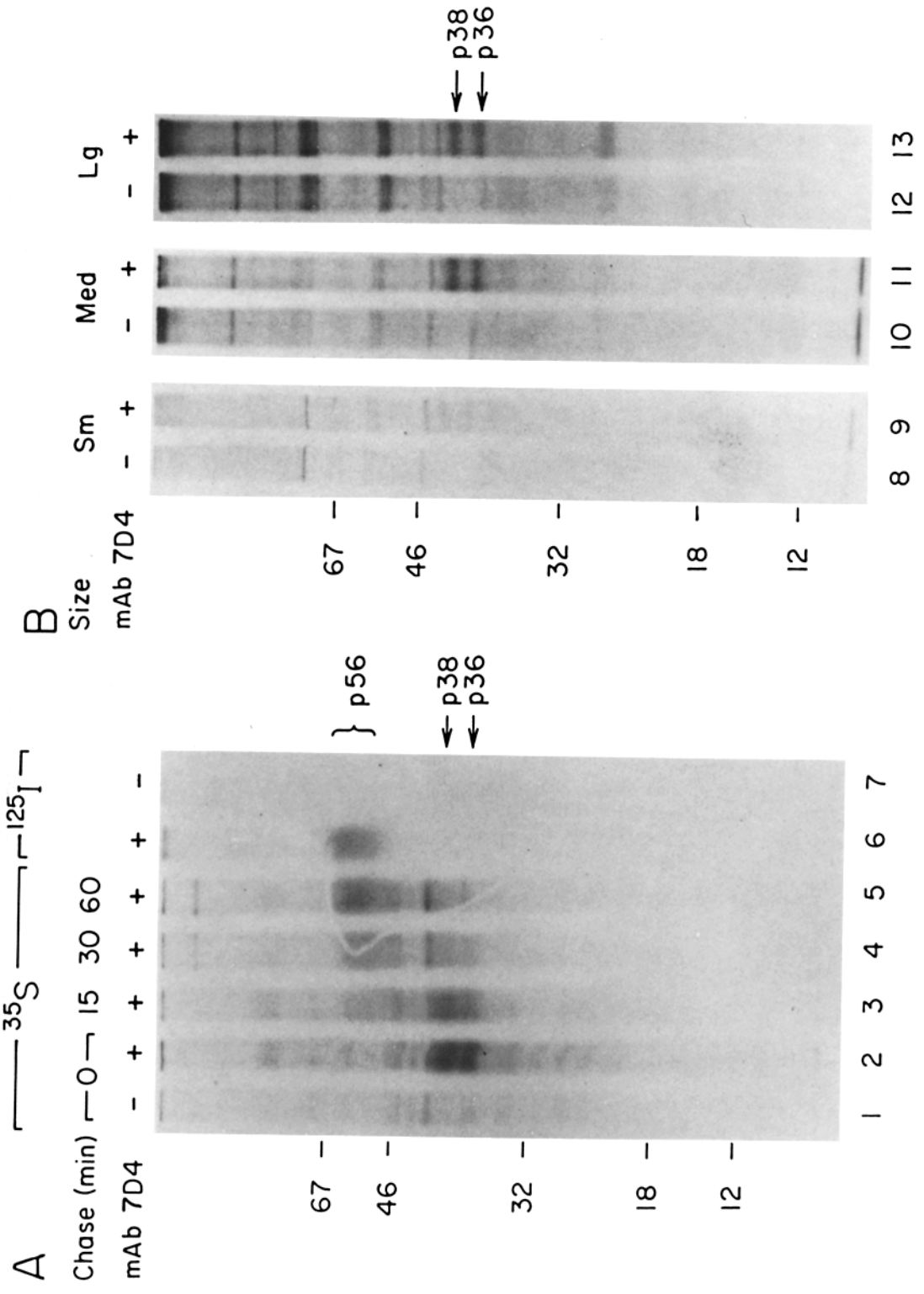


FIGURE 2. Immune precipitation of ^{125}I-labeled IL-2-R from the surfaces of thymocytes and MTL2.8.2 cells. Fractionated thymocytes and MTL2.8.2 IL-2-dependent cells were surface-labeled with Na^{125}I and lysed in detergent. Aliquots from each thymocyte lysate containing 2.5×10^6 cpm acid-precipitable ^{125}I were subjected to immune precipitation with antibody 7D4, and analyzed by sodium dodecyl sulfate gel electrophoresis (*2*, *4*, *6*, *8*, and *10*). Nonspecifically precipitated molecules were identified in parallel aliquots precipitated with the control antibody of AD4.15 (*1*, *3*, *5*, *7*, *9*). For comparison, aliquots of MTL2.8.2 cell lysates were precipitated with control antibody (*11*) or 7D4 (*12*, and *13*), using 5×10^5 (*11*, and *12*) or 6×10^4 (*13*) cpm of radioactive input in the immune precipitations. The diffuse band at 50–60 kD in the "+" samples is p56.

DNA content (Fig. 1, *H* and *I*) and their high probability of death. In these cells, as in the PNA$^+$ blast cells, the lack of IL-2-R was not an artifact of protein degradation in the lysates, since Lyt-2 could be precipitated from these samples in far better yield than from PNA$^-$ cell lysates (data not shown).

A Discrete Minority of PNA$^-$ Blasts Express IL-2-R. The experiments described


A
35S
125I
Chase (min) 0 15 30 60
mAb 7D4 - + + + + - + + + + -
p56
p38
p36
67
46
32
18
12
1 2 3 4 5 6 7
B
Size
mAb 7D4
Sm
Med
Lg
- + - + - +
p38
p36
67
46
32
18
12
8 9 10 11 12 13

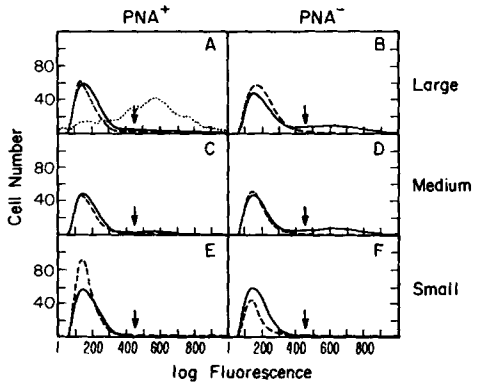


FIGURE 4. Flow-cytometric analysis of IL-2-R expression on fractionated thymocytes. Thymocytes were fractionated by size and PNA binding, then stained with 7D4 plus FITC-conjugaed goat anti-rat IgG (——), or with FITC-goat ant-rat IgG alone (- - -). After staining, the cells were fixed with 1% formaldehyde in isotonic phosphate-buffered saline, and analyzed for size (not shown) and fluorescence intensity (abscissa) by flow cytometry. Each histogram represents the signals from 20,000 cells, gated to exclude aggregates and debris by the correlated intensity of forward light scatter. To provide a positive control, spleen cells were cultured for 48 h with 3 μg/ml Con A, to enrich for activated T cells. The fluorescence histogram of these cells, stained with 7D4 and FITC-goat anti-rat IgG, is shown in panel A (····). The fluorescence intensity indicated by the arrow in each panel was taken as the threshold of positive staining (second antibody only giving ~5% "positive" cells). By this criterion, 70% of Con A-treated spleen cells were positive.

above establish that some PNA$^-$ thymic lymphoblasts express IL-2-R, but they do not indicate how the receptor is distributed within this population. To address this question, we stained fractionated thymocytes and concanavalin A (Con A)-activated spleen cells with 7D4 and FITC-conjugated goat anti-rat IgG, and analyzed the cells by flow cytometry.

As shown in Fig. 4, a distinct set of cells in the medium and the large PNA$^-$ populations displayed IL-2-R. The amounts expressed by this set of cells were virtually identical to those expressed by IL-2-R$^+$ spleen cells activated by Con A (cf. Fig. 4, *A*, *B*, and *D*). Still, even within the PNA$^-$ blast population, most cells lacked IL-2-R. Furthermore, at least 95% of the PNA$^+$ lymphoblasts also lacked IL-2-R, in good agreement with the biochemical data. As expected, small thymocytes contained few, if any, IL-2-R$^+$ cells (Fig. 4, *E* and *F*). The results were quantitatively indistinguishable, whether the cells were stained with 7D4 or with

---

FIGURE 3. De novo synthesis of IL-2-R in MTL2.8.2 cells and thymocytes. (*A*) MTL2.8.2 cells were labeled with [^{35}S]methionine for 15 min, and the label chased with excess cold methionine for 0–60 min, as indicated. The cells were lysed, and aliquots of the lysates containing 2.5 × 10^6 acid-precipitable cpm were subjected to immune precipitation with 7D4 (+) or control AD4.15 (−) antibody (*1–5*). For comparison, aliquots of ^{125}I-surface-labeled lysates of these cells were analyzed after precipitation with 7D4 (+) or AD4.15 (−) antibody (*6*, and *7*), both with 1.6 × 10^5 cpm input. The background band at 40–43 kD most likely represents actin. (*B*) Size-fractionated thymocytes were labeled for 15 min with [^{35}S]-methionine, lysed, and precipitated with 7D4 (*9*, *11*, and *13*) or AD4.15 (*8*, *10*, and *12*) from radioactive inputs of 2.1 × 10^6 cpm. In this experiment, the medium size fraction was divided in half. Data from one half are shown here, and the other half of the cells yielded quantitatively similar results (not shown).

a different mAb, 3C7, that reacts with the IL-2-binding site on the receptor (16) (data not shown).

Table I summarizes the results of several flow-cytometric analyses of fractionated thymocytes. We consistently found significant disparities between the percentage of cells in cycle and the percentage of IL-2-R$^+$ cells in the large PNA$^+$ and PNA$^-$ thymocyte populations. For mature T cells, IL-2-R expression precedes DNA replication and cell division by as much as 48 h (31, 32), and continues through at least one subsequent cell cycle (33). Therefore, even G_1 cells, once committed to cycle, ought to express receptors if their proliferation were mediated by IL-2. Instead, for thymic lymphoblasts, the percentage of IL-2-R$^+$ cells was considerably smaller even than the percentage of cells in S phase alone (Table I). This suggests that virtually all the PNA$^+$ and most of the PNA$^-$ blasts are dividing without first expressing IL-2-R. We therefore conclude that most intrathymic proliferation is IL-2-independent.

Neonatal Thymocytes Are Enriched for Proliferating IL-2-R$^+$ Cells. We wished to consider whether the IL-2-R$^+$ cells are also undergoing differentiation in the thymus, or whether they simply represent activated mature T cells. To rule out contamination by peripheral T cells, we assayed neonatal thymocytes for IL-2-R expression. These cells were obtained from mice <24 h after birth, a time when the thymus gland is accumulating large numbers of T lymphocytes for export, but before the emigration process has begun (34, 35). By immune precipitation with 7D4, ^{125}I-labeled lysates from neonatal thymocytes yielded 4–5 times more IL-2-R than the same radiolabeled input from adult thymocyte lysates (Fig. 5). This was not a result of 4–5-fold more proliferation in the neonatal thymus, since the fraction of proliferating cells estimated by flow cytometry was only twofold more than in the adult (data not shown). Furthermore, flow cytometric analysis revealed that 8.9% of the neonatal thymocytes expressed high levels of IL-2-R, in contrast to only 1.9% of total adult thymocytes (data not shown). Thus, far from being rarer in the neonatal thymus, IL-2-R appear to be expressed by a larger proportion of cells. These results exclude the possibility that all the lymphoblasts we detect expressing IL-2-R are actually posthymic cells. Moreover, they indicate a potential role for IL-2-R in the rapid growth of the thymus and peripheral T cell pool that takes place in early life (36).

Discussion

IL-2-R Expression in Thymic Lymphoblasts. The results presented here suggest that two different mitogenic mechanisms may drive the proliferation of different classes of thymic lymphoblasts. A discrete minority of lymphoblasts express IL-2-R at levels comparable to those expressed by activated peripheral T cells. This observation raises the possibility that IL-2-R could participate in the mitogenic activation of these thymocytes. At the same time, most of the T cell precursors dividing in the thymus do so without detectable expression of IL-2-R. This indicates that they must respond to signals other than those known to trigger mature T cells, and severely constrains the likelihood that their proliferation is driven by immune recognition.

The division of lymphoblasts into IL-2-R$^+$ and IL-2-R$^-$ classes is unlikely to result from a detection artifact. Within the blast cell population, those cells that

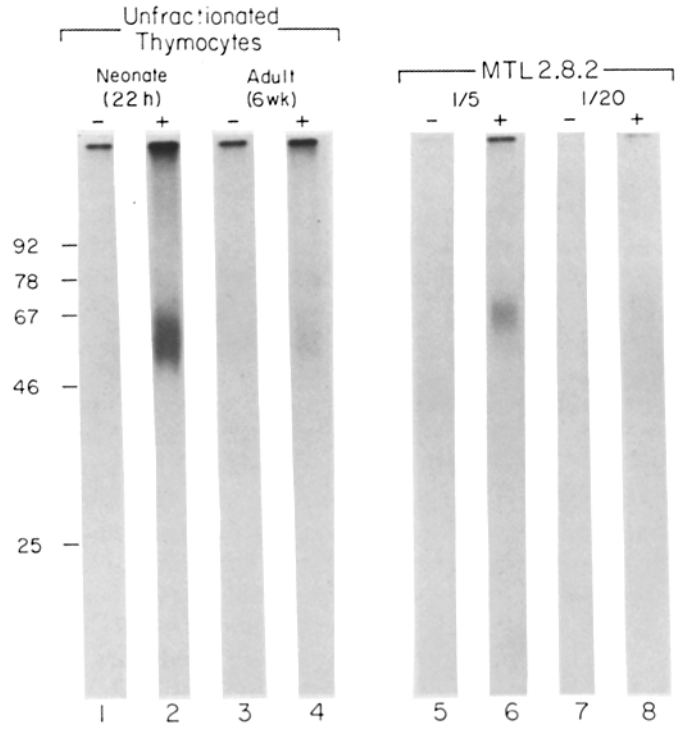


FIGURE 5. Expression of surface IL-2-R on neonatal and adult thymocytes. Total thymocytes from neonatal mice (≤22-h old) and from adult mice (6-wk old) were each surface-iodinated with ^{125}I, washed, and lysed. Aliquots of each lysate containing 5×10^6 cpm of acid-precipitable radiolabeled proteins were analyzed by immune precipitation with saturating amounts of 7D4 (+) or control antibody AD4.15 (−) in *1–4*. These samples were electrophoresed in parallel with immune precipitates from surface-labeled MTL2.8.2 cell lysates (*5–8*). In *5* and *6*, the radioactive input before immune precipitation was 10^6 cpm; in *7* and *8*, 2.5×10^5 cpm. The slight mobility difference between the IL-2-R in thymocytes and in MTL2.8.2 cells was reproducible.

do express IL-2-R are easily identified, and fall within a phenotypically distinct minority class that binds PNA weakly (although detectably; data not shown). The amount of IL-2-R that can be immune-precipitated from each of the cell lysates reflects the frequency of IL-2-R^+ cells found in that sample. Also, the mAb used here, 7D4, binds to the receptor distally from the IL-2-binding site (16). While it effectively blocks T cell proliferation (15, 32, 37), and binds both high- and low-affinity forms of the receptor (38), its own recognition of the receptor is not inhibited by the presence of bound IL-2. Therefore, we should be able to detect intrathymic IL-2-R whether or not they were engaged by ligand.

Antigen Recognition and IL-2-R Expression. There are several reasons why we expected proliferating thymocytes to express IL-2-R. IL-2-R are ubiquitous and probably rate-limiting in the proliferative response of T cell populations and cloned T cell lines. In these systems, contact with the correct antigen or a mitogenic lectin induces IL-2-R expression, after which IL-2 and only IL-2 can drive the cells into S phase (31, 33, 37, 39). Furthermore, the probability that a given dose of IL-2 can stimulate DNA synthesis in a cell is directly related to the

amount of IL-2-R expressed by that cell. For expected physiological concentrations of IL-2, the amount of receptor that is adequate for responsiveness is well above the threshold of detection (31). Thus, IL-2 is sufficient to drive T cell proliferation as long as IL-2-R expression is not limiting. Whether IL-2 and its receptor are absolutely necessary is less certain. mAb against IL-2-R may not completely block T cell proliferation under certain conditions of stimulation in vitro (32). However, it is technically difficult to saturate cell cultures with anti-receptor antibodies, since the binding constant of the high-affinity receptors for IL-2 is at least an order of magnitude greater than that for the antibody (38). In other experimental systems, it is clear that anti-receptor antibody can inhibit >99% of a T cell proliferative response (37). Finally, a striking point in all these studies is that antigen- or lectin-activated peripheral T cells invariably express IL-2-R before they enter S phase, whatever the mechanism that drives their proliferation (32, 33, 37, 39). Thus, while IL-2-R may be induced by stimuli other than antigen recognition (40), it is improbable that antigen can make T cells proliferate without inducing them to express IL-2-R.

Thymocytes that Express IL-2-R. If self antigen drives any thymocytes to divide, then the above arguments indicate that they would be among the cells expressing IL-2-R. This appreciably narrows the range of cell types to consider for antigen-driven selection. Still, the PNA^- blasts that include IL-2-R–bearing cells are a minuscule but complex population. They include medullary blast cells (41) as well as some cortical cells (1). Descendants of PNA^- blasts exhibit longer lifetimes than the average PNA^+ cortical cell, but which types of blast cells give rise to thymic emigrants is still controversial (42). The PNA^- population, which resembles peripheral T cells in many ways, is also likely to include any extrathymic T cell contaminants. The evidence that IL-2-R–bearing cells are not exclusively mature comes from their abundance in the neonatal thymus. Any members of this class that are demonstrably immature (for example, lack any number of T cell specific cell surface antigens) are the best candidates for cells undergoing repertoire selection during ontogeny.

High-level IL-2-R expression shortly after birth is provocative in its own right. Not only is the thymus undergoing a long-term size increase, but the periphery is also being seeded with thymic emigrants. The net T cell ouptut per thymic lymphoblast may be greater in the neonate than in the adult, where a larger number of lymphoblasts simply maintain the T cell population in steady state. Thus, IL-2-R expression on proliferating thymocytes, whether or not it is triggered by antigen, may be a characteristic of cell division that gives rise to functional progeny cells. This appears to be a rare event in the mature thymus.

It is not certain that the IL-2-R–bearing cells are actually proliferating in response to IL-2. We do not know whether IL-2 is available to these cells in the thymus, nor whether the receptor structures we detect here could bind IL-2 with a high affinity. In fact, isolated thymic lymphoblasts do not maintain their DNA synthesis in vitro in the presence of saturating levels of IL-2 (S. Krishnan, D. Triglia, and E. Rothenberg, unpublished observations). This may, however, have several explanations, only one of which is inadequate receptor function. IL-2 may be necessary but not sufficient for thymocyte growth, or receptor expression may need to be reinduced after each cell division, in contrast to expression

in mature cells (33). Thus, even if IL-2 is present in the thymus, receptor-bearing lymphoblasts may require additional stimulation in order to divide.

Predominance of IL-2-independent Proliferation in the Thymus. Considering the thymocyte population as a whole, novel mitogenic mechanisms are not only possible, but necessary. We have found that most of the proliferating cells in the thymus neither actively synthesize IL-2-R nor detectably express them on their surfaces. For these "receptor-negative" cells to be able to proliferate in response to IL-2, the background level of receptors they express would all have to be of very high affinity, even if IL-2 were present at high concentration. As discussed above, this is not the general case for stimulated T cells (31, 38). These results strongly suggest that most thymic lymphoblasts proliferate by one or more IL-2-independent mechanisms, in sharp contrast to activated peripheral T cells.

The clearest discrepancy between proliferation and IL-2-R expression is in the PNA^+ blast cells. These constitute the majority (~70%) of dividing cells in the thymus, are located in the cortex (1), and divide with average cell cycle times of 8–9 h (6). It is these cells which continually give rise to the small cortical thymocytes (27) that demonstrate neither immunological reactivity nor an ability to leave the thymus, and survive no more than 3–4 d after they appear (6, 9). It is also the PNA^+ cell class that expresses high levels of the potentially mutagenic DNA polymerase, terminal deoxynucleotidyl transferase (27, 28). If the thymus eliminates T cell clones that could be triggered by self antigens, one would assume that these were the self-reactive cells. The failure of PNA^+ cells to express IL-2-R, however, indicates that self antigen is unlikely to be driving their proliferation, whatever role it may play in bringing about their death. The implication is that thymic stromal cells may actively induce thymocytes to divide (5), rather than simply display antigenic targets for a stereotyped immune response. Such a mechanism would not only be a major aspect of normal T cell development, but could also represent a key factor in thymic leukemogenesis.

In summary, we have provided evidence that most adult thymic lymphoblasts are triggered to divide by a mitogenic mechanism or mechanisms unlike that used by mature T cells. Elucidation of this mechanism could help to explain why the thymus apparently overproduces and then destroys so many lymphocytes throughout life. We have also demonstrated that the neonatal thymus is enriched for cells which do express this receptor, where they most likely comprise a much higher proportion of the dividing cells than in the adult. It is interesting to speculate that this reflects the proportionally higher output of functional T cells by the neonatal thymus. Further research should determine whether or not IL-2-R expression discriminates between two distinct classes of lymphoblasts with different developmental fates.

Summary

We have tested the dividing cells in the mouse thymus for expression of interleukin 2 (IL-2) receptors (IL-2-R) using the rat monoclonal antibody 7D4. A discrete subpopulation of the lymphoblasts clearly expressed IL-2-R at levels comparable to those on mitogen-activated peripheral T cells. This subpopulation, however, represented a small minority of the proliferating cells. IL-2-R–bearing cells were depleted from the PNA^+ (peanut agglutinin) lymphoblast population,

which contains the direct precursors of most of the cells in the thymus. The majority of receptor-bearing cells were found in the PNA^- lymphoblast population, where they constituted only ~12% of the cells. Thus, virtually all the PNA^+ and most of the PNA^- blast cells were in cycle without detectable IL-2-R expression. This indicates that they were not dividing in response to IL-2, and implies that they were not dividing in response to antigen, but rather to novel thymus-specific mitogenic stimuli. On the other hand, the proliferating cells that do express IL-2-R were enriched 4–5-fold in the rapidly growing neonatal thymus, suggesting that they may also play a key role in T cell development.

We wish to thank Joseph Trotter, for his valuable help with the DNA content measurements, and Drs. Barry Caplan, Eric Davidson, Lee Hood, Mitchell Kronenberg, and Elias Lazarides for their helpful criticism of this manuscript.

Received for publication 2 January 1985 and in revised form 5 February 1985.

References

1. Scollay, R., and K. Shortman. 1983. Thymocyte subpopulations: an experimental review, including flow cytometric cross-correlations between the major murine thymocyte markers. *Thymus.* 5:245.
2. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, P. A. Klein, and J. Klein. 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J. Exp. Med.* 147:882.
3. Fink, P. J., and M. J. Bevan. 1978. H-2 antigens of the thymus determine lymphocyte specificity. *J. Exp. Med.* 148:766.
4. Metcalf, D. 1966. The structure of the thymus. *In* The Thymus, Recent Results in Cancer Research. D. Metcalf, editor. Springer-Verlag, Berlin. 5:1.
5. Kyewski, B. A., M. Travis, and H. Kaplan. 1984. Intrathymic lymphopoiesis: stromal cell–associated proliferation of T cells is independent of lymphocyte genotype. *J. Immunol.* 133:1111.
6. Bryant, B. J. 1972. Renewal and fate in the mammalian thymus. Mechanisms and inferences of thymocytokinetics. *Eur. J. Immunol.* 2:38.
7. Weissman, I. L. 1967. Thymus cell migration. *J. Exp. Med.* 126:291.
8. Scollay, R. G., E. C. Butcher, and I. L. Weissman. 1980. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur. J. Immunol.* 10:210.
9. McPhee, D., J. Pye, and K. Shortman. 1979. The differentiation of T lymphocytes. V. Evidence for intrathymic death of most thymocytes. *Thymus.* 1:151.
10. Smith, K. A. 1984. Interleukin-2. *Ann. Rev. Immunol.* 2:337.
11. Robb, R. J., A. Munck, and K. A. Smith. 1981. T-cell growth factor receptors: quantitation, specificity and biological relevance. *J. Exp. Med.* 154:1455.
12. Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur. J. Immunol.* 1:1.
13. von Boehmer, H., W. Haas, and N. K. Jerne. 1978. Major histocompatibility complex–linked immune-responsiveness is acquired by lymphocytes of low-responder mice differentiating in thymus of high-responder mice. *Proc. Nat. Acad. Sci. USA.* 75:2439.
14. Rock, K. L., and B. Benacerraf. 1984. The role of Ia molecules in the activation of

T lymphocytes. IV. The basis of the thymocyte IL-1 response and its possible role in the generation of the T-cell repertoire. *J. Immunol.* 132:1654.

15. Malek, T. R., R. J. Robb, and E. M. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin-2 receptor-ligand complex. *Proc. Nat. Acad. Sci. USA.* 80:5694.
16. Ortega, R. G., R. J. Robb, E. M. Shevach, and T. R. Malek. 1984. The murine IL-2 receptor. I. Monoclonal antibodies that define distinct functional epitopes on activated T cells and react with activated B cells. *J. Immunol.* 133:1970.
17. Osawa, H., and T. Diamantstein. 1984. A rat monoclonal antibody that binds specifically to mouse T lymphoblasts and inhibits IL-2 receptor functions: a putative anti–IL-2 receptor antibody. *J. Immunol.* 132:2445.
18. Osawa, H., and T. Diamantstein. 1983. The characteristics of a monoclonal antibody that binds specifically to rat T lymphoblasts and inhibits IL-2 receptor functions. *J. Immunol.* 130:51.
19. Hemler, M. E., B. Malissen, N. Rebai, A. Liabeuf, C. Marvas, F. M. Kourilsky, and J. L. Strominger. 1983. A 55,000 M_r surface antigen on activated human T lymphocytes defined by a monoclonal antibody. *Human Immunol.* 8:153.
20. Robb, R. J., and W. C. Greene. 1983. Direct demonstration of the identity of T cell growth factor-binding protein and the Tac antigen. *J. Exp. Med.* 158:1332.
21. Rothenberg, E. 1982. A specific biosynthetic marker for immature thymic lymphoblasts. Active synthesis of thymus leukemia antigen restricted to proliferating cells. *J. Exp. Med.* 155:140.
22. Caplan, B. and E. Rothenberg. 1984. High-level secretion of interleukin-2 by a subset of proliferating thymic lymphoblasts. *J. Immunol.* 133:1983.
23. Bleackley, R. C., C. Havele, and V. Paetkau. 1982. Cellular and molecular properties of an antigen-specific cytotoxic T lymphocyte line. *J. Immunol.* 128:758.
24. Gottlieb, P. D., A. Marshak-Rothstein, K. Auditore-Hargreaves, D. B. Berkoben, D. A. August, R. M. Rosche, and J. D. Benedetto. 1980. Construction and properties of new Lyt-congenic strains and anti-Lyt-2.2 and anti-Lyt-3.1 monoclonal antibodies. *Immunogenetics.* 10:545.
25. Siadak, A. W. and R. C. Nowinski. 1980. Identification of Ly-5 and T200 antigens on identical cell surface proteins. *J. Immunol.* 125:1400.
26. Rothenberg, E. and E. A. Boyse. 1979. Synthesis and processing of molecules bearing thymus leukemia antigen. *J. Exp. Med.* 150:777.
27. Rothenberg, E. 1980. Expression of differentiation antigens in subpopulations of mouse thymocytes: regulation at the level of de novo synthesis. *Cell.* 20:1.
28. Rothenberg, E. and D. Triglia. 1983. Clonal proliferation unlinked to terminal deoxynucleotidyl transferase synthesis in thymocytes of young mice. *J. Immunol.* 130:1627.
29. Leonard, W. J., J. M. Depper, G. R. Crabtree, S. Rudikoff, J. Pumphrey, R. J. Robb, M. Krönke, P. B. Svetlik, N. J. Peffer, T. A. Waldmann, and W. C. Greene. 1984. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature (Lond.).* 311:626.
30. Wano, Y., T. Uchiyama, K. Fukui, M. Maeda, H. Uchino, and J. Yodoi. 1984. Characterization of human interleukin-2 receptor (Tac antigen) in normal and leukemic T cells: coexpression of normal and aberrant receptors on Hut-102 cells. *J. Immunol.* 132:3005.
31. Cantrell, D. A. and K. A. Smith. 1984. The interleukin-2 T-cell system: a new cell growth model. *Science (Wash. DC).* 224:1312.
32. Malek, T. R., R. G. Ortega, J. P. Jakway, C. Chan, and E. M. Shevach. 1984. The

murine IL-2 receptor. II. Monoclonal anti–IL-2 receptor antibodies as specific inhibitors of T-cell function in vitro. *J. Immunol.* 133:1976.

33. Reske-Kunz, A. B., D. von Steldern, D. Rüde, H. Osawa, and T. Diamantstein. 1984. Interleukin 2 receptors on insulin-specific T-cell line: dynamics of receptor expression. *J. Immunol.* 133:1356.
34. Schwartz, D. H. and P. C. Doherty. 1981. Virus-immune and alloreactive response characteristics of thymocytes and spleen cells from young mice. *J. Immunol.* 127:1411.
35. Piguet, P.-F., C. Irlé, E. Kollatte, and P. Vassalli. 1981. Post-thymic T lymphocyte maturation during ontogenesis. *J. Exp. Med.* 154:581.
36. Mandel, T. E. and M. M. Kennedy. 1978. The differentiation of murine thymocytes in vivo and in vitro. *Immunology.* 35:317.
37. Kaye, J., S. Gillis, S. B. Mizel, E. M. Shevach, T. R. Malek, C. A. Dinarello, L. B. Lachman, and C. A. Janeway, Jr. 1984. Growth of a cloned helper T-cell line induced by a monoclonal antibody specific for the antigen receptor: interleukin-1 is required for the expression of receptors for interleukin-2. *J. Immunol.* 133:1339.
38. Robb, R. J., W. C. Greene, and C. M. Rusk. 1984. Low and high affinity cellular receptors for interleukin 2. Implications for the level of Tac antigen. *J. Exp. Med.* 160:1126.
39. Hemler, M. E., M. B. Brenner, J. M. McLean, and J. L. Strominger. 1984. Antigenic stimulation regulates the level of expression of interleukin-2 receptor on human T cells. *Proc. Nat. Acad. Sci. USA.* 81:2172.
40. Meuer, S. C., R. E. Hussey, M. Fabbi, D. Fox, O. Acuto, K. A. Fitzgerald, J. C. Hodgdon, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz. 1984. An alternative pathway of T-cell activation: a functional role for the 50 kd T11 sheep erythrocyte receptor protein. *Cell.* 36:387.
41. Goldschneider, I., K. Shortman, D. McPhee, S. Linthicum, G. Mitchell, F. Battye, and F. J. Bollum. 1982. Identification of subsets of proliferating low Thy 1 cells in thymus cortex and medulla. *Cell. Immunol.* 69:59.
42. Scollay, R. 1983. Intrathymic events in the differentiation of T lymphocytes: a continuing enigma. *Immunol. Today.* 4:282.