

PRECURSORS OF T CELL GROWTH FACTOR PRODUCING  
CELLS IN THE THYMUS:

Ontogeny, Frequency, and Quantitative Recovery in a Subpopulation of  
Phenotypically Mature Thymocytes Defined by Monoclonal Antibody  
GK-1.5

BY RHODRI CEREDIG, DENO P. DIALYNAS,\* FRANK W. FITCH,\* AND  
H. ROBSON MACDONALD

*From the Ludwig Institute for Cancer Research, Lausanne Branch, CH-1066 Epalinges,  
Switzerland; and Department of Pathology, University of Chicago, Chicago, Illinois 60637*

The thymus of adult mice is considered to be the major anatomical site at which immunocompetent T lymphocytes are generated (1). However, the proportion of thymocytes possessing demonstrable immunocompetence is rather low, representing a subpopulation of 10–15% of cells in the thymus (2). Recently, the phenotypic properties of thymocyte subpopulations have been characterized using monoclonal antibodies to cell surface determinants, and flow microfluorometry (FMF)<sup>1</sup> (3–5). When these techniques were combined with positive selection of thymocyte subpopulations and limit dilution analysis of mixed leukocyte microcultures, we were able to show that all thymic cytolytic T lymphocyte precursors (CTL-P) could be quantitatively recovered in a subpopulation of thymocytes that was medium-sized, Lyt-2<sup>+</sup>, H-2K<sup>+</sup> and weakly stained with Thy-1 (5). These results demonstrated that all thymic CTL-P were contained in a subpopulation of cells having a so-called “mature” phenotype.

Another functional property attributable to immunocompetent T lymphocytes in peripheral lymphoid organs is their ability to produce lymphokines, including T cell growth factor (TCGF), when stimulated by specific antigens or mitogens (6). Thymocytes are clearly capable of producing TCGF in bulk cultures (5, 6) but the activity detected is generally low compared with peripheral T cells and at the present time no data are available on the frequency of TCGF-producing cells among thymocyte subpopulations.

During mouse fetal development, the thymus anlage is colonized by blood-borne hematopoietic cells at around 10 d of embryonic development (7). This initial population of thymocytes has a blast-like morphology, but by 13 d begins

\* D. P. Dialynas and F. W. Fitch were supported by U. S. Public Health Service Grant Nos. AI-04197 and CA-19266.

<sup>1</sup> *Abbreviations used in this paper:* a.u., arbitrary unit; C, rabbit complement; Con A, concanavalin A; CRT, cortisone-resistant thymocytes; CTL-P, cytolytic T lymphocyte precursor; DME, 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; MHC, major histocompatibility complex; micro-MLC, mixed leukocyte microcultures; SN, supernatant; TCGF, T cell growth factor (also known as interleukin-2).

to proliferate and differentiate to generate the subpopulations of thymocytes present in the adult mouse thymus (8). Initially, embryonic thymocytes show no demonstrable immunocompetence but, depending on mouse strain, CTL-P begin to appear in the thymus around birth (9, 10), approximately the 20th day of embryonic development. At the present time, no data are available on the ontogeny of TCGF production by mouse embryonic thymocytes.

In the experiments reported herein, a recently described monoclonal antibody designated GK-1.5, developed by Dialynas et al.,<sup>2</sup> was used to define thymocyte subpopulations. Using this reagent and a limiting dilution microculture system in which the frequency of TCGF-producing cells may be enumerated (11), it was possible to quantitatively recover all precursors of alloreactive TCGF-producing cells in a subpopulation of phenotypically "mature" GK-1.5-positive Lyt-2-negative thymocytes. Furthermore, the appearance of this subpopulation of thymocytes during embryonic development was correlated with the ontogeny of TCGF production by the thymus.

### Materials and Methods

*Mice.* Adult male and female mice of strains CBA/JT6 (H-2<sup>k</sup>, Mls<sup>b</sup>), C57BL/6 (H-2<sup>b</sup>, Mls<sup>b</sup>), and DBA/2 (H-2<sup>d</sup>, Mls<sup>a</sup>) 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, ME. Fetal mice were obtained from timed matings as previously described (8). The date of finding a vaginal plug was designated as day 0 of embryonic development.

*Cell Suspensions.* Single cell suspensions were prepared in Dulbecco's Modified Eagle's medium (DME) containing 5% (vol/vol) fetal bovine serum (FBS) and 10 mM Hepes buffer. For the preparation of adult thymocyte suspensions, mice were killed by ether anesthesia and the thymuses removed free of parathymic lymph nodes. Fetal thymuses were removed using a dissecting microscope and fine watchmaker's forceps as previously described. Cortisone-resistant thymocytes (CRT) from adult mice were removed 48 h after a single intraperitoneal injection of 4 mg hydrocortisone acetate (Hydrocortifor, Vifar, Geneva).

*Antibodies.* Monoclonal rat IgG antibody (53-6.7) directed against nonpolymorphic determinants of the Lyt-2 molecule was provided by Dr. J. Ledbetter, Genetic Biosystems Inc., Seattle, WA. Monoclonal rat IgM antibody to nonpolymorphic determinants of Lyt-2 (3.168.8.1) and anti-Thy-1.2 antibody (AT 83) have been previously described (5). Monoclonal rat IgG2b antibody GK-1.5 was obtained from a fusion of a mouse nonsecretor hybridoma line SP2/0 and spleen cells from a rat hyperimmunized with an Lyt-2-negative T cell clone 243/2.5. Details of production and biological activity of GK-1.5 have been recently reported (12). Fluorescein isothiocyanate (FITC)-coupled rabbit anti-rat immunoglobulin was obtained from Nordic Immunological Laboratories, Tilburg, The Netherlands.

For negative selection experiments with anti-Lyt-2 antibodies, fetal thymocytes, normal adult thymocytes, or CRT ( $50 \times 10^6$ /ml) were incubated with a 1:10 dilution of anti-Lyt-2 (3.168.8.1) hybridoma culture supernatant together with a 1:20 dilution of rabbit complement (C) (Cedarlane Laboratories, Hornsby, Canada) for 45 min at 37°C. Treated cells were passed over Ficoll-Hypaque ( $d = 1.077$ ) and washed twice in DME. T cell depleted DBA/2 spleen cells were prepared as previously described (13).

*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,

<sup>2</sup> Dialynas, D. P., Z. S. Quan, K. A. Wall, A. Pierres, J. Quintans, M. R. Loken, M. Pierres, and F. W. Fitch. 1983. Identification of a non-LFA-1, non-Lyt-2,3 cell surface molecule, designated L3T4, which may play a role in the recognition of murine class II MHC antigen. *J. Immunol.* In press.

as previously described (5). Control samples were stained with the FITC-coupled second step reagent alone. Flow cytometric analysis was performed using a Spectra-Physics laser (Spectra-Physics Inc., Mountain View, CA) and a conventional FACS II electronics system modified to allow 3-parameter sorting (14). Viable cells were routinely gated by a combination of narrow-angle forward light scatter (FLS) and perpendicular light scatter (5).

*FACS Sorting.* This was carried out as previously described (5). Positively and negatively selected cells were collected in sterile plastic tubes whose inside surfaces had been coated with sterile FBS. In all instances in which the functional activity of sorted cells was tested, control cells were passed through the FACS and collected unseparated.

*Mixed Leukocyte Microcultures (Micro-MLC).* Micro-MLC were prepared as previously described (5, 11) in DME containing additional amino acids (15), 10 mM HEPES,  $5 \times 10^{-5}$  M 2-mercaptoethanol, and 10% (vol/vol) FBS, and 1/30 final concentration of a TCGF-containing supernatant (SN) was prepared from a subclone of an EL-4 thymoma subline, originally provided by Dr. J.J. Farrar (NIH, Bethesda, MD) (EL-4 SN) (16). Limiting numbers of CBA responder cells were cultured (24–36 microcultures per cell dose) with  $0.5\text{--}1.0 \times 10^6$ , 2,000 rad-irradiated T cell-depleted DBA/2 spleen stimulator cells in 200  $\mu$ l in round-bottomed microwells (Greiner, Nürtingen, West Germany) and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 10–14 d, cultures were scored microscopically for growth, and restimulated by washing each well three times to remove any residual TCGF and then adding  $0.5\text{--}1.0 \times 10^6$  irradiated T cell-depleted DBA/2 spleen cells in 200  $\mu$ l DME with 5% FBS. After 24 h of incubation, plates were centrifuged and 150  $\mu$ l of SN was removed from each well.

*Production of TCGF in Bulk Cultures.* Bulk cultures were established in the wells of Costar plates (3524; Costar, Cambridge, MA) using  $5 \times 10^6$  Lyt-2-negative responder cells and either  $5 \times 10^6$  T cell-depleted H-2 allogeneic 2,000-rad-irradiated spleen stimulator cells or an equal number of T cell-depleted irradiated syngeneic spleen cells together with 5  $\mu$ g/ml concanavalin-A (Con-A, Pharmacia Fine Chemicals, Uppsala, Sweden) in a total volume of 2 ml DME supplemented as for micro-MLC (see above). After 24 or 48 h of incubation at 37°C, supernatants (SN) from bulk cultures were harvested and assayed for their TCGF content.

*Assay of TCGF.* TCGF activity in bulk culture or microculture SN was carried out as previously described (11) using a cloned TCGF-dependent T cell line, CTLL, provided by Dr. K. A. Smith, Dartmouth Medical School, Hanover, NH. For the assay, CTLL were washed extensively and  $4 \times 10^5$  cells incubated with 75  $\mu$ l microculture SN in a total of 150  $\mu$ l DME with 5% FBS at 37°C in flat-bottomed microwells (3596; Costar, Data Packaging, Cambridge, MA). After 24 h, 1  $\mu$ Ci methyl-[<sup>3</sup>H]thymidine (2.0 Ci/mmol; Amersham International Ltd., Amersham, England) was added for a further 4–5 h. Cells were harvested onto filter paper strips using an automated cell harvester (Dynatech Produkte AG, Kloten, Switzerland) and counted in a liquid scintillation counter.

*Statistical Methods.* For TCGF assays of microcultures, positive microcultures were defined as those in which activity exceeded that in control wells (no responder cells) by more than 3 SD above the mean. Minimal estimates of precursor frequencies were obtained by the minimum chi-square method from the Poisson distribution relationship between the responding cell number and the logarithm of the percentage of nonresponding cultures (17).

## Results

*Phenotypic Properties of Adult Thymocytes and CRT Stained with GK-1.5.* When normal adult (>4 wk) thymocytes were stained by indirect immunofluorescence with GK-1.5 followed by FITC-conjugated second step reagent and analyzed by FMF, distinct subpopulations of positive and negative cells were observed (Fig. 1). In a series of 10 such experiments,  $91 \pm 3\%$  (mean  $\pm$  SD) of cells stained positively with this monoclonal reagent. By simultaneous analysis of forward

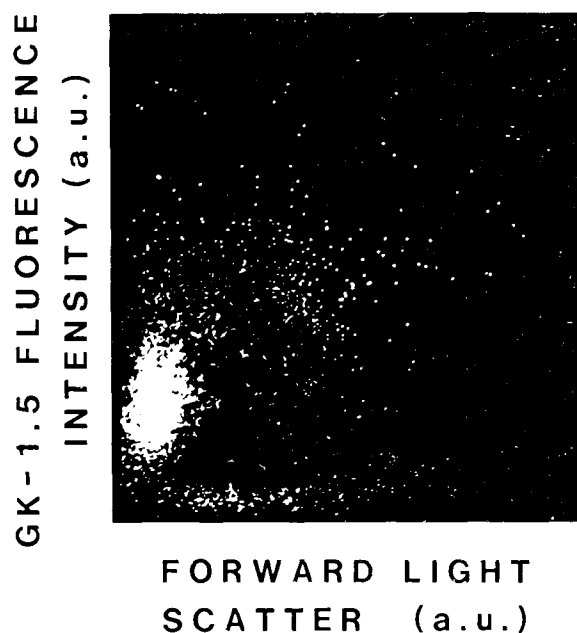


FIGURE 1. FMF analysis of adult CBA/J mouse thymocytes stained with monoclonal antibody GK-1.5. The photograph correlates the fluorescence intensity and forward light scatter (a cell size-related parameter) of 5,000 individual cells.

light scatter (FLS) and fluorescence intensity, GK-1.5-positive cells could be further subdivided into a major population of smaller, presumably cortical, cells having a mean FLS value of 100 arbitrary units (a.u.) and a minor population of larger blast cells with a mean FLS value  $>140$  (Fig. 1). GK-1.5-negative thymocytes had a heterogeneous FLS profile and in the adult thymus could be subdivided into smaller and larger subpopulations (*vide infra*).

Cortisone-resistant thymocytes (CRT) represent a subpopulation of thymocytes whose phenotypic and functional properties are indistinguishable from peripheral T lymphocytes (5, 18). When CRT were stained with GK-1.5, a distinct subpopulation of positive cells was seen (top right panel, Fig. 2). The mean FLS of GK-1.5-positive CRT (114) was lower than that of GK-1.5-negative cells (120). In a series of four experiments,  $64 \pm 6\%$  (mean  $\pm$  SD) of CRT stained positively with GK-1.5. In contrast, as shown previously (5), 34% of CRT stained positively with a monoclonal anti-Lyt-2 antibody (Fig. 2, top left panel).

Two approaches were taken in order to demonstrate that GK-1.5 and Lyt-2 were present on distinct subpopulations of CRT. Firstly, when stained with both antibodies essentially all CRT were stained (data not shown). Secondly, Lyt-2-negative CRT, obtained by treating the cells with the cytotoxic monoclonal anti-Lyt-2 antibody (3.168.8-1) and C, were subsequently stained with GK-1.5. Control staining of the cells recovered after this treatment indicated that no Lyt-2-positive cells remained (bottom left panel, Fig. 2). However, when stained with GK-1.5, 98% of Lyt-2-negative CRT were GK-1.5-positive (bottom right panel). The small number (2%) of GK-1.5-negative Lyt-2-negative CRT may

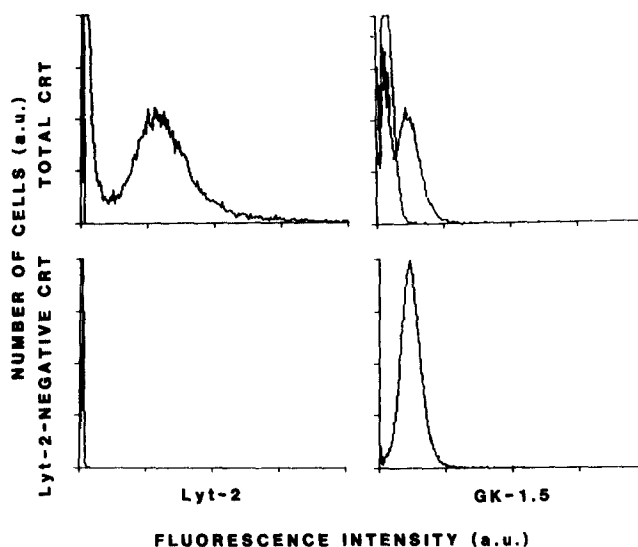


FIGURE 2. GK-1.5 and anti-Lyt-2 monoclonal antibodies stain mutually exclusive subpopulations of CRT. CRT (top two panels) or Lyt-2-negative CRT, obtained by monoclonal anti-Lyt-2 antibody plus  $C'$  treatment of CRT (bottom two panels), were stained by indirect immunofluorescence with either monoclonal anti-Lyt-2 (left two panels) or GK-1.5 (right two panels). CRT contain subpopulations of cells staining positively with either anti-Lyt-2 or GK-1.5 antibodies but following elimination of Lyt-2-positive CRT (bottom left panel) almost all (98%) of Lyt-2-negative CRT were GK-1.5-positive.

represent a contamination of CRT by small numbers of nonlymphoid cells or thymic blast cells, since a similar proportion of Lyt-2-negative CRT did not stain with a monoclonal anti-Thy-1.2 antibody (data not shown).

*Ontogeny of GK-1.5 Expression in the Developing Thymus.* Subpopulations of mouse thymocytes as defined by monoclonal antibodies to cell surface determinants and FMF are known to appear in the embryonic thymus at distinct times during ontogeny (8, 19). Therefore it was of interest to determine at what age GK-1.5-positive cells first appeared in the developing thymus. The 15-d thymus did not contain GK-1.5-positive cells (data not shown). As shown in Fig. 3, the 16-d embryonic thymus contained only 3% weakly-staining GK-1.5-positive cells, but by 17 d, a clearly distinct subpopulation of positive cells was seen. The proportion of positive cells at 17 d was  $53 \pm 18\%$  (Table I) and this increased to  $78 \pm 5\%$  by 18 d and  $89 \pm 2\%$  by 19 d. At birth, the 20th day of embryonic development in CBA mice,  $92 \pm 2\%$  of thymocytes stained positively with GK-1.5, a value similar to that found in adult mice (see above). As also shown in Table I, there was no dissociation between the ontogeny of GK-1.5-positive and Lyt-2-positive thymocytes in the early embryonic thymus.

When stained with both GK-1.5 and anti-Lyt-2 antibodies simultaneously the proportion of GK-1.5-negative, Lyt-2-negative blast cells was found to decrease from 83% at day 16 to 23% at day 17, 13% at day 18, 7% at day 19, and 3% in the adult thymus (Table I).

In the embryonic mouse thymus, changes in the FLS profile of thymocytes occur at distinct stages of development (8, 19). As shown in Fig. 3, the 16-d

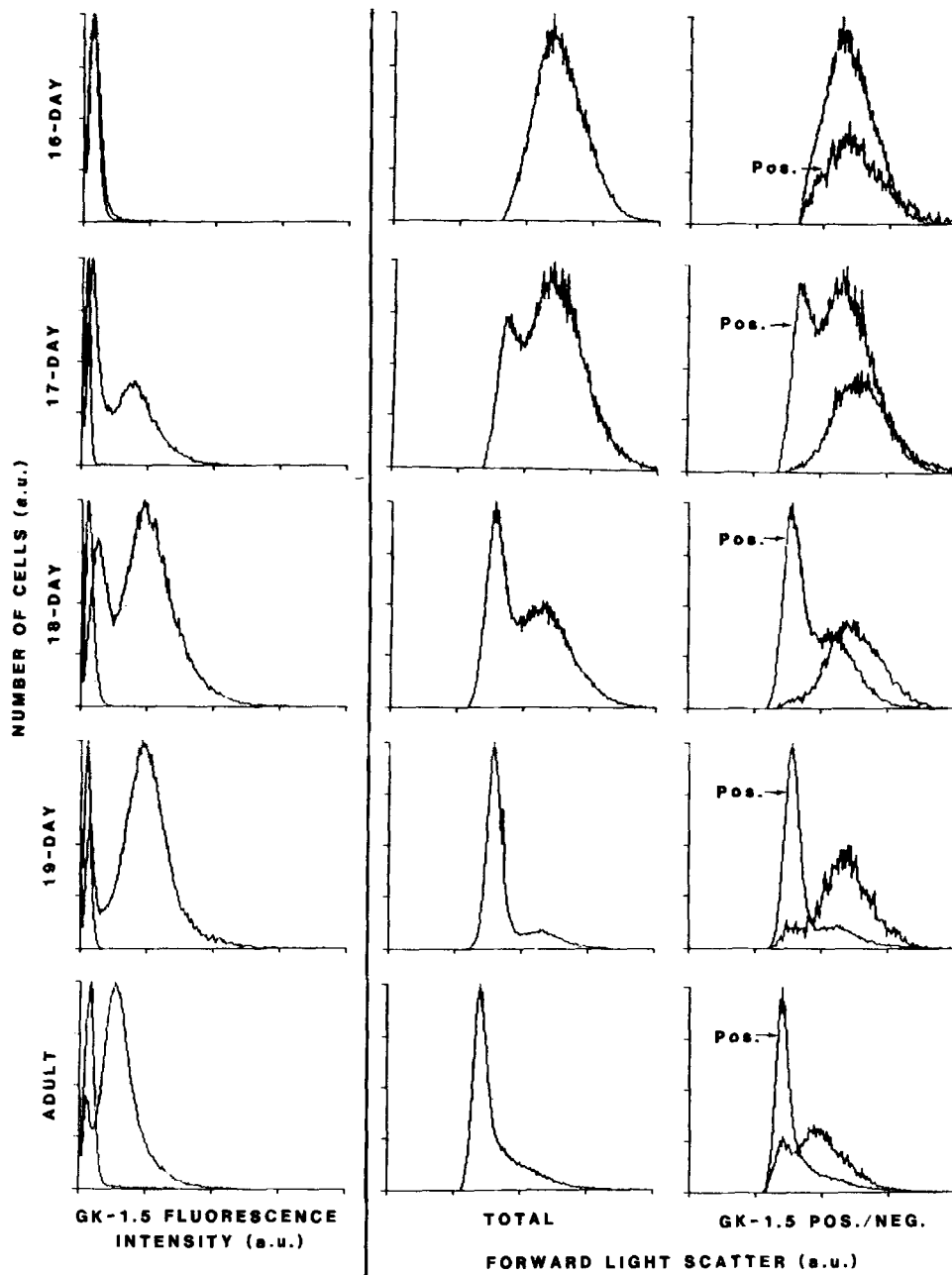


FIGURE 3. Ontogeny of GK-1.5 expression in the developing thymus. The left hand panels show the fluorescence histograms of 16-d, 17-d, 18-d, 19-d embryonic and adult CBA/J thymocytes stained by indirect immunofluorescence with monoclonal antibody GK-1.5. The 16-d embryonic thymus contains few GK-1.5-positive cells, but by 17-d a distinct peak of positive cells is seen. The middle histograms show the FLS distribution of all cells in the embryonic thymus. In each case, the FLS of embryonic thymocytes was normalized to that of the adult shown in the bottom panel. Starting from a homogeneous population of large cells at 16-d, a subpopulation of smaller (cortical) thymocytes appears to the left of the major peak on 17-d and the proportion of such smaller cells increases during thymus development. In the right-hand panels, the gated FLS profile of GK-1.5-positive and negative cells are shown superimposed, normalized arbitrarily to show the differences between each subpopulation. GK-1.5-positive cells are initially large but then contain both smaller and larger cells starting at 17-d. GK-1.5-negative cells remain homogeneously large until 19-d, when a second subpopulation of smaller GK-1.5-negative cells is observed.

TABLE I  
Ontogeny of GK-1.5 Expression in the Developing Thymus

Age	Percent cells		
	GK-1.5 <sup>+</sup>	Lyt-2 <sup>+</sup>	GK-1.5 <sup>-</sup> , Lyt-2 <sup>-*</sup>
15-d fetal	0	0	100
16-d fetal	3	16 ± 6	83
17-d fetal	53 ± 18	54 ± 12	23
18-d fetal	78 ± 5	75 ± 14	13
19-d fetal	89 ± 2	78 ± 9	7 ± 0.5
Birth	92 ± 2	87 ± 2	ND <sup>‡</sup>
7-d neonatal	94	89 ± 1	ND
4-wk adult	91 ± 3	82 ± 5	3 ± 0.5

\* Values obtained by staining with both antibodies simultaneously.

<sup>‡</sup> ND, not done.

thymus contains a homogeneous population of large cells but by 17 d, a distinct subpopulation of smaller cells (as measured by FLS) appears in the thymus. The proportion of such smaller thymocytes increases rapidly during subsequent thymus development with the adult organ containing predominantly smaller cells (Fig. 3). Using the fluorescence profiles shown on the left side of Fig. 3, the gated FLS profiles of GK-1.5-positive and -negative cells were obtained. Thus, the subpopulation of smaller thymocytes that first appeared on the 17th day appeared to be GK-1.5-positive. However, a distinct subpopulation of larger cells was also stained by this reagent at this time. The proportion of such large GK-1.5-positive cells decreased during thymus development. These larger GK-1.5-positive cells, which may be seen as a distinct subpopulation in Fig. 1, comprise a tail on the right-hand side of the one-dimensional FLS histogram in Fig. 3.

As with GK-1.5-positive cells, changes in the FLS profile of GK-1.5-negative cells were also taking place during thymus development. However, such changes started later and were not as dramatic as those of the positive subpopulation. At 17 d, when the thymus contained smaller GK-1.5-positive cells, GK-1.5-negative cells were still a homogeneous population of larger cells (Fig. 3). By 18 d and more clearly by 19 d, a subpopulation of smaller GK-1.5-negative cells appeared in the thymus. In adult mice, the FLS profile of GK-1.5-negative thymocytes was clearly bimodal with smaller and larger cells being present in approximately equal proportions (Fig. 2).

*Phenotypic Properties of TCGF-Producing Cells in the Adult Thymus and CRT.* In a previous publication (5), we showed that most alloantigen-induced TCGF production by mouse thymocytes was attributable to the Lyt-2-negative subpopulation. These results were obtained by stimulating purified responder cells in bulk cultures; the precursor frequency of TCGF-producing cells was not determined. Recently, a microculture system was described in which the precursor frequencies of cells producing various lymphokines, including TCGF, among subpopulations of peripheral T lymphocytes were determined (11). In this report, this method has been used to determine TCGF precursor frequencies among subpopulations of thymocytes.

Adult CBA thymocytes were treated with monoclonal anti-Lyt-2 antibody and C and the Lyt-2-negative cells, purified as described in Materials and Methods, used as responder cells in micro-MLC. In a series of four preliminary experiments the mean frequency of TCGF-producing cells among Lyt-2-negative thymocytes was 1/192 (range 1/72-1/1,869). In all instances, a linear semi-logarithmic relationship existed between the proportion of negative cultures and the dose of responder cells cultured (*vide infra*), thus indicating that only one cell, namely the precursor of TCGF-producing cells, was limiting the response.

The GK-1.5 phenotype of TCGF-producing cells among CRT was also determined. In these experiments, the control CRT population was stained and passed through the FACS but collected unseparated. As shown in Table II, in two experiments the frequency of TCGF-producing cells among GK-1.5-positive CRT (which represented 73% of the total) was significantly increased above that in the GK-1.5-negative subpopulation. Thus 92% and 97%, respectively of TCGF-producing precursor cells were recovered in the GK-1.5-positive subpopulation. Since essentially all (98%) of Lyt-2-negative CRT were GK-1.5-positive (Fig. 2), taken together these results suggest that the phenotype of the vast majority of TCGF-producing cells in the adult thymus is GK-1.5-positive, Lyt-2-negative.

*Ontogeny of GK-1.5-Positive Lyt-2-Negative Thymocytes and its Correlation with the Ontogeny of TCGF Production.* As shown above, TCGF-producing cells in the adult thymus were of the GK-1.5-positive Lyt-2-negative phenotype. The age at which cells of this phenotype appeared in the developing thymus was therefore determined. FMF analysis of embryonic thymocytes stained with monoclonal anti-Lyt-2 antibody and gated according to their Lyt-2 phenotype showed that FLS changes occurred among Lyt-2-negative cells during development (Fig. 4). At 17 d, when a subpopulation of smaller cells began to appear in the thymus (Fig. 2) Lyt-2-negative thymocytes were still homogeneously large cells (Fig. 4). At birth, Lyt-2-negative thymocytes had a broad FLS profile with subpopulations of larger and smaller cells being identifiable. In the adult, the smaller Lyt-2-negative subpopulation predominated.

The GK-1.5 phenotype of Lyt-2-negative embryonic and adult thymocytes was determined by staining such cells obtained following monoclonal antibody

TABLE II  
*Frequencies of TCGF-Producing Cells Among CRT Sorted According to GK-1.5 Phenotype\**

Experiment	Sorted population	Frequency	% Sorted	% Recovery
1	Total	1/890	100	100
	GK-1.5 <sup>+</sup>	1/295	73	92
	GK-1.5 <sup>-</sup>	1/1,237	27	8
2	Total	1/43	100	100
	GK-1.5 <sup>+</sup>	1/23	73	97
	GK-1.5 <sup>-</sup>	1/285	27	3

\* CRT from CBA mice were sorted according to GK-1.5 fluorescence intensity (see Fig. 2, top right panel) and assayed for their content of TCGF-producing cells.



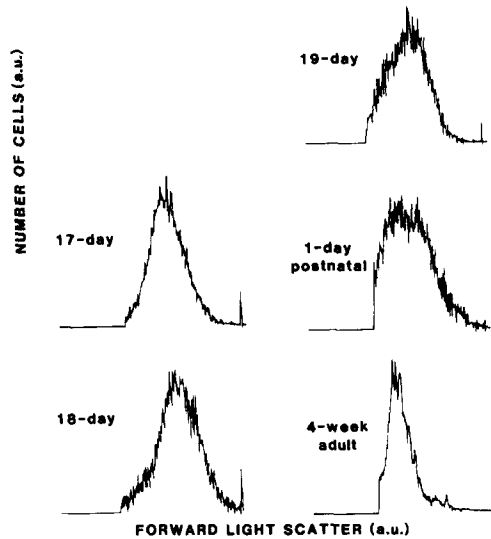


FIGURE 4. Changes in FLS of Lyt-2-negative thymocytes during mouse embryonic development. Each panel shows the gated FLS of Lyt-2-negative thymocytes from 17-d, 18-d, 19-d embryonic, 1-d postnatal and 4-wk adult CBA mice. Each histogram was normalized to the FLS of adult Lyt-2-negative thymocytes (bottom right). Starting as a homogeneous population of large cells at 17-d, a subpopulation of smaller Lyt-2-negative cells is seen at 19-d as a shoulder on the left side of the histogram. The 1-d postnatal thymus contains smaller and larger Lyt-2-negative cells in approximately equal proportions.

plus C treatment. GK-1.5-positive Lyt-2-negative thymocytes were first detected on day 18 of embryonic development (4%) and then increased to  $13 \pm 4\%$  at 19 d, 37% at birth, and 72% at 7 d. In the adult thymus,  $72 \pm 13\%$  of Lyt-2-negative thymocytes were GK-1.5-positive. Fig. 5 shows the phenotypic properties of Lyt-2-negative thymocytes obtained from 19-d embryonic and adult mice stained with GK-1.5. In this experiment, 14% of 19-d and 81% of adult cells were positively stained. As previously shown (Fig. 4), the FLS profiles of these two populations of Lyt-2-negative thymocytes differ, with adult cells being enriched in a smaller subpopulation of cells having a mean FLS value of 114. When the gated FLS profiles of GK-1.5-positive and negative Lyt-2-negative thymocytes were compared, the GK-1.5-positive cells were enriched in this smaller subpopulation. In contrast, GK-1.5-negative Lyt-2-negative thymocytes were homogeneously large cells with a mean FLS value  $>140$  at all stages of thymus development.

Since TCGF-producing cells in the adult thymus appeared to have the GK-1.5-positive, Lyt-2-negative phenotype, and since cells of this phenotype appeared in the developing thymus at  $\sim 18$ –19 d, it was of some interest to determine at what time TCGF production first appeared in the developing thymus. Using the TCGF assay as described herein, and separated Lyt-2-negative thymocytes stimulated in bulk cultures, it appeared that TCGF-producing cells were first detectable in the thymus on the 19th day of development (Fig. 6). Cells from the 18-d thymus did not produce detectable TCGF when stimulated with either allogeneic spleen stimulator cells (Fig. 6) or Con A in the presence

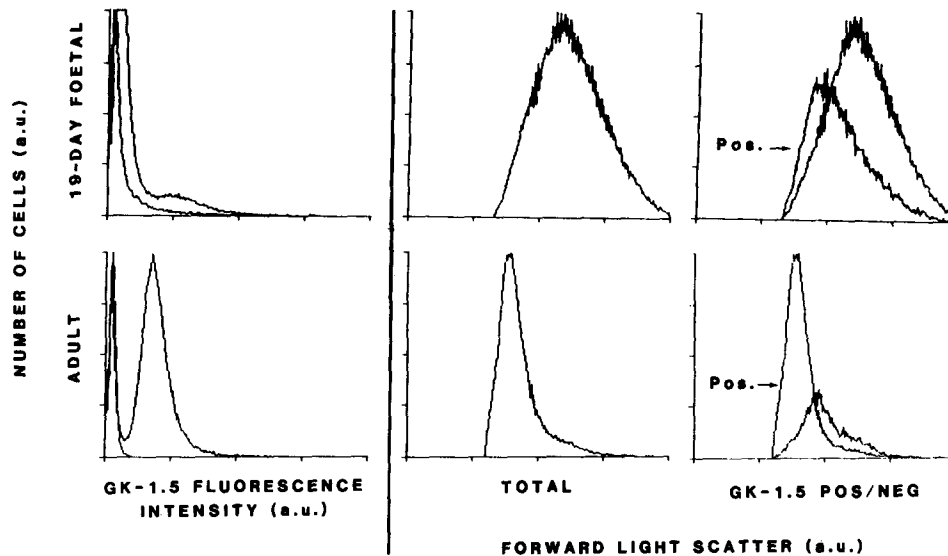


FIGURE 5. The GK-1.5 phenotype of Lyt-2-negative thymocytes from 19-d embryonic and adult CBA mice. The left-hand histograms show the fluorescence profiles of Lyt-2-negative cells obtained following antibody plus C' treatment of thymocytes, stained by indirect immunofluorescence with monoclonal GK-1.5 antibody. To the right, the FLS of total (middle panels) or cells gated according to GK-1.5 fluorescence (right panels) are shown superimposed. Among Lyt-2-negative thymocytes, GK-1.5-positive cells are smaller than the GK-1.5-negative subpopulation.

of T cell-depleted syngeneic (CBA) spleen cells (data not shown).

Limit dilution analysis of TCGF-producing cells among Lyt-2-negative thymocytes was carried out using cells from 18-d, 19-d, 1-d neonatal, and adult mice. As shown in Fig. 7, the 18-d thymus did not contain TCGF-producing cells at a frequency measurable by this system of analysis. However, by 19 d, the frequency of TCGF producing cells was 1/2,220 compared with an adult frequency of 1/223. Table III summarizes the data from experiments using unseparated Lyt-2-negative thymocytes. Clearly, Lyt-2-negative cells from the 19-d embryonic thymus contain TCGF-producing cells at a detectable frequency.

In order to further define the phenotypic properties of the subpopulation of 19-d embryonic thymocytes containing TCGF-producing activity, Lyt-2-negative cells from these mice were stained with GK-1.5 and sorted into positive and negative cells on the FACS. As shown in Fig. 8, when the supernatants from individual microwells set up using FACS-sorted responder cells were subsequently tested for TCGF, there was an obvious enrichment for TCGF-producing cells among the 15% GK-1.5-positive subpopulation. The frequency of TCGF-producing cells among the GK-1.5-positive subpopulation was 1/140, compared with  $<1/10^5$  for the 85% GK-1.5-negative subpopulation (Table IV). In terms of quantitative recovery of TCGF-producing cells,  $>99\%$  of such activity was recovered in a 15% positively selected subpopulation. A similar experiment was carried out using adult Lyt-2-negative cells separated into GK-1.5-positive and negative subpopulations (see Fig. 5). Clearly the GK-1.5-positive subpopulation contained essentially all precursors of TCGF-producing cells (Table IV).

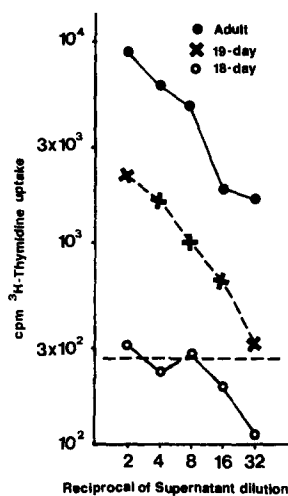


FIGURE 6. Ontogeny of TCGF production in the thymus. Thymocytes from 18-d, 19-d embryonic, or adult mice were treated with monoclonal anti-Lyt-2 antibody plus C' and the resultant Lyt-2-negative cells stimulated in bulk culture as described in Materials and Methods. After 48 h, SN were collected and assayed for their TCGF content using the TCGF-dependent CTLL cell line. The [<sup>3</sup>H]thymidine incorporated was then plotted against the reciprocal of the SN dilution. The dotted line (control) represents the mean cpm incorporated into CTLL cultured in the presence of medium alone. Whereas the SN from 18-d embryonic thymocytes (○) did not result in [<sup>3</sup>H]thymidine uptake above the control, those from the 19-d embryonic (×) and adult (●) were clearly positive.

### Discussion

In this report, the phenotypic properties of mouse thymocytes stained with a new monoclonal antibody GK-1.5 and analyzed by FMF are described. The ontogeny of GK-1.5 expression on unseparated and Lyt-2-negative embryonic thymocytes was characterized. In addition, subpopulations of embryonic and adult thymocytes were positively selected according to their GK-1.5 phenotype and the frequency of TCGF-producing cells among sorted populations determined using a recently described microculture system. By combining these methodologies it was found that essentially all TCGF-producing cells were present in a subpopulation of GK-1.5-positive Lyt-2-negative cells. Furthermore, the ontogeny of TCGF-producing cells in the fetal thymus appeared to correlate with the appearance of cells with this surface phenotype.

Previous studies have established that monoclonal antibody GK-1.5 appears to block all class II MHC antigen-specific T cell functions (12). In particular, class II MHC antigen-reactive clones and hybridomas express a determinant recognized by GK-1.5 and this antibody blocks cytotoxicity, proliferation, and lymphokine release by these cells in a fashion analogous to the effect of anti-Lyt-2 antibodies on class I MHC-reactive T cell clones (20).

When used to stain thymocytes, 91% of cells were positively stained with GK-1.5 (Fig. 1, Table I). Similarly, when stained with monoclonal anti-Lyt-2 antibody, 82% of thymocytes were positive (reference 5 and Table I). These high values together with the similarity in FLS distribution of Lyt-2-positive (5) and GK-1.5-positive (herein) thymocytes, where a major subpopulation of small

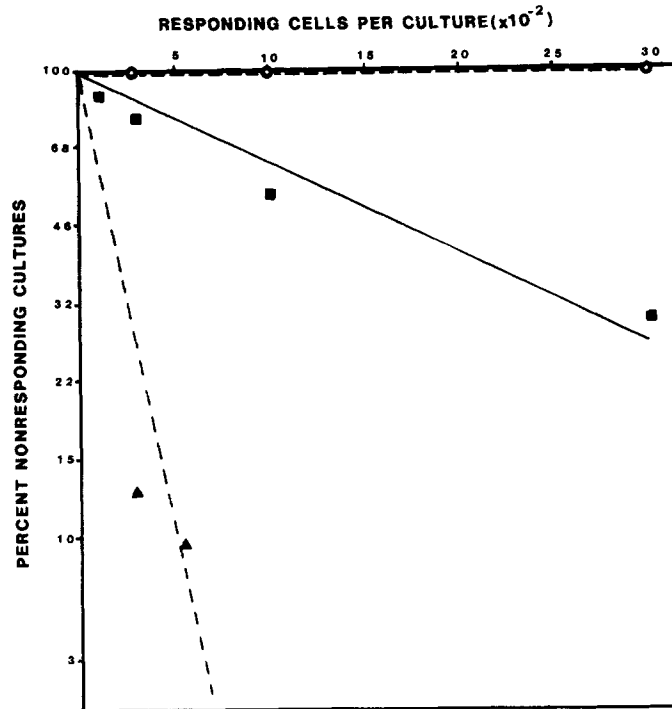


FIGURE 7. Minimal estimates of frequencies of TCGF-producing cells among Lyt-2-negative thymocytes from 18-d (○), 19-d (■) fetal, or adult (▲) mice. The data from an experiment in which SN from a series of micro-MLC were assayed for their TCGF content as outlined in Materials and Methods, are shown plotted semilogarithmically as the percent nonresponding (negative) cultures vs. the dose of responder cells. Frequencies determined by the method of minimum  $\chi^2$  were  $<1/10^5$  for 18-d fetal,  $1/2,220$  for 19-d fetal, and  $1/223$  for adult Lyt-2-negative thymocytes.

TABLE III  
*Ontogeny of TCGF-Producing Cells Among Lyt-2-Negative Thymocytes\**

Age	Frequency
18-d fetal	$<1/10^{5\ddagger}$
19-d fetal	$1/2,060$
	$1/2,220^\ddagger$
1-d neonatal	$1/637$
Adult	$1/192^\S$

\* Lyt-2-negative thymocytes, obtained following monoclonal anti-Lyt-2 antibody plus  $C'$  treatment were used as responders in micro-MLC.

$^\ddagger$  Results from Fig. 7.

$^\S$  Mean of four determinations.

thymocytes were stained, indicate that most (if not all) cortical thymocytes express both GK-1.5 and Lyt-2 determinants. However, a subpopulation of thymocytes, namely CRT, were either GK-1.5-positive or Lyt-2-positive (Fig. 2) and thus expressed the two antigens on mutually exclusive subpopulations in a manner similar to peripheral T lymphocytes (12). This further highlights the similarities

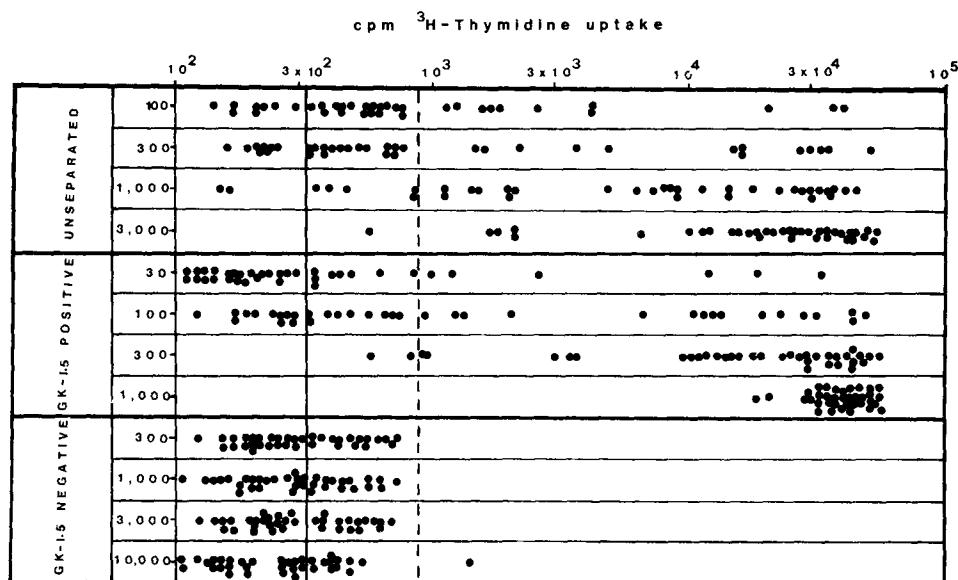


FIGURE 8. TCGF activity in SN's from individual micro-MLC using as responder cells Lyt-2-negative 19-d embryonic thymocytes separated according to GK-1.5 phenotype. Lyt-2-negative 19-d embryonic thymocytes were obtained following monoclonal anti-Lyt-2 antibody plus *C'* treatment as described in Materials and Methods and then stained by indirect immunofluorescence with monoclonal GK-1.5 antibody. The fluorescence profile obtained was similar to that shown in Fig. 5. The stained cells were then passed through an FACS II and collected either unseparated or separated into GK-1.5-positive or GK-1.5-negative subpopulations. The FACS-sorted cells were then used as responders in micro-MLC. Micro-wells were restimulated 14-d following initiation of culture, the SNs harvested at 24 h and then assayed for their TCGF content as described in Materials and Methods. Each point represents the cpm  $^3\text{H}$ thymidine uptake, on a logarithmic scale from individual microcultures initially containing the indicated number of responder cells. The dotted line represents 3SD above the mean (solid line)  $^3\text{H}$ thymidine uptake from cultures initially containing irradiated stimulator cells alone and defines the minimum positive  $^3\text{H}$ thymidine uptake value.

in phenotypic properties of CRT and peripheral T lymphocytes (5, 18).

During thymus development, rapid changes in the FLS profile and proportion of Lyt-2-positive cells occur (8, 19). The ontogeny of GK-1.5 expression during thymus development (Fig. 3, Table I) appeared to parallel that of Lyt-2 (8, 19) and no large difference in the proportion of cells staining positively with either monoclonal reagent was found. In the adult thymus, however, consistently more cells stained with GK-1.5 ( $91 \pm 3$ ) than with Lyt-2 ( $82 \pm 7$ ) (reference 5, Table I). Since a subpopulation of thymocytes, namely CRT (Fig. 2) expressed either GK-1.5 or Lyt-2, these results suggest, but do not prove, that the normal thymus contains both GK-1.5-positive, Lyt-2-negative and GK-1.5-negative, Lyt-2-positive thymocytes. By staining Lyt-2-negative thymocytes with GK-1.5 we were able to demonstrate the presence of the former cells (Fig. 5), but the presence of the latter must await dual parameter analysis of thymocytes stained with the two antibodies.

Another approach to the identification of the above minor subpopulations was by careful analysis of the FLS distributions of phenotypically defined subpopulations of thymocytes. Among either GK-1.5-negative (Fig. 3) or Lyt-2-negative

TABLE IV  
TCGF Precursor Frequencies Among Lyt-2-Negative Thymocytes Sorted According to GK-1.5 Phenotype\*

Age	Sorted population	Frequency	% Sorted	% Recovery
19-d fetal	Total	1/618	100	100
	GK-1.5 <sup>+</sup>	1/140	15	99
	GK-1.5 <sup>-</sup>	<1/10 <sup>5</sup>	85	1
4-wk adult	Total	1/256	100	100
	GK-1.5 <sup>+</sup>	1/152	83	99
	GK-1.5 <sup>-</sup>	1/7,636	17	1

\* Lyt-2-negative thymocytes were stained with monoclonal GK-1.5 antibody (see Fig. 5), passed through an FACS II, and the sorted populations assayed for their content of TCGF-producing cells.

(Fig. 4) thymocytes, distinct subpopulations of cells differing in their FLS distribution could be identified. Thus, the adult thymus contained smaller and larger (by FLS) subpopulations of GK-1.5-negative cells in approximately equal proportions, whereas for Lyt-2-negative thymocytes, smaller cells were the predominant subpopulation. When labeling was with both GK-1.5 and Lyt-2 antibodies simultaneously, the larger, (i.e. blast) cells were not stained by either antibody (Table I). During ontogeny, coincident with the appearance of the subpopulation of smaller Lyt-2-negative thymocytes (Fig. 4), GK-1.5-positive, Lyt-2-negative cells could be identified in the thymus (Fig. 5). The FLS profile of the latter cells (Fig. 5) was similar to that of the major subpopulation of Lyt-2-negative cells in the adult thymus. Similarly, starting at 18 d, a subpopulation of smaller GK-1.5-negative thymocytes was found in the embryonic thymus. Such GK-1.5-negative cells would be expected to express Lyt-2 antigen, but this has not been directly demonstrated at the present time.

TCGF production by GK-1.5-defined subpopulations of thymocytes was characterized by limit dilution analysis of mixed leukocyte microcultures using as responder cells FACS-sorted cells. As with our previous studies in which CTL-P frequencies of thymocyte subpopulations were determined by limit dilution analysis (5), the experimental procedure described herein allowed the quantitative recovery of immunocompetent thymocytes in phenotypically defined subpopulations of cells. Regardless of whether the sorted cells were Lyt-2-negative adult (Table IV), 19-d embryonic (Fig. 8, Table IV), or CRT (Table II), essentially all (95-99%) precursor cells capable of producing TCGF in response to H-2 and/or Mls alloantigens were recovered in the GK-1.5-positive subpopulation. In this regard, it must be emphasized that the phenotypic properties of TCGF-producing cells, namely cortisone-resistant, Lyt-2-negative, GK-1.5-positive cells, are those of a subpopulation of so-called "mature" thymocytes. Therefore, as with our quantitative studies with CTL-P (5) there was no evidence of functional maturation of "immature" thymocytes to "mature" functional cells in the presence of TCGF.

Regarding the ontogeny of TCGF production by the developing thymus, our initial studies using unseparated mouse thymocytes stimulated in bulk or limit dilution cultures gave equivocal results. This was probably due to the relative insensitivity of the TCGF assay being used and the finding that the presence of

Lyt-2-positive thymocytes or peripheral T cells among responder cells appeared to compete for TCGF released by Lyt-2-negative cells (A. Kelso, personal communication). With Lyt-2-negative embryonic thymocytes in bulk (Fig. 7) or limit dilution cultures (Fig. 8), it was found that TCGF-producing cells first appeared in the thymus at 19 d development in CBA mice. Furthermore, the ontogeny of this functional activity coincided with the appearance of a distinct subpopulation of GK-1.5-positive, Lyt-2-negative thymocytes. Previous studies in which the [<sup>3</sup>H]thymidine uptake by mitogen or alloantigen-stimulated (21) embryonic thymocytes was determined suggested that proliferative responses first appeared in the embryonic thymus at 15–16 d fetal development. However, between 15 and 18 d, thymus cellularity increases exponentially (8) and this together with the low amount of [<sup>3</sup>H]thymidine incorporated, brings into question the significance of apparent “helper” activity of very early embryonic thymocytes. Furthermore, at this time the thymus does not appear to contain any cells with a “mature” phenotype (reference 8 and herein).

In the human, a large number of monoclonal reagents exist that have been used to define subpopulations of thymocytes and peripheral T lymphocytes (22, 23). Two of these, namely OKT8 and OKT4, appear to recognize mutually exclusive subpopulations of peripheral T cells with the majority of T8<sup>+</sup> cells responding to class I MHC antigens and the majority of T4<sup>+</sup> cells responding to class II MHC antigens (24). In contrast to peripheral T cells, cortical thymocytes appear to express both antigens (23). Biochemically, GK-1.5 in the mouse<sup>2</sup> and OKT4 in the human (25) both precipitate a molecule that migrates as a single band and having an apparent molecular weight of ~50–60,000 daltons. These phenotypic, functional, and biochemical similarities between GK-1.5 and OKT4 are analogous to those found between Lyt-2 (26) and OKT8 (25) molecules.

By combining the present results with previous data (5), at least five subpopulations of cells in the adult mouse thymus may be identified according to their FLS and surface phenotype (Table V). Populations A and B are large cells, phenotypically distinguishable according to their lack of expression (A) or dual expression (B) of antigens recognized by anti-Lyt-2 and GK-1.5 monoclonal antibodies. Population C is the typical small, cortical thymocyte expressing both antigens. Populations D and E are both medium-sized, H-2K-positive, Thy-1-low “mature” cells with the two cell types being distinguishable both phenotypically, by Lyt-2 and GK-1.5 staining, and functionally, by their content of TCGF-producing cells or CTL-P, respectively.

TABLE V

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

Subpopulation	Percent total cells	Size (FLS)	Phenotype				Classification	Function
			GK-1.5	Lyt-2	H-2K	Thy-1		
A	3	Large (140)	–	–	–	High/–	Immature	None detected
B	20	Large (140)	+	+	–	High	Immature	None detected
C	60	Small (100)	+	+	–	Intermediate	Immature	None detected
D	10	Medium (114)	+	–	+	Low	Mature	TCGF production
E	5	Medium (120)	–	+	+	Low	Mature	CTL-P

Based on these five subpopulations, possible differentiation pathways within the thymus can be considered. The presence of large numbers of cells with phenotype A in the early embryonic thymus and their subsequent decrease to lower levels during ontogeny suggests that this subpopulation may represent cells at a very early stage of thymocyte differentiation. During thymus ontogeny, the order of appearance of the other subpopulations is B (day 16), C (day 17), D (days 18–19), and E (days 19–20). The possible lineage relationship between these subpopulations is unclear; however, using short-term *in vitro* culture, we have recently shown that subpopulation A can rapidly give rise to subpopulation B (27). Furthermore, in organ cultures of 13-d embryonic thymus rudiments, we have demonstrated that subpopulation C has arisen by division from subpopulations A and/or B (28). Because subpopulation C is itself nondividing (28, 29), it seems unlikely that these cells could be the precursors of functional thymocytes, namely subpopulations D and E. Thus dividing cells among subpopulations A or B may represent the precursors of subpopulations D and/or E. Unfortunately, unequivocal demonstration of the latter differentiation pathway(s) is lacking at the present time. The combination of monoclonal antibodies to GK-1.5 and Lyt-2 with functional assays and DNA staining techniques may make possible the study of such putative differentiation pathways.

### Summary

In this report, the ontogeny of precursors of T cell growth factor (TCGF)-producing cells in the mouse thymus was investigated using a recently described limiting dilution microculture system. In agreement with previous studies, in the adult thymus TCGF production by cells stimulated by alloantigens was largely the property of the Lyt-2-negative subpopulation. Furthermore, when Lyt-2-negative cells were stained with monoclonal antibody GK-1.5 and sorted according to fluorescence intensity, all precursors of TCGF-producing cells were quantitatively recovered in the GK-1.5-positive subpopulation. During ontogeny, TCGF production by Lyt-2-negative thymocytes was first detectable on the 19th day of embryonic development at which time the precursor frequency was 1/10th that found in the adult thymus. As in the adult thymus, all precursors of TCGF-producing cells had the GK-1.5-positive, Lyt-2-negative phenotype.

In parallel to these functional studies, the ontogeny of GK-1.5<sup>+</sup>, Lyt-2<sup>-</sup> cells was investigated. In the adult thymus, 80% of cells expressed both GK-1.5 and Lyt-2 antigens, whereas minor subpopulations of 10% and 5% (corresponding to phenotypically mature thymocytes as defined by cortisone-resistant thymocytes [CRT]) expressed GK-1.5 or Lyt-2 exclusively; 3% of cells expressed neither antigen. During ontogeny, thymocytes expressing both GK-1.5 and Lyt-2 first appeared on the 16th day of embryonic development and their proportion increased rapidly thereafter. Interestingly, the GK-1.5<sup>+</sup>, Lyt-2<sup>-</sup> subpopulation first appeared in significant numbers on day 19 in parallel with the appearance of functional TCGF activity. Taken together with our previous studies correlating cytolytic T lymphocyte precursor (CTL-P) activity with the Lyt-2<sup>+</sup>, GK-1.5<sup>-</sup> subpopulation, these results further emphasize the strict correlation between functional activity and mature surface phenotype of both embryonic and adult thymocytes.



We sincerely thank Pierre Zaech for operating the FACS, and Anne-Lise Peitrequin and Anita Cook for technical assistance.

Received for publication 16 June 1983.

### References

1. Miller, J. F. A. P., and D. Osoba. 1967. Current concepts of the immunological function of the thymus. *Physiol. Rev.* 47:437.
2. Blomgren, H., and B. Andersson. 1970. Characteristics of the immunocompetent cells in the mouse thymus: cell population changes during cortisone-induced atrophy and subsequent regeneration. *Cell. Immunol.* 1:515.
3. Mathieson, B. J., S. O. Sharrow, P. S. Campbell, and R. Asofsky. 1979. A Lyt differentiated thymocyte subpopulation detected by flow microfluorometry. *Nature (Lond.)* 277:478.
4. Scollay, R., S. Jacobs, L. Jarabek, E. Butcher, and I. Weissman. 1980. T cell maturation: thymocyte and thymus migrant subpopulations defined with monoclonal antibodies to MHC region antigens. *J. Immunol.* 124:2845.
5. Ceredig, Rh., A. L. Glasebrook, and H. R. MacDonald. 1982. 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. *J. Exp. Med.* 155:358.
6. Smith, K. A. 1980. T cell growth factor. *Immunol. Rev.* 51:337.
7. Moore, M. A. S., and J. J. T. Owen. 1967. Experimental studies on the development of the thymus. *J. Exp. Med.* 126:715.
8. Ceredig, Rh., H. R. MacDonald, and E. J. Jenkinson. 1983. Flow microfluorometric analysis of mouse thymus development *in vivo* and *in vitro*. *Eur. J. Immunol.* 13:185.
9. Ceredig, Rh. 1979. Frequency of alloreactive cytotoxic T cell precursors in the mouse thymus and spleen during ontogeny. *Transplantation (Baltimore)* 29:277.
10. Widmer, M. B., H. R. MacDonald, and J.-C. Cerottini. 1981. Limiting dilution analysis of alloantigen-reactive T lymphocytes. VI. Ontogeny of cytolytic T lymphocyte precursors in the thymus. *Thymus* 2:245.
11. Kelso, A., and H. R. MacDonald. 1983. Precursor frequency analysis of lymphokine-secreting alloreactive T lymphocytes. Dissociation of subsets producing interleukin 2, macrophage-activating factor, and granulocyte-macrophage colony-stimulating factor on the basis of Lyt-2 phenotype. *J. Exp. Med.* 156:1366.
12. Dialynas, D. P., D. B. Wilde, P. Marrack, A. Pierres, K. A. Wall, W. Havran, G. Otten, M. R. Loken, M. Pierres, J. Kappler, and F. W. Fitch. 1983. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK-1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol. Rev.* 74 (in press).
13. Maryanski, J. L., H. R. MacDonald, B. Sordat, and J.-C. Cerottini. 1981. Cell surface phenotype of cytolytic T lymphocyte precursors in aged nude mice. *Eur. J. Immunol.* 11:968.
14. Van den Engh, G. J. Visser, and B. Trask. 1979. Identification of CFU-s by scatter measurement on a light-activated cell sorter. In *Experimental Hematology Today*. S. J. Braun and G. D. Ledney, editors. Springer-Verlag, Inc., New York. New York. pp 19-26.
15. Cerottini, J.-C., H. D. Engers, H. R. MacDonald, and K. T. Brunner. 1974. Generation of cytotoxic T lymphocytes *in vitro*. I. Response of normal and immune mouse spleen cells in mixed leukocyte cultures. *J. Exp. Med.* 140:703.

16. Farrar, J. J., J. Fuller-Farrar, P. L. Simon, M. L. Hilfiker, B. M. Stadler, and W. L. Farrar. 1980. Thymoma production of T cell growth factor (Interleukin 2). *J. Immunol.* 125:2555.
17. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* 126:1614.
18. Ceredig, Rh., and D. E. Cummings. 1983. Phenotypic and functional properties of murine thymocytes. III. Kinetic analysis of the recovery of intrathymic cytolytic T lymphocyte precursors after *in vivo* administration of hydrocortisone acetate. *J. Immunol.* 130:33.
19. Mathieson, B. J., S. O. Sharrow, Y. Rosenberg, and U. Hämmerling. 1981. Lyt-1<sup>+</sup>23<sup>-</sup> cells appear in the thymus before Lyt-123<sup>+</sup> cells. *Nature (Lond.)*. 289:179.
20. MacDonald, H. R., A. L. Glasebrook, C. Bron, A. Kelso, and J.-C. Cerottini. 1982. Clonal heterogeneity in the functional requirement for Lyt-2/3 molecules on cytolytic T lymphocytes (CTL): possible implications for the affinity of CTL antigen receptors. *Immunol. Rev.* 68:89.
21. Mosier, D. E. 1974. Ontogeny of mouse lymphocyte function. I. Paradoxical elevation of reactivity to allogeneic cells and phytohemagglutinin in BALB/c fetal thymocytes. *J. Immunol.* 112:305.
22. Reinherz, E. L., P. C. Kung, G. Goldstein, and S. F. Schlossman. 1979. Separation of functional subsets of human T cells by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 76:4061.
23. Reinherz, E. L., P. C. Kung, G. Goldstein, R. H. Levey, and S. F. Schlossman. 1980. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-Cell lineage. *Proc. Natl. Acad. Sci. USA.* 77:1588.
24. Reinherz, E. L., S. C. Meuer, and S. F. Schlossman. 1983. The delineation of antigen receptors on human T lymphocytes. *Immunol. Today (Amst.)*. 4:5.
25. Terhorst, C., A. Agthoven, E. L. Reinherz, and S. F. Schlossman. 1980. Biochemical analysis of human T lymphocyte differentiation antigens T4 and T5. *Science (Wash. DC)*. 209:520.
26. Ledbetter, J. A., R. L. Evans, M. Lipinski, C. Cunningham-Rundles, R. A. Good, and L. A. Herzenberg, 1981. Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and cytotoxic/suppressor subpopulations in mouse and man. *J. Exp. Med.* 153:310.
27. Ceredig, Rh., R. P. Sekaly, and H. R. MacDonald. 1983. Differentiation *in vitro* of Lyt-2<sup>+</sup> thymocytes from embryonic Lyt-2<sup>-</sup> precursors. *Nature (Lond.)*. 303:248.
28. Sekaly, R. P., Rh. Ceredig, and H. R. MacDonald. 1983. Generation of thymocyte subpopulations in organ culture: correlated analysis of Lyt-2 phenotype and cell cycle status by flow microfluorometry. *J. Immunol.* Vol. 131.
29. 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.