

## THYMOCYTE CLONES FROM 14-DAY MOUSE EMBRYOS

### I. State of T Cell Receptor Genes, Surface Markers, and Growth Requirements

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The TCR for recognition of antigen is a disulphide-linked heterodimer made of two membrane glycoproteins,  $\alpha$  and  $\beta$ , which are expressed on the cell membrane in association with an invariant complex of proteins, T $\beta$  (1–3). Recent studies (4–9) have identified in both the thymus and peripheral blood a subset of T cells that carry on the cell membrane another type of TCR/T $\beta$  complex that includes the product of the T cell-specific gene  $\gamma$  (TCR- $\gamma$ /T $\beta$ ).

Bone marrow T cell progenitor clones may express germline transcripts for the  $\gamma$  but not for the  $\alpha$  or the  $\beta$  TCR genes before migrating to the thymus (10). Studies carried out with heterogeneous populations of 15-d fetal thymocytes (FT)<sup>1</sup> showed that some of these cells have rearranged and express  $\beta$  and  $\gamma$  genes (11, 13). Very little is known about the state of the TCR- $\alpha$  gene during ontogeny within the thymus. mRNA for the TCR- $\alpha$  gene could be detected in FT at day 17 of gestation (14, 15).

Mouse thymocytes at day 14 of gestation express Thy-1 and Ly-1 but not L3T4 or Lyt-2 T cell surface antigens (16, 17). The limited number of cells obtainable from 14-d embryonic thymuses has hampered the study of the configuration of the TCR- $\alpha$ , - $\beta$ , and - $\gamma$  genes in these cells.

FT can be induced to proliferate in vitro either with the combination of PMA, ionomycin, and the growth factor IL-2 (18) or with PMA plus the cytokine IL-4 (19, 20).

To study the cellular and molecular properties of 14-d FT and to further our knowledge of the effects of IL-4 on growth and differentiation of T cell precursors, we attempted to establish in culture FT clones. Here we report the establishment of 13 independently isolated thymocyte clones from a 14-d mouse embryo and the molecular, cellular, and functional characterization of eight of such clones.

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<sup>1</sup>Abbreviations used in this paper: FT, fetal thymocytes; GFCM, growth factor-conditioned medium; IMDM, Iscove's modified Dulbecco's medium.

### Materials and Methods

**Reagents.** PMA (Sigma Chemical Co., St. Louis, MO), ionomycin (Calbiochem-Behring Corp., San Diego, CA), [<sup>3</sup>H]thymidine (TRA 120; Radiochemical Centre, Amersham, United Kingdom), Iscove's modified Dulbecco's medium (IMDM Gibco Laboratories, Grand Island, NY), Con A (Pharmacia Fine Chemicals, Uppsala, Sweden), PHA (Wellcome Research Laboratories, Darford, United Kingdom), LPS (Sigma Chemical Co.), and FCS (Ready Systems Laboratories, Basel, Switzerland) were the reagents used.

**Mice.** C57BL/6, CBA/J, DBA/2, and B10.BR mice are bred and maintained in our animal facilities. B10.BR embryos were obtained from timed matings; the day of vaginal plug was designated day zero.

**Separation of Cells.** Thymocyte cell suspensions from fetal B10.BR mice (14 d of gestation) were prepared as described (21) and were kindly provided to us by Mrs. Katrin Hafen (Basel Institute for Immunology). The FT cell suspensions were treated with a mixture of anti-Ia (M5/114), Mac-1 (M1/70), and antigranulocyte (GM1.2) mAbs plus complement (22) to eliminate putative myeloid cells contained in these preparations of cells. Spleen cell suspensions were prepared as described (23) and T cells were eliminated from CBA/J spleen cells by treatment with a mixture of monoclonal anti-T cell antibodies and complement as detailed elsewhere (23). The cell preparations were washed and resuspended in culture medium (IMDM supplemented with 5% heat-inactivated FCS,  $5 \times 10^{-5}$  M 2-ME, and 50  $\mu$ g/ml of gentamycin) at the desired concentrations.

**Monoclonal Antibodies.** mAbs against the following surface antigens were used: Ly-1 (53-7-313), Lyt-2 (53-63-72, 3168.81), L3T4 (GK1.5, RL-172), Thy-1.2 (30 H12), J11d.1, Mac-1 (M1/70), Pgp-1 (I42/5), B-220 (14.8), IL-2-R (7D4, PC61), Ia (M5/114.15.2, 10.2.16), H-2D<sup>k</sup> (15-5-5S), LFA-1 (FD441-8). The reports describing the characteristics of all these mAbs are listed elsewhere (10, 18, 19, 22). The hamster anti-mouse T3 mAb 145-2CM (24) was a gift of Dr. J. Bluestone (National Institutes of Health, Bethesda, MD). FITC-conjugated goat anti-rat Ig (Nordic Immunological Laboratories, Tilburg, The Netherlands), FITC-labeled goat anti-mouse Ig (Southern Biotechnology Associates, Inc., Birmingham, AL), FITC-labeled goat anti-hamster Ig (Nordic Immunological Laboratories) and FITC-conjugated sheep anti-mouse Ig that crossreacts with rat Ig (a kind gift of Luciana Forni, Basel Institute for Immunology) were used as second-step reagents in immunofluorescence staining procedures.

**Cytokines.** Human rIL-2 (25), human rIL-1 (Cistrom Technologies, Pine Brook, NJ). Mouse rIL-4 (19, 26) produced by transfected HeLa H-109 cells was purified by gel filtration chromatography. The purified recombinant material has identical biochemical characteristics and biological activities to those of IL-4 purified from Th clones (26, 27, and Sideras, P., unpublished results). Supernatants from Con A-stimulated 2.19 Th line were obtained as described (27); they contain IL-3, IL-5, and IL-4 (80 U/ml, assessed in the IgG1-inducing factor assay [27]), and no detectable IL-2 or IFN- $\gamma$  activities (27 and our unpublished results).

**Establishment in Culture of Fetal Thymocyte Clones.** Single 14-d FT from a B10.BR mouse were placed by micromanipulation in round-bottomed microplates containing growth factor-conditioned medium (GFCM, culture medium supplemented with 5% supernatant from Con A-stimulated 2.19 Th line) and PMA (10 ng/ml). GFCM without PMA was used subsequently unless indicated otherwise. 3 d later, 100  $\mu$ l of fresh GFCM were added to each well, and thereafter, every 3 d 100  $\mu$ l of the medium was replaced with 100  $\mu$ l of fresh GFCM. After 15–20 d of culture, we observed that 13 of 172 wells contained confluent cell layers. Each of the 13 confluent cultures was transferred into macroculture plates (Linbro Chemical Co., Hamden, CT) and cultured in a final volume of 0.5 ml of GFCM per Linbro well. As the cultures reached confluency, they were subcultured in two to three new Linbro wells in a final volume of 0.5–1.0 ml of GFCM per well. Once each of the 13 FT clones were expanded up to 24 Linbro wells per clone, they were harvested, washed once, and then further expanded in 50-ml tissue culture plastic flasks at  $2\text{--}3 \times 10^5$  cells/ml GFCM. Samples of each FT clone were frozen by standard procedures (22) at this time. The FT clones could be transferred to new flasks with continued growth every 2–3 d and were therefore considered established clones

(September 1986). Eight FT clones were chosen at random for further study and the remaining five FT clones were frozen. The FT clones D11, H5, D5, H12, G12, E10, F1, and A2 have been maintained in culture with GFCM at the conditions indicated above.

*Other Cell Lines.* The LD1 Th line (23), the EL-4 T lymphoma, and the T hybrid HK30I (obtained by fusing 15-d FT with BW5147 T lymphoma cells) (Pelkonen, J., unpublished results) were maintained in culture medium. The 2.19 Th line (27) was maintained and expanded in culture medium supplemented with IL-2-containing supernatants and stimulation with x-ray irradiated C57BL/6 spleen cells as described (27). The 4E7 T killer line that recognizes minor histocompatibility antigens in the context of H-2<sup>k</sup> was derived from BALB/c spleen cells stimulated with BALB.K-irradiated spleen cells as described (28).

*Isolation and Analysis of Nucleic Acids.* DNA and RNA preparation, restriction enzyme digestions, agarose gel electrophoresis, DNA blotting procedures, probe preparations, hybridization procedures, and autoradiography were performed as described (29, 30).

*$\gamma$  Gene Complex Probe.* The C $\gamma$ 1 cDNA probe (900-bp Ava I-Eco RI cDNA clone containing all the C $\gamma$ 1 coding region and its 3' untranslated region) (14), the J $\gamma$ 1 probe (1.2-kb Ava I-Hind III genomic fragment) (30), and the C $\gamma$ 2 probe (1.0-kb Eco RI-Hind III genomic fragment) (30) were used.

*$\beta$  Gene Complex Probe.* The 4.1 cDNA probe specific for the constant region of the  $\beta$  gene complex (11), the pJ $\beta$ 1 probe (Bam HI-Eco RI genomic fragment) (31, 32), and the pJ $\beta$ 2 probe (Cla I-Eco RI genomic fragment) (31, 32) were used.

*$\alpha$  Gene Complex Probe.* The  $\alpha$  chain probe used is the 300-bp Nco I-Ava II fragment from the constant portion of the  $\alpha$  chain cDNA clone T1.2 (33).

*RNA-RNA In Situ Hybridization.* The FT clones and the LD1 T cell line stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) at 37°C for 2-4 h or unstimulated were assayed for the presence of mRNA for IL-4 by RNA-RNA in situ hybridization as described below and elsewhere (20).

*Cyto centrifuge Preparations.* Glass slides were immersed in 70% ethanol and air dried. The cells suspended in balanced salt solution containing 10% FCS were loaded on glass slides (10<sup>5</sup> cells/slide) with the aid of a cyto centrifuge. The cell smears were fixed in paraformaldehyde (4% diluted in PBS) for 1 min, were transferred into a solution of 70% ethanol, and were subsequently stored at +4°C until used.

*Preparation of the IL-4 Probe.* The Rsa I-Rsa I fragment from the cDNA clone pSP6KmIL4-293 (26) containing all the coding sequence for IL-4 was subcloned into the gemini, SP6/T7 vectors. The pUC18 vector containing the Rsa I-Rsa I fragment was kindly provided by Dr. T. Honjo (Kyoto University, Kyoto, Japan). Linearized plasmids were used as templates for the synthesis in vitro of radioactive RNA probes complementary to the cellular IL-4 mRNA. RNA was also transcribed from the opposite direction (sense) and used as a negative control.  $\sim 2 \times 10^8$  cpm were incorporated into RNA per microgram of RNA template. The size of the full-length probe mRNA was  $\sim 400$  nucleotides.

*In Situ Hybridization.* Fixed slides prepared as described above were rinsed twice in 2 $\times$  SSC (20 $\times$  SSC = 3 M NaCl, 0.3 M sodium citrate) and then acetylated (pH 8, 0.25% acetic anhydride) for 10 min. After this, the slides were rinsed in 2 $\times$  SSC and PBS, then immersed in 0.1 M Tris-HCl, 0.1 M glycine, pH 7.0, for 30 min before hybridization.

20  $\mu$ l of probe mixture (10  $\mu$ l formamide-20% dextran sulfate, 2  $\mu$ l sheared herring-sperm DNA [10 mg/ml], 2  $\mu$ l nuclease-free BSA [20 mg/ml], 1  $\mu$ l labeled RNA [10<sup>6</sup> cpm/ $\mu$ l]) were loaded on each slide and hybridization was performed at 50°C for 3 h. The slides were washed three times with 50% formamide, 2 $\times$  SSC at 52°C, rinsed in 2 $\times$  SSC, and treated with 30  $\mu$ l RNase solution for 30 min at 37°C (100  $\mu$ g/ml RNase A plus 1  $\mu$ g/ml RNase-T1). The slides were rinsed twice in 2 $\times$  SSC, once in 50% formamide, 2 $\times$  SSC at 52°C, rinsed again three times in 2 $\times$  SSC, were dehydrated in 70, 80, and 90% ethanol, respectively, were air dried, and finally were autoradiographed. Kodak nuclear track emulsion (NTB-2, Eastman Kodak Co., Rochester, NY) was melted at 43°C and diluted with an equal volume of water. The slides were dipped into the emulsion and allowed to solidify horizontally at room temperature for 3-4 h. The emulsion-coated slides were kept at +4°C for 3-5 d for exposure. The slides were developed in Kodak

developer-19 at +15°C for 5 min. After a rinse in tap water the fixation was carried out in Kodak fixer for 5 min and the slides were washed with water for 30 min. The slides were stained with 10% Giemsa stain for 2.5 min, washed twice with tap water, and were air dried. We considered as positive cells (cells expressing IL-4-mRNA) those cells that had >10 grains per cell. Elsewhere (20) we have shown that (a) cold IL-4-RNA synthesized in the sense orientation inhibits the binding of the radioactive probe to the cellular mRNA, and (b) no hybridization is observed either when the cytocentrifuge preparations are pretreated with RNase or when RNA probes synthesized in the wrong orientation are used (20), demonstrating the validity and specificity of the technique for the detection of IL-4-mRNA.

*Immunofluorescence Staining and Flow Cytometry Analysis (FACS).* This procedure was carried out as described in detail before (10, 22) using FITC-conjugated anti-mouse Ig, anti-rat Ig, and anti-hamster Ig as second-step reagents. Dead cells were excluded from analysis by using propidium iodide. The fluorescence emitted by single viable cells was measured using logarithmic amplification and the data collected from  $10^4$  cells were analyzed with a program in a Consort 30 computer and histograms were generated.

*Proliferative Responses to Cytokines.* The cells were harvested in log phase of growth, washed three times, and resuspended in culture medium. The cells ( $10^4$ ) were incubated in microculture wells in a final volume of 200  $\mu$ l of culture medium containing the following agents: rIL-1 (5–50 U/ml), rIL-2 (10–500 U/ml), rIL-4 (final dilutions, 1:4 to 1:256), PMA (10 ng/ml), or the combination of PMA + rIL-2 (100 U/ml). The cultures in triplicate were incubated at 37°C for 24 and 48 h. Cell growth was measured by both direct visualization of the cultures with an inverted microscope and [ $^3$ H]thymidine uptake (37 KBq/well) during the last 5–6 h of the culture period. The data are expressed as counts per minute and are the mean of triplicate samples.

*Helper and Cytotoxic T Cell Functions.* The capacity of the FT clones to assist T cell-depleted spleen B cells from CBA/J mice to generate antibody-secreting cells was studied in a Con A-facilitated microculture assay as detailed before (23). The number of antibody-secreting cells was determined by the reverse hemolytic protein A plaque assay after 5 d of culture at 37°C. The results are expressed as PFC per culture and are the mean of duplicate cultures.

The capacity of the FT clones to exert cytolytic activity was tested in a PHA-facilitated microcytotoxicity assay using as target cells  $^{51}$ Cr-labeled LPS-stimulated spleen cells from CBA/J and DBA/2 mice or EL-4 T lymphoma cells and were performed as described (28). The results are expressed as percent of specific  $^{51}$ Cr release.

## Results

### *Establishment in Culture of FT Clones*

Single FT from 14-d embryonic thymus of B10.BR mice were placed in microculture wells by micromanipulation and the cultures were supplemented with GFCM. 13 independently isolated clones were established out of 172 cultures as detailed in experimental procedures. Eight clones, called FT D11, FT H5, FT D5, FT G12, FT H12, FT E10, FT F1, and FT A2, were chosen at random for further study and the other five clones were frozen. The FT clones were established in September 1986 and maintained in culture up to present by culturing them in fresh GFCM every 2 d.

### *Configuration and Expression of the TCR- $\gamma$ , - $\beta$ , and - $\alpha$ Genes*

The eight FT clones chosen for further study were expanded and analyzed for the expression of the TCR- $\gamma$ , - $\beta$ , and - $\alpha$  genes and surface phenotype.

The expression of mRNA for TCR- $\gamma$ , - $\beta$ , and - $\alpha$  genes in the FT clones was studied by Northern blot analysis. The results show that all FT clones have

mRNA for the  $\gamma$  gene and that none of them contain detectable mRNA for the TCR- $\beta$  or - $\alpha$  genes (Fig. 1).

Next, we assessed the configuration of the TCR genes in the FT clones by Southern blot analysis. The state of the  $\gamma 1$ ,  $\gamma 3$ , and  $\gamma 4$  gene clusters (the nomenclature for the  $\gamma$  gene clusters is that proposed by Traunecker et al. [31]) was assessed using the PJ $\gamma 1$  probe and Hind III-digested DNAs from liver and the FT clones. This analysis shows that all eight clones contain the  $\gamma 1$  and the  $\gamma 4$  genes in germline configuration (Fig. 2C) and that the B10.BR mouse strain like other mice (DBA/2, B10.A, CBA/J) do not have the  $\gamma 3$  pseudogene (Fig. 2C). No evidence for rearrangement of the  $\gamma 2$  gene cluster was found using the C $\gamma 2$ -specific probe and Eco RI-digested DNAs (not shown).

Using probes specific for the J $\beta 1$  and the J $\beta 2$  regions, respectively, and Eco RI-digested DNAs we found that all eight FT clones contain both the  $\beta 1$  (Fig. 2A) and the  $\beta 2$  (Fig. 2B) genes in germline configuration.

Due to the long distance spanning the J $\alpha$  chromosomal region, the presence of rearrangements of the TCR- $\alpha$  gene is more difficult to determine. We are in the process of generating genomic probes that will cover the ~60-kb J $\alpha$  chromosomal region to be able to detect TCR- $\alpha$  gene rearrangements.

### *Surface Phenotype*

The phenotype of the FT clones was determined by immunofluorescence staining and FACS using a panel of mAbs against cells of the T cell, B cell, and myeloid cell lineages. Three distinct phenotypes were found.

*Phenotype I.* The FT clones E10, F1, D5, and H5 express Thy-1, Ly-1, Lyt-2, Pgp-1, and IL-2-R but no detectable L3T4 or T3 surface molecules (Fig. 3).

*Phenotype II.* The FT clones A2 and G12 resemble the FT clones with phenotype I except that they do not express detectable IL-2-R as assessed with the two anti-IL-2-R mAbs 7D4 (Fig. 3) and PC61 (not shown).

*Phenotype III.* The FT D11 and FT H12 clones differ from the FT clones with phenotypes I and II in that they express L3T4 (Fig. 3).

None of the eight FT clones have detectable IgM, Ia, B-220, or Mac-1 molecules on the cell membrane, and all of them are H-2k<sup>+</sup> and LFA-1<sup>+</sup>.

### *Growth Requirements*

The next set of experiments was designed to define the growth factors used by the FT clones to continuously proliferate in vitro. All eight FT clones grow in rIL-4 but not in rIL-1. All six IL-2-R<sup>+</sup> FT clones also proliferate in rIL-2, albeit to a lesser extent than in IL-4 (Table I). The FT clones A2 and G12 do not express receptors for IL-2 (Fig. 3). However, both FT A2 and FT G12 clones can be induced to express IL-2-R (assessed by FACS analysis with 7D4 and PC61 mAbs) by treating them with PMA (not shown), and the PMA-treated A2 and G12 FT clones proliferate in rIL-2 (Table I). PMA by itself is not mitogenic on the FT clones.

The PC61 and 7D4 anti-IL-2-R antibodies strongly inhibited the action of rIL-2 on the FT clones but did not significantly affect the action of rIL-4 (Fig. 4) indicating that IL-4 promotes growth of the FT clones via an IL-2-independent pathway. These results show that the FT clones can use both IL-4 and IL-2 as

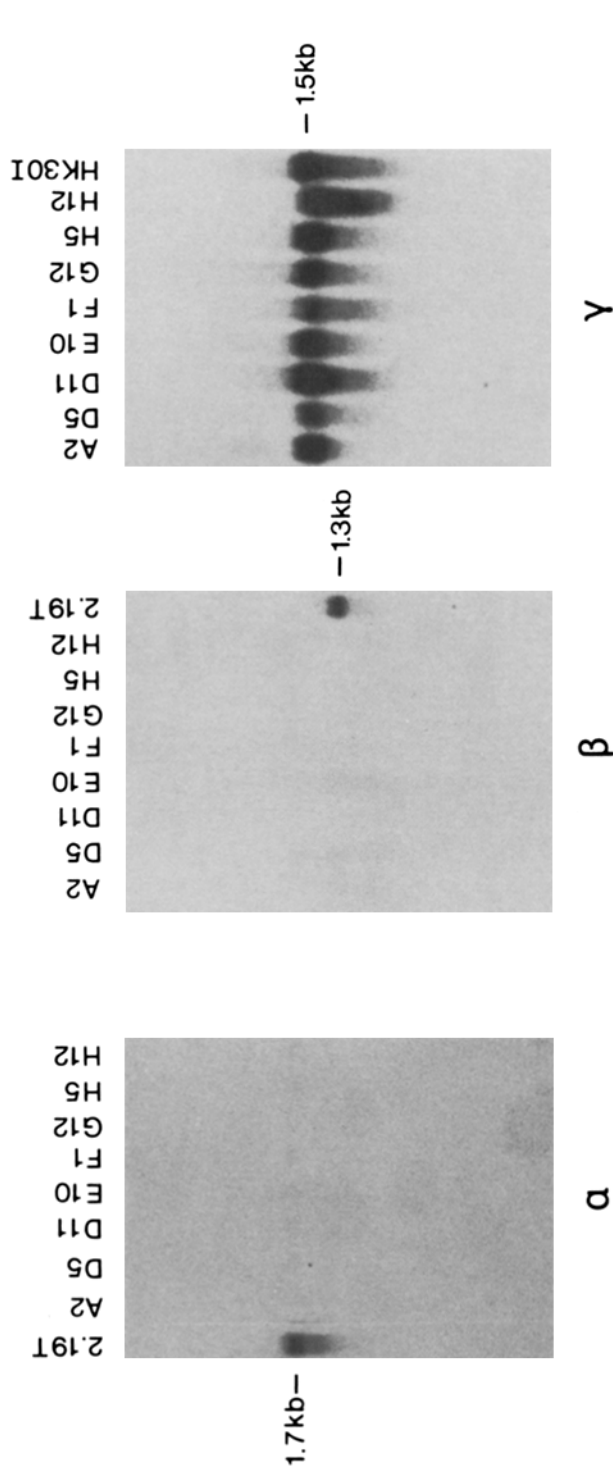


FIGURE 1. Analysis by Northern blot hybridization of mRNA for the TCR genes  $\alpha$ ,  $\beta$ , and  $\gamma$  in the FT clones and control mature T cell lines (2.19 and HK30I) was performed using probes specific for  $\gamma$  constant portion chain gene (probe C- $\gamma$ 1),  $\beta$  constant region gene (probe 4.1), and  $\alpha$  constant region gene (probe T1.2).

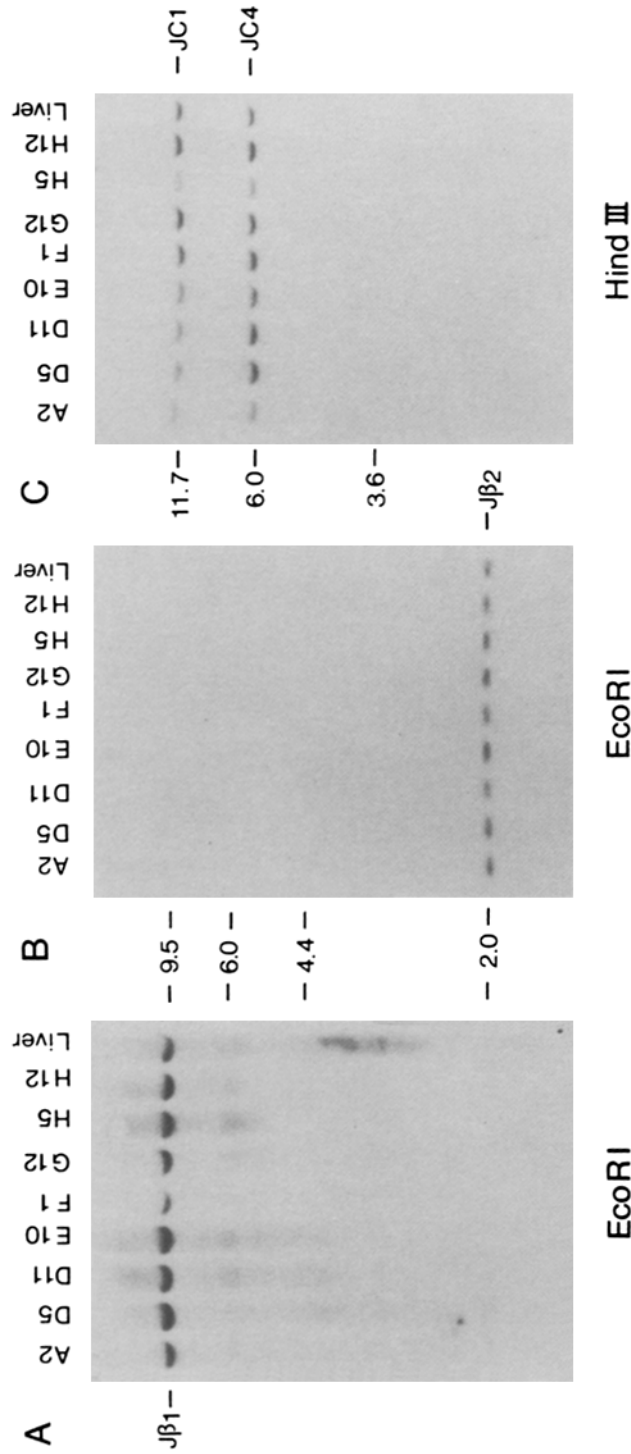


FIGURE 2. Analysis by Southern blot hybridization of the configuration of the  $\beta 1$  (A),  $\beta 2$  (B), and the  $\gamma$  (C) TCR genes in the FT clones using probes specific for: the  $J\gamma$  region gene (probe J $\gamma$ 1) and Hind III-digested DNAs, and the  $J\beta 1$  or the  $\beta 2$  regions (probes P $\beta$ 1 and P $\beta$ 2, respectively) and Eco RI-digested DNAs.

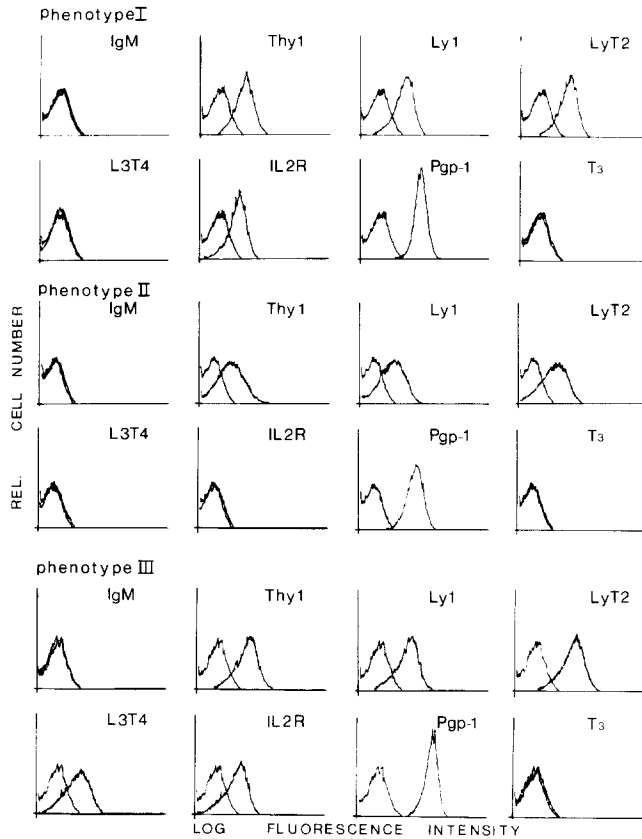


FIGURE 3. Phenotypic characterization of the FT clones. The presence of the various cell surface antigens indicated above on the FT clones was determined by direct or indirect immunofluorescence staining and FACS analysis. The surface characteristics of the FT clones fall into the following three phenotypes: I, illustrated by the FT clone H5; II, exemplified by the FT clone G12; and III, illustrated by the data obtained with the FT clone D11.

TABLE I  
Growth Factors for the FT Clones

Responding FT clone*	Cell proliferation driven by ( $^3\text{H}$ )thymidine uptake, cpm $\times 10^{-3}$ :					
	Medium	rIL-4	rIL-2	rIL-2 + PMA	PMA	rIL-1
A2 <sup>†</sup>	381	7,656	772	8,210	305	482
D5	280	16,532	8,404	—	—	305
F1	214	12,287	5,842	—	—	586
D11	299	10,248	4,671	—	—	350
H5	286	12,080	6,848	—	—	278
G12 <sup>†</sup>	272	10,332	968	7,120	311	304
H12	328	20,241	10,123	—	—	510
E10	201	13,775	5,065	—	—	322

\* The FT clones indicated above were cultured in medium containing rIL-1 (20 U/ml), rIL-2 (100 U/ml), rIL-4 (final dilution 1:16), PMA (10 ng/ml) or PMA + rIL-2, at 37°C for 24 h. Cell proliferation was assessed by [ $^3\text{H}$ ]thymidine uptake during the last 5 h of the culture period.

<sup>†</sup> The FT clones A2 and G12, unlike the other FT clones, do not express IL-2-R. PMA induces both A2 and G12 cells to express IL-2-R as determined by FACS analysis using 7D4 or PC61 mAbs specific for IL-2-R.



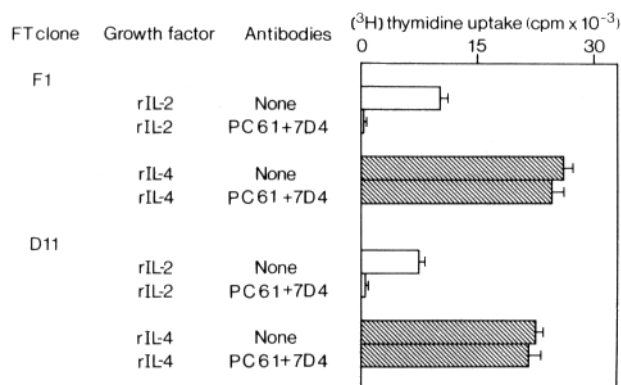


FIGURE 4. Anti-IL-2-R mAbs inhibit the proliferative response of the FT clones to rIL-2 but not to rIL-4. Both 7D4 and PC61 anti-IL-2-R mAbs were added to cultures (final concentration 10%) containing cells of the FT clones ( $5 \times 10^5$ /well) and either rIL-2 (50 U/ml) or rIL-4 (final dilution, 1:16). Cell proliferation was measured by [<sup>3</sup>H]thymidine uptake. The results are expressed as  $\text{cpm} \times 10^{-3}$  (mean  $\pm$  SE of triplicate samples). The results are illustrated with the data obtained with the FT clones F1 and D11.

growth factors in vitro. The FT clones self-renew in vitro in rIL-4 every 10–14 h and die within 48–72 h in the absence of exogenous growth factors.

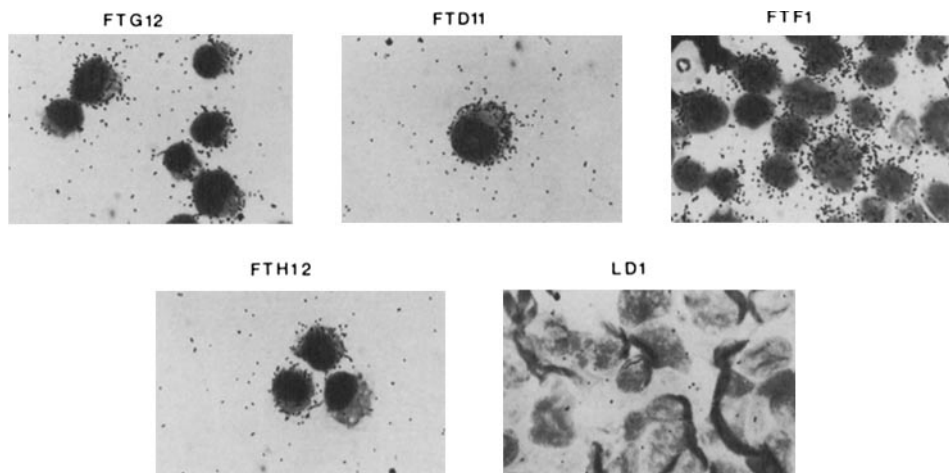
*Functions*

The FT clones were tested for three T cell functions: help to B lymphocytes, cytolytic activity, and synthesis of IL-4.

We studied whether the FT clones synthesize IL-4. This was of particular interest in light of our previous work (20) showing that 1 of 10 FT at day 14 of gestation synthesize mRNA for IL-4 after stimulation with PMA plus ionomycin, as assessed by RNA-RNA in situ hybridization. Thus the FT clones stimulated with PMA + ionomycin at 37°C for 2–4 h or unstimulated were assayed for the presence of mRNA for IL-4 by RNA-RNA in situ hybridization. The LD1 T cell line was included in these experiments and served as a negative control. We found that all eight FT clones synthesized IL-4-mRNA after stimulation by PMA + ionomycin. No IL-4-mRNA was detected in the unstimulated FT clones or in the stimulated LD1 T cells (negative control). No hybridization to the cellular RNA from stimulated FT clones was observed using radiolabeled probe synthesized in the wrong orientation (not shown). The amount of IL-4-mRNA produced by each clone (as judged by the number of grains counted per cell) varied among the FT clones, with clones A2, G12, and F1 producing the highest and clone H5 the lowest amount of IL-4-mRNA (Fig. 5).

We were confronted with technical drawbacks to assess in biological assays the production of IL-4 by the FT clones. While supernatants from PMA + ionomycin-stimulated FT clones supported proliferation of cells known to respond to IL-4 (e.g., C4-86 [10], HT-2, freshly isolated FT), we could neither rule out the contribution of PMA + ionomycin in the responses nor ascertain that the growth-promoting activity in such responses was due to IL-4 (the target cells can respond to other growth factors, e.g., IL-2, IL-3). Also, the presence of PMA + ionomycin in the supernatants made it very difficult to test them for IgG1-inducing activity on LPS-stimulated B cells, as the drugs inhibited such responses.

The capacity of the FT clones to assist B lymphocytes to mature into antibody-secreting cells was determined on a Con A-facilitated microculture assay that reveals Th activity for B cells regardless of the specificity of the effector and target cells (23). A Th line (LD1) was included in these experiments and served



**FIGURE 5.** Synthesis of mRNA for IL-4 by the FT clones after their stimulation with PMA + ionomycin as assessed by RNA-RNA in situ hybridization. The results obtained with cells from four FT clones and the LD1 T cell line (negative control) are shown above; similar results were obtained with the other FT clones. Unstimulated cells did not contain detectable IL-4-mRNA.

as positive control. The number of antibody-secreting cells (PFC) was determined by the protein A plaque assay after 5 d of culture at 37°C. We found that while the LD1 T cell line assisted B lymphocytes to generate antibody-secreting cells, none of the eight FT clones at the various concentrations tested exhibited this function (data not shown).

All FT clones were tested for cytolytic activity on <sup>51</sup>Cr-labeled target cells (LPS-activated syngeneic and allogeneic spleen cells or EL-4 T lymphoma cells) in a lectin-facilitated microcytotoxicity assay that reveals cytotoxic function regardless of the specificity of the effector cells. A T killer cell line (4E7) was included in the experiments as a positive control. None of the FT clones exert killer activity, whereas the 4E7 T cell line (positive control) efficiently killed the target cells (data not shown).

### Discussion

We have studied the molecular, cellular, and functional properties of the FT clones (D11, H5, D5, H12, G12, E10, F1, and A2) isolated from the thymus of a 14-d-old B10.BR mouse fetus.

The FT clones express mRNA for the  $\gamma$  chain gene, but no evidence for rearrangements of any of the  $\gamma$  genes was found. The expression of germline transcripts for the  $\gamma$  gene in the FT clones could reflect the accessibility of the chromatin to recombinases as proposed for the germline transcripts for the Ig heavy chain gene found in early B cell precursor lines (34). The finding that 1 d later in ontogeny (day 15 of gestation), FT with rearranged  $\gamma$  gene clusters are often found (11–13; Pelkonen, J., unpublished results) is consistent with this view.

The FT clones have neither rearranged the TCR- $\beta$  gene nor do they express

detectable mRNA for either the TCR- $\alpha$  or - $\beta$  genes, indicating that they are at a very early stage of development. The rearrangement of the  $\beta$  genes is often detected at day 15 of gestation (11, 13, 15).

The FT clones express the L3T4 and/or the Lyt-2 T cell differentiation antigens. It probably reflects the effect of IL-4 on the FT clones as we previously found (19) with heterogeneous populations of FT that rIL-4 induces some of these cells to express Lyt-2. The failure to detect L3T4<sup>+</sup> cells in such studies could be due to the sensitivity of the FACS analysis coupled to the lower frequency of cells induced to express L3T4 by IL-4 (2 of 10 FT clones analyzed) and to the constant exposure of the cells to PMA. PMA may inhibit the expression of L3T4 by FT cultured in vitro (Palacios, R., unpublished observations). The data obtained here with the FT clones show that the expression of the L3T4 and Lyt-2 surface molecules can precede that of the TCR- $\alpha$ , - $\beta$ /T3 complex on the cell membrane. Experiments to be reported elsewhere (Palacios, R., and J. Pelkonen, manuscript in preparation) showed that the FT clones with phenotypes I and II (Lyt-2<sup>+</sup>, L3T4<sup>-</sup>) can give rise to Lyt-2<sup>+</sup>, L3T4<sup>+</sup> thymocytes carrying  $\alpha$ , $\beta$ /T3 T cell antigen receptor on the cell membrane. Thus, during ontogeny, the expression of the Lyt-2 molecule appears to precede that of the L3T4 surface antigen. This view is consistent with the finding that in vivo Lyt-2<sup>+</sup>, L3T4<sup>-</sup> FT are found before Lyt-2<sup>+</sup>, L3T4<sup>+</sup>, or Lyt-2<sup>-</sup>, L3T4<sup>+</sup> thymocytes can be observed (35, 36).

The FT clones grow in rIL-4, which acts on these cells via an IL-2-independent pathway. Six FT clones also proliferate in rIL-2, albeit to a lesser extent than in rIL-4. Interestingly, the FT clones A2 and G12 do not have receptors for IL-2 but PMA induces both clones to express functional receptors for IL-2. Thus, the FT clones A2 and G12 resemble a subpopulation of freshly isolated FT (~30–50%) that can be induced with PMA to express IL-2-R (37, and Palacios, R., unpublished observations). The finding that the FT clones respond to both IL-2 and IL-4 suggests that single FT can use both interleukins as growth factor.

While the FT clones have not yet reached the stage to exhibit either helper function for B lymphocytes or cytolytic activity, they are already able to synthesize mRNA for IL-4 after stimulation in vitro. The latter functional property resembles that of freshly isolated 14-d fetal thymocytes (20) and strengthens the possible role of IL-4 in the process of T cell development within the thymus.

The FT clones should prove valuable to address the questions as to what determines that a given precursor gives rise to T cells that recognize antigen in the context of either class I or class II MHC products, whether a single precursor can generate both T cells having TCR made of  $\alpha$ , $\beta$ /T3 complex and T cells carrying TCR  $\gamma$ /T3 complexes on the cell membrane, and to study the regulation of the expression of the TCR- $\gamma$ , - $\beta$ , and - $\alpha$  genes.

### Summary

We have established in culture 13 clones from the thymus of a 14-d B10.BR mouse embryo and characterized 8 of them. All eight FT clones have the TCR- $\gamma$  and - $\beta$  genes in germline configuration. They express mRNA for the  $\gamma$ , but not for the  $\beta$  nor the  $\alpha$  genes. All eight FT clones are Thy-1<sup>+</sup>, Ly-1<sup>+</sup>, LFA-1<sup>+</sup>, Pgp-1<sup>+</sup>, H-2K<sup>+</sup>, and T3<sup>-</sup>. Three phenotypes could be distinguished on the basis

of Lyt-2, L3T4, and IL-2-R expression: Lyt-2<sup>+</sup>, L3T4<sup>-</sup>, IL-2-R<sup>+</sup> (I); Lyt-2<sup>+</sup>, L3T4<sup>-</sup>, IL-2-R<sup>-</sup> (II); and Lyt-2<sup>+</sup>, L3T4<sup>+</sup>, IL-2-R<sup>+</sup> (III) cells. All eight clones grow in rIL-4 and six clones also proliferate in rIL-2. Antibodies specific for IL-2-R inhibit their response to rIL-2 but not to rIL-4. The eight FT clones synthesize mRNA for IL-4 after stimulation in vitro and none of them exhibit cytolytic activity or helper function for B lymphocytes. We conclude that the FT clones are at a very early stage of T cell development, that the expression of Lyt-2 and L3T4 surface molecules can precede that of the antigen receptor, and that the same fetal thymocyte can use both IL-4 and IL-2 as growth factor.

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