MOLECULAR, CELLULAR, AND FUNCTIONAL PROPERTIES OF BONE MARROW T LYMPHOCYTE PROGENITOR CLONES

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Precursor cells from fetal liver and bone marrow colonize the thymus at day 11–12 of gestation, and the thymic microenvironment influences differentiation of the precursor cells along the T lymphocyte pathway (1–3). It is not known whether the colonizing precursor cell is a totipotential stem cell capable of giving rise to all types of blood cells, a common lymphoid stem cell incapable of myeloid differentiation but still able to generate both B and T cell lineages, or a restricted progenitor that is committed to T lymphopoiesis. While some evidence suggests the existence of totipotential stem cells (1–5), there is no direct proof that a common lymphoid stem cell or a T lymphocyte lineage–restricted progenitor (Pro–T lymphocyte) exists. Indeed, with the exception of B lineage–restricted progenitor cells (Pro–B lymphocytes) (6), self-renewing clones of the other putative progenitor cells have so far not been obtained in vitro.

The T cell receptor for recognition of antigen is a heterodimeric transmembrane glycoprotein consisting of disulfide-linked α and β subunits of molecular weight $40–50 \times 10^3$ each and the genes encoding these proteins were cloned (7–10). A third T cell–specific gene called γ was found (11, 12); the function of its products is still unknown. Mature functionally competent helper and cytotoxic T cells can develop with or without productive rearrangement at the γ locus (13).

Using heterogenous populations of thymocytes, it was found that embryonic thymocytes at day 15 of gestation start to rearrange D to J segments of the β gene complex. mRNA for α and β chains could be detected at day 17 of gestation when surface molecules with characteristics of α/β heterodimers began to be detectable on the cell membrane (14–16). The γ gene is transcriptionally active in the thymus at day 14 of gestation, reaches a peak steady-state level at day 15, and declines thereafter (12, 14).

By applying a criteria similar to that used for delineating the stages of B cell development, we call a cell that has both the α and the β chain genes in the

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germline configuration and gives rise only to mature T lymphocytes, a Pro–T lymphocyte; and we call a cell that has rearranged one of its α or β antigen-receptor genes and does not express T cell antigen-receptor on the cell membrane, a pre–T cell.

An ideal way of studying T cell development and the process of intrathymic differentiation is to establish Pro– and pre–T cell clones and follow their differentiation both in vivo and in vitro. Here we describe the establishment of continuous proliferating bone marrow clones whose cellular and molecular properties fit the criteria of Pro–T lymphocytes.

Materials and Methods

Materials. We used the following materials: PMA (Sigma Chemical Co., St. Louis, MO), LPS (Difco Laboratories Inc., Detroit, MI), Dextran-sulfate (DxS;[1] Pharmacia Fine Chemicals, Piscataway, NJ), Iscove's Dulbecco's modified medium (IMDM; Gibco Laboratories, Grand Island, NY), FCS (Boehringer Mannheim Biochemicals, Mannheim, Federal Republic of Germany), 5-Azacytidine (5-Aza; Sigma Chemical Co.), Retinoic acid (Sigma Chemical Co.), Ionomycin (Iono; Calbiochem-Behring Corp., La Jolla, CA), DMSO (Sigma Chemical Co.), low toxic rabbit complement (Cedarlane Laboratories Ltd., Ontario, Canada), [^{3}H]thymidine (TRA 120; The Radiochemical Centre, Amersham Corp., Amersham, United Kingdom).

Mice. CBA/J, C3H/HeJ, C57BL/6, DBA/2, ATH, and B10.A (4R) mice were from our animal facilities. Breeding pairs of CBA/N-t/*nu* and NIH Swiss II *nu/nu* mice were a gift of Dr. W. E. Paul (National Institute of Allergy and Infectious Diseases, Bethesda, MD). The CBA/N-*nu/nu* congenic strain, which carries both *xid* and *nu* mutations, was raised by a breeding strategy recommended by Azar and colleagues (17). The severe combined immunodeficiency (SCID) mutation occurred in the C.B.-17/*lcr* (C.B.-17) inbred strain (18). Breeding pairs of C.B.-17 SCID mice were donated by Dr. M. Bosma (Institute for Cancer Research, Philadelphia, PA) to our Institute. All mice are bred and maintained under pathogen-free conditions in the mouse barrier facility of our Institute and were housed in sterile isolators. All mice were 5–10-wk-old males or females at the time of study.

Monoclonal Antibodies. mAbs against the following surface antigens were used: Ly-1 (53-7-313), Lyt-2 (53-63-72, 3168.81, 3.155), L3T4 (GK1.5, H-129.19, RL-172), Thy-1.2 (jlj), Thy-1.1 (19E12,), jiid, Mac-1 (M1/70.15.11), PgP-1 (I42/5), IL-2-R (PC61, 7D4), antibody specific for IL-3-sensitive mouse cells (CC11), anti–mouse granulocytes (GM1.2; New England Nuclear, Boston, MA), B-220 (14.8, RA3-3A1/6B1), Ia (M5/114.15.2, 10.2.16), Lyb-2.1 (10.I.D2). The reports describing the characteristics of all these mAbs are listed elsewhere (6, 19–21).

mAbs 19E12, PC61, CC11, and 10.I.D2 were purified from hybridoma culture supernatants by affinity chromatography using protein A–Sepharose (Pharmacia Fine Chemicals) columns and were conjugated to biotin-*N*-hydroxisuccinimide (Calbiochem-Behring Corp.) as described (20). FITC-conjugated anti-Thy-1.2 mAb was purchased from New England Nuclear; FITC-labeled avidin was from Becton Dickinson & Co., Mountain View, CA; FITC-conjugated rabbit anti–mouse IgM was from Cappel Laboratories, Cochranville, PA; and FITC-conjugated sheep anti–mouse Ig that crossreacts with rat Ig was a generous gift of L. Forni (Basel Institute for Immunology).

Cytokines. Recombinant human IL-2 (22), recombinant mouse IL-3 (23), recombinant human IL-1 were from Cistrom Technologies (Boston, MA); purified IL-3 and granulocyte/macrophage colony-stimulating factor (GM/CSF) were purchased from Genzyme (Suffolk, England); recombinant mouse IL-4/B cell stimulatory factor 1 (BSF-1) (24) was a gift of Dr. P. Sideras (University of Stockholm, Stockholm, Sweden); synthetically

[1] *Abbreviations used in this paper:* 5-Aza, 5-Azacytidine; BSF-1, B cell stimulatory factor 1; CM, culture medium; DxS, dextran-sulfate; GM/CSF, granulocyte/macrophage colony-stimulating factor; IMDM, Iscove's Dulbecco's modified medium; SCID, severe combined immunodeficiency.

purified thymulin was a gift of Drs. M. Dardene and J. F. Bach (Hospital Necker, Paris, France).

Establishment of the C4 Line In Vitro. Bone marrow cell suspensions were obtained from CBA/N × NIH Swiss *nu/nu* male mice as described (25). The cells were exposed to a mixture of cytotoxic mAbs (j11d, M5/114, 10.2.16, GM1.2, and RA3-3A1/6B1) and rabbit complement treatment (20). After two cycles of treatment, viable cells were washed three times in culture medium (CM; IMDM + 2.5% FCS + 1% human AB serum + 5×10^{-5} 2-ME + 50 μg/ml gentamycin). The above procedure usually killed 93–98% of bone marrow cells. Viable cells were suspended in CM supplemented with IL-3-containing supernatants obtained from WEHI-3 cells as described (25), and supernatants obtained from Con A–stimulated mouse spleen cells (26) (final concentration 10%). Optimal concentrations of WEHI-3 supernatants were determined on the IL-3-dependent CB/Bm7 pro–B lymphocyte clone (6). Altogether, this CM is referred to as growth factor–conditioned medium (GFCM). Viable bone marrow cells (10^5) obtained as detailed above, suspended in 0.5 ml of GFCM, were cultured in Linbro tissue culture plates (Flow Laboratories, Irvine, Scotland) at 37°C for 4–5 d. Every 3 d, half of the medium was replaced with fresh GFCM. Confluent cultures were gently resuspended and split between two wells, each receiving 1 ml of fresh GFCM per Linbro well. After ~3–4 wk the cell lines could be transferred to 50-ml tissue culture flasks (Nunc, Roskilde, Denmark). Established cell lines can be harvested every 2.5–3 d and diluted 1:8 to 1:10 with fresh GFCM. Of the cell lines established, the C4 line was chosen for further study based on its phenotype. The C4 line was established in culture in October 1984 and was cloned by micromanipulation (25) in December 1984. The studies described here are those performed with the C4-77, C4-86, and C4-95 clones. They have been maintained in CM supplemented with IL-3-containing supernatants obtained from WEHI-3 cells (WEHI-CM) since January 1985 up to the present. The C4 clones can be frozen by standard procedures (25).

Immunofluorescence Staining and Flow Fluorometry Analysis (FACS). This was carried out as described in detail elsewhere (20) using a FACS-I analyzer (Becton Dickinson & Co.). Direct immunofluorescence staining was used for detection of Thy-1.2 and surface IgM. Indirect immunofluorescence staining was carried out using as second-step reagent FITC-conjugated avidin and FITC-conjugated sheep anti-Ig as required. Dead cells were excluded from analysis by propidium Iodide. Fluorescence emitted by single-viable cells was measured using logarithmic amplification, the data collected from 10^4 cells were analyzed with a program in a Consort 30 computer, and histograms were generated. Lyt-2^+ cells generated by the C4 clones in vitro (see below) were isolated by cell sorting with a FACS 440 based on staining (anti-Lyt-2 53-6-72 mAb and FITC-labeled sheep anti-Ig antibody) as described elsewhere (20).

Proliferative Responses to Cytokines. The cells were harvested in log phase of growth, washed three times, and resuspended in CM. The cells (10^4) were incubated in microplate wells in a final volume of 200 μl of CM containing the following agents: IL-3 (final dilution 1 to 20 U/ml), GM/CSF (10–100 U/ml), IL-2 (10–500 U/ml), IL-1 (5–50 U/ml), the combination of PMA (10 ng/ml) + Iono (500 ng/ml) and IL-2 (100 U/ml) plus or minus IL-1 (10 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 6 h of the culture period. The data are expressed as counts per minute and are the mean of triplicate samples.

Cell Surface Labeling, Immunoprecipitation, and SDS-PAGE Analysis. These procedures were carried out as described (19) using PC61 and CC11 mAbs coupled to CNBr-activated Sepharose 4B in the immunoprecipitations.

Isolation and Analysis of Nucleic Acids. DNA preparation, restriction enzyme digestions, agarose gel electrophoresis, DNA blotting procedures, probe preparations, hybridization procedures, and autoradiography were performed as described (10).

β Chain Gene Complex Probes. The 4.1 cDNA probe specific for the constant region of the β gene complex and the J15 probe specific for Jβ2 were described elsewhere (15).

α Chain Gene Complex Probes. The genomic probes Bam 7.5 (a 1.5-kb Bam HI fragment from cosmid clone BDFL7.5), Bam 2.5 (a 2.3-kb Bam HI fragment from cosmid clone BDFL2.5), and Kpn 7.5 (a 1.7-kb Kpn I fragment from cosmid clone BDFL7.5) spanning 30, 10, and 40 kb, respectively, 5′ to the α constant region (10) were used.

γ Gene Complex Probes. The Tγ5 cDNA specific probe for the constant regions of the γ gene complex (27) and the Jγ1 probe (1.2-kb Ava I–Hind III genomic fragment) (13, 28) were used.

Transfer of the C4 Clones into Sublethally Irradiated Mice. C4-77, C4-86, and C4-95 clones (5–10 × 10^6 in 0.1 ml PBS) were injected intravenously (lateral vein of the tail) into irradiated (600 rad) CBA/J or (300 rad) SCID mice. Control mice were injected with PBS only and the mice were maintained in sterile isolators. The thymuses were removed 2, 3, or 4 wk later and the spleens were removed at various times (2, 3, 4, 6, or 8 wk). Cell suspensions were prepared and the presence of Thy-1.2$^+$ and Thy-1.1$^+$ cells was determined by FACS analysis using FITC-labeled Thy-1.2 and biotin-conjugated Thy-1.1 (plus FITC-labeled avidin)-specific mAbs. The presence of PgP-1$^+$, L3T4$^+$, and Lyt2$^+$ cells in the thymocyte cell suspensions was analyzed by indirect immunofluorescence staining using FITC-conjugated sheep anti–murine Ig antibody as a second-step reagent.

Thy-1.1$^+$ cells from spleen of reconstituted mice were isolated by an immunorosetting technique using purified anti-Thy-1.1 mAb and protein A–coupled sheep erythrocytes, as detailed elsewhere (19), after 6–8 wk of transfer of the C4 clones. The erythrocytes in the rosette-forming cells were lysed with ammonium-Tris chloride buffer and the viable cells were washed three times and resuspended in CM. Aliquots of the cells were incubated at 37°C for 6–8 h, washed, and used to assess the presence of Thy-1.1$^+$, Thy-1.2$^+$, Ly-1$^+$, Lyt-2$^+$, and L3T4$^+$ cells by FACS analysis. The Thy-1.1$^+$ cells isolated from spleen were tested for both their proliferative response and the generation of cytotoxic T cells stimulated by several alloantigens as follows: 3–4 × 10^5 Thy-1.1$^+$ spleen cells were cocultured with 10^6 irradiated (2,200 rad) spleen cells from CBA/J, DBA/2, BALB/c, ATH, C57BL/6, and B10.A(4R) or with CM only, in flat-bottomed microplate wells in a final volume of 200 μl per microwell at 37°C. Cell proliferation was assessed by [^{3}H]-thymidine uptake (19.5 KBq/well) during the last 12 h of a 4–5-d culture period and cytotoxic activity was assessed after 6 d of culture using ^{51}Cr-labeled LPS-stimulated spleen cells from C57BL/6, BALB/c, and CBA/J mice as target cells, as described (21).

Induction of the C4 Clones to Differentiate In Vitro. The C4-77, C4-86, and C4-95 clones (3–5 × 10^6) suspended in 5 ml of WEHI-CM were exposed to several concentrations of the following agents: 5-Aza (1–100 μg/ml), rIL-2 (100 and 500 U/ml), rIL-1 (10–50 U/ml), thymulin (10 ng to 10 μg/ml), PMA (10–1,000 ng/ml), retinoic acid (0.01–1 mg/ml), DMSO (0.01–1%), and rIL-4/BSF-1 (final dilutions, 1:4 to 1:40). 7 ml of fresh WEHI-CM were added to the cultures in plastic flasks 24 h later and the cultures were incubated at 37°C for 1–7 d more. Aliquots of cells from the cultures were collected at various times and the cells were assayed for expression of Thy-1.1, Ly-1, Lyt-2, L3T4, Ig, Ia, PC61, and CC11 surface antigens by FACS analysis. The best and reproducible results were obtained with the following protocol: the C4 clones (5 × 10^6) were exposed to 5-Aza (20–25 μg/ml) in tissue culture flasks in a final volume of 5 ml of WEHI-CM at 37°C. 24 h later, 7 ml of fresh WEHI-CM were added to the cultures and incubated at 37°C for 3 d more. After 3–5 d of culture in the absence of 5-Aza, the cells were again treated with 5-Aza as indicated above. The cells were collected, washed, and cultured in WEHI-CM without 5-Aza, at 2–3 × 10^5 cells/ml in tissue culture flasks, changing them into fresh WEHI-CM every 2.5–3 d. In some experiments the C4 clones were treated with 5-Aza as above and half of the cells were cultured in CM supplemented with either rIL-2 (100 U/ml) or purified IL-3 (20 U/ml) at the same culture conditions as above. The C4 cells were collected at different times of culture and assayed for: (*a*) expression of surface antigens (indicated above) by FACS analysis, and (*b*) proliferative responses to cytokines as detailed above.

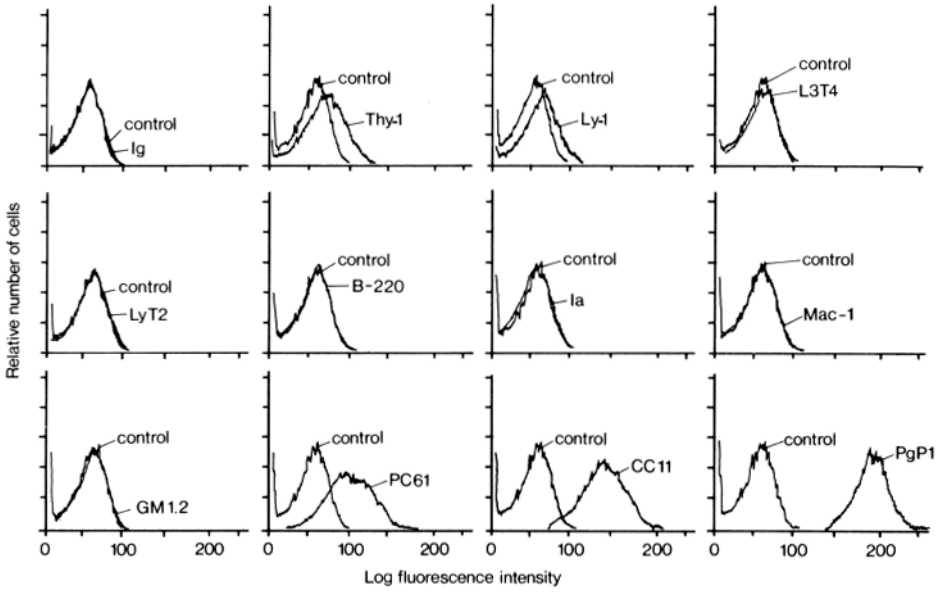

FIGURE 1. The presence of the surface antigens indicated above on the C4-77 clone was determined by direct or indirect immunofluorescence staining and FACS analysis. Control, histograms from C4-77 cells stained with second-step reagent only.

Results

Development and Establishment of the Clones C4-77, C4-86, and C4-95 In Vitro. We attempted to establish pro–T lymphocyte clones in vitro by using bone marrow cells from a *nu/nu* mouse that contains T cell precursors but no thymus. Most of the marrow cells belonging to other hematopoietic lineages were eliminated with a mixture of mAbs plus complement treatment. These procedures were performed to enrich for pro–T lymphocytes and to diminish possible negative regulatory effects on growth of these cells by other marrow cells, as encountered during the establishment of pro–B lymphocyte clones in vitro (6, 25). The details for the establishment of the C4 line obtained from bone marrow of a CBA/N × NIH Swiss II *nu/nu* mouse are given in Materials and Methods. The C4 line was obtained in October 1984 and chosen for further study because of its phenotype. Clones were obtained from this line by micromanipulation in December 1984. Here we report results of experiments carried out with the C4-77, C4-86, and C4-95 clones.

Phenotypic Characteristics. The phenotype of the C4 clones was determined by immunofluorescence staining and flow fluorocytometry (FACS) using a panel of mAbs against surface antigens expressed by cells of the T, B, or myeloid lineages. Fig. 1 shows the results of this analysis obtained with the C4-77 clone. These cells have low levels of Thy-1 and Ly-1 antigens, but no detectable Lyt-2 or L3T4 T cell differentiation surface markers present on most thymocytes and peripheral T lymphocytes from adult mice. The C4-77 cells do not carry on the cell membrane antigens normally present on cells of the B lymphocyte (B-220, Ig, Ia) or myeloid (GM1.2, Mac-1, Ia) lineages. They express the glycoproteins detected by the PC61 mAb against IL-2-R and by the C11 mAb specific for IL-

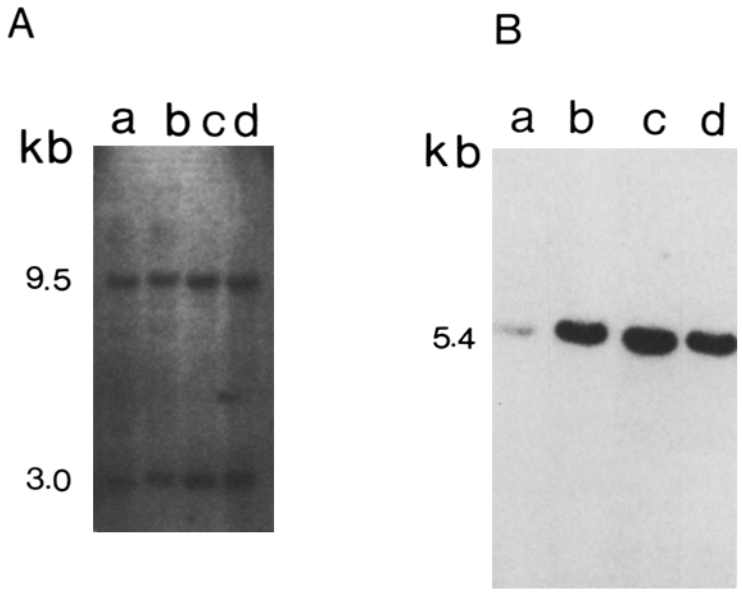

FIGURE 2. Southern blot analysis of the configuration of the β gene complex in the C4 clones using a β constant region probe (*A*) and a Jβ_2-specific probe (*B*) and Hind III–digested DNAs.

3-sensitive cells, which may bind to receptors for IL-3. The C4-77 cells have high levels of the PgP-1 glycoprotein normally present on marrow cells, fetal thymocytes, and Lyt-2$^-$ L3T4$^-$ thymocytes from adult mice. The phenotype of the C4-86 and C4-95 clones is very similar to that of the C4-77 clone.

The absence of markers normally found on cells of the B lymphocyte and/or myeloid lineages together with the presence of the Thy-1, Ly-1, as well as the PgP-1 glycoprotein, an antigen expressed by marrow cells that migrate to the thymus (29), on the C4 clones raised the possibility that they could represent early T cell precursors.

Configuration of the α, β, and γ T Cell–specific Genes. We began to explore the possibility that the C4-77, C4-86, and C4-95 clones could be early T cell precursors by assessing the presence of RNA transcripts for the α, β, and γ T cell genes by Northern blot analysis. We found that the three clones expressed shorter RNA transcripts (~1.0–1.2 kb) than the 1.5-kb size predicted for functional V-J-C transcripts of the γ gene, and no transcripts for either the β or the α genes. Next, we studied the configuration of these genes in the C4 clones by Southern blot analysis. The presence of rearrangement of the β1 gene complex was determined by using a cDNA constant region probe and Hind III–digested DNA from the clones, as well as from liver. Such a combination detects a 9.5-kb and a 3.0-kb band containing Cβ1 and Cβ2 germline elements, respectively (Fig. 2*A*, lane *a*). These analyses show that all three C4 clones contain the Cβ1 gene cluster in the germline configuration (Fig. 2*A*, lanes *b–d*). The presence of rearrangement of the β2 gene cluster in the clones was assessed by using a Jβ2-specific probe and Hind III–digested DNAs. The results (Fig. 2*B*) show that the

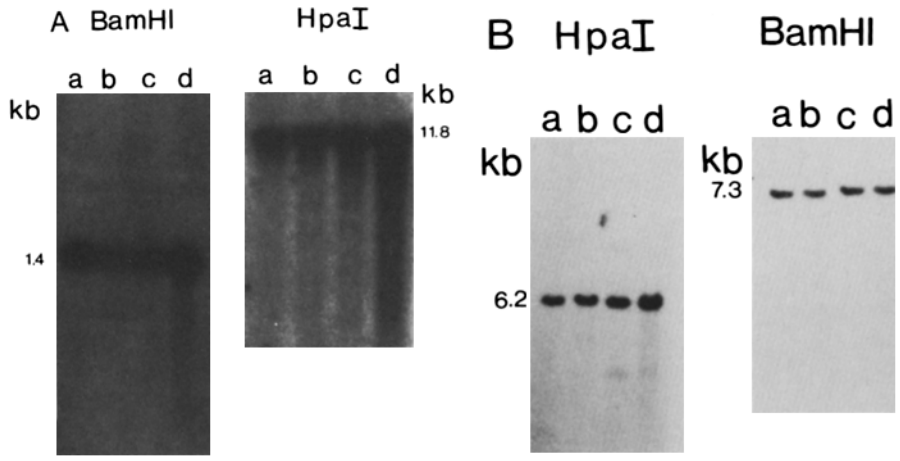

FIGURE 3. Southern blot analysis of the configuration of the α gene complex in the C4 clones using α chain–specific probes spanning 30 kb (*A*) and 40 kb (*B*) 5′ to the α constant region.

C4-77, C4-86, and C4-95 clones contain only the 5.4-kb germline band, indicating that the $\beta 2$ gene cluster in the C4 clones is in the germline configuration.

Owing to the distance spanning the Jα chromosomal region, rearrangement of the α chain gene is more difficult to analyze. Nevertheless, we addressed this issue by using α chain gene–specific genomic probes spanning 10 kb (not shown), 30 kb (Fig. 3*A*), and 40 kb (Fig. 3*B*) 5′ to the α constant region, respectively, and Bam HI– or Hpa I–digested DNAs. The results show no evidence for rearrangements of the α chain gene complex in the C4-77, C4-86, and C4-95 clones (Fig. 3).

The status of the γ gene complex in the clones was analyzed by using a cDNA constant region probe (Fig. 4) and Eco RI–digested DNAs. Such a combination detects a 13.4-, a 10.5-, and a 7.5-kb band containing constant germline regions of the $\gamma 4$, $\gamma 1$, and $\gamma 3$ gene clusters, respectively, in DNA from a BALB/c mouse (Fig. 4*A*); the nomenclature of the γ gene clusters used here is that of Traunecker et al. (13). These analyses show that all three C4 clones contain the $\gamma 1$ and the $\gamma 4$ gene clusters in the germline configuration (Fig. 4*A*, lanes *b–d*). In addition, these studies revealed that the mouse strains from which the C4 clones were derived like other strains of mice (e.g., DBA/2, CBA/J, C58) (13) lack the $\gamma 3$ pseudogene (Fig. 4*A*, lanes *a–d*). The same embryonic configuration of the $\gamma 1$ gene cluster in the C4 clones was found using a $\gamma 1$ constant region–specific probe (Fig. 4*B*). We conclude that the C4-77, C4-86, and C4-95 clones contain the α, β, and γ T cell genes in the germline configuration and that the γRNA transcripts detected in the C4 clones are germline transcripts.

Growth Requirements. As illustrated with the results depicted in Fig. 1, the C4-77, C4-86, and C4-95 clones have both receptors for IL-2, and the CC11 glycoprotein, as assessed by FACS analysis with the PC61 and the CC11 mAbs, respectively. We carried out immunoprecipitations from cell lysates of ^{125}I-labeled C4 clones with PC61 (30) or CC11 (19) mAbs and SDS-PAGE analysis. We found that the clones express both IL-2 receptors and the CC11 glycoprotein

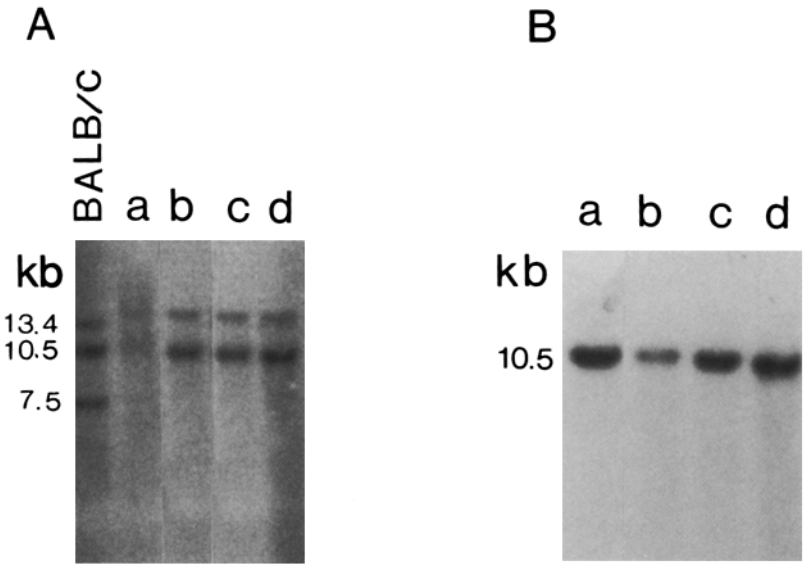

FIGURE 4. Southern blot analysis of the configuration of the γ gene complex in the C4 clones using cDNA-specific probes for the γ constant regions (*A*) and γ1-specific probe (*B*) and Eco RI–digested DNAs. *a*, liver; *b*, C4-77; *c*, C4-86; and *d*, C4-95 clones.

TABLE I

The Clones C4-77, C4-86, and C4-95 Grow in rIL-3

Growth factors	[^{3}H]thymidine uptake (cpm) of responding cells		
	C4-77	C4-86	C4-95
None	246	74	108
rIL-1	284	91	136
rIL-2	1,372	420	510
rIL-3	15,769	16,842	8,652
GM/CSF	207	101	117
PMA + ionomycin + rIL-2	1,010	453	446
PMA + ionomycin + rIL-1 + rIL-2	929	414	250

Proliferative responses to the agents indicated above by the C4 clones were assessed by [^{3}H]thymidine uptake during the last 6 h of a 2-d culture period conducted at 37°C. The data are the mean of triplicate samples (the SE in the experiment shown above was <8.6% of the mean). IL-1 (10 U/ml), IL-2 (100 U/ml), IL-3 (10 U/ml), GM/CSF (50 U/ml), PMA (10 ng/ml), Ionomycin (500 ng/ml).

of similar size to those precipitated by the same antibodies from IL-2-dependent and IL-3-dependent cell lines, respectively (data not shown).

The next set of experiments was designed to define the growth factor(s) used by the C4 clones in vitro. Table I shows that all three C4 clones proliferated significantly in IL-3 but not in rIL-1 or in purified GM/CSF. Saturating concentrations of recombinant human IL-2 promoted little DNA synthesis in the C4 clones (Table I). However, rIL-2 did not support actual growth of the C4 clones, i.e., increase in number of cells, whereas rIL-3 was very effective. Table I also shows that the combination of PMA, Ionomycin, and rIL-2 (± rIL-1), which we previously found very effective in inducing proliferation of intrathymic T cell precursors (21), did not increase the [^{3}H]thymidine uptake promoted by rIL-2

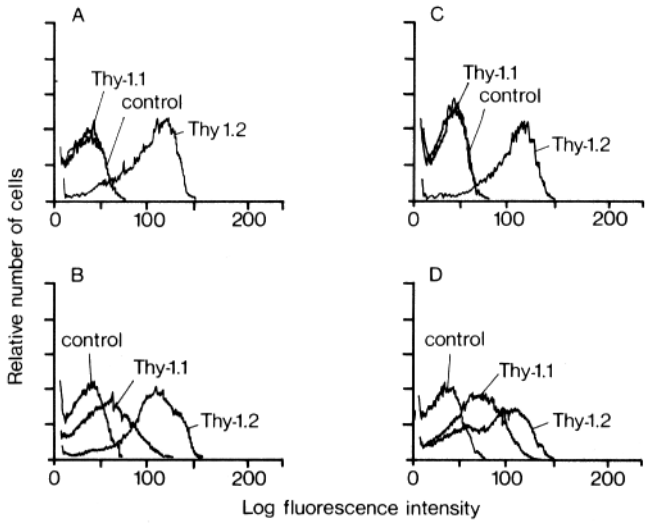

FIGURE 5. The presence of Thy-1.1$^+$ and Thy-1.2$^+$ cells in thymuses from irradiated mice that received either C4-77 cells (*B*), C4-86 (*D*), or PBS alone (controls) (*A* and *C*) 3 wk before, was determined by immunofluorescence staining and FACS analysis. *C*, histogram from cells stained with second-step reagent only.

alone. The data show that the growth requirements of the C4 clones are different from those of intrathymic T cell precursors from fetal and adult mice. The C4 clones cultured in IL-3 self-renew every 12–14 h and all three clones die within ~36–48 h in the absence of IL-3 (Table I and data not shown).

Differentiation of the C4 Clones. We next attempted to find out the functional potential of the C4-77, C4-86, and C4-95 clones both in vivo and in vitro.

In Vivo Studies. One property of a putative pro–T lymphocyte should be to home and colonize the thymus (see Introduction). We tested the C4-77, C4-86, and C4-95 clones for their ability to home and undergo differentiation in the thymus of sublethally irradiated mice. We used mAbs specific for Thy-1.1 or Thy-1.2 alleles and FACS analysis to distinguish cells of donor from cells of recipient origins. The C4 clones have the potential to express both Thy-1.1 (from NIH Swiss II) and Thy-1.2 (from CBA/N background) alleles. Thus, we transferred the C4 clones into irradiated mice whose T cells express Thy-1.2 only (CBA/J); the presence of Thy-1.1$^+$ cells in such hosts will indicate cells generated by the C4 clones. Fig. 5 shows that thymuses from irradiated CBA/J mice that received the C4-77 (Fig. 5*B*) or the C4-86 (Fig. 5*D*) clones 3 wk before analysis, contained Thy-1.1$^+$ thymocytes, while thymuses from mice that did not receive C4 cells (controls) contained Thy-1.2$^+$ but not Thy-1.1$^+$ thymocytes (Fig. 5, *A* and *C*). The C4-95 clone also has the property of homing and generating Thy-1.1$^+$ thymocytes. We have found between 13.5 and 67.4% Thy-1.1$^+$ C4-derived cells in thymuses of reconstituted mice. In two experiments, the clone C4-86 was injected into x ray–irradiated C3H/HeJ mice. The thymuses of these mice were not repopulated despite the fact that repopulation occurred after injection of AKR bone marrow cells. Unlike bone marrow cells, the intrathymic injection of C4-86 cells in either CBA/J or C3H/HeJ mice never resulted in any repopulation.

Normally the PgP-1 glycoprotein is expressed on 80–90% of fetal thymocytes at day 14 of gestation. The proportion of PgP-1$^+$ cells subsequently declines and reaches adult levels (5–10%) by day 19 (29, 31, and Palacios, R., unpublished

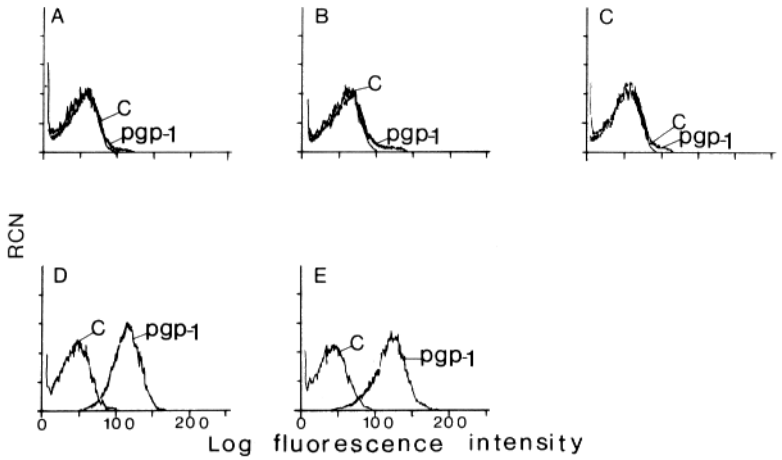

FIGURE 6. The presence of the PgP-1 glycoprotein on the C4-77 (*D*) and C4-86 (*E*) clones as well as on thymocytes from irradiated mice that received either C4-77 cells (*B*), C4-86 cells (*C*), or PBS alone (control) (*A*) 3 wk before, was assessed by immunofluorescence staining and FACS analysis. *C*, histograms from cells stained with second-step reagent only. *RCN*, relative cell number.

results). Using heterogenous populations of marrow cells it was found that the cells migrating to the thymus are PgP-1$^+$ (29, 31). Fig. 6, *D* and *E*, shows that the C4 clones are strongly PgP-1$^+$ before transfer into irradiated mice. Thus, one way of assessing differentiation of the C4 clones intrathymically in vivo was to determine the percentage of PgP-1$^+$ cells among the Thy-1.1$^+$ thymocytes generated by the C4 clones in vivo (Fig. 5). The same thymocyte cell suspensions analyzed in the experiments shown in Fig. 3 were also tested for the presence of PgP-1$^+$ cells by FACS analysis. The data (Fig. 6, *B* and *C*) show that there were <10% PgP-1$^+$ cells in thymuses from mice reconstituted with the C4-77 or C4-86 clones. A similar number of PgP-1$^+$ cells were detected in thymuses from irradiated mice that did not receive C4 cells (controls) (Fig. 6*A*). The results indicate that most Thy-1.1$^+$ thymocytes generated by the C4 clones have become PgP-1$^-$, i.e., they differentiated in vivo.

Further evidence that the C4 clones can undergo differentiation intrathymically in vivo comes from the results showing that the clones give rise to Thy-1.1$^+$ thymocytes expressing Lyt-2 and L3T4 antigens (see below). Thus, the data indicate that the C4 clones possess the property of homing and differentiating in the microenvironment of the thymus in vivo.

Next we wished to know whether the C4 clones give rise to functionally mature peripheral T lymphocytes in vivo. Thus, we studied the spleens of irradiated CBA/J mice that received the C4 clones 2, 3, 6, or 7 wk before, for the presence of Thy-1.1$^+$ splenocytes as assessed by FACS analysis. There were no detectable Thy-1.1$^+$ donor-derived cells in the spleens after 2 wk and very few or none after 3 wk of transfer, but significant numbers of Thy-1.1$^+$ splenocytes were detected after 6–7 wk of transfer of the C4 clones. We have found between 11 and 35% Thy-1.1$^+$ C4 clone–derived cells in the spleens of reconstituted mice.

We have begun to study the phenotype and functions of the Thy-1.1$^+$ cells in the spleen generated by the C4 clones in vivo. Thus, we isolated Thy-1.1$^+$ cells

TABLE II

The C4 Clones Give Rise to Mature T Lymphocytes In Vivo

C4-derived Thy-1.1$^+$ isolated responder spleen cells*		Proliferative cell responses to [^{3}H]thymidine uptake (cpm)				
Clone	Phenotype	Medium	CBA/J	C57BL/6	DBA/2	ATH
C4.86	93% Thy-1.1$^+$ 49% L3T4$^+$ 43% Lyt-2$^+$	2,104	2,801	33,516	53,571	47,892
		Medium	CBA/J	C57BL/6	BALB/c	B10.A (4R)
C4-77	91.5% Thy-1.1$^+$ 53% L3T4$^+$ 38% Lyt-2$^+$	4,814	5,613	67,920	40,391	32,973
C4-95	97% Thy-1.1$^+$ 56% L3T4$^+$ 39% Lyt-2$^+$	5,692	7,024	52,907	45,720	51,264

* Thy-1.1$^+$ cells were isolated from spleens of irradiated CBA/J after 7–8 wk of transfer of the C4-77, C4-86, or C4-95 clones. The isolated spleen cells were assayed for their phenotype by FACS analysis as well as for their proliferative responses to the alloantigens indicated above. Cell proliferation was determined by [^{3}H]thymidine uptake during the last 12 h of a 4–5-d culture period conducted at 37°C. The data are the mean cpm of triplicate samples (the SE in the experiments shown above was <11.4% of the mean).

from the spleens of reconstituted mice as detailed in Materials and Methods, and we assessed both their phenotype by FACS analysis and their capacity to proliferate in response to several alloantigens. Table II shows results consistent with the interpretation that the C4-77, C4-86, and C4-95 clones gave rise to both Thy-1.1$^+$, Lyt-2$^+$, L3T4$^-$, and Thy-1.1$^+$, Lyt-2$^-$, L3T4$^+$ mature T lymphocytes in vivo. Table II also shows that the isolated Thy-1.1$^+$ lymphocytes generated by the C4 clones proliferated in response to alloantigens from C57BL/6, DBA/2, ATH, BALB/c, and B10.A(4R) mice, but not to MHC antigens present on cells from CBA/J mice in which the C4 clones underwent differentiation. In addition, Fig. 7 shows that purified Thy-1.1$^+$ spleen cells give rise to cytotoxic T cells specific for H-2^b and H-2^d alloantigens but not against H-2^k MHC antigens after stimulation in vitro with irradiated spleen cells from C57BL/6, BALB/c, and CBA/J mice, respectively. The data indicate that the C4-77, C4-86, and C4-95 clones give rise in vivo to phenotypically and functionally mature T lymphocytes of different antigen specificities. In addition, the results show that the C4 clones became tolerant to the MHC antigens present on cells of the recipient mice in which they underwent differentiation.

Differentiation of the C4 Clones in the Environment of the SCID Mice. To further study the potential of the C4 clones to undergo differentiation in vivo, the C4-77 and the C4-86 clones were transferred into sublethally irradiated (300 rad) T cell– and B cell–deficient SCID mice (18). The cells from SCID mice have the potential to express Thy-1.2 and Lyb-2.2 surface antigens but not the Thy-1.1 or the Lyb-2.1 alleles. Thus, the presence of Thy-1.1 or Lyb-2.1 cells in reconstituted SCID mice will indicate that the T cells or the B cell lineage cells, respectively, were generated by the donor C4 cells. Fig. 8 shows the results of one of the two experiments carried out showing the same phenomena. 4 wk

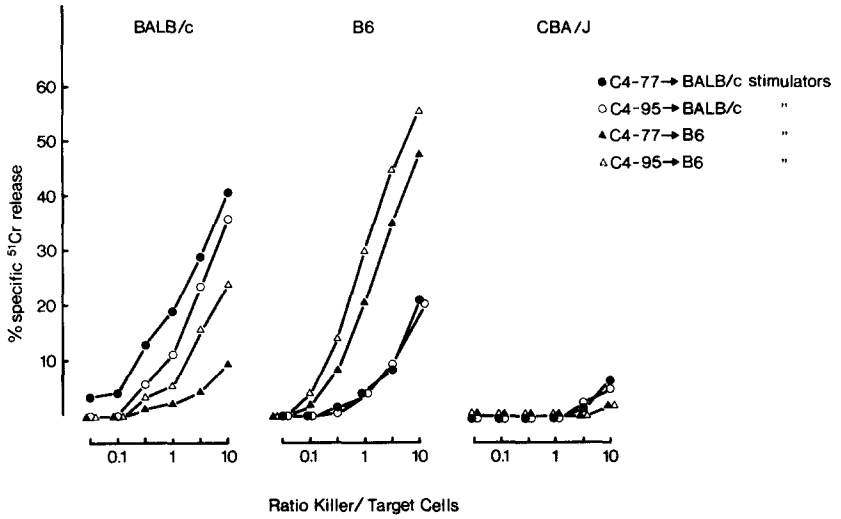

FIGURE 7. Thy-1.1$^+$ cells generated by the C4-77 and the C4-95 clones were isolated from spleens after 8 wk of transfer of the C4 clones into irradiated CBA/J mice. Purified Thy-1.1$^+$ (>98.5% Thy-1.1$^+$) splenocytes were stimulated in vitro with irradiated spleen cells from C57BL/6 (B6), BALB/c, and CBA/J mice at 37°C for 6 d and were tested for cytotoxic activity against ^{51}Cr-labeled LPS-activated B6, BALB/c, or CBA/J splenocytes.

after transfer of the C4-77 clone into SCID mice, the thymus contained ~2.5 × 10^7 cells. Virtually all of these thymic cells were Thy-1.1$^+$ and carried on their cell membrane the Lyt-2 and/or the L3T4 T cell surface markers (Fig. 8*A*). We found only ~0.45 × 10^6 cells from the thymic rudiment of the control SCID mice that received no C4 cells. 6 wk after transfer of the C4-77 clone into SCID mice, we found ~4.9–6.1 × 10^7 nucleated spleen cells. These populations clearly contained Thy-1.1$^+$, Lyt-2$^+$, and L3T4$^+$ T lymphocytes, but no IgM$^+$ mature B lymphocytes or B-220$^+$, Lyb-2.1$^+$ B cell precursors were detected (Fig. 8*B*). The spleens from control SCID mice that received no C4 cells had <12.0 × 10^6 nucleated cells, which comprised <8% Thy-1.2$^+$, 33–42% Mac-1$^+$, and no detectable (<1%) IgM$^+$, B-220$^+$, Lyb-2.1$^+$, L3T4$^+$ Lyt-2$^+$, or Thy-1.1$^+$ cells (Fig. 8*C*). Similar results to those found with the C4-77 clone were obtained with the C4-86 clone. The C4-95 clone has not yet been tested in this system. These results provide further direct evidence that the C4 clones can generate mature T lymphocytes but no cells of the B lymphocyte lineage in vivo.

In Vitro Studies. Due to the obvious potential to study cellular and molecular events during differentiation from pro–T lymphocytes to mature T lymphocytes, we undertook the task of finding out conditions that would induce the C4 clones to differentiate in vitro. Several agents (PMA, DMSO, retinoic acid, thymulin, GM/CSF, rIL-1, rIL-2, rIL-3, rIL-4/BSF-1, 5-Aza) reported to induce differentiation of cells of different origins were tested. We used as readout for induction of differentiation the expression of Lyt-2 and L3T4 surface antigens by the C4 clones as determined by FACS analysis. From all the agents tested, only the demethylating drug 5-Aza reproducibly induced differentiation of the C4 clones. Two examples illustrating these findings are shown in Fig. 9. After exposure to 5-Aza, the C4-77, C4-86, and C4-95 clones had higher levels of Thy-1 and Ly-1

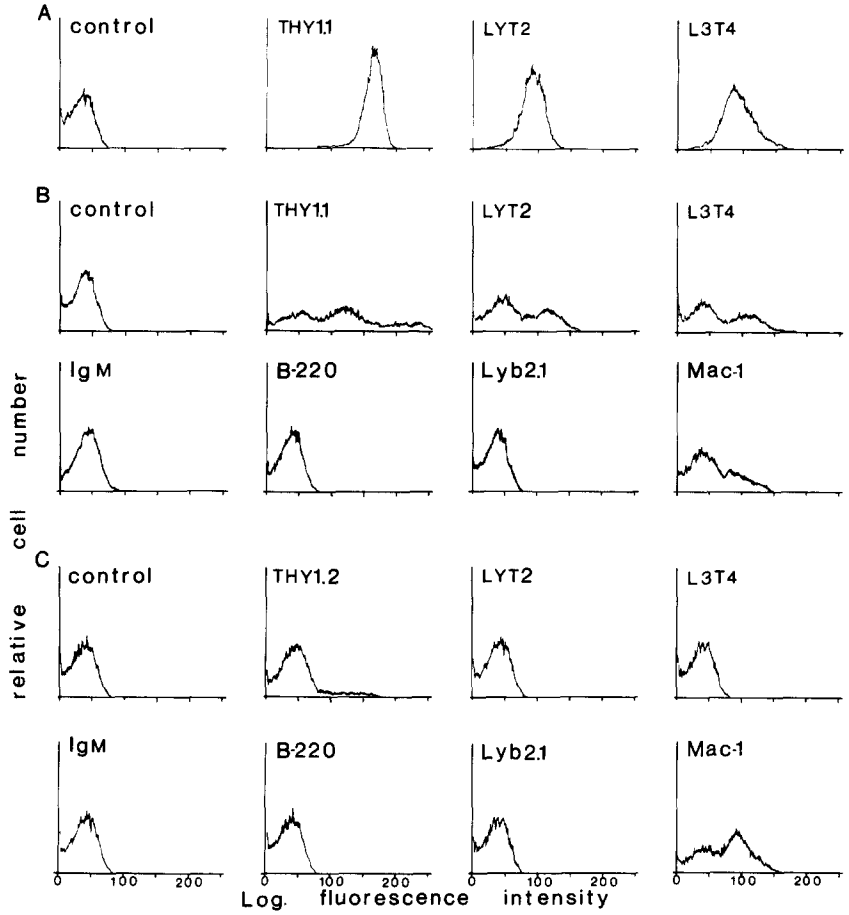

FIGURE 8. The thymus (*A*), and spleen (*B*) cells from SCID mice reconstituted with the C4-77 clone 4 and 6 wk, respectively, before analysis, and the spleen cells (*C*) from control SCID mice that received no C4 cells were studied for the presence of cells bearing the cell surface markers indicated above by immunofluorescence staining and FACS analysis. Control, cells stained with second FITC-labeled reagent only.

antigens and clearly expressed Lyt-2 and L3T4 antigens on their cell membrane. The presence of the latter two surface markers on 5-Aza-treated C4 cells was documented using three different mAbs against Lyt-2 (53-6-72 [IgG], 31688 [IgM], 3.155 [IgG]) and L3T4 (GK1.5 [IgG], RL-172 [IgM], H-129 [IgG]) antigens. None of the clones had detectable surface Ig or Ia antigens on their cell membrane after 5-Aza treatment, indicating that the drug induced the C4 clones to express products of certain genes only. The differentiation of the C4 clones upon treatment with 5-Aza became detectable after 7 d, reached plateau levels by ~14 d, and declined 50–80 d later. As 5-Aza induced the C4 clones to generate cells with a phenotype similar to that of thymocytes and since thymocytes use IL-2 but not IL-3 as growth factor in vitro, we were interested in knowing whether 5-Aza-treated C4 cells could be switched from IL-3 to IL-2 dependence. Thus, the C4 clones were exposed to 5-Aza and the cells were cultured and expanded in either IL-3 or IL-2. After 21 d of culture, cells from each group

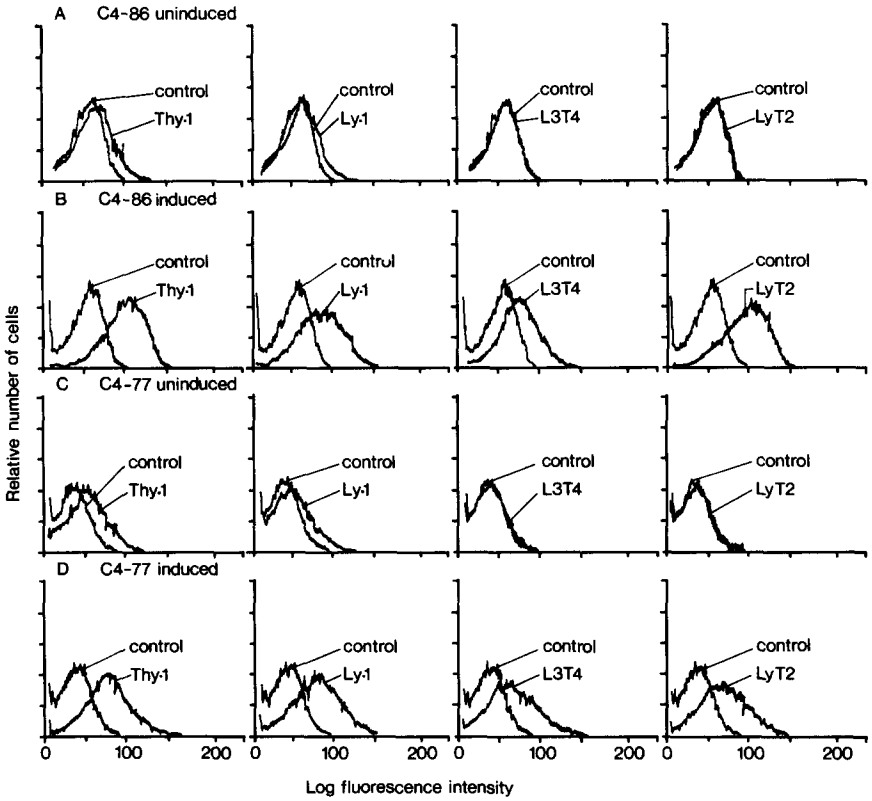

FIGURE 9. The presence of the surface antigens indicated above on the C4 clones either exposed (induced) or not exposed (uninduced) to 5-Aza was assessed by immunofluorescence staining and FACS analysis.

TABLE III

In Vitro-induced C4-86 and C4-77 Clones Can Be Switched from IL-3 to IL-2 Dependence

Responding cells		IL-supported proliferation [^{3}H]thymidine uptake (cpm)			
Clone treated with 5-Aza	Maintained in:	Medium	rIL-1	rIL-2	rIL-3
C4-77	IL-2	218	316	12,627	3,518
	IL-3	123	101	1,929	10,054
C4-86	IL-2	116	204	9,285	2,620
	IL-3	171	192	1,634	12,778

The C4 clones were treated with 5-Aza and kept in culture medium containing either rIL-2 or purified IL-3 for 21 d. The cells were washed and tested for their proliferative responses to the agents indicated above. Cell proliferation was measured by [^{3}H]thymidine uptake during the last 6 h of a 24-h culture period. The SE of the mean of the experiment shown above was <9.3%. IL-1 (10 U/ml), IL-2 (50 U/ml), and IL-3 (10 U/ml).

were tested for their proliferative response to rIL-1, rIL-2, and rIL-3. Table III shows that 5-Aza-treated C4-86 cells cultured in rIL-2 exhibited significant proliferative response to IL-2, little to IL-3, and none to IL-1. In contrast, 5-Aza-treated C4-86 cells that were cultured in IL-3 showed significant responses to IL-3, little to IL-2, and none to IL-1. The C4-86 cells cultured in rIL-2

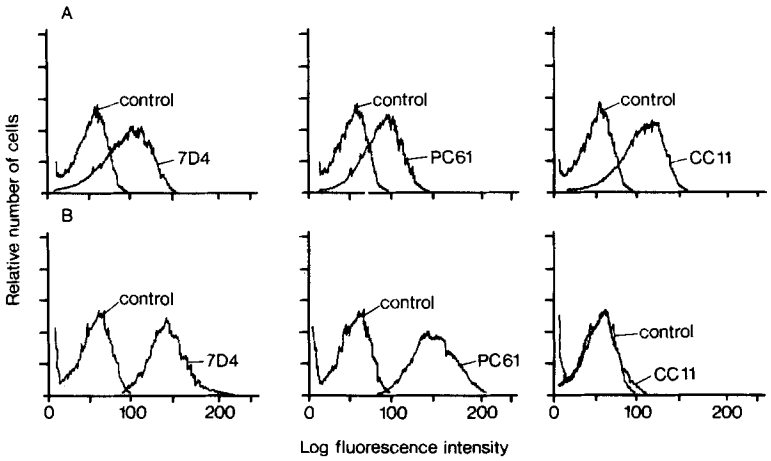

FIGURE 10. The expression of surface IL-2-R and the CC11 glycoprotein on C4-86 cells treated with 5-Aza and kept in medium containing purified IL-3 (*A*) or rIL-2 (*B*) was assessed by immunofluorescence staining and FACS analysis using mAbs specific for IL-2-R (7D4 and PC61) and the CC11 mAb specific for IL-3-sensitive cells.

without previous treatment with 5-Aza consistently died within ~3 d. Similar results were obtained with the C4-77 cells. The C4-95 clone has not yet been studied in this regard. These observations indicate that after their induction to differentiate by 5-Aza, the C4-86 and C4-77 clones can be switched from IL-3 to IL-2 dependence in vitro. Consistent with the functional data shown in Table III, most C4-86 and C4-77 cells exposed to 5-Aza and selected in rIL-2 had IL-2 receptors (assessed with both 7D4 and PC61 mAbs against IL-2-R by FACS analysis) and few cells still expressed the CC11 glycoprotein present on IL-3-sensitive cells (Fig. 10*B*). C4-86 and C4-77 cells treated with 5-Aza and cultured in IL-3 were both IL-2-R$^+$ and CC11$^+$ (Fig. 10*A*).

We wished to know whether treatment of the C4 clones with 5-Aza followed by selection in rIL-2 would induce them to further differentiate along the T lymphocyte pathway. We found that C4 cells subjected to such procedures became predominantly Thy-1$^+$, Ly-1$^+$, L3T4$^-$, Lyt-2$^+$ as assessed by FACS analysis (Fig. 11*B*). In several experiments performed, the C4 clones gave rise to Lyt-2$^+$, L3T4$^-$ cells but never to Lyt-2$^-$, L3T4$^+$ cells after 5-Aza treatment and subsequent selection in IL-2 in vitro. In one experiment we isolated the Lyt-2$^+$, L3T4$^-$ cells generated by the C4-86 clones by cell sorting and tested them for cytotoxic function in a lectin-facilitated cytotoxicity assay (21). We found that they did not possess killer function (data not shown).

Discussion

In the preceding section we described results of experiments aimed to characterize the continuous proliferating bone marrow clones C4-77, C4-86, and C4-95. These analyses indicate that the C4 clones do not express surface antigens normally found on cells of the B lymphocyte or myeloid lineages and have low levels of Thy-1 and Ly-1 antigens, but not surface markers present on thymocytes and peripheral T lymphocytes (Lyt-2, L3T4). They have the T cell antigen

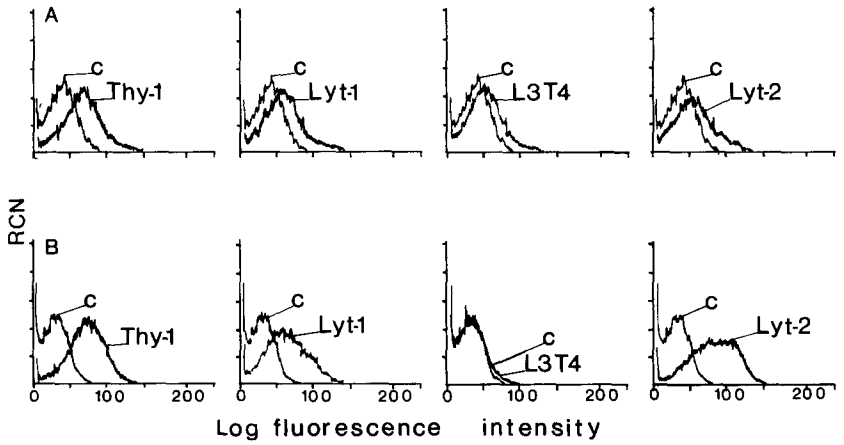

FIGURE 11. The expression of the surface antigens indicated by C4-86 cells that were treated with 5-Aza and kept in medium containing either IL-3 (*A*) or rIL-2 (*B*) was assessed by immunofluorescence staining and FACS analysis. *C*, histograms of cells stained with second-step reagent only. *RCN*, relative cell number.

receptor genes α and β, as well as the T cell–specific γ gene, in the germline configuration, and express germline transcripts for the γ but not for the α or the β T cell receptor genes. The C4 clones can home and undergo differentiation in the thymus and generate peripheral T lymphocytes with different antigen specificities in vivo. Repopulation of thymuses by the C4-86 clone failed for unknown reasons in two of twelve experiments. The C4 clones can be induced to differentiate through the T cell pathway with 5-Aza in vitro. However, the C4 clones did not give rise in vivo to B-220$^+$, Lyb-2$^+$ B cell lineage cells or to Ig$^+$ B lymphocytes capable of generating antibody-secreting cells after stimulation with polyclonal B cell activators in vitro. Nor were they induced in vitro by 5-Aza to express B cell lineage surface antigens (Ig, Ia). Moreover, the B cell precursor–specific gene pZ183 (32) is not expressed in the C4 clones, while Pro–B lymphocyte clones (6) clearly expressed it (Sakaguchi, N., and R. Palacios, unpublished results). Taken together the data indicate that the C4 clones do not undergo differentiation along the B lymphocyte pathway either in vivo or in vitro. Although we cannot formally exclude the possibility that their failure to generate lymphocytes of the B cell lineage could be due to the effects of the *xid* and the nude mutations on B cell development (20); it seems unlikely, because *xid/nu*-defective mice do have B-220$^+$ B cell precursors (33). Thus, the C4 clones functionally behave as progenitors committed to the T cell lineage, i.e., pro–T lymphocytes. The C4 clones, unlike unfractionated bone marrow cells, fail to grow in thymuses after intrathymic injection. This finding might indicate that they need to seed somewhere (bone marrow?) before being able to home in the thymus. Alternatively, the C4 clones might not be representative of all T cell precursors in the bone marrow (34).

Using bone marrow cells from mice treated with 5-fluorouracil and the site of integration of a vector introduced by retrovirus infection as a clonal marker, Lemischka et al. (35) failed to demonstrate the presence of lymphoid-restricted progenitors in those marrow preparations after transfer into irradiated mice.

This is not surprising as it is known that early lymphoid precursors are very sensitive cells to 5-fluorouracil treatment either in vivo or in vitro (36, 37, and Palacios, R., unpublished observations). Thus, the use of 5-fluorouracil-treated cell populations for the study of early stages of lymphocyte development can be misleading (35–37).

The C4-77, C4-86, and C4-95 clones express IL-3-R and use IL-3 as growth factor in vitro. Although they also express IL-2-R, these cells do not grow in the presence of saturating concentrations of rIL-2. Since it is known that IL-2 exerts its growth-promoting activity through its high-affinity class of receptors (38), the IL-2-R on the C4 clones are probably of low affinity. This is not a unique characteristic of the C4 clones, since other IL-3-dependent cells were also shown to express receptors with low affinity for IL-2 (39–41).

Interestingly, after induction in vitro with 5-Aza, the C4 cells could be switched from IL-3 to IL-2 dependence. Whether 5-Aza exerts this effect on the C4 clones by changing the affinity of the IL-2-R already present on these cells, by inducing *de novo* expression of functional IL-2-R or by other mechanisms remains to be determined.

The C4 clones are unresponsive to the growth factors IL-1 and GM/CSF as they neither proliferated nor underwent differentiation in the presence of these cytokines. It is worth stressing the similarities and differences in the requirements for growth in vitro of the pro–T lymphocyte C4 clones and those of intrathymic T cell precursors. The combination of PMA, Iono, and rIL-2 is very effective in inducing proliferation of intrathymic T cell precursors from fetal and adult mice (21), but ineffective on the C4 clones (Table I). Also, while the C4 clones express receptors for IL-3 and grow in IL-3 in vitro, most intrathymic T cell precursors from fetal and adult mice exhibit neither of these properties (19, 21). Presumably, when the pro–T lymphocyte gets into the thymus, the expression of IL-3-R is switched off and they use IL-2 and/or IL-4/BSF-1 as their growth factor (21, 42). This view would be supported by our results showing that after induction to differentiate with 5-Aza in vitro, the C4 cells can grow in IL-2 and most of these cells no longer express receptors for IL-3 (Figs. 10 and 11; Table III). Finally, it is interesting that both the C4 pro–T lymphocyte clones and freshly isolated intrathymic T cell precursors proliferate in recombinant IL-4/BSF-1, but this cytokine induces in vitro only intrathymic T cell precursors to differentiate into cytolytic T cells (this study, and references 41, 42). The latter finding could be explained by postulating that pre–T cells but not pro–T lymphocytes (see Introduction) may be able to differentiate under the influence of IL-4/BSF-1 in vitro. While it is clear that pro–T lymphocytes as well as pro–B lymphocyte clones express receptors for at least three known growth factors, namely, IL-2, IL-3, and IL-4/BSF-1, the biological significance of this is not yet understood (41). Also, we would like to stress that not all cells grown in IL-3 and expressing receptors for IL-2 behave as pro–T cells. Two independently derived clones of this phenotype had no measurable pro–T cell potential.

Another finding of fundamental interest in the present study is that 5-Aza induces the C4 clones to differentiate through the T cell pathway in vitro. 5-Aza induced the expression of certain genes in the C4 clones only, indicating that not all genes in these cells are susceptible to this drug. This is not a unique

feature of the C4 clones, as there are several reports in the literature showing that the expression of only some genes are activated by 5-Aza in various types of cells (reviewed in reference 43). How 5-Aza exerts its effects on the C4 clones is not yet understood. It is known that this drug causes demethylation of genes and that the state of methylation of genes correlates with their expression in many cases (43). Possibly, demethylation of the inducible genes (and/or of other genes required for their expression) caused by 5-Aza could have activated their expression in the C4 clones. If demethylation of genes were the only mechanism of action of 5-Aza one would expect that the expression of many more genes would be induced in the C4 clones, but clearly this is not the case. It follows that there must be a particular feature of the inducible genes that render them susceptible to 5-Aza.

Besides proving valuable as tools for developing mAbs specific for very early T cell precursors, the C4-77, C4-86 and C4-95 pro–T lymphocyte clones open new and direct ways of addressing several questions such as: the lineage relationship of the thymocyte subpopulations, mechanisms of tolerance to self MHC, the role of particular gene products in T cell development (e.g., Lyt2, L3T4, γ), what determines that a given pro–T lymphocyte gives rise to a lymphocyte with helper or with cytotoxic function recognizing foreign antigen in the context of class II and class I MHC products, respectively, and the regulation of the expression of the T cell α, β, and γ genes. We hope that the C4 clones will help to answer some of these questions in the near future.

Summary

The continuous proliferating bone marrow clones C4-77, C4-86, and C4-95 express low levels of Thy-1 and Ly-1 surface antigens, but no detectable surface antigens normally present on thymocytes, peripheral mature T lymphocytes, cells of the B lymphocyte or myeloid lineages. They contain the T cell antigen receptor genes α, β, and the T cell–specific gene γ in the germline configuration, and they express functional receptors for IL-3 and nonfunctional receptors for IL-2. The C4 clones are able to home and undergo differentiation in the thymus of sublethally irradiated mice and give rise in vivo to phenotypically and functionally mature peripheral T lymphocytes displaying several antigen specificities. In vitro 5-Azacytidine induces the C4 clones to express Lyt-2 and L3T4 T cell differentiation antigens, and renders them amenable to be switched from IL-3 to IL-2 dependence. However, the C4 clones seem incapable of giving rise to B lymphocytes either in vivo or in vitro. They self-renew in vitro in the presence of IL-3 every 12–14 h. We conclude that the C4 clones represent cells at the earliest stage of T cell development, i.e., Pro–T lymphocytes.

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