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Maturation or differentiation of human thymocyte precursors *in vitro*?

VERONIKA GROH, MARINA FABBI*, AND JACK L. STROMINGER

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138

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ABSTRACT **The differentiation or maturation potential of human thymocyte precursors has been studied by using a population of CD3/TCR$^-$, CD4$^-$, CD8$^-$ ("triple negative") thymocytes isolated by negative selection (TCR, T-cell receptor). This cell population, however, also contained 30–50% previously undescribed cells expressing very low levels of CD3/TCR$\gamma\delta$ (CD3/TCR$\gamma\delta^{low}$; ≈60% of which expressed the variable region gene Vδ1). Correspondingly, TCR γ and TCR δ gene rearrangements (predominantly Vδ1/joining region Jδ1) and full-length TCR γ and TCR δ transcripts (but only immature TCR β and no TCR α mRNAs) were found. These cells mobilized Ca^{2+} in response to ligation of CD3 but not following ligation of TCR$\gamma\delta$. When cultured in the presence of interleukin 7 or interleukin 2, these thymocytes gave rise to 30–60% CD3/TCR$\gamma\delta^{medium\ and\ high}$ cells (60–70% expressing Vδ1) seen as discrete populations. Thus, the proportion and Vδ phenotype of *in vitro* generated CD3/TCR$\gamma\delta$ cells closely resembled those of CD3/TCR$\gamma\delta^{low}$ cells in freshly isolated "thymocyte precursor" preparations. Small numbers of TCR$\alpha\beta^+$ cells also appeared. It is thus uncertain whether maturation, differentiation, or both account for the appearance of mature CD3/TCR$^+$ thymocytes, although the former appears most likely.**

Human T lymphopoiesis is still little understood. A putative chronology of human T-cell differentiation can be proposed based on the organization of T-cell receptor (TCR) genes and the expression of various cell surface molecules (reviewed in ref. 1). Several attempts have been made to identify human T-cell precursors, define stimuli promoting differentiation of these cells, and characterize their progeny. Cell surface CD7$^+$, CD2$^-$, CD3$^-$, CD4$^-$, CD8$^-$ cells isolated from *fetal liver* and *perithymic mesenchyme* gave rise to CD3/TCR$^+$ cells when cultured in the presence of T-cell conditioned medium (CM) and recombinant interleukin 2 (rIL-2) (2). Similarly, CD3/TCR$^-$ *postnatal thymocytes* also appeared to include T-cell precursors. Several different *in vitro* stimuli [i.e., mitogenic anti-CD2 monoclonal antibodies (mAbs) + rIL-2, CM + rIL-2, rIL-2 alone, rIL-4 + phorbol 12-myristate 13-acetate (PMA), or rIL-3 + anti-CD2 + rIL-2] induced differentiation of these thymocytes to CD3/TCR$^+$ cells (3). CD7$^+$, CD3$^-$ *adult peripheral blood* nuclear cells cultured in the presence of rIL-2 + phytohemagglutinin (PHA) gave rise to mature CD3/TCR$^+$ lymphocytes (4). Interestingly, the majority (when derived from fetal precursors) or a large proportion (when derived from adult peripheral blood) of emerging CD3/TCR$^+$ cells expressed TCR$\alpha\beta$ heterodimers (2, 4). In contrast, postnatal CD3/TCR$^-$, CD4$^-$, CD8$^-$ thymocytes gave rise predominantly to TCR$\gamma\delta$ cells under all culture conditions examined (3). Thus, in different precursor cell pools similar or identical stimuli resulted in different T-cell progenies. This observation might be due to different precursor frequencies for $\alpha\beta$ and $\gamma\delta$ T cells in precursor populations derived from different tissue sources. Alternatively, the effect of a given stimulus *in vitro* may depend on the developmental stage and tissue origin of T-cell precursors. This latter would imply that maturation of $\alpha\beta$ T-cell precursors in postnatal thymus requires stimuli different from those already studied.

The cytokine IL-7 was regarded originally as a pre-B-cell-specific growth factor (5, 6). However, IL-7 also acts on cells of the T-cell lineage (7) and might play a role in murine T-cell ontogeny. The most immature murine fetal thymic T-cell precursors and all thymocyte subpopulations derived from these cells in fetal thymus organ cultures proliferated in response to IL-7 (8). Thus, IL-7 may act on a wide variety of different target cells, potentially including precursors of both TCR$\alpha\beta$ and TCR$\gamma\delta$ lymphocytes.

The purpose of this study was (*i*) to characterize both the phenotype and the state of TCR gene organization in human postnatal CD3/TCR$^-$, CD4$^-$, CD8$^-$ thymocytes and (*ii*) to address the possibility that IL-7 mediates maturation and/or differentiation of these cells. In particular we wished to distinguish these two processes. Maturation is defined for this purpose as the *completion* of a genetic program already initiated, while differentiation necessarily involves the *initiation* of a new genetic program. A critical differentiation program in this case is the rearrangement of TCR genes.

MATERIALS AND METHODS

Thymi were obtained from normal children undergoing corrective cardiac surgery. Thymocyte suspensions were subjected to purification procedures for CD3$^-$, CD4$^-$, CD8$^-$ thymocytes involving various combinations of negative selection steps including mAb$^-$ [T3b (CD3), OKT4 (CD4), B9.4 (CD8); see ref. 9 for sources of mAbs] and complement-mediated cytolysis, "panning," and magnetic cell separation (3, 10). However, in a change from previous procedures, the purity of recovered thymocytes was tested by (*i*) direct immunofluorescence cytofluorographic analysis with fluorescein isothiocyanate (FITC)-conjugated mAbs against epitopes on CD3 (64.1; ref. 11), CD4 (anti-Leu3a), and CD8 (anti-Leu2a) different from those involved in the purification procedures (refs. 3, 10, and 12; S. A. Porcelli, personal communication); (*ii*) indirect immunostaining with FITC-F(ab')$_2$ goat anti-mouse immunoglobulin used to detect any residual mouse immunoglobulin-coated complement-resistant cells (3, 10). CD3$^{-,\,lo}$,† CD4$^-$, CD8$^-$ thymocytes were further analyzed for their cell surface and cytoplasmic expression of various lymphocyte antigens by one- and

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Abbreviations: TCR, T-cell receptor; IL, interleukin; rIL, recombinant IL; CM, T-cell conditioned medium; mAb(s), monoclonal antibody(ies); PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; FITC, fluorescein isothiocyanate; c-, cytoplasmic expression; V, variable; J, joining.

*Present address: Laboratorio di Immunobiologia, Istituto Nazionale per la Ricerca sul Cancro, 16132 Genova, Italy.

†Superscripts lo, med, and hi indicate low, medium, and high expression of the surface antigen.

two-color cytofluorographic analysis and by immunofluorescence and immunoperoxidase staining of cytocentrifuge preparations (9).

RESULTS AND DISCUSSION

Phenotypic and Functional Characterization of Human Postnatal Thymocytes Depleted of CD3^{med} and CD3^{hi} Cells. To purify cell surface CD3/TCR$^-$, CD4$^-$, CD8$^-$ thymocytes, protocols similar to those recently described were used (3, 10). Cytofluorography of a representative thymocyte preparation is shown in Fig. 1. No residual CD4$^+$ or CD8$^+$ cells were found in the negatively selected thymocyte population (Fig. 1 *A* and *B*). Exposure of these thymocytes to FITC-64.1 mAb (anti-CD3, not crossblocked by mAb T3b used for negative selection) gave ambiguous results: the profile was shifted slightly toward the right compared to the control and showed a shoulder above background as well as a small tailing beyond the vertical negative marker line (Fig. 1 *C* and *D*). This tailing was thought to represent authentic although dull staining of a small contaminant CD3$^+$ thymocyte population (≈2%). The larger but discrete shoulder was initially interpreted as an artefact caused by nonspecific binding or different affinities of the respective reagents. These assumptions were supported by data obtained from immunohistologic analyses of cytocentrifuge preparations of negatively selected thymocytes (Table 1). In accordance with previous results (3), a large proportion of cells (mean 76%) expressed cytoplasmic (c-) CD3 ε, while no surface CD3/TCR molecules were observed. cTCR β, γ, and δ proteins were detected in ≈2% of these cells. No cells containing cTCR α protein were found. These immunohistologic results were compatible with CD3/TCR$^-$ thymocyte preparations contaminated with ≈2% weakly surface CD3$^+$ cells, as deduced from the cytofluorographic analysis. Moreover, additional phenotypic properties of these thymocyte preparations were comparable to those of CD3/TCR$^-$ thymocytes previously isolated by the same purification protocol (3, 15) (see Tables 1 and 2).

However, 64.1 staining of negatively selected thymocyte suspensions and cytofluorographic analysis consistently showed profiles slightly brighter than background, despite

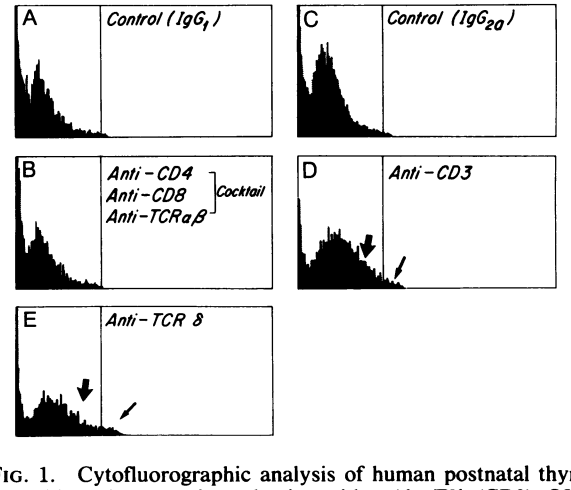

FIG. 1. Cytofluorographic analysis of human postnatal thymocytes subjected to negative selection with mAbs T3b (CD3), OKT4 (CD4), and B9.4 (CD8). Negatively selected cells were stained with FITC-mouse IgG1 (*A*); a cocktail of FITC-anti-Leu3a (CD4), FITC-anti-Leu2a (CD8), and FITC-WT31 or FITC-BMA031 (TCRαβ) (*B*); FITC-mouse IgG2a (*C*); FITC-64.1 (CD3) (*D*); and FITC anti-TCRδ1 (TCR δ) (*E*). Staining with the anti-Leu3a, anti-Leu2a, and WT31 cocktail gave negative results (*B*). Profiles with 64.1 and anti-TCRδ1 showed a small tailing (small arrows in *D* and *E*) and a shoulder above background (large arrows in *D* and *E*). Quantitative analyses were performed by subtracting control from test histograms.

Table 1. Cytoplasmic phenotype of surface CD3$^{-,\,lo}$ postnatal human thymocytes

	% cells expressing antigen		
		Two-week cultured cells† (n = 5)	
Antigen*	Freshly isolated cells (n = 4)	rIL-7	CM
cCD3 ε	76 (70–81)	80 (75–86)	75 (69–79)
cTCr α	ND	7 (5–9)	6 (5–7)
cTCR β	≈2	8 (6–9)	7 (5–8)
cTCR γ	≈2	76 (70–80)	73 (70–77)
cTCR δ	≈2	77 (71–82)	71 (69–75)
Keratin	≈2	NT	NT

Data are presented as percent of total cells expressing the antigen. Values represent means and numbers in parentheses represent range. ND, not detected; NT, not tested.

*mAbs were anti-Leu4 (CD3 ε), αF1 (TCR α chain; ref. 13); βF1 (TCR β chain); anti-CγM1 (TCR γ chain); anti-TCR-δ1 (TCR δ chain), and AE1 (anti-keratin; ref. 14); unless specifically noted see ref. 9 for sources of mAbs.

†Comparable results were obtained in cultures supplemented with rIL-7 + PMA or rIL-2 ± PMA. For culture conditions see † footnote to Table 2.

extensive titrations of control and specific mAbs and exposure of thymocytes to 25% human serum prior to mAb incubation. Furthermore, indirect immunofluorescence analysis using FITC-F(ab')$_2$ goat anti-mouse immunoglobulin showed similar profiles, indicating that residual mouse immunoglobulin (anti-CD3)-coated cells were present in the cell preparation (data not shown). Direct staining with FITC-anti-TCR-δ1 mAb (panreactive anti-TCR δ) and with mAb δTCS1 (Vδ1/Jδ1) also showed profiles above background (Fig. 1 *A* and *E* and data not shown). The panreactive anti-TCR γ mAb used in this study does not stain viable cells; thus, surface TCR γ expression was not determined. Staining results with mAbs specific for TCRαβ were negative (Fig. 1*B*). Together, these data suggested that the shoulders observed in the mAb 64.1, anti-TCRδ1, and δTCS1 profiles reflected surface expression of CD3 and TCR δ proteins at very low levels by a sizeable proportion of thymocytes (≈30–50%) obtained from the purification procedure and did not represent an artefact.

To test the functional potential of these CD3/TCR molecules, cells were analyzed for their capacity to mobilize Ca^{2+} in response to ligation of CD3 or TCRγδ. A transient increase in the cytoplasmic free Ca^{2+} concentration in the presence of crosslinking antiserum was induced by anti-CD3 but not anti-TCRδ1 in ≈30–50% of the cells (Fig. 2). Thus, these cells resembled a subpopulation of murine TCR^{lo} thymocytes, which lacked Ca^{2+} mobilization in response to anti-TCR mAb but mobilized Ca^{2+} following ligation of CD3 (21). These murine TCRαβ^{lo} thymocytes appeared resistant to negative selection (21). Human TCRγδ^{lo} thymocytes thus may provide a tool to study mechanisms of selection operating on the γδ T-cell lineage.

Two anti-CD3-reactive human thymocyte populations have been described (22). Anti-CD3 staining of unseparated human thymocytes, however, revealed three distinct anti-CD3-reactive cell populations. In addition to brightly CD3$^+$ cells (CD3^{hi}) and cells expressing medium levels of CD3 (CD3^{med}), a shoulder above background indicated the existence of a third CD3$^+$ population (CD3^{lo}) expressing less CD3 than CD3^{med} cells (Fig. 3). A similar distribution of expression levels was found for both TCRαβ and TCRγδ (data not shown). It thus appeared that the purification protocol applied in this study (and in earlier studies of others) did not yield a homogeneous CD3/TCR$^-$ population, although CD4$^+$, CD8$^+$ and CD3$^{med,\,hi}$ thymocytes were efficiently depleted. Instead, a cell population enriched for thymocytes without detectable CD3/TCR (some of which may express

Table 2. Cell surface phenotype of CD3$^{-, lo}$ postnatal human thymocytes

Antigen*	% cells expressing antigen		
	Freshly isolated cells (n = 10)	Two-week cultured cells† (n = 4)	
		rIL-7	rIL-2
CD4	ND	4 (3–4)	4 (3–5)
CD8	ND	14 (10–17)	18 (14–21)
CD3	42 (30–52)^{lo}	43 (32–61)	49 (35–57)
TCR$\alpha\beta$	ND	4 (3–4)	4 (3–6)
TCR δ	40 (29–50)^{lo}	45 (30–61)	44 (30–55)
Vδ1/Jδ1 (n = 5)	23 (17–29)^{lo}	30 (20–38)	32 (19–38)
Vδ2 (n = 5)	NT	5 (4–7)	7 (5–9)
Vγ2 (n = 5)	NT	8 (7–11)	7 (6–8)
CD7	77 (59–87)	75 (72–88)	71 (63–86)
CD2	73 (44–80)	79 (75–90)	85 (75–95)
CD1	NT	NT	NT
CD44	57 (49–64)	NT	NT
HLA-DR	40 (22–50)	34 (30–38)	45 (35–55)
IL-2Rp55 (n = 5)	5 (4–8) [3/5] ND [2/5]	21 (19–30)	23 (20–27)
IL-2Rp75 (n = 5)	6 (4–11) [3/5] ND [2/5]	NT	NT
CD45	98 (97–99)	NT	NT
CD34	20 (18–22)	NT	NT
CD14	ND	NT	NT

Data are presented as percent of total cells expressing the antigen noted. Values represent means and numbers in parentheses represent ranges. Numbers in brackets are number of samples exhibiting the noted phenotype/the total number analyzed. ND, not detected; NT, not tested. We thank J. A. Hansen, J. E. de Vries, L. J. Picker, K. Sugamura, K. A. Smith, W. J. M. Tax, W.-T. Tian, M. B. Brenner, T. Hercend, A. Moretta, and B. F. Haynes for the gifts of mAbs and K. Arai and A. E. Namen for the gift of recombinant lymphokines.

*mAbs were anti-Leu3a (CD4); anti-Leu2a (CD8); 64.1 (CD3); WT31 and BMA031 (TCR$\alpha\beta$ > $\gamma\delta$); anti-TCRδ1 (TCR δ chain); δTCS1 (variable region Vδ1/joining region Jδ1; ref. 16); BB3 (Vδ2; ref. 17); anti-Tiγa (Vγ2; ref. 18); anti-Leu9 (CD7); anti-Leu5b (CD2); anti-Leu6 (CD1); A3D8 (CD44; ref. 19); anti-HLA-DR; anti-IL-2Rp55 (CD25); TU27 (anti-IL-2Rp75; ref. 20); anti-HLe-1 (CD45); anti-HPCA-1 (CD34); and anti-LeuM3 (CD14). Unless specifically noted, see ref. 9 for sources of mAbs. Anti-Leu mAbs, anti-HPCA-1, anti-HLA-DR, and anti-IL-2Rp55 were purchased from Becton–Dickinson.

†Comparable results were obtained in cultures supplemented with rIL-7 + PMA, rIL-2 + PMA, or CM. For bulk cultures, cells were seeded at 0.5 × 10^6 per ml of RPMI 1640 medium containing 20% pooled human serum in round-bottom 96-well plates. Cultures were supplemented with (*i*) a 10^{-3} dilution of COS cell supernatant containing rIL-7 (2.5 × 10^5 units/ml) ± PMA (2.5 ng/ml final), (*ii*) rIL-2 (50 units/ml) ± PMA, or (*iii*) 10% CM. The cell surface phenotypes of bulk cultures were determined as described for freshly isolated thymocyte precursors.

cCD3 ε; see Table 1) and cells expressing low levels of CD3/TCR$\gamma\delta$ [a high proportion (≈60%) of which expressed Vδ1/Jδ1] was obtained. This population will be referred to as CD3$^{-, lo}$. No CD3/TCR$\alpha\beta^{lo}$ cells were detected in these preparations. Such cells may have coexpressed CD4 and/or CD8 and would thus have been eliminated by the purification procedure. Low levels of surface CD3/TCR δ were not detected by immunohistology in cytocentrifuge preparations of these cells, presumably because of the limited sensitivity of this technique.

TCR γ and δ Genes Are Functionally Rearranged in Postnatal Human Thymocytes Depleted of CD3$^{med, hi}$ Cells. Genomic DNA from nine different thymocyte preparations depleted of CD3$^{med, hi}$ cells was digested with *Kpn* I and *Xba* I to probe for Jγ and Jδ rearrangements, respectively. By

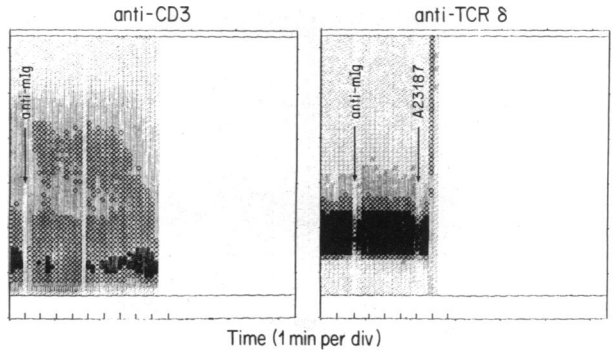

FIG. 2. Measurement of calcium responses to ligation of CD3 and TCR$\gamma\delta$ in CD3$^{-, lo}$ thymocytes. Analyses were performed as described (32). Anti-mouse immunoglobulin was added at the indicated time for crosslinking. Addition of calcium ionophore A23187 gave a maximum response. Crosslinking of CD3 but not TCR$\gamma\delta$ resulted in a transient increase in the cytoplasmic free Ca^{2+} concentration in ≈30% of cells.

using the Jγ1.3/2.3 genomic probe (23, 24), various TCR γ gene rearrangements were detected in all of the nine thymocyte preparations (data not shown). Similarly, each sample showed several Jδ1 rearrangements (25), of which the Vδ1/Jδ1 joining was the most prominent (Fig. 4). Lower intensity bands of 4.2 and 1.9 kb were also detected and corresponded to Vδ2 and Vδ3 rearrangements, respectively (Fig. 4) (25). Rearrangements of Jδ2 and Jδ3 were not detected. RNA from two CD3$^{-, lo}$ thymocyte samples was analyzed for the presence of TCR α, β, γ, and δ mRNAs, respectively, by blot hybridization using Cα (27), Cβ (28), Cγ (29), and Cδ (30) probes. No TCR α transcripts and only immature (1.0-kb) TCR β transcripts were found. In contrast, full-length TCR γ and δ mRNAs were detected in both samples (data not shown). These results strongly supported the cell surface staining and functional data. If, as initially assumed, thymocyte preparations had been contaminated with only ≈2% of CD3/TCR$\gamma\delta^+$ cells, no TCR γ and δ gene rearrangements would have been observed in this experiment. In DNA samples from control cells mixed with 2% TCR$\gamma\delta$ lymphocytes, no TCR γ or δ rearrangements were detectable (Fig. 4, lane 1). Presently the state of TCR gene organization in human CD3/TCR$^-$ lymphoid precursors is unknown. Thus, both CD3/TCR$^-$ thymocytes and CD3/TCR$\gamma\delta^{lo}$ cells could account for the observed TCR γ and δ gene rearrangements and mRNA transcripts.

Maturation or Differentiation of CD3$^{-, lo}$, CD4$^-$, CD8$^-$ Postnatal Human Thymocytes *in Vitro* in the Presence of IL-2 or IL-7? CD3/TCR$^{-, lo}$ thymocytes proliferated in the presence of rIL-7 with or without PMA, rIL-2 with or without PMA, or CM (unpublished data). To study the phenotype of cells generated under these conditions, bulk cultures and clones were derived from these cells exposed to rIL-7 ± PMA, rIL-2 ± PMA, or CM. The cell surface and cytoplasmic phenotypes of cultures/clones were monitored over a period of several weeks (6 weeks in the case of bulk cultures and up to 14 weeks for clones). In general, all five culture conditions gave similar results on bulk cultures and clones.

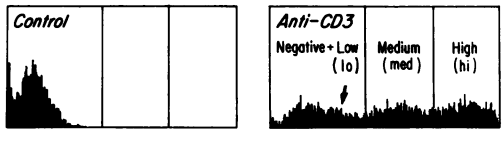

FIG. 3. Cytofluorographic analysis of unseparated human postnatal thymocytes stained with anti-CD3 (64.1) mAb. Three distinct cell surface CD3$^+$ populations can be distinguished: CD3^{lo} (see arrow), CD3^{med}, and CD3^{hi}.

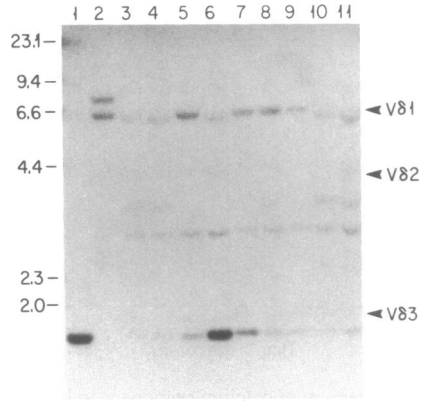

FIG. 4. Southern blot analysis of control cells (JY) mixed with 2% TCR$\gamma\delta^+$ cells (lane 1), Molt 13 cells (TCR$\gamma\delta^+$ leukemia cell line) (lane 2), and nine different preparations of CD3$^{-,lo}$ human postnatal thymocytes (lanes 3–11). Genomic DNAs were digested with *Xba* I and analyzed on Southern blots by standard procedures using a TCR Jδ1 probe (26). Lengths in kb are given on the left.

Thirty-six hours after the initiation of bulk cultures, CD3^{med} expression was found on ≈15–20% of the cells. CD3^{hi} cells were first visible after 3–4 days. Within about a week, a distribution of anti-CD3-staining intensities comparable to that observed with unfractionated thymocytes was found (Fig. 5). By 2 weeks, most CD3$^+$ cells were CD3^{hi}. The kinetics of TCR δ surface expression by these cells were almost identical. In contrast to these pronounced changes in intensity of surface CD3 and TCR δ expression, numbers of CD3$^+$ cells increased only slightly, if at all, from 30–52% in freshly isolated thymocyte preparations to 32–61% after 2 weeks of culture (Table 2). These results were consistent with the observation that CD3$^+$ and CD3$^-$ cells proliferated at similar rates (unpublished data). Summaries of the phenotypic properties of 2-week bulk cultures supplemented with rIL-7 or rIL-2 are shown in Tables 1 and 2. A large proportion of cells were CD3/TCR δ^+. TCR$\alpha\beta^+$ cells accounted for

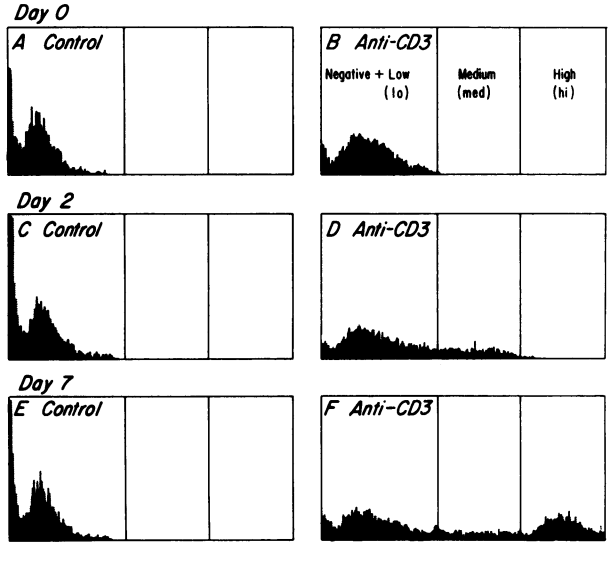

FIG. 5. Cytofluorographic analysis of freshly isolated CD3$^{-,lo}$ human postnatal thymocytes (day 0) and their *in vitro* (rIL-7 + PMA supplemented cultures) progeny (days 2 and 7) using FITC-anti-CD3 (64.1) mAb. On day 2, a significant number of cells expressed medium levels of CD3. On day 7, anti-CD3 staining intensities comparable to those observed on unfractionated thymocytes were found. The three levels of anti-CD3 staining intensity were deduced from those determined in Fig. 3. Quantitative analyses were performed by subtracting control from test histograms.

≈4% of the cells. Immunoprecipitation of ^{125}I surface-labeled cells with anti-CD3 revealed the known SDS/PAGE banding of TCR$\gamma\delta$ heterodimers, while only trace amounts of TCR$\alpha\beta$ heterodimers were found (data not shown). The majority of *in vitro* derived TCR$\gamma\delta^+$ thymocytes (≈60–70%) expressed Vδ1-encoded products, and ≈10–15% of the cells were Vδ2$^+$. Vγ2-encoded products were expressed by ≈15–20% of the TCR$\gamma\delta^+$ cells. The V gene usage of these *in vitro* generated thymocytes thus compared well with that observed *in vivo* (31). All CD4$^+$ cells expressed TCR $\alpha\beta$ by two-color cytofluorographic analysis. All CD8$^+$ cells coexpressed TCR$\gamma\delta$. About 80% of cells expressed cCD3 ε as well as TCR γ and δ proteins (Table 1). Phenotypic analyses of 4- and 6-week bulk cultures revealed similar results with a slight increase in the proportions of surface TCR$^+$ cells.

Phenotypes of clones derived from CD3$^{-,lo}$ thymocytes in the presence of IL-7 or CM are summarized in Table 3. After 3–5 weeks, 61 and 70 clones, respectively, developed from 576 seeded wells. A large proportion of clones, 46% and 59%, expressed surface CD3/TCR δ and were cTCR$\gamma\delta^+$. Of these clones, 74% and 66%, respectively, expressed Vδ1, and 7% and 10% were Vδ2$^+$. Three of the CD3/TCR δ$^+$, cTCR$\gamma\delta^+$ clones also contained cTCR β protein. Cells with cTCR β, γ, and δ proteins lacking TCR$\alpha\beta$ might express $\beta\delta$ receptors on their surface (32). Eight percent and 9% of clones, respectively, were CD3/TCR$\alpha\beta^+$. The remaining clones did not express cell surface CD3/TCR. About half of these clones contained cCD3 ε and cTCR γ and δ protein. All other clones were cCD3/TCR$\gamma\delta^-$. The majority of TCR δ$^+$ clones were double negative (CD4$^-$, CD8$^-$). No CD4$^+$, TCR δ$^+$ clone appeared. Some TCR δ$^+$ clones expressed CD8. Three CD4$^+$, CD8$^+$ (double-positive) TCR$\alpha\beta^+$ clones were generated, but they died within 5 weeks and were phenotyped only once. One double-negative $\alpha\beta$ T-cell clone was found. The phenotypes of all clones (except for the 3 double-positive clones and some of the initially CD3/TCR$^-$, cCD3/TCR$\gamma\delta^+$ clones, which eventually became surface CD3/TCR$\gamma\delta^+$) were stable over a period of 14 weeks. Thus, the overall pattern of phenotypes generated at the clonal level resembled closely that observed in bulk cultures, with the majority of cells belonging to the $\gamma\delta$ and small numbers of cells to the $\alpha\beta$ T-cell lineage. As with bulk cultures, the proportion of surface CD3$^+$ clones resembled closely that of CD3^{lo} cells in the freshly isolated thymocyte preparations. Bulk cultures and clones obtained in the presence of rIL-7 did not differ from those observed in cultures supplemented with rIL-2 or CM.

Table 3. Cell surface and cytoplasmic phenotypes of clones derived from CD3$^{-,lo}$ human postnatal thymocytes

Phenotype	Phenotype/total clones	
	rIL-7 (n = 61)	CM (n = 70)
CD3/TCR$\gamma\delta^+$	28/61 (46%)	41/70 (59%)
cTCR γ,δ^+	27/28 (96%)	39/41 (95%)
Vδ1/Jδ1$^+$	20/27 (74%)	26/39 (66%)
Vδ2$^+$	2/27 (7%)	4/39 (10%)
Other	5/27 (19%)	9/39 (23%)
cTCR β, γ, δ$^+$	1/28 (4%)	2/41 (5%)
CD3/TCR$\alpha\beta^+$	5/61 (8%)	6/70 (9%)
CD3/TCR$^-$	28/61 (46%)	23/70 (33%)
cTCRγ,δ^+	13/28 (46%)	10/23 (43%)
cTCRγ,δ^-	15/28 (54%)	13/23 (56%)

Numbers represent numbers of clones with the noted phenotype/total number of clones. Numbers in parentheses represent the clones with the noted phenotype as a percentage of the total number of clones. For limiting dilution cloning, cells were seeded at 0.3 cell per well onto allogenic feeders (peripheral blood mononuclear cells, Epstein–Barr virus-transformed lymphoblastoid B-cell lines) in the same supplemented media that were used for bulk cultures (see † footnote to Table 2).

Thymocytes depleted of $CD3^{med, hi}$ cells are phenotypically heterogeneous, including at least three major populations: (*i*) cells without detectable surface and cytoplasmic CD3/TCR; (*ii*) $CD3/TCR^-$ cells expressing cCD3ε but no cTCR proteins; (*iii*) $CD3/TCR\gamma\delta^{lo}$ cells. Additional, not necessarily overlapping, heterogeneity of these cells may exist at the level of TCR gene organization, transcription, and translation. Thus, precursor–product relationships between freshly isolated $CD3^{-, lo}$ thymocytes and cells generated in cultures cannot be inferred from this study. However, it is most likely that the majority of $CD3/TCR\gamma\delta^+$ cells in bulk cultures and most $CD3/TCR\gamma\delta^+$ clones stem from $CD3/TCR\gamma\delta^{lo}$ cells. Within about 2 weeks, these thymocytes appear to mature first to $CD3/TCR\gamma\delta^{med}$ and then $CD3/TCR\gamma\delta^{hi}$ cells when cultured in the presence of IL-7, IL-2, or CM. The predominant usage of Vδ1/Jδ1-encoded products both by freshly isolated $CD3/TCR\gamma\delta^{lo}$ thymocytes and by *in vitro* generated $CD3/TCR\gamma\delta^{med}$ and $TCR\gamma\delta^{hi}$ cells supports this conclusion. Whether maturation or differentiation of $CD3/TCR^-$, $cCD3/TCR^-$ cells and $CD3/TCR^-$, $cCD3^+/cTCR^-$ cells may also contribute to the appearance of phenotypically mature $\gamma\delta$ T cells remains speculative. $cTCR\gamma\delta^+$ cells without detectable cell surface CD3/TCR expression also appeared in bulk cultures and at the clonal level, although cells with this phenotype were not detected prior to culture. They may have matured or differentiated from $CD3/TCR^-$ thymocytes. $CD4^+$ $\alpha\beta$ T cells in bulk cultures, single-positive ($CD4^+$), double-positive, and double-negative $\alpha\beta$ clones may represent outgrowing contaminants that have escaped initial detection in the $CD3^{-, lo}$ thymocyte preparations. Alternatively, they may have originated from $CD3/TCR^-$ thymocytes.

Similar results have been described in earlier reports using the same or similar precursor purification protocols and culture conditions (2–4, 33, 34). The appearance of $\alpha\beta$ or $\gamma\delta$ T cells has been interpreted as differentiation of $CD3/TCR^-$ cells. However, since indirect staining techniques with mAbs also involved in the purification procedure were used to test the purity of the precursor cell preparations in some cases or the possibility of outgrowth of a small fraction of contaminating more mature cells cannot be excluded in others, such conclusions may be inappropriate.

This discussion illustrates some of the difficulties and limitations inherent in cell maturation/differentiation studies which use polyclonal, potentially heterogeneous, precursor populations. Phenotypically homogeneous, nonclonal precursor cell populations may be heterogeneous at the level of antigen receptor gene organization and at as-yet-undefined levels of cell lineage commitment. Well-characterized nontransformed T-cell progenitor clones and defined *in vitro* systems are needed to understand more fully precursor–product relationships in human T-cell development.

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