

IDENTIFICATION OF PRE-T CELLS IN  
HUMAN PERIPHERAL BLOOD  
Extrathymic Differentiation of CD7<sup>+</sup>CD3<sup>-</sup> Cells into  
CD3<sup>+</sup>  $\gamma/\delta$ <sup>+</sup> or  $\alpha/\beta$ <sup>+</sup> T Cells

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Pre-T cells that differentiate in the thymus into mature T cells are believed to arise in the bone marrow, although the circulating form of the putative T cell precursor has not been identified (1, 2). The defining characteristic of a mature T cell is the expression of an antigen-binding receptor (TCR), which is characteristically (if not universally) associated with CD3 proteins. The majority of T cells in peripheral blood express the  $\alpha/\beta$  form of the TCR (TCR-2), and a minority (1-3%) express the  $\gamma/\delta$  chains (TCR-1) (3). These receptors are encoded by genes that rearrange in T cell precursors, with  $\gamma$  chain rearrangement commonly occurring first. Although CD3 and the TCR are known to be acquired in the thymus, the possibility of extrathymic differentiation has not been excluded.

One of the earliest T lineage antigens, CD7 (gp40), is expressed before TCR- $\beta$  gene rearrangement and surface expression of CD1, CD2, and CD3 antigens and persists on most mature circulating T cells (4-6). CD7 has been demonstrated within the bone marrow (1) and on fetal lymphocytes before colonization of the thymus (4, 7). Depletion of CD7<sup>+</sup> cells from bone marrow results in the loss of T cell colony-forming capacity (2, 8). Cells that are CD7<sup>+</sup>CD3<sup>-</sup> (typically CD16<sup>+</sup>) are known to exist in peripheral blood and display natural killer activity (9).

These studies were designed to test the hypothesis that the circulating form of the T cell precursor has a surface phenotype of CD7<sup>+</sup>CD3<sup>-</sup>, and could be induced to differentiate into CD3<sup>+</sup> cells outside the thymus. We found that CD7<sup>+</sup>CD3<sup>-</sup> cells purified and cloned from normal human peripheral blood by FACS differentiate into CD3<sup>+</sup> cells in the presence of IL-2, PHA, and irradiated feeder cells. These CD3<sup>+</sup> cells include cells that are TCR-1<sup>+</sup> or TCR-2<sup>+</sup> and express various combinations of other T cell surface antigens.

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### Materials and Methods

**Cell Preparation.** Fresh peripheral venous blood (50–80 ml) from normal volunteers was collected in acid citrate dextrose solution (Vacutainer; Becton Dickinson & Co., Mountain View, CA) and mononuclear cells (PBL) were purified under sterile conditions by density gradient centrifugation (1.077 g/cm<sup>3</sup> Ficoll-Hypaque; Pharmacia Fine Chemicals, Piscataway, NJ). Normal human thymus was obtained from superfluous tissue removed at pediatric cardiac surgery.

**mAbs.** FITC or phycoerythrin (PE)<sup>1</sup>-conjugated mAbs were obtained from Becton Dickinson & Co.: CD1a (Leu-6), CD2 (Leu-5b), CD3 (Leu-4), CD4 (Leu-3a), CD5 (Leu-1), CD7 (Leu-9), CD8 $\alpha$  (Leu-2a), CD11b (Leu-15), CD16 (Leu-11), CD25 (anti-IL-2), CD45 (HLE-1), CD57 (Leu-7), Leu-8, CD56 (Leu-19), CD71 (transferrin receptor), HLA-DR, TCR-2- $\alpha/\beta$  (WT-31), and isotype controls (IgG1, IgG2a). The specificities of these reagents are described elsewhere (10). WT-31 showed little or no staining of TCR-1<sup>+</sup> cell lines at the concentrations utilized (11). mAbs that recognized constant epitopes of the TCR-1 chains were obtained from T Cell Sciences, Inc. (Cambridge, MA), including TCR-2  $\alpha$  chain (Identi-T  $\alpha$ F1), TCR-2  $\beta$  chain (Identi-T  $\beta$ F1), TCR-1  $\gamma$  chain (C $\gamma$ M1), and TCR-1  $\delta$  chain (Identi-T TCR- $\delta$ 1). Anti-CD7 (RT7.1) (12) and anti-CD3 (RT3.1) (10) mAbs were produced in our laboratory. Affinity-purified FITC-conjugated goat anti-mouse IgG (Tago Inc., Burlingame, CA) was used as a second step for the unconjugated mAbs.

**Staining.** All staining steps contained PBS with 0.1% normal human serum (NHS) to inhibit nonspecific binding. Cells washed in RPMI 1640/10% FCS were incubated in the PE-conjugated reagent for 15 min on ice in the dark and the FITC-conjugated mAb was added for an additional 30 min. For three-color staining, a third primary biotinylated reagent was added followed by streptavidin-APC (Becton Dickinson & Co.), or a monoclonal reagent directly conjugated to the fluorochrome cyanine 5.18-OSu (13) (CY-5 absorption  $\lambda_{\text{MAX}} = 652$  nm, kindly provided by A. Waggoner, Carnegie Mellon University, Pittsburgh, PA) for excitation by the Helium-Neon laser. Cells were washed twice in ice-cold PBS and strictly kept in an ice bath before and during the sorting procedure to prevent modulation or shedding of the labeled antigens (10). Cells taken from tissue culture for surface phenotype analysis were washed free of tissue culture media, fixed in 1% paraformaldehyde/PBS for 10 min, and resuspended in 4°C PBS.

For Identi-T  $\alpha$ F1,  $\beta$ F1 and C $\gamma$ M1 staining, cells were air dried on Histostick (Accurate Chemical & Scientific Corp., Westbury, NY)-treated glass slides for 1 h, fixed in acetone for 10 min, and air dried for 10 min. After 15 min of incubation with 1:50 normal goat serum, primary antibodies were applied for 1 h at 22°C, followed by three washes. FITC-labeled goat anti-mouse IgG was applied for 30 min, followed by three washes. Slides were cover-slipped and read on a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

**Sorting/Cloning.** Cell sorting and analysis was performed on a five-parameter FACS440 cell sorter (Becton Dickinson & Co.) equipped with a 5-W argon laser (Innova 90; Coherent Inc., Palo Alto, CA) tuned to 488 nm at 200 mW of power. Three-color immunofluorescence was performed with an additional 47 mW Helium-Neon laser (model 107A; Spectra-Physics Inc., Mountain View, CA) at 633 nm, intercepting the sample stream 254  $\mu$ m below the argon laser. Fluorescence emissions were collected by selective bandpass filtration of FITC (530  $\pm$  15 nm), PE (575  $\pm$  12.5 nm), propidium iodide (PI) (630  $\pm$  10 nm), and allophycocyanin (APC) or CY-5 (660  $\pm$  10 nm) signals. The overlap of FITC and PE fluorescence emissions were corrected by an electronic compensation network; APC or CY-5 emissions were both spectrally and temporally distinct from lower wavelength emissions. To omit cell clumps and doublets, unwanted dead cells, and debris from the sorted population, forward vs. right scatter gating and forward angle thresholding at low sorting rates (<1–2  $\times$  10<sup>3</sup> cells/s) were utilized, as described (14).

Upon completion of sorts, 5–10  $\times$  10<sup>3</sup> cells from purified populations were immediately analyzed with “open” forward vs. side scatter gates (ungated), forward angle threshold just

<sup>1</sup> Abbreviations used in this paper: APC, allophycocyanin; ET cells, extrathymic T cells; NHS, normal human serum; NT cells, natural T cells; PE, phycoerythrin; PI, propidium iodide.

above electronic noise, and live cell gating with PI through the fifth parameter channel of the cell sorter to determine the final purity of the sorted cells. This stringency was imposed because preliminary experiments showed that sorted CD7<sup>+</sup>CD3<sup>-</sup> populations could appear to be >99.99% pure if scatter gated, but still contain a few presumptive contaminating cells just outside the defined lymphocyte scatter gate. Data acquisition, reprocessing, and display were performed on Hewlett-Packard 217 and 310 computer systems running CONSORT 30 or LYSYS analysis software (Becton Dickinson & Co.).

**Cell Culture.** Cultures of cells sorted to >99.99% purity were initiated with a 10-fold excess ( $\sim 10^6$ ) of autologous x-irradiated (3,000 rad) feeders and optimal mitogenic concentrations (1–2  $\mu\text{g/ml}$ ) of PHA (Leukoagglutinin; Pharmacia Fine Chemicals) within the wells of 48-well culture plates. All cultures were grown at 37°C in 5% CO<sub>2</sub> in RPMI 1640 with L-glutamine supplemented with 10% FCS, Hepes (2.4 gm/liter), nonessential amino acids (1 mM/liter), sodium pyruvate (1 mM/liter), gentamicin (0.1 mg/ml) and 1,000 U/ml rIL-2 (Cetus Corp., Emeryville, CA). Control wells containing only feeder cells were established at the initiation of all cell cultures and restimulations (in all 24-, 48-, or 96-well plates) to exclude growth of feeder cells as a source of cell contamination. Cell cultures were subsequently restimulated with PHA and auto- or allogeneic irradiated feeder cells approximately every 3 wk to maintain cell growth. Cells sorted to >99.99% purity were also cloned with a single cell deposition unit (Becton Dickinson & Co.) (1 cell/well) into 96-well tissue culture plates containing  $2 \times 10^5$  autologous feeders/well and PHA as above. HLA serotyping was performed by standard techniques.

**Antigen Modulation.** Freshly isolated PBL from three separate donors were stained with direct antibody conjugates for CD7 (Leu-9) and CD3 (Leu-4). Aliquots from each donor were immediately fixed in 1% paraformaldehyde or maintained in the dark on ice or at room temperature for 1, 2, or 3 h.

**CD3-induced Proliferation.** Freshly sorted CD7<sup>+</sup>CD3<sup>-</sup> and CD7<sup>+</sup>CD3<sup>+</sup> cells were plated in triplicate ( $5 \times 10^4$ /well) with irradiated autologous feeder cells ( $2 \times 10^5$ /well). Irradiated cells alone served as negative controls. A mitogenic concentration (0.5  $\mu\text{g/ml}$ ) of anti-CD3 mAb RT3.1 was added to each well. Proliferation was assessed after 48 h of culture at 37°C in 5% CO<sub>2</sub> by incorporation of [<sup>3</sup>H]thymidine (1  $\mu\text{Ci/well}$ ) during a 4-h exposure.

**Statistical Analysis.** All data are given as means  $\pm$  SD; *p* values are calculated by paired student's *t* test. In the cloning experiments the probability that the observed number of CD3<sup>+</sup> clones was due to chance contamination by natural T (NT) cells was calculated from the Poisson formula (15):  $f(n) = [(Np)^n (1-p)^N] / n!$ ; where *N* = the number of seeded wells, *n* = the number of positive wells, and *p* = the frequency of positive cells in original material, corrected for the measured plating efficiency of the NT cells, which varied from 6 to 32%. For the purposes of this calculation the potential contamination was assumed to be one order of magnitude more (0.1%) than actually measured (<0.01%).

## Results

**CD7<sup>+</sup>CD3<sup>-</sup> Cells in Peripheral Blood.** In normal blood  $13.1 \pm 6.8\%$  (*n* = 9) of the lymphocytes were CD7<sup>+</sup>CD3<sup>-</sup> (Fig. 1 A). The separation between CD3<sup>-</sup> and CD3<sup>+</sup> cells in the blood was quite distinct, in contrast to the thymus, which had an intermediate CD3<sup>DIM+</sup> population (Fig. 1 D). The blood CD7<sup>+</sup>CD3<sup>-</sup> cells had significantly more intense staining for CD7 than the CD7<sup>+</sup>CD3<sup>+</sup> cells over the identical forward scatter range ( $528 \pm 250$  vs.  $350 \pm 161$  mean fluorescence intensity units, *p* = 0.02; Fig. 1 A).

The expression of selected cell surface antigens on CD7<sup>+</sup>CD3<sup>-</sup> cells is compared with CD7<sup>+</sup>CD3<sup>+</sup> cells in Fig. 2. By three-color FACS analysis, CD7<sup>+</sup>CD3<sup>-</sup> cells were uniformly CD45<sup>+</sup> and CD11b<sup>+</sup>, but expressed no detectable CD1, CD4, CD5, CD10, CD13, CD14, CD25, HLA-DR, CD71, TCR-1, or TCR-2. The CD7<sup>+</sup>CD3<sup>-</sup> cells were heterogenous in their expression of CD2, CD8, CD16, CD56, CD57, and Leu-8. Four subpopulations were evident in freshly sorted CD7<sup>+</sup>CD3<sup>-</sup> cells (Fig.

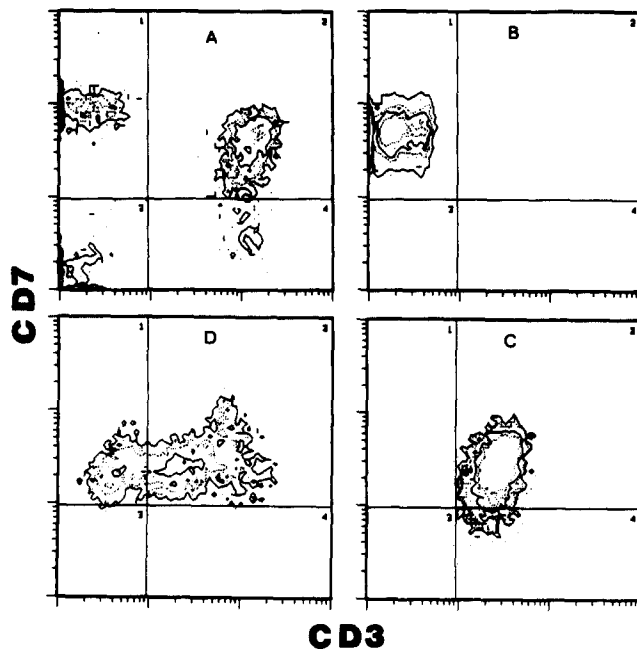


FIGURE 1. Pre- (A) and post-sort two-color flow cytometric analysis of fresh PBL from donor G stained with anti-CD7 (Leu-9 FITC) and anti-CD3 (Leu-4 PE). Post-sort analysis of CD7<sup>+</sup>CD3<sup>-</sup> (B) and CD7<sup>+</sup>CD3<sup>+</sup> (C) cells. Cells derived from a 6-mo-old infant and are seen in D. Cells were stained, sorted, and analyzed as described in Materials and Methods. The results are plotted by log fluorescence intensity as contours that enclose 1, 3, 10, and 30 cells.

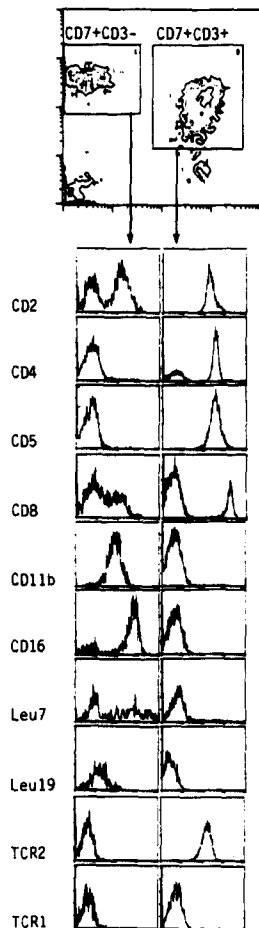


FIGURE 2. Triple-color flow cytometric analysis of fresh peripheral blood stained with anti-CD7 (Leu-9 FITC) and anti-CD3 (Leu-4 APC) mAbs (top) comparing the expression of a third antigen (usually PE conjugate) on either the CD7<sup>+</sup>CD3<sup>-</sup> (left) or CD7<sup>+</sup>CD3<sup>+</sup> (right) population. Leu-7 (CD57), WT31, and TCR- $\delta$ 1 are FITC conjugates combined with RT7.1 APC and Leu-4 PE. CD2 was directly conjugated to CY-5.

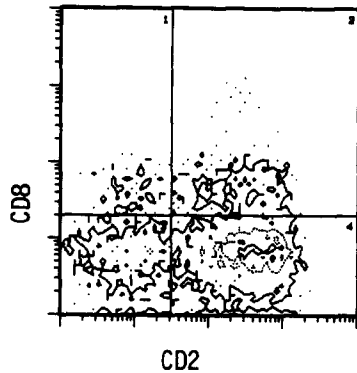


FIGURE 3. Expression of CD2 and CD8 on CD7<sup>+</sup>CD3<sup>-</sup> cells. Fresh peripheral blood was stained with anti-CD7 (Leu-9 FITC) and anti-CD3 (Leu-4 PE) and sorted on the FACS440. Freshly sorted CD7<sup>+</sup>CD3<sup>-</sup> (only FITC<sup>+</sup>) cells were immediately re-stained with anti-CD2 (Leu-5b CY-5) and CD8 (Leu-2 PE).

3): CD2<sup>+</sup>CD8<sup>-</sup> (51 ± 8%), CD2<sup>+</sup>CD8<sup>DIM+</sup> (18 ± 4%), CD2<sup>-</sup>CD8<sup>-</sup> cells (24 ± 4%), and CD2<sup>-</sup>CD8<sup>DIM+</sup> (7 ± 3%) (data from three donors).

*Differentiation of CD7<sup>+</sup>CD3<sup>-</sup> Cells in Culture.* Recovery of CD7<sup>+</sup>CD3<sup>-</sup> cells to >99.99% purity was usually achieved by two sorting cycles on the FACS440, with an average yield of 2.9 × 10<sup>5</sup> CD7<sup>+</sup>CD3<sup>-</sup> cells (Fig. 1 B; Table I). CD7<sup>+</sup>CD3<sup>-</sup> cells were initially cultured in rIL-2 (1,000 U/ml), PHA, and autologous irradiated PBL feeder cells. As a control, CD7<sup>+</sup>CD3<sup>+</sup> cells were sorted simultaneously and cultured under the same conditions (Fig. 1 C); these CD7<sup>+</sup>CD3<sup>+</sup> culture cells were termed NT cells.

CD7<sup>+</sup>CD3<sup>-</sup> cells in culture were periodically monitored for the presence of CD3 and other cell surface antigens. Analysis of all cultures was dependent upon cell growth (which could be variable) and not a sequential monitoring schedule. After a lag period in which no CD3 could be detected, eight of nine separate cultures of CD7<sup>+</sup>CD3<sup>-</sup> cells began to display CD3 on an increasing fraction of the cells (Fig. 4). In an av-

TABLE I  
Acquisition of Surface CD3 by CD7<sup>+</sup>CD3<sup>-</sup> Blood Pre-T Cells In Vitro

Exp.	Donor	Initial percent CD7 <sup>+</sup> CD3 <sup>-</sup>	Sort cycles*	Yield × 10 <sup>-3</sup>	Day <sup>†</sup> >5%	CD3 <sup>+</sup> >90%	TCR
1	A	4.2	2	372	60	88	TCR-1
2	B	10.6	2	500	34	50	TCR-1 + -2
3	C	9.8	2	300	14	107	TCR-2
4	D	16.4	3	80	38	105	TCR-1
5	E	5.6	4	ND	63	135	TCR-2
6	F	26.2	2	600	49	64	TCR-2
7	G	21.8	2	255	- <sup>§</sup>	- <sup>§</sup>	
8	G	12.8	2	196	37	- <sup>  </sup>	TCR-1
9	H	10.1	2	80	21	44	TCR-1 + -2
		13.1 <sup>¶</sup>		298	40	85	
		6.8 <sup>**</sup>		175	16	31	

\* All cells cultured were sorted to >99.99% final purity.  
<sup>†</sup> Data represent days in culture after initial CD7<sup>+</sup>CD3<sup>-</sup> purification.  
<sup>§</sup> CD3 was not detected during 90 d of culture.  
<sup>||</sup> 40% of the cells were CD3<sup>+</sup> at day 48.  
<sup>¶</sup> Mean.  
<sup>\*\*</sup> SD.

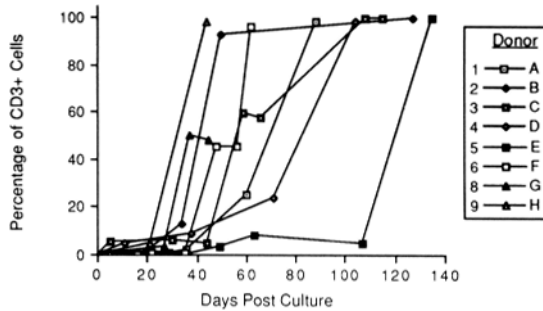


FIGURE 4. Time course of the appearance of CD3 in CD7<sup>+</sup>CD3<sup>-</sup> cultures.

erage of 40 ± 16 d (range, 14-63 d) at least 5% of the cells were CD3<sup>+</sup>, and by an average of 85 ± 31 d (range, 64-135 d) >90% of the cells were CD3<sup>+</sup> (Table I). Once CD3<sup>+</sup> was expressed, the CD3<sup>+</sup> cells gradually predominated, and the culture usually became >90% CD3<sup>+</sup> in 2-3 wk (Fig. 4). The rate of CD3<sup>+</sup> acquisition did not correlate with the percentage of CD7<sup>+</sup>CD3<sup>-</sup> cells in the blood of the donor. CD7<sup>+</sup>CD3<sup>-</sup> cells that acquired CD3<sup>+</sup> in culture are hereafter referred to as extrathymic T (ET) cells.

In five experiments (1, 2, 4, 8, and 9), the ET cells expressed TCR-1, and in five (2, 3, 5, 6, and 9), they expressed TCR-2 (Tables I and II). Both TCR-1<sup>+</sup> and TCR-

TABLE II  
Cell Surface Antigens Expressed on ET and NT Cells

Exp.	Donor	Cells	CD2	CD3	TCR-1	TCR-2	CD4	CD5	CD7	CD8	CD11b	CD16	CD56
1	A	NT	+	+	-	+	+	+b	+	+b	+		
		ET	+	+	+	-	-	-	+	+d	+		
2	B	NT	+	+	-	+	-	+b	+	+b			
		ET	+	+	+	-	+	+d	+	-	-		
		ET	+	+	+	-	-	+d	+	-	-		
		ET	+	+	-	+	-	+b	+	+b			
3	C	NT	+	+	-	+	+	+b	+	+b			
		ET	+	+	-	+	-	+b	+	+b			
4	D	NT	+	+	-	+	-	+b	+	+b			
		ET	+	+	+	-	-	+d	+	-			
5	E	NT	+	+	-	+	+	+b	+	+b			
		ET	+	+	-	+	-	+d	+	+b			
6	F	NT	+	+	-	+	+	+b	+	+b	-	+	+
		ET	+	+	-	+	+	+b	+	-	-	-	-
		ET	+	+	-	+	+	+d	+	-	-	-	-
7	G	NT	+	+	-	+		+b	+	+b			
		ET	+	-	-	-		-	+	+d			
8	G	NT	+	+	-	+	-	+b	+	+b			
		ET	+	+	+	-	-	+d	+	-		+	
9	H	NT	+	+	-	+	-	+d	+	+b			+
		ET	+	+	+	-	-	+d	+	-	-	-	+
		ET	+	+	-	+	-	+b	+	+b	-	-	+

d, dim stain; b, bright stain; scored + if >10% of cells were positive. Each distinct ET phenotype in the bulk culture is listed. TCR-1 reagents are TCR-δ1 and CγM1; TCR-2 reagents are βF1 and WT31.

2<sup>+</sup> cells were detected in experiments 2 and 9. Cultures of CD7<sup>+</sup>CD3<sup>-</sup> cells from all donors tested became CD3<sup>+</sup>, although in one of two experiments with donor G (experiment 7, Table I), CD7<sup>+</sup>CD3<sup>-</sup> cells were maintained for 90 d with no evidence of transition to CD3<sup>+</sup>; these cells remained CD7<sup>+</sup>CD3<sup>-</sup>, CD2<sup>+</sup>, CD5<sup>-</sup>, CD8<sup>DIM+</sup>, and TCR<sup>-</sup>. In another experiment (not shown) donor A CD7<sup>+</sup>CD3<sup>-</sup> cells were CD3<sup>+</sup> when first examined on day 14. Since the time course was atypical (no lag period was demonstrated) and both the NT and ET cells were TCR2<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup> contamination could not be excluded.

The extended phenotypes of the ET and NT cells are given in Table II. All ET and NT cultures expressed CD45, CD2, and CD7. The ET TCR-1<sup>+</sup> cells were CD5<sup>DIM+</sup> (5/6), CD4<sup>-</sup>CD8<sup>-</sup> (4/6), CD4<sup>-</sup>CD8<sup>DIM+</sup> (1/6), or CD4<sup>+</sup>CD8<sup>-</sup> (1/6) (Figs. 5 and 6). The ET TCR-2<sup>+</sup> cells expressed either CD5<sup>BRIGHT+</sup> (4/6) or CD5<sup>DIM+</sup> (2/6) and were CD4<sup>-</sup>CD8<sup>+</sup> (4/6) or CD4<sup>+</sup>CD8<sup>-</sup> (2/6). ET cultures in experiments 2 and 9 consisted of TCR-1<sup>+</sup> cells that were CD8<sup>-</sup> and TCR-2<sup>+</sup> cells that were CD8<sup>+</sup>. CD16, CD11b, and CD56, present on the starting population of CD7<sup>+</sup>CD3<sup>-</sup> cells, were sometimes detected on ET TCR-1<sup>+</sup> and TCR-2<sup>+</sup> cells.

The NT cells, tested at a time when the corresponding ET culture became CD3<sup>+</sup>, were all CD5<sup>+</sup> (9/9); four NT cultures were >90% CD8<sup>+</sup>CD4<sup>-</sup>, while the remaining four were mixtures of CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> cells (one was not tested for CD4). CD16, CD11b, and CD56 were variably present. The CD7<sup>+</sup>CD3<sup>+</sup> NT cells remained CD3<sup>+</sup> in association with TCR-2<sup>+</sup>; no detectable outgrowth of TCR-1<sup>+</sup> cells was detected over the same time periods ET cells were cultured.

*Controls.* In every experiment the ET cells differed from the NT established at the same time from the same donor by the presence or degree of expression of one to six surface molecules (TCR, CD4, CD8, CD5, CD7, CD11b, or CD56; Table II; Figs. 5 and 6). Wells containing irradiated feeder cells alone plus PHA and IL-2 were established to monitor their potential for growth; no cells grew from these cultures. To monitor for contamination with allogeneic feeder cells or other cells, four donors and their ET and NT cells were HLA typed. The HLA type of each ET and NT cell line tested was the same as the corresponding donor (data not shown).

The possibility of antigenic modulation of CD3 before or during sorting was tested by monitoring the change in CD3 intensity after staining under conditions equivalent to or less stringent than the sorting procedure. No antigenic modulation of CD3 could be demonstrated on cells maintained on ice for up to 3 h, as measured by mean fluorescence intensity after 3 h ( $293 \pm 19$  U), compared with the controls fixed immediately after staining ( $294 \pm 25$  U). Cells maintained at room temperature for 3 h had only a slight (<5%) and nonsignificant decrease in binding compared with the controls; no change in the percentage of positive CD7<sup>+</sup>CD3<sup>+</sup> cells was detectable (data not shown). The expression of CD7 on CD7<sup>+</sup>CD3<sup>-</sup> cells was stable over a 3 h period, either on ice or at room temperature. However, an appreciable reduction of staining intensity of CD7 did occur on CD7<sup>+</sup>CD3<sup>+</sup> cells after 2-3 h at room temperature ( $30 \pm 0.1$  U) compared with controls ( $46 \pm 5.8$  U;  $p < 0.001$ ). Addition of anti-CD3 (RT3.1) caused no increase in [<sup>3</sup>H]thymidine incorporation in freshly sorted CD7<sup>+</sup>CD3<sup>-</sup> cells cocultured with irradiated feeders, compared with feeders alone (data not shown).

*Cloning of CD7<sup>+</sup>CD3<sup>-</sup> Cells.* The main purpose of the cloning experiments was to determine whether the ET cells might simply represent outgrowth of residual

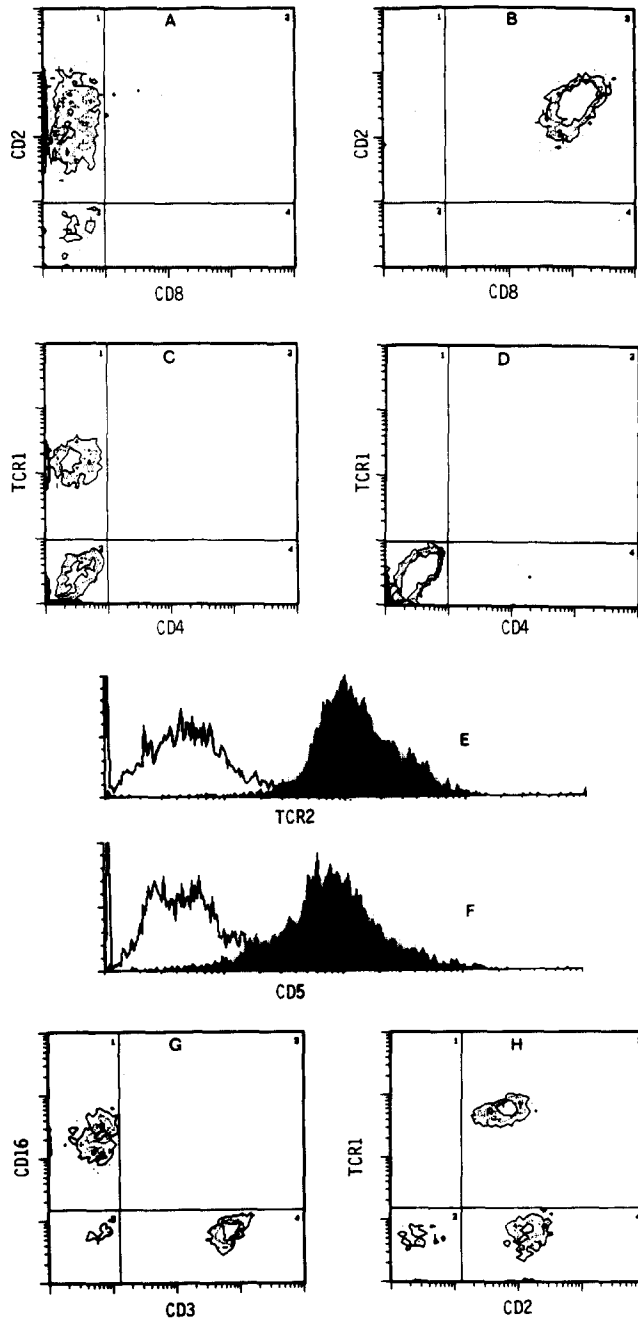


FIGURE 5. Comparative expression of cell surface antigens on cultured NT vs. ET cells after 48 d in culture (experiment 8). Subpopulations of cells sorted as CD7<sup>+</sup>CD3<sup>-</sup> (ET cells; A and C) are TCR-1<sup>+</sup> and CD2<sup>+</sup>, while all cells in the culture are CD4<sup>-</sup> and CD8<sup>-</sup>. CD7<sup>+</sup>CD3<sup>+</sup> cells (NT cells; B and D) purified from the same experiment are all CD2<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>+</sup>. All NT cells are TCR-2<sup>+</sup> and bright CD5<sup>+</sup> (E and F, dark histograms), whereas the ET cells are TCR-2<sup>-</sup> and CD5<sup>-</sup> or CD5<sup>dim</sup> (E and F, clear histograms). Multiparametric fluorescence analysis of the ET cells indicates that in addition to CD4<sup>-</sup>, CD8<sup>-</sup>, the TCR-1<sup>+</sup> cells are CD3<sup>+</sup>, CD16<sup>-</sup> (G), and CD2<sup>+</sup> (H). The remaining CD3<sup>-</sup> cells in the culture retain CD16<sup>+</sup> found on circulating CD7<sup>+</sup>CD3<sup>-</sup> cells, and are predominantly CD2<sup>+</sup>.

mature T cells. In five experiments, aliquots of the CD7<sup>+</sup>CD3<sup>-</sup> from the final purification step were cloned by the FACS440 (1 cell/well) into 96-well plates, under the culture conditions described. Cell growth was detected in 20 instances (of 1,440 wells seeded); sufficient numbers of cells were available for cell surface analysis in



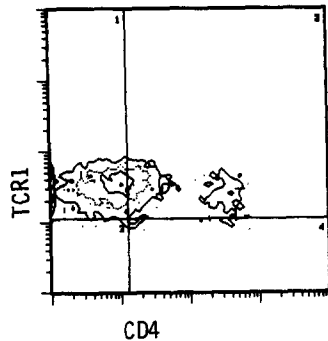


FIGURE 6. Expression of CD4 on ET TCR-1<sup>+</sup> cells. ET cells from experiment 2 were stained with anti-TCR-1 (TCR- $\delta$ 1 FITC) and anti-CD4 (Leu-4 PE). All TCR-1<sup>+</sup> cells are WT31<sup>-</sup>, CD8<sup>-</sup>, CD5<sup>DIM+</sup> (not shown), while a subpopulation of TCR-1<sup>+</sup> cells coexpress CD4.

14/20 wells. 10 of these 14 clones (71%) expressed CD3; one was 8% CD3<sup>+</sup> but was lost in culture before it could be retested, and 3/14 remained CD3<sup>-</sup>. Most (9/10) CD3<sup>+</sup> clones tested were TCR-2<sup>+</sup>, and either CD4<sup>+</sup> or CD8<sup>+</sup>. One well from experiment 8 grew out both TCR-1<sup>+</sup> and TCR-2<sup>+</sup> cells (in this experiment cell growth was observed in 1/288 wells); the TCR-2<sup>+</sup> cells were CD4<sup>-</sup>CD8<sup>+</sup> and the TCR-1<sup>+</sup> cells were CD4<sup>-</sup>CD8<sup>-</sup> or CD4<sup>+</sup>CD8<sup>-</sup>. Both TCR-1<sup>+</sup> and TCR-2<sup>+</sup> cells were CD5<sup>DIM+</sup>, as were the ET cells from the same experiment (Fig. 5 F); in contrast, the corresponding NT cells were CD5<sup>BRIGHT+</sup>. In control plates seeded with CD7<sup>+</sup>CD3<sup>+</sup> cells, growth was observed in 132/480 (28%) wells; all that were tested were CD3<sup>+</sup>, TCR-2<sup>+</sup>.

In three experiments with different donors (A, G, and H), data were obtained that permit a calculation of the probability of contamination. In these experiments, 8/288, 1/288, and 1/288 wells seeded with CD7<sup>+</sup>CD3<sup>-</sup> cells were CD3<sup>+</sup> (one well was CD3<sup>-</sup>). In the same experiments, wells seeded with CD7<sup>+</sup>CD3<sup>+</sup> cells grew in 31/96, 29/96, and 6/96 wells, respectively (all tested were CD3<sup>+</sup>). The probability (Poisson) that the CD3<sup>+</sup> cells in the CD7<sup>+</sup>CD3<sup>-</sup> wells were due to chance contamination with a CD7<sup>+</sup>CD3<sup>+</sup> cell is  $<10^{-12}$ ,  $<0.01$ , and  $<0.002$  in the three individual experiments, respectively.

### Discussion

This report identifies a hitherto unknown class of T cell precursors that circulate in normal peripheral blood. Highly purified (>99.99%) CD7<sup>+</sup>CD3<sup>-</sup> pre-T cells from all normal adults tested were capable of extrathymic differentiation into CD7<sup>+</sup>CD3<sup>+</sup> cells when cultured in the presence of IL-2, PHA, and irradiated feeder cells. In bulk culture the ET cells were predominantly TCR-1<sup>+</sup>, despite the much higher frequency of TCR-2<sup>+</sup> cells in normal blood. Purified CD7<sup>+</sup>CD3<sup>-</sup> cells developed the capacity to express CD3 in association with either TCR-1 or TCR-2, and expressed other mature T cell surface antigens, including CD4, which was generally absent on CD7<sup>+</sup>CD3<sup>-</sup> cells. The TCR-1<sup>+</sup> cells grown from these cultures were CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>DIM+</sup>, and CD4<sup>+</sup>CD8<sup>-</sup>, while TCR-2<sup>+</sup> cells were CD4<sup>+</sup> or CD8<sup>+</sup>. The TCR-1<sup>+</sup> CD4<sup>+</sup> phenotype has not been previously described, possibly because selection techniques utilized eliminated CD4<sup>+</sup> cells (16-18).

Alternative explanations for the results described here were excluded by several observations. The CD7<sup>+</sup>CD3<sup>-</sup> cells were sorted two to four times to give a popula-

tion that was >99.99% pure, even with no gating on the lymphocyte scatter gate. Mature T cells grown under the same conditions from the same donor always had a phenotype that differed by one or more markers from the ET cells. Artifactual conversion of CD3<sup>+</sup> to CD3<sup>-</sup> cells due to modulation before sorting was not demonstrable under the conditions of staining and sorting. Anti-CD3 did not induce proliferation of sorted CD7<sup>+</sup>CD3<sup>-</sup> cells after 2 d of culture. In addition, the lag period of ~40 d before 5% of the cells became CD3<sup>+</sup> could not be explained by simply recovery of modulated cells. The generation of TCR-1<sup>+</sup> cells from the majority of the CD7<sup>+</sup>CD3<sup>-</sup> bulk cultures adds strong additional evidence, since TCR-1<sup>+</sup> cells are a small fraction (<3%) of circulating T cells. The rare (CD5<sup>DIM+</sup>) and novel (TCR-1<sup>+</sup> CD4<sup>+</sup>) cell phenotypes observed in ET cells also argue against contamination by NT cells. Finally, differentiation of CD7<sup>+</sup>CD3<sup>-</sup> into either TCR-2<sup>+</sup> CD3<sup>+</sup> or TCR-1<sup>+</sup> CD3<sup>+</sup> cells was detected at a clonal level in three experiments with a frequency that was extremely unlikely to be due to chance contamination by CD3<sup>+</sup>. The lack of growth of feeder cells alone and the HLA typing of donors and their long-term cultured cell lines ruled out the possibility of contamination by feeder or unrelated cells.

The present results support prior observations that extrathymic fetal tissues contain CD7<sup>+</sup>CD3<sup>-</sup> T cell precursors that differentiate into CD3<sup>+</sup> T cells in the presence of IL-2 (4, 18-20). A committed CD7<sup>+</sup>CD3<sup>-</sup> T cell precursor has been identified in human fetal liver and yolk sac in the seventh week of gestation and soon thereafter in the thymic rudiment (4). Cells cultured from these fetal organs in the presence of IL-2 express mature T cell surface antigens *in vitro* (4). Intrathymic "prothymocytes" (CD1<sup>-</sup>CD2<sup>-</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD7<sup>+</sup>CD8<sup>-</sup>) have also been reported to differentiate into CD3<sup>+</sup> TCR-1<sup>+</sup> or TCR-2<sup>+</sup> populations *in vitro* (18). In the latter experiment prothymocytes were obtained by complement mediated lysis, and "matured" with IL-2 stimulation after 8 d of culture. This purification technique does not provide the purity of cell sorting, and hence, minor contamination (1%) is difficult to exclude. However, the shorter time for appearance of CD3<sup>+</sup> cells among culture thymic CD3<sup>-</sup> cells would be consistent with an enhanced capacity to grow and differentiate compared with pre-T cells in the blood.

Sequential acquisition of surface differentiation antigens has been described in the normal thymus (21), and in cultures of prothymocytes *in vitro* (18). CD2 is reported to be the first T cell antigen acquired by immature CD7<sup>+</sup> thymocytes after colonization of the thymic rudiment, preceding CD3 expression (4). Preliminary evidence suggests that blood CD2<sup>-</sup>CD7<sup>+</sup>CD3<sup>-</sup> cells may recapitulate the intrathymic maturation scheme by acquiring CD2 before CD3 (Preffer et al., unpublished results). Two differences in the ET pathway compared with the intrathymic pathway were the absence of detectable CD1 expression and the lack of intermediates with both CD4 and CD8. Furthermore, most ET cells expressed less CD5 (CD5<sup>-</sup> or CD5<sup>DIM+</sup>) than NT cells (CD5<sup>BRIGHT+</sup>), a phenotype shared with early thymocytes.

The optimum or essential conditions for the observed differentiation of ET cells was not determined, although IL-2 in combination with PHA and feeder cells was sufficient. High concentrations (>10 U/ml) of IL-2 may be required, as preliminary experiments with reduced levels of IL-2 failed to produce outgrowth of CD3<sup>+</sup> cells. In a prior study, CD3<sup>+</sup> cells were not detected after CD3<sup>-</sup> peripheral blood cells

were cultured in IL-2 for  $\leq 4$  wk. (9). The failure to detect CD3<sup>+</sup> cells may have been related to the limited interval of observation, or culture conditions (lack of PHA stimulation or feeder cell population). PHA stimulates production of a T cell differentiating factor from bone marrow cells depleted of T cells (22). The ET TCR-1<sup>+</sup> cells displayed a low efficiency of cloning (1/10 wells), compared with TCR-2<sup>+</sup> cells. The reason for the difference may be related to culture conditions, such as a requirement for as yet unidentified cell types or growth factor(s). It is notable that the culture conditions to generate ET cells (high dose IL-2) are similar to those used to generate lymphokine activated killer cells (23).

The CD7 molecule is a member of the Ig gene superfamily (24). An infant with a severe combined immunodeficiency and few circulating CD3<sup>+</sup> cells lacked expression of CD7, arguing that CD7 is necessary for the normal maturation of T cells (25). However, *in vitro* studies have not yet clearly identified a functional role of CD7. Antibodies (e.g., RT7.1) that modulate CD7 inhibit primary MLRs, but do not inhibit effector T cells or stimulate mitogenesis (26). As noted here, CD7 modulates more readily on CD3<sup>+</sup> cells than CD3<sup>-</sup> cells, which may be due to intrinsic differences in the cells or to perturbation of CD3. A substantial fraction of T cells derived from rejecting human renal allografts (12) and a subset of mature CD4<sup>+</sup> cells (27) lack CD7. The presence of CD7 in pre-T cells, and its decreased density on mature T cells, are consistent with the hypothesis that CD7 plays a critical role in homing to the thymus or in early ontogenetic events.

The actual frequency of T cell precursors in the blood was not directly demonstrated by these studies. Although the CD7<sup>+</sup>CD3<sup>-</sup> cells comprised 13% of the circulating blood lymphocytes, not every cell with this phenotype necessarily has the ability to differentiate into a T cell. Based on the cloning data, the T cell precursors capable of productive extrathymic differentiation under these conditions are  $\sim 5\%$  of CD7<sup>+</sup>CD3<sup>-</sup> cells (assuming a plating efficiency equal to T cells in the same experiment), or  $\sim 0.7\%$  of circulating lymphocytes.

A question arises as to whether the demonstrated ET pathway has any *in vivo* significance. Many studies have documented the presence of mature T lymphocytes in athymic mice, albeit at levels far below normal (28). These mice lack thymic epithelial cells, although their bone marrow T cell precursors are normal (29). T cells are not demonstrable at birth, but increase with age in the spleen and lymph nodes to detectable levels (30). The splenic T cells from athymic mice are unusual in having increased TCR-1 mRNA (31, 32), a several-fold increase in the proportion of CD4<sup>-</sup>CD8<sup>-</sup> T cells, and a reduced ( $<10\%$ ) capacity to proliferate in response to mitogens (30, 33). The TCR- $\beta$  gene rearrangements studied show limited diversity (28, 34). Together these data suggest the TCR-1 pathway is less dependent on intrathymic maturation than the TCR-2 pathway. In support of this view, extrathymic TCR- $\gamma$  gene rearrangement can be detected in the fetal mouse liver before thymic colonization (35), and patients who have received T cell-depleted bone marrow sometimes have increased levels of TCR-1<sup>+</sup> cells in the circulation (36). These data are in accord with our observation that populations of CD7<sup>+</sup>CD3<sup>-</sup> cells mature extrathymically into TCR-1<sup>+</sup> cells more frequently than expected from their rarity in the blood. The ET pathway may contribute proportionally more cells *in vivo* if the thymus is inactive or absent (e.g., aging or thymectomy), or if the stimuli for differen-

tiation are increased. It is not inconceivable that the increased frequency of TCR-1<sup>+</sup> cells isolated from sites of chronic autoimmune inflammation may be due to their derivation from the ET pathway (37, 38).

Our data do not address whether ET cells have previously been "educated" in the thymus or whether the TCR genes have begun rearrangement *in vivo*. Circulating CD7<sup>+</sup>CD3<sup>-</sup>CD2<sup>-</sup> lymphocytes might be unexposed to the thymic microenvironment, exposed but refractory to thymic processing, or partially processed by some alternative mechanism. In any case, the precursors of ET cells lack any detectable surface TCR that would permit thymic education by self-MHC antigen recognition (selection). Such T cells that mature under extrathymic conditions may have unusual, even "forbidden" specificities.

### Summary

CD7<sup>+</sup>CD3<sup>-</sup> cells purified (>99.99%) by FACS from the peripheral blood of healthy adults include precursors for mature T cells that have the capacity to differentiate into TCR-1<sup>+</sup> or TCR-2<sup>+</sup> CD3<sup>+</sup> cells. Extrathymic differentiation was demonstrable from all eight healthy donors in the presence of a high concentration of IL-2, mitogenic levels of PHA, and irradiated blood mononuclear feeder cells, after a lag of ~40 d *in vitro*. The extrathymic T (ET) cells were predominantly TCR-1<sup>+</sup>, although TCR-2<sup>+</sup> cells were also derived. ET TCR-1<sup>+</sup> cells were CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>DIM+</sup>, and CD4<sup>+</sup>CD8<sup>-</sup>, and were distinguished from natural T TCR-2<sup>+</sup> cells by a variety of cell surface markers. The ET cells had phenotypes generally displayed by normal mature T cells, although the CD5<sup>DIM+</sup> on ET cells was more typical of thymocytes. Acquisition of CD3 on purified CD7<sup>+</sup>CD3<sup>-</sup> cells was not due to antigenic modulation or growth of contaminants, and ET cells could be demonstrated at the clonal level. Studies in athymic mice and bone marrow recipients support the view that extrathymic maturation does occur *in vivo*. Whether the CD7<sup>+</sup>CD3<sup>-</sup> cell population was unexposed to the thymus, or exposed but not processed, is unknown. In any case, unusual or "forbidden" autoreactive specificities are predicted since ET cells differentiate without thymic selection of the TCR.

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