

# Differentiation of T Cell Lymphokine Gene Expression: The In Vitro Acquisition of T Cell Memory

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## Summary

A simple in vitro experimental system was devised to reflect the in vivo generation of a T cell anamnestic response so that T cell differentiation could be examined at the level of lymphokine gene expression. Comparison of neonatal and adult T cells revealed that both populations expressed the genes for interleukin 2 (IL-2) and its receptor, but only adult T cells were capable of transcribing mRNAs for IL-3, IL-4, IL-5, IL-6, interferon  $\gamma$ , and granulocyte/macrophage colony-stimulating factor. However, neonatal T cells could be induced to undergo functional differentiation in vitro, thereby acquiring the capacity to express the lymphokine gene repertoire characteristic for adult T cells. These data suggest that the T cells generated from neonatal blood by a primary stimulation in vitro are functionally indistinguishable from the T cells in adult blood that presumably have undergone primary stimulation in vivo. Therefore, we propose that the term "memory cell" be applied to those T cells that can be identified by their differentiated state of inducible effector-lymphokine gene expression.

Memory is the hallmark of an intact immune system, required for immunity against invading microorganisms and for the efficacy of vaccination in preventing infections. Immunologic memory is recognized by the capacity of a previously immunized host to respond more rapidly and with greater intensity to a secondary antigenic challenge (1). The cellular basis for memory lies in the selective clonal expansion of antigen-specific lymphocytes, a concept originally introduced by Burnet (2). Therefore, when compared with a naive host, a primed host responds to a secondary antigenic exposure more effectively simply because more antigen-reactive cells are present (3).

In addition to stimulating the expansion of antigen-reactive clones, the primary exposure to antigen has also been thought to initiate a permanent qualitative change in the cells. For example, B cells respond to antigen priming by undergoing molecular genetic changes that result in their differentiation to secretory cells, recognized morphologically as a change to a plasma cell phenotype. In addition, due to genetic recombination and deletion events, Ig H chain class switching occurs, resulting in a permanently differentiated genotype (4). Also, as a result of somatic hypermutation of V region genes, affinity maturation of the antigen-binding region takes place, ultimately resulting in the preferential selection and persistence of those cells secreting antibodies with the highest affinities (5, 6). As a consequence of these findings, the concept of B cell memory no longer includes only the quantitative expansion of specific B cell clones, but has progressed to a molecular genetic definition of a truly differentiated memory cell.

By comparison, T cell memory has thus far eluded a similarly distinctive molecular genetic analysis due to two fundamental experimental problems. First, it has been impossible to unequivocally discriminate between the cells responsible for the characteristic features of the naive and memory immune responses. Second, the molecules involved in T cell effector functions had not been identified until recently. Consequently, putative naive and memory T cell populations could not be prepared in sufficient quantity and purity to allow the application of biochemical and molecular genetic approaches that might delineate qualitative differences between them.

More recently, several investigators have made use of phenotypic markers to define the cellular compartment in adult peripheral blood that contains memory T cells. For instance, the T cell subsets identified by CD45RO, CD29, or CD58 have been shown to have adhesion and activation characteristics that are consistent with the concept of an accelerated secondary recognition event during a memory response (7-13). However, there is no way of knowing whether the subset defined by the absence of these putative memory markers is truly antigenically naive, particularly since adults have been exposed repeatedly and over a long period of time to a myriad of antigens in the environment. Functional comparisons between T cell subpopulations derived from the blood of immunologically experienced individuals and separated on the basis of their differential expression of these phenotypic markers may therefore not necessarily measure naiveté vs. memory. Indeed, an alternative interpretation of the dissimilarities found

in these populations has been proposed which holds that the CD45 isoform markers more appropriately distinguish between functionally diverse T cell subsets that evolve and persist as independent lineages (14–16).

To circumvent the uncertainties inherent in trying to identify and separate naive and memory T cells from adult blood, we have made use of a biological distinction between naiveté and memory and drew on the naturally occurring sources of the beginning and endpoints of the development of immunity. In this regard, neonatal T cells derived from umbilical cord blood are naive with respect to antigenic stimulation and represent a population of truly unprimed cells. By comparison, T cells derived from adult peripheral blood have had ample antigenic exposure and were used as a source of *in vivo* primed cells that have acquired “memory”.

With regard to a molecular characterization of T cell immune responsiveness, it is now established that T cell-derived lymphokines actually execute the cellular and molecular reactions that ultimately are recognized as cellular immunity: T cell proliferation, T cell help for B cell proliferation and antibody secretion, as well as the recruitment and activation of macrophages and leukocytes have all been assigned to distinct lymphokines (17). Moreover, sensitive assays are now readily available to detect all of these molecules.

Thus far, most studies directed toward uncovering functional disparities between naive and memory T cells have focused on potential differences in activation parameters (18–20). However, by analogy to the genetic differentiation process of B cell memory, we hypothesized that a fundamental functional distinction between naive and memory T cells might arise by differences in the expression of distinct genes, particularly those encoding the mediators of cellular immunity. Accordingly, we examined gene expression for a variety of lymphokines by taking advantage of the PCR as a highly sensitive method to detect mRNA transcripts (21–23). Our results, reported here, indicate that the differential capacity to express lymphokine genes underlies and determines the marked functional difference between unprimed and primed T cells. Moreover, during the course of these studies, we have developed the cellular and molecular methods to permit one to investigate the mechanisms responsible for this acquisition of an extended lymphokine repertoire.

## Materials and Methods

### Antibodies

T cell activation was performed using 64.1, an IgG2a mAb directed against CD3 (from Dr. Ellen Vitetta, Dallas, TX). Affinity-purified goat anti-mouse IgG (Fc specific) was purchased from Organon Teknika, West Chester, PA. For indirect fluorescent staining, the following mAbs were used at saturating concentrations:  $\alpha$ -T11 (clone 3PT2H9), reactive with CD2 (from Dr. Ellis Reinherz, Boston, MA); OKT3, OKT4, and OKT8, reactive with CD3, CD4, and CD8, respectively (Ortho Pharmaceuticals, Raritan, NJ); N901 and 3B8, IgG and IgM, respectively, directed against CD56 (from Dr. Jerome Ritz, Boston, MA); B1, reactive with CD20 (Coulter Immunology, Boston, MA); 3C10, reactive with CD14, and 9.3C9, reactive with HLA-DR and DQ (from Dr. Gilla Kaplan,

New York); 1HT4, reactive with CD25 (from Dr. Ellis Reinherz, Boston, MA). Rabbit anti-mouse FITC conjugate was from Zymed Laboratories, San Francisco, CA, and was used at 1:50 final dilution. Human gamma globulins used to block nonspecific FcR-mediated staining were purchased from Organon Teknika.

### Cell Culture Reagents

Complete medium consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 50 U/ml penicillin, 50  $\mu$ g/ml gentamycin, 200  $\mu$ g/ml L-glutamine, and 10% heat-inactivated calf serum (HyClone Laboratories, Logan, UT). Homogeneous rIL-2 was obtained from Takeda Chemical Industries, Osaka, Japan, and supplied as a 1-mg/ml solution in 5 mM ammonium acetate buffer (pH 5). In some experiments, 10  $\mu$ M aphidicolin (Sigma Chemical Co., St. Louis, MO) was used to completely inhibit [<sup>3</sup>H]TdR incorporation.

### Cell Purification

Umbilical cord blood was collected in preheparinized syringes from healthy full-term neonates immediately after vaginal delivery. Adult blood was drawn from healthy volunteers. Mononuclear cells were isolated by means of Ficoll-Hypaque discontinuous gradient centrifugation. In some instances, heavy red cell contamination of umbilical cord mononuclear cells necessitated further purification by centrifugation over a 65% Percoll (Pharmacia LKB Biotechnology, Piscataway, NJ) gradient. Interface cells were washed three times in normal saline and incubated at a final concentration of  $5 \times 10^6$  cells/ml in 5 mM L-leucine-methyl-ester hydrochloride (Sigma Chemical Co.), in serum-free medium at room temperature for 45 min. This procedure has been shown to effectively eliminate monocytes and a large proportion of NK cells (24, 25). The cell mixture was centrifuged at 450 g for 20 min over a 43.5% Percoll gradient to separate dead cells and residual low density NK cells and macrophages from the high density lymphocytes, as described (26). The cell pellet was washed twice in complete medium, and to eliminate residual Fc-Receptor positive cells,  $\sim 5 \times 10^7$  cells were incubated in a final volume of 2.5 ml for 20 min at 37°C in 100-mm bacteriological petri dishes (Fisher Scientific Co., Pittsburgh, PA) that had been coated with 10 mg/ml human gamma globulins in PBS immediately before use; nonadherent cells were then collected and the procedure repeated once. The remainder of the cells were incubated for 45 min at 37°C at a maximum concentration of  $2.5 \times 10^7$  cells/ml in Lympho-Kwik TH (One Lambda, Los Angeles, CA), to which 3B8 ( $\alpha$ CD56 mAb) was added to give a final dilution of 1:100 of ascites. Treatment with this cocktail of mAbs and complement selectively lyses HLA-DR<sup>+</sup> cells, B cells, residual granulocytes, NK cells, and CD8<sup>+</sup> cells. A final density centrifugation was performed to separate the dead cells, and the cell pellet was washed twice in complete medium.

### Assessment of Cell Purity

Cells at different stages of purification were incubated with saturating concentrations of mAbs specific for various surface markers for 60 min on ice. Indirect fluorescent staining was performed with 1:50 dilution of rabbit anti-mouse Ig FITC for 60 min on ice. Cells were then fixed in 2% paraformaldehyde-PBS until evaluation by FACScan (FACS is a registered trademark of Becton Dickinson & Co.).

Umbilical cord blood mononuclear cells from density centrifugation were 58–70% CD2<sup>+</sup>, 44–65% CD3<sup>+</sup>, 25–39% CD4<sup>+</sup>, 9–21% CD8<sup>+</sup>, 6–15% CD56<sup>+</sup>, 7–10% CD20<sup>+</sup>, 10–20% CD14<sup>+</sup>,

and 18–20% HLA-DR<sup>+</sup>. Umbilical cord blood cells purified by L-leucine-methyl-ester, FcR panning, and modified Lympho-Kwik TH were 97–99% CD2<sup>+</sup>, 96–99% CD3<sup>+</sup>, 95–98% CD4<sup>+</sup>, and 0–1% CD8<sup>+</sup>. Staining for B cells, NK cells, monocytes, and HLA-DR<sup>+</sup> cells was undetectable above background control levels. Less than 1/1,000 cells stained positive for nonspecific esterase ( $\alpha$ -naphthyl-acetate esterase; Sigma Chemical Co.). These highly purified umbilical cord blood-derived Th cells are referred to as neonatal T cells in the text.

Adult peripheral blood cells purified by L-leucine-ester, FcR panning, and modified Lympho-Kwik TH were 95–98% CD2<sup>+</sup>, 95–98% CD3<sup>+</sup>, 95–98% CD4<sup>+</sup>, 0–1% CD8<sup>+</sup>, and did not express detectable CD14, CD20, CD56, or HLA-DR. Less than 1/1,000 cells stained positive for nonspecific esterase. These cells are referred to in the text as adult T cells.

### Cell Stimulation and Culture

96-well, flat-bottomed tissue culture plates (Corning Glass Works, Corning, NY) were coated with 50  $\mu$ l of a 20- $\mu$ g/ml solution of goat anti-mouse IgG (Fc specific) in Tris-HCl (pH 9.5) for at least 3 h at room temperature. Immediately before use, wells were washed three times with normal saline. Unless indicated otherwise, cells were added to the wells at a final concentration of  $2 \times 10^5$ /well in the presence of 1  $\mu$ g/ml 64.1  $\alpha$ -CD3 mAb in complete medium. This procedure of crosslinking the CD3 structure is referred to throughout the text as stimulation via  $\alpha$ -CD3. For further expansion, cells were harvested after 48 h by vigorous pipetting and incubated at densities not exceeding  $10^6$ /ml in complete medium containing saturating amounts of rIL-2 (1 nM). On the day of restimulation, cells were washed twice, incubated at 37°C for 45 min, and washed once more to remove residual rIL-2. Secondary stimulation was performed identically to primary stimulation. In some experiments, the following mitogens were used to activate the cells: 1  $\mu$ g/ml PHA (Wellcome, Research Triangle Park, NC) or 1  $\mu$ M Ionomycin (Calbiochem-Behring Corp., San Diego, CA) with 5 ng/ml PMA (Sigma Chemical Co.).

### Thymidine Incorporation

After 24–72 h in culture, DNA synthesis was assessed by adding 0.5  $\mu$ Ci/well [<sup>3</sup>H-methyl]thymidine (10 Ci/mmol; ICN Biochemicals, Irvine, CA) for the final 2 h of culture. Triplicate cultures were harvested onto glass fiber filters, and radioactivity was counted by liquid scintillation. Under the specified conditions peak proliferation occurred at 48 h. [<sup>3</sup>H]TdR incorporation was calculated as cpm/10<sup>4</sup> cells/h.

### Measurement of IL-2, IFN- $\gamma$ and Granulocyte/Macrophage CSF (GM-CSF)<sup>1</sup>

Supernatants of stimulated cells were harvested after indicated time intervals, centrifuged to remove cells and debris, and stored at -70°C until protein determinations were performed. IL-2 concentrations were determined by the CTL-2 bioassay as described (27). INF- $\gamma$  and GM-CSF concentrations were determined by specific ELISA (Endogen, Boston, MA). ELISAs were performed exactly as specified by the manufacturers, and protein concentrations are listed in picomoles. The limits of detection were 2.5 pM for INF- $\gamma$  and 1 pM for GM-CSF.

<sup>1</sup> Abbreviation used in this paper: GM-CSF, granulocyte/macrophage colony-stimulating factor.

### PCR-assisted mRNA Amplification

**Preparation of cDNA.** Total RNA preparation was performed essentially as described (28). In brief, after indicated time intervals, supernatants were removed from the microtiter wells, and ice-cold PBS was added. Cells were removed by steady pipetting and wells were inspected for complete cell recovery. Cells were counted (Coulter Electronics Inc., Hialeah, FL) and aliquoted to 10<sup>6</sup>/Eppendorf tube (1.5 ml), pelleted for 10 s in a table-top Eppendorf microcentrifuge (Brinkmann Instruments Co., Westbury, NY), and lysed in 500  $\mu$ l lysis solution, consisting of 4 M guanidinium-thiocyanate (Boehringer Mannheim Biochemicals, Indianapolis, IN), 25 mM Na-citrate (pH 7), 0.5% N-lauroylsarcosine (Sigma Chemical Co.), and 100 mM 2-ME (Sigma Chemical Co.). Lysates were prepared at least in duplicate, vortexed, and stored at -70°C until further processing. After thawing, 50  $\mu$ l of 2 M Na-acetate (pH 4.0), 500  $\mu$ l of water-saturated acid phenol (Bethesda Research Laboratories, Bethesda, MD), and 100  $\mu$ l of chloroform-iso-amyl alcohol (49:1) were added to the lysates with thorough vortexing after each addition. The mixture was then chilled on ice for 15 min and spun at 10,000 g for 15 min at 4°C. The aqueous phase was recovered and RNA was precipitated in an equal volume of 2-propanol at -20°C for at least 90 min. Precipitates were pelleted at 4°C, washed once with 75% ethanol in diethylpyrocarbonate-treated double-distilled water (DEPC-ddH<sub>2</sub>O) and repelleted at 4°C at 10,000 g for 15 min. Vacuum-dried pellets were resuspended in 10  $\mu$ l DEPC-ddH<sub>2</sub>O containing 2  $\mu$ g oligo-dT (12–18 mer; United States Biochemical Corp., Cleveland, OH) and incubated at 65°C for 10 min. After cooling on ice, the mixture was incubated with 10  $\mu$ l of 2 $\times$  RT-buffer (100 mM Tris-Cl, pH 8.3, 150 mM KCl, 6 mM MgCl<sub>2</sub>, 20 mM DTT) and final concentrations of 200 U of MMLV reverse transcriptase (Bethesda Research Laboratories), 1 mM dNTPs (United States Biochemical Corp.), 100  $\mu$ g/ml acetylated BSA (Sigma Chemical Co.), and 25 U of RNasin (Promega Biotec, Madison, WI) for 60 min at 37°C. Tubes were then heated to 95°C for 5–10 min, and 80  $\mu$ l of dH<sub>2</sub>O was added to the 20- $\mu$ l reaction mixture. Samples were stored at 4°C until further use. RT reactions were performed simultaneously for lysates derived from adult and neonatal blood.

**PCR Conditions.** Lymphokine-specific primer pairs IL-2, -3, -4, -5, -6, IFN- $\gamma$ , and  $\beta$ -Actin(3') were purchased (Clontech, Palo Alto, CA) or synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, CA) and purified using NENSORB PREP columns (DuPont Co., Wilmington, DE):  $\beta$ -actin (5'), IL-1 $\beta$ , GM-CSF, and IL-2R p55. Sequences are specific as ascertained by computer assisted search of updated versions of GenBank. 5' and 3' primers were complementary to sequences in the first and last exons, respectively, or spanned exon-exon junctions and are therefore mRNA specific. 5' primers were designed to recognize sequences no farther than 1,250 bp upstream of the poly-T tract of the cDNA. The control  $\beta$ -actin 5' primer recognizes a sequence exactly 1,252–1,222 bp upstream. Primer sequences are shown in Fig. 1. In preliminary experiments, 10-fold serial dilutions of irrelevant DNA ( $\lambda$ -phage) were performed into cDNA samples from both adult and neonatal T cells and subjected to 25 cycles of PCR using primers amplifying a 500-bp fragment (GeneAMP kit; Perkin-Elmer Cetus, Emeryville, CA). The limit of detection was determined to be in the order of 10<sup>4</sup> molecules whose amplification product could reproducibly be visualized as a faint band after gel electrophoresis. Subsequently, 5  $\mu$ l of cDNA (representing RNA derived from  $5 \times 10^4$  cells to ensure a sensitivity of detection of at least one molecule per cell, allowing for losses during RNA preparation and reverse transcription) was amplified in 0.5 ml GeneAmp reaction tubes (Cetus Corp.,

mRNA	5' Sense Primer	3' Antisense Primer	Size of Amplified Fragment (bp)
$\beta$ -Actin	5'-TGACGGGGTCACCCACACTGTGCCATCTA-3'	5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'	(661 bp)
IL-1 $\beta$	5'-CTTCATCTTTGAAGAAGAACCTATCTTCTT-3'	5'-AATTTTTGGGATCTACACTCTCCAGCTGTA-3'	(331 bp)
IL-2	5'-ATGTACAGGATGCAACTCCTGTCTT-3'	5'-GTCAGTGTGAGATGATGCTTTGAC-3'	(458 bp)
IL-3	5'-ATGAGCCCGCTGCCCGTCTG-3'	5'-GCGAGGCTCAAAGTCGTCTGTTG-3'	(449 bp)
IL-4	5'-ATGGGTCTCACCTCCCAACTGCT-3'	5'-CGAACACTTTGAATATTTCTCTCAT-3'	(456 bp)
IL-5	5'-GCTTCTGCATTTGAGTTGCTAGCT-3'	5'TGGCCGTCAATGTATTTCTTTATTAAG-3'	(291 bp)
IL-6	5'-ATGAACTCCTTCTCCACAAGCGC-3'	5'-GAAGAGCCCTCAGGCTGGACTG-3'	(628 bp)
IFN $\gamma$	5'-ATGAAATATACAAGTTATATCTTGGCTTT-3'	5'-GATGCTCTCGACCTCGAAACAGCAT-3'	(501 bp)
GM-CSF	5'-ACACTGCTGAGATGAATGAAACAGTAG-3'	5'-TGGACTGGCTCCAGCAGTCAAAGGGGATG-3'	(286 bp)
IL-2R p55	5'-TTATCATTTCTGTGGTGGGCGAGATGGTTTA-3'	5'-TCTACTCTTCTCTGTCTCCGCTGCCAGGT-3'	(391 bp)

**Figure 1.** Primer sequences used for PCR-assisted mRNA amplification.

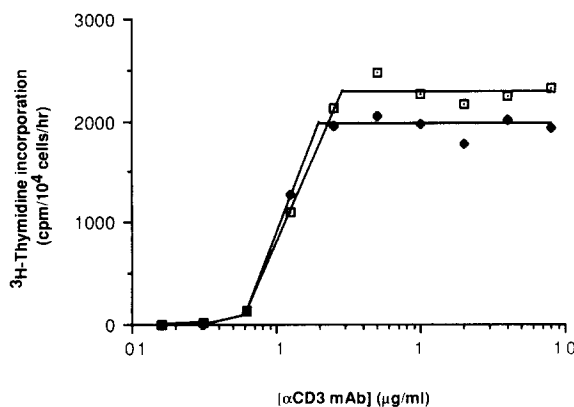
Emeryville, CA) in the presence of 200 nM final concentration of 5' and 3' primers, 200  $\mu$ M dNTPs, 0.5 U of Taq polymerase (Cetus Corp.), and PCR buffer containing 2.5  $\mu$ M MgCl<sub>2</sub>, 50 mM KCL, 10 mM Tris-CI (pH 8.3), and 0.001% gelatin in a final volume of 25  $\mu$ l. The reaction mixture was overlaid with a drop of light mineral oil, and PCR was performed in a DNA thermal cycler (Cetus Corp.) for 25 cycles: 45-s denaturation at 94°C, 45-s annealing at 60°C, and 1.5-min extension at 72°C. The reaction product was visualized by subjecting to electrophoresis 20  $\mu$ l of the reaction mix at 80 V for 70 min in 2% agarose in 0.5 $\times$  TBE buffer containing 0.5  $\mu$ g/ml ethidium bromide. 1  $\mu$ g of HaeIII-digested  $\phi$ x174 DNA (Clontech) was run in parallel as molecular weight markers (providing bands at 1,353, 1,078, 872, 603, 310, 281, and 234 bp). Specificity of the amplified bands was validated by their predicted size and restriction enzyme digests giving appropriately sized fragments. PCR-assisted mRNA amplification was repeated at least once for each RNA sample, and has been performed on six separate samples from adult T cells and >25 separate primary and secondary preparations from neonatal T cells to date, yielding identical results to the ones described in Results.

## Results

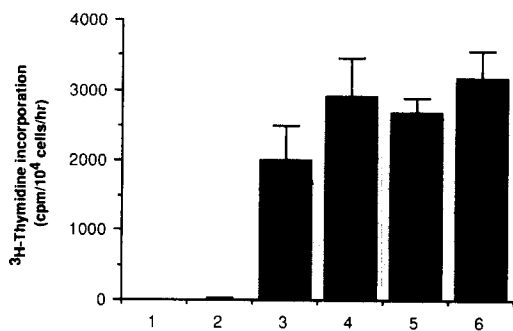
**Activation of Neonatal T Cells vs. Adult T Cells.** To meaningfully compare functional parameters of neonatal and adult T cells, it was important to ensure that both cell populations could be activated maximally and equally. To do so, we capitalized on the finding of Geppert and Lipsky (29) that purified T cells can be efficiently stimulated after depletion of accessory cells by crosslinking solely the TCR/CD3 complex, the  $\alpha$ -CD3 mAb 64.1 being particularly potent. A comparison of the concentrations of  $\alpha$ -CD3 required to activate neonatal and adult purified CD4<sup>+</sup> T cells when crosslinked via an immobilized bridging antibody is shown in Fig. 2. In three separate experiments, there were no differences in the response

thresholds of neonatal vs. adult T cells, so that an optimal dose of 1  $\mu$ g/ml  $\alpha$ -CD3 was chosen for all subsequent experiments.

Several approaches were used to evaluate whether activation by  $\alpha$ -CD3 delivered equally potent signals to the two cell populations via the TCR/CD3 complex. First, IL-2 production was monitored and found to be comparable (neonatal T cells: 40.3  $\pm$  22 pM vs. adult T cells; 43.3  $\pm$  21 pM,  $n = 8$ ). Second, IL-2R expression as measured by using IL-2R  $\alpha$ -p55 mAbs and flow cytometry showed equivalent and maximal levels after 72 h of culture, in that >97% of the cells stained positive. Third, consistent with these findings, proliferation monitored by [<sup>3</sup>H]TdR incorporation was com-

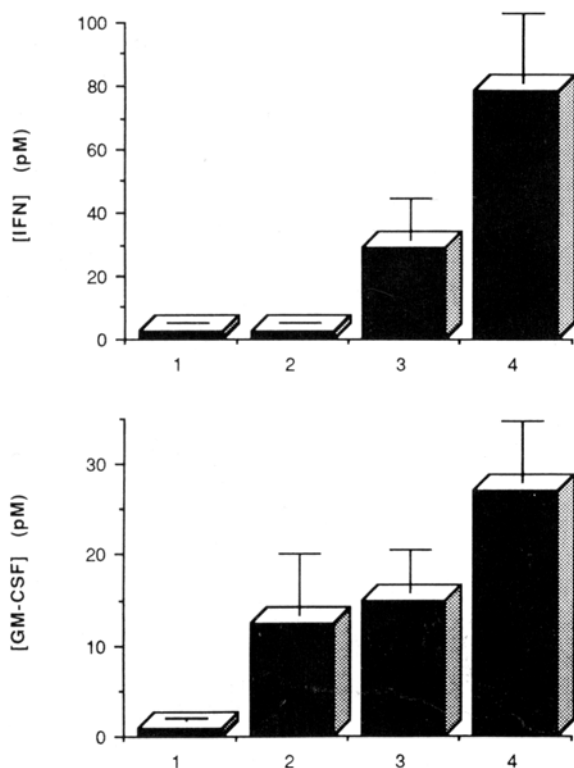


**Figure 2.** Proliferation of neonatal ( $\square$ ) and adult ( $\blacklozenge$ ) T cells after 48 h of activation with various doses of  $\alpha$ -CD3 crosslinked via immobilized goat anti-mouse IgG. [<sup>3</sup>H]TdR incorporation was assessed during the last 2 h of culture. Data are from an experiment representative of three performed. The SD of triplicate cultures were always <10%.



**Figure 3.** Proliferation of neonatal and adult T cells and neonatal mononuclear cells using various stimuli. (1) Adult T cells ( $n = 10$ ) stimulated with  $1 \mu\text{g/ml}$  soluble  $\alpha\text{-CD3}$ ; (2) neonatal T cells ( $n = 41$ ) stimulated with  $1 \mu\text{g/ml}$  soluble  $\alpha\text{-CD3}$ ; (3) adult T cells ( $n = 10$ ) stimulated via crosslinked  $\alpha\text{-CD3}$ ; (4) neonatal T cells ( $n = 41$ ) stimulated via crosslinked  $\alpha\text{-CD3}$ ; (5) neonatal T cells ( $n = 6$ ) stimulated with  $1 \mu\text{g/ml}$  PHA and  $5 \text{ ng/ml}$  PMA; (6) neonatal mononuclear cells ( $n = 8$ ) stimulated with  $1 \mu\text{g/ml}$  PHA. Background [ $^3\text{H}$ ]TdR incorporation  $\pm$  SD was  $5.3 \pm 2 \text{ cpm}/10^4/\text{h}$  for neonatal T cells,  $2.9 \pm 1$  for adult T cells, and  $9.5 \pm 1$  for neonatal mononuclear cells. [ $^3\text{H}$ ]TdR incorporation was assessed 48 h after activation during the last 2 h of culture. Error bars represent 1 SD.

parable in the two populations when measured at the peak of the response, which occurred after 48 h of culture (Fig. 3). As an additional test to ascertain whether neonatal T cells were optimally activated, PHA was used in place of  $\alpha\text{-CD3}$ .

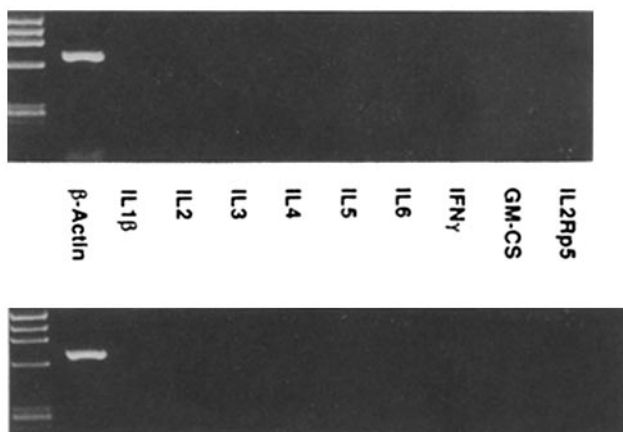


**Figure 4.** IFN- $\gamma$  (*top*) and GM-CSF (*bottom*) concentrations in supernatants of stimulated neonatal and adult T cells. Supernatants were harvested 24 and 48 h after activation via  $\alpha\text{-CD3}$ . Protein determinations were performed by specific ELISAs. (1) Neonatal T cells, 24 h ( $n = 25$ ); (2) neonatal T cells, 48 h ( $n = 25$ ); (3) adult T cells, 24 h ( $n = 10$ ); (4) adult T cells, 48 h ( $n = 10$ ). Error bars represent 1 SD.

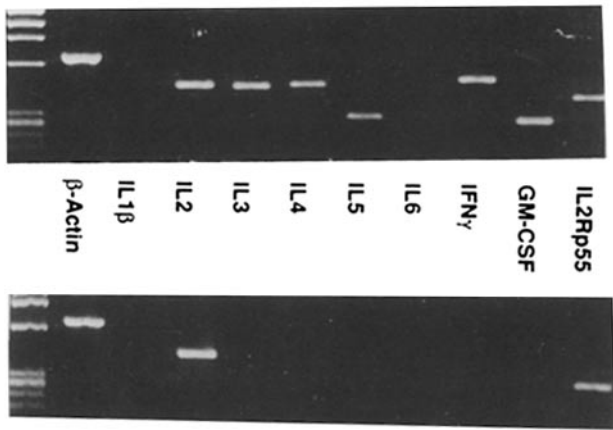
However, as the purified T cells did not proliferate in response to PHA, total mononuclear cells stimulated with PHA had to be compared with purified T cells stimulated with PHA and PMA. Maximal [ $^3\text{H}$ ]TdR incorporation occurred after 48 h and was found to be similar for all experimental groups (Fig. 3, columns 5 and 6).

**Production of IFN- $\gamma$  and GM-CSF during Primary Stimulation.** Having ensured equivalent activation results as defined by IL-2 production and responsiveness, the secretion of IFN- $\gamma$  and GM-CSF was monitored after 24 and 48 h of culture. As reported previously by Bryson et al. and Wilson et al. (30, 31), and shown in Fig. 4 (*top*), neonatal T cells did not produce IFN- $\gamma$ , even though adult T cells secreted readily detectable amounts. Neonatal T cell GM-CSF production also was undetectable after 24 h of culture in 24 of 30 samples examined, and at the limit of detection (1–1.7 pM) in the remaining six samples. (Fig. 4, *bottom*). By comparison, adult T cells secreted GM-CSF by 24 h and even higher levels by 48 h. It is noteworthy that in contrast to IFN- $\gamma$  secretion, neonatal T cell supernatants also contained GM-CSF when tested at 48 h. Therefore, neonatal T cells seem capable of mounting a GM-CSF secretory response, although it is delayed compared with the adult T cell response. In separate experiments (not shown), we have found the induction of GM-CSF secretion to be entirely IL-2 dependent.

**Lymphokine Gene Expression Detected by PCR-assisted mRNA Amplification.** Since our methods of TCR/CD3 triggering resulted in equivalent activation of neonatal and adult T cells as monitored by IL-2 production, yet resulted in deficient IFN- $\gamma$  and GM-CSF secretion, it appeared as though neonatal T cells might be unable to express some lymphokine genes. Therefore, to examine lymphokine gene expression more comprehensively, the PCR technique was chosen to provide the most sensitive assay for the presence or absence of mRNA. As shown in Fig. 5, before in vitro activation, both neonatal T cells and adult T cells were devoid of mRNA for any of the lymphokines tested. In contrast, as shown in Fig. 6, adult T cells contained readily detectable levels of mRNA for IL-2,



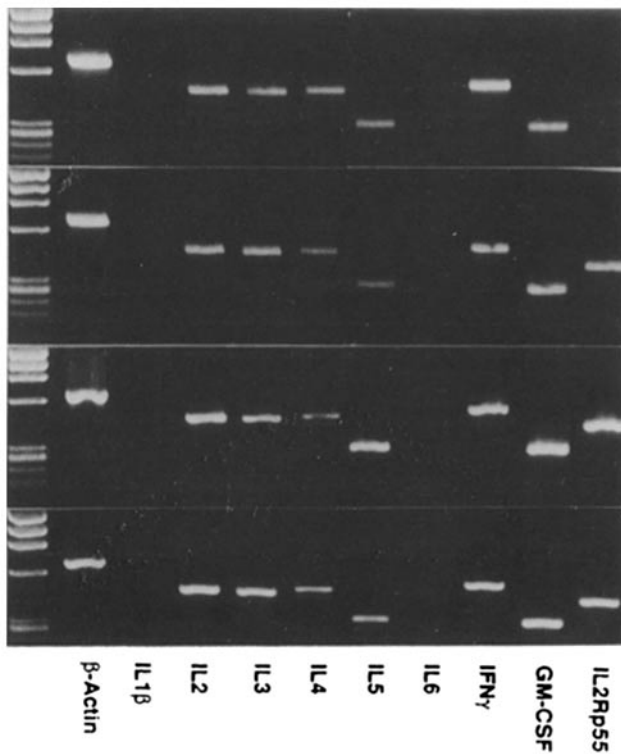
**Figure 5.** PCR-assisted mRNA amplification of adult (*top*) and neonatal (*bottom*) T cells immediately before stimulation.



**Figure 6.** PCR-assisted mRNA amplification of adult (*top*) and neonatal (*bottom*) T cells 3 h after primary activation via  $\alpha$ -CD3.

IL-3, IL-4, IL-5, IFN- $\gamma$ , GM-CSF, and IL-2R p55 as early as 3 h after stimulation. By comparison, neonatal T cells only expressed detectable mRNAs for IL-2 and the IL-2R p55 chain at this time interval.

To examine the kinetics of lymphokine mRNA expression, cells were harvested after 3, 6, 9, 24, and 48 h of stimulation. Adult T cells showed the same lymphokine expression profile throughout the study period (Fig. 7), IL-6 mRNA being only faintly detectable and only after prolonged cul-

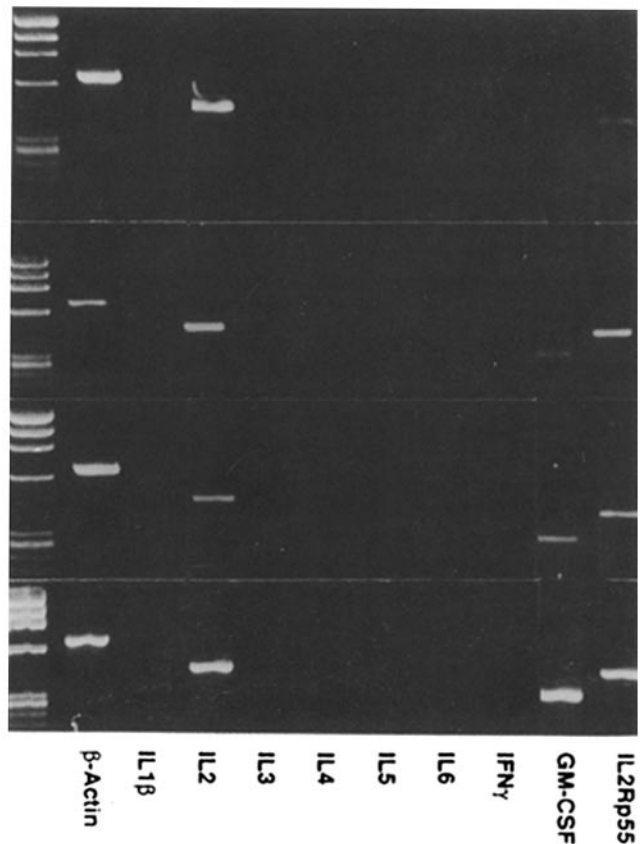


**Figure 7.** PCR-assisted mRNA amplification of adult T cells at various times after stimulation via  $\alpha$ -CD3. From top to bottom: 6, 9, 24, and 48 h after activation.

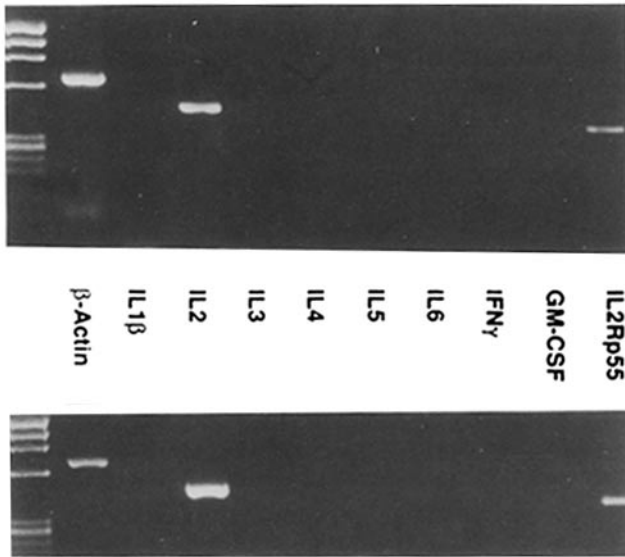
ture (24 h). By comparison, neonatal T cells were incapable of producing any of the lymphokines, with the notable exceptions of IL-2 and GM-CSF (Fig. 8). GM-CSF mRNA was reproducibly detected in neonatal T cells after 24 h of culture, and in some experiments it appeared as early as 9 h after stimulation. However, the remainder of the lymphokines remained undetectable up to 48 h of culture.

*Lymphokine mRNA Expression in Response to Alternative Stimuli.* Some reports have indicated that naive T cells might have a higher activation threshold than primed T cells (18–20). Therefore, it was important to test alternative pathways of stimulation to determine whether neonatal T cells were simply stimulated suboptimally, or whether they were actually incapable of expressing most of the lymphokine genes. The combinations of PHA/PMA and Ionomycin/PMA at concentrations that resulted in optimal proliferation had no effect on the inducibility of any of the lymphokine mRNAs tested (Fig. 9). Again, only IL-2 and IL-2R p55 mRNAs were detected under these conditions, thereby confirming the results obtained using  $\alpha$ -CD3 for stimulation.

*Comparison of Neonatal T Cells with Neonatal Mononuclear Cells.* Since the T cells were extensively purified before stimulation, it was possible that cells exhibiting a lymphokine profile typical of adult cells could have been lost during the



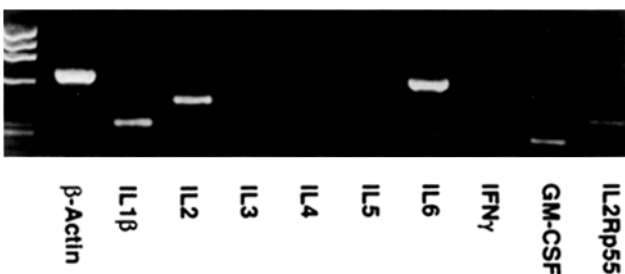
**Figure 8.** PCR-assisted mRNA amplification of neonatal T cells at various times after stimulation via  $\alpha$ -CD3. From top to bottom: 6, 9, 24, and 48 h after activation.



**Figure 9.** PCR-assisted mRNA amplification of neonatal T cells after primary stimulation with PHA and PMA (*top*) or Ionomycin and PMA (*bottom*).

purification procedure. Alternatively, costimuli originating from accessory cells might be necessary for neonatal T cells to express lymphokine genes other than IL-2. Therefore, total mononuclear cells were stimulated with 1  $\mu\text{g}/\text{ml}$  PHA and tested for cytokine mRNA expression. As shown in Fig. 10, the mRNAs for IL-3, IL-4, IL-5, and IFN- $\gamma$  still remained undetectable under these conditions, while IL-1 $\beta$ , IL-6, and GM-CSF mRNAs were easily amplified, as expected from the presence of monocytes and NK cells.

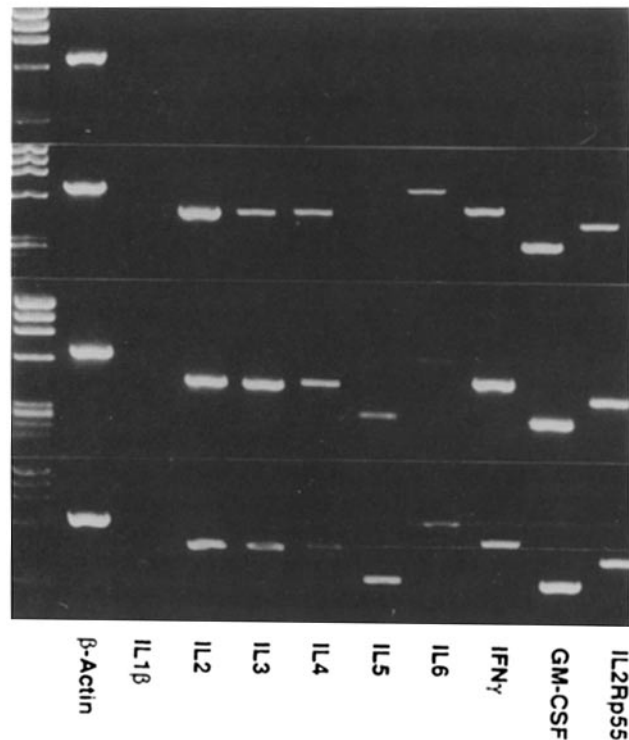
*The Differentiation of Neonatal T Cells In Vitro.* The foregoing experiments served to illustrate a distinct difference between neonatal and adult T cells in their capacities to express lymphokine genes. However, this inability to express lymphokine genes upon a primary stimulation in vitro might be explained simply by an immaturity on the part of the neonatal cells. Consequently, it was crucial to ascertain whether the primary in vitro stimulation could actually mimic the in vivo situation, promoting a switch, so that upon secondary stimulation, an adult-type lymphokine repertoire could be expressed. Therefore, neonatal T cells were stimulated as before for 48 h, then washed out of  $\alpha$ -CD3 and cultured with



**Figure 10.** PCR-assisted mRNA amplification of neonatal mononuclear cells after 3 h of primary stimulation with PHA. Mononuclear cells were prepared by a 65% Percoll-gradient centrifugation.

a saturating concentration of rIL-2. At varying intervals, the cells were restimulated via  $\alpha$ -CD3 using the same protocol developed for the primary stimulation.

After 7–10 d of culture, the cells cease proliferating, and express no lymphokine mRNAs (Fig. 11, *top*). Then, upon restimulation via the TCR/CD3 complex, the in vitro primed neonatal T cells express within 3 h the mRNAs for all of the lymphokines produced by adult T cells, with the exception of IL-5. As shown in Fig. 11, a detailed time course after restimulation revealed that IL-5 mRNA does appear, but only after a lag period of  $\sim 9$  h. It then persists along with the other lymphokine mRNAs for as long as 48 h. Also, when tertiary stimulation of neonatal T cells was performed, IL-5 mRNA was apparent after 3 h. No lymphokine transcripts were detected in the absence of restimulation. Also, when IL-2 was replenished without restimulation of the TCR/CD3 complex, only GM-CSF mRNA was apparent. Control neonatal T cells placed only in medium and then stimulated after 5 d via  $\alpha$ -CD3 still produced only IL-2 and IL-2R mRNA, in a fashion identical to neonatal cells that were stimulated when freshly isolated. Moreover, the capacity to express an extended lymphokine mRNA profile was also reflected in the secretion of the gene products: all supernatants harvested 24 h after secondary stimulation contained readily detectable levels of IFN- $\gamma$  ( $19.2 \pm 4.1$  pM,  $n = 25$ ) and GM-CSF ( $71.4 \pm 7.8$  pM,  $n = 25$ ).



**Figure 11.** PCR-assisted mRNA amplification of secondary neonatal T cells. Neonatal T cells were primarily stimulated with  $\alpha$ -CD3 and expanded in rIL-2 for 7 d. From top to bottom: immediately before restimulation, 3 h after restimulation via  $\alpha$ -CD3, 9 h after restimulation, and 48 h after restimulation.

The switch in the capacity to express the lymphokine genes first becomes detectable after 3 d of culture and is stable for as long as 14 d, after which the cells begin to die if not restimulated. These results suggested that the switch could not be explained by the selective outgrowth of a minor subset of already differentiated cells present at the initiation of culture. However, to confirm this interpretation, neonatal T cells were stimulated in the presence of aphidicolin to prevent DNA replication. As aphidicolin specifically blocks DNA polymerase I and II without affecting RNA synthesis or blastogenesis, it is an ideal agent to prevent proliferation without overall metabolic inhibition (32). After 5 d of stimulation in the presence of aphidicolin, viable cells were recovered and restimulated via  $\alpha$ -CD3. Again, a lymphokine profile typical of differentiated T cells was detected.

### Discussion

Primary antigenic exposure *in vivo* initiates changes in the immune system that can ultimately lead to immunity. One crucial feature of immunity is the capacity of the host to "remember" the priming antigen, and thereby respond to it more efficiently; this property of the immune system is conventionally termed immunologic memory (1, 33). The precise nature of the events governing the acquisition of memory *in vivo* has escaped direct experimental scrutiny, partly because of the inaccessibility and complexity of the cellular components involved. As a first step toward an adequate *in vitro* representation of the processes determining T cell memory, we devised a simple experimental model system that makes use of the biologically defined cellular sources of naiveté and memory, yet applies a reductionist approach with respect to T cell activation and the measurement of T cell responsiveness. This *in vitro* system demonstrates a clear qualitative difference between unprimed and primed T cells, in that antigenically virgin cells can respond to activation by proliferating, yet cannot secrete effector lymphokines other than IL-2. By comparison, antigenically experienced cells are shown to be capable of expressing many, if not all, of the lymphokines involved in an immune/inflammatory response immediately upon stimulation. Most important, naive T cells can and do respond to a primary *in vitro* activation by a differentiation process, which then enables them to express the entire repertoire of effector lymphokines immediately upon subsequent restimulation. Therefore, this *in vitro* system of T cell differentiation mimics the accelerated nature and functional superiority of the *in vivo* anamnestic immune response.

It should be emphasized that the early studies by Tedder et al., (34, 35) comparing putative naive and memory T cell subsets separated from adult blood found that memory cells could provide help for the generation of antibody-forming cells, while naive T cells were deficient in this regard. However, at this time, T cell help had not yet been ascribed to distinct lymphokines, so that it was impossible to proceed to a molecular dissection of the phenomenon. Subsequently, Sanders et al. (7, 18), Salmon et al. (36), and Lewis et al. (37) did examine the production of lymphokines, notably IFN- $\gamma$  and IL-4, and found that the putative naive cell subset

was deficient in both the mRNA expression and the secretion of these lymphokines relative to the putative memory cell subset. Separate studies by Lewis et al. (38) using Northern blot analysis to examine IFN- $\gamma$  mRNA expression by neonatal T cells supported the impression that antigenically naive T cells were somehow defective in their capacity to respond to stimulation by the production of at least some lymphokines. However, the mechanism underlying such a functional deficiency has remained controversial and ill defined, and thus far, it has not been approached with a molecular genetic hypothesis in mind. Rather, most investigators have focused on uncovering differences in the activation parameters between naive and memory T cells, and have not directly posed the question as to whether primed T cells might differ from unprimed T cells qualitatively by their ability or inability to express multiple effector lymphokine genes.

To devise an experimental approach that would allow an unambiguous interpretation of the results in terms of gene expression, several conceptual and technical problems had to be taken into consideration.

(a) It was crucial to begin the analysis with a homogeneous population of antigenically virgin T cells, since any contamination of the naive T cells with already primed cells would have precluded a meaningful analysis. However, the exact cellular compartments responsible for memory and naiveté have remained uncertain, so that it has been difficult to identify representative cell subsets in adult blood and to separate them from one another. In this regard, several phenotypic markers have recently been proposed to distinguish between naive and memory T cells. For instance, it has been shown that the T cell subsets defined by CD26, CD29, CD45RO, or CD58 are capable of responding to recall antigens and therefore, by definition, contain memory T cells (7-9). A number of investigators have made use of these markers to separate cells and have reported differences in activation parameters and functional and phenotypic characteristics of the subsets obtained (7, 10, 18-20, 36, 37, 39-43). A distinct disadvantage of this strategy lies in the inevitable crosscontamination with the reciprocal subset, which essentially precludes the application of sensitive molecular genetic approaches that might detect the products of those few contaminating cells. In addition, the marker CD45RO, for example, does not *per se* qualitatively delineate "memory", since many other cell types of the hematopoietic lineage express it, including immature thymocytes (44, 45). Accordingly, to circumvent the ambiguities inherent in identifying and separating putative naive and memory T cells in adult blood, we capitalized on the only known natural source of purely naive cells, those found in newborns. By definition, neonatal T cells derived from umbilical cord blood represent a population comprised primarily of virgin T cells, in that antigenic exposure *in utero* is rare. This state of functional immune naiveté is reflected in the fact that neonates are less efficient than adults at mounting an effective immune response to a variety of infectious agents (46). By comparison, a considerable proportion of T cells derived from adult blood have memory, in that normal individuals have had extensive exposure to en-



environmental microbes and have developed long-lasting immunity to most infectious agents.

(b) To interpret any differences in the lymphokine gene expression between neonatal and adult T cells as attributable to the T cells themselves, it was critical to rigorously purify them from contaminating granulocytes, B cells, monocytes, and NK cells, which are also known to produce some cytokines (47–49).

(c) In view of several reports that have suggested that neonatal monocytes are relatively immature or might even have suppressive effects, it was important to use a system depleted of accessory cells (50, 51). Moreover, Inaba and Steinman (52) have provided evidence that unprimed and primed T cells have distinct requirements for APC. As well, phenotypic studies have suggested that primed cells have a higher density of a number of activation molecules on their surface (10, 11). Most of these molecules facilitate cell-cell interaction, while some have also been implicated in auxiliary signaling. Since most of these structures find their counterparts on monocytes, it has been suggested that a complex of triggering molecules involving the TCR and these accessory structures is more easily formed with primed than unprimed cells (11, 53). Thus, only an accessory cell-depleted system avoids the experimental ambiguity and difficulties in the interpretation of results that arise when stimulation is performed in the presence of monocytes and other non-T cells.

(d) Some investigators have emphasized that primed T cells express a higher density of ancillary molecules, such as CD2, CD18/CD11a, CD28, CD29, CD44, CD54, CD58, etc., contributing to their lower threshold for activation (8, 11, 53, 54). Therefore, to compare neonatal and adult T cells meaningfully, it was mandatory to devise an efficient and equal system for activating the cells, one that functioned solely via a triggering structure present in equal densities on both populations, and one that would be indifferent to any dissimilarities in T cell accessory molecules. This was accomplished by cross-linking exclusively the CD3/TCR complex via immobilized  $\alpha$ -CD3, which has been shown to be an effective stimulus for T cells even in the absence of accessory cells (29).

(e) It was necessary to demonstrate that the signals transduced through the TCR/CD3 complex were equivalent for both neonatal and adult T cells, particularly since several reports have implied that neonatal T cells are somehow immature (55–59). When monitored by IL-2/IL-2R-mediated proliferation, crosslinking with  $\alpha$ -CD3 yielded similar results with identical  $\alpha$ -CD3 dose-response relationships for both neonatal and adult T cells (Figs. 2 and 3). Moreover, when triggering via the TCR/CD3 complex was bypassed entirely using phorbol ester and calcium ionophore, identical lymphokine patterns were observed as with  $\alpha$ -CD3-mediated activation (Fig. 9). Therefore, in this experimental activating system, it is unlikely that differential lymphokine mRNA expression by neonatal and adult T cells is attributable to differing activation thresholds or due to differences in known intracellular signal transduction pathways.

(f) To implicate a switch in gene expression, it was important to use a sensitive method such as the PCR to mon-

itor mRNA expression (21–23). The particular reaction conditions chosen were designed to detect as few as one mRNA molecule/cell, given that all the cells in the population under study were responding to activation. Within these limits of detection, neonatal T cells expressed easily detectable levels of IL-2 and IL-2R p55 mRNA transcripts, yet did not express any of the other lymphokines tested for. Therefore, at this sensitivity level, it can only be concluded that the vast majority of neonatal CD4<sup>+</sup> T cells cannot express the genes encoding most of the T cell lymphokines upon only a primary stimulation.

Finally, having uncovered a fundamental difference between neonatal and adult T cell lymphokine gene expression, it was obligatory to demonstrate that a primary stimulation of neonatal T cells would lead to their differentiation *in vitro*, allowing them to express an adult-type lymphokine pattern upon restimulation. Otherwise, the lack of the capacity to express the majority of lymphokine genes could have been interpreted as merely a sign of immaturity on the part of the neonatal T cells. As secondary stimulation of neonatal T cells did result in the expression of a lymphokine repertoire typical of T cells exposed to antigenic stimulation *in vivo*, our data support the interpretation that neonatal T cells really are mature: they can respond to TCR/CD3 activation *in vitro* by proliferating, and also by differentiating to fully competent, lymphokine-secreting effector cells. It is important to note that our data are derived from whole cell populations and therefore contribute no information as to functions of single cells. However, it is worthy of emphasis that all attempts to demonstrate clonal segregation or restriction of lymphokine gene expression by human T cell clones similar to that reported with murine T cell clones have failed to date (36, 60–62).

When considering the nature of a primary immune response compared with an anamnestic response, our results are consistent with the characteristics of the phenomenon recognized *in vivo* as immunologic memory. Thus, if our *in vitro* findings are reflective of what actually occurs upon introduction of antigen *in vivo*, the first several days are taken up by the IL-2-promoted clonal expansion of lymphocytes. Assuming that antigen persists during this period, the expanded clones of naive T cells that have undergone the differentiative switch would then be capable of receiving a secondary stimulation from persisting antigen. Ultimately, the secretion of the rest of the lymphokines would direct the recruitment and activation of the remainder of cells that participate in a fully developed immune response. By comparison, a previously primed host that already has an expanded antigen-experienced population is capable of secreting all of the lymphokines immediately upon antigenic stimulation. Consequently, the tempo of the anamnestic response is more rapid compared with a primary response because of two fundamental differences in the T cell populations: a primed host has an expanded number of antigen-reactive cells, and these cells are already differentiated so that they can release all of the immune-inflammatory lymphokines immediately after antigen triggering.

With respect to additional mechanisms thought to be involved in the phenomenon of immunologic memory, several studies have indicated that memory cells also differ from naive cells in their tissue distribution and recirculation patterns (12, 13, 63). These characteristics may help explain the observed rapid accumulation of primed cells at inflammatory foci and may also contribute to the known longevity of cell-mediated immunity (11–13). Furthermore, the response to antigen during a secondary exposure may begin more rapidly because primed T cells may be more sensitive to lower antigen concentrations (10, 11, 18–20). However, in addition to these characteristics of memory, the data presented here imply that much of the functional superiority of the anamnestic immune response lies in the differentiated state of a primed T cell that enables it to immediately secrete precisely those effector lymphokines that are required for a full-fledged, coordinate immune defense.

From the way our *in vitro* experiments were planned and performed, differences in activation thresholds, known signalling pathways, and accessory cell contributions could be excluded as responsible for the two distinct functional T cell phenotypes. Instead, all of our data are consistent with the interpretation that by analogy to B cells, T cells also undergo an activation-dependent differentiation event that permits their secretion of multiple effector lymphokines upon restimulation. Accordingly, we propose that the term “memory T cell” be reserved for those T cells identified by their differentiated state of lymphokine gene inducibility. T cell memory thus entails not only the clonal expansion of specific T cells, but also encompasses a differentiation event that allows additional lymphokine gene expression.

This tentative definition provides for a testable working hypothesis of T cell memory. In particular, it can now be

compared with and complemented by other criteria that have been advanced, especially for the phenotypic delineation of memory T cells. In this regard, most data derived from CD45RO- or CD58-based separations of adult T cells are congruent with this hypothesis, although a similarly comprehensive analysis of lymphokine gene expression in the complete absence of accessory cells has not yet been attempted (36, 39). Further proof for the validity of this definition is to be expected from *in vivo* experimental systems that afford a detailed examination of successive stages in the physiologic development of cell-mediated immunity. Clinical data from pathophysiologic conditions may prove even more useful, in that a possible cause for immunodeficiencies could be the failure to acquire, or alternatively, the loss of, memory T cell function. Similarly, the concept of T cell tolerance may benefit from the hypothesis advanced here: it is entirely conceivable that an anergic T cell genetically resembles a naive cell, but one that cannot undergo the switch to a functional memory phenotype. It is also attractive to speculate that some pathogens may have evolved strategies that selectively interfere with this differentiation process of the T cell as a means to evade cellular immunity.

At this stage, it is premature to speculate in detail as to the molecular mechanisms that might account for the apparent coordinate change in lymphokine gene expression that underlies the switch from naiveté to memory. However, since the model system described in this report provides all of the components necessary for the differentiation of T cells *in vitro*, thereby recapitulating the developmental history of the anamnestic T cell response *in vivo*, a molecular dissection of the mechanisms that determine and govern the development of T cell memory is feasible.

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