INFECTION AND IMMUNITY, Sept. 1991, p. 3053–3059
0019-9567/91/093053-07$02.00/0

Vol. 59, No. 9

Increase of T-Cell Receptor γ/δ-Bearing T Cells in Cord Blood of Newborn Babies Obtained by In Vitro Stimulation with Mycobacterial Cord Factor

IZUO TSUYUGUCHI,[1]* HIROMI KAWASUMI,[1] CHISATO UETA,[1] IKUYA YANO,[2] AND SUSUMU KISHIMOTO[1]

Osaka Prefectural Habikino Hospital, Habikino-shi, Osaka 583,[1] and Department of Bacteriology, Osaka City University Medical School, Abeno-ku, Osaka 545,[2] Japan

Received 25 March 1991/Accepted 10 June 1991

Cord blood T lymphocytes proliferated in vitro in response to mycobacterial organisms but did not proliferate in the presence of tuberculin purified protein derivative. Components recognized by cord blood T cells were resistant to protease digestion. In contrast, T lymphocytes derived from tuberculin-positive adult peripheral blood proliferated when stimulated by the protease-sensitive component of mycobacterial organisms or purified protein derivative, confirming that adult T cells respond to protein components whereas cord blood T cells respond to the nonpeptide component of mycobacteria. In vitro culture of cord blood lymphocytes stimulated by either mycobacterial lysates or the lipid fraction showed increases in the numbers of T-cell receptor (TcR) γ/δ T lymphocytes with no changes in the numbers of TcR α/β T lymphocytes in contrast to the in vitro cultures of adult blood lymphocytes stimulated with mycobacterial ligands in which no increase of TcR γ/δ cells was observed. Interleukin-2 receptor (CD25) and Ia antigen (HLA-DR) analyses evidenced the activation of a large proportion of cord blood γ/δ T cells which had increased after stimulation with mycobacteria in vitro. Further characterization of mycobacterial ligand suggested that the lipid fraction of mycobacterial lysate or trehalose dimycolate-cord factor was the most plausible cause for T-cell proliferation in cord blood. These results suggest that when the γ/δ T cells in a newborn infant not yet sensitized to any pathogenic organisms are confronted by a mycobacterium, they respond nonspecifically to the mycobacterial organism or its lipid component (cord factor). γ/δ T cells may therefore play a distinct role in forming the first line of the host defense system against certain microorganisms.

Protective immunity against microbial organisms is mediated by both humoral and cellular immunity. During the newborn period in humans, immunoglobulin G antibodies which are passively transferred into the fetus from the mother through the placenta play an important role in protective immunity. To date, no evidence of passive transfer of lymphocytes from the mother to the fetus via the placenta has been reported, making cord blood lymphocytes the logical choice for investigating the cellular mechanism of the first line of the host defense against various pathogenic microorganisms in humans.

Protective immunity against mycobacteria, which are intracellular pathogens, is dependent on antigen-specific T lymphocytes. Most T cells recognize foreign antigenic peptides bound to major histocompatibility complex (MHC) class I or class II molecules via a T-cell receptor (TcR) that is composed of α and β chains (23). Recently, a small subpopulation of T cells was found to bear a distinct TcR composed of γ and δ subunits (5, 12, 15). In humans, γ/δ T cells account for 1 to 5% of adult peripheral blood T cells, whereas they are almost nonexistent in the cord blood of newborn infants (25).

The nature of the specificity and immunologic functions of the γ/δ T cells remains unclear. Because of the relatively small number of Vδ, Jδ, Vγ, and Jγ segments present in the human genome, there is perhaps only a limited diversity for TcR γ/δ molecules in contrast to the situation for α/β T cells (9). Recently, evidence of γ/δ T cells playing a role in the immune response to mycobacteria has been reported by several groups. In humans, γ/δ T-cell lines reactive with mycobacterial antigen and tuberculin purified protein derivative (PPD) have been generated from the synovial fluid of a rheumatoid arthritis patient (11, 26), leprosy skin lesions (16), and a healthy individual immune to PPD (10). In mice, γ/δ T cells have been shown to accumulate in draining lymph nodes of mice immunized with *Mycobacterium tuberculosis* (13). Recently, Yoshikai et al. reported an increase in the number of γ/δ T cells in athymic nude mice treated with complete Freund's adjuvant (29).

Several papers reported that a bacterial heat shock protein of 65 kDa was identified as a ligand for the γ/δ T cell (1, 13, 19). On the other hand, Kabelitz et al. (14) and Pfeffer et al. (22) reported that in human peripheral blood T cells, a frequent set of γ/δ T cells are stimulated by the protease-resistant components of *M. tuberculosis* but not by its 65-kDa heat shock protein.

In the present study, we demonstrated that the cord blood of newborn infants contains γ/δ T cells which proliferate in vitro upon stimulation with either heat-killed mycobacterial organisms or their lipid fractions and far less with tuberculin PPD. Active mycobacterial components are resistant to protease digestion. Trehalose 6,6'-dimycolate, one of the mycobacterial cell wall components and well known as a toxic glycolipid cord factor, is a most plausible ligand for γ/δ T cells in cord blood. Cord factor is a common constituent of the cell walls of bacteria belonging to the order *Actinomycetales*, such as *Mycobacterium*, *Nocardia*, and *Rhodococcus* spp. (8). It is suggested that γ/δ T cells may participate in immune surveillance at the early stage of life as a first line of defense against the invasion of various pathogens, including mycobacteria.

* Corresponding author.

TABLE 1. In vitro proliferative responses of PBMC and CBMC

Stimulator (concn)	[^{3}H]thymidine incorporation (10^3 cpm)[a]	
	PBMC (n = 8)	CBMC (n = 10)
Medium	0.5 ± 0.1 (1.0)	1.1 ± 0.2 (1.0)
Mycobacterial lysate (10 μg/ml)	7.5 ± 0.4[b] (18.1 ± 6.8)[c]	8.4 ± 1.1[c] (8.9 ± 1.5)[c]
Lipid fraction (100× dilution)	0.5 ± 0.1 (1.2 ± 0.2)	4.1 ± 0.6[c] (4.3 ± 0.8)[c]
PPD (10 μg/ml)	20.2 ± 0.6[c] (44.9 ± 10.1)[c]	1.5 ± 0.3 (1.5 ± 0.3)

[a] Numbers in parentheses are the SI (cpm in stimulator/cpm in medium, where cpm is counts per minute).
[b] $P < 0.05$ compared with culture in medium.
[c] $P < 0.01$ compared with culture in medium.

MATERIALS AND METHODS

Antigens. *Mycobacterium avium* organisms (ATTC 15769, serovar 16) were provided by Kobayashi Pharmaceutical Co. Ltd., Osaka, Japan. They were treated at 60°C for 2 h before use. Stock suspensions of 10 mg/ml in physiological saline were sonicated, kept at −20°C, and then diluted in RPMI 1640 medium for use. The lipid component was extracted in chloroform-methanol (2:1) as described by Bligh and Dyer (2). Finally, lipid extract from 2.0 g of dried *M. avium* was suspended in 10 ml of chloroform-methanol. This suspension was chosen as the original dilution and used in the culture after having been diluted in methanol to the concentrations mentioned in Results. Various glycolipids from *Nocardia rubra* were obtained by the method described by Yano et al. (28). The lipids were extracted from cultured and harvested *N. rubra* 1311 with chloroform-methanol and developed on thin-layer plates of silica gel G (Analtech Inc., Newark, Del.) with the solvent system chloroform-methanol-acetone-acetic acid (90:10:6:1 by volume). Each glycolipid was recovered from the gels with chloroform-methanol (2:1 by volume), and the purification procedure was repeated until a single spot was obtained. Each purified glycolipid was chemically identified by several analytical procedures. Finally, three preparations were obtained; trehalose dimycolate (TDM), mannose monomycolate (MM) and glucose monomycolate (GM). PPD of tuberculin from culture filtrates of *M. tuberculosis* was obtained from the Institute for Microbial Diseases, Osaka University, Osaka, Japan. Proteolytic digestion of mycobacterial lysate and PPD was performed by the method described by K. Pfeffer et al. (22). Mycobacterial lysate was incubated with 25 μg of proteinase K (Sigma) for 15 min at 37°C and then heated to 60°C for 15 min. The same incubation procedure was used for PPD (1 mg/ml, pH 7.5). This procedure was repeated twice, incubating the sample with freshly added proteinase K for another 15 min and then for 60 min at 37°C. Proteinase K digests were subsequently treated with 100 μg of pronase (Sigma) solubilized in 50 mM NaH_2PO_4 (pH 6.5) for 90 min at 25°C.

Mononuclear cell separation and preparation of T cells and monocytes. Cord blood samples from umbilical cords were obtained with a heparin-coated syringe at delivery. All babies were born of healthy, tuberculin skin test-positive mothers. Cord blood mononuclear cells (CBMC) were isolated by the Ficoll-Hypaque method, as described previously (24). Peripheral blood mononuclear cells (PBMC) from tuberculin-positive, healthy volunteers were obtained from heparinized venous blood by a similar method with Ficoll-Hypaque. The resultant mononuclear-cell suspension was separated into adherent and nonadherent cells as follows. A 5-ml sample of 2 × 10^6 PBMC per ml of RPMI 1640 medium supplemented with 10% fetal calf serum (M. A. Bioproducts, Walkersville, Md.) was placed in a plastic petri dish (60 by 15 mm; Kotai Kagaku Co. Ltd., Tokyo, Japan) and incubated at 37°C in 7.5% CO_2 in air for 2 h. Adherent cells were then extensively washed and were removed by adding 3 ml of cold EDTA solution and further incubating the dish at 4°C for 30 min. Adherent cells thus prepared were used as monocytes. In the experiments in which monocytes were added into T-cell proliferation culture, previously irradiated (2,000 rads) monocytes were used. T cells were obtained by passing nonadherent cells through a nylon wool column as described previously (27). The cellular compositions of monocytes and T cells thus prepared were examined by fluorescence-activated cell sorter analysis. The monocyte population contained more than 95% LeuM3$^+$ cells. The T-cell population contained less than 0.5% LeuM3$^+$ and more than 85% Leu4$^+$ cells.

Generation of monocyte culture supernatant. Adherent mononuclear cells (1.0 × 10^6/ml) were cultured in the presence of mycobacterial lipid (100× dilutions). After 24 h, the supernatants were collected, sterile filtered, and frozen until assay for T-cell mitogenic activity.

Immunofluorescence staining and two-color analysis of mononuclear cells. Immunofluorescence analysis was performed by using FACScan (Becton Dickinson and Co., Mountain View, Calif.). Cells were stained either with phycoerythrin (PE)-coupled anti-Leu4 (CD3; Becton Dickinson) plus fluorescein isothiocyanate-conjugated anti-TcR γ/δ-1 (Becton Dickinson) or with anti-Leu4 (anti-CD3) plus anti-TcR-1 (α/β WT31; Becton Dickinson) for 30 min at 4°C and then washed twice. Anti-TcR γ/δ-1 monoclonal antibody was shown to react with the human TcR γ/δ framework. Data were presented as two-dimensional contour maps. To obtain the subpopulation percentage, total counts were integrated in selected areas of contour plots.

In vitro assay of proliferative response. Mononuclear cells were cultured at a density of 2.5 × 10^4 cells per ml in flat-bottomed tissue culture plates (Microtest II, Falcon no. 3000) with an appropriate concentration of antigens or lipid samples. The culture medium used was RPMI 1640 supplemented with 10% pooled human serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cultures were maintained in humidified 7.5% CO_2 in air at 37°C. Eighteen hours before culture termination, 0.2 μCi of [^{3}H]thymidine was added to each well. At the end of culture, the cells were harvested and washed with a semiautomated microharvester (Laboscience Co. Ltd., Tokyo, Japan), and then [^{3}H]thymidine incorporation was evaluated. Each determination was performed in triplicate, and the data were expressed as counts per minute ± standard error of the mean. In the experiment to evaluate the effect of anti-HLA-DR monoclonal antibody on the mycobacterial-ligand-stimulated in vitro proliferative responses of mononuclear cells, appropriately diluted monoclonal antibody (anti-HLA class II, immuno-

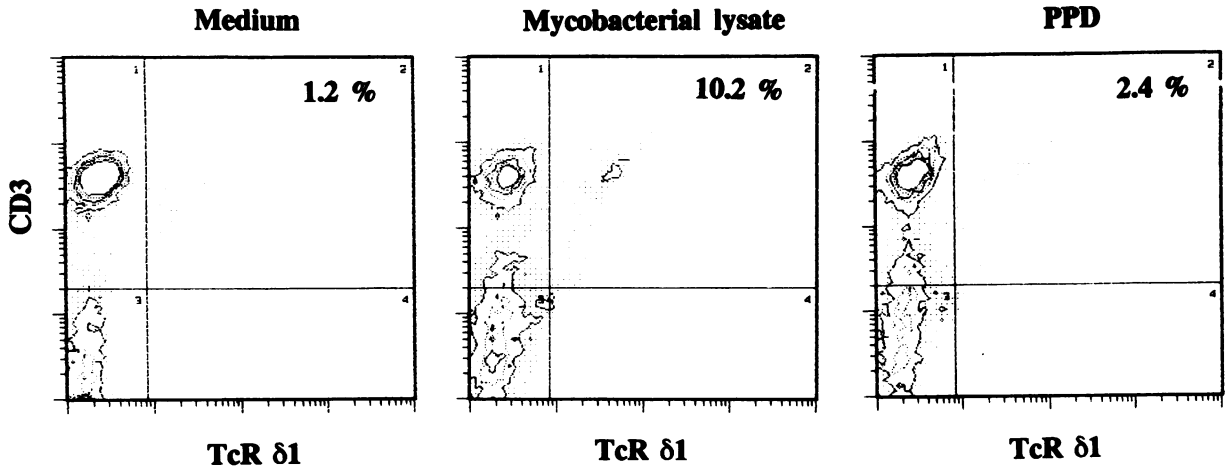

FIG. 1. Two-color cytometric analysis of CD3 and TcR γ/δ$^+$ cells on CBMC after in vitro stimulation with mycobacterial lysate, lipid fraction, and PPD. The CBMC were stained with PE-conjugated anti-CD3 plus fluorescein isothiocyanate-conjugated anti-TcR δ1 monoclonal antibodies.

globulin G2b; Cosmo Bio Co., Ltd., Tokyo, Japan) was added to the culture at the initiation of cultivation.

Assay of cell viability. At the time of harvest, the cells in the culture were diluted and gently suspended in eosin-Y solutions (final concentration, 0.2%), the total number was determined, and the percentage of viable cells was determined. At least 200 cells were examined in each culture.

Statistical analysis. Experimental data were expressed as mean ± standard error of the mean. The significance of the difference between groups was calculated by Student's *t* test. Computer-assisted evaluation of the results was used to calculate the probability value in the data. A *P* of 0.05 was used as the limit of statistical significance.

RESULTS

In vitro proliferative responses of mononuclear cells to mycobacterial stimulation. CBMC and PBMC were cultured in vitro for 6 days together with various concentrations of whole mycobacterial lysates, lipid fractions, or PPD, and the extent of proliferation was then assessed by measuring the radioactivity of [^{3}H]thymidine incorporated into the cultured cells. From the kinetic and dose-response studies, in vitro culture was carried out for 6 days at a concentration of 10 μg of mycobacterial lysate per ml, 100 dilutions of lipid, or 10 μg of PPD per ml. As shown in Table 1, CBMC proliferated in vitro in response to mycobacterial lysates and their lipid fractions and far less in response to PPD. The stimulation indexes (SI) were 8.9 ± 1.5 with mycobacterial lysates, 4.3 ± 0.8 with lipid fractions, and 1.5 ± 0.3 with PPD. In one CBMC subject of the 10 examined, a significantly high response to PPD stimulation was observed (Δcpm, 13,400; SI, 12.1). PBMC, on the other hand, proliferated in vitro when stimulated by the mycobacterial lysates (SI, 18.1 ± 6.8) and PPD (SI, 44.9 ± 10.1) but did not respond to lipid fraction (SI, 1.2 ± 0.2). The viability of cultured cells in CBMC and PBMC was as follows: 80 and 80% in control cultures, 83 and 85% in mycobacterial lysates, 75 and 78% in lipid, and 85 and 83% in PPD cultures, respectively.

Phenotype characterization of mycobacterium-stimulated T cells. The phenotypes of the mycobacterium-stimulated cord blood T cells were analyzed by using two-color flow cytometry. Cultured cells were stained with both PE-coupled anti-CD3 and either fluorescein isothiocyanate-coupled anti-TcR γ/δ-1 or anti-TcR α/β lymphocytes. A representative two-color analysis is shown in Fig. 1, and the summarized data are shown in Table 2. The data are expressed as the percentage of TcR γ/δ1$^+$ or TcR α/β$^+$ cells in relation to total CD3$^+$ cells after in vitro culture with various stimulants. The percentages of TcR γ/δ-1$^+$ cells in CD3$^+$ cells were increased in vitro in CBMC after stimulation with either mycobacterial lysates or lipid fraction; however, the PBMC percentages did not change. Two-color analysis also showed that the γ/δ T cells which increased in number in CBMC after in vitro stimulation with mycobacterial lysates expressed interleukin 2 receptor (CD25) and/or Ia antigen (HLA-DR), suggesting that these γ/δ T cells were activated T cells (Table 3). On the other hand, no such increase in the frequency of

TABLE 2. Percentage of α/β and γ/δ T cells in PBMC and CBMC after in vitro stimulation with mycobacterial ligands

Stimulator (concn)	% positive cells in CD3$^+$ cells			
	PBMC (*n* = 7)		CBMC (*n* = 8)	
	α/β	γ/δ	α/β	γ/δ
Medium	86.9 ± 2.4	6.2 ± 1.4	91.9 ± 1.1	2.1 ± 0.4
Mycobacterial lysate (10 μg/ml)	78.8 ± 4.0	5.3 ± 1.2	78.9 ± 3.2[a]	10.7 ± 2.1[a]
Lipid fraction (100× dilution)	85.5 ± 11.0	7.8 ± 2.2	68.4 ± 5.3[a]	8.1 ± 1.4[b]
PPD (10 μg/ml)	77.9 ± 5.3	3.8 ± 0.3	89.6 ± 1.2	3.3 ± 0.6

[a] $P < 0.01$ compared with culture in medium.
[b] $P < 0.05$ compared with culture in medium.

TABLE 3. $CD25^+$ and $HLA\text{-}DR^+$ TcR γ/δ- and α/β-bearing T cells in cord blood after in vitro stimulation with mycobacterial lysate or PPD tuberculin

Stimulator[a] (concn)	% Positive cells in γ/δ T cells		% Positive cells in α/β T cells	
	$CD25^+$	$HLA\text{-}DR^+$	$CD25^+$	$HLA\text{-}DR^+$
Medium	14.3	11.3	11.3	5.5
Mycobacterial lysate (10 μg/ml)	38.3	51.2	19.8	8.3
PPD (10 μg/ml)	12.6	15.5	13.3	5.7

[a] Cells were cultured for 2 days in vitro with stimulator.

CD25- or HLA-DR-bearing α/β T cells was observed in CBMC after stimulation with mycobacterial lysates.

Protease treatment of mycobacterial ligands stimulating CBMC. Mycobacterial lysates and PPD were treated with proteinase K and pronase and later used to stimulate CBMC and PBMC. As shown in Table 4, after being treated with protease, mycobacterial lysates and PPD lost their abilities to stimulate PBMC. On the other hand, the CBMC which responded to mycobacterial lysate still gave a significant response to protease-digested mycobacterial lysates. These results indicate that PBMC responded to peptides involved in both mycobacterial lysates and PPD preparations, whereas CBMC responded to a nonpeptide component in mycobacterial lysate, the candidate probably being carbohydrate or lipid. PPD preparation contains both carbohydrate and protein. In the present study, PPD, whether protease treated or not treated, had no stimulatory effect on cord blood T cells.

Stimulation of cord blood lymphocytes by mycobacterial cord factor. The data mentioned above suggested that the stimulatory agent contained within the mycobacterium is not a peptide and that this agent may possibly be retained in the lipid fraction. Therefore, our next step was to ascertain whether or not the cord factor was able to stimulate CBMC or γ/δ T cells in CBMC. We examined the stimulating activity of cord factor-TDM and several other mycoloyl glycolipids which differ in their carbohydrate moiety. As shown in Fig. 2, both trehalose TDM and GM sufficiently stimulated the CBMC to proliferate in vitro, whereas MM showed no significant activity. In contrast, neither TDM nor GM stimulated PBMC to proliferate. The mode of action of TDM and GM paralleled the mode of action of lipid fractions, as described above. The percentages of TcR γ/δ^+ cells in $CD3^+$ cells were also increased in vitro after stimulation with either TDM or GM but not with MM, which parallels the proliferative responses of CBMC to stimulation with these glycolipids (data not shown).

TABLE 4. Effect of protease treatment of mycobacterial ligands on stimulation of PBMC and CBMC

Treatment	[^{3}H]thymidine incorporation (10^3 cpm ± SE)	
	PBMC	CBMC
Medium	0.2 ± 0.0	0.9 ± 0.1
Mycobacterial lysate		
Nontreated	7.5 ± 0.3	7.3 ± 0.6
Protease treated	0.9 ± 0.2	7.2 ± 1.1
PPD		
Nontreated	23.7 ± 1.7	1.3 ± 0.1
Protease treated	1.1 ± 0.5	1.3 ± 0.0

Requirement for adherent cells in mycobacterium-stimulated in vitro proliferation of cord blood T cells. To see whether accessory cells are required in CBMC proliferation in vitro in response to mycobacterial lysates and lipid fraction and to cord factor-TDM stimulation, we next carried out adherent-cell depletion and cell-mixing experiments with T cells. Nonadherent, nylon wool column-passed cord blood T cells were cultured in the presence of autologous adherent cells and stimulated with either mycobacterial lysates or lipid fraction. A representative result from three independent experiments is shown in Table 5. Cord blood T cells alone did not respond at all to stimulation with either mycobacterial lysates or lipid fraction. The response of T cells was restored by the addition of adherent cells. These results show that T cells were responsible for the mycobacterium-induced proliferative response observed in CBMC and that adherent cells were essential for T cells to respond to lipid fraction and to mycobacterial lysates.

The possibility of involvement of HLA class II antigen is small, since the addition of anti-HLA-DR monoclonal antibody caused no significant suppression of CBMC response to mycobacterial lysate and lipid stimulation. This was in contrast to the significant suppression noted in the PBMC response to PPD. A representative result is shown in Table 6.

In vitro proliferation of cord blood T cells in the presence of lipid-stimulated-monocyte culture supernatant. Finally, we tried to discover whether the stimulatory action of mycobacteria on cord blood T cells is brought about by a soluble factor(s) from monocytes cocultured with mycobacterial lipid fraction. Figure 3 shows that culture supernatants obtained from adherent cells cocultured for 24 h with lipid fraction were capable of stimulating cord blood T cells. Supernatants of monocytes cultured in the absence of lipid

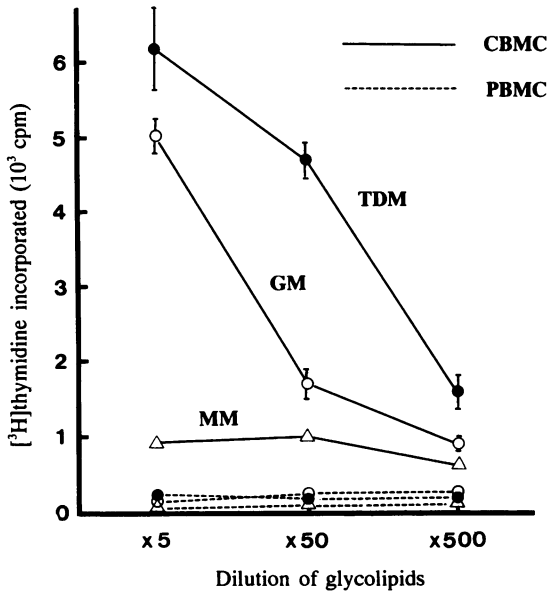

FIG. 2. In vitro proliferation of CBMC in response to mycoloyl glycolipids. CBMC and PBMC were cultured in vitro for 6 days in the presence of various dilutions of TDM, MM, or GM. The extent of cell proliferation was assessed by measuring the radioactivity of [^{3}H]thymidine incorporated into the cultured cells.

TABLE 5. Requirement for adherent cells in in vitro proliferation of cord blood T cells in response to mycobacterial ligands

Type of cells	[^{3}H]thymidine incorporation (10^2 cpm ± SE)				
	Medium	Mycobacterial lysate (10 μg/ml)	Lipid fraction (100× dilution)	TDM (100× dilution)	PPD (10 μg/ml)
CBMC	7.3 ± 0.5	61.9 ± 1.5	85.7 ± 4.4	81.6 ± 5.2	17.7 ± 2.8
T cells	1.9 ± 0.0	8.6 ± 1.3	2.6 ± 0.0	3.9 ± 1.1	4.4 ± 1.1
T cells + adherent cells[a]	7.0 ± 0.5	69.8 ± 5.4	33.2 ± 1.3	30.0 ± 2.9	6.4 ± 1.4

[a] Adherent cells were previously irradiated with 2,000 rads.

fraction, whether reconstituted with lipid or not, gave a distinct but lesser stimulation to cord blood T cells. Monocytes obtained from tuberculin-positive adult peripheral blood gave an activity similar to that of monocytes from cord blood. These monocyte culture supernatants, however, exerted no stimulating activity on T cells from adult peripheral blood (data not shown).

DISCUSSION

In our present study, we demonstrated that the γ/δ T cells which exist in the cord blood of newborn infants proliferate in vitro after stimulation with whole mycobacterial organisms or their lipid fractions in the presence of adherent cells. CBMC proliferated in vitro when stimulated by whole mycobacterial organisms, their lipid fractions, or cord factor. However, CBMC did not respond significantly to PPD stimulation. Previously, we reported that CBMC proliferated in vitro after stimulation with PPD (24), although the optimum dose of PPD required for CBMC to show the maximum response was 10 times higher than that required for PBMC from tuberculin skin test-positive donors. In the present study, we noted less proliferation of CBMC in response to PPD stimulation than in our former study. It may have been that the PPD preparation employed in the previous study was from a different lot than the PPD employed in the present study and was contaminated with more lipid. It is also of note that whole mycobacterial organisms and lipid fractions, both of which were stimulatory to CBMC proliferation, were less stimulatory or nonstimulatory to the proliferative responses of PBMC from tuberculin skin test-positive individuals. These results suggest that CBMC responded differently from PBMC, i.e., different in responding cell types and/or different in stimulating epitopes of mycobacterium.

Protective immunity against mycobacteria is dependent on antigen-specific T cells. It has been assumed that upon recognition of antigen-MHC, these T cells secrete macrophage-activating factors that enable effector macrophages to eliminate the intracellular pathogens. The majority of T cells involved in antimycobacterial immunity bear the TcR α/β complex (α/β T cells) (23). Recently, a small subpopulation of T cells was found to bear a distinct TcR composed of γ and δ subunits (γ/δ T cells) (5, 12, 15). Evidence suggesting that γ/δ T cells may participate in the immune response to mycobacteria and other infectious organisms has accumulated (3, 4, 9). A large fraction of human peripheral blood γ/δ T cells are activated by *M. tuberculosis* organisms (10). In mice infected with viable *Listeria* organisms, γ/δ T cells in the peritoneal exudate cells increased to the maximal level as early as day 3, whereas α/β T cells increased on day 8 after infection (20). It has been suggested that the appearance of broadly reactive TcR γ/δ-bearing T cells in microbial infections may serve as a first line of defense against the invasion of various pathogens (14). In humans, γ/δ T-cell lines reactive with mycobacterial PPD have been generated from a rheumatoid arthritis patient (11, 26). In our present study, PBMC from tuberculin-sensitized individuals responded in vitro to PPD stimulation, and in this response, the α/β rather than the γ/δ T cells were thought to be the responding cells. Considering that newborn infants had not been primed with mycobacteria, the absence of mycobacterium- or PPD-reactive α/β T cells was not surprising. However, the increased number of mycobacterium-reactive γ/δ T cells was unexpected.

What are the ligands of proliferating γ/δ T cells in cord blood? Several reports have indicated that some γ/δ T cells respond to mycobacterial antigens, such as the PPD of *M. tuberculosis*, and mycobacterial 65-kDa heat shock protein (1, 13, 15). On the other hand, Pfeffer et al. reported that mycobacterium-reactive human γ/δ T cells responded to the nonpeptide constituents of mycobacterium and not to the 65-kDa heat shock protein (22). Our study indicates that mycobacterial fractions capable of stimulating T cells in cord blood were resistant to protease digestion, whereas those stimulating PBMC were protease sensitive. In our study, cord blood γ/δ T cells increased after in vitro culture with lipid fractions as well as whole mycobacterial organisms. The most plausible stimulus is the cord factor isolated from mycobacterial cell walls. Cord factor is one of the toxic

TABLE 6. Effect of anti-HLA-DR monoclonal antibody on mycobacterial-ligand-induced in vitro proliferative responses of CBMC and PBMC

Stimulator (concn)	[^{3}H]thymidine incorporation (10^3 cpm)					
	CBMC			PBMC		
	Medium	40×[a]	20×	Medium	40×	20×
Medium	1.1 ± 0.2	0.8 ± 0.1	0.9 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
Mycobacterial lysate (10 μg/ml)	6.7 ± 0.3	7.6 ± 0.6	6.1 ± 0.1	5.7 ± 0.3	1.3 ± 0.0	0.3 ± 0.0
Lipid fraction (100× dilution)	3.0 ± 0.4	3.7 ± 0.6	3.2 ± 0.4	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.0
PPD (10 μg/ml)	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	30.9 ± 1.5	12.9 ± 4.1	1.8 ± 0.2

[a] Dilution of anti-HLA-DR antibody.

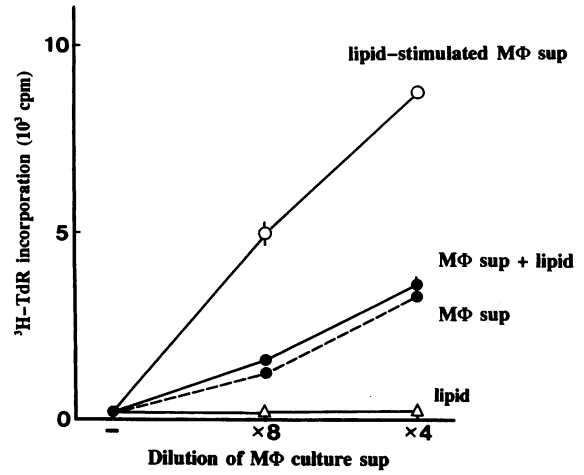

FIG. 3. In vitro proliferation of cord blood T cells in the presence of monocyte (MΦ) culture supernatants or lipid. Culture supernatants were obtained from cord blood adherent cells cocultured with lipid (○), without lipid (●----●), or without lipid and reconstituted with lipid (●——●).

glycolipid constituents of the cell walls of bacteria belonging to the order *Actinomycetales*, such as *Mycobacteriuum*, *Nocardia*, and *Rhodococcus* spp. (8). It has been widely assumed that cord factor is directly responsible for the wasting associated with mycobacterial infections. Recently, cord factor has also been found to be a potent immunomodulator, possessing granuloma-forming activity and antitumor activity (7, 18, 28).

An intriguing hypothesis is that γ/δ T cells have evolutionally selected to respond to certain common microbial antigens, thus enabling this population to respond quickly while the population of antigen-specific α/β T cells begins to expand. The secondary response may involve a switch in receptor usage, with γ/δ participating early in the response and α/β eventually taking over. Consistent with this notion is the virtual absence of expansion of the γ/δ T-cell population after secondary *M. tuberculosis* infection (13). In the case of tuberculosis infection, the lipid component of mycobacterium organisms may stimulate γ/δ T cells in the primary response, acting as a bacterial superantigen to induce polyclonal γ/δ T-cell proliferation. PPD, on the other hand, acts in the secondary response as a nominal antigen to induce monoclonal proliferation of sensitized α/β T cells. When an individual is first confronted with pathogenic microorganisms, large numbers of cells with the same specificity may provide a pool for rapid immune response to certain critical antigens without the need for prior clonal expansion. In the case of mycobacterial infection, cord factor plays a role as the ligand for γ/δ T cells. Cord factor has a high morphological homogeneity among several cord factor preparations from bacteria. Thus, the γ/δ T cells may play a role in covering the gap between the phagocytic system and the highly evolved type immune responses mediated by α/β T cells in host defense against mycobacterial infection (4, 20). Indeed, Munk et al. demonstrated that in vitro stimulation of peripheral blood T cells from healthy donors with *M. tuberculosis* induced marked expansion of γ/δ T cells, which acquired specific cytolytic activation against *M. tuberculosis*-pulsed target cells (17).

No significant increase of γ/δ T cells was observed in PBMC from tuberculin-positive donors after in vitro stimulation with PPD or whole mycobacterial lysate was carried out, although the baseline value of the percentage of γ/δ T cells was greater in PBMC than in CBMC. This is in keeping with the data reported by Smith et al. (25). It is possible that γ/δ T cells in PBMC did increase after mycobacterial-antigen stimulation, but the increase may have been masked by the greater increase of PPD-sensitized α/β T cells. Another possibility is that the γ/δ T-cell response was suppressed while the α/β T-cell response was occurring, possibly through the action of a soluble factor(s) produced by activated α/β T cells. Parker et al. recently reported extrathymic changes in the TcR γ/δ repertoire after birth (21). With increasing age, the number of Vδ2-expressing TcR γ/δ-bearing T cells in peripheral blood increased, whereas the number of Vδ1-bearing cells did not change with age, resulting in a dramatic rise in the ratio of Vδ2/Vδ1-bearing cells after birth. Smith et al. also compared γ/δ T-cell subsets in cord and adult blood (25). They showed that the neonate had a different overall repertoire of γ/δ T cells than the adult. This change in the TcR γ/δ repertoire after birth may account for the difference in γ/δ T-cell responses after mycobacterial stimulation between CBMC and PBMC observed above. The anti-TcR γ/δ-1 monoclonal antibody we employed for the detection of γ/δ T cells identifies T cells bearing a TcR γ/δ framework. According to Happ et al. (9), none of 28 murine PPD-reactive γ/δ T-cell hybridomas expressed Vδ1, whereas 10 of 22 PPD-nonreactive hybridomas expressed Vδ1. In the present study, some CBMC proliferated in vitro in response to PPD stimulation. This may imply that some of the γ/δ T cells in CBMC expressing Vδ2 responded to PPD as well as to mycobacterial lipid.

As to the involvement of MHC class II antigen in γ/δ T-cell response in the recognition of mycobacterium by T cells, anti-HLA-DR monoclonal antibody did not block the responses of CBMC to mycobacterial lysate and lipid component, while the responses of PBMC to PPD and mycobacterial lysate were significantly blocked by anti-Ia monoclonal antibody. These data indicate that, in contrast to mycobacterium-reactive α/β T cells, mycobacterium-reactive γ/δ T cells do not require MHC class II molecules for the recognition of mycobacteria. The failure of monoclonal antibody to block the responses of γ/δ T cells contrasts with the response of α/β T cells, where the recognition of soluble exogenous antigens is generally predicted by MHC class II presentation. Fisch et al. demonstrated that TcR γ/δ T lymphocytes might be involved in the distinct pattern of non-MHC-restricted cytolysis mediated by γ/δ T-cell clones on certain tumor target cells such as the Daudi cell line, possibly at the level of effector-target cell recognition (6). Taken together, these studies and our present study indicate that γ/δ T cells and α/β T cells recognize antigen differently and suggest that γ/δ T cells have distinct roles in the primary immune response. In the first 6 months of the newborn period, during which α/β T-cell-mediated immunity does not occur, γ/δ T cells may play a transient defense role in reactions against pathogenic microorganisms.

As to the mechanism by which cord blood γ/δ T cells are activated by stimulation with mycobacterial cord factor, the data in Fig. 3 suggest a possible role for a soluble factor produced by mycobacterium-induced macrophages that stimulates cord blood but not adult peripheral blood T cells to proliferate. In preliminary experiments, we have observed that neither recombinant interleukin-1α nor tumor necrosis factor alpha stimulated cord blood γ/δ T cells. This issue is currently under investigation.

REFERENCES

1. **Augustin, A., R. T. Kubo, and G. K. Sim.** 1989. Resident pulmonary lymphocytes expressing the γ/δ T-cell receptor. Nature (London) **340**:239–241.
2. **Bligh, E. C., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
3. **Born, W., L. Hall, A. Dallas, J. Boymel, T. Shinnick, D. Young, P. Brennan, and R. O'Brien.** 1990. Recognition of a peptide antigen by heat shock-reactive γδ T lymphocytes. Science **249**:67–69.
4. **Born, W., M. P. Happ, A. Dallas, C. Reardon, R. Kubo, T. Shininck, P. Brennan, and R. O'Brien.** 1990. Recognition of heat shock proteins and γδ cell function. Immunol. Today **11**:40–43.
5. **Brenner, M. B., J. McLean, D. P. Dialynas, J. L. Strominger, J. A. Smith, F. L. Owen, J. G. Seidman, S. Ip, F. Rosen, and M. S. Krangel.** 1986. Identification of a putative second T-cell receptor. Nature (London) **322**:145–149.
6. **Fisch, P., M. Malkovsky, E. Braakman, E. Sturm, R. L. H. Bolhuis, A. Prieve, J. A. Sosman, V. A. Lam, and P. M. Sondel.** 1990. γ/δ T cell clones and natural killer cell clones mediate distinct patterns of non-major histocompatibility complex-restricted cytolysis. J. Exp. Med. **171**:1567–1579.
7. **Fujita, T., N. Sugimoto, F. Yokoi, Y. Ohtsubo, M. Ikutoh, Y. Kato, Y. Natsuhara, S. Oka, and I. Yano.** 1990. Induction of interferons (IFNs) and tumor necrosis factor (TNF) in mice by a novel glycolipid trehalose 2,3,6′-trimycolate from *Rhodococcus aurantiacus* (Gordona aurantiaca). Microbiol. Immunol. **34**:523–532.
8. **Goren, M. B.** 1972. Mycobacterial lipid: selected topics. Bacteriol. Rev. **36**:33–64.
9. **Happ, M. P., R. T. Kubo, E. Palmer, W. K. Born, and R. L. O'Brien.** 1989. Limited receptor repertoire in a mycobacteria-reactive subset of γδ T lymphocytes. Nature (London) **342**:696–698.
10. **Haregewoin, A., G. Soman, R. C. Hom, and R. W. Finberg.** 1989. Human γδ T cells respond to mycobacterial heat-shock protein. Nature (London) **340**:309–313.
11. **Holoshitz, J., F. Koning, J. E. Coligan, J. D. Bruyn, and S. Strober.** 1989. Isolation of $CD4^+$ $CD8^+$ mycobacteria-reactive T lymphocyte clones from rheumatoid arthritis synovial fluid. Nature (London) **339**:226–229.
12. **Janeway, C. A., Jr., B. Jones, and A. Hayday.** 1988. Specificity and function of T cells bearing γδ receptors. Immunol. Today **9**:73–76.
13. **Janis, E. M., S. H. E. Kaufmann, R. H. Schwartz, and D. M. Pardoll.** 1989. Activation of γδ T cells in the primary immune response to *Mycobacterium tuberculosis*. Science **244**:713–716.
14. **Kabelitz, D., A. Bender, S. Schondelmaier, B. Schoel, and H. K. Kaufmann.** 1990. A large fraction of human peripheral blood γ/δ^+ T cells is activated by *Mycobacterium tuberculosis* but not by its 65-kD heat shock protein. J. Exp. Med. **171**:667–679.
15. **Kaufmann, S. H. E.** 1990. Heat shock proteins and the immune response. Immunol. Today **11**:129–136.
16. **Modlin, R. L., C. Pirmez, F. M. Hofman, V. Torigian, K. Uyemura, T. H. Rea, R. R. Bloom, and M. B. Brenner.** 1988. Lymphocytes bearing antigen-specific γδ T-cell receptors accumulate in human infectious disease lesions. Nature (London) **339**:544–548.
17. **Munk, M. E., A. J. Gatrill, and S. H. E. Kaufmann.** 1990. Target cell lysis and IL-2 secretion by γ/δ T lymphocytes after activation with bacteria. J. Immunol. **145**:2434–2439.
18. **Natsuhara, Y., J. Yoshinaga, T. Shogaki, Y. Sumi-Nishikawa, S. Kurano, Y. Kato, K. Kaneda, S. Oka, and I. Yano.** 1990. Granuloma-forming activity and antitumor activity of newly isolated mycoloyl glycolipid from *Rhodococcus terrae* 70012 (Rt. GM-2). Microbiol. Immunol. **34**:45–53.
19. **O'Brien, R. L., M. P. Happ, A. Dallas, E. Palmer, R. Kubo, and W. K. Born.** 1989. Stimulation of a major subset of lymphocytes expressing T cell receptor γδ by an antigen derived from Mycobacterium tuberculosis. Cell **57**:667–674.
20. **Ohga, S., Y. Yoshikai, Y. Takeda, K. Hiromatsu, and K. Nomoto.** 1990. Sequential appearance of γ/δ- and α/β-bearing T cells in the peritoneal cavity during an i.p. infection with *Listeria monocytogenes*. Eur. J. Immunol. **20**:533–538.
21. **Parker, C. M., V. Groh, H. Band, S. A. Porcelli, C. Morita, M. Fabbi, D. Glass, J. L. Strominger, and M. B. Brenner.** 1990. Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. J. Exp. Med. **171**:1597–1612.
22. **Pfeffer, K., B. Schoel, H. Gulle, S. H. E. Kaufmann, and H. Wagner.** 1990. Primary responses of human T cells to mycobacteria: a frequent set of γ/δ T cells are stimulated by protease-resistant ligands. Eur. J. Immunol. **20**:1175–1179.
23. **Saito, T., A. Weiss, J. Miller, M. A. Norcross, and R. N. Germain.** 1987. Specific antigen-Ia activation of transfected human T cells expressing murine Ti αβ-human T3 receptor complexes. Nature (London) **325**:125–130.
24. **Shiratsuchi, H., and I. Tsuyuguchi.** 1981. Tuberculin purified protein derivative-reactive T cells in cord blood lymphocytes. Infect. Immun. **33**:651–657.
25. **Smith, M. D., C. Worman, F. Yuksel, B. Yukusel, L. Moretta, E. Ciccone, C. E. Grossi, L. Mackenzie, and P. M. Lydyard.** 1990. Tγδ-cell subsets in cord and adult blood. Scand. J. Immunol. **32**:491–495.
26. **Söderström, K., E. Halapi, E. Nilsson, A. Grönberg, J. van Embden, L. Klareskog, and R. Kiessling.** 1990. Synovial cells responding to a 65-kDa mycobacterial heat shock protein have a high proportion of a TcRγδ subtype uncommon in peripheral blood. Scand. J. Immunol. **32**:503–515.
27. **Tsuyuguchi, I., H. Kawasumi, T. Takashima, T. Tsuyuguchi, and S. Kishimoto.** 1990. *Mycobacterium avium-Mycobacterium intracellulare* complex-induced suppression of T-cell proliferation in vitro by regulation of monocyte accessory cell activity. Infect. Immun. **58**:1369–1378.
28. **Yano, I., I. Tomiyasu, K. Kaneda, Y. Kato, Y. Sumi, S. Kurano, N. Sugimoto, and H. Sawai.** 1987. Isolation of mycolic acid-containing glycolipids in *Nocardia rubra* and their granuloma forming activity in mice. J. Pharmacobio-dyn. **10**:113–123.
29. **Yoshikai, Y., G. Matsuzaki, T. Inoue, and K. Nomoto.** 1990. An increase in number of T-cell receptor γ/δ-bearing T cells in athymic nude mice treated with complete Freund's adjuvants. Immunology **70**:61–65.