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Tuberculin Purified Protein Derivative-Reactive T Cells in Cord Blood Lymphocytes

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Lymphocytes obtained from cord blood of newborn babies who were born of healthy mothers were studied in vitro for their responsiveness to purified protein derivative (PPD) of tuberculin. Cord blood lymphocytes proliferated in vitro by stimulation with PPD, despite wide variations in the results. Studies with fractionated lymphocytes revealed that PPD-responding cells belonged to E-rosetting, nylon wool-nonadherent T lymphocytes. Non-E-rosetting B lymphocytes alone did not proliferate at all after stimulation with PPD. In addition, bromodeoxyuridine and light treatment of in vitro PPD-stimulated lymphocytes eliminated the responsiveness to PPD. These results suggest that T lymphocytes do exist in cord blood and respond in vitro to stimulation with PPD. A possible role for PPD-reactive T lymphocytes in cell-mediated protective immunity in newborns is discussed.

Antimicrobial protective immunity is brought about not only by humoral immunity, but also by cell-mediated immunity. Immunity against tuberculosis and some viral diseases is mediated mostly by the action of cellular immunity. In humans, protective immunity mediated by humoral immunoglobulin G, which is passively transferred into the fetus from the mother through the placenta, is known to be operating during the newborn period. On the other hand, cell-mediated immunity has not been reported to play an important role in protective immunity during that period.

Several studies on the immunological functions of cord blood lymphocytes (CBL) have been reported previously (7, 9, 15, 16, 21, 25). Most of them were concerned with regulatory activities in immune responses. The in vitro proliferative response to purified protein derivative (PPD) of tuberculin, which is recognized to reflect delayed-type hypersensitivity in vivo (12, 13), has been investigated in some experimental systems in CBL (3, 5, 22) or in peripheral blood lymphocytes (PBL) obtained from tuberculin skin test-positive or -negative individuals (5, 18). However, no evidence has been reported so far about the existence of cell-mediated protective immunity during the newborn period.

In this present study we demonstrate that cord blood of newborn infants contains T lymphocytes which proliferate specifically in vitro by stimulation with tuberculin PPD. Our results suggest that cell-mediated protective immunity, as well as humoral immunity, may be operating during the newborn period.

MATERIALS AND METHODS

Lymphocyte preparation. Cord blood samples were obtained from umbilical cords at delivery with a heparin-coated syringe. All babies were born of healthy, tuberculin skin test-positive mothers. Lymphocytes were isolated by the Ficoll-Hypaque method, as described previously (24). By the E-rosetting method with sheep erythrocytes, it was revealed that approximately 46% of the CBL thus prepared were E-rosetting T lymphocytes, in contrast to 74% of adult PBL being E-rosetting T lymphocytes. PBL from healthy volunteers were obtained from heparinized venous blood by a similar method, using Ficoll-Hypaque. The age distributions of PBL donors were 4 months to 10 years (mean, 3.8 years) in tuberculin skin test-negative individuals and 22 to 67 years (mean, 38.4 years) in tuberculin skin test-positive individuals. Skin testing was performed by intradermal injection of appropriate doses of PPD, and reactions were measured 48 h after injection. Individuals who showed >10-mm-diameter erythema and induration with 0.05 μg of PPD were regarded as positive; those who showed no reaction with as much as 2.5 μg of PPD were regarded as negative. Blood samples were taken before skin testing.

Purification of T lymphocytes. For the preparation of purified T lymphocytes, lymphocytes were first rosetted with sheep erythrocytes (E-rosette-forming cells), followed by isolation by the Ficoll-Hypaque sedimentation technique as described previously (24). Briefly, E-rosette formation was carried out by incubating lymphocytes with sheep erythrocytes for 15 min at 37°C, followed by centrifugation and another 120-min incubation on ice. The mixture of lymphocytes and sheep erythrocytes was gently resuspended and sedimented over a Ficoll-Hypaque gradient. The E-rosette-forming cells were collected, and rosetting and free sheep erythrocytes were lysed in 0.83% am-

monium chloride. The E-rosette-forming cell-rich fraction, after lysis of erythrocytes, was then filtered through a nylon wool column. The cell suspension was applied to the column, incubated at 37°C for 1 h in 5% CO_2 in air, and then slowly eluted, washed twice with RPMI 1640 culture medium (M.A. Bioproducts, Walkersville, Md.), and resuspended in the appropriate medium. More than 95% of the purified T lymphocytes thus prepared rosetted with sheep erythrocytes. They contained less than 2% surface immunoglobulin-positive cells. Functional depletion of adherent cells in the T-cell fraction was ascertained by the evidence that they did not proliferate at all after stimulation with concanavalin A or phytohemagglutinin unless reconstituted with plastic petri dish-adherent cells. The non-E-rosetting fraction, which floated on the top of the Ficoll-Hypaque gradient, was used as a B-lymphocyte-enriched fraction. Adherent cells were obtained by recovering the plastic petri dish (Falcon Plastics, Oxnard, Calif.; no. 3002)-adherent non-E-rosetting cells with a rubber policeman.

Antigens and lectins. Tuberculin PPD was kindly donated by H. Fujii, Institute for Microbial Diseases, Osaka University. Tuberculin-active peptide (TAP), an acid-extracted product of mycobacteria (27), was a gift of T. Tawara, Toneyama National Tuberculosis Sanatorium. TAP is characterized as having the same antigenicity as PPD but as lacking immunogenicity when tested with guinea pigs (2, 27, 28). Concanavalin A was purchased from Miles Yeda Ltd., Rehovot, Israel, and pokeweed mitogen was obtained from GIBCO Laboratories, Grand Island, N.Y.

In vitro assay of proliferative response. Lymphocytes were cultured at a density of 10^6 cells per ml in flat-bottomed tissue culture plates (Microtest II, Falcon no. 3042) with an appropriate concentration of PPD, TAP, or lectins. The culture medium used was RPMI 1640 supplemented with 10% pooled human AB serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cultures were performed for various times in humidified 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, using a semiautomated microharvester (Laboscience Co. Ltd., Tokyo, Japan), and the radioactivity was counted in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). Results with cord blood samples whose background counts per minutes without PPD or lectin stimulation were as high as 5,000 were excluded from the data. Results were expressed as mean counts per minute of triplicate determinations, Δ counts per minute = counts in culture without stimulant subtracted from counts in culture with stimulant, or as a stimulation index as follows: counts per minute in culture with stimulant/counts in culture without stimulant.

BUdR and light treatment. To eliminate PPD-reactive lymphocytes, a negative selection procedure with 5-bromo-2-deoxyuridine (BUdR; P-L Biochemicals, Inc., Milwaukee, Wis.) and light treatment was used. A total of 2×10^6 CBL in 1 ml of culture medium were placed in a polystyrene culture tube (Falcon no. 2054, 12 by 75 mm) and cultured in the simultaneous presence of PPD (100 μg/ml) and BUdR (5 μg/ml) at 37°C in 5% CO_2 in air. On day 4 of culture, cells were illuminated for 90 min by fluorescent light to eliminate the PPD-induced, deoxyribonucleic acid-synthesizing cells during the culture period.

RESULTS

PPD-induced proliferative response of CBL. Time course studies of the PPD-induced in vitro proliferative response of CBL and PBL from tuberculin skin test-positive individuals showed that the maximum proliferative response of CBL after stimulation with PPD (100 μg/ml) was observed on day 5 or 6, which was parallel with PPD-induced proliferation of PBL from tuberculin-positive donors, although the peak response of CBL was lower than that of PBL. PPD doses as high as 200 μg/ml resulted in a maximum proliferative response of CBL assayed on day 6 of culture, whereas PBL from tuberculin reactors showed the highest response with PPD doses of 50 μg/ml. The same doses of TAP (200 μg/ml) were required for CBL to give the maximum response. TAP was prepared from tubercle bacilli known to have the same antigenicity as PPD. PBL from tuberculin skin test-negative donors showed no significant response regardless of the concentration of PPD or TAP used.

PPD- or TAP-induced [^{3}H]thymidine incorporation by CBL or by PBL from tuberculin skin test-positive and -negative individuals at 6 days of culture is shown in Fig. 1. The highest mean response was observed in PBL from tuberculin-positive individuals stimulated by either PPD or TAP. Counts per minute of $>3 \times 10^4$ were observed in nearly half of the PBL from tuberculin-positive donors. Two thirds of the CBL subjects tested responded significantly to PPD or TAP ($>10^4$ cpm), in contrast to the reactions of most of the PBL from tuberculin nonreactors ($<10^3$ cpm). The mean background counts without stimulant were 2,545 ± 1,749 in CBL, 1,951 ± 1,120 in tuberculin-positive PBL, and 1,050 ± 865 in tuberculin-negative PBL. Age distributions of PBL donors were given in Materials and Methods. These results show that CBL certainly reacted to PPD or TAP, although the strength of CBL reactivity was lower than that of PBL from tuberculin-positive donors.

Proliferative response of CBL to mitogenic stimulation. Table 1 shows the response of CBL to nonspecific mitogen stimulation. PPD high- and low-responding CBL were compared. Lymphocytes were cultured for 3 days with optimal concentrations of mitogens. No significant differences were observed between the PPD high- and low-responder CBL (Table 1).

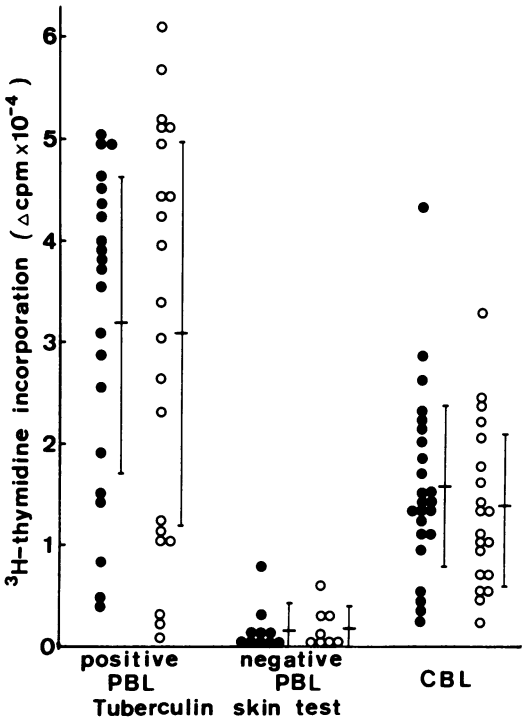

FIG. 1. *PPD- or TAP-induced proliferative response of CBL and PBL from tuberculin skin test-positive or -negative donors. Lymphocytes were cultured for 6 days with optimal doses of PPD (●) or TAP (○), and [^{3}H]thymidine incorporated during the last 18 h was counted and presented as Δ counts per minute. The mean background counts were 2,545 ± 1,749 in CBL, 1,951 ± 1,120 in tuberculin-positive PBL, and 1,050 ± 865 in negative PBL.*

PPD-induced proliferative response by fractionated lymphocytes. In humans, the PPD-induced proliferative response has been reported to be due to the reactions of tuberculin-sensitized T lymphocytes to PPD (12, 13), and nonspecific B-cell mitogenicity has not been reported so far. To determine which cell population was proliferating after stimulation with PPD in CBL, experiments were performed with fractionated lymphocytes. E-rosette-enriched, nylon wool-nonadherent cells (T-lymphocyte fraction) and E-rosette-depleted cells (B-lymphocyte fraction) were obtained from CBL by the method described in Materials and Methods. A total of 2×10^5 T lymphocytes were cultured together with various numbers of B lymphocytes in the presence of PPD (50 μg/ml). Treatment of lymphocytes with mitomycin C (40 μg/ml) abolished their ability to proliferate. Figure 2a shows that PPD-induced proliferating cells were E-rosetting T lymphocytes and that non-E-rosetting cells (B lymphocytes) were required for T lymphocytes to respond to PPD stimulation.

Next, 2×10^5 B lymphocytes were cultured with various numbers of T lymphocytes. The reactivity increased as the number of T lymphocytes increased (Fig. 2b). The T- or B-lymphocyte fraction alone did not proliferate at all after stimulation with PPD. The lack of responsiveness of isolated B lymphocytes to PPD stimulation could be due to the absence of helper T cells known to be required for B-cell mitogenesis (11). However, addition of mitomycin C-treated T lymphocytes did not have an augmenting effect on the B-lymphocyte response to PPD (Fig. 2a and b).

To examine the role of non-E-rosetting cells in the PPD-induced proliferative response of T lymphocytes, adherent cells were obtained by the method described in Materials and Methods. Cord blood T lymphocytes were stimulated with PPD or TAP in the presence of adherent cells (Table 2). T lymphocytes alone did not respond at all to stimulation with either PPD or TAP. The response of T lymphocytes was restored by the addition of adherent cells (T lymphocyte/adherent cell raio, 10:1). These results showed that T lymphocytes were responsible for the PPD-induced proliferative response observed in CBL and that adherent cells were essential for T lymphocytes to respond to PPD.

Negative selection of PPD-reactive lymphocytes in CBL. The negative selection procedure with BUdR and light treatment was used to determine whether the CBL response to PPD stimulation represented antigenic or nonspecific mitogenic reactivity. Depletion of PPD-reactive lymphocytes was carried out by preculturing CBL for 4 days with PPD in the presence of BUdR. On day 4 of culture, cultured lymphocytes were illuminated for 90 min with a fluorescent light, washed, and resuspended in fresh medium. Samples of cells were again stimulated in vitro for as long as 6 days with PPD or 3 days with concanavalin A, and the proliferative response was assessed by measuring the amount of [^{3}H]thymidine radioactivity incorporated during the last 18 h. The results obtained from the 6-day culture with PPD are shown in Table 3, and the data are expressed as a stimulation index. Lymphocytes precultured with PPD in the presence of BUdR followed by light treatment lost reactivity to a second PPD stimulation. Lymphocytes treated with PPD only gave seemingly no response when the result was expressed as a stimulation index (Table 3). They actually incorporated considerable amounts of [^{3}H]thymidine without addition of PPD in the second culture, however, probably because they

TABLE 1. *Proliferative response of CBL*[a]

Donors	Δcpm (stimulation index)			
	3-day culture		6-day culture	
	PWM	ConA	PPD	TAP
PPD high responders				
1	51,655 (19.82)	53,142 (20.26)	43,632 (16.38)	
2	38,517 (34.82)	20,355 (18.87)	9,504 (10.66)	3,953 (5.02)
3	27,473 (12.91)	11,674 (6.06)	11,358 (3.98)	14,887 (4.91)
4	36,047 (36.37)	11,378 (12.17)	16,932 (11.59)	12,256 (8.66)
5	21,468 (6.43)	15,604 (4.95)	23,338 (17.05)	11,310 (8.78)
Mean	35,032 (22.07)	22,430 (12.46)	20,965 (11.93)	10,601 (6.84)
PPD low responders				
6	21,832 (7.04)	3.030 (1.84)	4,002 (2.84)	(0.89)
7	25,120 (4.86)	15,214 (3.34)	3,053 (2.44)	382 (1.18)
8	25,895 (7.16)	16,054 (4.82)	2,265 (1.82)	1,496 (1.54)
9	38,000 (15.52)	50,801 (20.41)	1,078 (1.50)	800 (1.37)
Mean	27,711 (8.64)	21,274 (7.60)	2,599 (2.15)	669 (1.24)

[a] CBL (2×10^6) were cultured in vitro for 3 days with pokeweed mitogen (PWM) (diluted ×400) or concanavalin A (ConA) (10 μg/ml) or for 6 days with PPD (100 μg/ml) or TAP (200 μg/ml). [^{3}H]thymidine incorporation was expressed as Δ counts per minute or stimulation index, which is given in parentheses (see text).

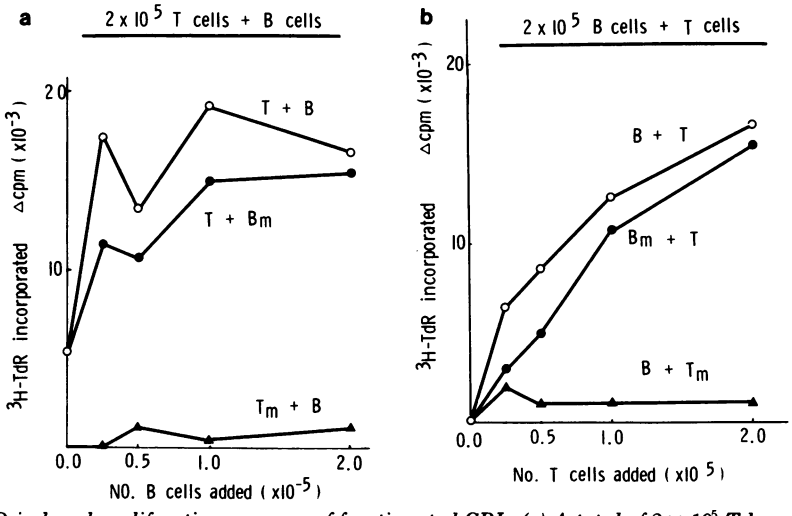

FIG. 2. *PPD-induced proliferative response of fractionated CBL. (a) A total of 2×10^5 T lymphocytes were cocultured with various numbers of B lymphocytes (○) or mitomycin C-treated B lymphocytes (B_m) (●) for 6 days in the presence of PPD (50 μg/ml). Mitomycin C-treated T lymphocytes (T_m) were also cultured with B lymphocytes (▲). (b) A total of 2×10^5 B lymphocytes were cultured with various numbers of T lymphocytes (○) or mitomycin C-treated T lymphocytes (▲) for 6 days in the presence of PPD (50 μg/ml). Mitomycin C-treated B lymphocytes were also cultured with T lymphocytes (●).*

were already stimulated in the preculture with PPD, and they showed a secondary immune response in the second culture to the small amount of PPD which was transferred from the first culture. The reactivity of lymphocytes to concanavalin A stimulation, whether treated with BUdR or not, remained intact, although the responsiveness was slightly decreased by treatment with BUdR. This result argues for the existence of specifically PPD-reactive lymphocytes in CBL.

DISCUSSION

In our study we have demonstrated that T lymphocytes do exist in cord blood, which proliferated in vitro after stimulation with PPD or

TABLE 2. *PPD- or TAP-induced proliferative response of fractionated CBL*[a]

Cell population	PPD		TAP	
	SI	Δcpm	SI	Δcpm
Unfractionated	1.53	7,642	2.58	13,318
T lymphocytes alone	0.54		0.96	
T lymphocytes + adherent cells	2.38	17,130	2.28	10,204
Adherent cells alone	0.68			

[a] Purified T lymphocytes (2×10^6/ml) were stimulated in vitro with an optimal concentration (200 μg/ml) of PPD or TAP in the presence or absence of 2×10^5 adherent cells per ml. As controls, unfractionated or adherent cells (2×10^6/ml) were cultured with PPD or TAP alone. Cells were cultured for 6 days. Results are expressed as a stimulation index (SI) or as Δ counts per minute incorporated during the last 18 h of culture.

TABLE 3. *Negative selection of PPD reactivity*[a]

First culture		[^{3}H]Thymidine incorporation (stimulation index)			
BudR	PPD	Expt 1	Expt 2	Expt 3	Expt 4
−	−	2.82	2.58	5.92	3.10
−	+	1.84	1.23	3.77	1.11
+	−	4.10	4.89	5.93	3.63
+	+	1.52	1.49	1.96	0.79

[a] CBL were cultured in vitro in the presence (+) or absence (−) of BUdR (5 μg/ml) or PPD (100 μg/ml) (first culture) for 4 days, On day 4, cultures were illuminated for 90 min with a fluorescent light. Cells were restimulated with PPD (100 μg/ml) for 6 days in vitro (second culture).

TAP in the presence of adherent cells. Although the optimum dose of PPD required for CBL to show the maximum response was higher than that required for PBL from tuberculin skin test-positive donors, the culture period required to reach the maximum response was the same in CBL and PBL, 5 to 6 days. The antigenic specificity of the reaction was shown by the fact that CBL responded to TAP as well as to PPD. TAP, a peptide prepared from tubercle bacilli, is known to have the same antigenicity as PPD but does not have mitogenicity. Furthermore, the responsiveness of CBL to PPD in vitro was specifically eliminated by preculture with PPD and BUdR treatment.

PPD has been known as a B-cell mitogen in mice (1, 23) and guinea pigs (14) and also as a polyclonal B-cell activator producing immunoglobulins in mice (1) and humans (6, 8, 19). No evidence has been reported so far about B-cell mitogens of PPD in humans. Our results with fractionated lymphocytes showed that the PPD-induced proliferative response in CBL was elicited by E-rosetting T lymphocytes and not by non-E-rosetting B lymphocytes. TAP is known to have the same antigenicity as PPD (2, 27). We examined the mitogenic activity of TAP in mice. No proliferative response was noted when normal mouse spleen cells were cultured together with TAP, in contrast to PPD, which showed strong mitogenic activity with mouse spleen cells (data not shown). However, both TAP and PPD stimulated a proliferative response of PBL from tuberculin skin test-positive donors, but not from negative donors. CBL showed similar responsiveness to TAP and PPD. Fractionated non-E-rosetting cells (B-cell fraction) alone did not respond to either PPD or TAP. These observations favor the proposal that CBL proliferated by antigenic PPD stimulation and not by PPD nonspecific mitogenic stimulation. The mitogenic activity of PPD in CBL proliferation is also excluded by the finding that CBL, whether responding to PPD well or poorly, responded equally to nonspecific stimulation with concanavalin A or pokeweed mitogen. We also observed that PPD-nonresponder PBL proliferated well after concanavalin A stimulation.

One plausible explanation of the PPD-induced proliferative response of CBL in vitro is that lymphocytes were primed in vitro and proliferated after stimulation with PPD, as has been reported by Ellner et al. (5). Experiments on in vitro sensitization of human PBL to soluble protein antigens have been reported previously (10, 17, 20). According to these reports, optimum priming conditions required at least 6 or 7 days of in vitro culture. Our time course study revealed that the maximum response occurred on day 5 to 6 of cultivation, which was comparable to the PPD-induced proliferation curve of PBL obtained from tuberculosis patients. The delay in a peak response of CBL in kinetics, as reported by Ellner et al. (5), was not observed in our study. Another argument against in vitro priming is that TAP, which is known to lack immunogenicity (28), was able to generate proliferation of CBL in vitro, whereas PBL from tuberculin skin test-negative donors did not respond to stimulation with either PPD or TAP.

Another possibility which cannot completely be excluded is that PPD or TAP is functioning as a nonspecific mitogen on cord blood T lymphocytes; no findings have been reported so far on PPD as a T-cell mitogen in humans and in other animals.

Protective immunity against microbial organisms is mediated by both humoral and cellular immunity. During the newborn period in humans, immunoglobulin G antibodies which were passively transferred into the fetus from the mother through the placenta played an impor-

tant role in protective immunity. Our observation that PPD-reactive T lymphocytes did exist in cord blood cells strongly suggests that cell-mediated immune mechanisms are also operating in the antimicrobial protection during that period.

Generally, a correlation is found between in vitro lymphocyte transformation and in vivo delayed-type skin reaction to tuberculin PPD (4, 12, 13, 19). In newborn babies whose cord blood cells were studied, skin tests were not performed, although all of the mothers gave positive delayed-type skin reactions with ordinary doses of tuberculin PPD. It remains to be elucidated whether PPD reactivity of CBL was passively transferred from the mother directly, although no evidence of passive transfer of lymphocytes from the mother to the fetus via the placenta has been reported. A soluble factor might be transferred from the tuberculin-positive mother into the fetus, providing the fetal lymphocytes with reactivity to tuberculin. Studies with CBL from babies born to tuberculin-negative mothers will aid in elucidating this question, although Japanese are usually vaccinated with BCG and therefore are tuberculin positive. Our preliminary study showed that no correlation exists between the reactivity of PPD-induced proliferation of PBL from mothers and that of CBL from their babies. PBL in infants which showed distinct proliferative responses to PPD at birth might lose their PPD reactivity soon after birth. As stated in Materials and Methods, children as young as 3 months of age, unless vaccinated with BCG, were PPD nonresponders.

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