Transfer of Tuberculin Immunity from Mother to Infant

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ABSTRACT. The purpose of our study was to investigate transfer of tuberculin immunity from mother to infant via breast milk by studying newborn lymphocyte blastogenesis induced by purified protein derivative antigen at 1–5 days of age, 4–6 wk of age, and 3 months of age. Our study consisted of four mother-infant groups: breast-feeding and bottle-feeding infants of tuberculin-positive and tuberculin-negative mothers. A difference in the groups was found only at 4–6 wk of age where 17% (4/23) of breast-feeding infants and 13% (2/15) of bottle-feeding infants of tuberculin positive mothers had lymphocyte blastogenesis to purified protein derivative. None of the infants of tuberculin negative mothers had purified protein derivative-induced blastogenesis. Analysis of covariance with tests for equality of slopes showed that the responses of tuberculin-positive mothers were significantly different from the responses of tuberculin-negative mothers ($p < 0.05$). These studies suggest transplacental transfer of tuberculin immunity evident at 4–6 wk of age which wanes by 3 months of age. We could not find evidence of transfer via human milk. (*Pediatr Res* 22: 277–281, 1987)

Abbreviations

PPD, purified protein derivative (Connaught)
HBSS, Hanks' balanced salt solution
PHA, phytohemagglutinin
SI, stimulation index
cpm, counts per minute
HI, heat inactivated

Previous investigators have suggested that transfer of tuberculin immunity may occur via human breast milk (1–3). Others have found evidence for transplacental transfer of tuberculin immunity (4), measles immunity (5) and cellular immunity to other infectious antigens (4).

Mohr's (1) study was based on tuberculin skin tests performed in 11 children of five tuberculin-positive mothers years after birth. Five of the nine breast-feeding infants of tuberculin-positive mothers had positive tuberculin skin tests. The two infants that were not breast-fed had negative skin tests. As was suggested by the author, these preliminary results did not exclude transplacental transfer of tuberculin immunity. Environmental exposure can also not be excluded. Studies have examined *in vitro* lymphocyte blastogenesis to PPD by studying lymphocytes obtained from cord blood or neonatal blood. Both Schlessinger and Cov-

elli's (3) study and the study of Ogra *et al.* (2) found no evidence of cord blood lymphocyte response to PPD antigen but did find PPD-induced blastogenesis responses for lymphocytes obtained from infants 4–5 wk of age. Since maternal PPD-induced lymphocyte blastogenesis has been found to be depressed in the last 4 wk of pregnancy and at the time of delivery (6), it is possible that cord blood lymphocytes may not respond optimally in a blastogenesis assay. For this reason we chose to examine neonatal lymphocyte response instead of cord blood lymphocyte response to PPD.

The purpose of our study was to investigate transfer of tuberculin immunity via human breast milk by studying newborn lymphocyte blastogenesis induced by PPD antigen at 1–5 days of age, 4–6 wk of age, and 3 months of age. In addition to blastogenesis we wished to study functional capability transfer from the mother to the infant as evidenced by lymphokine production. For this purpose, PPD-induced production of monocyte chemotactic factor was examined for neonatal lymphocytes obtained at 4–6 wk of age. We chose this time for lymphokine studies since previous studies had shown transfer of a blastogenesis capability evident at 4–5 wk of age.

We also wished to examine the mechanism of transfer of this cellular immunity. Transfer of lymphocytes from the mother to the infant either via the placenta or human milk must be considered. We studied male infants of tuberculin positive breast-feeding mothers at 4–6 wk of age. The sex of the dividing lymphocytes responding to PPD stimulation was examined.

METHODS

Study population. Healthy, postpartum adult females with known tuberculin status participated in the study with their healthy infants. Mothers were classified as tuberculin-positive or tuberculin-negative on the basis of skin test results shortly after delivery, during pregnancy, or prior to pregnancy. Repeat skin testing was not done for tuberculin-positive mothers. For this study, a subject with ≥ 12 mm of induration at the skin test site after 48–72 h was defined as tuberculin positive. We purposely used such a large amount of induration to eliminate borderline readings. Tuberculin-negative subjects had no induration at the skin test site. Tuberculin-negative mothers were skin tested at time of entry into the study (1–5 days postpartum) and again at the first return visit 4–6 wk postpartum. All tuberculin negative mothers without confirmatory negative skin testing at 4–6 wk postpartum were excluded from the study since pregnancy and delivery have been associated with decreased tuberculin immunity (6). In addition, all tuberculin-negative mothers with a history of BCG immunization were excluded from the study.

Our study consisted of four mother-infant groups: infants of tuberculin-positive breast-feeding mothers, infants of tuberculin-positive bottle-feeding mothers, infants of tuberculin-negative breast-feeding mothers, and infants of tuberculin-negative bottle-

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feeding mothers. Mothers and infants were excluded if the infant had any serious illness in the first 3 months of life. Breast-feeding infants received at least 80% of their feeding as human breast milk. Bottle-feeding infants never received any human breast milk.

Informed consent was obtained from all mothers and this study was approved by the human subjects committee.

Lymphocyte studies. Infant lymphocytes from peripheral blood were examined at 1–5 days of age, 4–6 wk of age, and 3 months of age. The mean ages for the four groups (PPD + breast-feeding, PPD + bottle-feeding, PPD – breast-feeding, PPD – bottle-feeding) were quite similar. At 1–5 days, the mean ages ($\pm$ SE) were 1.5 ± 0.1 , 1.3 ± 0.1 , 1.9 ± 0.5 , 1.6 ± 0.2 days. At 4–6 wk the mean ages were 31.2 ± 0.8 , 31.5 ± 1.0 , 32.6 ± 1.6 , 31.9 ± 1.5 days, and at 3 months the mean ages were 86.9 ± 4.6 , 90.1 ± 4.1 , 91.2 ± 2.7 , 83.0 ± 2.5 days. Peripheral blood and colostrum/milk specimens were obtained from mothers at 1–5 days postpartum and 4–6 wk postpartum.

Mononuclear cell isolation for lymphocyte cultures. Peripheral whole blood was diluted in HBSS, Ca⁺⁺, Mg⁺⁺ free, and the mononuclear cell fraction separated by Ficoll-Hypaque differential centrifugation (7). After washing, mononuclear cells were resuspended for counting in RPMI-1640, containing gentamicin (10 μ g/ml) and an antibiotic-antimycotic preparation (Gibco) and additional L-glutamine. Cell viability was determined using trypan blue exclusion and the differential calculated by staining cells with 0.025% methyl violet in 0.5% acetic acid. Breast milk cells were first centrifuged at 1400 RPM ($250 \times g$) to remove the fatty top layer and washed twice in HBSS to eliminate additional fat. Lymphocytes were then harvested in the same manner as peripheral blood cells and counted using toluidine blue stain in a wet-preparation differential. When cell yield did not permit Ficoll-Hypaque separation, total washed milk cells were used.

Lymphocyte stimulation by PPD antigen. PPD, 2 mg/ml concentrate was diluted with HBSS containing 0.1% fetal bovine serum (heat inactivated) to achieve the following final concentrations for lymphocyte cultures: 200, 100, 50, 25, 10, 5, 2.5, 0.5, 0.1, 0.05, μ g/ml PPD. Fifty μ l of appropriate antigen dose was added to triplicate wells of a 96-well, U-bottom microtiter plate, (Linbro). In addition, a dose of 0.83 μ g/ml final concentration of PHA (Burroughs Wellcome) was also assayed as a control for culture viability. Lymphocytes were resuspended in RPMI-1640 containing 5% heat inactivated human AB sera for a final concentration of 1×10^6 lymphocytes/ml. 1×10^5 lymphocytes were added to each well stimulated with antigen as well as nonstimulated control wells. Microtiter plate cultures were placed at 37° C, 5% CO₂ for 7 days. Additional lymphocyte cultures were also included for incubation periods of 3, 5, and 6 days when cell yield permitted. After incubation, cells were radiolabeled with ³H-thymidine (Amersham) and 0.4 μ Ci were added to each well. Twenty-four h later, the cells were harvested onto glass fiber filter discs and upon addition of scintillant cocktail, counted in a liquid scintillation counter. SI was calculated by dividing the cpm from stimulated wells by the cpm from nonstimulated wells. The stimulated cpm minus the nonstimulated cpm was also calculated as Δ cpm. Maximum responses of infant lymphocytes at any dose at any time of culture harvest were used for data analysis.

Generation of lymphocyte-derived monocyte chemotactic factor. Lymphocyte cultures (1×10^6 lymphocytes/ml) were incubated at 37° C with 5% CO₂ for 72 h in serum-free RPMI-1640 with additional L-glutamine. Stimulated cultures were incubated with 10 μ g/ml PPD antigen. Nonstimulated control cell cultures were incubated without antigen which was added at the end of the incubation period. Supernatants were harvested by centrifugation and frozen at –70° C until assay.

Monocyte chemotaxis assay. Test supernatants from PPD stimulated and nonstimulated cultures were diluted 1:2 and 1:3 in RPMI-1640 and 5% fetal bovine serum (HI) for assay. Each

assay included a lymphocyte culture supernatant from a tuberculin positive adult control. Monocyte chemotaxis was assayed using the method of Altman *et al.* (8) and Snyderman *et al.* (9). Blind well chambers (Bio-Rad Laboratories) were used with upper and lower compartments separated by a 5.0 μ m Nucleopore filter. Human peripheral blood monocytes from adult laboratory volunteers were used as test monocytes and were prepared by Ficoll-Hypaque differential centrifugation. The wet-preparation differential was done using 0.025% methyl violet, and the mononuclear cells were suspended at a concentration of 1.5×10^6 monocytes/ml in RPMI-1640 with 5% fetal calf serum. For each chamber and filter, 0.2 ml of monocyte resuspension, containing 1.5×10^6 monocytes/ml, was added to the top of the filter. Appropriate dilutions of test supernatants were placed in the lower chambers (0.2 ml). The chemotaxis chambers were then incubated for 90 minutes at 37° C with 5% CO₂. To measure chemotactic activity, the filters were removed at the end of the incubation period, fixed in ethanol, and stained in hematoxylin and Wright's stain. Under oil immersion, the average number of monocytes per field migrating to the opposite side of the filter was determined by counting 20 fields for each filter. Each supernatant dilution was assayed in triplicate. Chemotactic activity was calculated by comparing the monocyte migration in nonstimulated supernatants to those stimulated by PPD. The number of monocytes migrating toward stimulated supernatants minus the number of monocytes migrating toward control supernatants was used as Δ migrating monocytes.

Statistical methods. To investigate the effect of breast-feeding status on lymphocyte blastogenesis, several approaches were used. In one approach infants were classified as lymphocyte blastogenesis responders if they satisfied a definition based on cutpoints for SI and Δ cpm and on comparison of stimulated and nonstimulated counts. A positive monocyte response was defined by a cutpoint for Δ monocytes. To determine the cutpoints for SI, Δ cpm and Δ monocytes, we determined with 99% confidence tolerance limits between which will lie 99% of infant responses from tuberculin-negative mothers (10). The upper tolerance limit was then used as a cutpoint to define positive and negative responses. These tolerance limit cutpoints varied for the three time periods at which infants were studied: Δ cpm $\geq$ 6325, SI $\geq$ 3.6 at entry; Δ cpm $\geq$ 3613 SI $\geq$ 3.1 at 4–6 wk of age, Δ cpm $\geq$ 3261, SI $\geq$ 2.9 at 3 months of age. An infant was considered to have a positive lymphocyte blastogenic response if tolerance limit criteria were met for either SI or Δ cpm. Also, the cpm in stimulated wells had to be statistically significantly different from the counts per minute in control wells using Student's *t* test on logarithmically transformed data (11). Tolerance limit criteria were also used to analyze Δ monocytes for monocyte chemotactic factor and a Δ monocytes $\geq$ 18.9 was considered to be a positive response. In both blastogenesis and monocyte chemotaxis assays, Fisher's exact test (12) was used to compare breast and bottle feeders for percent of positive responders. This was done for infants of PPD-positive mothers separately from infants of PPD-negative mothers to determine effect of feeding method on results. In addition, infants of tuberculin-positive breast-feeding mothers were compared to infants of tuberculin-negative breast-feeding mothers. Similar comparisons were performed on bottle-feeding infants.

In another statistical approach, SI and Δ cpm data were logarithm transformed and a two-way analysis of variance (13) was used to determine if there were significant effects of feeding, tuberculin status, or interaction of these on blastogenesis data at each time period. When the analysis of variance indicated a significant effect of tuberculin status or feeding, the Bonferroni approach to controlling the overall level of significance at 0.05 was used to do six pairwise comparisons (each at α of 0.008).

Another statistical approach consisted of two-way analysis of covariance on stimulated counts with nonstimulated counts as a covariate (13). Correlation analysis was used to investigate the linear relation between stimulated and nonstimulated counts.

Chromosome analysis. Lymphocytes from male infants (4–6 wk of age) were cultured with PPD or without antigen for 7 days. Colcemid treatment was used to arrest cells in metaphase and standard air-dried chromosome preparations were made. Chromosome analyses, primarily for sex chromosome constitutions were performed with the aid of Q-banding.

RESULTS

Lymphocyte blastogenesis. Table 1 summarizes the data in terms of infants meeting tolerance limit criteria for SI and Δ cpm in addition to having cpm statistically significantly ($p < 0.05$) different in stimulated and nonstimulated wells. At 4–6 wk of age, 17% of infants of tuberculin-positive breast-feeding mothers and 13% of infants of tuberculin-positive bottle-feeding mothers had lymphocyte blastogenesis to PPD. One of the breast-feeding infants of a tuberculin-positive mother with a positive response at 4–6 wk of age had also had a positive blastogenic response at age 1–5 days. One of the bottle-feeding infants of a tuberculin-positive mother with a positive response at 4–6 wk of age also continued to have a positive response at 3 months of age. When Fisher's exact test (12) was used to compare the number of responders in each of the four groups at each of the times studied, there were no statistically significant differences. However, if all infants of tuberculin-positive mothers were compared to all infants of tuberculin-negative mothers at 4–6 wk regardless of feeding method, the number of responders 6/38 (16%) *versus* 0/33 (0%) was statistically significantly different ($p < 0.05$). Table 2 presents the mean stimulation index and mean Δ cpm for each of the four groups at each of the study times.

For analysis, SI and Δ cpm data were transformed logarithmically so as to satisfy better the assumption of equal variances in the four groups. A two-way analysis of variance showed that at 4–6 wk of age there was a significant effect of both mother's PPD status and feeding method on SI and Δ cpm data. The interaction of PPD status and feeding method was not significant. Use of the Bonferroni pairwise test showed that this difference was between the infants of PPD-positive breast-feeding mothers and infants of PPD-negative bottle-feeding mothers. These results were not helpful in determining the influence of breast-feeding *versus* bottle-feeding on transferred immunity. Analysis of variance revealed no effect of feeding or PPD status on SI or Δ cpm at 1–5 days of age. At 3 months of age, there was no significant effect of PPD status or feeding on Δ cpm, but there was a significant interaction for the SI data. Thus, one cannot make any conclusion about effect of feeding or PPD on SI. The

Table 1. Infants with positive lymphocyte blastogenesis to PPD

Age 1–5 days (Definition of responder: SI ≥ 3.6 , or Δ cpm ≥ 6325 , $p \leq 0.05$)			
Infants of PPD-positive mothers		Infants of PPD-negative mothers	
Breast-feeding	Bottle-feeding	Breast-feeding	Bottle-feeding
2/33 (6%)	0/26 (0%)	1/24 (4%)	0/14 (0%)
Age 4–6 wk (Definition of responder: SI ≥ 3.1 , or Δ cpm ≥ 3613 , $p \leq 0.05$)			
Infants of PPD-positive mothers		Infants of PPD-negative mothers	
Breast-feeding	Bottle-feeding	Breast-feeding	Bottle-feeding
4/23 (17%)	2/15 (13%)	0/19 (0%)	0/14 (0%)
Age 3 mo (Definition of responder: SI ≥ 2.9 , or Δ cpm ≥ 3261 , $p \leq 0.05$)			
Infants of PPD-positive mothers		Infants of PPD-negative mothers	
Breast-feeding	Bottle-feeding	Breast-feeding	Bottle-feeding
0/12 (0%)	1/9 (11%)	0/14 (0%)	0/9 (0%)

Table 2. Infant PPD-induced lymphocyte blastogenesis

Age	Mean stimulation index ($\pm$ SE)			
	PPD+ mother breast-feeding	PPD+ mother bottle-feeding	PPD- mother breast-feeding	PPD- mother bottle-feeding
1–5 days	1.9 $\pm$ 0.2 ($n = 33$)	1.5 $\pm$ 0.1 ($n = 26$)	1.5 $\pm$ 0.1 ($n = 24$)	1.7 $\pm$ 0.2 ($n = 14$)
4–6 wk	1.9 $\pm$ 0.1 ($n = 23$)	1.6 $\pm$ 0.1 ($n = 15$)	1.6 $\pm$ 0.1 ($n = 19$)	1.3 $\pm$ 0.1 ($n = 14$)
3 mo	1.6 $\pm$ 0.1 ($n = 12$)	1.9 $\pm$ 0.2 ($n = 9$)	1.5 $\pm$ 0.1 ($n = 14$)	1.2 $\pm$ 0.1 ($n = 9$)
Age	Mean Δ cpm ($\pm$ SE)			
	PPD+ mother breast-feeding	PPD+ mother bottle-feeding	PPD- mother breast-feeding	PPD- mother bottle-feeding
1–5 days	1293 $\pm$ 361 ($n = 33$)	707 $\pm$ 191 ($n = 26$)	1212 $\pm$ 367 ($n = 24$)	717 $\pm$ 176 ($n = 14$)
4–6 wk	2380 $\pm$ 692 ($n = 23$)	1377 $\pm$ 505 ($n = 15$)	916 $\pm$ 196 ($n = 19$)	221 $\pm$ 155 ($n = 14$)
3 mo	660 $\pm$ 433 ($n = 12$)	591 $\pm$ 252 ($n = 9$)	721 $\pm$ 210 ($n = 14$)	14 $\pm$ 51 ($n = 9$)

correlation analysis showed a strong dependence of stimulated counts on nonstimulated counts. The linear regression analysis for stimulated cpm *versus* nonstimulated cpm for all the different groups studied showed slopes ranging from 0.81 to 2.20. The Pearson correlation coefficient (14) was always > 0.84 with $p < 0.0005$.

Analysis of covariance (14) was performed on the stimulated cpm (as the dependent variable) with the nonstimulated cpm as a covariate. The test for equality of slopes of the regression of stimulated cpm on nonstimulated cpm at both 1–5 days and 3 months of age showed no significant difference in the slopes for any of the feeding or PPD groups. Analysis of covariance showed no effect of feeding or PPD group in the blastogenesis results at 1–5 days or 3 months of age.

The data at 4–6 wk of age are strikingly different. When the assumption of equality of slopes in the four groups was tested for the analysis of covariance, the hypothesis of equal slopes was rejected ($p < 0.05$). These results are summarized graphically in Figure 1. The slopes (stimulated *versus* nonstimulated cpm) for PPD positive breast-feeding infants *versus* PPD-positive bottle-feeding infants did not differ. Similarly, the slopes for PPD-negative breast-feeding and PPD-negative bottle-feeding infants did not differ. However, each positive group differed from each negative group ($p < 0.0005$). The test for equality of slopes suggests strongly that the transfer of tuberculin immunity from mother to infant evident at 4–6 wk of age is transplacental, not via human breast milk.

Maternal lymphocyte blastogenesis. Mothers were classified as tuberculin positive or negative based on skin test, but blastogenesis data correlated well with skin test results. Linear regression analysis was performed to determine if there was any correlation between baby's SI or Δ cpm and mother's SI or Δ cpm. The strongest correlation was for the PPD + bottle-feeding group. The correlation coefficient for SI (baby at 4–6 wk *versus* SI (mother 4–6 wk postpartum) was 0.82 ($p < 0.005$). The corresponding correlation coefficient for PPD + breast-feeding mothers was only $r = 0.03$.

Milk data. Analysis of covariance was performed on milk stimulated cpm with nonstimulated cpm as a covariate. There was no effect of maternal PPD status on the human milk PPD-induced blastogenesis probably due to the wide variability in the cpm. The mean ($\pm$ SE) SI for milk lymphocytes from PPD + mothers was 2.7 (± 1.1). The mean ($\pm$ SE) for PPD-mothers was 1.8 (± 0.4).

Monocyte chemotactic factor. A total of fourteen assays were performed on supernatants from PPD-stimulated lymphocyte cultures from infants 4–6 wk of age. In each assay specimens

Fig. 1. Cpm for PPD-stimulated cultures versus cpm for control cultures for each of four groups studied at 4-6 wk of age.

from infants of both tuberculin-negative and tuberculin-positive mothers were assayed to control for variability of the assay. Δ monocytes for specimens obtained from infants of tuberculin-negative mothers was used to determine with 99% confidence the tolerance limit below which 99% of negative values (Δ monocytes) should fall. This tolerance limit cutpoint (Δ monocytes ≥ 18.9) was used to define a positive response.

Two of 17 (12%) infants of breast-feeding tuberculin-positive mothers had positive results for PPD-induced lymphokine production while one of 11 (9%) infants of tuberculin-positive bottle feeding infants were also positive. None of the 14 infants of tuberculin-negative breast-feeding or three infants of tuberculin-negative bottle-feeding mothers had evidence of lymphokine production. These results might also suggest transplacental transfer of tuberculin immunity since the PPD + breast-feeding and PPD + bottle-feeding infant groups are not significantly different (12 versus 9%), but the response rate of the tuberculin-positive groups are not significantly different from the tuberculin-negative control groups.

Of the three infants with lymphokine production, none had been defined as a responder in the blastogenesis assay performed at the same time. There did not appear to be much correlation between the two assays although the results are similar in suggesting transplacental transfer.

Chromosome data. Two male infants with PPD-induced lymphocyte blastogenesis had metaphase spreads analyzed for the sex of the dividing cells. All 179 and 132 metaphase spreads examined from these infants were male (XY) suggesting that transferred maternal cells are not responsible for the blastogenesis.

DISCUSSION

The main purpose of our study was to determine if tuberculin immunity can be transferred from the mother to the infant via human breast milk. If we could demonstrate an effect of mother's tuberculin status on tuberculin immunity in the infant and no

effect of feeding method, we would conclude that transfer occurred via the placenta. If both mother's tuberculin status and method of feeding influenced results, transplacental transfer and transfer via milk must be considered.

We first analyzed the lymphocyte blastogenesis data using the traditional SI and Δ cpm. We used strict tolerance limit criteria to define a positive response in both blastogenesis and monocyte chemotaxis assays and analyzed the number of positive responders in each group using Fisher's exact test. This analysis allowed no firm conclusion about transplacental versus milk transfer and only suggested transplacental transfer.

We then analyzed the data using the traditional analysis of variance with SI or Δ cpm as the variable studied. At 4-6 wk there was evidence of a significant effect of PPD status and feeding group on the lymphocyte blastogenesis. Unfortunately, the difference was between the infants of PPD-positive breast-feeding mothers and the bottle-feeding infants of PPD-negative mothers. This result was not helpful in dissecting transplacental versus milk transfer of immunity. Again, we were left with no clear answer. Since our correlation analysis showed a strong dependence of stimulated cpm on nonstimulated cpm, we used another statistical approach, the two-way analysis of covariance on stimulated counts with nonstimulated counts as a covariate. In order to perform analysis of covariance, the slopes (stimulated cpm versus nonstimulated cpm) must be equal. At 1-5 days and 3 months of age, the assumption of equality of slopes was satisfied and the analysis of covariance did not show any effect of PPD status or feeding group on lymphocyte blastogenesis. When the data were examined at 4-6 wk of age, the assumption of equality of slopes was not satisfied. Hence, analysis of covariance could not be done. The slopes for stimulated cpm versus nonstimulated cpm in Figure 1 are quite striking in that the slope for PPD-positive breast-feeding infants did not differ from that of PPD-positive bottle-feeding infants. Similarly, the slopes for PPD-negative breast-feeding and PPD-negative bottle-feeding infants did not differ. However, each positive group differed from each negative group. For a given nonstimulated cpm, the stimulated cpm was determined solely by the tuberculin status of the mother. The feeding method had no effect. These results suggest strongly that the transfer of tuberculin immunity from mother to infant evident at 4-6 wk of age is transplacental, not via human breast milk.

In addition, this transplacental transfer of immunity appears to be transient since it wanes between 4-6 wk and 3 months of age. This disappearance of transferred immunity agrees with the previous studies of Ogra *et al.* (2) and Pabst *et al.* (4). Presumably, the lack of evidence for transferred immunity at 1-5 days of age is the result of immunoincompetence of the neonate or residual effects from the stress of delivery. However, Shiratsuchi and Tsuyuguchi (15) have demonstrated cord blood lymphocyte blastogenesis to PPD in specimens from infants of tuberculin-positive mothers, suggesting transplacental transfer of immunity.

Most difficult is to reconcile our results with those of Schlesinger and Covelli (3) since their prospective study showed an effect of breast-feeding on transfer of tuberculin immunity. This was evidenced at 4 wk of age when eight of 13 infants of PPD-positive breast-feeding mothers had a stimulation index ≥ 2.0 for PPD-induced blastogenesis. Only one of 13 infants of PPD-positive bottle-feeding mothers and none of nine infants of PPD-negative breast-feeding mothers met similar criteria. In Schlesinger and Covelli's (3) study, the mean nonstimulated cpm for the breast-feeding negative control group at age 4 wk (16,449) was higher than the nonstimulated cpm for the breast-feeding-positive group (3751) and bottle-feeding-positive group (2759). We also found a pattern of higher nonstimulated cpm's in breast-feeding infants at 1-5 days, 4-6 wk, and 3 months of age compared to bottle-feeding infants but did not find such large differences between the groups. The large differences in the nonstimulated cpm of the previous data may have influenced the results. We are very comfortable with our analysis of cova-

riance and tests of equality of slopes since the dependence of stimulated cpm on nonstimulated cpm is considered.

Recently, Stephens *et al.* (16) showed that breast-fed infants have higher spontaneous proliferation than bottle-fed infants at age 6 days and 6 wk of age, but by age 6 months spontaneous proliferation was higher in bottle-fed than breast-fed infants. Transfer of immunologically active factors in the milk were proposed as an explanation for the higher results in breast-feeding infants of young age. Older bottle-feeding infants may have higher nonstimulated counts due to a higher antigenic exposure via the gastrointestinal tract. Whatever the reasons for these observed immunologic differences between breast-feeding and bottle-feeding infants, our study does not show a difference in transfer of immunity to a specific antigen such as PPD between breast-feeding and bottle-feeding infants.

Another difference between our study and the previous study (3) is the number of patients and the inclusion of a PPD-negative bottle-feeding group in the control groups. At 4–6 wk of age our study included 38 infants of tuberculin-positive mothers and 33 infants of tuberculin-negative mothers compared to 26 infants of tuberculin-positive mothers and nine infants of tuberculin-negative mothers in the previous study. This sample size difference, particularly in control groups, may also explain our different conclusions.

A confounding variable that may influence our results is the ethnicity of the population studied. Our population is largely an indigent population with many Mexican-American mothers participating in the study. The percentage of Mexican-American women in the tuberculin-positive groups was higher (90%) than in the tuberculin-negative groups (27%). However, if such a difference influenced our results, it did not affect results at 1–5 days or 3 months of age. Another possible confounding variable could be sex differences in the infant groups. This was not a factor for our study since the male-female ratio was similar in the four groups.

Our results agree with studies of other authors suggesting transplacental transfer of cell-mediated immunity. Gallagher *et al.* (5) found cord blood lymphocyte blastogenesis to measles antigen in six of 12 infants of seropositive mothers. Unlike our study, this study showed persistence of this blastogenesis for several months in a few infants. Thong *et al.* (17) examined rubella cytotoxicity in 11 newborn cord specimens and found positive results from three infants. Russell (18) examined cord blood lymphocyte blastogenesis to PPD in infants of mothers with a positive PPD-induced lymphocyte blastogenesis. One of the five such infants studied had a positive lymphocyte blastogenic response. He also showed that two of 14 infants of mothers responding to herpes virus antigen were positive for blastogenesis to herpes antigen.

Other investigators have examined antigen-stimulated lymphokine production as an index of transplacental transfer of cellular immunity from the mother to the infant. Field and Caspary (19) demonstrated production of a PPD-induced macrophage inhibition factor in infants of tuberculin positive mothers. Pabst *et al.* (4) have also shown production of lymphokine in infants of mothers with reactivity to several antigens. In our study, it was interesting that the infants with production of lymphokine did not respond with blastogenesis. However, this additional assessment of transfer of immunity also suggested transplacental transfer.

Our work does not answer the mechanism of transfer. Both transfer of lymphocytes from mother to fetus across the placenta during pregnancy or at the time of delivery and transfer of subcellular factors from mother to infant such as lymphokines are possibilities. We tried to examine transfer of maternal cells but our study was not adequate to eliminate this possibility. One cannot rule out transfer of maternal cells which are eliminated from the infant quickly but persist as subcellular factors. Transfer

of maternal lymphocytes may occur rarely to infants (20–22). However, it is clear that maternal cells are not the cells responding to the PPD with blastogenesis. Antigen exposure *in utero* has been shown in both mouse and human systems (23) to influence the cellular immune response of the developing fetus. We believe that this is an unlikely possibility for our study since all subjects were in good health and did not have or had not had active tuberculosis.

In summary, our study clearly supports the hypothesis that cellular immunity to PPD antigen as evidenced by blastogenesis and lymphokine production is transferred from the mother to her infant. We find no evidence to suggest a role for breast milk in this transfer, but find clear evidence for transplacental transfer of tuberculin immunity.

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