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Interferon-Gamma and Interleukin-10 Production Among HIV-1–Infected and Uninfected Infants of HIV-1–Infected Mothers

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ABSTRACT

Immunologic consequences of exposure to HIV-1 *in utero* are still poorly understood. This study investigates relationships between type-1 [interferon-γ (IFN-γ)] and type-2 (IL-10) cytokine production and maternal-infant HIV-1 transmission. Cord blood leukocytes from deliveries of 71 HIV-1–infected and 11 uninfected mothers were tested for *in vitro* IFN-γ and IL-10 production after phytohemagglutinin (PHA) stimulation. The infants of these HIV-1–infected mothers were followed prospectively after birth to determine HIV vertical transmission, and IFN-γ and IL-10 production was measured again at 6 mo. Median PHA-stimulated IFN-γ production was 210 pg/mL in cord blood cells from infected and 73 pg/mL from uninfected mothers ($p = 0.12$), and median PHA-stimulated IL-10 production was 491 pg/mL in cord blood cells from infected and 161 pg/mL from uninfected mothers ($p = 0.004$). PHA-stimulated IFN-γ and IL-10 production alone were not significantly associated with transmission, but relationships between the two cytokines differed among infected and uninfected infants of HIV-1–infected mothers. PHA-stimulated IFN-γ and IL-10 production was positively correlated among infected ($r = 0.7$, $p = 0.12$ in cord blood and $r = 0.66$, $p = 0.03$ at 6 mo) but not uninfected infants, and stronger relative production of IFN-γ to IL-10 was observed among exposed uninfected than among infected infants ($p = 0.04$). Exposure *in utero* to HIV-1 may augment production of IL-10 detectable in fetal cord blood. Stronger relative production of IFN-γ to IL-10 in cord blood cells from infants of HIV-1–infected mothers may be associated with protection against perinatal HIV infection. **(*Pediatr Res* 50: 412–416, 2001)**

Abbreviations

IFN-γ, interferon-γ
PHA, phytohemagglutinin
CTL, cytotoxic T lymphocyte
PBMC, peripheral blood mononuclear cells

Most infants of HIV-1–infected mothers do not acquire HIV-1 infection themselves. Protection from maternal-infant HIV-1 transmission is associated with less advanced maternal disease, particularly lower maternal viral loads (1), antiretroviral treatment (2), cesarean delivery before rupture of membranes (3), and absence of breast-feeding (4). The most likely explanation for the benefits of these factors is reduction in the duration or dose of viral exposure, although other mechanisms may also be involved.

Less well elucidated are fetal and neonatal host factors that may be critical for protection against vertical transmission upon viral exposure. More than a third of uninfected infants of HIV-1–infected mothers elicit memory helper T-cell responses to HIV-1 peptides at birth, and these cell-mediated responses have been correlated with protection from perinatal infection (5–7). Helper T-cell responses to HIV-1 have also been observed in uninfected adult populations sexually exposed to HIV-1 (8, 9), including those repeatedly exposed and thought to be resistant (10). HIV-1–specific CTL responses have also been observed in uninfected adults exposed to HIV-1 (11, 12). However, HIV-specific CTL responses are more difficult to elicit in infants but have been detected in some uninfected infants of infected mothers (13–18). The reports that protection against both maternal-infant (19) and sexual transmission in female sex workers (20) is associated with HLA class I and II alleles suggest that HLA complex will also contribute to protection. Overall, the balance of evidence supports the notion

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that acquired cellular, rather than humoral, immune responses (at least at a systemic level) are needed for protective immunity to HIV-1. Other processes may be operational at local mucosal sites (9).

Development of a strong cellular response to an antigen is influenced by the cytokine milieu. Type-1 cytokines, including IFN-γ, IL-2, and IL-12, enhance the development of cellular responses, whereas type-2 cytokines, including IL-4, IL-5, IL-6, and IL-10, stimulate humoral responses (21). Hence, we hypothesized that type-1 predominance in cytokine production may be one of the factors associated with protection from HIV-1 infection in HIV-1–exposed populations, including infants of HIV-1–infected mothers. In this study, we investigated whether IFN-γ (type 1) and IL-10 (type 2) production in cord blood of infants of HIV-1–infected mothers would predict which infants were subsequently found to have acquired infection and which were not.

METHODS

Study population. The mother-child pairs included in this study were participants in a randomized clinical trial of vitamin A supplementation that is described in more detail elsewhere (22, 23). In brief, HIV-1–seropositive women attending prenatal clinics at two hospitals in Durban, South Africa, were recruited with written informed consent during pregnancy. Women were counseled about the risks of breast-feeding transmission and about the health risks of formula-feeding and were encouraged to make an informed choice. No woman in the study was treated with antiretroviral therapy. This study was done before the results of the short-course antiretroviral drug trials were available. The study was approved by the Institutional Review Boards of the University of Natal and Columbia University.

Samples collected. As part of the clinical trial protocol, a maternal blood sample was drawn at enrollment for CD4+ and CD8+ T-lymphocyte counts and for quantification of HIV-1 RNA in plasma. Neonatal and obstetric information was recorded at delivery. Mother-child pairs were followed after delivery with regular clinical examinations. Venous blood was drawn from infants on the day of birth, at 1 wk, 6 wk, and 3 mo of age and thereafter every 3 mo until 18 mo for HIV-1 diagnostic tests. If children were breast-fed beyond 15 mo, an additional sample was drawn at least 3 mo after complete cessation of breast-feeding.

Over a specified period of time, cord blood was collected when possible depending on staff availability from the clinical trial participants. Cord blood was collected by cordocentesis to avoid maternal contamination immediately after delivery of the placenta. Cytokine production was measured in cord blood samples and in venous blood samples collected from infants at 6 mo of age if sufficient sample remained after the sample for the HIV-1 diagnostic tests had been prepared. All blood samples were drawn into ethylenediamine tetraacetate tubes. Cord blood was also collected from a sample of infants of HIV-seronegative women as controls, but these infants were not followed after birth. Sample volumes were not sufficient to complete *in vitro* cytokine measurement on all samples. The number of samples included in each analysis is recorded in "Results."

In vitro *IFN-γ and IL-10 production.* Within 24 h of sample collection, mononuclear cells were separated on Ficoll-Hypaque, washed twice in PBS, and the number of viable leukocytes determined by trypan blue exclusion. Cells were resuspended at 3×10^6/mL in RPMI 1640 containing 100 U/mL penicillin and 2 mM glutamine. Three $\times 10^6$ cells in suspension were placed in flat-bottom wells of a microtiter culture plate. For each sample, stimulated and unstimulated (control) wells were set up in triplicate. PHA at a final concentration of 1:100 was used as the stimulus. Pooled human plasma was added to each well an hour after sensitization. The cells were cultured at 37°C in a moist 7% CO_2 atmosphere for 48 h. Culture supernatants were harvested, frozen, and stored at −20°C until assayed for IFN-γ and IL-10 production. Cytokine production was measured using commercially available ELISA assays for human IFN-γ and IL-10 (R&D Systems, Minneapolis, MN, U.S.A.) run according to the manufacturer's instructions. The numerical value for the amount of cytokine was calculated from the standard curve. For all statistical analyses, the level of stimulated IFN-γ and IL-10 was estimated by subtracting from the stimulated value the unstimulated value (truncated to 0 if unstimulated exceeded stimulated values).

Determination of infant HIV-1 status. Plasma from infant samples collected at 6 mo or younger was tested for HIV-1 RNA using PCR (Roche Molecular Systems, Branchburg, NJ, U.S.A.) with an analytic limit of detection of ~10 RNA copies (approximately 400 copies/mL). Samples collected at older ages were tested for HIV-1 antibody (Abbott Laboratories, Chicago, IL, U.S.A.). Infants with at least one positive PCR test were classified as HIV-1 infected. All children classified as infected in this study, except one who was lost to follow-up, tested PCR positive on two or more different specimens. Cord blood was not used for HIV diagnosis. Infants testing negative on their last available sample and who had no positive PCR results were classified as uninfected. Infected children were further stratified into those presumed to have acquired infection intrauterine if the PCR test on their venous blood sample collected on the day of birth was positive, those presumed to have acquired infection intrapartum or during the early postnatal period if the PCR test on the day of birth was negative but was positive by 6 wk of age, and those presumed to have acquired infection during the late postnatal period through breast-feeding if the PCR at 6 wk was negative but was positive at older ages.

Statistical methods. Nonparametric statistics were used throughout because IFN-γ and IL-10 levels were not normally distributed. Differences in IFN-γ and IL-10 levels were tested using the Mann-Whitney *U* test for 2-group comparisons or the Kruskal-Wallis test for greater than 2-group comparisons. Differences between cord blood and 6-mo results were tested with a paired Wilcoxon signed rank test. Spearman's rank correlation coefficient was used to estimate associations between IFN-γ and IL-10 production. Proportions with IFN-γ and IL-10 levels above threshold levels were tested using the χ^2 test. All *p* values were 2-sided.

RESULTS

Infants of HIV-1–infected and uninfected mothers. The median level of PHA-stimulated IFN-γ measured was 210 pg/mL (25th–75th percentile, 17–1479) in cord blood leukocytes of 71 infants of HIV-1–infected mothers compared with 73 pg/mL (2–196) among 11 infants of uninfected mothers, but this trend did not reach significance ($p = 0.12$). PHA-stimulated IFN-γ production in cord blood cells ranged from 0 to more than 500,000 pg/mL among the infants of HIV-1–infected mothers, with 18 (25.4%) having levels above 1396 pg/mL—the maximum value observed in the infants of uninfected mothers ($p = 0.059$). PHA-stimulated IL-10 production was significantly higher ($p = 0.004$) in cord blood leukocytes from 70 infants of HIV-1–infected mothers (median, 491 pg/mL) than among 11 controls (median, 161 pg/mL) (Table 1). After excluding HIV-infected infants, significantly higher levels of PHA-stimulated IL-10 ($p = 0.004$) production in cord blood cells was observed among uninfected infants of HIV-infected mothers than among uninfected infants of uninfected mothers.

HIV-1–infected and uninfected infants of HIV-1–infected mothers. PHA-stimulated IFN-γ production in cord blood leukocytes was not statistically significantly different among seven infants later established to be infected and 62 uninfected infants ($p = 0.27$), although median levels tended to be higher in the uninfected (217 pg/mL) than in the infected group (89 pg/mL). Two of the seven infected infants were presumed to have acquired infection intrauterine because they had detectable HIV-1 RNA in peripheral blood samples collected on the day of birth. The other five were presumed to have acquired infection intrapartum or during the early postnatal period: four had positive PCR results by 6 wk of age, and one was negative at 6 wk but positive when next tested at 3 mo. PHA-stimulated IL-10 production in cord blood cells was similar among eight infected (median, 653 pg/mL) and 60 uninfected (median, 491 pg/mL) infants of HIV-infected mothers ($p = 0.96$) (Table 1).

HIV-1–infected and uninfected infants at 6 mo. Quantities of PHA-stimulated IFN-γ production in PBMC collected at 6 mo of age among the cohort of infants of HIV-1–infected mothers were similarly high to quantities observed in cord blood cells ($p = 0.85$), but quantities of IL-10 production had significantly declined at 6 mo ($p = 0.001$) (Table 1).

No significant differences in PHA-stimulated IFN-γ or IL-10 production in PBMC from infected and uninfected infants of HIV-infected mothers at 6 mo of age were observed (Table 1). One child with 6-mo cytokine results of the 12 who were classified as infected was PCR negative at 6 mo but positive when retested at 7 mo. The others had tested PCR positive on samples collected at ages younger than 6 mo. No significant differences were observed in PHA-stimulated IFN-γ or IL-10 production in PBMC between breast-fed and never breast-fed children, but numbers of nonbreast-fed children were small.

Correlation between IFN-γ and IL-10 production. The relationship between PHA-stimulated IFN-γ production and IL-10 production in cord blood cells differed among HIV-infected and uninfected infants. In cord blood cells, production of these two cytokines was positively correlated among six infected infants of HIV-1–infected mothers with both cytokines measured ($r = 0.7$, $p = 0.12$), *i.e.* individuals with high IFN-γ production *in vitro* after PHA stimulation tended to have high IL-10 production and individuals with low IFN-γ production had low IL-10 production. However, no association between production of the two cytokines was observed among 58 uninfected infants ($r = 0.06$, $p = 0.66$) with these two cytokines measured in cells from the same blood specimen. A similar pattern was observed at 6 mo: $r = 0.66$, $p = 0.028$ among 11 infected and $r = 0.27$, $p = 0.083$ among 44 uninfected (Fig. 1). No correlation between IFN-γ and IL-10 production in cord blood cells was observed among control infants of HIV-seronegative mothers ($r = 0.08$, $p = 0.821$).

Relative production of IFN-γ to IL-10. Because a predominant type-1 cytokine milieu supportive of cell-mediated immunity may depend not only on individual cytokine production but also on relative production of type-1 to type-2 cytokines, we also investigated the relative production of IFN-γ to IL-10. Relative production was measured by calculating the ratio of PHA-stimulated IFN-γ:IL-10 production. The number 1 was added to the cytokine quantity to avoid division by 0. Among

Table 1. *IFN-γ and IL-10 production among HIV-infected and uninfected infants of HIV-infected mothers and among uninfected control infants of uninfected mothers*

	Cord blood			6 mo		
	*n**	Median	25th–75th percentile	*n**	Median	25th–75th percentile
PHA-stimulated IFN-γ						
Infants of HIV-infected mothers	71†	210	17–1479	57	520	81–976
HIV-infected infants	7	89	0–591	12	775	101–1105
Uninfected infants	62	217	32–1880	45	467	33–848
Infants of uninfected mothers	11	73	2–196	–	–	–
PHA-stimulated IL-10						
Infants of HIV-infected mothers	70†	491	203–762	58	263	31–508
HIV-infected infants	8	653	114–730	11	334	11–654
Uninfected infants	60	491	202–780	47	256	38–425
Infants of uninfected mothers	11	161	62–333	–	–	–

* Cord blood and 6-mo measurements were conducted in the same cohort of children. The actual no. of children with adequate sample volumes available for each assay is shown.

† Two of these children were lost to follow-up before their HIV status could be determined.

–, indicates not tested.

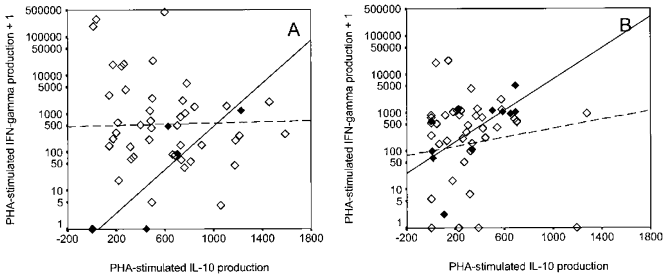

Figure 1. Scatterplot of IFN-γ and IL-10 production after stimulation with PHA in cord blood cells or in PBMC at 6 mo of age among infected (*solid dots*) and uninfected (*open dots*) infants of HIV-infected mothers. The lines indicate the best-fitting lines through the data points based on a linear regression model among the infected (*solid line*) and the uninfected (*dashed line*) infants. (*A*) Cord blood results; (*B*) venous blood at 6 mo.

infants of HIV-1–infected mothers, the ratio of PHA-stimulated IFN-γ to IL-10 production in cord blood cells was higher (median = 0.563, 25th–75th percentile, 0.08–2.80) in those 58 infants who escaped infection compared with those six who acquired infection (median = 0.16, 25th–75th percentile, 0.002–0.808). Two of the six were presumed intrauterine infections and four intrapartum or early postnatal infections. Cord blood cells from none of the six infants of HIV-1–infected mothers who became infected produced more IFN-γ than IL-10 *in vitro* after PHA stimulation, whereas 25/58 (43.1%) of the uninfected infants did ($p = 0.04$).

Association of vertical transmission with maternal HIV RNA levels. The quantity of maternal HIV-1 RNA in plasma was significantly higher among infants who acquired infection than among those who remained uninfected ($p = 0.002$). Maternal viral load did not, however, appear to explain any associations with the cytokines measured because HIV-1 RNA copy numbers did not correlate with *in vitro* production of PHA-stimulated IFN-γ, IL-10, or the ratio of these two cytokines in cord blood cells of infants of HIV-infected mothers.

DISCUSSION

We observed elevated levels of IL-10 production in response to mitogen stimulation in cord blood leukocytes from infants of HIV-1–infected mothers compared with infants from uninfected control mothers. Levels of IFN-γ tended to be elevated, too, but did not reach significance. Elevations could not be explained by HIV-1 infection *per se* among the infants and were observed among the majority of infants of HIV-1–infected mothers who did not themselves acquire infection. Elevations in IL-10 production among the HIV-exposed infants declined by 6 mo of age. These results suggest that the intrauterine experience with an HIV-1–infected mother may be associated with augmented IL-10 (and possibly IFN-γ) production detectable in fetal cord blood. A limitation of our study is the small control group. Hence, we are unable to definitively rule out conditions other than HIV that may account for these differences. However, the control group was an unselected sample of uninfected women delivering at the same hospital during the same period and from the same communities as the HIV-1–infected women.

These results are consistent with other studies that have observed immunologic changes in newborns exposed to HIV *in utero*. A recent study reported reduced *Staphylococcus aureus* Cowan (SAC)-stimulated IL-12 production by cord blood from uninfected deliveries of HIV-infected mothers, demonstrating the influence of the HIV-infected maternal environment on *in utero* immunologic development (24). Another study reported increased proportions of activated (HLA-DR+ CD38+) and memory (CD45RA- RO+) CD4+ T lymphocytes among uninfected infants of HIV-1–infected mothers compared with infants of uninfected mothers (25). Antigen stimulation *in utero* may decrease the pool of naive lymphocytes enhancing cytokine production in the newborn.

The relative production of IFN-γ to IL-10 appeared to be associated with maternal-infant HIV-1 transmission rather than the independent production of either cytokine alone. In the infected, IFN-γ and IL-10 production was positively correlated, *i.e.* either both high or both low production of the two cytokines tended to occur together in the same individual. No infant subsequently found to be infected produced more IFN-γ than IL-10 after PHA stimulation regardless of maternal viral load, whereas 43% of uninfected infants did. These results are consistent with the hypothesis that the failure to establish a polarized type-1 response is associated with an increased risk of transmission. Although fully mature type-1 responses can be achieved in neonates, neonatal T cells tend to be poor producers of type-1 cytokines under neutral conditions (26, 27). More costimulation and augmentation of antigen-presenting cell function, which tends to be immature in fetal cord blood, are necessary to achieve vigorous type-1 responses in newborns (26, 27). Further understanding of the conditions promoting robust type-1 responses is critical to elucidating protective immunity to HIV-1 among infants of HIV-1–infected mothers.

Type-1 and type-2 cytokine production has been investigated in several studies of HIV-1–infected children, mostly to consider associations with disease progression (28–32). Similar to studies in adults, progression of pediatric HIV-1–infection tends to be associated with declines in type-1 cytokines and elevations in type-2 cytokines, although these profiles are not consistently observed depending on the methods used to measure cytokine production (28–32). Even if type-1 predominance can be shown to be associated with slower disease progression among infected children, it does not necessarily follow that a nonspecific type-1 predominance will help protect against infection in the first place. Given the dynamic changes in cytokine production that occur over time among infected children, it is difficult on the basis of these previous studies (even those that included some samples from uninfected infants of HIV-1–infected mothers) to make any inferences about transmission. A strength of our study is that samples from both infected and uninfected children were collected at the same ages and that all the offspring of HIV-1–infected mothers were followed prospectively to determine transmission. However, we cannot rule out that the associations we observed are secondary to HIV-1 infection rather than relevant to transmission because we had insufficient numbers of cord blood samples from study subjects who acquired infection intrapartum or during the postnatal period to consider

these subgroups separately. An inherent difficulty in studying immunologic factors associated with vertical transmission is that immunologic data need to be collected before transmission occurs because HIV infection itself has multidimensional immunologic consequences.

Our findings suggest intrauterine exposure to HIV-1 may result in detectable increases in IL-10 production after PHA stimulation in fetal cord blood leukocytes and suggest that, among infants of HIV-1–infected mothers, greater IFN-γ production relative to IL-10 production in cord blood leukocytes after PHA stimulation may be associated with lack of HIV infection. These findings are consistent with that observed in a study of adult i.v. drug users who were known to have shared needles with HIV-infected partners but who, despite intensive follow-up for many years, remained seronegative. Among this high-risk exposed uninfected population, increased PHA-stimulated IFN-γ production in PBMC and decreased IL-4 and IL-10 production were observed (33). Further study of a wider range of type-1 and type-2 cytokines and of chemokines and their associations with HIV-specific cell-mediated immune responses is needed to clarify these relationships. Better understanding of the spectrum of immune responses that may occur after HIV-1 exposure among individuals who do not themselves develop a chronic HIV infection may assist the development of an effective HIV-1 vaccine. Uninfected infants of HIV-1–infected mothers are a special case of such an exposed uninfected population and are an important group to study to better understand processes of natural immunity to HIV.

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