MAJOR ARTICLE

Breast Milk HIV-1 RNA Levels and Female Sex Are Associated With HIV-1–Specific CD8$^+$ T-Cell Responses in HIV-1–Exposed, Uninfected Infants in Kenya

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Background. Although evidence supports a relationship between human immunodeficiency virus (HIV)–1 exposure and HIV-1−specific CD8$^+$ T cell responses, studies have not demonstrated a direct association between the quantity of HIV-1 to which a person is exposed and the presence or absence of a response.

Methods. From 1999 to 2005, maternal HIV-1 RNA levels were measured in blood, cervical secretions, and breast milk at delivery and 1 month after delivery. HIV-1−specific interferon (IFN)–γ Elispot assays were conducted to determine infant CD8$^+$ T-cell responses at 3 months of age.

Results. Among 161 infants tested with Elispot assays, 23 (14%) had positive results. Mothers whose infants had a positive assay had higher breast milk HIV-1 RNA levels at month 1 compared with mothers whose infants had negative Elispot assays (3.1 vs 2.5 $\log_{10}$ copies/mL; $P = .017$). Female infants were also more likely to have positive Elispot assays than male infants ($P = .046$), and in multivariate analyses, both female sex and high breast milk HIV-1 levels remained important predictors of a positive response ($P = .022$ and $P = .015$, respectively).

Conclusions. Exposure to breast milk HIV-1 and sex were associated with development of HIV-1−specific CD8$^+$ T-cell responses in infants. These data support a role for mucosal exposure via the oral route in induction of systemic HIV-1−specific cellular immunity.

Cytotoxic T lymphocytes (CTLs) play a critical role in the immune response against human immunodeficiency virus type 1 (HIV-1) during active infection. For acutely infected adults, HIV-1−specific CTLs appear shortly after onset of viremia and have been associated with control of viral replication. The presence of systemic virus has been an important predictor of HIV-1−specific CTL induction, and studies in adults and children demonstrate that treatment with antiretroviral drugs and reduction of systemic HIV-1 to undetectable levels result in decreased CTL responses. Treatment interruptions or failure, with subsequent increases in viral load [1], as well as exposure to exogenous virus from untreated HIV-1−infected partners with HIV-1 viremia [2], have been accompanied by return of HIV-1−specific CTLs.

HIV-1−specific CTLs have also been identified among HIV-1−uninfected adults exposed to HIV-1 occupationally or through sexual contact and in infants exposed to maternal HIV-1 perinatally [3, 4]. Although viral exposure is believed to be critical for induction of HIV-1−specific T cells in exposed, uninfected individuals, studies have not described a direct relationship between HIV-1 exposure and these responses. It is

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difficult to assess and quantify viral levels in sexual partners when studying high-risk cohorts; thus, indirect evidence has been used to establish an association between the extent of viral exposure and the T-cell response. HIV-1−uninfected commercial sex worker cohort studies have determined that increased frequency of unprotected sex, longer duration of sex work, and current sex work were associated with increased likelihood of systemic HIV-1−specific T-cell responses [5]. In addition, women stopping sex work lost HIV-1−specific CTL responses. Among women who stopped, those who later resumed sex work were reported to have HIV-1−specific CTL responses return [5].

Using a mother-to-child HIV-1 transmission model, one can more readily evaluate associations between HIV-1 exposure and HIV-1−specific immune responses, as was done in a study by Kuhn et al [6]. In a cohort of nonbreastfeeding infants with in utero exposure to HIV-1, they found no association between maternal plasma viral load and T-helper responses in cord blood [6]. Our goal was to evaluate predictors of HIV-1−specific $CD8^+$ T-cell responses among breastfeeding infants born to HIV-1−infected mothers, specifically addressing whether exposure to HIV-1 in genital secretions at delivery and in breast milk at 1 month of age correlated with a positive HIV-1−specific interferon (IFN)–γ response in infants who remained HIV-1 uninfected during 12 months of follow-up. Maternal HIV-1 RNA levels in plasma, cervical secretions, and breast milk compartments and factors associated with increased viral shedding or greater HIV-1 exposure at these mucosal sites were evaluated for their role in subsequent induction of CTLs in infants.

METHODS

Participant Follow-up and Specimen Collection

As previously described by John-Stewart et al [4], pregnant HIV-1−infected women were enrolled in Nairobi, Kenya, at 32 weeks' gestation and followed for 12 months after delivery with their infants. Mothers were counseled antenatally regarding infant feeding, and oral zidovudine was initiated at 34−36 weeks' gestation. Cervical samples were collected at delivery; and postpartum follow-up occurred at 2 weeks and then monthly. Blood and breast milk were collected from mother−infant pairs at these visits to measure HIV-1 RNA, define HIV-1−specific IFN-γ Elispot responses, and determine infant HIV-1 status.

Laboratory Assays

HIV-1 RNA was measured in maternal plasma, cervical secretions, and breast milk supernatant using the Gen-Probe viral load assay [7]. The lowest reliable limit for this assay was defined as 30 copies/mL for plasma and breast milk samples and 18 copies/swab for cervical samples. Infant HIV-1 infection status was determined using polymerase chain reaction (PCR) to detect HIV-1 *gag* DNA in filter paper blood specimens or HIV-1 RNA in infant plasma using the Gen-Probe assay. Human leukocyte antigen (HLA) typing was performed using amplification refractory mutation system PCR (ARMS-PCR), and sodium and potassium concentrations were measured in whole breast milk using ion-selective electrodes (Olympus Diagnostica).

A modified Elispot assay specific for detection of IFN-γ was used to define epitope-specific $CD8^+$ T-cell responses [4, 8]. Ninety-six-well plates were coated with 7.5 μg IFN-γ monoclonal antibody (MabtechAB), and 2×10^5 fresh peripheral blood mononuclear cells were added. Individual class I HLA-restricted peptides, 8–11 amino acids in length, were derived from HIV-1 clades A and D and added at a concentration of 20 μM. Plates were incubated in the presence of biotinylated anti–IFN-γ antibody (MabtechAB), and individual IFN-γ−producing cells were detected as blue spots using streptavidin-alkaline phosphatase and an alkaline phosphatase-conjugate substrate (Bio-Rad Laboratories). Assays were defined as positive or negative based on a predetermined computer algorithm using published criteria (≥50 HIV-1−specific spot-forming units (SFU)/10^6 cells and experimental SFU ≥ twice background); 20 HIV-1−unexposed Kenyan infants were assayed as negative controls [4].

Statistical Analysis

To define associations between dichotomous variables, we used χ^2 and Fisher exact tests; to evaluate continuous variables, we performed independent *t* tests. Multivariate logistic regression models were used to assess the independent effects of univariate correlates. Covariates of interest were predetermined, and all HIV-1 RNA data were $\log_{10}$ transformed for analyses.

RESULTS

Cohort Characteristics

Among 270 HIV-1−uninfected breastfeeding infants in the larger cohort described by John-Stewart et al [4], we included 161 (60%) in this analysis who had been HLA-typed and had a $CD8^+$ T-cell IFN-γ Elispot assay performed at 3 months of age. Baseline characteristics for the 161 mother−infant pairs are presented in Table 1. Two (1%) infants died during follow-up and 133 (83%) infants completed 12 months of follow-up.

Correlates of Infant IFN-γ Elispot Responses

At 3 months of age, 23 (14%) of 161 infants had positive IFN-γ Elispot responses, which on average had a maximum HIV SFU of 78 HIV SFU/10^6 cells (range, 50–225). Median number of peptides tested per assay was 11 (range, 5–19), and the most prevalent infant HLA types were HLA A*02 (36%), A*30 (32%), A*6802 (27%), Cw*06 (35%), and Cw*07 (37%).

To determine predictors of positive IFN-γ Elispot responses, we evaluated maternal HIV-1 RNA, as well as maternal and infant factors that might influence exposure to HIV-1 or affect infant immune responses (Table 2). We found a positive

Table 1. Characteristics of Breastfeeding Mother–Infant Pairs With Infant $CD8^+$ T-Cell Assays

Characteristic	Mother–infant pairs		
	No. (%) or mean (SD)		
Mothers			
Age (years)	161	24.9	(4.3)
CD4 count (cells/μL) at 32 weeks' gestation	154	505	(251)
Plasma HIV-1 RNA (copies/mL) at 32 weeks' gestation	155	4.5	(0.8)
Cervical HIV-1 RNA (log_{10} copies/mL)			
Delivery	129	2.1	(1.1)
Breast milk HIV-1 RNA (log_{10} copies/mL)			
Month 1 after delivery	136	2.6	(1.0)
Genital ulceration at 32 weeks' gestation	161	10	(6%)
Zidovudine use			
During pregnancy (weeks)	161	3.9	(2.2)
Intrapartum doses	161	3.0	(1.60)
Spontaneous vaginal delivery	161	138	(86%)
Nonemergent cesarean	161	17	(11%)
Duration of ruptured membranes (hours)	161	2.7	(6.8)
Mastitis between delivery and month 1	159	13	(8%)
Mastitis between months 1 and 3	160	12	(8%)
Breast milk Na/K ratio >1	117	49	(42%)
Infants			
Female sex	159	81	(51%)
Birth weight (kg)	157	3.2	(0.4)
Gestational age at delivery (weeks)	140	40	(1.7)
Duration of breastfeeding (months)	161	8.4	(3.6)
Infant deaths	161	2	(1%)
Completed 12 months of follow-up	161	133	(83%)

association between HIV-1 in month 1 breast milk specimens and positive IFN-γ Elispot responses in infants. Three-month-old infants with positive assays were more likely to have mothers with HIV-1 detected in their breast milk at >250 copies/mL (cohort median) than those with negative responses. Thirteen of the 18 (72%) mothers of infants with a positive Elispot assay had HIV-1 RNA >250 copies/mL in their breast milk compared with 55 of 118 (47%) mothers of infants with negative Elispot responses ($P = .04$). Breast milk HIV-1 RNA was also significantly higher among mothers whose infants had a positive assay compared with mothers with infants having negative $CD8^+$ T-cell IFN-γ Elispot assays (3.1 vs 2.4 log_{10} copies/mL; $P = .02$). Associations between breast milk HIV-1 at 1 month after delivery and infant IFN-γ Elispot responses measured contemporaneously in 1-month-old infants were not observed (data not shown). We also found no association between plasma or cervical HIV-1 RNA and a positive infant CTL response ($P > .05$ at months 1 and 3 for both compartments).

When we evaluated factors related to breast milk virus exposure, we found a trend toward statistical significance for clinical mastitis in mothers to be associated with positive infant Elispot results. Clinical mastitis was diagnosed between 1 and 3 months postpartum in 4 of 23 (17%) women with positive infant responses and in only 8 of 138 (6%) women whose infants had negative responses at 3 months of age ($P = .07$). This was not observed in a multivariate model adjusting for breast milk viral load and infant sex. Subclinical mastitis and other markers of inflammation, the CC and CXC chemokines (MIP-1α, MIP-1β, RANTES, and SDF-1α), which were measured in 154 (82%) month 1 breast milk specimens as part of another study [9], were not associated with infant $CD8^+$ T-cell responses at 3 months of age (data not shown).

Maternal $CD4^+$ T-cell count at 32 weeks' gestation did not correlate with infant $CD8^+$ T-cell responses at 3 months of age, nor were there associations with other risk factors for increased HIV-1 exposure (Table 2). We did find that female sex was associated with a positive CTL response. Among 23 infants with positive Elispot responses, 16 (70%) were female compared with 67 of 138 (49%) infants with negative responses ($P = .046$). In multivariate analyses adjusting for breast milk virus, both female sex and high breast milk HIV-1 levels remained significant predictors of a positive response ($P = .022$ and $P = .015$, respectively).

Table 2. Correlates of HIV-1–Specific CD8$^+$ T-Cell Interferon-γ Elispot Responses Among 161 HIV-1–Exposed, Uninfected Breastfeeding Infants

	3-month-old infants with CD8$^+$ IFN-γ Elispot assays				
	Negative response (n = 138 [86%]), mean (SD) or no. (%)		Positive response (n = 23 [14%]), mean (SD) or no. (%)		*P* value
Mothers					
Age (years)	25	(4.3)	24.2	(4.1)	.43
CD4 count (cells/μL)					
32 weeks' gestation	504	(256)	507	(204)	.96
Month 1	612	(295)	650	(309)	.60
Plasma HIV-1 RNA (copies/mL)					
32 weeks' gestation	4.5	(0.80)	4.6	(0.8)	.79
Cervical HIV-1 RNA (copies/mL)					
Delivery	2.0	(1.1)	2.2	(1.3)	.6
Breast milk HIV-1 RNA (copies/mL)					
Month 1 after delivery (log_{10} copies/mL)	2.4	(0.9)	3.1	(1.1)	.02[a,b]
>250 copies/mL[c]	55	(47%)	13	(72%)	.04[a]
Zidovudine use					
During pregnancy (weeks)	3.8	(2.2)	4.2	(2.2)	.37
Intrapartum doses	2.9	(1.6)	3.2	(1.5)	.43
Genital ulceration at 32 weeks' gestation	10	(7%)	0	(0%)	.19
Spontaneous vaginal delivery	119	(86%)	19	(83%)	.80
Duration of ruptured membranes (hours)	2.6	(7.0)	3.5	(6.5)	.54
Episiotomy performed	17	(12%)	1	(9.3%)	.47
Genital laceration during delivery	37	(26.6%)	5	(21.7%)	.62
Mastitis before 1 month after delivery	9	(6%)	4	(17%)	.09
Mastitis between 1 and 3 months after delivery	8	(6%)	4	(33%)	.07
Infant					
Female sex	67	(49%)	16	(70%)	.05[b]
Birthweight (kg)	3.2	(0.45)	3.1	(0.41)	.39
Gestational age at delivery (weeks)	39.5	(1.8)	39.8	(1.3)	.53
Oral candidiasis before 1 month of age	10	(7.0)	1	(4.3)	1.0

[a] Breast milk HIV-1 RNA testing was performed on specimens from 118 mother–infant pairs with negative Elispot assays and from 18 with positive assays.

[b] In a multivariate analysis including breast milk HIV-1 RNA levels and female sex, both remained associated with a positive Elispot response ($P = .015$ and $P = .022$, respectively).

[c] Median breast milk HIV-1 RNA.

DISCUSSION

In this study we systematically evaluated correlates of HIV-1–specific CD8$^+$ T-cell IFN-γ responses among breastfeeding HIV-1–uninfected infants with ongoing exposure to maternal HIV-1. We found induction of HIV-1–specific T cells in uninfected breastfeeding infants was positively associated with HIV-1 in breast milk. Mother–infant pairs with breast milk HIV-1 above the median (250 copies/mL) at 1 month of age were more likely to have positive infant responses at 3 months of age. Higher HIV-1 concentrations in breast milk were also associated with increased risk of a positive infant assay at the subsequent visit. Thus, our data provide direct evidence that HIV-1–specific responses among exposed, uninfected individuals are related to HIV-1 exposure. These findings validate predictions from other studies that have demonstrated an association between positive HIV-1–specific CTL assays in exposed, uninfected female sex workers and factors that would be expected to increase exposure to virus over time [5]. Furthermore, these data suggest that systemic responses can be induced by HIV-1 exposure at oral mucosal surfaces, an observation made recently by Perez et al [10] that may be relevant to development of mucosally administered vaccines.

We did not observe a contemporaneous association between exposure and immune responses. Month 1 breast milk viral RNA levels were not associated with positive Elispot responses in samples collected from infants at the same visit (data not shown). That breast milk virus levels only impact later responses is biologically plausible and consistent with development of a cellular response over the course of 1–2 weeks, as has been

demonstrated in studies examining HIV-specific $CD8^+$ T-cell responses in animal models [11]. In a study of primary HIV infection among adults, CTL responses using Elispot assays also developed well after exposure at a median of 22 days after onset of symptoms [12]. The relevant predictor of a positive Elispot response in 1-month-old infants would therefore be breast milk virus from an earlier time point, one closer to the time of delivery, which was not collected in this study.

Other important correlates were clinical mastitis and infant sex. The trend for an association between clinical mastitis and a positive IFN-γ response was not present after we adjusted for HIV-1 concentrations in breast milk, suggesting that mastitis influenced infant HIV-specific $CD8^+$ T-cell activity by increasing breast milk viral load. Female sex remained predictive of a positive infant response independent of breast milk HIV-1 RNA. The observation that female infants were more likely than male infants to mount a positive response at 3 months of age is intriguing because several investigators have found risk for HIV-1 acquisition in utero to be higher for girls compared with boys. Mechanisms for this association have not been defined, and hormonal, genetic, and immunologic factors may play a role [13]. In addition, humoral and cellular responses to a variety of vaccines differ between females and males [14], and differences in Toll-like receptor results in increased CD8 immune activation in females [15].

In conclusion, we found breast milk HIV-1 levels and infant sex to be associated with HIV-1−specific CD8+ T-cell responses among exposed, uninfected breastfeeding infants. There has been speculation that such responses among HIV-1−uninfected individuals are not directed at HIV-1 antigens but target cross-reacting non-HIV epitopes. Our findings indicate that these responses are related to HIV-1 exposure and are less likely to be the result of chance events or nonspecific T-cell responses. These data support the hypothesis that HIV-1−specific T-cell responses in exposed uninfected infants result from HIV-1 exposure and exposure across mucosal surfaces, and may provide insight into the design of vaccines targeting promotion of CTL responses in infants, including orally administered vaccines.

Notes

Acknowledgments. Written informed consent was obtained from all study participants. This study received ethical approval from the institutional review boards of the University of Washington and the University of Nairobi and was conducted according to the guidelines set forth by the US Department of Health and Human Services.

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Potential conflicts of interest. All authors: No reported conflicts.

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