Prevalence and Magnitude of Human Immunodeficiency Virus (HIV) Type 1–Specific Lymphocyte Responses in Breast Milk from HIV-1–Seropositive Women

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Human immunodeficiency virus (HIV) type 1–specific cell-mediated immunity of breast milk may influence the likelihood of mother-to-child transmission of HIV-1 via breast-feeding. In breast-milk specimens collected during the first month postpartum from HIV-1–seropositive women in Nairobi, HIV-1 gag–specific cellular responses were detected in 17 (47%) of 36, and env–specific cellular responses were present in 20 (40%) of 50. Peripheral blood lymphocyte responses against either gag or env were detected in 35 (66%) of the 53 subjects, 18 (51%) of whom had positive gag or env responses in their breast milk. In paired analyses of blood and breast milk, the mean magnitude of responses to env or gag stimulation in breast milk was significantly higher than that in blood and remained higher in breast milk after normalization of responses according to CD8+ lymphocyte count. These results suggest that CD8+ lymphocytes present in breast milk have the capacity to recognize HIV-1–infected cells and may be selectively transported to breast milk to reduce either viral replication or transmission in breast milk.

Breast-feeding infants of human immunodeficiency virus (HIV) type 1–seropositive women have ~16% risk of acquiring HIV-1 through breast-feeding [1]. Despite that risk, breast-feeding remains common among HIV-1–infected women throughout the world because of concerns about the safety of replacement formula feeds and the potential loss of confidentiality regarding HIV-1 infection status. Antiretroviral drugs administered peripartum can reduce the risk of HIV-1 transmission, yet the infant remains at risk of acquiring HIV-1 from breast milk. HIV-1 has been demonstrated in both the cell-free and cell-associated fractions of breast milk [2–4], and neonatal tissues susceptible to HIV-1 include the tonsils and oral and intestinal mucosa. Because the infant remains vulnerable to HIV-1 infection, additional intervention strategies designed to reduce the risk of transmission of HIV-1 via breast milk are needed.

Breast milk is part of the mucosal immune system, providing the neonate immunologic protection against a variety of infectious agents (reviewed in [5]). Maternal antigenic exposure to enteric organisms or respiratory pathogens results in the appearance of antigen-specific IgA and T helper cells in breast milk, the specificities of which do not always reflect what is present in peripheral blood [6–10]. A recent study demonstrated that lymphocytes with specific recognition of HIV-1 antigens are present in the breast milk of HIV-1–infected women [11]; all 12 HIV-1–seropositive wom-
en from the United States and Zambia had HIV-1–specific responses to gag in their breast milk, as measured by ELISPOT assays, with fewer women responding to env, pol, and nef. The detected responses were consistent with cytotoxic T lymphocytes (CTLs), on the basis of decreased response following depletion of CD8+ lymphocytes and detection of HIV-1–specific cytolytic response in a chromium-release assay.

Further characterization of the cell-mediated immune responses in breast milk will be helpful in designing strategies to reduce the risk of mother-to-child transmission of HIV-1. We evaluated 53 HIV-1–seropositive women in Nairobi, Kenya, for HIV-1–specific cellular immune responses in paired breast-milk and blood specimens during the first month postpartum, to determine the population prevalence and magnitude of HIV-1–specific responses in breast milk.

SUBJECTS AND METHODS

Cohort. Breast-milk specimens were obtained as part of an ongoing perinatal cohort of HIV-1–infected women and their infants [12]. Women were recruited during pregnancy and received zidovudine, beginning at 34–36 weeks of gestation and continuing through delivery, for prevention of infant HIV-1 infection [13]. Mothers were advised about feeding options, and their infants were breast- or formula-fed according to maternal preference. Mother-infant pairs were examined by clinicians at 2 and 4 weeks postpartum. Written informed consent was obtained from all subjects. Human experimentation guidelines of the US Department of Health and Human Services and the Institutional Review Boards of the University of Washington and the University of Nairobi were followed in the conduct of this study.

Collection of breast milk and blood. Paired samples of maternal blood and breast milk were collected via venipuncture and manual expression, respectively, at 2 or 4 weeks postpartum from 43 HIV-1–infected women and at both time points from 10 HIV-1–infected women. Breast milk was fractionated into the lipid, plasma, and cellular components by centrifugation for 20 min at 710 g [2]. The cellular component was collected and washed twice in PBS (Sigma). Breast-milk lymphocytes were enumerated on the basis of trypan blue exclusion, size, and shape under a hemocytometer. Thus, the total number of breast-milk lymphocytes added to the ELISPOT assay wells was normalized; however, the total number of nonlymphoid cells per well was variable. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated blood by ficoll gradient separation (Lymphocyte Separation Medium; Organon Teknika), washed in RPMI 1640 medium (Sigma), and counted as described above.

HIV-1 DNA polymerase chain reaction (PCR). HIV-1 infection status of the infant was determined by PCR assays for HIV-1 DNA, as described elsewhere [14]. In brief, DNA was extracted from the dried blood spot, and quadruplicate independent PCRs were done on each blood spot by use of nested primers to amplify HIV-1 gag. The sensitivity and specificity of this method have been shown to be 96% and 100%, respectively.

Plasma HIV-1 RNA load. Quantitative analysis of HIV-1 RNA load in maternal plasma 1 month postpartum was done by use of the Gen-Probe HIV-1 Viral Load Assay, a transcription-mediated amplification method sensitive for detection of multiple HIV-1 subtypes [15].

Depletion of CD8+ lymphocytes. Breast-milk cells and PBMCs were depleted of CD8+ T lymphocytes by use of anti-CD8 monoclonal antibody–coated magnetic beads, according to the manufacturer’s instructions (Dynal). Depletion of the CD8+ subset was verified by flow cytometry after staining of depleted and undepleted populations with the following antibodies: anti–CD3–fluorescein isothiocyanate, anti–CD8–phycoerythrin, and anti–CD45–peridinin chlorophyll protein antigens (Tristet; Becton Dickinson) and analyzing the lymphocyte populations by use of CellQuest software on a FACScan flow cytometer (both from Becton Dickinson).

Percentage of lymphocytes in blood and breast milk. Paired specimens of peripheral blood and cryopreserved breast milk were used to determine the percentage of lymphocytes and percentage of CD3+CD8+ lymphocytes in each compartment. The percentages of lymphocytes in whole blood and breast milk were determined by flow cytometry on the basis of forward- and side-scatter parameters, in conjunction with anti-CD45 staining. The percentage of CD3+CD8+ lymphocytes in blood and breast milk was determined from the percentage of dually positive events present within the lymphocyte gate.

Interferon (IFN)–γ ELISPOT assay. An ELISPOT assay was used to detect HIV-1–specific IFN-γ release from the blood and breast-milk lymphocytes after overnight incubation with recombinant vaccinia virus vectors expressing HIV-1 gene products as a marker for CTL activity [16–18]. In brief, 96-well Millipore plates (MAIP45; Millipore) were coated with 7.5 µg of monoclonal antibody to IFN-γ (1-DIK; Mabtech Ab) for 2 h at 37°C. Excess antibody was removed by washing 6 times with RPMI 1640 medium and was blocked with RPMI 1640 medium containing l-glutamine and supplemented with antibiotic/antimycotic and 10% fetal cell serum (all from Gibco), designated R10, for 30 min at room temperature before cells were added. CD8+ lymphocyte–depleted or undepleted PBMCs or breast-milk lymphocytes (2 × 10⁴) were added to wells of the blocked plates. Vaccinia virus (WR strain, negative control) or recombinant vaccinia viruses expressing either HIV-1 clade A gp160env (env) or p55S (gag) (S.R.-J., National Institutes of Health AIDS Research and Reference Reagent Program, Be-
for addition of alkaline phosphatase substrate (Bio-Rad Laboratories) 1000; Mabtech) for 1.5 h at room temperature. After the final addition of streptavidin-conjugated alkaline phosphatase (1: 0.05% Tween 20 (Sigma), followed in sequence by the addition of biotinylated anti–IFN-γ antibody (1:1000, 7-B6-1 biotin; Mabtech) for 3 h at room temperature, washing, and addition of streptavidin-conjugated alkaline phosphatase (1: 1000; Mabtech) for 1.5 h at room temperature. After the final washing, spot-forming units (sfu) were visualized by the addition of alkaline phosphatase substrate (Bio-Rad Laboratories) for ∼10 min or until an intense blue reaction was visible in the wells stimulated with PHA. Additional color development was prevented by washing the plates under running water. Plates were allowed to dry overnight before reading on an automated ELISPOT reader (Autoimmun Diagnostika). HIV-specific sfu/10^6 cells was defined as the average number of sfu/10^6 cells from gag- or env-stimulated wells minus the average number of sfu/10^6 cells in wells containing strain WR (negative control). The following criteria were used to determine a positive assay: 350 HIV-specific sfu/10^6 cells and gag or env sfu ≥2 times the strain WR negative control [20]. By comparing the HIV-1–specific response to gag or env with the response stimulated by the vaccinia wild-type vector alone, confounding additional cell types present in the preparation of breast-milk lymphocytes was minimized. The HIV-1 specificity of the ELISPOT method with use of recombinant vaccinia virus vectors has been confirmed elsewhere [17, 21].

**Statistical methods.** To avoid duplicate assessment of the 10 subjects with testing at both time points, only the 4-week data were used from this group of subjects for comparison of responses. Independent-samples t tests were used to compare clinical and virological data presented in table 1, between subjects who had detectable HIV-1–specific IFN-γ ELISPOT responses and those who lacked detectable responses. A paired nonparametric comparison (sign test) of positive and negative assays was used to compare the prevalence of env- and gag-specific responses in blood versus breast milk. χ^2 tests were used to compare prevalence of gag- and env-specific responses at 2 and 4 weeks postpartum in blood and breast milk. Among subjects with responses in both blood and breast milk, mean magnitudes of the HIV-1–specific responses in blood and breast milk were compared by means of paired-samples t tests. Comparisons of lymphocyte and CD3^+CD8^+ T lymphocyte percentages in blood and breast milk were conducted with paired-samples t tests. McNemar’s test for related samples was used to compare the number of positive responses detected with use of 2 criteria: (1) ≥50 HIV-specific sfu/10^6 cells and gag- or env-specific sfu of ≥2 times the strain WR negative control; and

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>All women (n = 53)</th>
<th>Women with HIV-1–specific lymphocytes in breast milk (n = 24)</th>
<th>Women without HIV-1–specific lymphocytes in breast milk (n = 29)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 cell count,^c^ cells/mm^3</td>
<td>521 (499) [155–1167]</td>
<td>533 (559) [117–1167]</td>
<td>510 (473) [155–1137]</td>
<td>.8</td>
</tr>
<tr>
<td>CD4 cell %^d^</td>
<td>22 (22) [7–39]</td>
<td>23 (22) [9–39]</td>
<td>22 (23) [7–39]</td>
<td>.7</td>
</tr>
<tr>
<td>CD8 cell count,^c^ cells/mm^3</td>
<td>1165 (1084) [451–2448]</td>
<td>1111 (1019) [451–2139]</td>
<td>1211 (1112) [567–2448]</td>
<td>.5</td>
</tr>
<tr>
<td>CD8 cell %^d^</td>
<td>50 (51) [22–79]</td>
<td>48 (46) [22–79]</td>
<td>52 (52) [32–70]</td>
<td>.2</td>
</tr>
<tr>
<td>CD4:CD8 cell ratio^d^</td>
<td>0.50 (0.48) [0.11–1.25]</td>
<td>0.55 (0.53) [0.11–1.15]</td>
<td>0.47 (0.44) [0.11–1.25]</td>
<td>.3</td>
</tr>
<tr>
<td>Plasma HIV-1 RNA load, log_{10} copies/mL^b^</td>
<td>4.8 (4.8) [2.2–7.9]</td>
<td>4.6 (4.8) [2.3–6.7]</td>
<td>5.0 (4.8) [2.2–7.9]</td>
<td>.2</td>
</tr>
<tr>
<td>History of HIV-related signs and symptoms,^c^ no/total (%)</td>
<td>8/53 (15)</td>
<td>3/24 (13)</td>
<td>5/29 (17)</td>
<td>.6</td>
</tr>
<tr>
<td>HIV-1 transmission to infant,^d^ no/total (%)</td>
<td>7/53 (13)</td>
<td>3/24 (13)</td>
<td>4/29 (14)</td>
<td>.6</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean (median) [range], except where noted.

^a P values reported from t tests for differences in means between women who had cellular HIV-1–specific interferon-γ responses in breast milk vs. those who lacked detectable breast milk responses.

^b Data from 1 month postpartum were missing for 3 subjects, 1 of whom had HIV-1–specific lymphocytes in breast milk and 2 of whom lacked HIV-1–specific lymphocytes in milk.

^c HIV-1–related signs and symptoms of disease include fever, diarrhea, cough, itchy skin rash, or thrush lasting >1 month within the last year or weight loss of >5 kg within the last year. History was taken at enrollment at ~28 weeks of gestation.

^d HIV-1 infection in the infant was diagnosed on the basis of presence of HIV-1 gag DNA from infant blood specimens collected within the first 48 h of birth, at months 1 and 3 of life, and at 3-monthly intervals thereafter.
at 4 weeks postpartum (\(P\)). The prevalence of HIV-1–specific responses 2 weeks postpartum and 63% had positive responses. The prevalence of HIV-1–specific responses was similar at either gag–
gag–
env–
env–
env–
2). In breast milk, there was a trend for more frequent detection of responses in breast milk, 6 (25%) had no detectable responses in their peripheral blood. The HIV-1–specific cellular responses in breast milk were strong for these 6 subjects: all had gag-specific responses, the mean magnitude of which was 658 HIV-specific sfu/10^6 cells (median, 352 sfu/10^6 cells; range, 52–2418 sfu/10^6 cells), and 5 had concurrent env-specific responses, with a mean magnitude of 564 HIV-specific sfu/10^6 breast milk cells (median, 538 sfu/10^6 cells; range, 228–1192 sfu/10^6 cells).

**RESULTS**

**Characteristics of the cohort.** Fifty-three HIV-1–seropositive women had specimens evaluated for HIV-1–specific cellular immune responses during the first month postpartum. Forty-three were tested either at 2 or 4 weeks postpartum, and 10 were tested at both 2 and 4 weeks postpartum. For the subjects tested twice, only 4-week data were used in analyses. The median age of the subjects was 25 years (range, 18–37 years). The median duration of zidovudine treatment was 28.5 days (range, 7–72 days). Lymphocyte subset analysis was done at 4 weeks postpartum and was available for 50 women from the cohort (table 1). The mean and median CD4 cell counts were 521 cells/mm^3 and 499 cells/mm^3 (range, 155–1167 cells/mm^3). In this group, 4 subjects (8%) had CD4 cell counts of <200 cells/mm^3. The mean and median CD4 cell percentages were 22.3% and 22.5% (range, 7%–39%). The mean and median CD8 cell counts were 1165 and 1084 cells/mm^3 (range, 451–2448 cells/mm^3). The mean and median CD8 cell percentages were 50% and 51% (range, 22%–79%).

The mean and median CD4:CD8 counts were 1165 and 1084 cells/mm^3 (range, 451–2448 cells/mm^3). The mean and median CD8 cell percentages were 50% and 51% (range, 22%–79%). The mean and median CD4:CD8 cell ratios were 0.50 and 0.48, respectively (range, 0.11–1.25).

**Prevalence of HIV-1–specific lymphocyte responses in breast milk and blood.** Overall, 24 (45%) of the 53 HIV-1–infected women had detectable anti-HIV-1 gag– or env–specific lymphocyte responses in early breast milk and 35 (66%) had anti-HIV-1 gag– or env–specific responses in peripheral blood (table 2). In breast milk, there was a trend for more frequent detection of gag– or env–specific responses at 2 weeks postpartum (55%) than at 4 weeks postpartum (33%), although this difference did not reach significance (\(P = .11\)). In peripheral blood, the prevalence of HIV-1–specific responses was similar at either time point: 69% of subjects had either gag– or env–specific responses 2 weeks postpartum and 63% had positive responses at 4 weeks postpartum (\(P = .42\)). The prevalence of HIV-1–specific responses did not differ significantly between blood and breast milk for env (\(P = .50\)) or gag (\(P = .79\)).

Detection of HIV-1–specific cellular responses in the blood did not always predict detection of responses in breast milk. Of 35 women with HIV-1–specific cellular responses in peripheral blood, 18 (51%) had responses in their breast milk. Conversely, of 24 subjects with HIV-1–specific lymphocytes in breast milk, 6 (25%) had no detectable responses in their peripheral blood. The HIV-1–specific cellular responses in breast milk were strong for these 6 subjects: all had gag-specific responses, the mean magnitude of which was 658 HIV-specific sfu/10^6 cells (median, 352 sfu/10^6 cells; range, 52–2418 sfu/10^6 cells), and 5 had concurrent env-specific responses, with a mean magnitude of 564 HIV-specific sfu/10^6 breast milk cells (median, 538 sfu/10^6 cells; range, 228–1192 sfu/10^6 cells).

**HIV-1–specific cellular immune responses and relationship to plasma HIV-1 RNA levels and HIV-1 transmission.** Women who had peripheral blood responses to either HIV-1 gag or env had a mean virus load of 4.5 log_{10} HIV-1 RNA copies/mL (median, 4.5 log_{10} HIV-1 RNA copies/mL), compared with 5.4 log_{10} HIV-1 RNA copies/mL (median, 5.3 log_{10} HIV-1 RNA copies/mL) in subjects without peripheral blood HIV-1–specific responses (\(P < .005\)). Similarly, subjects with HIV-1–specific responses in breast milk had lower plasma HIV-1 RNA loads than did subjects without HIV-1–specific responses in breast milk; however, this difference was not statistically significant (4.6 vs. 5.0 log_{10} HIV-1 RNA copies/mL; \(P = .2\)) (table 1). Breast-milk virus load data are not available for this cohort. Women with HIV-1–specific IFN-\(\gamma\) secretion from peripheral blood lymphocytes tended to have higher median CD4 cell counts (556 vs. 405 cells/mm^3; \(P = .14\)), CD4 cell percentages (24% vs. 19%; \(P = .07\)), and CD4:CD8 cell ratios (0.52 vs. 0.41; \(P = .21\)) than did subjects who lacked detectable responses in breast milk, whereas differences in CD8 cell count, CD8 cell percentage, and rate of HIV-1 transmission to their infants were not significantly different (\(P = A\), \(P = A\), and \(P = .7\), respectively). There were no significant differences between CD4 cell count, CD4 cell percentage, CD8 cell count, CD8 cell percentage, CD4:CD8 cell ratio, HIV-1–related signs and symptoms, plasma HIV-1 RNA load, or rates of HIV-1 transmission among subjects who possessed HIV-1 gag– or env–specific lymphocyte responses in breast milk and those who lacked detectable responses (table 1).

**Phenotype of the HIV-1–specific cellular responses in breast milk.** The cell population responsible for the HIV-1 gag– and env–specific responses in breast milk was determined by depleting CD8^+ T lymphocytes. In 3 independent depletion experiments, removal of CD8^+ lymphocytes resulted in a 78%–94% reduction in responses to either gag or env (from 313 to 20 HIV-specific sfu/10^6 cells, from 1500 to 311 HIV-specific sfu/10^6 cells, and from 190 to 42 HIV-specific sfu/10^6 cells).
Table 2. Prevalence of human immunodeficiency virus (HIV) type 1–specific cellular responses in breast milk and peripheral blood from HIV-1–seropositive women.

<table>
<thead>
<tr>
<th>Time postpartum</th>
<th>Breast milk HIV-1 gag</th>
<th>Breast milk HIV-1 env</th>
<th>Breast milk Either gag or env</th>
<th>Peripheral blood HIV-1 gag</th>
<th>Peripheral blood HIV-1 env</th>
<th>Peripheral blood Either gag or env</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>9/17 (53)</td>
<td>13/29 (45)</td>
<td>16/29 (55)</td>
<td>12/28 (43)</td>
<td>15/29 (52)</td>
<td>20/29 (69)</td>
</tr>
<tr>
<td>4 weeks</td>
<td>8/19 (42)</td>
<td>7/21 (33)</td>
<td>8/24 (33)</td>
<td>13/24 (54)</td>
<td>10/23 (43)</td>
<td>15/24 (63)</td>
</tr>
<tr>
<td>Overall</td>
<td>17/36 (47)</td>
<td>20/50 (40)</td>
<td>24/53 (45)</td>
<td>25/52 (48)</td>
<td>25/52 (48)</td>
<td>35/53 (66)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. of positive results/no. tested (%). Peripheral blood or breast-milk lymphocytes were stimulated overnight with recombinant vaccinia virus expressing either HIV-1p55gag or gp160env. Production of interferon-γ was detected in an ELISPOT assay. Positive assays were defined as HIV-specific spot-forming units (sfu)/10^9110915 and gag- or env-specific sfu/10^91109152 times the background.

The depletion of CD8+ lymphocytes was verified by flow cytometry and was 99.9%, 99.9%, and 84.3%, respectively.

Magnitude of the HIV-1–specific lymphocyte responses in blood and breast milk. The relationship between the magnitude of paired HIV-1–specific responses, both positive and negative, in blood compared with that in breast milk from each subject is shown in figure 1. In subjects with positive responses in both blood and breast milk, the mean number of HIV-specific sfu/10^6 lymphocytes was compared with gag- or env-specific sfu by means of paired-samples t tests (figure 2). The magnitude of HIV-1–specific responses against gag and env present in blood or breast milk did not vary between the 2 time points tested (data not shown). Responses in peripheral blood and breast milk did not differ from each other in terms of positive and negative control stimuli. The mean ± SE background response to vaccinia virus strain WR was 54 ± 25 sfu/10^6 cells in blood and 106 ± 30 sfu/10^6 cells in breast milk (P = .20). The mean response to PHA had a trend for increased levels in blood versus breast milk: 1809 ± 149 sfu/10^6 cells in blood versus 1384 ± 216 sfu/10^6 cells in breast milk (P = .09). In 11 subjects with positive gag-specific responses in both blood and breast milk, the mean number of HIV-specific sfu/10^6 lymphocytes was 888 ± 244 in breast milk versus 376 ± 64 in peripheral blood (P = .05). Similarly, in 12 subjects with positive env-specific responses in blood and breast milk, the mean env response in breast milk was 707 ± 189 HIV-specific sfu/10^6 cells, compared with 373 ± 128 HIV-specific sfu/10^6 cells in peripheral blood (P = .03). Thus, despite a lower background and higher response to PHA in blood, the HIV-1 gag– and env–specific responses were significantly greater in breast milk.

Percentage of CD8+ lymphocytes in blood and breast milk. Blood and breast milk differ in cellular composition, and, to directly compare the results from the 2 compartments, we determined the percentage of total lymphocytes and percentage of CD3+CD8+ lymphocytes in blood and breast milk of 13 HIV-1–infected women from our cohort (figure 3). This subset was reflective of the larger cohort with respect to CD4 cell count. The mean and median CD4 cell counts were 502 and 396 cells/mm^3 (range, 170–1167 cells/mm^3). One subject (7.6%) had a CD4 cell count of <200 cells/mm^3. The mean and median CD4 cell percentages were 22.5% and 22.0% (range, 7%–39%). The women tested had a mean (±SE) total lymphocyte percentage in peripheral blood of 41% ± 2.7%, whereas the paired breast-milk samples contained a mean of 76% ± 1.4% lymphocytes (P < .01). Although the percentage of total lymphocytes in the cellular fraction of blood was significantly higher than in breast milk, there was no significant difference between the

Figure 1. Magnitude of human immunodeficiency virus (HIV) type 1–specific responses in blood and breast milk. Paired samples of blood and breast-milk lymphocytes were tested for either HIV-1 gag or HIV-1 env. Horizontal axis indicates the level of the response in peripheral blood, and the vertical axis represents the magnitude of the breast milk response, both expressed at HIV-1–specific spot-forming units (sfu)/10^6 lymphocytes. Dashed line indicates cutoff level of 50 HIV-1–specific sfu.
Figure 2. Magnitude of human immunodeficiency virus (HIV) type 1–specific cellular responses in peripheral blood and breast milk of HIV-1–infected women. Mean ± SE HIV-specific spot-forming units (sfu)/10⁶ lymphocytes is plotted for 3 stimuli: phytohemagglutinin (PHA; positive control), HIV-1p55gag (gag), and HIV-1gp160env (env). Paired t test for comparisons of mean response in blood vs. breast milk was conducted on data points from women who had positive responses in both compartments to either gag or env. *nP11; nP12; P < .05.

mean (±SE) percentages of CD3⁺CD8⁺ lymphocytes contained within the lymphocyte subset of blood versus breast milk: 46% ± 3.4% versus 54% ± 4.8% (P = .22).

A direct comparison of the mean HIV-specific sfu expressed either per 10⁶ lymphocytes or per 10⁶ CD3⁺CD8⁺ lymphocytes was possible within this subset of subjects. The mean (±SE) number of gag-specific HIV-specific sfu/10⁶ lymphocytes from 6 paired samples was 424 ± 81 in blood and 856 ± 196 in breast milk–2-fold higher in breast milk. When expressed per 10⁶ CD8⁺ lymphocytes, the mean (±SE) number of HIV-specific sfu increased to 911 ± 186 in blood and 2292 ± 944 in breast milk–2.5-fold higher in breast milk. Positive responses to env were present in paired samples from 4 subjects. The mean (±SE) env response was 505 ± 379 HIV-specific sfu/10⁶ lymphocytes in blood and 1197 ± 368 HIV-specific sfu/10⁶ lymphocytes in breast milk–2.4-fold higher in breast milk. When expressed per 10⁶ CD8⁺ lymphocytes, the env HIV-specific sfu were 1088 ± 854 in blood and 3078 ± 1327 in breast milk–2.8-fold higher in breast milk. Although the difference in mean responses per 10⁶ CD8⁺ cells between blood and breast milk did not reach significance because of a smaller sample size, the trend was maintained for increased magnitude of HIV-1–specific lymphocyte responses in breast milk than in blood.

Comparison of 2 criteria for positive responses. The prevalence of detection of HIV-1–specific responses is dependent on the criteria used to determine a positive assay. The prevalence of positive responses was compared by use of a minimum number of HIV-1–specific spots of ≥50 HIV-specific sfu/10⁶ cells and ≥100 HIV-specific sfu/10⁶ cells. No significant difference in the prevalence of positive responses for the different criteria in peripheral blood or breast milk specific for either gag or env was detected at either 2 or 4 weeks postpartum. The overall prevalence of HIV-1 gag– or env–specific lymphocyte responses decreased by ~5% when the cutoff of ≥100 HIV-specific sfu/10⁶ cells was used, to 40% in breast milk and 60% in peripheral blood. HIV-1 env–specific responses remained significantly higher in breast milk (874 ± 231) than in blood (508 ± 175) (P = .05). In addition, there continued to be a trend for a 2-fold increase in HIV-1 gag–specific responses in breast milk (308 ± 63) compared with those in blood (406 ± 264) (P = .10).

DISCUSSION

The results of the present study confirm the presence of lymphocytes in breast milk that respond to HIV-1–specific antigens in a manner consistent with CD8⁺ CTL activity. Lymphocytes obtained from early breast milk of HIV-1–infected women were capable of producing IFN-γ after incubation with recombinant vaccinia virus expressing either HIV-1 clade A p55gag or gp160env. Depletion of CD8⁺ lymphocytes diminished these responses by...
Within our study, we were able to directly compare the level of HIV-1–specific cellular responses in peripheral blood with those found in breast milk and to characterize the lymphocyte populations in breast milk of HIV-1–infected women. In paired analysis of women with responses in both blood and breast milk, the magnitude of response to HIV-1 antigens was significantly greater in breast milk than in blood, and this difference was enhanced when the response was adjusted to the number of CD8+ lymphocytes per well. To our knowledge, this is the first report of lymphocyte levels in breast milk of HIV-1–seropositive women. The finding that the percentages of CD8+CD3+ lymphocytes within the lymphocyte fraction are not significantly different between blood and breast milk, whereas HIV-1–specific responses are of significantly higher magnitude in breast milk than blood, strongly suggests selective transport of HIV-1–specific lymphocytes to breast milk, perhaps for protection of infants of HIV-1–seropositive women. In this study of 50 women, we were unable to determine an effect of HIV-1–specific cellular responses in breast milk on transmission of HIV-1 to the neonate via breast milk. Larger population studies will be important to determine whether HIV-1–specific immune responses in breast milk influence the risk of HIV-1 transmission via breast milk.

The finding that HIV-1–specific IFN-γ responses in peripheral blood were associated with significantly decreased HIV-1 RNA levels in plasma in this cohort is consistent with the studies suggesting that HIV-1–specific CTL responses control virus burden [22, 23], a finding demonstrated in the simian immunodeficiency virus (SIV) model [24, 25]. This area is not without controversy, because others have demonstrated a positive association with HIV-1–specific CTL and IFN-γ responses and plasma levels of HIV-1 [26, 27]. Women who had detectable HIV-1–specific IFN-γ secretion from their peripheral blood lymphocytes had \( \sim 1 \log_{10} \) lower HIV-1 burden than did women who lacked any detectable HIV-1–specific response in peripheral blood. In our previous studies, HIV-1 RNA levels in breast milk paralleled HIV-1 RNA levels in plasma and were \( \sim 1 \log_{10} \) lower [28]. However, without data on HIV-1 RNA in breast milk, it is not possible to determine the role of the immune responses we detected on control of virus secreted in breast milk. It will be important to determine whether HIV-1–specific immune responses in breast milk are associated with decreased levels of virus in breast milk.

A recent report by Sabbaj et al. [11] described the presence of CD8+ T lymphocytes in breast milk of 12 HIV-1–infected women from the United States and Zambia. As determined by use of overlapping peptide pools reflecting sequences derived from either clades B or C to detect HIV-1–specific responses in an ELISPOT assay, all 11 women had lymphocyte responses directed against gag, and 2 (40%) of 5 had detectable responses to env, with use of a cutoff for a positive response of \( \geq 100 \).
HIV-specific sfu/10⁶ cells. In a sample from 1 HIV-1–infected woman, a cytolytic response was detected against a vaccinia virus construct expressing multiple HIV-1 antigens, conclusive of breast-milk CTL activity. Our study differed from that of Sabbaj et al. [11] in the method of ELISPOT stimulation and in the criteria for a positive assay. We used recombinant vaccinia virus vectors expressing clade A HIV-1 gag and env, the predominant HIV-1 clade circulating in Kenya. These vectors have been previously shown to be specific for HIV-1 and fail to stimulate a primary HIV-1–specific response in HIV-unexposed, uninfected persons [17]. However, vaccinia virus vector expression is dependent on infection of monocytes present in ELISPOT wells, a variable we were unable to control. Thus, we may have observed a lower frequency of virus-specific responses than if we stimulated with clade A–derived pools of overlapping peptides for HIV-1 gag and env in our assays. A potential confounding factor in comparing ELISPOT responses in blood and breast milk is the different cellular composition of the 2 compartments. Lymphocytes are difficult to enrich from breast milk, as opposed to peripheral blood, and, as a result, additional cell types are present in breast milk assays that are absent from peripheral blood assays. It is unlikely, however, that the contaminating cells (predominantly neutrophils) contribute to the HIV-1–specific secretion of IFN-γ measured in these assays.

To date there is little consensus on the definition of a positive ELISPOT assay result. We chose to apply the criterion of ≥50 HIV-specific sfu and ≥2 times the background to this data set on the basis of the published use of this definition and on a statistical analysis of 3 different criteria in other studies in infants within this cohort (authors’ unpublished data). When we applied a more stringent criterion of ≥100 HIV-specific sfu to our data, the prevalence of responses decreased by ~5%, and mean breast-milk responses remained higher than blood responses. Thus, with either definition, in women with responses detected in both compartments, mean HIV-1–specific responses in breast milk were higher than in blood. Our study complements and extends the findings of Sabbaj et al. [11] in providing population assessment of HIV-1–specific responses in breast milk by use of a different stimulation technique, with normalization of CD8+ lymphocyte numbers to compare levels of response in blood and breast milk.

Passive transfer of maternal antibody is a cornerstone of the infant’s immunity to infectious diseases (reviewed in [5]). The presence of lymphocytes in breast milk suggests that they also play a role in infant immunity. The precise nature of that role in mother-to-child transmission of HIV-1 is not clear but could conceivably be through reduction of cell-associated virus load in breast milk or via passive immunization of the neonate with maternal lymphocytes. CD8+ CTLs may be recruited to breast tissues in response to the presence of virus-infected cells, which, in turn, may result in a reduction in cell-associated virus load in milk. Alternatively, passive transfer of maternal lymphocytes may provide the neonate with partial immunity. Animal models have shown that maternally derived breast-milk lymphocytes are not recognized as non-self in the neonate and can cross the gut epithelium and traffic to lymphoid tissues [29–32]. In vitro studies and clinical observations support the hypothesis that maternal lymphocyte transfer also takes place in human newborns [33–35]. Transfer of HIV-1–specific CD8+ cells to the neonatal oral and gut mucosal surfaces may provide enhanced immunity to oral HIV-1 exposure before infection. HIV-1–specific CD8+ CTL responses have been detected in mucosal surfaces of HIV-1–exposed uninfected persons [20] and SIV-exposed seronegative rhesus macaques [36]. The rhesus macaque model has shown that SIV-specific CTL responses in gut-associated lymphoid tissue are correlated with protection against colonic or rectal SIV challenge [37, 38]. The exact mechanism of protection has not been clearly shown, but CTLs present at mucosal sites may be necessary for protection from HIV or SIV infection. Our study demonstrates that HIV-1–specific cellular immune responses in breast milk are present in nearly half of HIV-1–infected women and suggests that there is selective recruitment of HIV-1–specific lymphocytes to the breast. These results emphasize the need to determine the relevance of HIV-1–specific lymphocyte responses in breast milk to shedding of cell-associated HIV-1 in breast milk and to the risk of HIV-1 infection of the breast-feeding neonate.

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