MAJOR ARTICLE

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

Barbara L. Lohman,[1,4] Jennifer Slyker,[1] Dorothy Mbori-Ngacha,[2] Rose Bosire,[1] Carey Farquhar,[4,5] Elizabeth Obimbo,[2] Phelgona Otieno,[3] Ruth Nduati,[2] Sarah Rowland-Jones,[6] and Grace John-Stewart[1,4,5]

Departments of [1]Medical Microbiology and [2]Paediatrics, University of Nairobi, and [3]Kenya Medical Research Institute, Nairobi, Kenya; Departments of [4]Epidemiology and [5]Medicine, University of Washington, Seattle; [6]Institute of Molecular Medicine, Oxford University, Oxford, United Kingdom

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-

Received 20 March 2003; accepted 13 June 2003; electronically published 14 November 2003.

Presented in part: Keystone Symposia on HIV-1 Protection and Control by Vaccination, Keystone, Colorado, 5–11 April 2002 (abstract X8-306).

Financial support: National Institutes of Health (NIH; grants HD-23412 and HD-42949); B.L., J.S., C.F., E.O., and P.O. were scholars in the International AIDS Research and Training Program, which is supported by the Fogarty International Center (NIH grant D43-TW00007).

Reprints or correspondence: Dr. Grace John-Stewart, International AIDS Research and Training Program, University of Washington, 325 Ninth Ave., Box 359909, Seattle, WA 98104 (gjohn@u.washington.edu).

The Journal of Infectious Diseases 2003;188:1666–74

0022-1899/2003/18811-0008$15.00

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 (Tritest; 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 calf 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 (2×10^5) were added to wells of the blocked plates. Vaccinia virus (WR strain, negative control) or recombinant vaccinia viruses expressing either HIV-1 clade A $gp160^{env}$ *(env)* or $p55^{gag}$ *(gag)* (S.R.-J., National Institutes of Health AIDS Research and Reference Reagent Program, Be-

thesda, MD) were added to duplicate wells containing cells at an MOI of 2. HIV-1 clade A is the predominant HIV-1 clade in Nairobi [19]. Some breast-milk samples contained insufficient lymphocyte numbers to allow testing of both *env* and *gag* constructs. Assay control wells were stimulated with 10 μg/mL phytohemagglutinin (PHA; Murex Biotech) as a positive control and with R10 media alone as an additional negative control, to assess the contribution of background IFN-γ secretion in the absence of vaccinia virus stimulation. After overnight stimulation in a humidified incubator at 37°C with 5% CO_2 , cells were removed from the plates by washing with PBS containing 0.05% Tween 20 (Sigma), followed in sequence by the application 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: $\geqslant$50 HIV-specific sfu/10^6 cells and *gag* or *env* sfu $\geqslant$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) $\geqslant$50 HIV-specific sfu/10^6 cells and *gag*- or *env*-specific sfu of $\geqslant$2 times the strain WR negative control; and

Table 1. Clinical characteristics of human immunodeficiency virus (HIV) type 1–seropositive women studied for HIV-1–specific responses in breast milk.

Clinical parameter	All women (n = 53)	Women with HIV-1–specific lymphocytes in breast milk (n = 24)	Women without HIV-1–specific lymphocytes in breast milk (n = 29)	P[a]
CD4 cell count,[b] cells/mm^3	521 (499) [155–1167]	533 (559) [117–1167]	510 (473) [155–1137]	.8
CD4 cell %[b]	22 (22) [7–39]	23 (22) [9–39]	22 (23) [7–39]	.7
CD8 cell count,[b] cells/mm^3	1165 (1084) [451–2448]	1111 (1015) [451–2139]	1211 (1112) [567–2448]	.5
CD8 cell %[b]	50 (51) [22–79]	48 (46) [22–79]	52 (52) [32–70]	.2
CD4:CD8 cell ratio[b]	0.50 (0.48) [0.11–1.25]	0.55 (0.53) [0.11–1.15]	0.47 (0.44) [0.11–1.25]	.3
Plasma HIV-1 RNA load, $\log_{10}$ copies/mL[b]	4.8 (4.8) [2.2–7.9]	4.6 (4.8) [2.3–6.7]	5.0 (4.8) [2.2–7.9]	.2
History of HIV-related signs and symptoms,[c] no./total (%)	8/53 (15)	3/24 (13)	5/29 (17)	.6
HIV-1 transmission to infant,[d] no./total (%)	7/53 (13)	3/24 (13)	4/29 (14)	.6

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.

(2) ⩾100 HIV-specific sfu/10^6 cells and *gag*- or *env*-specific sfu of ⩾2 times the strain WR negative control.

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 cell ratios were 0.50 and 0.48, respectively (range, 0.11–1.25). Plasma HIV-1 load measurements were conducted 1 month postpartum for 50 women. The mean virus load was 4.8 $\log_{10}$ HIV-1 RNA copies/mL (median, 4.8 $\log_{10}$ HIV-1 RNA copies/mL), ranging from 2.24 to 7.94 $\log_{10}$ HIV-1 RNA copies/mL. Signs and symptoms related to HIV-1 infection (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) were assessed at enrollment to the study at ~28 weeks' gestation. The majority of the subjects (45/53 [85%]) in the cohort denied any history of HIV-1–related symptoms within the preceding year, whereas 8 (15%) reported experiencing either fever ($n = 1$), cough ($n = 3$), or itchy skin rash ($n = 4$) during the past year.

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-γ 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 blood, whereas differences in CD8 cell count, CD8 cell percentage, and rate of HIV-1 transmission to their infants were not significantly different ($P = .4$, $P = .4$, 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.

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

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-1p55^{gag} or gp160^{env}. Production of interferon-γ was detected in an ELISPOT assay. Positive assays were defined as HIV-specific spot-forming units (sfu) $\geq$50 and *gag*- or *env*-specific sfu $\geq$2 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 $\pm$ SE background response to vaccinia virus strain WR was 54 $\pm$ 25 sfu/10^6 cells in blood and 106 $\pm$ 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 $\pm$ 149 sfu/10^6 cells in blood versus 1384 $\pm$ 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 $\pm$ 244 in breast milk versus 376 $\pm$ 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 $\pm$ 189 HIV-specific sfu/10^6 cells, compared with 373 $\pm$ 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 ($\pm$SE) total lymphocyte percentage in peripheral blood of 41% $\pm$ 2.7%, whereas the paired breast-milk samples contained a mean of 7.6% $\pm$ 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

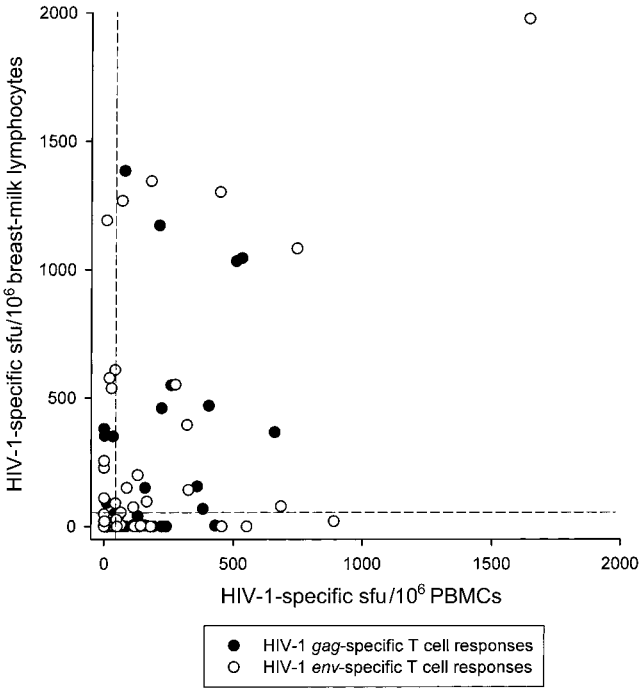

Figure 1. Magnitude of human immunodeficiency virus (HIV) type 1–specific responses in blood and breast milk. Paired samples of blood and breast-milk lymphocytes were tested for either HIV-1 *gag* or HIV-1 *env*. Horizontal axis indicates the level of the response in peripheral blood, and the vertical 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.

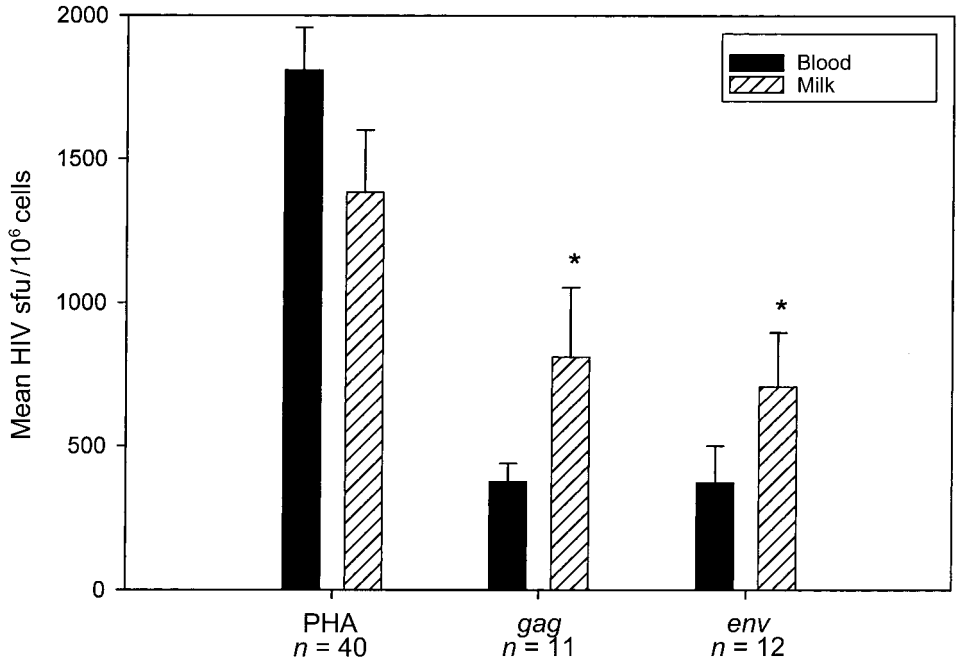

Figure 2. Magnitude of human immunodeficiency virus (HIV) type 1–specific cellular responses in peripheral blood and breast milk of HIV-1–infected women. Mean ± SE HIV-specific spot-forming units (sfu)/10^6 lymphocytes is plotted for 3 stimuli: phytohemagglutinin (PHA; positive control), HIV-1p55^{gag} *(gag),* and HIV-1gp160^{env} *(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* ($n = 11$) or *env* ($n = 12$). *$P \leq .05$.

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 10^6 lymphocytes or per 10^6 $CD3^+CD8^+$ lymphocytes was possible within this subset of subjects. The mean (±SE) number of *gag*-specific HIV-specific sfu/10^6 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 10^6 $CD8^+$ lymphocytes, the mean (±SE) numbers 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/10^6 lymphocytes in blood and 1197 ± 368 HIV-specific sfu/10^6 lymphocytes in breast milk–2.4-fold higher in breast milk. When expressed per 10^6 $CD8^+$ 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 10^6 $CD8^+$ 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/10^6 cells and ⩾100 HIV-specific sfu/10^6 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/10^6 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 (838 ± 264) compared with those in blood (406 ± 63) ($P = .10$).

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 p55^{gag} or gp160^{env}. Depletion of $CD8^+$ lymphocytes diminished these responses by

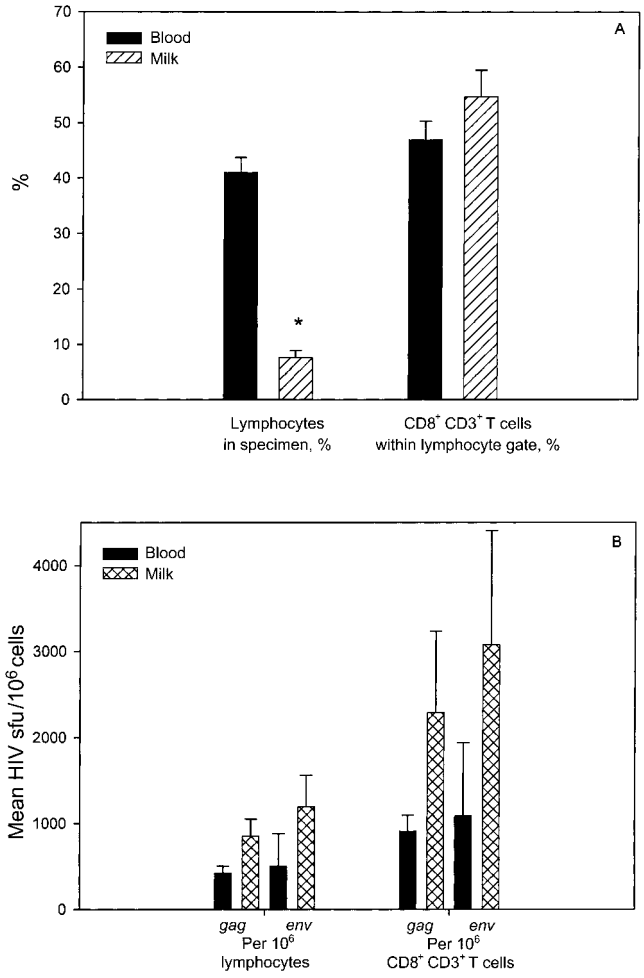

Figure 3. Comparison of total lymphocyte and $CD3^+CD8^+$ lymphocyte responses in breast milk and peripheral blood. *A,* Mean ± SE percentage of lymphocytes in blood and breast milk and percentage of $CD3^+CD8^+$ lymphocytes within the lymphocyte subset were determined by flow cytometry on 13 paired samples of blood and breast milk. $^*P < .05$. *B,* Cell-surface phenotype data were available from paired blood and breast-milk specimens with positive responses to either *gag* ($n = 6$) or *env* ($n = 4$). Mean ± SE human immunodeficiency virus–specific spot-forming units (sfu)/10^6 cells is plotted for paired samples of blood and breast milk.

~80%. Breast-milk responses were present in ~45% of women, with the highest prevalence of HIV-1–specific responses detected against *gag* at 2 weeks postpartum. Peripheral blood responses to either HIV-1 *gag* or *env* were present in ~65% of women, half of whom had detectable HIV-1–specific responses in their breast milk. Interestingly, 25% of women with HIV-1–specific responses in breast milk did not have responses detected in peripheral blood, consistent with possible compartmentalization of immune responses in breast milk. The prevalence and magnitude of HIV-1–specific lymphocyte responses in breast milk and peripheral blood were stable during the first month postpartum, a time period during which pregnancy-associated changes in hormone levels return to baseline.

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 ~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 ~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 ⩾100

HIV-specific sfu/10^6 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 $\geqslant$50 HIV-specific sfu and $\geqslant$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 $\geqslant$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.

Acknowledgments

We thank Julie Overbaugh (Fred Hutchison Cancer Research Center, Seattle) and her laboratory for providing data on maternal virus load and infant infection status; the women and infants of the cohort for their participation in this study; Nairobi City Council Antenatal clinic staff and Kenyatta National Hospital for their support; the clinicians, nurses, and peer counselors of the study for their dedication; and Anne Maingi for laboratory assistance.

References

1. Nduati R, John GC, Mbori-Ngacha D, et al. Effect of breast-feeding and formula feeding on transmission of HIV-1: a randomized clinical trial. JAMA **2000**; 283:1167–74.
2. Lewis P, Nduati R, Kreiss JK, et al. Cell-free human immunodeficiency virus type 1 in breast milk. J Infect Dis **1998**; 177:34–9.
3. Pillay K, Coutsoudis A, York D, Kuhn L, Coovadia HM. Cell-free virus in breast milk of HIV-1-seropositive women. J Acquir Immune Defic Syndr **2000**; 24:330–6.
4. Van de Perre P. Breast milk transmission of HIV-1. Laboratory and clinical studies. Ann NY Acad Sci **2000**; 918:122–7.
5. Lawrence RM. Host-resistance factors and immunologic significance of human milk. In: Fathman EM, ed. Breastfeeding: a guide for the medical profession. St. Louis: Mosby, **1999**:159–95.
6. Ogra SS, Ogra PL. Immunologic aspects of human colostrum and milk. II. Characteristics of lymphocyte reactivity and distribution of E-rosette

forming cells at different times after the onset of lactation. J Pediatr **1978**; 92:550–5.
7. Keler MA, Rodgriguez AL, Alvarez S, Wheeler NC, Reisinger D. Transfer of tuberculin immunity from mother to infant. Pediatr Res **1987**; 22:277–81.
8. Fishaut JM, Murphy D, Neifert M, McIntosh K, Ogra PL. Bronchomammary axis in the immune response to respiratory syncytial virus. J Pediatr **1981**; 99:186–91.
9. Ruben FL, Holzman IR, Fireman P. Responses of lymphocytes from human colostrum or milk to influenza antigens. Am J Obstet Gynecol **1982**; 143:518–22.
10. Losonsky G, Fishaut JM, Strussenber J, Ogra PL. Effect of immunization against rubella on lactation products. I. Development and characterization of specific immunologic reactivity in breast milk. J Infect Dis **1982**; 145:654–60.
11. Sabbaj S, Edwards BH, Ghosh MK, et al. Human immunodeficiency virus–specific $CD8^+$ T cells in human breast milk. J Virol **2002**; 76: 7365–73.
12. Farquhar C, VanCott TC, Mbori-Ngacha DA, et al. Salivary secretory leukocyte protease inhibitor is associated with reduced transmission of human immunodeficiency virus type 1 through breast milk. J Infect Dis **2002**; 186:1173–6.
13. Shaffer N, Chuahchoowong R, Mock PA, et al. Short-course zidovudine for perinatal HIV-1 transmission in Bangkok, Thailand: a randomized clinical trial. Bangkok Collaborative Perinatal HIV Transmission Study Group. Lancet **1999**; 353:773–80.
14. DeVange Panteleeff D, John GC, Nduati R, et al. Rapid method for screening dried blood samples on filter paper for human immunodeficiency virus type 1 DNA. J Clin Microbiol **1999**; 37:350–3.
15. Emery S, Bodrug S, Richardson BA, et al. Evaluation of performance of the Gen-Probe human immunodeficiency virus type 1 viral load assay using primary subtype A, C, and D isolates from Kenya. J Clin Microbiol **2000**; 38:2688–95.
16. Lalvani A, Brookes R, Wilkinson S, et al. Human cytolytic and interferon gamma–secreting $CD8^+$ T lymphocytes specific for mycobacterium tuberculosis. Proc Natl Acad Sci USA **1998**; 95:270–5.
17. Larsson M, Jin X, Ramratnam B, et al. A recombinant vaccinia virus based ELISPOT assay detects high frequencies of Pol-specific CD8 T cells in HIV-1–positive individuals. AIDS **1999**; 13:767–77.
18. Kaul R, Dong T, Plummer FA, et al. $CD8^+$ lymphocytes respond to different HIV epitopes in seronegative and infected subjects. J Clin Invest **2001**; 107:1303–10.
19. Neilson JR, John GC, Carr JK, et al. Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. J Virol **1999**; 73:4393–403.
20. Kaul R, Plummer FA, Kimani J, et al. HIV-1–specific mucosal $CD8^+$ lymphocyte responses in the cervix of HIV-1–resistant prostitutes in Nairobi. J Immunol **2000**; 164:1602–11.
21. Scott ZA, Chadwick EG, Gilbson LL, et al. Infrequent detection of HIV-1–specific, but not cytomegalovirus-specific, $CD8^+$ T cell responses in young HIV-1–infected infants. J Immunol **2001**; 167:7134–40.
22. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MBA. Virus-specific $CD8^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol **1994**; 68:6103–10.
23. Koup RA, Safrit JT, Cao Y, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol **1994**; 68:4650–5.
24. Kuroda MJ, Schmitz JE, Charini WA, et al. Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. J Immunol **1999**; 162:5127–33.
25. Schmitz JE, Kuroda MJ, Santa S, et al. Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. Science **1999**; 283:857–60.
26. Addo MM, Yu XG, Rathod A, et al. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)–specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol **2003**; 77:2081–92.
27. Buseyne F, Scott-Algara D, Porrot F, et al. Frequencies of ex vivo–activated human immunodeficiency virus type 1–specific gamma-interferon–producing $CD8^+$ T cells in infected children correlate positively with plasma viral load. J Virol **2002**; 76:12414–22.
28. Rousseau CM, Nduati RW, Richardson BA, et al. Longitudinal analysis of human immunodeficiency virus type 1 RNA in breast milk and of its relationship to infant infection and maternal disease. J Infect Dis **2003**; 187:741–7.
29. Zhang L, van Bree S, van Rood JJ, Claas FHJ. Influence of breast feeding on the cytotoxic T cell allorepertoire in man. Transplantation **1991**; 52:914–6.
30. Weiler IJ, Hickler W, Sprenger R. Demonstration that milk cells invade suckling neonatal mice. Am J Reprod Immunol **1983**; 4:95–8.
31. Jain L, Vidyasagar D, Xanthou M, Ghai V, Shimada S, Blend M. In vivo distribution of human milk leucocytes after ingestion by newborn baboons. Arch Dis Child **1989**; 64:930–23.
32. Nepomnaschy I, Deroche A, Pasqualini CD, Piazzon I. Maternal influence on the immune response: SMLC reactions between identical and reciprocal F1 hybrids and the role of lactation. Immunol Lett **1988**; 18:19–26.
33. Schlesinger JJ, Covelli HD. Evidence for transmission of lymphocyte responses to tuberculin by breastfeeding. Lancet **1977**; 2:529–32.
34. Ogra SS, Weintraub D, Ogra PL. Immunologic aspects of human colostrum and milk. III. Fate and absorption of cellular and soluble components in the gastrointestinal tract of the newborn. J Immunol **1977**; 119:245–8.
35. Michie CA, Tantscher E, Rot A. The long term effects of breastfeeding: a role for the cells in breast milk. J Trop Pediatr **1998**; 44:2–3.
36. Wilson LA, Murphey-Corb M, Martin LN, Harrison RM, Ratterree MS, Bohm RP. Identification of SIV *env*–specific CTL in the jejunal mucosa in vaginally exposed, seronegative rhesus macaques *(Macaca mulatta)*. J Med Primatol **2000**; 29:173–81.
37. Murphey-Corb M, Wilson LA, Trichel AM, et al. Selective induction of protective MHC class I–restricted CTL in the intestinal lamina propria of rhesus monkeys by transient SIV infection of the colonic mucosa. J Immunol **1999**; 162:540–9.
38. Fuller DH, Rajakumar PA, Wilson LA, et al. Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. J Virol **2002**; 76:3309–17.