High Cell-Free Virus Load and Robust Autologous Humoral Immune Responses in Breast Milk of Simian Immunodeficiency Virus-Infected African Green Monkeys


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The design of immunologic interventions to prevent postnatal transmission of human immunodeficiency virus (HIV) will require identification of protective immune responses in this setting. Simian immunodeficiency virus (SIV)-infected rhesus monkeys (RMs), a species that develops an AIDS-like illness following experimental infection, transmit the virus at a high rate during breastfeeding. In contrast, postnatal transmission of SIV occurs rarely or not at all in natural, asymptomatic primate hosts of SIV. These contrasting transmission patterns provide a unique opportunity to study mechanisms that evolved to protect suckling infants from SIV infection. We compared the virologic and immunologic properties of milk of SIV-infected and uninfected natural hosts of SIV, African green monkeys (AGMs), to that of RMs. Interestingly, despite a low number of milk CD4+ T lymphocytes in uninfected AGMs, milk virus RNA load in SIV-infected AGMs was comparable to that of SIV-infected RMs and that in AGM plasma. This observation is in contrast to the relatively low virus load in milk compared to that in plasma of SIV-infected RMs and HIV-infected women. Milk of SIV-infected AGMs also displayed robust virus-specific cellular immune responses. Importantly, an autologous challenge virus-specific neutralization response was detected in milk of five of six SIV-infected AGMs that was comparable in magnitude to that in plasma. In contrast, autologous challenge virus neutralization was not detectable in milk of SIV-infected RMs. The autologous virus-specific adaptive immune responses in breast milk of AGMs may contribute to the rarity of postnatal transmission in the infant oral/gastrointestinal tract and the rarity of maternal infection.

Human immunodeficiency virus (HIV) transmission through breastfeeding remains an important mode of infant HIV acquisition in the developing world, accounting for nearly half of the 350,000 new infant HIV infections occurring annually (31). While studies of maternal or infant antiretroviral therapy during the period of breastfeeding are promising for reduction of infant HIV infections (11, 27, 52, 55), a small number of postnatal virus transmissions continue to occur in the setting of optimal antiretroviral prophylaxis. Furthermore, maternal and infant toxicities, barriers to implementation, and the impact of the development of antiretrovirus-resistant virus strains during maternal or infant antiretroviral prophylaxis have not been evaluated. Therefore, development of immunologic strategies to reduce HIV transmission via breast milk remains critical to improving HIV-free survival of infants born to HIV-infected mothers in the developing world.

Interestingly, approximately only 10% of HIV-infected mothers will transmit the virus via breastfeeding in the absence of antiretroviral prophylaxis, despite up to 2 years of daily low-dose oral exposure of the infant (16, 31, 43). This low rate of transmission suggests that the immunologic milieu in milk of HIV-infected women may contribute to protection against virus acquisition. HIV/simian immunodeficiency virus (SIV)-specific cellular and humoral immune responses have been identified in milk (2, 5, 15, 26, 37, 40, 46); however, the role of these responses in protection against virus transmission is not known. Further, as the cell-associated and cell-free virus loads in milk both correlate with the risk of infant HIV acquisition (22, 45, 51), it is not known which pool of virus initiates infection. Therefore, defining the role of virus-specific immune responses and the contribution of cell-free and cell-associated virus to HIV transmission via breastfeeding is crucial for the design of immunologic interventions to prevent infant HIV infection.

The nonhuman primate pathogenesis model of HIV/AIDS, SIV infection of rhesus monkeys (RMs), allows investigation of virus pathogenesis and transmission, as this species develops an AIDS-like illness within 2 years of infection and vertically transmits the virus in utero (12) and via breastfeeding (1). SIV is transmitted to the majority of suckling infants of SIV-infected RMs during both acute and chronic infection (1, 2), despite a milk virus load that remains 1 to 2 logs lower than that in plasma throughout the infection (2, 37). While robust virus-specific cellular immune responses are detected in the milk of SIV-infected RMs, virus-specific antibody responses...
are of low magnitude in milk of RMs (37, 40). Furthermore, we have detected evidence of transient local virus replication and virus escape of cytotoxic T lymphocyte responses in the breast milk compartment of RMs (39), virus mechanisms that may contribute to the high rate of postnatal infant virus transmission in this species.

African-origin nonhuman primate hosts of SIV, monkeys that are naturally infected with SIV in the wild, have evolved to sustain a nonprogressive SIV infection and maintain a normal life span (36, 53, 54). Importantly, in contrast to the high rate of postnatal SIV transmission in RMs, natural hosts of SIV do not or only extremely rarely transmit the virus via breastfeeding (32, 35, 48). This lack of postnatal transmission is apparent despite high levels of virus in blood and milk of lactating maternal natural hosts of SIV (35). The lack of breastfeeding transmission of SIV to the infants of African green monkeys (AGMs) has been described observationally in the wild (32), where infants remain uninfected until sexual maturity. Furthermore, acute SIV infection of lactating mandrill monkeys, a natural host species of SIV, resulted in no SIV transmission to the infants despite high level of virus RNA in the milk (35).

The distinct infant transmission patterns of nonpathogenic and pathogenic SIV infection comprise a unique feature in which to investigate mechanisms that naturally evolved to block infant virus acquisition, despite chronic oral virus exposure. Experimental infection of AGMs with an endemic SIV strain is a robust model in which to study the virus pathogenesis and immune responses of natural SIV infection (33). Furthermore, we have established a protocol to induce lactation in nonpregnant RMs and validated this model for assessment of the immunologic and virologic parameters of milk during SIV infection, avoiding reliance on primate breeding patterns (37–40). In this study, we employed this lactation model in uninfected and SIV-infected female AGMs and compared the SIV-specific immune responses and virus loads to those of postnatally transmitting symptomatic hosts of SIV, RMs. Defining the mechanisms that evolved to protect infants of natural hosts of SIV against virus transmission via breastfeeding is important to the design of strategies to prevent infant HIV acquisition.

### MATERIALS AND METHODS

#### Animals, infection, and induction of lactation.

Four cohorts of monkeys underwent induction of lactation as previously described (37): six uninfected female AGMs, six female AGMs chronically infected with SIVab9351BR as previously described (63) (3 years after infection), six uninfected female RMs, and six female AGMs chronically infected with SIVmac251 as previously described (59). All reactions were performed in duplicate on the 7700 fast real-time PCR system (Applied Biosystems) with a 2× SYBR Green Master Mix (Ambion), quantitated by optical density (OD), and serially diluted to generate a standard curve. The sensitivity of the assay is 450 copies per milliliter. To quantify milk and plasma SIV RNA loads in RMs, SIVmac251 quantitative real-time PCR was similarly performed as previously described (37). RNA copies per milliliter were determined by dividing the copy number by the volume of milk used for the extraction.

To calculate cell-associated proviral loads in AGMs and RMs, PBMC and breast milk cell DNA was isolated using the QIAaMP DNA kit and eluted in 100 μl of RNase-free water. A total of 30 μl of the RNA suspension was used in a reverse transcriptase (RT) reaction mixture containing SuperScript III RT enzyme (Invitrogen) and the SIVab9351BR long terminal repeat (LTR) primer 5'-AACGCTTGGAGCTGATATTA-3' (37), according to the manufacturer’s protocol. A quantity amounting to 10 μl of the resulting cDNA was used in a real-time PCR using the TaqMan EZ RT-PCR kit (Applied Biosystems) as well as an LTR-specific labeled probe [FAM]-AGGTGCTTGGCCCGTGTAAACAGTC-Black Hole Quencher (BHQ) and a reporter primer (5’-CCCCCGTCTGAAGTTAAACAG-3') (10). An RNA standard was transcribed from a plasmid containing the SIVab9351BR LTR gene by using the Megascript 7 Kit (Ambion), quantitated by optical density (OD), and serially diluted to generate a standard curve. The sensitivity of the assay was 450 copies per milliliter. To quantify milk and plasma SIV RNA loads in RMs, SIVmac251 quantitative real-time PCR was similarly performed as previously described (37). RNA copies per milliliter were determined by dividing the copy number by the volume of milk used for the extraction.

#### Phenotyping and intracellular cytokine staining of mucosal, breast milk, and PBMC.

Lymphocyte phenotyping was performed by staining milk, colon, vaginal, and bronchial alveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMC) with anti-CD4-peridinin chlorophyll protein (PerCP)-Cy5.5 (L200 [BD Biosciences]), anti-CD3-Pacific Blue (SP34.2 [BD Biosciences]), anti-CD8-allophycocyanin (APC)-H7 (560179 [BD Biosciences]), and anti-CCR5-phycocerythrin (PE) (IA9 [BD Biosciences]).

Intracellular cytokine staining was performed after stimulation of PBMC and breast milk cells of AGMs with 15-mer peptides overlapping by 11 amino acids spanning the SIVab9351BR Gag protein, as well as the positive control, Staphylococcus enterotoxin B (SEB), as previously described (37), and intracellular staining with the following additional antibodies: anti-tumor necrosis factor alpha (TNF-α)-Pacific Blue (Mab11 [eBiosciences]), anti-gamma interferon (IFN-γ)-PE-Cy7 (B27 [Becton Dickinson]), and anti-interleukin-2 (IL-2)-APC (MQ1-17H12 [Becton Dickinson]). The proportion of cytokine-producing cells was determined by subtracting the proportion of unstimulated cytokine-producing cells from the proportion of Gag-stimulated or SEB-stimulated cytokine-producing cells. A positive response was defined as two times that of the unstimulated control. An amine dye (Red Amine) was used to distinguish live from dead cells in all flow cytometric analyses. Only milk samples with a CD8+ cell count of >100 cells for each condition were used in the analyses. Adequate numbers of CD4+ cells were not present in milk samples to include in the analysis. Data were collected on an LSRII flow cytometer (BD Biosciences) with FACSDiva software and analyzed with FlowJo software.
lymphocytes expressing the virus coreceptor CCR5 was also extremely low in the uninfected AGMs (median, 0.01%; range, 0 to 0.2 CD4⁺ CCR5⁺ T lymphocytes/μl) compared to that in uninfected RMs (median, 0.2%; range, 0.1 to 6.1 CCR5⁺ CD4⁺ T lymphocytes/μl; P = 0.008).

Despite the very low proportion of CD4⁺ T lymphocytes in milk of AGMs, the proportion of CD8⁺ T lymphocytes in milk of AGMs (median, 55%; range, 41.9 to 84.1%) was similar to that of RMs (median, 32.9%; range, 29.1 to 67.2%; P = 0.13) (Fig. 2C). Accordingly, the absolute numbers of CD8⁺ T lymphocytes in milk were also comparable in AGMs (median, 16,330; range, 471 to 31,700 CD8⁺ T lymphocytes/μl) and RMs (median, 3,941; range, 817 to 11,680 CD8⁺ T lymphocytes/μl; P = 0.25). Therefore, we examined the milk CD4⁺ CD8⁻ T lymphocyte population, which was previously noted to comprise a considerable proportion of the T lymphocytes in mucosal compartments of natural hosts of SIV (4). Importantly, the proportion of T lymphocytes that did not express the CD4 or CD8 molecule was significantly higher in milk of AGMs (median, 42.7%; range, 14 to 54.6% CD4⁻ CD8⁻ T lymphocytes) than in that of RMs (median, 5.9%; range, 0 to 10.5% CD4⁻ CD8⁻ T lymphocytes; P = 0.004) (Fig. 2E). Furthermore, the absolute number of double-negative T lymphocytes in milk of AGMs (median, 2,511; range, 616 to 49,250 CD4⁻ CD8⁻ T lymphocytes/μl) trended significantly higher than that of RMs (median, 346; range, 0 to 3,934 CD4⁻ CD8⁻ T lymphocytes/μl; P = 0.08) (Fig. 2F).

The low proportion of CD4⁺ T lymphocytes in milk of uninfected AGMs is not unique to the breast milk compartment in this species (4, 34), as the median proportion of CD4⁺ T lymphocytes in milk (2.0%; range, 1.1 to 10.5%) was similar to that in other mucosal compartments (median proportion in colon, 2.9% [range, 1.9 to 7.4%]; duodenum, 5.5% [range, 2.7 to 12.5%]; vagina, 1.8% [range, 0.4 to 6.2%]; BAL, 1.2% [range, 0.8 to 9.3%]; P > 0.43 for all) but trended lower than that in peripheral blood (median proportion, 23.6%; range,
15.1 to 28.8%; \( P = 0.06 \)) (Fig. 3A). Accounting in part for this low proportion of CD4\(^+\) T lymphocytes in mucosal compartments of natural hosts of SIV is the large population of CD4\(^+\)CD8\(^-\) T lymphocytes present in all mucosal compartments (4). The median proportion of T lymphocytes that did not express the CD4 or CD8 molecule trended significantly higher in milk (42.7%; range, 14 to 54.7%) than in blood (15.7%; range, 2.2 to 23%; \( P = 0.06 \)) as well as in colon (10.9%; range, 1.3 to 30.4%), duodenum (7.5%; range, 1.9 to 21%), and vagina (23.8%; range, 11.3 to 45.2%; \( P = 0.06 \) for all) (Fig. 3B).

We next investigated the virus target cell population present in milk of chronically SIV-infected AGMs by inducing lactation in six SIV-infected female AGMs (63). Despite the well-described CD4\(^+\) T lymphocyte depletion during acute and chronic SIV infection of pathogenic hosts (9, 29, 57), chronically SIV-infected RMs maintained a significantly high proportion of CD4\(^+\) T lymphocytes in milk during chronic infection (median proportion, 4.1%; range, 1.6 to 11.8%) compared to SIV-infected AGMs (median proportion, 0.6%; range, 0.1 to 2.3%; \( P = 0.02 \)) (Fig. 4A). However, due to lower absolute number of T lymphocytes in milk of SIV-infected RMs, the absolute numbers of CD4\(^+\) T lymphocytes did not differ significantly in milk of chronically SIV-infected AGMs (median, 182 cells/\( \mu l \); range, 93 to 2,011 cells/\( \mu l \)) and RMs (median, 264 cells/\( \mu l \); range, 113 to 3,335 cells/\( \mu l \); \( P = 0.76 \)) (Fig. 4B).

**High SIV RNA load in milk of SIV-infected AGMs.** We reasoned that the low proportion of CD4\(^+\) T lymphocytes in milk of AGMs may result in low cell-free and cell-associated virus loads in milk of this species, possibly accounting for the lack of virus transmission via breastfeeding. Surprisingly, despite a low SIV RNA load in peripheral blood of chronically SIV-infected AGMs (median, 1.8 \( \times 10^4 \); range, 4.4 \( \times 10^3 \) to 4.8 \( \times 10^5 \) copies/ml) compared to what was seen for RMs (median, 6.9 \( \times 10^6 \); range, 8.8 \( \times 10^5 \) to 3.7 \( \times 10^7 \) copies/ml; \( P = 0.009 \)) (Fig. 5A), we observed virus RNA loads that were similar in milk of AGMs (median, 3.8 \( \times 10^4 \); range, 7.1 \( \times 10^2 \) to 7.6 \( \times 10^5 \) copies/ml) and RMs (median, 3.1 \( \times 10^5 \); range, 6.8 \( \times 10^4 \) to 7.9 \( \times 10^5 \) copies/ml; \( P = 0.17 \)) (Fig. 5A). Moreover, while the virus RNA loads in milk of RMs and humans remain 1 to 2 logs lower than that in plasma throughout acute and chronic infection (37, 45), the SIV RNA load in milk of chronically infected AGMs trended toward a magnitude higher than that in blood (median, 1.8 \( \times 10^6 \); range, 4.3 \( \times 10^5 \) to 4.8 \( \times 10^6 \) copies/ml; \( P = 0.09 \)) (Fig. 5A).

The proportion of SIV-infected cells in milk of AGMs (median, 46; range, 4 to 124 SIV\(^+\) cells/10\(^5\) cells) was equal to that in blood (median, 58; range, 34 to 139 SIV\(^+\) cells/10\(^5\) cells; \( P = 0.84 \)) (Fig. 5B).
RMs. Consistent with the low proportion of CD4\(^+\) T lymphocytes in milk of SIV-infected AGMs, the proportion of SIV-infected cells was 1 log lower in the milk of AGMs than that in the milk of RMs (\(P = 0.04\)) (Fig. 5B).

**SIV-specific cellular immune responses are present in breast milk of SIV-infected, lactating AGMs.** HIV/SIV-specific CD8\(^+\) T lymphocyte immune responses are known to be critical in containment of systemic virus replication of pathogenic HIV/SIV infection (13, 20, 49) and may contribute to partial containment of systemic virus replication in natural hosts of SIV (18, 25, 50, 62, 63). However, the role of mucosal virus-specific cellular immune responses in the containment of local virus replication and protection against virus transmission is not known. Therefore, we assessed the SIV Gag-specific CD8\(^+\) T lymphocyte response in milk of chronically SIV-infected AGMs by intracellular cytokine staining in three AGMs with a milk T lymphocyte number adequate to perform this analysis. SIV Gag-specific CD8\(^+\) T lymphocyte cytokine responses were detectable only in the blood of one of three AGMs (Fig. 6A), despite detectable superantigen cytokine responses against SEB in blood of all three AGMs (Fig. 6C). In contrast, both SEB and SIV Gag-specific CD8\(^+\) T lymphocyte cytokine responses were detectable in milk of all three AGMs (Fig. 6B and D). In fact, the proportion of milk CD8\(^+\) T lymphocytes producing IFN-\(\gamma\) and TNF-\(\alpha\) following SIVsab Gag stimulation in the single AGM with detectable SIV-specific CD8\(^+\) T lymphocyte responses in blood was approximately 10-fold higher than the responses measured in the blood of this animal (Fig. 6A and C). Therefore, despite a virus load in milk that is similar to that in plasma of AGMs, the virus-specific cellular immune responses appear to be considerably more robust in milk than in blood of these natural SIV hosts.

**High-titer autologous virus-specific neutralizing antibody responses in milk of SIV-infected AGMs.** Finally, we investigated the humoral immune responses in milk of chronically SIV-infected AGMs that may contribute to the protection of suckling infants from virus transmission. We first assessed the neutralizing antibody responses in milk and plasma of AGMs and RMs against a tier 1 T cell line-adapted (TCLA) SIVmac251 in an envelope pseudovirus TZM.bl neutralization assay. The neutralizing antibody responses against this neutralization-sensitive heterologous SIVmac251 virus were 1 log lower in milk of AGMs (median, 383; range, 91 to 716 ID\(_{50}\)) (Fig. 7A) than in that of RMs (median, 3,281; range, 1,998 to 17,456 ID\(_{50}\); \(P = 0.009\)) (Fig. 7B) and were approximately 2 logs lower than that in the plasma of each species. We then assessed the autologous challenge virus (SIVsab9351BR)-specific neutralizing antibody responses in milk and plasma of AGMs. Remarkably, autologous challenge virus-specific neutralization responses were detectable in milk of five of six chronically SIV-infected AGMs (median, 383; range, 91 to 716 ID\(_{50}\)) (Fig. 7A) than in that of RMs (median, 3,281; range, 1,998 to 17,456 ID\(_{50}\); \(P = 0.009\)). The neutralization responses were normalized for total antibody.

**FIG. 3.** High proportion of CD4\(^+\) T lymphocytes in milk of uninfected AGMs compared to blood and mucosal gastrointestinal compartments. (A) Proportion of CD4\(^+\) T lymphocytes in milk of uninfected AGMs (open symbols) compared to blood and other mucosal compartments (closed symbols). (B) Proportion of CD4\(^+\)CD8\(^-\) T lymphocytes in milk of uninfected AGMs (open symbols) compared to blood and other mucosal compartments (closed symbols). Solid lines indicate medians. Paired comparisons with milk values reaching the lowest obtainable \(P\) value (\(n = 5, P = 0.06\)) are indicated.

**FIG. 4.** CD4\(^+\) T lymphocyte proportion is significantly lower in milk of chronically SIV-infected AGMs than RMs. Proportions (A) and absolute numbers (B) of CD4\(^+\) T lymphocytes in milk (open symbols) and blood (closed symbols) of AGMs (circles) and RMs (squares). Solid lines indicate medians. Comparisons of blood and milk between species with \(P\) values of <0.05 are indicated.
content in milk and plasma, the heterologous TCLA SIVmac251 neutralization responses in milk of AGMs [median, 50.2; range, 9.7 to 336.4 ID50/(mg/ml)] remained significantly lower than that in plasma [median, 2,887; range, 1,942 to 4,638 ID50/(mg/ml); \(P = 0.03\)] (Fig. 7E). The heterologous TCLA SIVmac251 neutralization response in milk [median, 2,380; range, 1,280 to 2,951 ID50/(mg/ml)] was also consistently, yet nonsignificantly, lower than that in plasma of RMs [median, 8,156; range, 6,450 to 11,429 ID50/(mg/ml); \(P = 0.12\)] (Fig. 7F). However, the autologous challenge virus-specific neutralization responses in AGMs remained equal in magnitude in both milk [median, 26.7; range, <10 to 116 ID50/(mg/ml)] and plasma [median, 76.2; range, 2.5 to 422.9 ID50/(mg/ml); \(P = 0.31\)] (Fig. 7G) after normalization for antibody content.

The similarity of the magnitudes of the high-titer autologous neutralization responses in milk and plasma of SIV-infected AGMs is quite surprising, as the total IgG concentration in milk of SIV-infected AGMs (median, 0.36; range, 0.15 to 0.38 mg/ml) was >1 log lower than that in plasma (median, 10.7; range, 5 to 14.2 mg/ml; \(P = 0.03\)) (Fig. 8A). However, the total IgA concentration in milk of SIV-infected AGMs (median, 3.4; range, 1 to 31.2 mg/ml) was similar to that in plasma (median, 4.3; range, 3.9 to 8.3 mg/ml; \(P = 1.0\)) (Fig. 8C). The comparability of IgA content in milk and plasma of SIV-infected AGMs is in contrast to what was seen for SIV-infected RMs, as RM milk contains approximately 1 log less total IgA (median, 1.1; range, 0.85 to 7 mg/ml) than RM plasma does (median, 17.7; range, 14.9 to 26 mg/ml; \(P = 0.06\)) (Fig. 8D). Furthermore, milk of SIV-infected AGMs (median, 3.8; range, 1.4 to 31.4 mg/ml) has a total immunoglobulin content that is similar to that in plasma (median, 14.7; range, 9.4 to 22.5 mg/ml; \(P = 0.31\)) (Fig. 8E), whereas milk of SIV-infected RMs (median, 79.3; range, 72.6 to 86.3 mg/ml) has nearly 2 logs less total immunoglobulin compared to plasma (median, 1.4; range, 1.2 to 8.4 mg/ml; \(P = 0.06\)) (Fig. 8F). Therefore, we assessed the contribution of the IgG and IgG-depleted fractions of milk to neutralization of the autologous challenge virus in two AGMs with milk volume adequate to perform the IgG isolation. Neutralization of the autologous challenge virus was detected in the milk IgG fractions of both AGMs (IC50, 7.37 μg/ml and

![FIG. 5. SIV RNA load in milk of chronically SIV-infected AGMs is similar to that in plasma and that of chronically SIV-infected RMs. SIV RNA load (A) and proportion of SIV-infected cells (B) in milk (open symbols) and plasma (closed symbols) of SIV-infected AGMs (circles) and RMs (squares). Solid, horizontal lines indicate medians; dashed lines indicate milk and blood values from the same monkey. Comparisons with \(P\) values of <0.05 are indicated.](http://jvi.asm.org/)

![FIG. 6. Robust SIV-specific CD8+ T lymphocyte responses in breast milk of three chronically SIV-infected AGMs, measured by intracellular cytokine staining. SIV-specific CD8+ T lymphocyte responses in blood (A) and milk (B) of SIV-infected AGMs. Global T lymphocyte responses in blood (C) and milk (D) of SIV-infected AGMs. SEB, Staphylococcus endotoxin B superantigen, positive control and indicator of global T lymphocyte responses.](http://jvi.asm.org/)
28.3 μg/ml) at IC_{50} values similar to those of AGM plasma IgG (IC_{50}, 63.6 μg/ml and 42.2 μg/ml). However, no autologous virus neutralization was detected in the IgG-depleted fraction of milk, indicating that the milk IgA of AGMs does not appear to mediate the majority of the autologous neutralization response detected in milk of SIV-infected AGMs.

**DISCUSSION**

The lack of postnatal virus transmission in natural hosts of SIV offers an opportunity to define mechanisms that evolved to protect infants against virus acquisition via breastfeeding. Similar to the strong link between HIV plasma RNA load and heterosexual virus transmission (41), the maternal plasma and milk virus load has been associated with the risk of HIV transmission via breastfeeding (22, 45, 51). However, the apparent lack of SIV transmission via breastfeeding in AGMs is not attributable to low-level virus exposure of the infants, as our study revealed that maternal milk virus RNA load in non-pathogenic SIV infection of AGMs is comparable to that of pathogenic SIV infection in RMs. Therefore, the breastfeeding infants of SIV-infected AGMs are a highly exposed, uninfected population, warranting evaluation of the factors contributing to the impediment of virus transmission in this setting. Elucidation of mechanisms that evolved to protect breastfeeding infants of natural hosts of SIV from postnatal SIV infection will inform immunologic strategies to reduce HIV transmission via breastfeeding.

There are a number of infant and maternal factors that could contribute to the low probability of HIV transmission via breastfeeding in humans. Breast milk not only contains robust virus-specific adaptive immune responses but also contains a wealth of innate immune factors, including cytokines (58), antiviral glycoproteins such as lactoferrin (6), and secretory leukocyte peptidase inhibitor (SLPI) (30), which could contribute to the impediment of virus transmission in the infant gut. While the site of virus entry is not defined, the virus must remain viable and retain the ability to traverse a mucosal barrier upon exposure to the infant saliva, the gastric environment, and/or the mucus-covered intestinal tract. Finally, a virion that successfully traverses the infant gastrointestinal mucosal barrier must evade the infant innate and adaptive
mucosal immune responses and contact a CD4+ target cell in order to establish a systemic infection. Therefore, it is not surprising that HIV transmission via breast milk in humans is not a common occurrence, despite daily, chronic virus exposure. However, as HIV transmission continues to occur at a steady rate throughout breastfeeding (23) despite all of the possible impediments, it is striking that with exposure to a similar quantity of virus in milk, infants of AGMs are subject to negligible or nonexistent risk of virus acquisition (32, 35, 48).

It has been suggested that natural SIV host infants do not become vertically infected due to the low-level expression of the virus coreceptor CCR5 in infant CD4+ T lymphocyte populations (35). However, SIV infection of the natural host species sooty mangabeys has been initiated in the setting of CCR5-null mutations (44). Moreover, primary SIV infection is known to occur in juvenile natural hosts through bite wounds or the onset of sexual behavior in the wild (32). Therefore, the infant and maternal factors contributing to protection against vertical transmission are not clear. While the susceptibility of infants of natural SIV hosts to SIV infection is worthy of investigation, these investigations are limited by access to infants, expense, and breeding cycles. However, the induction of lactation in nonbreeding monkeys has been shown to produce milk that is immunologically similar to that of naturally lactating monkeys (37). Therefore, initiating investigations of the factors associated with the lack of postnatal transmission in the maternal natural hosts of SIV will guide future investigations of the susceptibility of infants.

The comparable milk virus RNA loads of SIV-infected AGMs and RMs was unexpected, as we detected few CD4+ T lymphocytes that could sustain local virus replication in the breast milk compartment of uninfected AGMs. This unexpected finding of equivalent SIV RNA loads in milk of AGMs and RMs indicates that the lack of virus transmission via breastfeeding in natural hosts is not accounted for by low cell-free virus load in milk. Furthermore, the similarity of magnitudes of the virus RNA loads in milk and plasma of AGMs is in contrast to the relatively low magnitude of the milk virus load in RMs and humans, suggesting origin and replication kinetics of virus in milk of natural SIV hosts that are distinct from those of postnatafly transmitting hosts (17, 21, 37). In contrast, the SIV cell-associated virus load was higher in the milk of postnatally transmitting RMs than in that of the nontransmitting AGMs. The high proportion of SIV-infected cells in the milk of RMs compared to that of AGMs mirrors the difference in the proportion of infected cells in the blood of these species. The low cell-associated virus load in milk of the AGMs may account for the lack of virus transmission via breastfeeding in this species. Moreover, this finding may suggest that the cell-associated virus pool in milk is more likely to be responsible for postnatal infant HIV acquisition. Interestingly, the milk of AGMs contained a high proportion of CD4+ CD8- T lymphocytes. This double-negative T lymphocyte population prevalent in mucosal compartments of uninfected AGMs may have evolved in this species in the setting of endemic SIV to reduce the number of target cells of SIV (4). This mechanism of maintaining nonprogressive SIV infection could also result in an inability of cell-associated SIV to be transmitted via breastfeeding. Finally, while there are similar absolute numbers of CD4+ T lymphocytes in milk of SIV-infected RMs and AGMs, the gastrointestinal tract of uninfected AGMs contains a low proportion of CD4+ T lymphocyte target cells (4), which may account for the low likelihood of virus transmission via oral exposure in this species.

HIV-specific cellular immune responses have been identified in milk of HIV-infected women and SIV-infected RMs (37, 46). However, the role of these mucosal immune responses in protection against virus transmission is not known. As milk appears to be partially populated by virus produced by transient virus replication in the breast milk compartment (17, 21, 39, 47), the milk virus-specific CD8+ T lymphocyte response has the potential to target locally replicating virus. We observed a robust virus-specific cellular immune response in milk of chronically SIV-infected AGMs similar to that described for milk of chronically SIV-infected RMs (37). However, as both the transmitting and nontransmitting species display robust cellular immune responses in milk, this response may not confer the observed protection against infant SIV transmission in the natural hosts.

Despite evidence that neutralization-resistant viruses are vertically transmitted both perinatally (14) and postnatally (42), the function of virus-specific humoral immune responses in milk and their roles in virus transmission via breastfeeding are not well characterized. Passive infusion of broadly neutralizing antibody to infant rhesus monkeys was protective against oral challenge with simian-human immunodeficiency virus (3), suggesting that effective virus neutralization in the infant systemic or gastrointestinal compartment is protective against virus acquisition. Interestingly, we detected an autologous challenge virus-specific neutralization response in the milk of five of six AGMs that had a titer similar to that in plasma. In contrast, no autologous challenge virus neutralization was detected in milk of chronically SIV-infected, postnatally transmitting RMs. It is possible that detection of this robust autologous challenge virus neutralization response milk of AGMs is due to the neutralization sensitivity of this challenge virus, as a heterologous virus neutralization response against a neutralization-sensitive SIV was detected in milk of both RMs and AGMs. Furthermore, the relationship of the SIVsab931BR challenge virus stock envelope used in these neutralization assays to the chronic virus variants circulating in these AGMs is not known.

The similar autologous neutralization potencies of milk and plasma against the challenge virus of SIV-infected AGMs are in contrast to the divergent neutralization potencies of milk and plasma of SIV-infected RMs. Furthermore, the neutralization potencies of milk and plasma against the heterologous neutralization-sensitive SIV derived from an SIVmac251 virus stock are disparate in the AGMs and RMs. IgG appears to predominantly mediate HIV/SIV neutralization by breast milk of RMs (40) and humans (S. R. Permar, unpublished data). Although AGM milk had high IgA content compared to RMs, we did not detect neutralization by the non-IgG fraction of milk of AGMs. Therefore, the comparable autologous neutralization responses in milk and plasma of AGMs despite the lower milk IgG content are remarkable. It is possible that local, mucosally produced IgG directed against the autologous virus contributes to the autologous neutralization response in milk of AGMs. Furthermore, the comparable neutralization potencies of purified milk and plasma IgG of AGMs are in contrast
to the poor neutralization potency of milk IgG compared to that of plasma IgG of RMs during chronic SIV infection (40). Although plasma IgG transudate predominantly contributes to the pool of milk IgG in primates (7, 8), IgG-producing B cells are found in milk and contribute to a portion of the IgG found in milk (7, 56). Therefore, the locally produced IgG in milk of AGMs may be more effective at autologous virus neutralization than that of RMs.

The autologous neutralization response detected in milk of chronically SIV-infected AGMs is a possible mechanism of the impedance of postnatal virus transmission in natural hosts of SIV. However, further investigation of the neutralization response in milk of AGMs infected with a cloned transmitted/founder virus (19) may further delineate the effectiveness of this response without concerns of variable envelope neutralization sensitivity within a challenge virus stock. Further evidence that neutralizing mucosal antibody responses in milk mediate protection against SIV transmission to suckling infants of natural hosts of SIV would have major implications for the design of vaccines to prevent postnatal transmission of HIV.

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