Published in final edited form as:

J Infect. 2014 March ; 68(3): 264–272. doi:10.1016/j.jinf.2013.11.002.

Neutralization of HIV subtypes A and D by breast milk IgG from women with HIV infection in Uganda

Jana M. Palaia^{a,b}, Michelle McConnell^c, Jenna E. Achenbach^a, Claire E. Gustafson^{a,b}, Kristina A. Stoermer^a, Monica Nolan^c, Laura A. Guay^d, Thomas K. Leitner^e, Flavia Matovu^d, Allan W. Taylor^c, Mary Glenn Fowler^d, and Edward N. Janoff^{a,b,*}

^aMucosal and Vaccine Research Colorado (MAVRC), University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA ^bDenver Veterans Affairs Medical Center, Denver, CO 80220, USA ^cCenters for Disease Control and Prevention, Atlanta, GA 30333, USA ^dMakerere University – Johns Hopkins University, Kampala, Uganda ^eLos Alamos National Laboratory, Los Alamos, NM 87544, USA

Summary

Objectives: Among HIV-exposed infants in resource-limited countries, 8–12% are infected postnatally by breastfeeding. However, most of those uninfected at birth remain uninfected over time despite daily exposure to HIV in breast milk. Thus, we assessed the HIV-inhibitory activity of breast milk

Methods: We measured cross-clade neutralization in activated PBMC of Ugandan subtype A (92UG031) and D (92UG005) primary HIV by breast milk or purified milk IgG and IgA from 25 HIV-infected Ugandan women. Isotype-specific antigen recognition was resolved by immunoblot. We determined HIV subtype from envelope population sequences in cells from 13 milk samples by PCR.

Results: Milk inhibited p24 production by ~50% (dose-dependent) by subtype A (21/25; 84%) and subtype D (11/25; 44%). IgG consistently reacted with multiple HIV antigens, including gp120/gp41, but IgA primarily recognized p24 alone. Depletion of IgG ($n = 5$), not IgA, diminished neutralization (mean $78 \pm 33\%$) that was largely restored by IgG repletion. Mothers infected with subtype A more effectively neutralized subtype A than D.

Conclusions: Breast milk from HIV-infected women showed homotypic and cross-subtype neutralization of HIV by IgG-dependent and -independent mechanisms. These data direct further investigations into mechanisms of resistance against postnatal transmission of HIV to infants from their mothers.

For personal use only. No other uses without permission.

*Corresponding author. University of Colorado Denver, Mucosal and Vaccine Research Colorado (MAVRC), Box B-168, 12700 E. 19th Ave., Aurora, CO 80045, USA. Tel.: +1 303 724 4936; fax: +1 303 724 4926. Edward.Janoff@ucdenver.edu (E.N. Janoff).

Conflict of interest

The authors have no conflict of interest.

Disclaimer

The findings and conclusions in this paper are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

Keywords

Breast milk; HIV; Neutralization; IgG, IgA; Uganda; Subtype A; Subtype D; Mucosal immunity

Introduction

Breastfeeding accounts for 25–44% of the up to 330,000 cases of mother-to-child transmission (MTCT) of HIV infection annually, particularly in sub-Saharan Africa.^{1,2} The risk of postnatal MTCT by breastfeeding is 8–12%,^{1,3} despite daily exposure to HIV in breast milk. Breast milk also lowers morbidity and mortality of children from malnutrition, respiratory and diarrheal diseases.^{3–5} As a result, current WHO recommendations for infant feeding among HIV-infected mothers in resource-poor nations encourage continued exclusive breastfeeding for up to 6 months.⁶

The primary vehicles for MTCT of HIV infection are cell-free and cell-associated HIV virions found in maternal blood, vaginal fluids and breast milk.⁷ The amount of virus per liter of milk is low, so cumulative exposure of the infant to breast milk over time is more likely to lead to transmission of the virus in mothers that shed more cell-free viral particles in their breast milk.⁸ Both colostrum⁹ and milk^{10–13} contain HIV-specific IgG and IgA as well as innate immune constituents, each of which shows inhibition of HIV inhibition *in vitro*.^{14–19} Therefore, we characterized the ability of breast milk antibodies from HIV-infected women in Uganda, where HIV seroprevalence and MTCT are high, to neutralize the predominant local HIV subtypes A and D. We also characterized the inhibitory role of antibodies by isotype in cross-subtype neutralization.

Materials and methods

Study subjects

We tested milk from a random sample of 25 of 150 HIV-infected mothers enrolled in the Pathobiology of Breast Milk study in Kampala, Uganda²⁰ from November 2003–November 2004. These antiretroviral (ARV)-naïve pregnant women were 18 years old, 28 weeks gestation, and intended to breastfeed for 6 weeks. Each received single dose nevirapine at birth. All milk samples had undetectable levels of nevirapine by 4 weeks postpartum,²⁰ and no women received ARV's postnatally per local guidelines at that time. The median age of women ($n = 25$) was 23 years (range 20–30). Median values for CD4+ T-cells were 410/ μ L (range 123–934), plasma HIV RNA 85,992 copies/mL (range <400–750,000) and breast milk HIV RNA 77 copies/mL (range 4–43,363). Mothers whose newborns tested positive for HIV by PCR between birth and six months were termed transmitters and mothers of newborns testing negative were termed non-transmitters. Exclusion criteria and study approval is as previously described.²⁰

Breast milk samples

Breast milk samples were obtained between 4 and 14 weeks postnatally. Mothers manually expressed breast milk after washing with water. Samples were centrifuged at 3000 rpm for 15 min at room temperature, visible lipid removed and lipid-poor milk (heretofore “milk”)

aliquoted. Milk was not heat-inactivated. Cells were washed twice with phosphate buffered saline (PBS), re-suspended in 0.5 mL PBS, and frozen at -70°C .

Neutralization

With modifications of reported assays,²¹ we incubated HIV subtype A (92UG031) or subtype D (92UG005) (AIDS Research and Reference Reagent Program, NIAID, NIH:U-NAIDS Network for HIV Isolation and Characterization) at 50 TCID₅₀ per well with milk diluted 1:4–1:16 in RPMI 1640 (Invitrogen; Carlsbad, CA), 5% IL-2 (Roche, Basel, Switzerland), heat-inactivated 10% FBS (Hyclone, Logan, UT) and gentamicin (Invitrogen) for 30 min at 37°C . This mixture was added for 2 h to peripheral blood mononuclear cells (PBMCs) activated for 3 days with phytohemagglutinin (PHA)(Sigma, St. Louis, MO), washed and incubated at 37°C with media changed on day 4. We tested supernatants on day 6 for p24 by ELISA (Perkin Elmer, Waltham, MA). Percent neutralization was calculated as $100 - ([\text{p24 with virus and milk}/\text{p24 with virus alone}] \times 100)$.

We determined breast milk toxicity by counting viability of PHA-activated PBMCs at 2 h and 4 days by trypan blue exclusion after incubation with milk diluted 1:4 for 2 h at 37°C and washing. Samples showing toxicity were treated with Lipid Removal Agent (LRA, 2 mg in 0.5 mL milk; Suppelco, St. Louis, MO) for 4 h at 4°C , centrifuged, and reassayed.

Fractionation and purification of antibodies in breast milk

We prepared purified IgG milk fractions by incubating milk (500 μL) and 350 μL magnetic protein G beads (Miltenyi-Biotec, Auburn, CA) as modified from the manufacturer's protocol. After collecting the non-IgG flow-through, the antibody-bound beads were washed with radioimmunoprecipitation (RIPA) buffer, low salt buffer and PBS, and IgG was eluted with pH shift buffer (0.1 M Triethylamine and 0.1% triton-X, pH 11.0) and adjusted to pH 7 with 1 M MES (pH 3.0). We restored the 500 μL volume with PBS, buffer exchanged with PBS to avoid toxicity using Amicon ultra-0.5 centrifugal filter devices (Millipore; Billerica, MA).

For IgA, we incubated milk (500 μL) with 400 μL of washed SSL7 agarose (Invivogen, San Diego, CA) in a spin filter (Pierce, Rockford, IL) rocked overnight at 4°C . The non-IgA flow-through was collected, the antibody-bound agarose washed with buffer (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2), and IgA eluted with 500 μL of 0.1 M glycine buffer (pH 2.7), adjusted to pH 7 using 1 M tris-HCL (pH 9.0) and buffer exchanged. Purified IgG and IgA milk fractions contained $<1\%$ of other isotypes by ELISA using affinity-purified anti-human antibodies (Jackson ImmunoResearch; West Grove, PA)⁵

Antibody-depleted non-Ig milk fractions were prepared by covalently linking purified goat anti-human IgA, IgG and IgM (Southern Biotech, Birmingham, AL) to CNBr-activated sepharose 4B (Sigma). Antibody-linked sepharose (450 μL IgA, 300 μL IgG, 100 μL IgM) was packed into a spin filter, spun and washed 3 times at 4000 g with binding buffer (150 mM NaCl, 0.02% NaN₃, and 20 mM Na₂HPO₄, pH 7.4). Milk (500 μL) was added, rocked overnight at 4°C , centrifuged at 4000 g for 2 min, flow-through collected and buffer exchanged.

Total IgG and IgA in each milk fraction were adjusted to pre-processing levels. Whole and fractionated milk (1–3 µg protein) were resolved with 15% SDS-polyacrylamide gel (Bio-Rad Laboratories; Hercules, CA) and silver stained (Bio-Rad), with purified IgG, IgM, IgA (Jackson ImmunoResearch), lactoferrin and lysozyme (MP biomedical, Solon, OH) as controls.

Protein immunoblots

The presence of HIV-specific antibodies in milk and purified antibody fractions were determined by immunoblot (ImmuneticsQualiCode HIV-1/2 Kit; Boston, MA) per manufacturer's instructions for IgG or with anti-human IgA-biotin (1:50, Jackson Immuno Research) with streptavidin-alkaline phosphatase (1:1000, Invitrogen).

Sequencing and subtyping

We amplified a 416 bp fragment spanning the HIV-1 C2–V3–C3 region of the *env* gene by nested PCR (nucleotides 6959-7375) using the outer primers JA167/JA170 and inner primers JA168/JA169.²² Genomic DNA (DNeasy Blood and Tissue Kit Qiagen, Valencia, CA) was isolated from milk cell lysates of 13 mothers, for which we had cell pellets, collected at 14–24 weeks postpartum. First round amplification using the outer primer pair was carried out at 95 °C for 2 min, 95 °C for 20 s, 48.8 °C for 10 s, and 70 °C for 10 s for 30 cycles, and the second round at 95 °C for 2 min, 95 °C for 20 s, 51 °C for 10 s, and 70 °C for 10 s for 40 cycles, each with 0.5 µL KOD Hot Start Polymerase (Novagen, Gibbstown, NJ). We extracted amplified fragments from a 1.5% agarose gel (MinElute Gel extraction kit, Qiagen) and sequenced using forward primer JA168. HIV subtype was determined using a maximum likelihood tree (PhyML V3.0) the LANL HIV-1 subtype 2010 reference set (<http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html>). All sequences were deposited in GenBank (accession numbers: KC329755–KC329769).

Statistics

We compared differences in demographic variables between HIV-transmitters and -nontransmitters and in neutralization of HIV subtypes A and D by breast milk using unpaired student's *t* test and Wilcoxon matched-pairs signed rank test. We tested differences between neutralization in milk and milk fractions by ANOVA and if $p < .05$, we then analyzed the data by Tukey's multiple comparison test (Prism 5.0d; GraphPad Software, Inc., La Jolla, CA).

Results

Neutralization by breast milk

Most breast milk samples from 25 women showed dose-dependent neutralization of HIV-1 subtype A or D strains (Fig. 1a). Subtype A strain 92UG031 was more readily neutralized than subtype D. For subtype A, 84% of milk samples supported 50% reduction in p24 production compared with 44% with the subtype D strain 92UG005 ($p = .0025$) (Fig. 1a). Toxicity of milk for target cells, present in 3 of 25 samples and abrogated by LRA treatment, did not relate to inhibition. Neutralization of neither subtype A nor D correlated with HIV

RNA levels in plasma or breast milk, but neutralization of subtype A did correlate inversely with CD4⁺ T cell number ($p = .0083$; $R^2 = 0.266$).

Based on HIV-1 subtype analysis, milk samples from 8 of 9 mothers infected with subtype A showed greater neutralization of a local primary subtype A than a local subtype D virus ($p = .01$) (Fig. 1b). Neutralizing activity from women infected with subtype D showed similar activity against both subtypes.

Of the 25 milk samples, 19 were obtained from non-transmitting and 6 from mothers who transmitted HIV-1 to their infants in the prenatal ($n = 4$; HIV PCR-positive at birth-2 weeks) or perinatal period ($n = 2$; HIV PCR-negative at 0-2 weeks, positive at 6 weeks). No samples were obtained from women who transmitted HIV postnatally through breast milk. Neutralization was similar in transmitters and non-transmitters, but HIV RNA in milk was higher in transmitters (9002 ± 6979 vs. 103 ± 25 copies/mL; $p < .03$).

Homology among clinical and target virus populations

Consistent with the ability of milk from mothers to neutralize the local subtypes, sequence analysis revealed extensive homology between the target strains and those in milk cell pellets (Fig. 2). This 416 bp envelope segment, one of the two most variable regions in the entire HIV genome, encodes the V3 loop and 2 important conformational CD4 binding sites (D loop and CD4 binding loop, an early target for CD4 binding). These two sites include residues that bind the broadly-neutralizing human monoclonal antibodies IgG b12 and VRC01²³⁻²⁵ and predicted V3 loop targets for the monoclonal antibody PG16.²⁶ Each subtype A sequence contained the V3 crown motif GPGQ, whereas subtype D isolates shared the sequence GPGR or GPGQ (Fig. 2). Among neutralizing epitopes in the V3 loop crown,^{27,28} those sequences having the GPGQ motif, 8 of the 9 subtype A HIV sequences (Fig. 1b) had broader cross-neutralizing activity than viruses with the GPGR motif²⁹ identified in the more resistant subtype D viruses. As expected, our subtype A and D sequences displayed restricted amino acid variation in the CD4-binding region (Fig. 2), particularly at positions 369 and 371 (amino acids D and E), which forms a relatively conserved discontinuous neutralizing epitope and is conserved in most of our isolates.²⁸ Sequencing analysis also shows that of 13 women with known subtype in our study, 9 had the 332 glycan (5 subtype A and 4 subtype D), which is a known N-linked glycosylation neutralizing target, and 66% showed both homologous and heterologous neutralizing activity.

Neutralization by antibody isotypes and non-antibody milk fractions

Antibodies appeared to support the majority of HIV neutralization in breast milk. Purified antibody isotypes showed distinct contributions to neutralization. Milk from five women infected with subtype A virus supported a mean of 57% neutralization (range 33-99%) of the subtype A virus and 42% (range 13-86%) of the subtype D virus (Fig. 3). The IgG purified from these 5 samples supported the majority of neutralization against subtype A (range 78-99%) and D viruses (range 63-97%). The remaining non-IgG fractions retained some of the original activity against subtype A (range 0-43%) and subtype D (range 0-51%)

viruses, but less than that of the IgG fractions ($p < .01$). Restoration of purified IgG to the IgG-depleted milk largely reconstituted the inhibitory activity to that of intact milk (Fig. 3).

Among milk samples from women infected with HIV subtype A, purified IgA showed less activity than intact milk (Fig. 3). Indeed, the percent neutralization by purified IgG exceeded that of IgA ($p < .05$) in milk samples even though the concentration of total IgG was lower than that of IgA (222 ± 60.3 vs. 307.2 ± 70.1 $\mu\text{g/mL}$, respectively [$p = .03$]). Both purified IgA and the removal of IgA had only a modest impact on neutralization (Fig. 3).

Innate factors in milk supported only a very limited role in HIV inhibition. After depletion of antibodies, the non-Ig fraction showed 1–8% neutralization of subtype A (1–17% of neutralizing activity of intact milk) (data not shown), despite an abundance of lactoferrin and lysozyme (>850 $\mu\text{g/mL}$ and >10 $\mu\text{g/mL}$, respectively), factors associated with inhibitory activity.

Antigen specificity of HIV-specific IgG and IgA in breast milk

IgA in all milk samples reacted predominantly with p24 antigen alone (Fig. 4). In contrast, IgG recognized multiple HIV antigens (Fig. 4), including the envelope gp120 and gp41 fusion domains. IgG was more likely than IgA to recognize gp120 (68% vs. 0% of samples, respectively, $p < .0001$), gp160 (100% vs. 24%, respectively, $p < .0001$), and gp41 (80% vs. 0%, respectively, $p < .0001$), with similar trends for p66, p51, and p31. In contrast, the more conserved internal antigen, p24, was recognized at comparable frequencies. Although IgG comprised $40\% \pm 17\%$ of all milk immunoglobulins (IgA $55\% \pm 18\%$ and IgM $5\% \pm 4\%$), IgG did not mask the reactivity of IgA. Depletion of IgG did not increase the number or intensity of bands recognized by IgA (not shown).

Discussion

We have shown that breast milk from HIV-1-infected women in Uganda supports dose-dependent neutralization of two local HIV subtypes A and D in primary human immune cells. Although this inhibition extends to both HIV subtypes, cross-subtype neutralization appeared greater for subtype A than subtype D with the two strains utilized in this study. We confirmed that, although IgA is the predominant isotype in milk,^{5,11,30,31} IgG, not IgA, mediates most neutralization. IgG comprises a larger fraction of HIV-specific antibodies³² and reacted in all milk samples with multiple HIV proteins, including gp120 and gp41 proteins, which were recognized by IgA in only a minority of samples (6 of 25; 24%). These results are important because prevention of post-natal HIV transmission among breastfed infants has remained one of the most difficult challenges in controlling the HIV epidemic in children. The problem has been how to limit postnatal HIV transmission while retaining the nutritional and immunologic benefits of milk against the morbidity and mortality from diarrhea and pneumonia in infants at risk.

Protective effects of mucosal IgG may also be mediated through antibody-dependent cellular cytotoxicity (ADCC). Clinically, women with high titers of HIV-specific IgG antibodies in their cervicovaginal lavage fluids have lower genital viral loads than women with specific IgG only in serum, and the majority of these fluids that also have ADCC activity exhibited

gp120-specific IgG, not IgA.³³ Similarly, HIV-specific IgG-mediated ADCC titers in milk from women infected with subtype C correlated with the specific-IgG binding responses, as did *env*-specific IgG binding and neutralization of tier 1 pseudoviruses.¹³ In other settings, the human monoclonal antibody (MAb) IgG 2F5 inhibited transcytosis of HIV across both polarized epithelial cell monolayers and rectal tissue,³⁴ as did gp160-specific IgG from colostrum and milk^{9–11} and the human MAb IgG b12.²¹ These data and ours highlight the important functional activity of HIV-specific IgG in a variety of mucosal fluids. The higher levels of local HIV-specific IgG than IgA,^{10,11} observed in other mucosal fluids, likely derive from transudation or active transport from serum, and, to a lesser extent, from the predominance of specific IgG-rather than IgA-secreting cells in milk,³⁵ although the IgA cells should be more prominent in the gland tissue itself.³⁵

Although HIV-specific IgA has been detected in a range of mucosal fluids, its reactivity with envelope glycoproteins is quite limited.³² The infrequent reactivity with gp160, gp120 and gp41 in our study is lower than that reported from other mucosal fluids, including breast milk.^{13,32,36} These differences may relate to the sensitivity of the detection method (immunoblot vs. ELISA or antigen-coated bead binding). Similar to our results in humans, HIV-specific IgA responses in the mucosa of HIV-infected chimpanzees³⁷ and SIV-infected rhesus macaques is either decreased or absent.^{12,38} The predominance of human p24 gag-specific IgA is recapitulated with SIV p27 gag-specific IgA,¹² highlighting the impaired recognition of HIV and SIV envelope by IgA in milk.

Even when HIV envelope-specific IgA was present in milk, we confirm that such purified IgA had limited but detectable neutralizing activity, as previously reported in milk¹³ and other fluids.^{11,12,32,39,40} Similarly, in milk samples from subtype C-infected Malawian women, IgA supported neutralization in only 1 of 35 milk samples¹³ and did not correlate with ADCC, results that are comparable to those with IgA from milk from SIV-infected rhesus macaques.¹² HIV-specific IgA, particularly polymeric IgA derived from a potent human monoclonal antibody (IgG b12), can neutralize the virus *in vitro*,²¹ but clinical mucosal fluids show low levels and limited activity *in vivo*. Indeed, HIV-specific IgA has been detected more, rather than less often in milk from transmitting than from non-transmitting women.⁴¹ However, specific IgA and IgG may support different activities in colostrum and milk particularly in their ability to inhibit viral transcytosis.^{10,11}

We and others have shown that IgA congeners of human monoclonal antibodies that bind the CD4 binding site (IgG b12) and the conserved membrane proximal external region of gp41 (2F5) can substantially inhibit epithelial uptake and transcytosis of HIV as well as transfer from epithelial cells to PBMCs.^{21,34} Clinically, mucosal IgA, but not IgG, from cervicovaginal fluids of Cambodian women infected with HIV subtype E blocked binding to the alternative galactosylceramide receptor on epithelial cells and transcytosis.⁴² Thus, mucosal IgA, particularly that derived *in vivo* rather than *in vitro*, may have a more prominent impact on local binding, uptake and transfer of HIV by epithelial cells at primary sites of infection, rather than neutralization.

In this context, innate components of breast milk also showed limited neutralizing activity (non-Ig fraction) in this study and others.¹³ However, multiple individual constituents of

breast milk, such as lactoferrin, lysozyme, mucin, lipids, secretory leukocyte protease inhibitor (SLPI), bile salt-stimulated lipase, lewis X component, and milk oligosaccharides each show such activity.^{14,16,17,19,30,43,44} Complement components present in breast milk may also affect infectivity or neutralization. Moreover, breast milk from HIV-seronegative women is also capable of blocking transmission of HIV in BLT humanized mice.⁴⁵ Thus, multiple components of breast milk, including neutralization by IgG, likely act in concert by complementary mechanisms to block post-natal transmission of HIV-1.

Preferred targets for inhibition would be shared by multiple strains and subtypes. Our sequencing results suggest that the cross-subtype neutralization we observed with these milk samples infected with subtypes A and D viruses may be associated with binding to a highly conserved, broadly neutralizing epitope in the CD4-binding region of gp120. Residues in the C2, C3 and V5 regions of the HIV CD4 binding site also comprise antibody recognition sites.²⁸ The C3 region also includes several HIV N-linked glycosylation neutralizing targets at positions 332, 332, and 339^{46,47} that are associated with production of broadly cross-neutralizing antibodies, activity that is highly dependent on single amino acid changes and glycan binding.⁴⁷

One limitation of this study is that we did not have access to autologous viruses from these patients. Fouda et al. reported that neutralization of autologous subtype C viruses by breast milk was infrequent as was neutralizing activity with subtype C pseudoviruses in cell lines.¹³ However, we used primary human cells due to substantial differences in neutralization of subtype A virus between these cells and the TZMbl cell line.⁴⁸ Our primary local viruses and target cells pooled from ten healthy U.S. donors, although not from local Ugandans, may better model physiologic events. Finally, we only looked at cross-subtype neutralization with two local strains, one subtype A and one subtype D. Based on these results, we will expand our virus panel to better elucidate the ability of breast milk and its constituents to neutralize more diverse viruses and subtypes.

In summary, the frequency of HIV transmission to infants is unacceptably high without antiretroviral therapy.^{1,3} Our findings of high frequencies of neutralization by breast milk, particularly the IgG fraction, are consistent with observations that the majority of infants who are uninfected at birth remain uninfected during breastfeeding despite daily exposure to the virus in breast milk. This inhibition was independent of CD4+ T cell number and levels of HIV RNA in blood or milk. Current work is directed to integrate the ability of breast milk to neutralize HIV in PBMC with that in other targets (e.g., epithelial and dendritic cells). Such immune defenses interact with the levels of cell-free or cell-associated virus in milk, with antibody and innate factors, and genetic and immunologic characteristics of both infant and the founder virus(es) to determine the risk of post-natal infection. Antiretroviral therapy of mothers and prolonged prophylaxis of infants provides substantial protection against MTCT of HIV,⁴⁹ but, to date, only about 60% of eligible women in SubSaharan Africa are receiving one or more drugs in this vulnerable period.² Indeed, a potentially clinically protective role for inducible virus-specific responses at local mucosal sites, including breast milk, has recently been proposed with 3 different vaccine strategies.^{50–52} Thus, understanding the range of mechanisms of defense that are or are not elicited by natural infection, and that can be stimulated experimentally, particularly in breast milk and other

mucosal sites, will advance our efforts to prevent postnatal MTCT of HIV through breastfeeding from women in resource-limited settings.

Acknowledgments

This work supported by NIH R01-HD059527, R01-AI41361, R01 AI097265, the United States Centers for Disease Control (CDC; “Pathobiology of Breast Milk among HIV-1 infected Ugandan women receiving intrapartum nevirapine” study), the Elisabeth Glaser Pediatric AIDS Foundation (EGPAF) MV-00-9-900-01432-0-00, University of Colorado Denver’s Office of Interdisciplinary Women’s Health Research Grant, the Mucosal and Vaccine Research Colorado Program (MAVRC) and the University of Colorado Cancer Center DNA Sequencing and Analysis Core (Grant #P30 CA046934). The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the United States Centers for Disease Control and Prevention. We thank Jacinta Cooper for technical support and advice and the women in Kampala, Uganda for their participation.

Portions of this work has been presented in part at the 14th International Congress on Mucosal Immunity, Boston, MA. July 5–9, 2009, and the 47th Annual Meeting of the Infectious Disease Society of America, Philadelphia, PA. October 29–November 1, 2009.

References

1. Fowler MG, Lampe MA, Jamieson DJ, Kourtis AP, Rogers MF. Reducing the risk of mother-to-child human immunodeficiency virus transmission: past successes, current progress and challenges, and future directions. *Am J Obstet Gynecol* 2007 9;197(3 Suppl.):S3–9. [PubMed: 17825648]
2. UNAIDS. UNAIDS report on the global AIDS epidemic 2012. Available at: http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_UNAIDS_Global_Report_2012_en.pdf; 2012.
3. Nduati R, John G, Mbori-Ngacha D, Richardson B, Overbaugh J, Mwatha A, et al. Effect of breastfeeding and formula feeding on transmission of HIV-1. *JAMA* 2000;283: 1167–74. [PubMed: 10703779]
4. Mortality WCsTotRoBotPoI. Effect of breastfeeding on infant and child mortality due to infectious diseases in less developed countries: a pooled analysis. *Lancet* 2000;355(9202): 451–5. [PubMed: 10841125]
5. Shapiro RL, Lockman S, Kim S, Smeaton L, Rahkola JT, Thior I, et al. Infant morbidity, mortality, and breast milk immunologic profiles among breast-feeding HIV-infected and HIV-uninfected women in Botswana. *J Infect Dis* 2007 8 15; 196(4):562–9. [PubMed: 17624842]
6. WHO. Guidelines on HIV and infant feeding 2010. Principles and recommendations for infant feeding in the context of HIV and a summary of evidence; 2010.
7. Lehman DA, Farquhar C. Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission. *Rev Med Virol* 2007 Nov-Dec;17(6):381–403. [PubMed: 17542053]
8. Neveu D, Viljoen J, Bland RM, Nagot N, Danaviah S, Coutoudis A, et al. Cumulative exposure to cell-free HIV in breast milk, rather than feeding pattern per se, identifies postnatally infected infants. *Clin Infect Dis* 2011 3 15; 52(6):819–25. [PubMed: 21367736]
9. Becquart P, Hocini H, Garin B, Sepou A, Kazatchkine MD, Belec L. Compartmentalization of the IgG immune response to HIV-1 in breast milk. *AIDS* 1999 7 30;13(11):1323–31. [PubMed: 10449284]
10. Chomont N, Hocini H, Gody JC, Bouhlal H, Becquart P, Krief-Bouillet C, et al. Neutralizing monoclonal antibodies to human immunodeficiency virus type 1 do not inhibit viral transcytosis through mucosal epithelial cells. *Virology* 2008 1 20; 370(2):246–54. [PubMed: 17920650]
11. Becquart P, Hocini H, Levy M, Sepou A, Kazatchkine MD, Belec L. Secretory anti-human immunodeficiency virus (HIV) antibodies in colostrum and breast milk are not a major determinant of the protection of early postnatal transmission of HIV. *J Infect Dis* 2000 2;181(2): 532–9. [PubMed: 10669336]
12. Permar SR, Wilks AB, Ehlinger EP, Kang HH, Mahlokozera T, Coffey RT, et al. Limited contribution of mucosal IgA to Simian immunodeficiency virus (SIV)-specific neutralizing antibody response and virus envelope evolution in breast milk of SIV-infected, lactating rhesus monkeys. *J Virol* 2010 8;84(16): 8209–18. [PubMed: 20519381]

13. Fouda GG, Yates NL, Pollara J, Shen X, Overman GR, Mahlokozera T, et al. HIV-specific functional antibody responses in breast milk mirror those in plasma and are primarily mediated by IgG antibodies. *J Virol* 2011 9;85(18): 9555–67. [PubMed: 21734046]
14. Kourtis AP, Jamieson DJ, de Vincenzi I, Taylor A, Thigpen MC, Dao H, et al. Prevention of human immunodeficiency virus-1 transmission to the infant through breastfeeding: new developments. *Am J Obstet Gynecol* 2007 9;197(3 Suppl.): S113–22. [PubMed: 17825642]
15. Villamor E, Koulinska IN, Furtado J, Baylin A, Aboud S, Manji K, et al. Long-chain n-6 polyunsaturated fatty acids in breast milk decrease the risk of HIV transmission through breastfeeding. *Am J Clin Nutr* 2007 9;86(3):682–9. [PubMed: 17823433]
16. Hong P, Ninonuevo MR, Lee B, Lebrilla C, Bode L. Human milk oligosaccharides reduce HIV-1-gp120 binding to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN). *Br J Nutr* 2009 2;101(4):482–6. [PubMed: 19230080]
17. Naarding MA, Dirac AM, Ludwig IS, Speijer D, Lindquist S, Vestman EL, et al. Bile salt-stimulated lipase from human milk binds DC-SIGN and inhibits human immunodeficiency virus type 1 transfer to CD4+ T cells. *Antimicrob Agents Chemother* 2006 10;50(10):3367–74. [PubMed: 17005819]
18. Kourtis AP, Butera S, Ibegbu C, Beled L, Duerr A. Breast milk and HIV-1: vector of transmission or vehicle of protection? *Lancet Infect Dis* 2003 12;3(12):786–93. [PubMed: 14652204]
19. Naarding MA, Ludwig IS, Groot F, Berkhout B, Geijtenbeek TB, Pollakis G, et al. Lewis X component in human milk binds DC-SIGN and inhibits HIV-1 transfer to CD4+ T lymphocytes. *J Clin Invest* 2005 11;115(11):3256–64. [PubMed: 16239964]
20. Aizire J, McConnell MS, Mudiope P, Mubiru M, Matovu F, Parsons TL, et al. Kinetics of nevirapine and its impact on HIV-1 RNA levels in maternal plasma and breast milk over time after perinatal single dose nevirapine. *J Acquir Immune Defic Syndr* 2012 8 15;60(5):483–8. [PubMed: 22217678]
21. Mantis NJ, Palaia J, Hessel AJ, Mehta S, Zhu Z, Corthesy B, et al. Inhibition of HIV-1 infectivity and epithelial cell transfer by human monoclonal IgG and IgA antibodies carrying the b12 V region. *J Immunol* 2007 9 1;179(5):3144–52. [PubMed: 17709529]
22. Leitner T, Korovina G, Marquina S, Smolskaya T, Albert J. Molecular epidemiology and MT-2 cell tropism of Russian HIV type 1 variant. *AIDS Res Hum Retroviruses* 1996 11 20; 12(17):1595–603. [PubMed: 8947294]
23. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 1998 6 18; 393(6686):648–59. [PubMed: 9641677]
24. Zhou T, Georgiev I, Wu X, Yang ZY, Dai K, Finzi A, et al. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. *Science* 2010 8 13;329(5993):811–7. [PubMed: 20616231]
25. Zhou T, Xu L, Dey B, Hessel AJ, Van Ryk D, Xiang SH, et al. Structural definition of a conserved neutralization epitope on HIV-1 gp120. *Nature* 2007 2 15;445(7129):732–7. [PubMed: 17301785]
26. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, et al. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* 2009 10 9;326(5950):285–9. [PubMed: 19729618]
27. Gorny MK, Conley AJ, Karwowska S, Buchbinder A, Xu JY, Emini EA, et al. Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. *J Virol* 1992;66(12):7538–42. [PubMed: 1433529]
28. Pestano G, Prince A, Guyden J, Ntambi JM, Atkin A, Boto WM. Independent divergences in the CD4 binding site and V3 loop encoded in two seroprevalent Ugandan HIV-1 clinical isolates. *J Acquir Immune Defic Syndr* 1993 8;6(8):872–80. [PubMed: 7686222]
29. Gorny MK, Williams C, Volsky B, Revesz K, Wang XH, Burda S, et al. Cross-clade neutralizing activity of human anti-V3 monoclonal antibodies derived from the cells of individuals infected with non-B clades of human immunodeficiency virus type 1. *J Virol* 2006 7;80(14):6865–72. [PubMed: 16809292]
30. Alexander R, Mestecky J. Neutralizing antibodies in mucosal secretions: IgG or IgA? *Curr HIV Res* 2007 11;5(6):588–93. [PubMed: 18045115]

31. Van de Perre P Transfer of antibody via mother's milk. *Vaccine* 2003 7 28;21(24):3374–6. [PubMed: 12850343]
32. Mestecky J, Jackson S, Moldoveanu Z, Nesbit LR, Kulhavy R, Prince SJ, et al. Paucity of antigen-specific IgA responses in sera and external secretions of HIV-type 1-infected individuals. *AIDS Res Hum Retroviruses* 2004 9;20(9):972–88. [PubMed: 15585085]
33. Nag P, Kim J, Sapiega V, Landay AL, Bremer JW, Mestecky J, et al. Women with cervicovaginal antibody-dependent cell-mediated cytotoxicity have lower genital HIV-1 RNA loads. *J Infect Dis* 2004 12 1;190(11):1970–8. [PubMed: 15529262]
34. Shen R, Drelichman ER, Bimczok D, Ochsenbauer C, Kappes JC, Cannon JA, et al. GP41-specific antibody blocks cell-free HIV type 1 transcytosis through human rectal mucosa and model colonic epithelium. *J Immunol* 2010 4 1;184(7): 3648–55. [PubMed: 20208001]
35. Tuaille E, Valea D, Becquart P, Al Tabaa Y, Meda N, Bollere K, et al. Human milk-derived B cells: a highly activated switched memory cell population primed to secrete antibodies. *J Immunol* 2009 6 1;182(11):7155–62. [PubMed: 19454712]
36. Janoff EN, Scamurra RW, Sanneman TC, Eidman K, Thurn JR. Human immunodeficiency virus type 1 and mucosal humoral defense. *J Infect Dis* 1999 5;179(Suppl. 3):S475–9. [PubMed: 10099123]
37. Israel ZR, Marx PA. Nonclassical mucosal antibodies predominate in genital secretions of HIV-1 infected chimpanzees. *J Med Primatol* 1995 2;24(2):53–60. [PubMed: 8613973]
38. Jackson S, Moldoveanu Z, Mestecky J, Pitts AM, Eldridge JH, McGhee JR, et al. Decreased IgA-producing cells in the gut of SIV-infected rhesus monkeys. *Adv Exp Med Biol* 1995;8: 1035–8.
39. Bélec L, Dupre T, Prazuck T, Tevi-Benissan C, Kanga JM, Pathey O, et al. Cervicovaginal overproduction of specific IgG to human immunodeficiency virus (HIV) contrasts with normal or impaired IgA local response in HIV infection. *J Infect Dis* 1995;172(3):691–7. [PubMed: 7658060]
40. Soderlund J, Hirbod T, Goh LE, Andersson J, Broliden K. Presence of HIV-1 neutralizing IgA antibodies in primary HIV-1 infected patients. *Scand J Infect Dis* 2004;36(9):663–9. [PubMed: 15370653]
41. Kuhn L, Trabatttoni D, Kankasa C, Sinkala M, Lissoni F, Ghosh M, et al. Hiv-specific secretory IgA in breast milk of HIV-positive mothers is not associated with protection against HIV transmission among breast-fed infants. *J Pediatr* 2006 11;149(5):611–6. [PubMed: 17095329]
42. Alfsen A, Iniguez P, Bouguyon E, Bomsel M. Secretory IgA specific for a conserved epitope on gp41 envelope glycoprotein inhibits epithelial transcytosis of HIV-1. *J Immunol* 2001; 166(10): 6257–65. [PubMed: 11342649]
43. Kazmi SH, Naglik JR, Sweet SP, Evans RW, O'Shea S, Banatvala JE, et al. Comparison of human immunodeficiency virus type 1-specific inhibitory activities in saliva and other human mucosal fluids. *Clin Vaccine Immunol* 2006 10; 13(10):1111–8. [PubMed: 16928883]
44. Lee-Huang S, Huang PL, Sun Y, Kung HF, Blithe DL, Chen HC. Lysozyme and RNases as anti-HIV components in beta-core preparations of human chorionic gonadotropin. *Proc Natl Acad Sci U S A* 1999 3 16;96(6):2678–81. [PubMed: 10077570]
45. Wahl A, Swanson MD, Nochi T, Olesen R, Denton PW, Chateau M, et al. Human breast milk and antiretrovirals dramatically reduce oral HIV-1 transmission in BLT humanized mice. *PLoS Pathog* 2012 Jun;8(6):e1002732.
46. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP, et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* 2011 9 22;477(7365): 466–70. [PubMed: 21849977]
47. Moore PL, Gray ES, Wibmer CK, Bhiman JN, Nonyane M, Sheward DJ, et al. Evolution of an HIV glycan-dependent broadly neutralizing antibody epitope through immune escape. *Nat Med* 2012 11;18(11):1688–92. [PubMed: 23086475]
48. Binley JM, Wrinn T, Korber B, Zwick MB, Wang M, Chappey C, et al. Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. *J Virol* 2004 12;78(23):13232–52. [PubMed: 15542675]
49. Coovadia HM, Brown ER, Fowler MG, Chipato T, Moodley D, Manji K, et al. Efficacy and safety of an extended nevirapine regimen in infant children of breastfeeding mothers with HIV-1

- infection for prevention of postnatal HIV-1 transmission (HPTN 046): a randomised, double-blind, placebo-controlled trial. *Lancet* 2012 1 21;379(9812):221–8. [PubMed: 22196945]
50. Bomsel M, Tudor D, Drillet AS, Alfsen A, Ganor Y, Roger MG, et al. Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. *Immunity* 2011 2 25; 34(2):269–80. [PubMed: 21315623]
51. Fouda GG, Amos JD, Wilks AB, Pollara J, Ray CA, Chand A, et al. Mucosal immunization of lactating female rhesus monkeys with a transmitted/founder HIV-1 envelope induces strong Env-specific IgA antibody responses in breast milk. *J Virol* 2013 4 17;87(12):6986–99. [PubMed: 23596289]
52. Wilks AB, Christian EC, Seaman MS, Sircar P, Carville A, Gomez CE, et al. Robust vaccine-elicited cellular immune responses in breast milk following systemic simian immunodeficiency virus DNA prime and live virus vector boost vaccination of lactating rhesus monkeys. *J Immunol* 2010 12 1;185(11):7097–106. [PubMed: 21041730]

Figure 1.

(A) Neutralization of HIV subtype A (92UG031, triangles) and D (92UG005, circles) target viruses by breast milk from 25 Ugandan women with HIV infection. Differences in neutralization of subtype A vs. subtype D were compared using unpaired student's *t* test, $**p = .002$. The right side of the graph shows dose-dependent neutralization of subtype A virus by 6 milk samples at various dilutions. (B) Neutralization of homologous and heterologous HIV target virus subtypes by breast milk. Milk from 9 mothers infected with subtype A virus and 4 mothers with subtype D virus was incubated with target virus subtype A (92UG031, black bars) and D (92UG005, grey bars).

Figure 3.

Neutralization of subtype A and D viruses by milk fractions with subtype A mothers. Milk from five Ugandan mothers infected with subtype A virus was separated into antibody (IgG and IgA) and non-antibody (non-Ig) fractions. Milk fractions were incubated with subtype A (92UG031) and D virus (92UG005). Values represent mean $\pm$ SEM. Differences in neutralization between milk fractions were determined using one-way ANOVA and Tukey's multiple comparison test. * $p < .05$, ** $p < .01$, *** $p < .001$.

Figure 4. HIV-specific antibody to HIV proteins in milk of infected Ugandan mothers. Reactivity of milk IgA and IgG with HIV antigens was detected by immunoblot. Yellow = no reactivity, green = weak reactivity, blue = strong reactivity. The ability of milk to neutralize subtype A and D target viruses is shown (grey = ≥50% neutralization; right panel), and nested PCR of the C2–V3–C3 region of the *env* gene was done on 13 of 25 milk cell pellets to determine HIV viral subtype.