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HIV-Specific Functional Antibody Responses in Breast Milk Mirror Those in Plasma and Are Primarily Mediated by IgG Antibodies▽

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Despite months of mucosal virus exposure, the majority of breastfed infants born to HIV-infected mothers do not become infected, raising the possibility that immune factors in milk inhibit mucosal transmission of HIV. HIV Envelope (Env)-specific antibodies are present in the milk of HIV-infected mothers, but little is known about their virus-specific functions. In this study, HIV Env-specific antibody binding, autologous and heterologous virus neutralization, and antibody-dependent cell cytotoxicity (ADCC) responses were measured in the milk and plasma of 41 HIV-infected lactating women. Although IgA is the predominant antibody isotype in milk, HIV Env-specific IgG responses were higher in magnitude than HIV Env-specific IgA responses in milk. The concentrations of anti-HIV gp120 IgG in milk and plasma were directly correlated ($r = 0.75$; $P < 0.0001$), yet the response in milk was 2 logarithm units lower than in plasma. Similarly, heterologous virus neutralization ($r = 0.39$; $P = 0.010$) and ADCC activity ($r = 0.64$; $P < 0.0001$) in milk were directly correlated with that in the systemic compartment but were 2 log units lower in magnitude. Autologous neutralization was rarely detected in milk. Milk heterologous virus neutralization titers correlated with HIV gp120 Env-binding IgG responses but not with IgA responses ($r = 0.71$ and $P < 0.0001$, and $r = 0.17$ and $P = 0.30$). Moreover, IgGs purified from milk and plasma had equal neutralizing potencies against a tier 1 virus ($r = 0.65$; $P < 0.0001$), whereas only 1 out of 35 tested non-IgG milk fractions had detectable neutralization. These results suggest that plasma-derived IgG antibodies mediate the majority of the low-level HIV neutralization and ADCC activity in breast milk.

Breast milk transmission of human immunodeficiency virus (HIV) accounts for almost half of the 400,000 pediatric HIV infections occurring annually in resource-limited areas (68), where replacement feeding is associated with high infant mortality due to respiratory and diarrheal infections (1, 42). Antiretroviral interventions can significantly reduce the risk of HIV transmission through breastfeeding (7, 58). However, little is known about the effects of long-term antiretroviral prophylaxis on infant development or maternal health, including the emergence of antiretroviral-resistant strains of viruses in this setting. Therefore, there is a need to develop alternative preventive strategies, such as maternal or infant vaccination, that would allow safe breastfeeding of infants born to HIV-infected women.

Breast milk transmission of HIV has been correlated with high milk levels of viral RNA, cell-associated viral DNA, and mastitis (25, 51–52), but these associations do not fully explain the low level of virus transmission through breastfeeding. In the absence of antiretroviral prophylaxis, less than 10% of infants born to HIV-infected women and breastfed during the first 6 months of life become infected postnatally, despite daily breast milk exposure (9). This low level of transmission suggests that breast milk may contain protective antiviral factors. Identifying these factors would provide important insights into the type of immune responses required to protect against infant HIV acquisition. As breast milk is a rich source of antibodies, the potential antiviral activity of breast milk could be mediated by adaptive humoral immune responses (20, 65). Antibodies in milk are either transferred from plasma by transudation or locally produced by plasma cells that migrate to the mammary gland from other mucosal sites, in particular, the gut-associated lymphoid tissues (19). Antibodies against HIV Env glycoproteins have been detected in the breast milk of HIV-infected women (5, 31, 64). However, no quantitative differences in milk HIV-specific antibody responses of transmitting and nontransmitting mothers have been identified (5, 31). In simian immunodeficiency virus (SIV)-infected rhesus

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monkeys, although a lack of antibody response was associated with early breast milk transmission, the levels of the SIV-specific antibodies were similar in late transmitters and nontransmitters (54). These findings indicate that the specificity and the functionality of breast milk antibodies may be stronger correlates of protection against infant virus acquisition than the magnitude of the responses.

Systemic administration of neutralizing antibodies can protect neonatal rhesus monkeys against oral challenge with simian-human immunodeficiency virus (SHIV) (14, 53), indicating a potential role for neutralizing antibodies in preventing vertical transmission of HIV. Interestingly, previous studies have reported an association between neutralization activity in maternal sera and protection against infant virus acquisition (3, 11, 56). Furthermore, viruses that are resistant to neutralization by maternal plasma have been associated with HIV transmission via breastfeeding (48). However, in a recent study, there was no association between the magnitude and breadth of heterologous virus neutralization in HIV-exposed infants at birth and postnatal HIV acquisition (33). Thus, the role of neutralizing antibodies in vertical transmission of HIV is unclear. Viruses isolated from the breast milk and plasma of HIV-infected women have similar sensitivity to neutralization by broadly HIV-neutralizing monoclonal antibodies (MAbs) (55), but the ability of breast milk antibodies to neutralize autologous and heterologous HIVs has not been studied. Moreover, nonneutralizing antibodies, such as antibodies that mediate antibody-dependent cellular cytoxicity (ADCC) responses, may contribute to better clinical outcome and lower HIV/SIV load (2, 17, 41) and to the protection of vaccinated rhesus macaques against SHIV challenge (71). However, little is known about the ability of breast milk antibodies to mediate this function. Therefore, it is necessary to further investigate the neutralizing and nonneutralizing functions of breast milk HIV Env-specific antibodies that could contribute to the protection of infants against HIV acquisition via breastfeeding.

Plasma and mucosal HIV Env-specific IgA antibody responses directed against gp140 and gp41 are detected in the majority of individuals during acute HIV infection (72), yet in studies of chronic infection, the magnitude and frequency of HIV-specific IgA antibodies were low (30, 35). HIV-specific mucosal IgA responses have been detected in the genital tracts of highly exposed uninfected women, and these antibodies were shown to neutralize HIV primary isolates (6, 8, 10, 27, 43). However, other studies have reported little or no evidence of HIV-specific mucosal IgA responses in HIV-exposed uninfected subjects (12, 37). Furthermore, HIV-specific IgA antibodies were rarely detected in the saliva of exposed uninfected infants (13). In SIV-infected rhesus monkeys, SIV-specific IgA antibodies isolated from milk had limited neutralization potency compared to that of IgG (45). Breast milk secretory IgA (sIgA) can inhibit virus transcytosis (5, 24), but this function does not appear to correlate with protection (5). Thus, the antiviral functions of breast milk IgA need to be further investigated in order to elucidate the potential roles of mucosal IgA in protection against mucosal HIV transmission.

In this study, HIV Env-specific antibody responses were measured in breast milk and plasma from 41 HIV-infected lactating women from Blantyre, Malawi. The magnitude and breadth of the HIV Env-binding IgG and IgA responses, the autologous and heterologous neutralization responses, and the ADCC activity were characterized in the systemic and breast milk compartments. Further, the IgG and IgA fractions of breast milk and plasma were isolated and assessed for heterologous neutralization, providing insight into the functional role of each HIV Env-specific antibody isotype in milk.

MATERIALS AND METHODS

Study population. Pregnant women testing HIV positive by a rapid antibody test were recruited from two rural health clinics outside Blantyre, Malawi, as part of the CHAVI 009 protocol. Women were enrolled at delivery if HIV infection was confirmed by HIV RNA and breastfeeding was initiated. All mothers and infants in this study received single-dose nevirapine at delivery. Maternal plasma and breast milk samples from 41 women who remained off antiretroviral therapy during lactation (except nevirapine at delivery) were included. The breast milk samples were centrifuged at low speed and separated into a breast milk cell layer, supernatant, and a fat layer. The breast milk supernatant was frozen at −20°C until it was used. The median CD4 count was 360 (range, 80 to 833). Samples were collected at 4 to 6 weeks, at 3 months, and then every 3 months after delivery until the cessation of breastfeeding or the infant reached 18 months of age. Additionally, breast milk and plasma samples collected from nine lactating uninfected women recruited from the same rural Malawian health clinics and four uninfected women recruited in Boston, MA, were used as negative controls. This study was approved by the College of Medicine Research and Ethics Committee in Malawi and institutional review boards at each of the participating institutions where samples were received or processed for end user analysis.

HIV plasma and breast milk virus load. Viral loads in breast milk and plasma were measured using the Roche Cobas Ampliprep/Cobas TaqMan 48 for HIV-1 load assay. The breast milk supernatant was diluted 1:5 in phosphate-buffered saline (PBS) prior to analysis, the optimal dilution for the most accurate quantitation of virus RNA based on preliminary assays with milk containing a known quantity of virus. The detection limit of this assay for breast milk is 240 RNA copies/ml and 48 RNA copies/ml for plasma. Viral RNA was detected in 17 of 34 tested breast milk samples and in 31 of 34 plasma samples. If virus RNA was detected in the sample but below the minimum for quantitation, a value of half the minimum for detection was assigned. The median virus load in breast milk was 770 RNA copies/ml (range, not detectable to 155,000 RNA copies/ml) and 5,340 RNA copies/ml in plasma (range, not detectable to 202,000 RNA copies/ml). The laboratory performing these assays was enrolled in the National Institute of Allergy and Infectious Diseases Division of AIDS Virology Quality Assessment program and certified for HIV load determinations.

HIV *env* gene single-genome amplification and cloning. Plasma aliquots containing approximately 10,000 RNA copies were extracted using a QIAamp viral RNA minikit (Qiagen). For plasma samples with low viral loads (<10,000 RNA copies/ml) and all breast milk supernatants, aliquots of 1 ml were concentrated 10 times by centrifugation at 23,600 × *g* for 1 h at 4°C (final volume, 100 μl) before RNA extraction. Depending on the virus RNA load, between 200 and 10,000 RNA copies were reverse transcribed. Single-stranded cDNA was synthesized using the SuperScript III protocol according to the manufacturer's instructions (Invitrogen Life Technologies) and either used immediately for PCR or stored frozen at −80°C. Full-length *env* genes were PCR amplified by single-genome amplification methods as previously described (28). Briefly, cDNA was titrated by endpoint dilution (undiluted to 1:40 dilution for breast milk cDNA) in 96-well PCR plates to a concentration that yielded no more than 30% PCR-positive wells and conformed to a Poisson distribution of a single template per reaction. The reaction mixture for the first round of PCR (total volume, 20 μl) consisted of 1× High Fidelity platinum PCR buffer, 2 mM $MgSO_4$, 0.2 mM each deoxynucleoside triphosphate, 0.2 μM primers Vif1 (5′-GGGTTTATTACAGG GACAGCAGAG-3′; nt 4900 to 4923) and OFM19 (5′-GCACTCAAGGCAA GCTTTATTGAGGCTTA-3′), and 0.025 U/μl platinum *Taq* High Fidelity polymerase (Invitrogen). The following PCR conditions were used: 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 4 min, with a final extension of 68°C for 10 min. The second-round PCR was carried out using 2 μl of the first-round product and 0.2 μM primers EnvA (5′-GGCTTAGGCA TCTCCTATGGCAGGAAGAA-3′; nt 5954 to 5982) and EnvN (5′-CTGCCA ATCAGGGAAGTAGCCTTGTGT-3′; nt 9145 to 9171) with the same PCR mixture as the first round. The conditions were 94°C for 2 min, followed by 45 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 4 min, with a final extension at 68°C for 10 min. The amplicons were sized on precast 1% agarose E-gel 96 (Invitrogen Life Technologies) and gel purified according to the manufacturer's instructions (Qiagen). Amplicons derived from cDNA dilutions yielding less than

30% PCR positivity were DNA sequenced using BigDye Terminator v.3.1 chemistry and the protocols recommended by the manufacturer (Applied Biosystems). The sequences were determined by using an ABI 3730xl DNA analyzer (Applied Biosystems) and edited by using the Sequencher program, version 4.9 (Gene Codes). The purified amplicons were ligated into pcDNA3.1 Directional Topo vectors (Invitrogen) and used to transform TOP10 *Escherichia coli* cells according to the manufacturer's instructions. Cultures were grown at 30°C, and selected colonies were screened by miniprep and restriction digested with XhoI and BamHI (New England BioLabs). Plasmids with fragments of the correct size after restriction digestion were sequenced to confirm sequence identity to the original *env* amplicon. Confirmed *env* clones were grown in large culture at 30°C. Plasmids were prepared by Megaprep (Qiagen) and resequenced to again confirm sequence identity to the original *env* amplicon.

Pseudovirus preparation. Env pseudoviruses were prepared by transfection in 293T cells with 4 μg of *env* plasmid DNA and 8 μg of *env*-deficient HIV plasmid DNA using the FuGene 6 transfection reagent (Roche Diagnostics). Two days after transfection, the culture supernatant containing pseudoviruses was harvested, filtered, aliquoted, and stored at −80°C. An aliquot of frozen pseudovirus was used to measure the infectivity in TZM-bl cells. Serial 5-fold dilutions of pseudovirus were distributed in quadruplicate to 96-well flat-bottom plates (Costar) in a total volume of 100 μl per well. Then, freshly trypsinized TZM-bl cells were added (10,000 cells/well in Dulbecco's modified Eagle's medium [DMEM]-10% fetal bovine serum [FBS] containing HEPES and 10 μg/ml of DEAE-dextran). After 48 h of incubation at 37°C, 100 μl of cells/well was transferred to a 96-well black solid plate (Costar), and the luminescence was measured using the Britelite Plus luminescence reporter gene assay system (Perkin-Elmer Life Sciences). Wells producing relative luminescence units (RLU) >3 times background were scored as positive, and the $TCID_{50}$ was calculated by the method of Reed and Muench, as previously described (26).

Quantification of total and HIV Env-specific IgG and IgA antibodies in breast milk and plasma. The concentrations of total IgA and IgG antibodies in plasma and milk were measured by commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Immunology Consultants Laboratory, Inc.). Levels of HIV Env-specific IgA and IgG were determined with a customized HIV-1 binding antibody multiplex assay as previously described (61) with some modifications. Briefly, a total of 5×10^6 carboxylated fluorescent beads (Luminex Corp.) were covalently coupled to 25 μg of purified HIV antigen and incubated with patient samples at various dilutions. HIV-specific antibody isotypes were detected with phycoerythrin (PE)-conjugated goat anti-human IgA (Jackson ImmunoResearch) or PE-conjugated mouse anti-human IgG (Southern Biotech). The beads were then washed and acquired on a Bio-Plex instrument (Bio-Rad). The results were expressed as mean fluorescence intensity (MFI). For enhanced detection of IgA antibodies, IgG was removed using protein G columns, as described below. The positive controls in each assay included purified IgG and IgA proteins (Sigma-Aldrich), HIVIG (Quality Biological), and a constant HIV-positive serum titration. Normal human plasma was utilized as a negative control, and unconjugated beads were used to control for nonspecific binding of sample to the beads. HIV-1 Env-specific antibodies were captured using the following proteins: a clade C consensus Env gp120 constructed from alignments of full-length subtype C Env sequences available in 2001 in the Los Alamos HIV sequence database (29) (ConC gp120, provided by Lynn Morris and Elin Gray, National Institute of Communicable Diseases, Johannesburg, South Africa); an artificial multiclade consensus Env gp120 described previously (18) (Con6 gp120); a multiclade gp140 consensus Env constructed from alignments of full-length subtype C Env sequences available in 2002 in the Los Alamos HIV sequence database with deletions in the gp41 cleavage site, fusion domain, and immunodominant region (ConS gp140); a clade C consensus Env gp140 with deletions in the gp41 cleavage site and fusion domain (ConC gp140); a previously described (69) gp140 Env from a clade C primary isolate (DU123 gp140); a gp140 trimer from a transmitted/founder virus obtained from a single-genome amplified sequence by Ronald Swanstrom, University of North Carolina, Chapel Hill, NC (1086C gp140); and a recombinant gp41 Env (Immunodiagnostics). Con6 gp120, ConS gp140, ConC gp140, 1086C gp140, and DU123 gp140 envelope proteins were all provided by Hua-Xin Liao and Barton Haynes, Duke Human Vaccine Institute, Durham, NC. Antibody concentrations were determined by applying standard curves generated by titrating HIV Env-specific MAbs in a 4PL curve analysis: 2G12 IgG (62), provided by Herman Katinger, Polymune Scientific, Vienna, Austria (for Con6 gp120 IgG and ConS gp140 IgG), b12 IgA (34), provided by Anne Hessell and Dennis Burton, Scripps Research Institute (for Con6 gp120 IgA and ConS gp140 IgA); and 2F5 IgG and IgA (40), obtained from Polymune (for gp41 IgG and IgA).

Breast milk delipidization. To reduce breast milk cell toxicity, the samples were delipidized prior to functional assay testing. Milk samples were centrifuged at 25,000 × *g* at 4°C for 30 min and then filtered using spin X filter tubes (Fisher). The efficiency of the procedure was tested by incubating serial dilutions of nondelipidized and corresponding delipidized milk samples with TZM-bl cells in the absence of virus for 48 h and measuring luminescence. Cell toxicity was observed by microscopy in wells containing the three lowest dilutions of nondelipidized milk, and the MFI in these wells was lower than in control wells. No toxicity was observed based on microscopy or MFI deflection with delipidized samples. To ensure that the procedure did not affect antibody function, a negative breast milk sample was spiked with either b12 IgG or HIVIG prior to delipidization and assayed for neutralization of a tier 1 virus. The delipidization procedure did not affect the neutralization potency of b12 or HIVIG.

IgG and non-IgG fraction purification. IgG was purified from plasma and breast milk using protein G resin prepacked into 96-well depletion plates (GE Healthcare). Plasma samples were clarified by centrifugation at 10,000 × *g* for 10 min and diluted 2-fold with PBS (pH 7.5). A total of 200 μl of diluted plasma or delipidized breast milk was added to each well, and the plates were incubated at room temperature, with shaking, for 1 h. Unbound fractions were removed by centrifugation at 1,200 × *g* for 3 min. The wells were then washed 3 times with 200 μl of Tris-buffered saline (TBS). IgG bound to the resin was eluted with 2.5% glacial acetic acid and immediately neutralized with 120 μl of 1 M Tris-HCl, pH 9.0. The unbound fractions of milk were then run over Protein L Spin Columns according to the protocol (Pierce) to purify the non-IgG antibody fraction. The eluted IgG and non-IgG fractions were concentrated using Amicon Ultra centrifugal filters with a 30,000-Da cutoff (Millipore). The protein concentration was determined by spectrophotometry at A_{280} using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Extinction coefficients of 1.36 (the extinction coefficient for monomeric IgG) and 1.26 (the extinction coefficient of sIgA) (44) were used to calculate the antibody concentrations for the IgG and non-IgG fractions, respectively. The purities of the IgG and non-IgG fractions were tested in a subset of samples by ELISA. No IgA was detected in the IgG fraction, and the non-IgG fraction contained <2% IgG. The presence of sIgA in the non-IgG fraction was confirmed by commercial ELISA (Antibodies-Online Inc.).

IgA purification. IgA was purified from the 6 breast milk-plasma pairs with the highest milk HIV Env-specific IgA magnitudes and breadths by binding to peptide M resin (Invitrogen). Briefly, peptide M gel slurry (250 μl) was loaded into spin columns (Millipore); then, IgG-depleted milk preparations were loaded onto the columns and incubated with rocking at room temperature for 45 min. After incubation, the columns were washed and the flowthrough fractions were collected. The bound antibodies were then eluted with elution buffer (Thermo Scientific) in small fractions and neutralized immediately with neutralization buffer (Thermo Scientific). The columns were subsequently regenerated by washing with regeneration buffer and used for another round of purification of the flowthrough to increase the yield. Finally, the eluted fractions from the two consecutive purification processes were combined for each sample, buffer exchanged with PBS, and concentrated using Amicon Ultra-4 filters. The protein concentration was determined by spectrophotometry at A_{280} using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The purified IgA fractions were tested in the binding antibody multiplex assay to confirm the presence of HIV-specific IgA and the efficient removal of IgG.

Autologous and heterologous virus neutralization. Autologous and heterologous neutralization assays were performed in TZM-bl reporter cells. For autologous virus neutralization assays, five to nine breast milk- and plasma Env-pseudotyped viruses from each of five HIV-infected women were tested. The autologous Env clones were sequenced and cloned from milk and plasma collected 4 to 6 weeks postdelivery. Plasma and milk samples collected from that time point and all subsequent available time points were assayed in these five subjects. For heterologous virus neutralization assays, one clade C tier 1 virus (HIV MW965.26) and two clade C tier 2 viruses (HIV DU422.1 and HIV CAP45.200.G3) were used. Breast milk and plasma samples from all 41 women collected 4 to 6 weeks after delivery were assayed. Purified milk IgG, non-IgG, and IgA fractions were subsequently tested against the tier 1 virus. Prior to neutralization assessment, the plasma samples were heat inactivated at 56°C for 1 h and the breast milk samples were delipidized and concentrated 4 times using Amicon Ultra centrifugal filters with a 100,000 cutoff. Neutralization was measured by the reduction in luciferase reporter gene expression after a single round of infection in TZM-bl cells, as previously described (32). Briefly, diluted virus preparations (a predetermined dilution yielding RLU at least 10 times above the cell control background) were incubated with 3-fold serial dilutions of plasma, concentrated milk supernatant, or purified immunoglobulin-milk preparations in duplicate for 1 h at 37°C in 96-well flat-bottom culture plates. TZM-bl cells were then added (1×10^4/well in a 100-μl volume) in 10% FBS-DMEM growth medium containing DEAE-dextran (Sigma) at a final concentration of 10 μg/ml.

Assay controls included replicate wells of TZM-bl cells alone (cell control) and TZM-bl cells with virus (virus control). In addition, negative-control assays were performed using a murine retrovirus (simian virus amphotropic murine leukemia virus [SVA.MLV]) Following 48 h of incubation at 37°C, luminescence was measured using the Britelite Plus luminescence reporter gene assay system (Perkin-Elmer Life Sciences). The 50% inhibitory dose (ID_{50}) titer was calculated as the plasma or milk dilution that caused a 50% reduction in RLU compared to the virus control wells after subtraction of cell control RLU. The 4× concentration of breast milk was taken into account in calculating the breast milk dilution. A dilution of sample was considered positive for neutralization when the ID_{50} was higher than 3 standard deviations above the median of the negative controls and 3 times above the SVA.MLV control. The 50% inhibitory concentration (IC_{50}) titer was calculated as the purified Ig concentration that caused a 50% reduction in RLU.

ADCC. ADCC activities in breast milk and plasma were measured using an adapted GranToxiLux (OncoImmunin, Inc.) protocol (45a). The assay is based on the cleavage of a cell-permeable green fluorogenic peptide substrate by the serine-protease granzyme B (GzB) within target cells. GzB is synthesized and stored in cytotoxic cells in an inactive form. When effector cells recognize target cells through antibody (Ab)-Fc-receptor interactions, their granule content, including GzB, is released into target cells. In this assay, GzB cleaves its fluorogenic substrate within the target cells, thereby generating a green fluorescent signal that can be quantified by flow cytometry. $CEM.NKR_{CCR5}$ target cells were coated with recombinant HIV-1 DU422.1 gp120 to saturation, counted, washed, and resuspended at 1×10^6 cells/ml in complete medium (RPMI 1640 with 10% FBS). The target cells were labeled with a red fluorescent maker and a viability marker. Viable cells were counted with a Guava PCA system (Millipore) and then adjusted to a concentration of 0.4×10^6 cells/ml. Cryopreserved peripheral blood mononuclear cells (PBMCs) were used as effector cells. The PBMCs were thawed and rested overnight at 2×10^6 cells/ml in complete medium and then counted and resuspended at 12×10^6 viable cells/ml. Effector and target cell suspensions (25 μl of each) were dispensed into each well of a 96-well V-bottom plate and incubated with 75 μl of GzB substrate at room temperature for 5 min. Diluted milk and plasma samples (25 μl) were then added. The final effector-to-target ratio was 30:1, and the final GzB substrate concentration was 0.25×. After 15 min of incubation at room temperature, the plates were centrifuged for 1 min at $300 \times g$, incubated for 1 h at 37°C and 5% CO_2, and then washed twice with PBS containing 1% FBS. The cells were then resuspended in a final volume of 225 μl of PBS-1% FBS and placed at 4°C until they were analyzed. A minimum of 2,500 events representing viable target cells were acquired within 5 h. The maximal cytotoxic potency was defined as the maximal proportion of gp120-coated target cells positive for GzB activity after incubation with 5-fold serial antibody dilutions and effector cells. The results were expressed as percent GzB activity, defined as the percentage of GzB-positive cells out of the total viable target population. For final results, the background (percent GzB activity in wells containing effector and target cell populations and no test samples) was subtracted. The ADCC-mediating antibody titer was defined as the reciprocal of the lowest dilution indicating a positive GzB response (>8.1% GzB activity) after background subtraction.

Statistical analysis. The paired nonparametric Wilcoxon signed rank test was used to compare the magnitudes of HIV Env-specific IgG and IgA antibodies in milk and plasma. The Spearman correlation coefficient was used to determine the correlation between the binding, neutralizing, and ADCC responses in milk and plasma. This test was also used to determine the correlation of milk neutralizing and ADCC responses with HIV-specific IgG and IgA responses and the correlation between the viral load and antibody responses. The lowest tested dilution of plasma/milk was used to compute undetectable levels of functional antibodies. Fisher's exact test was used to compare the proportions of women with detectable responses in plasma and milk. All statistical tests were performed with Graphpad (La Jolla, CA) Prism 5.

Nucleotide sequence accession numbers. All *env* sequences determined in this study were deposited in GenBank (accession numbers HM070449 to HM070824 and HQ595810 to HM596189).

RESULTS

IgA is the predominant antibody isotype in milk, yet the HIV Env-specific humoral response in milk is primarily IgG isotype. We first sought to assess the relationship between the magnitude of the HIV-specific humoral immune responses and the total IgG and IgA content in breast milk of HIV-infected lactating women. To examine the IgG and IgA content of breast milk compared to that in plasma, we quantified total IgG and IgA in plasma and milk samples collected 4 to 6 weeks after delivery from chronically HIV-1-infected women from Blantyre, Malawi (Fig. 1). The median level of IgG was 18.7 mg/ml (range, 5.1 to 62 mg/ml) in plasma and 0.08 mg/ml (range, 0.02 to 0.4 mg/ml) in milk (Fig. 1). Thus, plasma contains 2 log units more IgG than milk ($P < 0.0001$). The total IgA content was 0.5 to 1 log unit higher in plasma than in milk (median, 3.9 mg/ml versus 0.71 mg/ml; $P < 0.0001$); however, it is possible that a portion of the milk IgA could be associated with the fatty fraction of the milk that is removed with the delipidization procedure. Consistent with previous characterizations of antibody isotypes in milk (60), breast milk samples contained 0.5 to 1 log unit more total IgA than IgG ($P < 0.0001$), whereas plasma samples contained approximately 1 log unit less IgA than IgG ($P < 0.0001$) (Fig. 1).

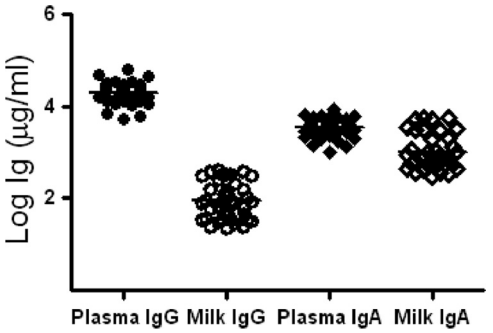

FIG. 1. Total IgG and IgA are lower in breast milk than in plasma. Shown is the total IgG and IgA contents in plasma and breast milk of 41 HIV-infected lactating women. In Fig. 1 to Fig. 6, the lines in the panels represent the medians.

The magnitudes and breadths of the HIV Env-binding IgG and IgA binding responses in milk were then assessed using a panel of HIV Env antigens, including two consensus gp140 proteins (ConS gp140 and ConC gp140), one gp140 Env protein from a clade C primary isolate (DU123 gp140), two consensus gp120 proteins (Con6 gp120 and ConC gp120), a consensus gp41 Env protein, and a transmitted/founder gp140 Env trimer (1086c gp140). To ensure that high-affinity IgG did not interfere with IgA binding, samples were depleted of IgG prior to measurement of HIV-specific IgA. HIV Env-specific IgG responses against all of the above-mentioned Env antigens were detected in plasma and breast milk of all subjects (41/41), but the seroprevalence of IgA responses to gp120 proteins was higher in plasma samples than in milk samples (Table 1). Similarly, IgA antibodies against gp41 were detected in 41 out of 41 plasma samples and in only 4 out of 41 milk samples ($P < 0.0001$). IgA responses against gp140 proteins were detected in the plasma and milk of most subjects, but as with the other antigens, the frequency of positive IgA gp140 responses was significantly higher in plasma than in milk (Table 1). Moreover, IgA antibodies to the transmitted/founder Env trimer were detected in 40 out of 41 plasma and in 30 out of 41 breast milk samples ($P = 0.003$). This difference in the seroprevalence of HIV Env-specific IgA responses between plasma and milk might indicate partial compartmentalization of the milk IgA response.

TABLE 1. Seroprevalence of HIV Env-binding IgG and IgA in plasma and milk

HIV Env antigen	IgG prevalence [% (no.)]		IgA prevalence [% (no.)]		*P* value (Fisher's exact test), plasma vs. milk IgA
	Plasma	Milk	Plasma	Milk	
ConS gp140	100 (41/41)	100 (41/41)	97.5 (40/41)	71 (29/41)	0.0015
ConC gp140	100 (41/41)	100 (41/41)	95 (39/41)	68 (28/41)	0.0032
DU123 gp140	100 (41/41)	100 (41/41)	90 (37/41)	71 (29/41)	0.048
Con6 gp120	100 (41/41)	100 (41/41)	76 (31/41)	39 (16/41)	0.0016
ConC gp120	100 (41/41)	100 (41/41)	73 (30/41)	27 (11/41)	<0.0001
gp41	100 (41/41)	100 (41/41)	100 (41/41)	10 (4/41)	<0.0001
1086c gp140	100 (41/41)	100 (41/41)	97.5 (40/41)	73 (30/41)	0.0033

The levels of gp120, gp140, and gp41 IgG and IgA antibody responses in milk did not correlate with the milk viral load (IgG, *r* values between 0.07 and 0.24 and *P* values between 0.15 and 0.56; IgA, *r* values between 0.10 and 0.24 and *P* values between 0.19 and 0.79). However, there was a strong correlation between the levels of IgG and IgA antibodies to interclade and clade C consensus gp120 proteins ($r = 0.69$ to 0.78 and $P < 0.0001$ for all) and between the levels of interclade and clade C (either consensus or primary isolate) antibodies to gp140 proteins ($r = 0.56$ to 0.93 and $P < 0.0001$ for all) in both plasma and milk. There was also a very strong correlation between binding antibodies to the consensus clade C and the clade C primary isolate DU123 gp140 proteins ($r = 0.84$ to 0.97 and $P < 0.0001$) (data not shown), indicating that the IgG and IgA binding antibody responses in the plasma and milk may be mainly directed against conserved epitopes.

The concentrations of antibodies directed against gp140 (ConS), gp120 (Con6), and gp41 in plasma and milk were estimated by assaying the plasma and milk HIV Env binding compared to that of known concentrations of antigen-matched HIV Env-specific MAbs. After plotting the MFI of each sample on the MAb standard curve, the results were expressed in ng/ml equivalents (eq). When detected in plasma and milk samples, the magnitudes of the HIV Env-specific IgG binding responses were higher than those of the HIV Env-specific IgA binding responses for all antigens in both compartments, except for gp41 in milk (gp140, $P < 0.0001$ in plasma and $P = 0.0003$ in milk; gp120, $P < 0.0001$ in plasma and milk; and gp41, $P < 0.0001$ in plasma and $P = 0.62$ in milk) (Fig. 2A to C). Thus, the HIV Env-specific IgG response appears to be predominant in both the breast milk and systemic compartments. This observation in milk is surprising, as breast milk contains approximately 1 log unit more total IgA than total IgG antibodies (Fig. 1). However, these results are in agreement with previous reports of an IgG-biased HIV Env-specific response in breast milk and other mucosal compartments (35, 49, 64).

As plasma has a higher total IgG and IgA content than milk, we expected the concentrations of HIV Env-binding antibodies to be higher in plasma than in milk. As predicted, the concentrations of IgG antibodies against all Env antigens were higher in plasma than in milk (median plasma versus milk Env antigen-specific antibody concentrations: gp140, 2.6×10^6 versus 1.1×10^4 2G12 IgG ng/ml eq; gp120, 6.6×10^5 versus 1.8×10^3 2G12 IgG ng/ml eq; and gp41, 7.8×10^6 versus 2.1×10^4 2F5 IgG ng/ml eq; $P < 0.0001$ for all) (Fig. 2A to C). When detectable, there was no difference in the concentrations of IgA antibodies in plasma and milk against gp120 (median concentration, 0.14×10^3 versus 0.29×10^3 b12 IgA ng/ml eq; $P = 0.33$) or gp41 (median concentration, 5.0×10^3 versus 5.4×10^3 2F5 IgA ng/ml eq; $P = 0.87$). However, the concentration of IgA antibodies to gp140 was significantly higher in plasma than in milk (median concentration, 0.23×10^3 versus 0.16×10^3 b12 IgA ng/ml eq; $P = 0.0075$). Interestingly, there was a strong correlation between the concentration of HIV Env-binding IgG responses against gp120 and gp140 antigens in the two compartments (gp140, $r = 0.71$ and $P < 0.0001$; gp120, $r = 0.75$ and $P < 0.0001$) (Fig. 2D and E). However, these correlations may be driven by the subset of women with high Env-specific IgG levels in both milk and plasma. There was also a statistically significant trend toward a correlation of the concentrations of IgG antibodies against gp41 in milk and plasma ($r = 0.45$; $P = 0.0027$) (Fig. 2F). In contrast, the concentrations of HIV Env-binding IgA responses did not correlate between plasma and milk (gp140, $r = 0.26$ and $P = 0.16$; gp120, $r = 0.10$ and $P = 0.72$) (Fig. 2G and H). These results suggest that the majority of the HIV-specific IgG antibodies in milk originate from plasma and further indicate that milk and plasma HIV Env-specific IgA responses are at least partially compartmentalized.

Breast milk neutralizes heterologous HIV virions with lower potency than plasma. We then investigated the ability of the HIV Env-specific antibody responses in breast milk to neutralize the virus, as virus neutralization by breast milk is a possible mechanism for blocking HIV infection in the infant oral/gastrointestinal tract. The abilities of plasma and milk samples to neutralize heterologous viruses were measured against one tier 1 clade C (HIV MW965.26) and two tier 2 clade C (HIV DU422.1 and HIV CAP 45.2.00.G3) viruses. All of the chronically HIV-infected mothers had potent neutralizing activity in plasma against the tier 1 virus, with ID_{50} titers ranging from 583 to 116,990 (median ID_{50} titer, 20,583). This virus was neutralized by a significantly lower frequency of milk samples (23/41 subjects; $P < 0.0001$) (Fig. 3A). Furthermore, the tier 1 virus neutralization titer of milk was severalfold lower than that of plasma (median ID_{50} titer, 103; range, 37 to 549). A positive correlation was observed between tier 1 virus neutralization titers in milk and plasma ($r = 0.39$; $P = 0.01$) (Fig. 3D), suggesting that milk neutralization may be partially mediated by antibodies that cross from the systemic circulation into the breast milk. There was no correlation between milk tier 1 neutralization and the milk viral load ($r = 0.27$; $P = 0.14$) (data not shown). Neutralization of the tier 2 viruses was detected in plasma of 56 to 61% of the subjects (23/41 for HIV CAP45.2.00.G3 and 25/41 for HIV DU422.1) (Fig. 3B and C).

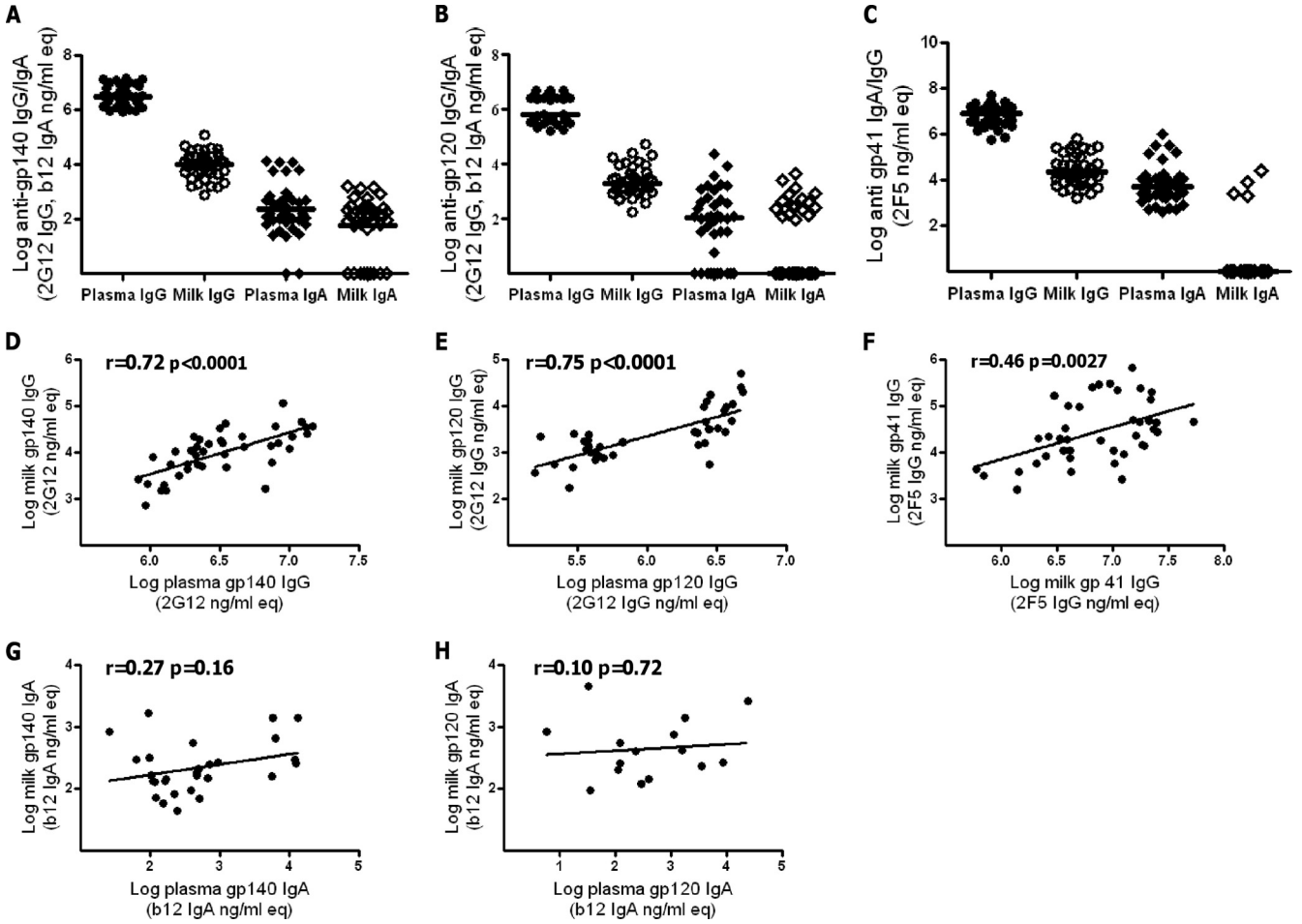

FIG. 2. The HIV Env-binding antibody response in breast milk is predominantly IgG isotype. (A) Anti-gp140 IgG and IgA concentrations in plasma and corresponding milk of 40 HIV-infected women. (B) Anti-gp120 IgG and IgA concentrations in plasma and corresponding milk of 40 HIV-infected women. (C) Anti-gp41 IgG and IgA concentrations in plasma and milk. (D to H) There is strong correlation between anti-gp140, anti-gp120, and anti-gp41 IgG concentrations, but not between anti-gp140 or anti-gp120 IgA concentrations, in plasma and milk.

In contrast, only 22% (9/41) of the HIV-infected lactating women had detectable neutralization of HIV CAP45.2.00.G3 in milk ($P = 0.0030$), and none of the subjects' milk neutralized HIV DU422.1 ($P < 0.0001$). This low level and frequency of neutralization in milk against both tier 1 and tier 2 HIV is probably related to the small amount of HIV Env-specific antibodies in milk compared to that in plasma (Fig. 2). Although it is likely that a portion of immunoglobulin was lost during the delipidization procedure, the procedure did not interfere with neutralization in control assays. As most circulating HIV strains display a tier 2 neutralization phenotype (57), our findings may indicate that the amount of HIV Env-specific antibodies in breast milk is not sufficient for autologous virus neutralization.

Plasma and milk IgG antibodies have equal neutralization potencies. To determine if the weak neutralization potency of milk was due to the low concentration of HIV Env-specific antibodies, we isolated IgG from milk and plasma of HIV-infected lactating women. We measured the neutralization potencies of the purified milk and plasma IgG fractions against the tier 1 HIV MW965.26 (Fig. 4A). Neutralizing activity was detected in the plasma IgG fractions of all HIV-infected lactating women (41/41) and in the milk IgG fractions of 95% of the subjects (39/41). Notably, there was no difference in the median IC_{50}s of the plasma (1.55 μg/ml; range, 0.24 to 40.75 μg/ml) and milk (1.13 μg/ml; range, 0.17 to 36.94 μg/ml; $P = 0.38$) IgG fractions. Moreover, there was a strong correlation between milk and plasma IgG IC_{50}s ($r = 0.65$; $P < 0.0001$) (Fig. 4B). Thus, the IgG fractions of milk and plasma have the same capacity to neutralize HIV, after adjusting for their antibody contents.

Limited neutralization potency of the non-IgG fractions of breast milk. The non-IgG fraction of breast milk was purified from 35 of the breast milk samples by binding of the IgG-depleted fraction to protein L columns. The fractions were then tested for neutralization of the tier 1 virus. Neutralizing activity was detected in 1 out of the 35 non-IgG milk fractions (subject 1209; IC_{50}, 19.01 μg/ml). As protein L binds to kappa light chains, our preparations could contain both IgA and IgM antibodies. Therefore, we specifically purified IgA from the six milk samples with the highest HIV Env-specific IgA breadths and magnitudes and from their corresponding plasma by affinity binding to peptide M, which binds monomeric and di-

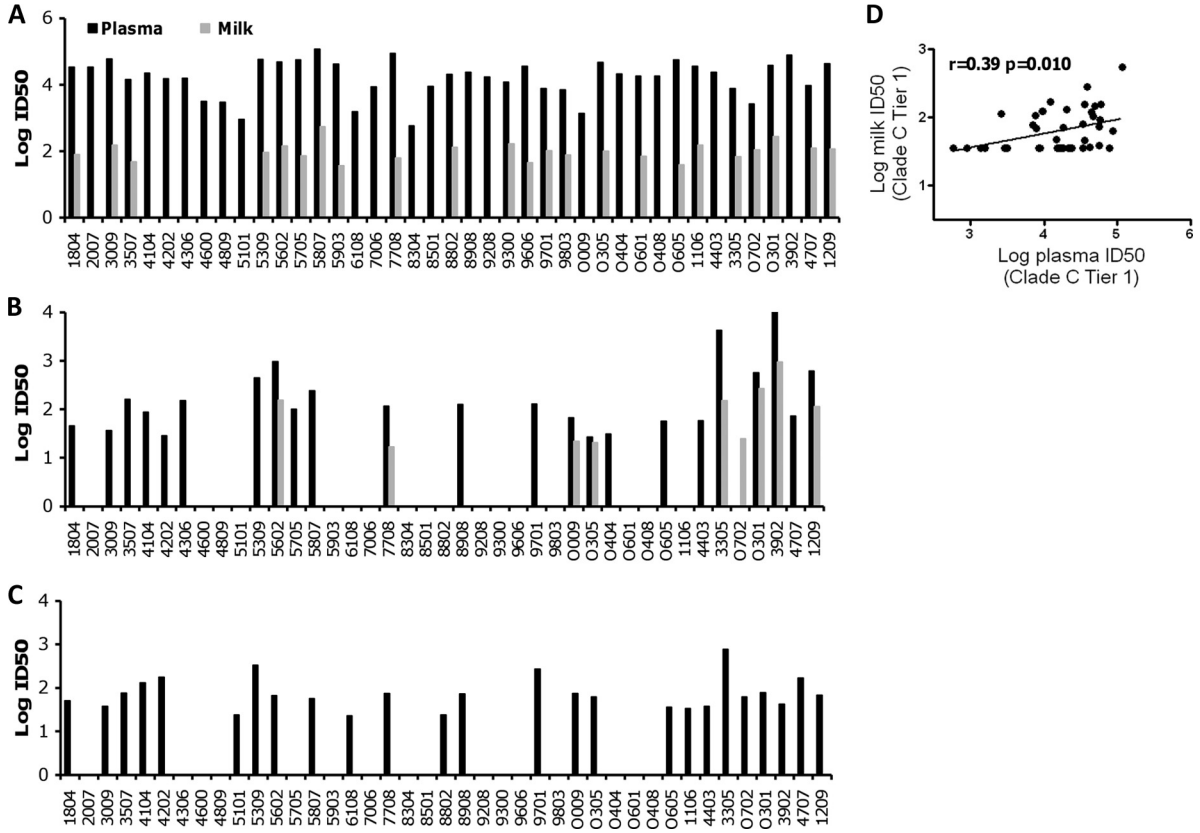

FIG. 3. Breast milk neutralizes heterologous HIV-1 with low potency, yet the neutralizing titer in milk is correlated to that in plasma. (A to C) Neutralization titers of 41 plasma and corresponding breast milk samples against HIV MW965.26, a clade C tier 1 virus (A); HIV CAP45.2.00.G3, a clade C tier 2 virus (B); and HIV DU422.1, a clade C tier 2 virus (C). Only samples that had a neutralization titer greater than 3 standard deviations above the mean of three HIV-negative plasma samples or six HIV-negative milk samples are presented. (D) A direct correlation was observed between the neutralization titers of plasma and corresponding milk samples for the tier 1 virus.

meric human IgA1 and IgA2. Testing of the IgA fractions of breast milk indicated that IgA from subject 1209 was responsible for the neutralization detected in the non-IgG milk fraction (Table 2). In addition, the plasma IgA fraction from 1209 also neutralized the tier 1 virus. The plasma, but not the milk, IgA fraction of subject 0605 also neutralized (IC_{50}, 23.76 μg/ml). Interestingly, although comparable levels of HIV Env-specific IgA (Table 2) were detected in other plasma and breast milk samples tested for IgA neutralization, no neutralizing activity was detected. In contrast, the IgG fractions from all six samples neutralized the tier 1 virus (IC_{50} range, 0.49 to 20.47 μg/ml and 0.6 to 12.5 μg/ml for plasma and milk, respectively) (Table 2), demonstrating that potent IgA-mediated neutralization of HIV is not frequent in breast milk.

Lack of detectable autologous virus neutralization in breast milk of HIV-infected lactating women. The ability of breast milk to neutralize milk and plasma autologous viruses was measured over the period of lactation (6 to 12 months) in five

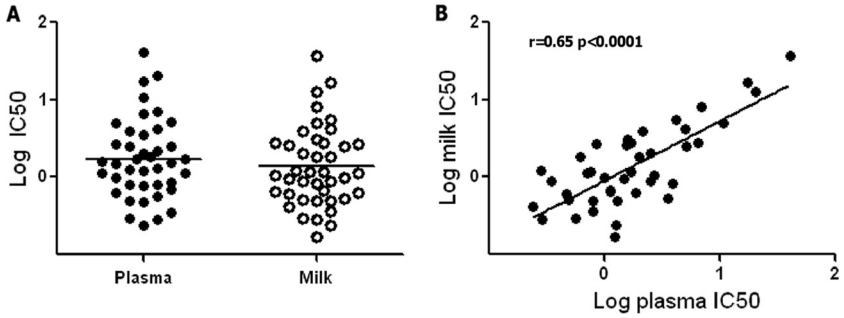

FIG. 4. Plasma and breast milk IgGs of HIV-infected women neutralize HIV-1 with the same potency. (A) IC_{50}s of plasma and milk IgG fractions from 41 HIV-infected women against a tier 1 virus (HIV MW965.26) are not statistically different ($P = 0.36$). (B) Direct correlation of the IC_{50}s of paired plasma and milk IgG fractions.

TABLE 2. IgA neutralization in plasma and breast milk

Subject	Sample	Anti-ConS gp140 IgA (no. of b12 IgA ng/ml eq)	IC_{50} (μg/ml)	
			IgA	IgG
3902	Plasma	707	>50	0.95
	Breast milk	252	>50	2.67
605	Plasma	13,331	23.76	0.79
	Breast milk	1,394	>50	0.6
8304	Plasma	93	>50	20.47
	Breast milk	1,641	>50	12.5
8802	Plasma	26	>50	1.11
	Breast milk	828	>50	0.67
1209	Plasma	5,785	8.79	0.49
	Breast milk	1,417	19.62	0.51
9701	Plasma	6,371	>50	2.01
	Breast milk	646	>50	1.92
b12			0.13	0.57

untreated, HIV-infected, lactating women. The autologous virus neutralization of plasma and milk was assessed against five to nine autologous Env-pseudotyped viruses amplified from plasma or milk collected at week 4 following delivery. Nonspecific neutralization was assessed in plasma and milk from uninfected, lactating women against a control murine retrovirus (SVA.MLV). In general, there was no difference in the abilities of plasma samples to neutralize autologous plasma versus milk viruses (Table 3). However, patient 4403 plasma neutralized all the milk but none of the plasma viruses. Weak neutralization titers were detected in the plasma of each woman at most visits, with ID_{50} titers generally lower than 100 (Table 3). However, patient 3902 showed moderate to high neutralization potency at 9 months, with ID_{50}s as high as 463. These results confirm that milk and plasma virus Envs have comparable neutralization sensitivities, as previously indicated by assessment of milk and plasma virus Env neutralization sensitivity using broadly neutralizing MAbs (55).

The majority of the breast milk samples from uninfected lactating women displayed low levels of nonspecific HIV-neutralizing activity. This activity could be mediated by previously reported antiviral proteins in the milk, such as lactoferrin or secretory leukocyte protease inhibitor (20, 66). To account for the nonspecific activity, only samples with ID_{50}s higher than 3 standard deviations above the mean ID_{50} of the uninfected milk samples were considered positive. Based on this criterion, only one milk sample neutralized a milk virus (4707.BMF8) (Table 3). Thus, milk from HIV-infected lactating women does not potently neutralize autologous virus.

Breast milk antibodies mediate ADCC with a lower titer than but potency equal to that in plasma. We next determined the ability of milk antibodies to mediate another important virus-specific effector function, ADCC (Fig. 5). Target cells were coated with recombinant clade C gp120 (HIV DU422.1), and antibody-mediated cytotoxicity was measured by the release of GzB from NK cell granules. ADCC activity was detected in the milk of all subjects, with titers ranging from 19 to 3,272 (median titer, 385), but this response was severalfold lower than that in plasma (median, 81,132; range, 12,644 to 912,152) ($P < 0.0001$) (Fig. 5A). Nevertheless, similar to the neutralizing antibody responses, the titers in the two compartments were directly correlated ($r = 0.65$; $P < 0.0001$) (Fig. 5B). In addition, the maximal cytotoxic potency (the maximal proportion of target cells positive for GzB after incubation with antibodies and effector cells) was directly correlated between plasma and milk ($r = 0.60$; $P < 0.0001$) (Fig. 5C). In contrast, there was no correlation between the ADCC response and the milk virus load ($r = 0.27$; $P = 0.14$) (data not shown). Interestingly, although all HIV-infected lactating mothers had ADCC activity detected against HIV DU422.1 gp120 in plasma and milk, only a portion of subjects had detectable plasma neutralization, and no milk neutralization response was detected against the virus. These discrepant results may indicate some differences in the specificities of neutralization and ADCC-mediating antibodies.

Neutralization and ADCC in milk correlate with HIV Env-specific IgG responses. Based on our findings, the functional anti-HIV Env antibodies in milk appear to be predominantly IgG isotype. To further confirm this observation, we correlated the functional antibody responses in milk with the milk IgG and IgA HIV Env-binding responses (Fig. 6). There was a strong correlation between HIV Env-specific IgG binding responses and tier 1 neutralization in milk (ConS gp140, $r = 0.62$ and $P < 0.0001$; 1086c gp140, $r = 0.56$ and $P = 0.0001$; DU123 gp140, $r = 0.53$ and $P = 0.0003$; ConC gp140, $r = 0.63$ and $P < 0.0001$; Con6 gp120, $r = 0.71$ and $P < 0.0001$; ConC gp120, $r = 0.59$ and $P < 0.0001$; and gp41, $r = 0.43$ and $P = 0.0049$) (Fig. 6A and data not shown). Interestingly, a weak but significant correlation was observed between anti-ConC gp140 IgA antibodies and neutralization ($r = 0.30$; $P = 0.049$), whereas there was no correlation between neutralization and IgA response to all other antigens ($r = 0.17$ to 0.25; $P = 0.10$ to 0.30) (Fig. 6B and data not shown) (Fig. 6B). The correlation with anti-gp41 IgA antibodies was not determined, as only 10% of breast milk had detectable IgA of this specificity.

Finally, ADCC titers in milk correlated with milk HIV Env-specific IgG binding responses. Since the ADCC assay used NK cells that do not express Fcα receptors, we did not compare ADCC and HIV Env-specific IgA responses. However, there was a highly significant, robust direct correlation between the concentration of IgG antibodies to consensus HIV Env glycoproteins and ADCC titers in milk (ConS gp140, $r = 0.61$ and $P < 0.0001$; Con6 gp120, $r = 0.59$ and $P < 0.0001$) (Fig. 6C). Similarly, the magnitude of the IgG response to clade C Env glycoproteins and ADCC activity in milk were strongly correlated (1086c gp140, $r = 0.71$ and $P < 0.0001$; DU123 gp140, $r = 0.71$ and $P < 0.0001$; ConC gp140, $r = 0.70$ and $P < 0.0001$; and ConC gp120, $r = 0.64$ and $P < 0.0001$) (Fig. 6C and data not shown). Thus, IgG antibodies directed against HIV Env glycoproteins appear to primarily contribute to the antiviral functional humoral immune responses of breast milk.

DISCUSSION

Maternal antibodies transferred to the infant during breastfeeding are important for protection against pathogens encountered during the first months of life. Although HIV Env-

TABLE 3. Autologous neutralization titers in plasma and milk

Virus	Origin[a]	Titer[b]										
		Plasma					Breast milk					Uninfected breast milk (+3 SD)[c]
		6 wk	3 mo	6 mo	9 mo	12 mo	6 wk	3 mo	6 mo	9 mo	12 mo	
3305.A7	Plasma	23	26	36			<4	4	6			48
3305.A10	Plasma	35	104	124			<4	<4	5			51
3305.C4	Plasma	25	<20	30			<4	<4	5			72
3305.E9	Plasma	<20	30	23			<4	<4	<4			53
3305.BMC1	BMS	<20	26	20			<4	<4	<4			26
3305.BMC9	BMS	26	<20	38			<4	<4	<4			42
3305.BMC11	BMS	27	32	22			<4	<4	<4			25
3305.BMF4	BMS	20	30	<20			<4	<4	5			85
3305.BMF6	BMS	30	36	30			<4	<4	6			66
SVA.MLV		<20	<20	27			<4	<4	<4			51
3902.A7	Plasma	<20	30	59	463		9	<20	13	6		55
3902.B10	Plasma	<20	<20	<20	138		13	<20	6	7		64
3902.G3	Plasma	<20	51	44	159		13	<20	5	6		63
3902.G10	Plasma	22	35	27	66		11	<20	5	4		25
3902.G12	Plasma	<20	33	40	75		13	<20	12	11		37
3902.BMD7	BMS	<20	<20	<20	NA		<4	<20	6	5		53
3902.BME6	BMS	<20	<20	21	51		13	<20	8	11		69
3902.BMG14	BMS	<20	24	47	108		<4	<20	5	10		116
3902.BM.H9	BMS	<20	<20	39	26		<4	<20	<4	<4		10
SVA.MLV		<20	25	<20	<20		<4	<20	<4	<4		51
4403.A18	Plasma	<20	37	<20			<4	<4	<4			28
4403.D1	Plasma	<20	<20	<20			<4	<4	<4			34
4403.H2	Plasma	<20	27	<20			<4	<4	<4			25
4403.H7	Plasma	<20	23	<20			<4	<4	<4			32
4403.BMB6	BMS	<20	22	28			<4	<4	<4			22
4403.BMB10	BMS	23	91	30			<4	<4	<4			22
4403.BMC5	BMS	<20	41	25			<4	<4	<4			15
4403.BMG1	BMS	<20	22	22			<4	<4	<4			31
SVA.MLV		<20	21	<20			<4	<4	<4			51
0301.A8	Plasma	32	24	27			15	14	<4			76
0301.D1	Plasma	25	30	50			16	15	12			24
0301.E8	Plasma	<20	<20	<20			16	19	14			79
0301.E9	Plasma	31	21	27			18	22	17			97
0301.BMA2	BMS	36	22	45			14	13	5			26
0301.BMA6	BMS	29	22	24			19	17	4			68
0301.BMA12	BMS	50	56	83			29	43	31			163
SVA.MLV		<20	<20	<20			13	<4	<4			51
4707.E1	Plasma	<20	<20	27	39	61	7	15	29	18	23	52
4707.G3	Plasma	32	21	22	23	26	16	16	29	12	19	45
4707.H8	Plasma	27	26	37	42	50	8	14	24	17	26	49
4707.BMF8	BMS	31	32	42	46	62	16	18	56	25	47	37
4707.BM H2	BMS	27	23	32	47	67	8	16	27	17	25	32
SVA.MLV		<20	<20	<20	<20	<20	<5	<5	17	<5	18	51

[a] BMS, breast milk sample.

[b] A sample was considered positive (shaded) if the ID_{50} was greater than 3 times the SVA-MLV background and greater than 3 standard deviations above the mean of the HIV-negative control samples. All negative controls from plasma had ID_{50} values of <20.

[c] The mean ID_{50} of uninfected milk controls plus 3 standard deviations.

specific antibodies are detected in breast milk of infected mothers, their role in the prevention of infant HIV acquisition is still unclear (5, 31, 64). As viruses resistant to neutralization by maternal plasma have been associated with transmission through breastfeeding (48), it is possible that the virus neutralization activity of breast milk is important in protection against postnatal infant HIV acquisition. In this study, we investigated the magnitudes and breadths of HIV Env-binding IgG and IgA responses in breast milk, as well as the HIV-specific functional humoral responses of breast milk compared to those of plasma. Our results indicate that low levels of neutralizing and ADCC-mediating antibodies are present in the breast milk of chronically HIV-infected lactating mothers. Furthermore, breast milk responses parallel plasma responses, suggesting that functional HIV-specific antibodies in breast milk are mostly derived from the systemic compartment. This low level of neutralization detected in breast milk may indicate that nonneutralizing antibodies or innate immune factors are involved in protecting infants from postnatal HIV acquisition. However, we cannot exclude the potential benefit of inducing high levels of potent neutralizing antibodies in breast milk by maternal immunization.

Antibodies in breast milk are mainly in the form of sIgA, a dimeric antibody containing the J chain peptide and the secretory component (60). Breast milk contains at least 1 log unit more IgA than IgG (36, 60), but a bias toward the IgG isotype

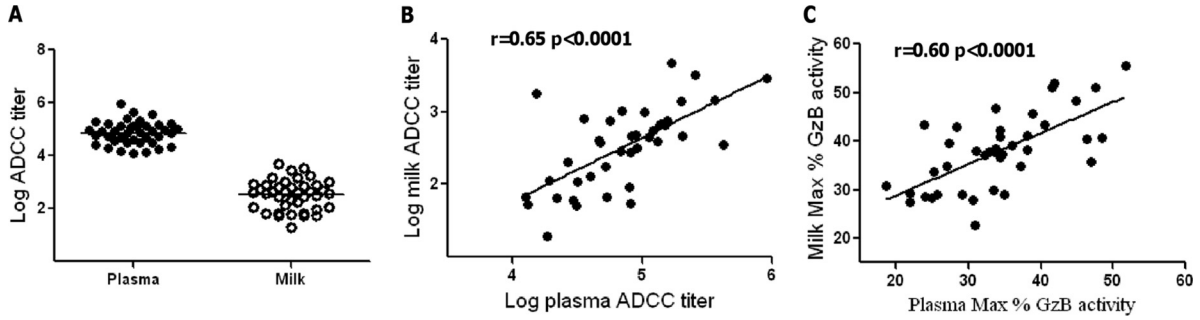

FIG. 5. ADCC titers are 2 log units lower in milk than in plasma, but the maximal cytotoxic potencies are similar between the two compartments. (A) The ADCC titer in milk is 2 log units lower than that in plasma. (B) A strong correlation is observed between ADCC titers in milk and plasma. (C) The maximal cytotoxic potency, measured by the maximal proportion of gp120-coated target cells positive for GzB activity, is correlated between paired milk and plasma samples.

of the antibody response to HIV Env glycoproteins has been reported in breast milk and other mucosal sites (35, 49, 64). Accordingly, in this study, although we detected HIV Env-specific IgG antibodies in the breast milk of all the women, the seroprevalence of HIV Env-specific IgA antibodies was variable between HIV Env antigens, ranging from 10% to 73% (Table 1). Furthermore, milk IgG responses directed against gp120 and gp140 were significantly higher than the milk HIV Env-specific IgA responses, despite the 10-fold-higher total IgA than IgG content of breast milk. A similar lower magnitude of breast milk SIV Env-specific IgA than IgG responses has also been reported in rhesus monkeys (45, 54). The weak IgA response to HIV Env glycoproteins in breast milk contrasts with the milk IgA response against other pathogens. In fact, antibodies against rotavirus (47) and respiratory syncytial virus (15) in breast milk are predominantly of the IgA isotype. While HIV-specific IgA responses are detected during acute HIV infection (72), previous studies have also reported that the systemic IgA response against HIV Env proteins in chronically infected individuals is of low magnitude (30, 35). Identifying pathways for induction of potent HIV-1-specific mucosal IgA antibody responses may be critical for an effective HIV transmission-blocking vaccine.

The role of mucosal HIV Env-specific IgA antibodies in

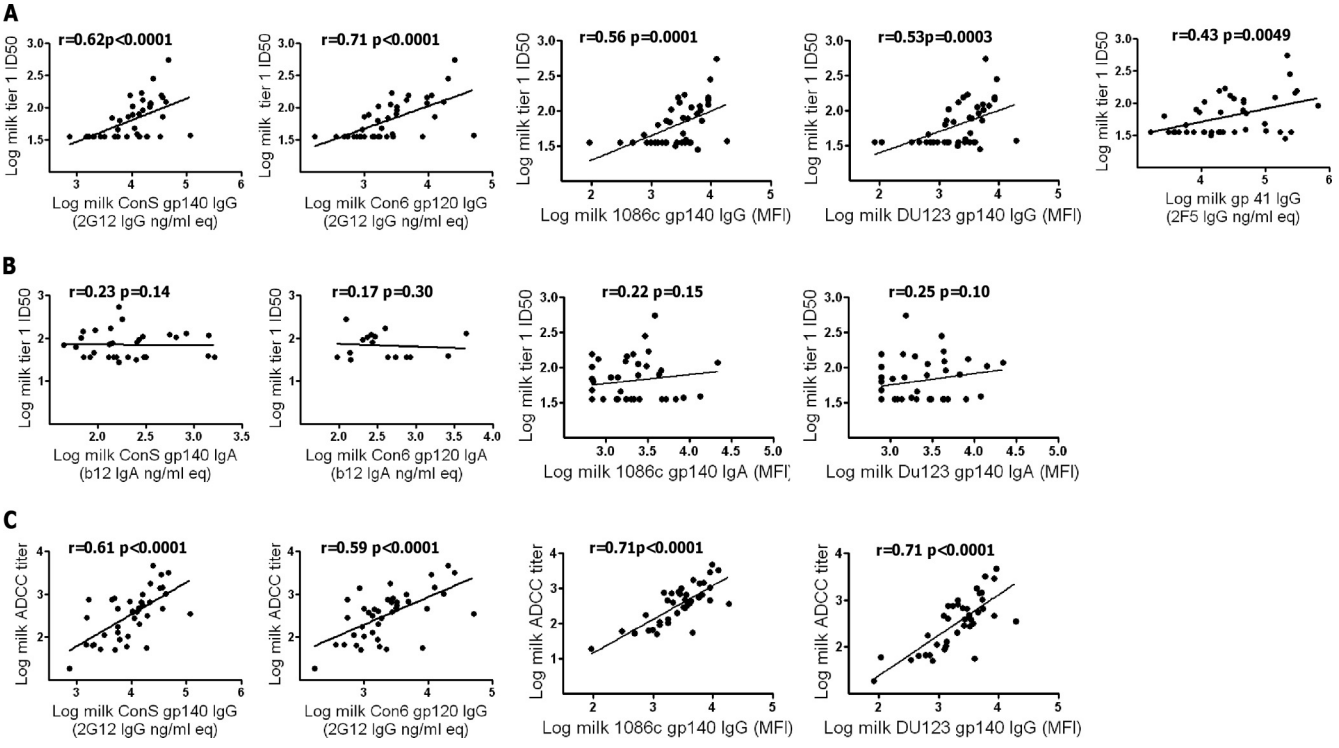

FIG. 6. The magnitude of HIV Env-binding IgG antibody responses correlate with neutralization and ADCC responses in breast milk. (A) Direct correlation between the magnitudes of the HIV Env-binding IgG antibody responses and the neutralizing antibody responses in milk. (B) The magnitudes of HIV Env-binding IgA antibody responses do not correlate with the neutralizing antibody response in milk. (C) Direct correlation between the magnitudes of the HIV Env-binding IgG antibody responses and the ADCC titer in milk.

protection against virus transmission is still unclear. Previous studies have reported that IgA antibodies isolated from mucosal secretions are able to neutralize HIV virions (38, 59, 70). These observations were mainly based on studying heavily HIV-exposed but persistently seronegative (HEPS) subjects (8, 10, 22). However, a recent cross-laboratory analysis reported that HIV-Env specific IgA antibodies are rarely detected in the cervicovaginal fluid of HEPS women (37). In this study, we detected neutralization in only 1 out of 35 (3%) non-IgG breast milk fractions (subject 1209). This sample also had one of the most robust HIV Env-specific IgA binding responses. As the ratio of the milk HIV Env-specific IgA to total IgA appears to be lower than that of IgG, larger amounts of milk IgA than IgG might be required to neutralize. Accordingly, the milk IgA IC_{50} of subject 1209 was more than 1 log unit higher than the IgG IC_{50}.

Most previous reports of the neutralization potency of HIV-specific mucosal IgA have used PBMCs as reporter cells. This assay is highly variable and is dependent on the donor cells used in each assay (46). Therefore, we chose to use the TZM-bl pseudovirus neutralization assay, an assay that has emerged as a standard assessment of neutralization in the HIV field (39). However, this assay may not be sufficiently sensitive for detection of IgA neutralization. It might therefore be necessary to assess neutralization by mucosal IgA in alternative cell lines to truly establish its neutralization potency. Aside from neutralization, sIgA could also mediate nonneutralizing effector functions that may play protective roles in mucosal HIV-1 transmission. For example, sIgA antibodies may aggregate virions and inhibit their movement through mucous and epithelial layers (23), and IgA antibodies have been demonstrated to inhibit virus transcytosis (24). More studies of the function of HIV Env-specific mucosal IgA are needed to determine if these types of responses are required for blocking HIV acquisition.

Although passive immunization with nonneutralizing ADCC-mediating immune sera did not protect neonatal infants from oral challenge with SIV (16), the removal of the Fc portion of the broadly neutralizing antibody b12 significantly impaired its *in vivo* protective function (21). Thus, Fc-mediated antibody functions could act synergistically with neutralization to confer protection. In this study, HIV-specific ADCC responses were detected in the breast milk of all HIV-infected women. ADCC-mediating antibodies could potentially be important in preventing the establishment of HIV infection in the infant gut following ingestion of cell-free or cell-associated virions. The assay used in this study specifically measured IgG-mediated ADCC. It is also possible that HIV-specific IgA could mediate cytotoxicity through interaction with phagocytes in breast milk and the infant gut. The development of an assay to measure IgA-mediated ADCC may be important to explore all potential anti-HIV functions of mucosal IgA.

Plasma cells secreting HIV-specific IgG antibodies can be detected in breast milk (63), and differences in the specific activity of IgG between milk and plasma have been described, suggesting a compartmentalization of the IgG response in breast milk (4). This difference was marked by lower anti-gp160 and higher anti-gp120 responses in breast milk than in plasma. In this study, the magnitude of the responses was consistently lower in breast milk than in plasma for all the antigens tested. Furthermore, there was a strong correlation in the magnitude of both neutralizing and ADCC responses in breast milk and plasma, suggesting that the majority of the functional antibodies in milk are derived from plasma. Moreover, IgGs isolated from breast milk and plasma had similar neutralization potencies (Fig. 4), further supporting the hypothesis that the neutralization responses in milk are mainly attributable to plasma-derived IgG. The similar neutralization potencies of HIV-specific IgG in milk and plasma differ from results in rhesus macaques, where breast milk IgG antibodies were slightly less potent at neutralizing a laboratory-adapted strain of SIV than plasma IgG (45). These discordant results may indicate a difference in the transfer of IgG from plasma to breast milk between humans and nonhuman primates.

The neutralization potency of breast milk was significantly lower than that of plasma, and autologous neutralization was not detected in the majority of the breast milk samples tested in this study (Table 3). Furthermore, although more than half of the breast milk samples neutralized a tier 1 HIV, tier 2 virus neutralization was scarcely detected (Fig. 3). As breast milk neutralizing antibodies appear to be mostly derived from plasma, the lack of detection of autologous response in this compartment may be due to the weak response observed in plasma and the low total antibody content of breast milk. Autologous neutralizing responses usually develop over several months, and plasma samples do not typically neutralize contemporaneous viruses (50, 67). The autologous viruses were derived from milk or plasma collected 4 weeks after delivery, and the samples used in the studies were collected up to 12 months after delivery. Similarly, in rhesus monkeys, breast milk samples collected 1 year after SIV inoculation were not able to neutralize the inoculated virus, but they neutralized laboratory-adapted strains of the virus (45). These results indicate that the level of neutralizing antibodies present in breast milk might not be sufficient for *in vivo* HIV virion inhibition.

The observation that HIV-specific functional antibody responses appear to be primarily derived from the plasma could have important implications for the development of a vaccine to prevent mother-to-child HIV transmission through breastfeeding. Our results suggest that an efficient maternal vaccine to prevent breast milk HIV transmission may only need to induce strong systemic humoral responses that would translate into potent breast milk humoral responses. Passive immunization studies may help determine the magnitude of the systemic responses required to achieve potent neutralization in breast milk.

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