Transmission of Macrophage-Tropic HIV-1 by Breast-Milk Macrophages via DC-SIGN

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Recent findings suggest that macrophage-tropic human immunodeficiency virus type 1 (HIV-1) produced in colostrum/early breast milk may hold a clue to determine the mechanisms of transmission of HIV-1 via breast-feeding. Here, we show that the majority of CD4+ cells in the colostrum are CD14+ macrophages expressing both chemokine receptors and DC-SIGN, a dendritic cell–specific receptor for HIV-1. The R5-type macrophage-tropic HIV-1 isolate NL(AD8) infected such breast-milk macrophages and caused them to secrete virus particles efficiently; however, the secreted virions showed only a weak transmissibility to their susceptible target, MAGIC-5 cells. When stimulated with interleukin-4, the breast-milk macrophages demonstrated a striking enhancement of expression of DC-SIGN and showed a strong capacity to transmit NL(AD8) virions to MAGIC-5 cells, which was specifically blocked by anti–DC-SIGN–specific antibody. These results suggest that HIV-1 virions captured by DC-SIGN, but not secreted cell-free virions, may be more efficiently transmitted to other compartments, such as the gastrointestinal tract, through acidic gastric juice.

Although the benefits of breast-feeding, including decreased infant morbidity and mortality, are well recognized, it may cause a substantial increase in the risk of transmission of HIV-1 from an infected mother to her child. Indeed, in the absence of prophylactic antiretroviral therapy, approximately one-third to one-half of HIV-1 infections in infants are acquired via breast-feeding [1]. The magnitude of breast-milk infectivity is significantly higher for mothers with a more advanced disease status, as measured by prenatal plasma HIV-1 RNA loads and CD4 cell counts [2, 3], indicating the strong correlation between the risk of transmission via breast-feeding and maternal plasma HIV-1 load. In addition, an association between breast-milk HIV-1 load and mother-to-child transmission of HIV-1 has been reported [4]. Thus, both plasma and breast-milk HIV-1 loads in infected mothers seem to be relevant indicators with which to assess mother-to-child transmission of HIV-1.

The origin of HIV-1 virions in breast milk remains unclear. Although the size of the study [5] was small, it has recently been reported that there is a possible compartmentalization of HIV-1 between blood and breast milk, suggesting that the actual virions for transmission via breast-feeding may be produced within the breast milk itself. Also, it has been reported that HIV-1 can infect human mammary epithelial cells and that HIV-1 virions are productively secreted from them [6]. Such mammary epithelial cell–derived HIV-1 may have selective advantages for the infection of mucosal cells and may determine the HIV-1 tropism for transmissible target cells situated in the gastrointestinal tract when they encounter HIV-1 after breast-feeding. Indeed, apart from macrophages that express CD4 but do not express either CCR5 or CXCR4, resident lamina propria lymphocytes that express CD4 as well as CCR5 and CXCR4 are speculated to be the target mononuclear cells for HIV-1 infection in the intestinal mucosa during early HIV-1 infection [7, 8]. Also, the risk of HIV-1 infection via breast-feeding in infants has been confirmed to be...
influenced by breast-milk HIV-1 load, which is significantly higher in colostrum than in mature breast milk obtained 14 days after delivery [9]. Moreover, as indicated elsewhere [10, 11], the principal HIV-1 variants identified in breast milk were R5-type [5]. Collectively, R5-type macrophage-tropic HIV-1 produced in colostrum/early breast milk may hold a clue to determine the mechanisms of transmission of HIV-1 via breastfeeding. Therefore, we have examined the actual components that organize transmission in colostrum/early breast milk. On the basis of the findings obtained by analyzing cells in the colostrum for their susceptibility to HIV-1, a possible mechanism for mother-to-child transmission of HIV-1 via breastfeeding will be discussed in the present study.

MATERIALS AND METHODS

Isolation of breast milk–derived cells and breast-milk macrophages. Breast milk was collected from healthy women within 3–6 days of delivery, after informed consent had been obtained under a protocol approved by the Institutional Review Board of the Nippon Medical School and in accordance with the human-experimentation guidelines of the US Department of Health and Human Services. Breast-milk cells were isolated from freshly obtained breast milk by Ficoll-Paque (Amersham Pharmacia Biotech) gradient centrifugation methods, as described elsewhere [12]. Breast-milk macrophages were isolated from freshly obtained breast-milk cells, followed by adherence to polystyrene tissue-culture dishes for 1 h at 37°C. The adherent cells were then removed by incubation with 5 mmol/L EDTA for 30 min at 4°C. The obtained adherent cells were confirmed to express homogeneous CD14+ cells at ≥ 95% by use of a FACScan cytometer (Becton Dickinson).

Cytokine treatment of breast-milk macrophages. For the treatment of breast-milk macrophages with either interleukin (IL)–2 or IL-4, cells were plated at 10^5 cells/mL in RPMI 1640 medium–based complete culture medium (CCM) [13] supplemented with 10% fetal calf serum (FCS), 20 mmol/L HEPES (Invitrogen), 50 µmol/L 2-mercapto-ethanol (Sigma), 2 mmol/L L-glutamine (Sigma), and 100 U of penicillin-streptomycin solution (Sigma) and were cultured for 5 days at 37°C in the presence or absence of either IL-2 (50 U/mL) (Shionogi Pharmaceutical) or IL-4 (1000 U/mL) (Biosource International). The culture medium was changed every 2 days.

Antibodies and flow-cytometric analysis. Fluorescein isothiocyanate–conjugated anti-human monoclonal antibodies (MAbs) to CD3 (UHCT1), CD4 (SK3), and CD14 (M5E2); phycoerythrin (PE)–conjugated anti-human MAbs to CD3 (UHCT1), CCR5 (2D7), and CXCR4 (12G4); biotin-labeled MAb to anti-human CD4 (RPA-T4); and PE-conjugated isotype-matched control antibody (MOPC-21) were all purchased from BD Biosciences. PE-conjugated and unlabeled anti-human DC-SIGN (120507) MAbs were purchased from R&D Systems, and CD4 (OKT4) and control isotype-matched polyclonal antibody for the blocking experiments were purchased from American Type Culture Collection.

Cells were stained with the relevant antibody for 30 min on ice in PBS with 2% FCS and 0.01 mol/L sodium azide (PBS-based medium), were washed twice, and were resuspended in the PBS-based medium. Then, the labeled cells were analyzed by use of a FACScan cytometer with CellQuest software (version 3.1F; BD Biosciences). Live cells were gated on the basis of propidium-iodide gating.

Measurement of virus titer. NL(AD8) infectious viral particles [14] were obtained by transfection of 293T cells with pNL(AD8) (gift from Malcolm A. Martin, National Institute of Allergy and Infectious Diseases, National Institutes of Health [NIH]). Polyethyleneimine (25 kDa; Sigma) was used for transfection. The virus titer was determined on the basis of the HIV-1 p24 antigen concentration (picograms per milliliter) in the culture supernatant, by ELISA, as described elsewhere [15]. In brief, Immulon II plates (Dynex Technologies) were coated with anti–HIV-1 p24 MAb (183-H12-5C) [16, 17], and the samples were incubated in the plates for 2 h. After washing, biotinylated human anti-HIV immunoglobulin was added for detection of p24. HIV-1 p25/p24 Gag protein (NIH AIDS Research and Reference Reagent Program) [18] was used as a standard.

Infection of cultured breast-milk macrophages with NL(AD8). Breast-milk macrophages cultured for 5 days with or without IL-4 were harvested and added to a flat-bottom 48-well microplate (Corning), at 1–2 × 10^5 cells/well, in a total volume of 200 µL of CCM. After incubation for 2 h at 37°C, the media were gently removed, and the remaining cells were further incubated with 200 µL of CCM containing 2 ng/mL HIV-1 p24 of NL(AD8) for 2 h at 37°C. Then, the cells were washed 3 times with RPMI 1640 medium containing 2% FCS and were cultured with CCM (total volume, 400 µL) for an additional 7 days. The culture supernatant was replaced with fresh CCM every other day, and the collected culture supernatant was stored at −80°C for further analysis.

HIV-1 transmission assay. An indicator cell line, named MAGIC-5 [19] (provided by M. Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan), was used to examine the capacity of NL(AD8)-sensitized cells to transmit that isolate. MAGIC-5 cells (10^4 cells/well) were plated in a flat-bottom 96-well microplate (Corning) with CCM the day before coculturing with the target cells. After removal of the medium from each well, 5 × 10^4 intensively washed NL(AD8)-infected breast-milk macrophages were added to 10^4 preincubated MAGIC-5 cells. After 16 h, the loaded cells were removed by gentle washing with warmed PBS. Then, 50 µg of the substrate X-gal was added, to identify the β-gal–expressing infected cells, by use of a β-gal staining kit (Invitrogen). The stained cells were counted, to estimate transmissibility.

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Acid treatment of NL(AD8)-infected cells. The NL(AD8)-infected breast-milk macrophages were washed twice with PBS, incubated with adjusted HCl (pH 3.0) for 1–3 min at room temperature, and then promptly neutralized with 0.1 mol/L Tris-buffer (pH 8.0). Cells were washed 3 times with CCM, and 5 × 10^4 infected cells were added to the wells seeded with 10^5 MAGIC-5 cells. After 16 h, each well was gently washed 3 times, to remove loaded, infected breast-milk macrophages, and the remaining MAGIC-5 cells were stained with X-gal, as described above. The number of β-gal–positive blue-colored cells was counted, to estimate infectivity.

RESULTS

Flow-cytometric analysis of cells in breast milk. On the basis of recent findings showing that the risk of HIV-1 infection via breast-feeding in infants is influenced by breast-milk HIV-1 load, which is significantly higher in colostrum than in mature breast milk [9], we first analyzed the surface phenotype of the cells in freshly isolated colostrum/early breast milk. We could detect only a few CD3+ cells in the early breast milk (figure 1A), and the majority of CD4+ cells did not express CD3 (figure 1B) but rather expressed CD14 molecules (figure 1C), which we have named “breast-milk macrophages.” Thus, we focused on the R5-type macrophage-tropic HIV-1 isolate NL(AD8) [14] for further analysis.

DC-SIGN and expression of chemokine receptors on CD4+ breast-milk macrophages. Next, we examined whether CD4+ breast-milk macrophages could be infected with NL(AD8). Contrary to intestinal macrophages, which lack expression of CCR5 [8], freshly isolated CD4+ breast-milk macrophages did express both CXCR4 and CCR5 (figure 2). Also, they weakly but spontaneously expressed DC-SIGN, a dendritic cell (DC)–specific receptor for HIV-1 [20, 21] that might capture cell-free HIV-1 virions (figure 2). On the basis of previous observations showing that surface expression of DC-SIGN on breast-milk macrophages was markedly enhanced when breast-milk macrophages were cocultured with IL-4 for 5 days [12], we compared the surface expression of chemokine receptors and DC-SIGN on breast-milk macrophages cultured for 5 days either in the absence or in the presence of IL-4. Without IL-4 stimulation, the expression of CXCR4, CCR5, and DC-SIGN on breast-milk macrophages cultured for 5 days was almost unchanged, compared with that in freshly isolated breast-milk macrophages (figure 2). In contrast, when breast-milk macrophages were cocultured with IL-4 for 5 days, a dramatic reduction of expression of both CXCR4 and CCR5, together with strong enhancement of expression of DC-SIGN, was observed (figure 2). However, we did not observe any enhancement of expression of DC-SIGN when breast-milk macrophages were incubated with IL-2 for 5 days (figure 2). Also, as we have reported elsewhere [12], the IL-4–stimulated breast-milk macrophages became CD1a-positive DC-like cells that lost CD14 (data not shown).

Analysis of susceptibility of R5-type HIV-1 to cultured breast-milk macrophages. Next, we compared the susceptibility of HIV-1 to breast-milk macrophages cultured for 5 days, using the R5-type macrophage-tropic HIV-1 isolate NL(AD8). Cultured breast-milk macrophages were infected with NL(AD8) (2 ng/mL p24 antigen) for 2 h at 37°C and were washed extensively, to remove cell-free HIV-1 virions, and the quantity of HIV-1 p24 Gag protein in the supernatant of the further-cultured breast-milk macrophages was measured. As expected, a marked increase of production of HIV-1 p24 antigen was observed in the supernatant of NL(AD8)–infected, cultured breast-milk macrophages on days 3 and 5 (figure 3A). Unexpectedly, although a striking reduction of expression of CCR5 was mediated by treatment with IL-4, the IL-4–stimulated breast-milk macrophages could also be fairly infected with...
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Figure 2. Expression of chemokine receptors and DC-SIGN on CD14+ breast-milk macrophages. Both CXCR4 and CCR5 (left middle and lower panels), as well as DC-SIGN molecules (left upper panel), were expressed on freshly isolated breast-milk macrophages. Without stimulation with interleukin (IL)-4 (second 3 panels from the left) or IL-2 (third 3 panels from the left), surface expression of CXCR4, CCR5, and DC-SIGN molecules on breast-milk macrophages cultured for 5 days was almost unchanged, compared with that on freshly isolated breast-milk macrophages. In contrast, breast-milk macrophages cocultured with IL-4 for 5 days showed a dramatic reduction of expression of both CXCR4 and CCR5, whereas strong enhancement of expression of DC-SIGN was observed (right 3 panels). The nos. indicate the percentage of cells in each quadrant. The data shown are representative of 3 distinct experiments.

NL(AD8), and they secreted good amounts of p24 antigen on day 5 (figure 3A). This may be because of the enhancement of virus acquisition induced by the cis effect of DC-SIGN, as reported elsewhere [22]. It is important to note that, on day 3, the production of HIV-1 p24 antigen by IL-4–stimulated breast-milk macrophages was much less than that by unstimulated macrophages, indicating the substantial difference of intracellular replication of NL(AD8) between those distinct conditioned breast-milk macrophages at that time point. Nevertheless, on day 3, the ability of IL-4–stimulated breast-milk macrophages to transmit the virions to NL(AD8)-sensitive MAGIC-5 cells was far stronger than that of unstimulated macrophages (figure 3B), suggesting that the high transmissibility was mediated through the virions captured via DC-SIGN but not through cell-free virus particles released by those infected cells.

Inhibition of transmission of NL(AD8) by IL-4–stimulated breast-milk macrophages with anti–DC-SIGN antibody but not with isotype-matched antibodies. The above results reveal that DC-SIGN–mediated capture and transmission of HIV-1 virions may be a major pathway for vertical transmission via breast-feeding in infants. Therefore, we asked whether blocking of DC-SIGN with specific antibody would reduce the capacity for transmissibility of HIV-1 virions in IL-4–stimulated breast-milk macrophages. Pretreatment of the IL-4–stimulated breast-milk macrophages with 1–5 μmol/L anti–DC-SIGN MAb for 30 min on ice and subsequent infection with NL(AD8) for 2 h at 37°C, in the presence of the antibody, showed remarkable inhibition of transmission of NL(AD8) to MAGIC-5 cells (figure 4). In contrast, pretreatment with either isotype-matched control IgG or anti-human CD4-specific MAb (OKT4) (5 μmol/L) did not induce significant inhibition (figure 4). Also, the DC-SIGN–specific MAb was not toxic—treatment of control breast-milk macrophages cultured for 5 days with the same or higher concentrations of anti–DC-SIGN MAb did not affect the transmissibility of virus at all (data not shown). Thus, in breast-feeding, DC-SIGN–mediated capture of HIV-1 virions seems to be critical for transmission of cell-associated viral particles to target cells. Moreover, the effect of pretreatment with the anti–DC-SIGN MAb (5 μmol/L) on the secreted virus titer of IL-4–stimulated breast-milk macrophages cultured for 5 days was examined, and ~70% reduction in the amount of p24 was observed (data not shown). This result indicates that DC-
Figure 3. Macrophage-tropic HIV-1 infectivity to breast-milk macrophages cultured for 5 days. Cultured breast-milk macrophages were infected with the R5-type macrophage-tropic HIV-1 isolate NL(AD8) (2 ng/mL p24 antigen) for 2 h at 37°C and washed extensively to remove cell-free HIV-1 virions, and the quantity of HIV-1 p24 Gag protein in the supernatant of further-cultured breast-milk macrophages was measured by ELISA. A. Production of HIV-1 p24 antigen in the supernatant of NL(AD8)-infected interleukin (IL)–4–unstimulated breast-milk macrophages (●) and IL-4–stimulated breast-milk macrophages (■). The data shown are mean ± SD and are representative of 4 independent experiments. B. Fifty thousand intensively washed NL(AD8)-infected breast-milk macrophages were added to 10^5 MAGIC-5 cells. After 16 h, the loaded cells were removed by gentle washing 3 times with warmed PBS. Then, 50 μg of the substrate X-gal was added, to identify the β-gal–expressing cells, by use of a β-gal staining kit (Invitrogen), and stained cells were counted. Data shown are mean ± SD and are representative of 3 independent experiments.

SIGN–mediated transcytosis of HIV-1 virions may contribute to acquisition of the virus by IL-4–stimulated breast-milk macrophages.

Effect of acidification on virus infectivity. There are 2 modes of transmission of virus: (1) the cell-free situation and (2) the cell-associated pattern. Breast milk from HIV-1–infected mothers contains both forms, and breast milk with virions, when ingested, must encounter gastric juice with low pH (pH 3.0–4.0 in infants [23, 24]) before meeting with intestinal target cells that have high susceptibility to macrophage-tropic HIV-1. Thus, we first examined the effect of acidification on virus infectivity, using cell-free NL(AD8). In our preliminary data, we observed that 1 min of treatment with medium whose pH was adjusted to <4.0 produced a profound inhibition of virus infectivity (data not shown). Therefore, we treated the virions in the cell-free form with HCl (pH 3.0) for 1 min at 37°C, followed by subsequent neutralization with Tris-buffer (pH 8.0), and found that the infectivity of HIV-1 virions was lost almost completely (figure 5A), indicating that most HIV-1 virions may not reach intestinal targets in their cell-free form via breastfeeding. Then, we investigated the effect of acidification on transmission of DC-SIGN–mediated cell-associated HIV-1 virions.

To our surprise, at least 10%–20% (mean, ~40%) of the infectivity to MAGIC-5 cells remained when NL(AD8)-infected
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**DISCUSSION**

In the present study, we found that we could detect only a few CD4+ T cells in colostrum/early breast milk and that the majority of CD3+ T cells were CD14+ breast-milk macrophages. Therefore, cells in breast milk can produce and transmit predominantly macrophage-tropic HIV-1, which seems to be a major reason for the dominant spread of R5-type macrophage-tropic virions in vertical transmission of HIV-1 via breast-feeding, if the cellular part contains a critical element for spread of HIV-1. As expected, such CD4+ breast-milk macrophages did express CCR5, were susceptible to NL(AD8), and efficiently secreted the virions at least 3–5 days after infection. In addition to this observation, it has been reported that HIV-1 can infect human mammary epithelial cells that productively release macrophage-tropic HIV-1 virions [6]. Also, possible compartmentalization between blood and breast milk has been shown [5]. Taken together, these findings strongly suggest that R5-type macrophage-tropic HIV-1 might be dominantly produced in the breast milk, and, thus, macrophage-tropic HIV-1 seems to predominantly spread among infants via breast-feeding, probably through breast-milk macrophages.

When breast-milk macrophages were cocultured with IL-4, surface expression of CCR5 was markedly down-regulated, but susceptibility to NL(AD8) was not significantly decreased. This might be because IL-4–stimulated breast-milk macrophages showed a striking augmentation of expression of DC-SIGN, and, thus, the enhancement of virus acquisition would be induced by the *cis* effect of DC-SIGN, as reported elsewhere [22]. However, on day 3, the production of HIV-1 virions by IL-4–stimulated breast-milk macrophages was far less than that by unstimulated macrophages. Nonetheless, on day 3, the transmissibility of the virions to NL(AD8)-sensitive MAGIC-5 cells was dominantly observed in IL-4–stimulated breast-milk macrophages, compared with that in unstimulated macrophages. Therefore, we speculated that high transmissibility was mediated through the virions captured by DC-SIGN but not through cell-free virus particles released by infected cells, although some reports indicate that cell-free HIV-1 virions in breast milk may contribute to vertical transmission of HIV-1 [25]. Moreover, that cell-free HIV-1 virions may lose their infectivity when treated with HCl (pH 3.0) for 1 min suggests that the transmission of cell-free virions in breast milk may be impeded by gastric acidification, although it may be possible for cell-free virions to infect via oral or esophageal mucosa, which are subjected less to such acidification [26]. In contrast, the infectivity of cell-associated virions captured by DC-SIGN unexpectedly remained after the same acidification procedure. This might be a protective effect mediated by DCs, similar to the previous observation that IL-4–stimulated breast-milk macrophages were incubated with HCl (pH 3.0) for 1–3 min at 37°C (figure 5B).

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**Figure 5.** Effect of acidification on transmissibility of cell-free and cell-associated virions. A, Cell-free NL(AD8) virions (2 ng/mL p24 antigen) were treated with HCl (pH 3.0) for 1 min at room temperature and immediately neutralized with 0.1 mol/L Tris-buffer (pH 8.0). Then, the acid-treated virions were added to 10⁶ MAGIC-5 cells and further cultured, for an additional 16 h, before X-gal staining. The infectivity of the treated virions was evaluated by comparing the no. of blue-stained MAGIC-5 cells with the untreated virions. The data shown are of 3 experiments. B, NL(AD8)-infected breast-milk macrophages were washed twice with PBS and incubated with adjusted HCl (pH 3.0) for either 1 or 3 min at room temperature and then promptly neutralized with 0.1 mol/L Tris-buffer (pH 8.0). Cells were washed 3 times with complete culture medium, and then 5 x 10⁴ HIV-1–infected cells, either acid treated or untreated, were added to the wells seeded with 10⁵ MAGIC-5 cells. After 16 h, each well was gently washed 3 times, to remove loaded, infected breast-milk macrophages, and the remaining MAGIC-5 cells were stained with X-gal, as described above. The no. of β-gal–positive blue-colored cells was counted, to estimate infectivity. Results are expressed as the percentage change of acid-treated cell no. from each untreated control. The data shown are mean ± SD of 4 experiments, each from a separate donor.
HIV-1 captured by follicular DCs is highly infectious, even when attached to neutralizing antibody [27]. Collectively, our present findings strongly suggest that vertical transmission of HIV-1 via breast-feeding may be mediated through cell-associated virions retained by breast-milk macrophages through DC-SIGN, rather than through cell-free virus particles.

That the striking enhancement of expression of DC-SIGN on breast-milk macrophages was induced by stimulation with IL-4 indicates the requirement of local inflammatory changes with Th2 dominancy for the acceleration of transmission of HIV-1 via breast-feeding. Indeed, it has recently been reported that mastitis is linked with a higher breast-milk HIV-1 load, as well as with a greater risk of transmission of HIV-1 via breast-feeding [4]. Thus, DC-SIGN-mediated vertical transmission may be prevented by the establishment and preservation of Th1 dominancy in the breast milk, which would be mediated either by treatment of milk-borne infections with appropriate antibiotics, by direct injection of Th1-type cytokines—such as IL-2, IL-12, or interferon-γ—by manipulating the internal Th1/Th2 balance by use of the polarization of innate NK T cells [28], or by stimulating DCs with poly (I:C) by use of Toll-like receptor (TLR) 3 [29] or with a CpG DNA fragment by use of TLR9 [30]. Those treatments may modify breast-milk macrophages into cells with less ability to transmit HIV-1 virions and with lower-level expression of DC-SIGN. The establishment of methods to reduce expression of DC-SIGN on breast-milk macrophages by maintaining Th1 dominancy in the breast milk may offer another strategy to prevent mother-to-child transmission of HIV-1 via breast-feeding.

References