HIV

HIV-1 Reservoirs in Breast Milk and Challenges to Elimination of Breast-Feeding Transmission of HIV-1

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By compensating for the relative immaturity of the neonatal immune system, breast milk and breast-feeding prevent deaths in children. Nevertheless, transmission of HIV-1 through breast-feeding is responsible for more than half of new pediatric HIV infections. Recent studies of possible HIV-1 reservoirs in breast milk shed new light on features that influence HIV-1 transmission through breast-feeding. The particular characteristics of breast milk CD4+ T cells that distinguish them from circulating blood lymphocytes (high frequency of cell activation and expression of memory and mucosal homing markers) facilitate the establishment of HIV-1 replication. Breast milk also contains a plethora of factors with anti-infectious, immunomodulatory, or anti-inflammatory properties that can regulate both viral replication and infant susceptibility. In addition, CD8+ T lymphocytes, macrophages, and epithelial cells in breast milk can alter the dynamics of HIV-1 transmission. Even during efficient antiretroviral therapy, a residual stable, CD4+ T cell–associated reservoir of HIV-1 is persistently present in breast milk, a likely source of infection. Only prophylactic treatment in infants—ideally with a long-acting drug, administered for the entire duration of breast-feeding—is likely to protect HIV-exposed babies against all forms of HIV transmission from breast milk, including cell-to-cell viral transfer.

INTRODUCTION

The advantages of breast-feeding for the human neonate are immense. Breast milk satisfies the infant’s nutritional and hydration needs and provides immunological protection against mucosal pathogens and likely against allergy and cancer as well. It also guards the integrity of the infant’s gut by delivering immune and nonimmune innate factors with antimicrobial (lactoferrin, oligosaccharides, lysozyme, antibodies, and complement) or anti-inflammatory properties (lactoferrin, adiponectin, and various cytokines). Human milk also contains cytokines and chemokines that can directly modulate the immunological development of the newborn (1–3). In addition to these health benefits, the act of breast-feeding promotes psychological development (4, 5). If practiced in the 42 countries in which 90% of under-five deaths occurred in 2000, good breast-feeding habits would be a tremendously cost-effective public health intervention, able to prevent 13% of under-five deaths, or 15 million deaths in a single decade (6).

Newborn babies are especially vulnerable to disease because they are suddenly and newly exposed to a large number of microorganisms at a time when their immune defenses are incomplete and immature. Breast milk compensates for this immaturity by conferring passive defenses and facilitating immune maturation. Secretory antibodies in breast milk target infectious agents from the mother’s environment, which are also likely to be encountered by the infant during the first weeks of life (7). Thus, the mammary gland is as an integral part of the mother-offspring immune dyad (8).

Despite its beneficial effects, breast milk can also be a vehicle for the transmission of viruses to the infant. The intestinal mucosa—a simple columnar epithelium—has an outsized surface area of about 200 m² when all of its folds, crypts, villi, and microvilli are taken into account. In addition, achlohydria (lack of hydrochloric acid secretion in the gastric juice) of the stomach is frequent in the first weeks or months of life. Hence, the gut mucosa forms an accessible portal of entry for HIV-1.

Recent advances in prophylactic HIV treatment have decreased perinatal transmission (20 weeks of gestation to 28 days after birth) of HIV-1 from mother to child so that transmission through breast-feeding is now responsible for more than half of the estimated yearly 400,000 new pediatric infections worldwide (9). The risk of late postnatal transmission of HIV-1 by breast-feeding has been estimated at 3.2 per 100 child-years of breast-feeding. Despite the seriousness of these statistics, the vast majority of HIV-exposed babies remain uninfected (10), likely because protective components in breast milk and host susceptibility factors prevent obstacles to transmission (11, 12).

Three clinical factors confer increased risk of HIV-1 transmission from a woman to her breast-fed child. The first is a high rate of viral replication in the mother. When the mother acquires HIV-1 at any time during lactation, profuse viral replication causes an elevated viral load in all body fluids—including breast milk—which causes an extremely high risk of HIV-1 transmission to the breast-fed child, about 30 to 40% of breast-fed babies (13–15). During the later stages of maternal infection, immunodeficiency related to AIDS also exacerbates the risk of transmission by accelerating HIV-1 replication in tissues and body fluids (16). Second, the length and intensity of breast-feeding are major determinants of the risk of postnatal transmission of HIV-1 (10). Infants exclusively breast-fed for the first 6 months, with no other liquid or solid food, acquire HIV-1 at a much lower rate than do infants fed a mixed diet or one that is predominantly breast milk, but that includes early introduction of a liquid or solid (17, 18). Consequently, exclusive breast-feeding, regardless of maternal HIV-1 status, is recommended by the World Health Organization (WHO) for the first 6 months of life. The third factor that increases risk of HIV transmission is inflammation in the mammary gland. Mastitis, breast abscess, or simple engorgement is accompanied by...
increased HIV-1 shedding in breast milk and, in some but not all studies, by postnatal transmission to the infant (19). One recent study reported that mastitis is associated with breast-feeding transmission of HIV-1 only from women with high plasma viral load (20). The facilitating effect of mastitis on transmission could also be a result of inflammation and an impaired immune climate in the mammary gland and breast milk, possibly through exposure to bacterial lipopolysaccharides (LPS) or debris, as observed in the gut submucosae during HIV infection (21).

Beyond these known risk factors, the mechanisms of HIV-1 transmission through breast-feeding are poorly understood. The complex and evolving nature of the developing infant, of breast milk, and of the HIV-1 reservoir itself presents obstacles to easy elucidation.

**PREVENTION OF HIV-1 TRANSMISSION THROUGH BREAST-FEEDING**

The WHO estimates that on December 2009, the global mother-to-child HIV transmission rate was 27%. WHO goals are to reduce, by 2015, all forms of mother-to-child HIV transmission to a rate below 5% and to decrease the annual number of new pediatric infections to a value below 40,000 (9). WHO also recommends that HIV-exposed babies be breast-fed for 12 months (22).

Women eligible for antiretroviral therapy (ART) are encouraged to initiate or continue their treatment during pregnancy and lactation. This regimen reduces mother-to-child HIV-1 transmission: In an observational cohort of ART-treated, HIV-1-infected pregnant women from the Kesho Bora trial with fewer than 200 CD4+ cells per microliter or with WHO stage 4 AIDS, the 18-month probability of HIV-1 transmission was 7.5% (16). Nevertheless, some women are not eligible for ART for their own health because of their HIV-associated symptoms or low CD4+ cell count (23). On the basis of the goals for 2015 and the results of proof-of-concept trials (24–31), the WHO offers two recommendations for prevention of HIV transmission to breast-fed infants in these untreated, HIV-infected mothers.

Option A: Azidothymidine (AZT) is given daily to the mother during pregnancy and delivery (and discontinued after birth). Immediately after birth, nevirapine (NVP) is given daily to the exposed infant for a minimum of 4 to 6 weeks until 1 week after all exposure to breast milk has ended.

Option B: Triple antiretroviral (ARV) prophylaxis is given to the mother starting from as early as 14 weeks of gestation and is continued until 1 week after all infant exposure to breast milk has ended.

These two options can not be combined presently, because ARV drugs diffuse into breast milk and there is a risk of overdose to the infant. The choice of one or the other is generally made at the national level, according to local strategies for combating HIV-1, but the operational difficulties in implementing these prevention programs are considerable.

Achieving the goal to reduce mother-to-child transmission to a rate below 5% by 2015 will be challenging. Correct identification of all HIV-1-infected pregnant women is difficult, so some offspring cannot benefit from prevention interventions. Also, very few clinical trials and observational studies that have evaluated the efficacy of either WHO-recommended option have shown reduction of mother-to-child transmission below 5% (24–32). We need to learn more about mother-to-child transmission of HIV, particularly through breast-feeding, to develop better preventive approaches.
Breast milk also contains numerous factors involved in the anti-
microbial innate immune response, although to date only a few have
been fully characterized. These include a soluble form of CD14
(sCD14) and several components of complement and various non-
immune innate factors. These substances protect the infant

(i) by bacterial lysis or inactivation. Lactoferrin, found in significant
concentrations in human colostrum and breast milk, has broad-
spectrum antimicrobial activity against a wide range of Gram-positive
and Gram-negative bacteria, a function of its ability to sequester iron
and its direct lytic effect on microbial cell membranes (37). Lactoferrin
removes LPS from the outer cell membrane so that lysozyme, another
major whey enzyme, can penetrate and degrade the inner proteoglycan
matrix of the membrane of invading cells (38). Milk fats are also anti-
microbial (39). Breast milk contains 3 to 4 g of fat per liter, with 93 to
97% in the form of triglycerides. The mechanism for antimicrobial
effects of fatty acids and monoglycerides has not been established, but
free fatty acids may damage bacteria by disrupting their cell membranes
or by changing intracellular pH.

(ii) by boosting the cellular immune response to bacteria. sCD14,
produced by mammary epithelial cells, is present in breast milk in
concentrations 20 times higher than in serum (35). This glycoprotein
receptor, along with Toll-like receptor 4 (TLR4), detects Gram-negative
bacteria by binding LPS (40), conferring LPS responsiveness to cells that
do not express CD14. sCD14 is believed to regulate microbial growth in
the neonatal gut. The exact role of maternal soluble TLRs, also found in
breast milk, is still debated (34, 41). Because TLRs are lacking in the
neonatal gut, soluble TLRs may allow response to pathogens by activating
transcription of early innate immune genes (42). Another breast milk
component, β-defensin-1, has antimicrobial activity against Escherichia
coli and may up-regulate the adaptive immune system in the infant
gut (43).

(iii) by blockade of pathogen attachment and entry to host cells.
Nondigestible oligosaccharides are also important antibacterial constituents
of human milk. Changing in type during lactation, oligosaccharides are
produced by an antigen-independent mechanism in mammary epithelial
cells. They resist hydrolysis by gastrointestinal enzymes and remain intact
in the baby’s small intestine. These compounds function like bacterial
analogues and compete with pathogens for binding sites on epithelial
intestinal cell surfaces. They also directly bind pathogens present on the
mucosal epithelia, such as E. coli, Campylobacter jejuni, and Streptococcus pneumoniae. Lactadherin, a mucin-related glycoprotein
produced by mammary epithelial cells during lactation, can bind hu-
man rotavirus and prevent viral attachment to host cell receptors; its
concentration is inversely associated with rotavirus-related symptoms
(44). Lactadherin may act similarly to prevent entry of other viruses, including HIV-1. The secretary leukocyte protease inhibitor (SLPI), present at potentially active concentrations in colostrum and transition milk, can inhibit HIV-1 entry into host cells in vitro (45). Lactoferrin too can block HIV-1 entry and inhibit the
attachment of bacteria to intestinal cells (37). Several components (C3
and C4), receptors (CF2 and CD21), and activation fragments of complement in human milk likely participate in the innate immune re-
response against bacteria by multiple mechanisms: immune bacteriolysis,
normalization of viruses, immune adherence, cytokisis, and enhanced
phagocytosis in the infant’s intestine (35, 46).

(iv) by mitigation of gut inflammation. Breast milk also down-
regulates inflammation in the infant’s gut. Immediately after birth,
the immature newborn digestive tract is exposed to new antigens and
LPS from pathogens and colonizing commensal bacteria, which can
induce an extensive mucosal inflammatory response, as shown in vitro (47). In severe cases, this response may contribute to necrotizing
enterocolitis in preterm infants (48). Later, the infant gut is exposed to
unfamiliar dietary antigens and enteric pathogens, also a potential
source of inflammation and injury. Factors in breast milk may mitigate
the T helper 1 (Th1) or inflammatory response and thereby preserve the
gut mucosal barrier, likely accounting for the lower frequency of nec-
rotizing enterocolitis in breast-fed than formula-fed infants (48). For
example, in addition to its antibacterial functions, lactoferrin is anti-
flammatory by inhibiting the production of proinflammatory mediators,
such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α),
and IL-6 through a decrease in nuclear factor κ light-chain enhancer
of activated B cell (NFκB) or through its capacity to bind iron, a po-
tent oxidizer (34, 49). Similarly, in a human intestinal model in which
IL-1β triggers an inflammatory IL-8 response, colloidal proteins re-
duced both IL-8 and the luminal expression of TLR4 (50). Erythro-
poietin (EPO), transforming growth factor–β (TGF-β), and IL-10 at
concentrations found in breast milk reduce IL-8 secretion from a fetal
human enterocyte cell line in vitro (51). The association between low
EPO concentration in breast milk and HIV-1 transmission may re-
fect an active antiviral role for EPO (52). Other compounds with anti-
-inflammatory functions, such as hydrocortisone or adipopectin, are also
found in breast milk at concentrations sufficiently high to affect the in-
fant gut (53).

Epithelial cells and progenitor cells
Breast milk also contains numerous living cells (see Table 1), but their
functions are not always clear (35). Particularly in mature milk, epithelial
cell adhesion molecule–positive (EpCAM+) epithelial cells from the
mammary gland are the most abundant cells (54). Stem and progenitor
cells have also been identified by their specific surface markers in breast
milk (55, 56). The function of either of these cell types, if any, after
ingestion by breast-fed infants remains unknown.

Leukocytes
Differing from colostrum and transition milk (where leukocytes are
abundant), mature breast milk contains a small and inconsistent
concentration of leukocytes estimated at 1 × 10^5 to 5 × 10^6 cells per milliliter of
mature milk. Among these, neutrophils account for 80%, macro-
phages for 15%, and lymphocytes for less than 5% (54).

Lymphocytes. Various lymphocyte types coexist in breast milk:
CD3+ T cells (representing about 83% of lymphocytes, almost equally
distributed between CD4+ and CD8+ lymphocytes), γδ T cells (11%),
CD16+ natural killer cells (3 to 4%), and B cells (2%). CD4+ T cells,
one of the main target cells for HIV-1, represent almost 40% of the total
lymphocyte population with 1 ml of breast milk containing about 2000
CD4+ T lymphocytes (by comparison, blood contains almost 1 million
CD4+ T lymphocytes/ml). During feeding, the mucosal area of the tonsil
and the gut is exposed to, on average, 700 ml of maternal milk each day,
exposing the infant to more than 1 million maternally derived CD4+ T
 cells. After 6 months of life, a baby will have ingested about 2 × 10^8
breast milk–derived CD4+ T cells. HIV-1 infection of the mother
depletes CD4+ T cells more rapidly in blood than in breast milk, so that
CD4+ T cells, particularly CCR5+ CD4+ T cells, persist longer in breast
milk than in other mucosal sites (57, 58).

Breast milk T and B lymphocytes are distinct from circulating blood
lymphocytes (Fig. 1). First, breast milk contains almost exclusively
memory T and B lymphocytes, which have previously encountered antigens. Indeed, very few breast milk cells express the CD45RA receptor that characterizes naïve T cells (59, 60). Likewise, more than 70% of breast milk B cells are IgD− CD27+ memory B cells (61), most of which carry somatically mutated variable region genes and are class-switched B lymphocytes expressing surface IgG or IgA molecules. Therefore, most breast milk T and B cells are antigen-experienced and so can respond efficiently to bacterial and viral pathogens.

Second, many T and B lymphocytes from breast milk are activated, frequently expressing activation markers such as human leukocyte antigen (HLA)−DR, CD38, and CD69 (57, 59–64). Many of these activated cells in breast milk are effector memory cells (which are therefore primed to respond to antigen exposure) (65, 66), in contrast to blood T cells, which are primarily central memory cells. An average of 42% of CD4+ memory T lymphocytes are activated in breast milk (59), a proportion 5 to 10 times higher than in blood. HIV-1–specific CD8+ T cells are more frequent in breast milk than in blood, where they may help to limit HIV-1 production by infected CD4+ T cells (64).

The high frequency of activated immune cells in breast milk is paradoxical because human milk per se does not confer immune activation and is in fact anti-inflammatory. Indeed, blood lymphocytes incubated in human milk, in contrast to plasma, display fewer activation markers on their surface, indicating that breast milk can limit lymphocyte activation (67). Breast milk lymphocytes most likely become activated through extravasation or during transepithelial migration (61, 63). In addition, breast milk B cells include mainly large-sized B cells, plasmablasts, and plasma cells (61), which do not express complement receptor but are switched memory B cells primed to secrete antibodies.

Third, most breast milk T (57, 60, 64) and B cells (61) express the mucosal homing markers αE integrin (CD103), α4 integrin (CD49d), β7 integrin, and CCR9, confirming that they were primed in mucosal-associated lymphoid tissues (MALTs) and migrated to the mammary gland as an effector site. Milk B cells seem to have migrated preferentially from the gut-associated lymphoid tissues (GALTs). The CD103 (α4β7) homing receptor—a useful marker for in vivo–activated regulatory T (Treg) cells—is a hallmark of breast milk T cells (68–70) but is rarely found on blood T cells (71). The α4β7 integrin, which is preferentially expressed on activated CD4+ T lymphocytes of mucosal origin, interacts with the V2 loop of HIV-1 gp120, thus facilitating HIV-1 infection of these cells (72, 73). Furthermore, most breast milk CD4+ T lymphocytes express high levels of chemokine receptors CCR5 and CXCR4, the major co-receptors required for HIV-1 attachment and entry.

These characteristics of breast milk lymphocytes reinforce the idea that human milk provides neonates and infants with supplemental, highly immunologically active components designed to protect the
mother-infant dyad from potential pathogens. Nevertheless, some of the same cells that provide these functions are ideal targets for HIV-1 infection and transmission: They are memory cells, of mucosal origin, with a high level of activation and abundant cell surface expression of HIV-1 co-receptors.

Macrophages. Macrophages, another cellular target for HIV-1, likely represent only 15% of breast milk leukocytes (54, 63). Breast milk macrophages differ from their blood counterparts in that they have a higher phagocytic capacity and a more effective defense against pathogens (74). More frequently activated (62), their motility is also higher. Breast milk macrophages spontaneously secrete granulocyte macrophage colony-stimulating factor, a cell growth factor that enhances effector functions and cell signaling pathways (74). They express the dendritic cell–specific intercellular adhesion molecule 3 gr–abbing nonintegrin (DC-SIGN), used by HIV-1 for transport between tissues, and, after incubation with IL-4, differentiate into CD11c+ dendritic cells, which can transport antigens (and HIV-1 particles) (74). In addition, these cells contain sigA, which can be released during phagocytosis (75), and profusely secrete soluble factors such as lactoferrin and complement factors C3 and C4 (76). Breast milk macrophages and dendritic cells probably facilitate antigen transport, cell signaling, and cell-to-cell antigen trafficking (including HIV-1 antigens).

HIV-1 RESERVOIRS IN BREAST MILK

The mammary gland environment

The stroma of the lactating mammary gland is an effector site for mucosal immunity that interacts with the MALT (35). Its resident immune cells also have activation and cytokine profiles different from those of blood that can influence the dynamics of HIV-1 replication (11, 77, 78). This environment could encourage initiation of the viral cycle or promote ongoing replication in CD4+ T lymphocytes harboring HIV-1 DNA (79, 80). In contrast, an antiviral TH11 environment in breast milk could limit HIV-1 replication through direct effects of cytokines such as interferon-γ (IFN-γ) and by promoting cytotoxic T cell responses (64, 65).

Large amounts of IL-1β, IL-6, TNF-α, and IFN-γ are found in breast milk from healthy lactating mothers, as are cytokines of the CXC and CC chemokine families. These small chemotactic factors are mediators of inflammation that can activate leukocytes. Breast milk also contains IL-8, a member of the CXC chemokine family; monocyte chemotactic protein-1 (MCP-1); RANTES; and macrophage inflammatory protein-1α (MIP-1α). These cytokines are transcribed and secreted by both mammary epithelial cells and breast milk leukocytes.

Subclinical mastitis, diagnosed by an elevated sodium/potassium ratio in milk, is a frequent asymptomatic event in both HIV-1–infected and HIV-1–uninfected lactating mothers (81). Mastitis increases proinflammatory cytokines in the mammary gland. Subclinical mastitis also results in higher milk concentrations of proinflammatory cytokines such as IL-8 (82). Leakage of HIV-1 from plasma because of an inflammation-induced increase in mammary epithelial permeability could favor HIV-1 shedding into breast milk. The increased risk of mother-to-child HIV-1 transmission in women with mastitis or subclinical mastitis (19, 20, 82) might also be related to an imbalance between antiviral and proinflammatory cytokines that facilitates HIV-1 replication in the mammary gland.

Mammary epithelial cells

Mammary gland epithelial cells, the major cellular component of breast milk (54), are also susceptible to HIV-1 infection (83). These cells express CCR5, CXCR4 (co-receptors necessary for HIV-1 entry), galactosyl ceramide (GalCer), and, unexpectedly, CD4 surface markers (84). When these cells are exposed to HIV-1 in vitro, HIV-1 is taken up into endosomal vacuoles (84). Coculture of activated CD4+ T cells with HIV-1–exposed mammary epithelial cells can result in their productive infection, suggesting that epithelial cells can enhance infection in vivo, probably by transcytosis (84). Mammary epithelial cells also express several potentially protective factors against HIV-1 transmission, such as the apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3 (APOBEC3), the mucin 1 antigen (MUC1), and CCL28 (85–87). Therefore, mammary epithelial cells may transport HIV-1 across the epithelial surface of mammary gland acini and lactiferous ducts to contribute to HIV-1 shedding in breast milk, but because HIV-1 does not replicate in these cells, they are not likely to be an active reservoir for the virus.

CD4+ T lymphocytes and cell-associated HIV-1 RNA

Latently HIV-1–infected, resting CD4+ T lymphocytes harbor HIV-1 proviral DNA. These cells are very rare, estimated at 105 to 106 for an entire infected individual (88). Although the decay characteristics of this latent reservoir remain uncertain, these cells have a very long half-life of about 44 months and are not affected by conventional ART (89, 90). They constitute an inducible reservoir of HIV-1–producing cells, although the efficiency of DNA transcription and translation into viral proteins is low in blood CD4+ T cells (91, 92). Indeed, these latently infected, resting CD4+ T cells in both breast milk and blood of HIV-1–infected women can transcribe HIV DNA and generate viral particles (93, 94). Even when the HIV-1 DNA viral load is comparable in blood and breast milk, polyclonal activation results in 10 times more HIV-1 antigen–secreting cells (Ag SCs) in breast milk than in blood (500 versus 45). If one assumes that one to three copies of HIV-1 are integrated in latently infected cells, the efficiency of transcription and translation after activation is 1 to 2% in blood and 10 to 30% in breast milk (93). Thus, the CD4+ T cells in breast milk are potentially 17 times more effective than their blood counterparts in producing HIV-1 antigens. The trafficking route and functional role of breast milk lymphocytes in the recipient infant remain unclear. Nevertheless, these cells likely produce HIV-1 if they become activated in the mammary gland or later in the infant’s digestive tract. Indeed, latently infected, resting CD4+ T cells in breast milk are probably an HIV sanctuary from which the virus can be released after activation.

The pronounced differences between CD4+ T cells in the blood and the breast milk may arise from several nonmutually exclusive causes. First, as suggested by the absence of correlation between HIV-1 Ag SCs in blood and breast milk, T cells in milk may be a different functional cell population from those in peripheral blood. Most breast milk CD4+ T cells exhibit markers of the MALT system, showing that they originate from, differentiate within, or migrate through mammary gland tissue, where they may acquire properties different from those of blood T cells. Second, the HIV-1 quiescent cells in milk may differ from their counterparts in peripheral blood (95). At least some breast milk HIV-1 in CD4+ T lymphocytes originates from maternal epithelial cells; this HIV-1 can invade local CD4+ T lymphocytes with more accurate proviral integration than can blood HIV-1; and it is likely to be better adapted to mucosal transmission than is blood HIV-1 (73). Thus, breast
milk HIV-1 is particularly prone to transmission to the infant. Third, the cytokines IL-1β, IL-6, TNF-α, and TNF in human milk (96) may stimulate latently infected lymphocytes to produce HIV-1 virions. Finally, protein S100, present in high concentrations in breast milk (97), may induce HIV-1 transcription from latently infected human CD4+ T lymphocytes by up-regulating NFκB through a viral enhancer sequence that positively modulates HIV replication (98).

In blood, almost all of the HIV-1 RNA originates from functional, activated CD4+ T cells that are in a productively infected state. These cells are short-lived, with a half-life of only 24 to 36 hours, and in viremic subjects, they spontaneously secrete HIV-1 antigens, as measured by enzyme-linked immunospot (ELISPOT), and can produce HIV-1 RNA in culture (99). Even in ARV-treated individuals, these functional, activated CD4+ T cells can support residual viral replication that can infect new susceptible cells and perpetuate infection (100).

In women with successful responses to ART, undetectable HIV-1 RNA in plasma and breast milk has been interpreted to mean that breast milk HIV-1 is no longer being replenished by lymphoid tissue viral replication (57) and that HIV-1 replication has been suppressed in the mammary gland (101). But this may not be the case. Although ART causes a marked decrease of HIV-1 RNA and to a lesser extent HIV-1 DNA in breast milk (102), cell-associated HIV-1 RNA is not, or is only moderately, affected (103) (Fig. 2). Indeed, CD4+ T cells spontaneously secreting HIV-1 antigen can be detected by ELISPOT in both breast milk and blood of all HIV-1-infected women, whether untreated or successfully treated with ART (59). More than half of these patients also show cell-associated HIV-1 RNA in blood and breast milk. Further, when these cells are cultured, HIV-1 RNA could be detected and quantified in the supernatant, and this harvested HIV-1 was infectious. Thus, cells that can secrete HIV-1 antigens are present in breast milk of ART-treated and untreated women, and these cells may be responsible for a residual mother-to-child viral transmission in the treated patients. The fact that HIV antigen-producing T cells can be identified in samples that have no detectable HIV-1 RNA suggests that these cells may only release tiny amounts of HIV-1 RNA or that their residence time in breast milk is very short.

Thus, HIV-1-secreting CD4+ T cells in breast milk, which can be detected in vitro by their HIV-1 antigen or HIV-1 RNA production, are the most plausible source of HIV-1 transmission by breast-feeding from women successfully treated with ARV regimens (59, 104).

**Breast milk macrophages**

HIV-1 infection does not kill macrophages but severely impairs their function. It is not
fully established whether HIV-infected macrophages in milk contribute to HIV-1 replication and release of viral particles. The DC-SIGN surface receptor, frequently expressed on breast milk macrophages (74), may bind HIV-1 and aid its transport in breast milk, and expression of DC-SIGN on the mucosal surfaces of the breast-fed infant could also facilitate transmission. In vitro, expression of DC-SIGN in breast milk macrophages is decreased by TLR3 stimulation, with consequent inhibition of cell-to-cell transmission of HIV-1 from macrophages to T lymphocytes (105). In colostrum and transition milk from HIV-1–infected women, 0.1 to 1% of macrophages are infected, and some can actively produce viral particles. These macrophages have a longer half-life than T lymphocytes, are resistant to apoptosis, and could contribute to transmission (106). It is likely that breast milk macrophages contribute minimally to HIV-1 replication in mammary gland. Nevertheless, breast milk macrophages expressing DC-SIGN may augment HIV-1 transport in the infant mucosa and cell-to-cell infection of infant T lymphocytes.

Indeed, HIV-1 may behave similarly to other viruses transmitted through breast milk. The lentiviruses maedi-visna virus (MVV) and the related caprine arthritis-encephalitis virus (CAEV) are transmitted to newborn lambs through colostrum and milk. The virus is excreted from highly productive germinal centers in the vicinity of the lactiferous ducts and is propagated in macrophages (107). The human T cell leukemia virus type I (HTLV-I) is also transmissible by breast-feeding; in this model, macrophages are thought to play a central role in viral propagation since an infected breast milk macrophage cell line can efficiently transmit the virus to activated T lymphocytes in vitro (108).

Cell-free HIV-1 particles

Seventy percent to 80% of HIV-1–infected, lactating women not treated by ART have detectable HIV-1 RNA in the whey, more if breast milk sampling is repeated because most women have intermittent viral shedding in breast milk (109–112). In addition, up to one-third of the HIV-1 RNA in milk may be sequestered in the lipid fraction (113), and HIV-1 particles can be passively carried on the surface of breast milk cells. Thus, the frequency of HIV-1 RNA shedding in breast milk has probably been underestimated in studies testing only the liquid fraction of milk. The relationship between the level of HIV-1 RNA in breast milk and that in blood and the origin of cell-free HIV-1 particles in breast milk remain uncertain. Although correlated with plasma HIV-1 RNA levels (109), breast milk viral load is most frequently lower (by about 2 log10) than plasma viral load (10). In addition, HIV-1 RNA levels may differ in milk collected from the right and left breasts (10), suggesting that local factors in the mammary gland contribute to viral production. The association of mammary gland inflammation (clinical or subclinical mastitis, breast abscess, engorgement, and systemic or multiorgan inflammation) with elevated breast milk HIV-1 RNA supports this conclusion (19, 109, 114, 115). Thus, cell-free HIV-1 particles in human milk, as measured by HIV-1 RNA, originate at least partly from local replication in the mammary gland (95, 116).

CELL-FREE AND CELL-ASSOCIATED HIV-1 IN MOTHER-TO-CHILD TRANSMISSION

High concentrations of cell-free HIV-1 RNA in breast milk, although an imperfect reflection of infectiousness, are associated with postnatal HIV-1 transmission by breast-feeding (104, 117). Infants infected with HIV-1 by breast-feeding have been exposed to 17 times more cell-free HIV-1 RNA in milk than age-adjusted exposed but uninfected controls (10). Each log10 increase in breast milk cell-free HIV-1 RNA doubles postnatal transmission risk (117). Postnatal transmission risk also increases during the rebound of virus concentrations in milk after ARV treatment is interrupted in the mother (104). Nevertheless, two studies show that 15% of HIV-1–infected mothers who transmitted the virus to their offspring by breast-feeding had undetectable HIV-1 RNA in the breast milk samples collected before transmission occurred (104, 118), indicating that cell-free HIV-1 in breast milk is not the sole viral reservoir that contributes to transmission. Indeed, both cell-free and cell-associated HIV-1 have been shown to mediate transmission events (119–122).

For HTLV-I as well as for bovine leukemia virus and other animal retroviruses transmissible by breast milk, cell-to-cell transfer is considered the predominant mechanism of transmission from mother to infant. One milliliter of human mature breast milk from an HTLV-I–infected mother contains 1000 infected cells but very few virions (123). HTLV-I infection can be experimentally transmitted to susceptible animals by ingestion of breast milk from infected mothers (124) or by oral inoculation with cultured mononuclear cells from a patient with acute T cell leukemia (123). Converging arguments suggest that similar mechanisms apply to HIV-1.

Detection of HIV-1 proviral DNA in human breast milk indicates that infants are exposed to HIV-1–infected cells and, indeed, proviral DNA is associated with breast milk transmission of HIV-1 (119, 120). The proportion of HIV-1–infected cells in breast milk is strongly and independently (from cell-free viral load) associated with postnatal transmission; each log10 increase in number of infected cells per milliliter triples the risk of transmission (121). Therefore, cell-associated HIV-1 in milk is at least as important as cell-free virus in transmitting HIV-1 to infants. In fact, transmission probably arises from multiple pathogenic pathways of varying importance during the lactation process and according to breast-feeding practices. For example, in a study conducted in Botswana, the comparison of C2 to C5 env fragment sequences among cell-free HIV-1, cell-associated HIV-1 in breast milk, and the virus transmitted to the infants suggested that before infants are 9 months old, HIV-1 is mainly transmitted by cells containing HIV-1 provirus, whereas cell-free virus is frequently the culprit later on (120).

Some babies breast-fed by HIV-1–infected women taking ART or ARV prophylactic treatment become infected despite undetectable levels of HIV-1 RNA in their mother’s plasma and breast milk (104, 125). A stable HIV-1 reservoir in breast milk within CD4+ T lymphocytes, which have a much higher propensity to enter the viral cycle after activation than do blood CD4 cells (93), and within infected macrophages of HIV-1–infected mothers with immune activation, likely fuels cell-to-cell transmission. In vitro infectivity of HIV-1 is 100 to 1000 times higher from cell-associated virus than from cell-free virus stocks (126).

HIV-1–secreting cells (59) in breast milk have direct access to infants’ intestinal and respiratory mucosae, and active immune cells from breast milk can infiltrate the intestinal mucosa of the breast-fed infant (127). Cell-associated viral particles can also penetrate to the submucosa of the infant gut through mucosal breaches or via transcytosis. Viral transcytosis occurs through a virological synapse scaffold and integrin- and agrin-dependent molecular machinery in epithelial cells (Fig. 3) (128, 129). For HTLV-I, the viral protein tax participates in its own transfer by
reorienting intracytoplasmic microtubules, thus favoring microvacuole transport (130). Filopodia and nanotubes may also facilitate cell transfer of HIV-1 (131, 132). HIV-1 transmission can occur across polysynapses between one infected cell and multiple recipient cells (133). These structures may facilitate exponential viral growth and sustain sufficient viral propagation to establish infection from a very small inoculum. Virological synapses and polysynapses also allow the virus to avoid host immune cells (11, 119) and the innate protective substances present in breast milk. Indeed, although soluble factors in milk can prevent cell-free HIV-1 propagation in vitro, they cannot prevent cell-associated virus propagation (134).

An infant breast-fed by an HIV-1–infected woman ingests an average of 178 HIV-1–secreting cells per day during the first 4 months of life (59). Because one cell with replicating HIV-1 produces at least 1000 viral particles (126), the infant’s daily exposure could be as high as 178,000 cell-associated viruses, with a high capacity for cell-to-cell transfer. It is therefore likely that cell-associated HIV-1 in breast milk transferred by mother-infant cell-to-cell contact contributes substantially to transmission of HIV-1 from breast milk to infant.

**Fig. 3.** Mechanisms of HIV-1 transfer from breast milk to the infant’s intestinal mucosae. Cell-free HIV-1 and infected cells producing viruses encounter GalCer⁺ CCR5⁺ CXCR4⁻ epithelial cells of the gut mucosal surface. In the upper small intestine, cell-free virus enters epithelial cells through endocytosis at the luminal surface in a GalCer/CCR5 receptor–mediated mechanism (center of illustration). HIV-1–infected cells may also bind to the epithelial cell and induce the polarized budding of newly formed viruses that are rapidly endocytosed via GalCer (left side of illustration). HIV viruses able to penetrate into the lamina propria infect CCR5⁺CXCR4⁺CD4⁺ T lymphocytes. The capacity of human M cells to translocate HIV-1 remains unclear (right side of illustration). Adapted from (129).

**BOTTLENECKS TO PREVENTING HIV-1 TRANSMISSION VIA BREAST-FEEDING**

**Incomplete understanding of the role of activated T cells**

Although it is clear that both T cells latently infected with HIV and activated HIV-producing T cells persist in breast milk and contribute to transmission of HIV to breast-feeding infants, we do not understand the respective roles of these two reservoirs. This is important to clarify because maternal ART only minimally reduces HIV in these cells. Consequently, because these reservoirs contribute to HIV transmission, approaches other than maternal ART should be considered to eliminate this source of pediatric infection. For example, if immune activation facilitates HIV-1 transmission, strategies such as prevention of inflammation and subclinical...
mastitis in the breast, both causes of immune cell activation, could prove useful.

To test whether activated CD4+ T cells from breast milk contribute to HIV-1 transmission, these cells should be enumerated in breast milk samples from transmitting and nontransmitting mothers. Such studies are ongoing on limited numbers of frozen samples, but conclusive findings may require fresh cells and rigorous freezing procedures. Identifying a proxy of cell activation by measuring the activation-prone environment in breast milk and soluble factors could well prove more informative.

Incomplete understanding of the role of immune factors and co-infections

Innate, anti-infectious factors such as lactoferrin, lactadherin, mucins, and anti-secretory lectins, as described above, may prevent bacterial adherence to the gut epithelial surface and therefore protect against alteration of the vulnerable newborn’s gut mucosal barrier. Because bacterial translocation and consequent immune activation may boost HIV-1 replication in CD4+ T cells and maybe macrophages, it is important to know whether breast-fed HIV-1–infected infants have a slower disease progression or a better ART response than infants deprived of their mother’s milk. Other factors, such as SLPI, lysozyme, or lactoferrin, are under scrutiny in studies comparing breast milk composition in transmitting and nontransmitting mothers. If proven protective, these factors could be included in an intervention package aimed at defending the infant’s mucosa against HIV-1. Such studies will also reveal the influence on transmission of co-infections with viruses like herpesviruses (135), GB virus C, hepatitis C virus, or transfusion-transmitted virus or of viral reactivation in the mammary gland. Finally, we need to determine whether the humoral immune response, mainly sIgA and sIgM, or the local T cell response protects against HIV-1 transmission; positive findings would indicate that maternal immunization eliciting such responses may prove beneficial in preventing breast milk–mediated transmission.

Unclear efficacy of prophylaxis

In HIV-1–infected mothers not eligible for ART, triple combination ART administered during lactation reduces transmission by only 50 to 60% (25). This poor response will hamper considerably efforts to achieve the WHO objective of reducing mother-to-child transmission of HIV-1 worldwide to ~10% of present levels. The prophylactic efficacy of the WHO-recommended option B (maternal triple prophylaxis) has been assessed by the Kesho Bora trial (25). In this randomized trial, prophylactic ARV therapy with three drugs during pregnancy and breast-feeding for a maximum of 6 months was compared to a short perinatal AZT/single-dose NVP prophylaxis to prevent mother-to-child transmission of HIV-1. In infants whose mothers declared they intended to breast-feed, the cumulative rate of HIV-1 transmission at 12 months was 5.6% in the triple ARV group and 10.7% in the AZT/single-dose NVP group, corresponding to an intervention efficacy of 52%. This lower than expected efficacy of the triple combination prophylaxis could be a result of suboptimal maternal adherence, breast milk exposure after maternal prophylaxis had been stopped, or transmission via cell-associated viruses not suppressed by maternal prophylaxis. An unexpected adverse effect of the “option B” maternal triple prophylaxis is development of a high rate of resistance to multiclass ARV drugs in babies that become infected despite maternal prophylaxis (136–138). In a study from Uganda in which mothers initiated ART immediately after delivery while breast-feeding, six of seven HIV-1–infected babies harbored multiclass-resistant viruses at 12 months of age, jeopardizing the success of further ARV therapies (139, 140). This high rate of resistant mutants in these untreated babies is likely a result of exposure to suboptimal concentration of ARV drugs in ingested milk caused by variable diffusion of maternal drugs into breast milk (139, 140).

The prophylactic efficacy of WHO option A (infant peri-exposure prophylaxis) has been demonstrated in two proof-of-concept trials (30, 31). In addition, prophylaxis by treatment of the infant with daily NVP from 6 weeks to 6 months has been evaluated in South Africa in a randomized placebo-controlled trial (32). Of the infants receiving this treatment, 1.1% acquired HIV-1 between 6 weeks and 6 months, whereas 2.4% of the placebo controls became infected, a 54% reduction in transmission. However, mortality at 6 months did not differ between the two groups.

No study has evaluated the efficacy of WHO option A applied for the entire 12-month duration of breast-feeding. In addition, the optimal drug of choice for infant prophylaxis remains unclear. The ideal drug should have excellent efficacy and a very good safety profile because the vast majority of infants will not be infected with HIV and so cannot ethically be given drugs with problematic side effects. The drug should not compromise or complicate the future HIV-1 treatment of infants who may acquire HIV despite the treatment. NVP satisfies the first two points (although its efficacy could be improved), but most infants who acquire HIV will become resistant to the who class of nonnucleoside reverse transcriptase inhibitor (NNRTI) drugs. Lamivudine (3TC), which proved as efficacious and safe as NVP (29, 141), with a similar rate of resistance, may be a better choice. In this case, resistance would be limited to 3TC, and so use of this drug would not compromise the successful use of other nucleosidic reverse transcriptase inhibitors. Finally, other drugs could prove useful, such as lopinavir/ritonavir (LPV/r), which is more potent and has a high genetic barrier to resistance, with a good safety profile in preliminary studies of young infants (142–144).

To address these questions, we are conducting a phase 3 multicenter, randomized trial comparing two alternative drugs to NVP. The French National Agency for AIDS Research (ANRS) 12174 trial (National Institutes of Health registration: NCT00640263) compares the efficacy and safety of prolonged infant preexposure prophylaxis with LPV/r to that of lamivudine in preventing HIV-1 transmission through breast milk in children born to HIV-1–infected mothers not eligible for ART. Before entering the trial, all mothers and babies have benefited from perinatal HIV-1 prophylaxis according to national and international recommendations. We have enrolled 1300 mother-infant pairs from four African countries and are assessing the efficacy and safety of treatment during the entire duration of breast-feeding, as well as the resistance profile of infants who become infected.

These data should inform decision-makers on the best choice of drug for infant preexposure prophylaxis, including economic considerations and the ability of the approach to achieve the WHO targets. Should we treat infants of mothers already receiving treatment? After further evaluation, it may be possible to administer infant preexposure prophylaxis (option A) to babies whose mothers are already on ART as a protection against residual transmission. But this could only be done safely, without risk of overdosing, if the drug for prophylaxis is different from the ones used by the mother or if the mother’s drug does not diffuse into breast milk.

Lack of longer-acting drugs

Infant protection against transmission of HIV through breast-feeding may be improved through the use of longer-acting ARV drugs for infant preexposure prophylaxis. It is difficult for many mothers to administer to their infants a twice-daily oral drug prophylactic regimen, and in the...
real world, the adherence is likely to be worse than in a research setting. Many mothers have not disclosed their HIV status to their partner and will therefore give the drug to the infant only if it can be done secretly. When this is not possible, they may not administer the drug at all. Besides affecting program implementation and management, poor adherence can also alter the efficacy of prevention of postnatal mother-to-child transmission considerably.

Longer-acting prophylactic drugs could help solve this problem. Given to the baby by subcutaneous or intramuscular injections and requiring a limited number of doses during the course of breast-feeding (two to four times, possibly matched with immunization visits), such drugs can be administered under the supervision of a health care worker (directly observed short-course treatment). Longer-acting agents would alleviate adherence problems and better protect the infants. In addition, the baby could be systematically screened for HIV-1 infection during these visits before the next injection of the prophylactic drug, which would limit the chances of the baby receiving a single drug while infected and risking development of resistance.

Such long-acting ARV drugs already exist. The diarylpyrimidine analog rilpivirine, an NNRTI active against wild-type and NNRTI-resistant HIV-1 strains, is available as a nanosuspension for long-acting injectable formulation (145). Preclinical studies in rats and dogs show that the drug is well tolerated and results in stable plasma concentrations for more than 6 weeks (145). Rilpivirine seems to be taken up by macrophages and concentrated in lymph nodes and lymphoid tissues (145). Satisfactory phase 1/2 trials in human adult volunteers have been reported (146). Other long-acting pharmacological preparations of ARV drugs are in development; these drugs are good candidates for clinical evaluation in HIV-1–exposed breast-fed infants to achieve peri-exposure prophylaxis with improved adherence.

CONCLUSIONS

Although HIV-1 can enter breast milk by transudation from the vascular compartment, HIV-1 can also replicate in mammary gland tissues and breast milk. Transmission of HIV-1 by breast-feeding is the result of multiple factors: the nature and size of the viral reservoir, host susceptibility, and the complex interplay of numerous breast milk factors that may be anti-infectious, immunomodulatory, and anti- or proinflammatory.

Although cell-free HIV-1 particles can mediate HIV-1 transmission from breast milk to infant, especially late in lactation (120), cell-associated HIV-1—either latently infected or activated, virus-producing T cells—is predominantly responsible for breast milk–mediated HIV transmission. Compared with those in blood, breast milk B and T cells are activated more frequently and express higher levels of memory and transmission. Compared with those in blood, breast milk B and T cells reservoir in breast milk, which is not eliminated by maternal ART but does not apply to breast-feeding transmission. The residual HIV-1 cell-associated virus in breast milk, which is not eliminated by maternal ART, can also alter the efficacy of prevention of postnatal mother-to-child transmission.

REFERENCES AND NOTES

STATE OF THE ART REVIEW


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HIV-1 Reservoirs in Breast Milk and Challenges to Elimination of Breast-Feeding Transmission of HIV-1

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Editor's Summary
The breast milk of HIV-infected mothers contains reservoirs of HIV, even when they are successfully treated with antiretroviral therapy; new approaches to prophylactic therapy are needed to prevent HIV transmission to their infants through breast-feeding.