Transformation of breast milk macrophages by HTLV-I: implications for HTLV-I transmission via breastfeeding

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ABSTRACT

Human T cell leukemia virus type I (HTLV-I), a causative agent of adult T-cell leukemia (ATL), is transmitted from mother to child predominantly by breastfeeding. The source of HTLV-I-infected cells in breast milk has been thought to be T cells, however, the majority of cells in breast milk are CD14+ macrophages but not CD3+ T lymphocytes, and no data are available regarding HTLV-I transmission through breast milk macrophages (BrMMΦ). To explore the potential of BrMMΦ as a possible source of infection in mother to child transmission (MTCT) of HTLV-I, an immortalized cell line (HTLV-BrMMΦ) has been established from BrMMΦ by infection with HTLV-I. HTLV-BrMMΦ retained macrophage characteristics and did not express a complete dendritic cell (DC) phenotype; nevertheless, HTLV-BrMMΦ efficiently promoted T cell proliferation in primary allogeneic mixed lymphocyte reaction (MLR) like DC. Moreover, HTLV-I infection could be transmitted from HTLV-BrMMΦ to activated T cells in the peripheral blood. These findings suggested that BrMMΦ might be an appropriate HTLV-I reservoir involved in MTCT transmission via breastfeeding.

Human T cell leukemia virus type I (HTLV-I) is a delta-retrovirus that infects 10–20 million people worldwide. It is estimated that 2–5% of infected individuals develop adult T cell leukemia/lymphoma (ATL) during their lifetime after a long latent asymptomatic period (1). Further, another small proportion (0.1–2%) will develop a clinically distinct progressive neurological disease known as HTLV-I-associated myelopathy or tropical spastic paraparesis (21, 22). HTLV-I predominantly exists as a cell-associated provirus and can infect various types of cells, such as T cells, B cells, monocytes, and fibroblasts (7). Cell-to-cell contact seems to be required for transmission because naturally infected cells with HTLV-I produce very few amount of cell-free virions (2). HTLV-I is transmitted primarily in three ways: mother to child (8), man to woman (29), and blood transfusion (4). Among these routes, mother-to-child transmission (MTCT) of HTLV-I is the most important issue to stop, because infection early in life is associated with a subsequent risk of ATL (8). The risk of infection in infant from a seropositive mother correlates with provirus load in breast milk (17, 18), the concordance of HLA class I type between mother and child (3), and the duration of breastfeeding (25). So far, the source of HTLV-I-infected cells in breast milk has been thought to be CD4+ T cells (16, 27), however, the majority of cells in breast milk particularly in earlycolostrum turned out to be CD14+ macrophages but not CD3+ T cells (23), and there has been no report regarding HTLV-I transmission via breast milk macrophages...
Peripheral blood mononuclear cells (BrMMΦ). Therefore, we have developed experimental systems for studying the biological roles of breast milk macrophages in MTCT of HTLV-I.

Recently, we have reported that BrMMΦ were capable of producing granulocyte-macrophage colony-stimulating factor (GM-CSF) spontaneously and differentiating into CD1⁺ dendritic cells (DCs) by stimulation with exogenously added interleukin-4 (IL-4) (11). DCs are essential for the initiation of primary acquired/adaptive immune responses against viral infections by taking up antigens, migrating to lymphoid organs, and presenting selected antigens to T cells in association with their surface MHC molecules. Conversely, some viruses, like human immunodeficiency virus type 1 (HIV-1), hijack the trafficking properties of DCs to be transported them from the peripheral blood to lymph nodes (28). Also, we have found that DCs derived from BrMMΦ gained a strong capacity to transmit HIV-1 to susceptible cells. These findings suggest that BrMMΦ, involved in the initiation and modulation of local immune responses, may provide an opportunity for the invaded pathogen to spread and transmit via breastfeeding.

Here, we have successfully established and characterized an immortalized macrophage cell line (HTLV-BrMMΦ) from BrMMΦ by infection with HTLV-I, and provide a possible mechanism of MTCT of HTLV-I by using the HTLV-BrMMΦ.

MATERIALS AND METHODS

Establishment of a BrMMΦ cell line. Early colostrum breast milk was collected from a healthy woman within 5 days of delivery after informed consent under a protocol approved by the Institutional Review Board of Nippon Medical School and in accordance with the human-experimental guidelines of the US Department of Health and Human Services. Fresh BrMMΦ were isolated from breast milk by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, followed by adherence to polystyrene tissue culture dishes for 1 h at 37°C. The adherent cells were then removed by incubation with 5 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 4°C. Freshly isolated BrMMΦ (1 × 10⁵) were infected with 0.5 mL of supernatants containing 354 ng/mL of HTLV-I p19 from MT-2, an HTLV-I-producing T cell line, for 18 h at 37°C. After incubation, cells were washed with phosphate-buffered saline (PBS) to remove unabsorbed free virus and were further propagated in RPMI 1640 medium-based complete culture medium (10) supplemented with 10% fetal calf serum (FCS), 20 mmol/L HEPES (Invitrogen, Carlsbad, CA), 50 μmol/L 2-mercapto-ethanol (Sigma-Aldrich, St. Louis, MO), 2 mmol/L L-glutamine (Sigma), and 100 U penicillin-streptomycin solution (Sigma).

Cell preparation. Peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells were isolated from heparinized blood using Ficoll-Hypaque density gradient centrifugation. For mixed lymphocyte reaction (MLR) assays, non-T cells were removed from PBMCs using the Lympho-quick (One Lambda, Canoga Park, CA) according to the manufacturer’s instructions.

Cell staining. Cytospin preparations of HTLV-BrMMΦ were fixed with 2% paraformaldehyde and then stained with May-Grünwald-Giemsa solution or non-specific esterase staining solution (1.1 mM α-naphthyl butyrate and 3.4 mM hexazotized para-nitroanilin in PBS, pH 5.0. For immunochemical analysis, cells were incubated with anti-HTLV-I p24 antibody (ZeptoMetrix Buffalo, NY). After washing, they were overlaid with Texas red-conjugated anti-mouse IgG antibody (Jackson Immuno Research Lab. Inc., Baltimore, MD). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 antibody (eBioscience Inc. San Diego, CA) was used to detect T cells. For electron microscopic analysis, cells were pelleted by centrifugation and fixed with modified Karnovsky’s solution (2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.1 M phosphate buffer). After fixed with 1% OsO₄, cells were dehydrated through a series of ethanol and n-butyl-glycidyl ether, and embedded in Epon 812 (TAAB, Berks, England). Ultrathin sections were cut and stained with uranyl acetate and lead citrate for examination by H-700 electron microscope (Hitachi, Tokyo, Japan). For flow cytometric analysis, FITC-conjugated antibodies to CD4, CD14, CD19, HLA-DR, CD40, CD80, CD1b, as well as phycoerythrin (PE)-conjugated CD11b, CD83, CD86, CXCR4, CCR5, C1d, were purchased from BD Bioscience Pharmingen (Franklin Lakes, NJ). PE-conjugated antibody to CD1c was from Immunotech (Marseille, France) and PE-conjugated antibody to DC-SIGN was from R&D Systems (Minneapolis, MN). FITC-conjugated antibodies to mouse immunoglobulins were purchased from Beckman Coulter (Fullerton, CA). 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), washed twice, and re-suspended in PBS-based medium. Labeled cells...
were analyzed using a FACScan (Becton Dickinson, Mountain View, CA) with propidium iodide gating for viable cells.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted from HTLV-BrMMΦ using a commercial RNeasy kit (QIAGEN, Hilden, Germany), and first-strand DNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Transcripts of HTLV-I-Tax were amplified by PCR reactions using primer: sense, 5’-tgtttggagactgtgacctgta caagggc-3’; antisense, 5’-gttgtatgattggcggggtaa-3’. After 35 cycles of PCR reactions, the PCR products were resolved by electrophoresis in agarose gels and visualized by ethidium bromide staining using a UV light source.

**Southern hybridization analysis.** Aliquots (15 μg) of high-molecular weight DNA were digested with EcoRI. The cleavage products were size-separated on 0.6% agarose gels and transferred to nylon-membranes. The HTLV-I-specific primer pair 5’-caacccagaagtccctaa-3’ and 5’-gggtgtacaggttttgggc-3’ was used to amplify 200 base pair fragment from MT-2 cells for use as a probe. Based on the previous report (24, 26), Southern hybridization was performed with digoxigenin labeled probe using DIG DNA labeling kit (Roche Applied Science, Mannheim, Germany) and the band pattern was visualized with DIG nucleic acid detection kit (Roche Applied Science).

**MLR assays.** For assessment of T cell stimulation in allogeneic MLR, both HTLV-BrMMΦ and freshly isolated BrMMΦ were irradiated (9000 rads) and used as antigen-presenting cells (APCs). Purified allogeneic T cells (5 × 10⁴ cells/well) as well as allogeneic naive cord blood T cells (5 × 10⁴ cells/well) were cultured with the indicated numbers of irradiated APCs in 96-well U-bottomed tissue culture plates at 37°C for 5 days. Proliferation was measured by addition of 0.5 μCi ³H-thymidine/well for the last 16 h, and the plates were harvested and counted using a β-counter (1450 Microbeta Trilux; Wallac, Gaithersburg, MD).

**HTLV-I infection by HTLV-BrMMΦ.** PBMC-derived lymphocytes (1 × 10⁶/mL) were stimulated or unstimulated with either 10 μg/mL phytohemagglutinin (PHA)-P (Sigma) or 5 μg/mL concanaravin A (ConA) (Sigma), and co-cultured with HTLV-BrMMΦ (2 × 10⁵/mL) or MT-2 cells (2 × 10⁵/mL) for 4 days. The percentage of HTLV-I-infected T cells was determined by intracellular staining of HTLV-I-p24 gag antigen using BD Cytofix/Cytoperm (BD Bioscience Pharmingen). To expose HTLV-BrMMΦ to an acidic environment, HTLV-BrMMΦ were treated with culture medium adjusted to pH 3 for 3 min, followed by neutralization with 0.1 mol/L Tris-buffer (pH 8.0), and then co-cultured with either Con A-activated or PHA-activated T cells.

**RESULTS**

**Establishment of a BrMMΦ cell line**

Although BrMMΦ are thought to have an important role in MTCT of various pathogens, the precise roles of BrMMΦ remain unclear; therefore, we have established a BrMMΦ cell line persistently infected with HTLV-I, termed HTLV-BrMMΦ. The appearance of HTLV-BrMMΦ (Fig. 1A left) was different from freshly isolated BrMMΦ, which contained numerous lipid particles in the cytoplasm (Fig. 1A middle; see arrows) as reported previously (11). Moreover, HTLV-BrMMΦ constitutively expressed high levels of intracellular HTLV-I antigens p24 (Fig. 1A right). These virus particles stained with uranyl acetate and lead citrate could apparently be observed in thin-section electron micrographs of fixed HTLV-BrMMΦ (Fig. 1B). Moreover, induced HTLV-I infection was confirmed by RT-PCR using a primer to Tax of HTLV-I (Fig. 1C), and Southern blot hybridization analysis revealed the integrated-status of the HTLV-I provirus (Fig. 1D). To examine whether HTLV-I virions are released from HTLV-BrMMΦ into culture supernatants, we measured the extracellular HTLV-I antigen p19 by ELISA. Indeed, culture supernatants from HTLV-BrMMΦ harvested on day 2 contained higher amounts of p19 (623.6 ± 38.7 ng/mL) compared to that from MT-2 (354.1 ± 13.1 ng/mL).

**Characterization of HTLV-BrMMΦ**

To define the physiological properties of HTLV-BrMMΦ, we examined phagocytic activity of HTLV-BrMMΦ by flow cytometry and fluorescence microscopy, as well as by electron microscopy using fluorescent latex particles. The majority of HTLV-BrMMΦ displayed a remarkable phagocytosis (Fig. 2A, 2B). In addition, cytochemical staining of HTLV-BrMMΦ showed strong positive reactions for nonspecific esterase (Fig. 2C). These findings indicated that HTLV-BrMMΦ retained macrophage phagocytic activity to uptake and process foreign antigens.
Next, we analyzed the expression of surface antigens on HTLV-BrMMΦ and compared those on freshly isolated BrMMΦ. Both T and B cell markers, CD3 and CD19, were not expressed on HTLV-BrMMΦ as well as BrMMΦ. Monocyte/macrophage lineage markers, CD14 and CD11b, were expressed on original BrMMΦ but not on transformed HTLV-BrMMΦ. Nevertheless, both types of cells expressed antigen-presenting molecules, such as HLA-DR and class I MHC, as well as various co-stimulatory molecules, such as CD80, CD86 and CD40, involved in T cell stimulation (Fig. 3A). Moreover, CD83, a
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glycoprotein expressed on mature DCs was also expressed on both cells (Fig. 3A). These findings strongly suggested that, similar to uninfected BrMMΦ that showed strong capacity to induce allogeneic MLR as demonstrated previously, the virally transformed HTLV-BrMMΦ might also have the ability to stimulate T cells via allogeneic MLR.

Therefore, we examined whether virus-infected HTLV-BrMMΦ could stimulate T cells to proliferate in a primary allogeneic MLR. As responders for primary MLR, we used purified T cells from PBMCs and naïve cord blood T cells based on our previous findings, in which a higher number of uninfected BrMMΦ generated stronger proliferative responses (11). In contrast to our previous results, although an appropriate number of HTLV-BrMMΦ induced proliferation of allogeneic T cells, a higher number of HTLV-BrMMΦ did not (Fig. 3B). This might be because infected HTLV-BrMMΦ may secrete some growth inhibitory factors for T cells. We are currently investigating to compare cellular factors between uninfected BrMMΦ and HTLV-BrMMΦ.

Because freshly isolated uninfected BrMMΦ expressed the C-type lectin DC-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN) (9) that will tightly capture free HIV virions and transmit them to HIV-1-susceptible infant CD4-positive cells (23), we examined DC-SIGN expression on HTLV-BrMMΦ. Unexpectedly, DC-SIGN was not detected on transformed HTLV-BrMMΦ at all (Fig. 3C) and the lack of its expression was further confirmed at the transcriptional level by RT-PCR (data not shown), although CD4 and CXCR4 but not CCR5 were observed on their surface (Fig. 3C). These findings indicate that transformed HTLV-BrMMΦ had lost the capacity to capture HIV-1 virions while they may retain HIV-1 susceptibility to X4-type HIV-1.

Transmission of HTLV-I to T cells via HTLV-BrMMΦ

To confirm whether HTLV-I is transmittable from HTLV-BrMMΦ to PBMCs, viable HTLV-BrMMΦ were co-cultured with lectin-stimulated or un-stimulated PBMCs. As controls, PBMCs were also co-cultured with a known HTLV-I producing cell line, MT-2. FACS analysis showed the intracellular expression of HTLV-p24 gag antigen; about 9% positive for p24 antigen in stimulated T cells and below 1% positive in un-stimulated T cells at day 4. Thus, transmission of HTLV-I to PBMCs was mediated only to the activated T cells and the transmissibility by HTLV-BrMMΦ was more potent than that by MT-2 cells (Fig. 4A). Also, transmission of HTLV-p24 antigen was confirmed in the lectin-stimulated T cells by fluorescent laser microscopy (data not shown).

These results suggest that breast milk of the HTLV-I-infected mothers who may have virus-infected BrMMΦ might transmit the virions to gastro-intestinal targets in the newborn baby through breastfeeding. The ingested milk with virus-infected BrMMΦ must encounter gastric juice with low pH (pH 3.0–4.0) before meeting with intestinal target cells (19). Also, there is a possibility that BrMMΦ may die under the acidic condition. Therefore, we conducted an experiment in which HTLV-BrMMΦ
The evidence of HTLV-I transmission via breast milk has been well documented since the 1980s and recent reports have also shown the importance of proviral load in breast milk as a predictor of HTLV-I transmission from mother to child (18). Particularly, in Japan, screening of pregnant women and avoiding breastfeeding by those infected resulted in profound reduction of the prevalence of HTLV-I (14).

HTLV-I infection requires cell-to-cell contact before the virions are transmitted. Pre-treatment of culture medium adjusted to pH 3 for 3 min were further co-cultured with either concanavalin-A (Con A) or phytohemagglutinin (PHA)-stimulated PBMCs (Fig. 4B). The results showed that medium acidity had little influence on HTLV-I transmissibility for lectin-stimulated PBMCs. Taken together, the HTLV-I-infected BrMΦ in the colostrums milk of HTLV-I-infected mothers may stimulate mucosal infant T cells and transmit them the virions via breastfeeding.

**DISCUSSION**

The evidence of HTLV-I transmission via breast milk has been well documented since the 1980s and recent reports have also shown the importance of proviral load in breast milk as a predictor of HTLV-I transmission from mother to child (18). Particularly, in Japan, screening of pregnant women and avoiding breastfeeding by those infected resulted in profound reduction of the prevalence of HTLV-I (14). HTLV-I infection requires cell-to-cell contact be-
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(15) and surface heparin proteoglycan serves as a receptor for HTLV-I (12). Both GLUT1 and heparin proteoglycan are ubiquitously expressed on the surface of many types of cells, hence, HTLV-I can infect not only CD4+ T cells but also macrophages. Second, we have previously reported that BrMMΦ were capable of producing GM-CSF spontaneously and differentiating into immature DCs by stimulation with exogenous IL-4 alone, indicating that BrMMΦ maintain the developmental potential to differentiate into DCs and efficiently respond to environmental stimuli. DCs act not only as sentinels for the detection of, but also as target cells for intruded viruses, and this can be important for viral transport and spread.

We have demonstrated that BrMMΦ stimulated with IL-4 showed a strong capacity to transmit HIV-I with enhanced expression of DC-SIGN (23). Moreover, Jones et al. demonstrated that HTLV-I can efficiently infect myeloid and plasmacytoid DCs, and DCs exposed to HTLV-I can rapidly, efficiently, and reproducibly transfer a virus to autologous primary CD4+ T cells (13). Therefore, to explore the potential of BrMMΦ to be a HTLV-I reservoir involved in transmission during breastfeeding, we have established and characterized an immortalized cell line of HTLV-BrMMΦ, which lost monocyte/macrophage lineage markers CD14 and CD11b. Nevertheless, they expressed DC marker CD83, antigen-presenting molecules HLA-DR, and co-stimulatory molecules such as CD80, CD86, and CD40. These phenotypes were consistent with immature DCs (iDCs) derived from monocytes co-cultured with both GM-CSF and IL-4. However, DC-SIGN and human group 1 CD1 molecules (CD1a, CD1b, CD1c), expressed mainly on professional APCs, were not detected on HTLV-BrMMΦ. These findings indicate that HTLV-BrMMΦ were not equipped with a complete DC phenotype; however, an appropriate number of them efficiently induced T cell proliferation in an allogeneic MLR like DCs. The ability of HTLV-BrMMΦ to stimulate T cells is of considerable significance, because stimulated T cells are highly susceptible to virus infections; thus, HTLV-BrMMΦ can serve as both a T cell stimulator and a source of infection. Collectively, HTLV-BrMMΦ derived from colostrum milk seem to be an excellent reservoir as well as a transmitter for HTLV-I.

In this study, we showed the ability of HTLV-BrMMΦ to transmit HTLV-I virions to stimulated T cells derived from PBMCs but few un-stimulated T cells could be infected by contact with HTLV-BrMMΦ, which may be due to the inappropriate cause lymphocytes naturally infected with HTLV-I produce very few cell-free HTLV-I virions (6). HTLV-I-infected T cells in breast milk have been proposed as a source of HTLV-I infection. However, the following evidence has led us to hypothesize that BrMMΦ would be more likely to be responsible for MTCT of HTLV-I. First, HTLV antigen-positive T cells from seropositive mothers were not always detected in their breast milk (27). The majority of breast milk cells are CD14+ macrophages (23). Also, it should be noted that the HTLV-I receptor is the glucose transporter 1 protein (GLUT1)
culture conditions for T cell activation. It is possible that when HTLV-I-infected BrMMΦ are ingested with the maternal breast milk and arrive at the infant intestinal tracts through exposure to acidic gastric juice, they might encounter intestinal intraepithelial lymphocytes (IEL). Since most IELs at the surface mucosal compartment are 'partially activated' T cells waiting for appropriate signals to put them into a state of full activation (20), IELs might be more susceptible to HTLV-I infection. Indeed, Bourinbaiar et al. proposed that ATL might be derived from the intestinal lymphocytes of breastfed infant, because ATL-derived CD4+ T cell lines were positive for HML-1 monoclonal antibody that recognizes human intestinal lymphocytes (5). Although we have not yet shown that BrMMΦ were actually infected with HTLV-I, if effective infection of intestinal lymphocytes with HTLV-I is necessary for the development of ATL, HTLV-I infected BrMMΦ might be an appropriate HTLV-I reservoir with regard to a potent T cell stimulator.

We showed here that BrMMΦ could be differentiated into DC-like cells by HTLV-I infection. Since breastfeeding allows the invasion of foreign antigens into breast milk, BrMMΦ may have an opportunity to encounter the antigens. In some cases, BrMMΦ might be stimulated and undergone phenotypic and functional modifications leading to a beneficial or deteriorate influence on infant immunity after breastfeeding. Therefore, to further clarify the biological roles of individual BrMMΦ may be helpful to understand the formation of immune systems of breastfed infant.

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