Identification of the factors which impact on the transmission of human immunodeficiency virus type 1 (HIV-1) from an infected mother to her infant is essential for the development of effective strategies to prevent perinatal HIV-1 infection. The current study was designed to determine if unstimulated human neonatal cord blood mononuclear cells (CBMC) differ from adult peripheral blood mononuclear cells (PBMC) in susceptibility to HIV-1 infection. Both cell populations were challenged with two laboratory and two clinical HIV-1 isolates with different phenotypic properties. Infection was evaluated by quantitation of p24 antigen production and p24 antigen expression by an enzyme immunoassay and immunofluorescence, respectively. T-cell markers were determined by flow cytometry. Unstimulated CBMC were preferentially infected by macrophage-tropic, non-syncytium-inducing (non-SI) laboratory and clinical isolates, whereas PBMC were more susceptible to T-lymphotropic, SI HIV-1 strains. The macrophage-tropic strain HIV-1 Ba-L, replicated to 100-fold higher titers in CBMC than a similar inoculum of the SI isolate HIV-1 LAI. The opposite occurred in unstimulated PBMC, which replicated HIV-1 LAI to eightfold higher titers than the macrophage-tropic isolate. These findings indicate that a selection of viral phenotype may occur with unstimulated CBMC displaying a predominant susceptibility to infection by macrophage-tropic, non-SI HIV-1 strains and that this selection may influence mother-infant transmission of HIV-1.

Mother-infant transmission of human immunodeficiency virus type 1 (HIV-1) is an increasing problem worldwide. Perinatal transmission is estimated to occur in approximately 25% of infants born to HIV-1-infected mothers (7, 16, 22), with intrapartum transmission thought to occur more frequently than congenital infection (2–4). Information concerning the intrapartum transmission thought to occur more frequently of infants born to HIV-1-infected mothers (7, 16, 22), with natal transmission is estimated to occur in approximately 25% of maternal virus that may preferentially infect the infant with no evidence for transmission of more prevalent genotypes (25). Thus, mother-infant HIV-1 transmission could involve in vivo selection for specific viral strains with distinctive biological phenotypes.

The physiologic immaturity of neonatal cells and the low level of lymphocyte activation in newborn infants are host factors that could influence infection of neonatal cells. Activated or memory CD4⁺ T cells that express the CD45RO marker are much less frequent in the neonate, and they have been reported to be the major target cell type for HIV-1 infection (18). The studies described here were designed to compare the susceptibilities of unstimulated cord blood mononuclear cells (CBMC) and unstimulated adult peripheral blood mononuclear cells (PBMC) to infection with HIV-1 isolates of different phenotypes. Our findings suggest that CBMC can be preferentially infected with macrophage-tropic, non-SI HIV-1 strains and that this susceptibility may play a role in HIV-1 strains that are transmitted from an infected mother to her infant.

MATERIALS AND METHODS

Cell separation and culture conditions. Umbilical cords of full-term newborns born to HIV-1-seronegative mothers were cleaned of maternal blood, and cord blood samples were collected under sterile conditions by puncture of the umbilical vein. Peripheral blood samples were obtained from healthy, HIV-1-seronegative adults. CBMC and PBMC were separated from heparinized whole-blood samples by density centrifugation on Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo.). The isolated mononuclear cells were washed twice in sterile phosphate-buffered saline (PBS) and once in RPMI 1640 (JRH Biosciences, Lenexa, Kans.) and maintained in RPMI 1640 supplemented with 20% heat-inactivated, low-endotoxin fetal bovine serum (JRH Biosciences), 1 mM L-glutamine, 100 U of penicillin per ml, and 1 µg of streptomycin per ml (basic medium). Neither phytohemagglutinin A (PHA) nor interleukin-2 was added to cells that were maintained unstimulated. In parallel, the same CBMC and PBMC samples were stimulated with PHA (3.5 µg/ml) (Sigma) in the basic medium described above for 48 h prior to infection. Unstimulated or stimulated cells were challenged with HIV-1 immediately following Ficoll-Hypaque gradient separation or PHA stimulation, respectively. Following HIV-1 infection, the unstimulated cells were maintained in basic medium while PHA-stimulated cells were maintained in basic medium supplemented with interleukin-2 (10 U/ml) (Boehringer Mannheim, Indianapolis, Ind.). At every harvest (see below), half of the medium was removed and replaced with the same fresh medium.

Cell viability was determined by trypan blue exclusion. Wright/Giemsa differential blood stains were performed on CBMC and PBMC samples imme-
from the National Institutes of Health AIDS Research and Reference Reagent Program and was expanded in monocyte-derived macrophages (9). HIV-1 clinical isolates were propagated and their phenotypes were characterized at the University of California, San Diego, Retrovirology Laboratory. Viruses were expanded in PHA-stimulated PBMC (6). One clinical isolate was high replication and T lymphotropic, induced syncytia in MT-2 cells, and did not infect monocyte-derived macrophages. This virus was isolated from a symptomatic patient with a CD4+ lymphocyte count of 562/mm^3. The other clinical isolate was low replication, did not induce syncytia in MT-2 cells, and replicated well in primary monocyte-derived macrophages. This virus was isolated from a patient with a CD4+ lymphocyte count of 352/mm^3. MT-2 cell assays were performed as previously described (20). All HIV-1 stocks were stored at −70°C as cell-free virus isolates after filtration through a 0.2-μm-pore-size filter. The tissue culture infective dose for each isolate was determined in PBMC or macrophages (HIV_{Ba-L}) as previously described (9, 11).

### Susceptibility experiments

Unstimulated CBMC or unstimulated PBMC (5 × 10^6) were incubated overnight with 5 × 10^6 tissue culture infective doses of cell-free viral inoculum. Infection was performed in polypropylene tubes to prevent adherence of monocytes during viral exposure. After being washed five times with PBS, the cells were cultured either in 24-well flat-bottom tissue culture plates (Costar, Boston, Mass.) or in 4-well tissue culture chamber slides (New England Nuclear, Boston, Mass.). The last wash was assayed for carryover p24 antigen. Supernatants were collected on days 3, 7, and 11 or 14 postinfection for quantitation of p24 antigen detection (enzyme immunoassay; Abbott, Abbott Park, Ill.). Cultures were considered positive when increasing quantities of p24 antigen greater than 25 pg/ml were obtained on consecutive culture supernatants. Simultaneous infection of PHA-stimulated PBMC from a healthy adult donor was carried out to confirm the infectivity of viral stocks. All cultures were screened on days 3, 7, 10, and 14 for cytopathic effect by phase-contrast microscopy at a 100-fold magnification. Mock-infected cells from all CBMC and PBMC samples were cultured and evaluated as negative controls.

### Flow cytometry

Representative unstimulated or PHA-stimulated CBMC and PBMC samples were stained with monoclonal antibodies to evaluate T-cell subset and activation parameters immediately prior to infection and 1 and 3 days after HIV-1 or mock infection. Two hundred thousand CBMC and PBMC were harvested, washed three times with sterile PBS, and resuspended in 10 μl of Hank’s balanced salt solution (without phenol red) containing 3% heat-inactivated fetal bovine serum, 0.1% sodium azide, and 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) (staining buffer) before selected concentrations of directly labelled antibodies were added. After incubation for 30 min at 4°C, cells were washed and resuspended in an ice-cold staining buffer. Human cell surface molecules and corresponding murine monoclonal antibodies were as follows: CD3, fluorescein isothiocyanate and phycoerythrin-labeled antibodies; CD45RA, fluorescein isothiocyanate; CD45RO, fluorescein isothiocyanate; CD25, fluorescein isothiocyanate; CD69, fluorescein isothiocyanate (Becton Dickinson, Mountain View, Calif.). To inactivate infectious virus, all CBMC and PBMC samples were fixed with 1% paraformaldehyde prior to analysis. Two-color flow cytometryemployed a FACScan flow cytometer (Becton Dickinson) and LYSIS II research software. A total of 10^5 events per sample were collected. Debris and dead cells were excluded by gating according to forward and side scatter properties.

### Cell staining and immunofluorescence

CBMC and PBMC cultured in chamber slides were depleted of nonadherent cells by vigorous washing and evaluated by nonspecific esterase, Wright/Giemsa staining, and immunostaining. For immunostaining, adherent cells were fixed in acetone–100% methanol and incubated with 10% human plasma (HIV seronegative) in PBS. Adherent cells were then incubated in order to slot a mouse anti-HIV p24 antigen antibody (immunoglobulin G) (0.02 mg/ml) (DuPont, Boston, Mass.) and the M01 (Coulter, Hialeah, Fla.) mouse anti-CD11b (immunoglobulin M) (0.1 mg/ml) in PBS with 2% human plasma. After repeated washing with PBS, the fluorescein isothiocyanate-labeled rabbit-anti-mouse immunoglobulin G antibody (0.05 mg/ml) (Tago, Burlingame, Calif.) and the rhodamine-labeled goat anti-mouse immunoglobulin M antibody (0.05 mg/ml) (Chemicon, Los Angeles, Calif.) were added. The slides were washed after 30 min of incubation, mounted, and viewed with a Nikon Optiphot microscope.

### RESULTS

#### Assessment of CBMC and PBMC populations

A total of 26 unstimulated CBMC samples and 15 unstimulated PBMC samples were analyzed for infectivity by the laboratory-adapted HIV-1 isolates HIV_{LAI} and HIV_{Ba-L}. Wright/Giemsa stains of the samples revealed normal blood differentials, with monocytes constituting 4 to 6% of the leukocyte populations in both cord blood samples and adult blood samples. After Ficol-Hypaque separation of mononuclear cells, the viability was >98%, as determined by trypan blue dye exclusion, and the percentage of monocytes was 7 to 13% in both CBMC samples and PBMC samples; the remaining cells belonged almost exclusively to the lymphocyte population.

To characterize the differences between the fetal and adult mononuclear-cell samples, flow-cytometric analysis was performed on six CBMC and six PBMC samples with and without PHA stimulation. Samples were also analyzed before and after challenge with either HIV_{LAI} or HIV_{Ba-L} (Table 1). The distribution of T-cell subsets and T-cell maturation and activation parameters was assessed before and after HIV-1 infection. The levels of CD4+ T cells for CBMC and PBMC samples were similar. The majority (mean ± standard deviation, 90% ± 3.8%) of cord blood T cells expressed CD45RA (naive subset), while only 13% ± 2.1% expressed CD45RO (memory subset) molecules on their surface. In contrast, T cells in the PBMC samples had a higher concentration of CD45RO cells (47% ± 6.1%) with an almost equal division between memory and naive T cells (Table 1). Three days after infection with HIV-1, no significant difference in CD4+ T-cell levels was observed either between infected and uninfected cultures or between CBMC and PBMC cultures. Stimulating CBMC or PBMC with PHA 48 h after infection substantially increased the CD45RO+ population, as well as CD25 and CD69 (T-cell activation markers) expression (Table 1), signifying that both populations were competent for activation. To determine if T-cell activation of unstimulated cells occurs during the period of HIV-1 exposure,
the expression of CD25 and CD69 was evaluated before and after challenge with virus as well as 3 days postinfection. No significant upregulation of CD25 or CD69 in the CBMC and PBMC samples was observed, indicating that T-cell activation had not occurred during acute infection with HIV-1 (Table 1). Thus, the major difference between CBMC and PBMC was the reduced numbers of memory T cells in CBMC.

CBMC and PBMC differ in HIV-1 susceptibility and the rate of replication of HIV. To investigate differences in HIV-1 susceptibility between CBMC and PBMC, unstimulated cultures were challenged with either HIV<sub>LAI</sub> or HIV<sub>Ba-L</sub>, and p24 antigen was measured from the supernatant at 3, 7, and 14 days postinfection. Whereas HIV<sub>Ba-L</sub> preferentially replicated in CBMC, HIV<sub>LAI</sub> replicated at high levels in PBMC (Fig. 1).

All 26 (100%) unstimulated CBMC samples were infected by the macrophage-tropic isolate HIV<sub>Ba-L</sub>, whereas the lymphotropic isolate HIV<sub>LAI</sub> replicated in only 18 (69%) of 26 challenged CBMC samples. In contrast, while all 15 unstimulated PBMC samples replicated HIV<sub>LAI</sub>, only 11 (73%) of 15 were susceptible to infection by HIV<sub>Ba-L</sub>. There was a significant difference between these two cell populations in susceptibility to infection by HIV<sub>LAI</sub> and HIV<sub>Ba-L</sub> ($P = 0.003; \chi^2$). Of the 26 CBMC samples that were challenged with the two HIV-1 isolates, 24 (92%) HIV-1<sub>Ba-L</sub>-infected samples produced 10- to 50-fold higher p24 antigen levels than the HIV-1<sub>LAI</sub>-infected CBMC. The remaining two CBMC samples did not exhibit a difference in the ability to replicate either virus. The opposite was found for the unstimulated PBMC population, in which 14 (93%) of 15 samples showed enhanced replication of the isolate HIV<sub>LAI</sub> with three- to fivefold-higher p24 antigen levels than the levels for infection with HIV<sub>Ba-L</sub> (Table 2). The one remaining PBMC sample did not display a difference in the ability to replicate either virus. Thus, CBMC were preferentially infected by macrophage-tropic isolates, while PBMC were more susceptible to infection by lymphotropic HIV-1 isolates.

Replication of clinical isolates of different phenotypes in CBMC and PBMC cultures. To determine if selective susceptibility to infection was also observed with clinical isolates, six unstimulated CBMC samples and six unstimulated PBMC samples were challenged with two clinical isolates of previously established phenotypes. One clinical isolate was high replica-
tion, T lymphotropic, and SI, while the other was low replication, macrophage-tropic, and non-SI. As in the experiments with the laboratory HIV-1 isolates, a higher susceptibility of unstimulated CBMC was found for the macrophage-tropic clinical HIV-1 isolate, which infected four (67%) of six samples, while the SI, T-cell-tropic clinical isolate replicated in only one (17%) of six CBMC samples. The reverse was observed with the PBMC cultures, in which the SI, T-cell-tropic clinical isolate produced high levels of p24 antigen in five (83%) of six samples and the macrophage-tropic isolate failed to replicate in any of the six unstimulated PBMC samples. Thus, the same dichotomy of infection that was observed with laboratory strains was also observed with clinical isolates.

**PHA stimulation increases the replication of HIV-1 in CBMC.** PHA stimulation is known to affect T-cell maturation and activation (13). Because we observed that the maturation and activation markers CD45RO, CD25, and CD69 were increased 10- to 40-fold following PHA stimulation, we studied whether PHA stimulation of cells would alter the HIV susceptibility pattern. For these experiments six PHA-stimulated and unstimulated CBMC and PBMC samples were concomitantly challenged with HIV-1LAI or HIV-1Ba-L. HIV-1LAI infected 100% of unstimulated PBMC, with replication being increased 10- to 40-fold following PHA stimulation by day 14 (Fig. 1). While only four of six unstimulated CBMC samples could be infected with HIV-1LAI, PHA stimulation resulted in 100% infectivity of samples. The rate of replication of HIV-1LAI in CBMC was also influenced by PHA stimulation, with a 100-fold increase in viral replication observed by day 14. The impact of PHA stimulation was less dramatic in HIV-1Ba-L-challenged cell samples, leading primarily to accelerated infection kinetics (Fig. 1). No increase in susceptibility in stimulated PBMC challenged with HIV-1Ba-L was observed, with only four of six PBMC samples being susceptible to infection, regardless of stimulation. All unstimulated CBMC samples were infectable with HIV-1Ba-L; therefore, the influence of PHA on CBMC susceptibility to this virus could not be determined.

**HIV-1Ba-L causes cytopathic effects in adherent unstimulated CBMC.** The morphology of HIV-1-infected and mock-infected CBMC and PBMC samples was examined during the 14 days of culture. Within 2 to 5 days, a monolayer of adherent cells was observed, consisting of rounded as well as spindleshaped cells. Multinucleated giant cells developed among the adherent cells of cord blood samples within 7 days of challenge with HIV-1Ba-L and reached 20 to 50% of adherent cells by 14 days postinfection with the macrophage-tropic isolate. No cytopathic changes were seen in HIV-1LAI-challenged or mock-infected CBMC samples or among the adherent cells of the adult blood samples, regardless of viral challenge (Fig. 2).

To determine the predominant cell type of adherent cells in CBMC and PBMC cultures, nonspecific esterase staining and immunofluorescence staining against the macrophage epitope CD11b were performed. Adherent cells from HIV-1-challenged unstimulated CBMC and PBMC cultured on chamber slides were also evaluated for HIV-1 p24 antigen expression. More than 98% of adherent cells of the uninfected control CBMC and PBMC samples were esterase positive, and more than 80% stained positive with CD11b, while p24 antigen was undetectable in these samples. Positive staining for p24 antigen was not observed in adherent cells of CBMC or PBMC challenged with HIV-1LAI, while the staining pattern for CD11b was similar to that for the mock-infected control. After challenge with HIV-1Ba-L, PBMC remained negative for intracytoplasmic p24 antigen. However, 30 to 50% of the adherent CBMC stained positive for p24 antigen, and viral-protein-containing cells were mainly multinucleated giant cells that also stained positive for esterase and CD11b (data not shown). Thus, the adherent CBMC producing HIV-1 were predominantly macrophages.

**DISCUSSION**

These results demonstrate an enhanced susceptibility of unstimulated CBMC to macrophage-tropic, non-SI HIV-1 isolates, while unstimulated adult PBMC were more readily infected by T-lymphotropic, SI isolates. Moreover, unstimulated CBMC were more susceptible to infection by macrophage-tropic strains than were PBMC and allowed for significantly higher viral replication as reflected by higher release of p24 antigen into culture supernatants. Additionally, HIV-1Ba-L-infected adherent CBMC demonstrated intracytoplasmic staining for p24 antigen, while no such staining was observed in adherent CBMC challenged with HIV-1LAI. The high p24 antigen production in HIV-1Ba-L-infected CBMC was accompanied by adherent multinucleated giant cells, which were not observed in the HIV-1-infected PBMC. The reasons for differences in susceptibility between CBMC and PBMC are unclear. The discrepancy in susceptibility to macrophage-tropic HIV-1 isolates cannot be explained by differences in the percentages of monocytes in CBMC and PBMC, which were equivalent in both sources of cells. It is possible that interactions between cord blood T cells and monocytes may lead to production of cytokines that differ from those seen with adult PBMC. Previous in vitro studies have shown that gamma interferon can down regulate viral replication in monocytes/macrophages (8). In this regard, the markedly lower gamma interferon production of neonatal CD4+ and CD8+ T cells compared with analogous adult T cells (10) may explain, in part, the significantly higher levels of HIV-1 in CBMC cultures after infection with HIV-1Ba-L.

Recently Ho et al. (5) described higher replication of laboratory-adapted, macrophage-tropic HIV-1 isolates in cord blood-derived adherent macrophages compared with macrophages derived from adult peripheral blood. Similarly, Sperduto et al. have recently shown that differentiated CBMC-derived macrophages are more susceptible to both macrophage-tropic and T-lymphotropic strains of HIV-1 (21). However, there are important differences between these studies and our present study. In these reports (5), cord blood monocytes were adhered and differentiated into macrophages before challenge with HIV-1, thus making them more susceptible to HIV-1 infection. In the current study, in order to more closely assess the susceptibility of circulating neonatal cells to HIV-1 infection, unstimulated CBMC were challenged with HIV-1 without prior differentiation of cells. Therefore, high levels of undifferentiated monocytes and naive, nonactivated T cells (high CD45RA+, low CD25+) were exposed to infection by HIV-1.
In parallel to the enhanced susceptibility of unstimulated CBMC to HIV Ba-L, CBMC were less permissive to infection and replication of the lymphotropic, SI isolate HIV LAI than PBMC. The difference in lymphocyte maturation between neonatal and adult cells could explain these findings, whereas flow-cytometric analysis before and after infection with HIV LAI did not reveal differences in CD4+ T-cell levels or rates of CD4+ cell depletion. Only a minority of neonatal T cells express CD45RO, the memory subset marker, whereas 50 to 60% of adult T cells belong to the memory T-cell subset. Furthermore, PHA stimulation of CD45RA+ T cells leads to activation with increased CD25 expression and conversion to CD45RO+ T cells and, thus, to an increased susceptibility to HIV-1 infection. Therefore, addition of T-cell-activating stimuli (i.e., PHA) abolished the difference in HIV-1 susceptibility and the rate of replication between CBMC and PBMC. A difference in intrinsic cell properties (19, 24), such as the state of activation or differentiation, may be responsible for these observations.

The enhanced susceptibility of CBMC to infection with macrophage-tropic isolates suggests that a selective process in favor of such isolates might be involved in mother-infant transmission. Moreover, the finding of HIV-1-infected macrophages in the cervixes of infected women (12) is of interest, because transmission of HIV-1 may occur during vaginal delivery when comes in contact with a non-SI, macrophage-tropic HIV-1 strain. Our laboratory and others have recently reported that perinatally infected infants are predominantly infected by non-SI strains of HIV-1 (17, 20). Of interest is that, in contrast to adults, among whom those infected with non-SI strains of HIV-1 usually have slowly progressive disease, infants infected with non-SI variants can rapidly develop AIDS within the first year of life (20).

In summary, we have found that unstimulated CBMC are more susceptible to productive infection with macrophage-tropic, non-SI HIV-1 strains than are adult PBMC and that SI, T-lymphotropic virus more readily infects adult PBMC than neonatal cells. These data support the hypothesis that certain viral phenotypes of HIV-1 may be preferentially transmitted from an infected mother to her infant and that these phenotypes may replicate more efficiently in infants than in similarly infected adults. Further studies will be required to support these hypotheses.

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