**In Vitro Susceptibility of Thai Seronegative Donor CD8+ T Lymphocytes to Human Immunodeficiency Virus-1 (HIV-1) Infection**

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While the CD4 molecule serves as the primary receptor for human immunodeficiency virus (HIV-1), the CXCR4 and CCR5 chemokine receptors are the co-receptors for virus entry into target cells.¹⁻⁶ After virus attachment, high affinity interactions between CD4, a chemokine coreceptor and gp120 of the HIV-1 envelope initiate conformational changes, resulting in exposure of a fusogenic domain of the transmembrane envelope protein (gp41) which allows entry of the virus into the cytoplasm. Consequently, the targets for HIV-1 are cells that express CD4 molecules and chemokine coreceptors, such as CD4+ T lymphocytes and macrophages.⁴⁻⁵,⁷⁻¹⁰ Infected CD4+ cells have been found in both lymphoid and nonlymphoid organs, such as brain, skin, lungs, liver, lymph nodes and bone marrow.¹¹⁻¹⁵ However, HIV-1 has also been found to infect cell lineages that do not express CD4 molecules on the cell membrane, including bone marrow precursor cells, colorectal cells, astrocytes, neurons and fibroblastoid cells.¹⁶⁻²¹ Of particular interest is the demonstration that CD8+ lymphocytes can harbor HIV-1.²²⁻³¹

In the present work we provide evidence that mature peripheral blood CD8+ T lymphocytes from Thai donors are susceptible to HIV-1 infection in vitro in the presence of stimulated CD4+/CD8+ and CD8+ T lymphocytes. We postulate that CD8+ T lymphocytes from Thai donors can be infected with HIV-1 subtypes B and E in vitro.

SUMMARY To determine whether CD8+ T lymphocytes from Thai donor cells are susceptible to HIV-1 infection, undepleted peripheral blood mononuclear cells (PBMC) and CD8-enriched PBMC were infected with HIV-1 Thai subtype B and CRF01_AE (E) primary isolates. Virus kinetics in HIV-1 infection of CD4+ and CD8+ T lymphocytes peaked at day 7 or 10 post infection (pi); the TCID₅₀ used for cell infection was proportional to the level of p24 production in the cultures. We also found that the level of p24 antigen in the supernatants of infected undepleted PBMC was significantly higher than that of infected CD8-enriched PBMC. Interestingly, both single positive T lymphocytes (CD4+ and CD8+ T lymphocytes) as well as double positive CD4+/CD8+ T lymphocytes were infected with HIV-1. The double positive T lymphocytes in PBMC were found only in the presence of both CD4+ and CD8+ T lymphocytes. The majority of p24+/CD4-/CD8- T lymphocytes were HIV-1 infected CD4 down-modulated PBMC. This report provides direct evidence that single positive CD8+ T lymphocytes and double positive CD4+/CD8+ T lymphocytes from Thai donors can be infected with HIV-1 subtypes B and E in vitro.

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cytes may function as a reservoir for HIV-1 in vivo, and that HIV-1 has a broader tropism for different cell types than previously thought. Infection of CD8+ T lymphocytes may contribute to the decline of this subset upon disease progression in HIV-infected persons and may perturb the CD8 arm of the immune response. HIV-1 infection of CD8+ T lymphocytes may occur through a non-CD4-dependent mechanism of virus entry. These results provide evidence for additional mechanisms for HIV immunopathogenesis.

MATERIALS AND METHODS

HIV-1 strains

The 4971 (CM237, Thai subtype B') and 9667 (CM235, Thai subtype E) are non-syncytium-inducing (NSI) primary isolates of Thai patients in the north (kindly provided by Dr. Francine McCutchan, the Henry M. Jackson Foundation for the Advancement of Military Medicine, Rockville, MD).

Blood donors and peripheral blood mononuclear cells (PBMC) separation

Peripheral blood was drawn by venipuncture from HIV-seronegative healthy Thai donors registered at the Blood Bank in the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital. PBMC were separated by gradient centrifugation on Ficoll Hypaque (Histopaque 1077; Sigma, St. Louis, MO). After the centrifugation step, PBMC were harvested and washed three times in phosphate-buffered saline (PBS; GIBCO, Grand Island, NY). The pellet was resuspended in complete RPMI (cRPMI; RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum [FCS], 1% penicillin-streptomycin, and 20 mM L-glutamine [all reagents from GIBCO, Grand Island, NY]) plus 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) at 2 x 10^6 cells/ml. The cell suspension was aliquoted (1 ml/cryogenic tube) and frozen in a liquid nitrogen freezer.

Preparation of CD8-enriched PBMC

Frozen cells were rapidly thawed in water bath at 37°C, and washed once with cRPMI. CD8+ T lymphocytes were isolated by negative selection from PBMC by using magnetic bead separation. According to the manufacturer’s instructions, CD4+ T lymphocytes were depleted to prepare CD8-enriched PBMC using CD4 antibody-coated immunomagnetic beads (DyanaBeads; Dynal, Lake Success, NY). The ratio of beads-to-cells was kept at 1:10. The separation process was performed at 4°C for 30 minutes. The CD8-enriched PBMC were then harvested by using a magnetic particle concentrator (Dynal), washed and resuspended in cRPMI.

Phytohemagglutinin-P (PHA) stimulation

After the depletion step, CD8-enriched PBMC were centrifuged and resuspended at 1 x 10^6 cells in cRPMI containing 5 μg/ml PHA (DIFCO, Detroit, MI). After incubating with PHA overnight, cells were centrifuged and cultured in cRPMI supplemented with 10% (10 international units [IU]/ml) recombinant interleukin-2 (Boehringer-Mannheim, Indianapolis, USA) (IL-2 medium) at 37°C and 5% CO₂ for subsequent experiments. The depletion efficiency was determined by staining 0.5 x 10^6 cells with phycoerythrin (PE)-conjugated CD4 monoclonal antibody (mAb) (Becton Dickinson, CA) and peridinin chlorophyll protein (PerCP)-conjugated CD8 mAb. The percent of CD8+ enriched cells in the PBMC were analyzed using a FACSort flow cytometer (Becton Dickinson Biosciences, San Jose, CA). On the day of infection (after 3-4 days of culture in IL-2 medium) the depleted population was comprised of more than 95% CD8+ T cells. Undepleted normal PBMC were PHA stimulated (PHA-PBMC) and cultured as described for the depleted cells.

Virus stock preparation

Primary isolates were propagated to produce high titered stocks. Five million PHA-PBMC were resuspended in 1 ml of viral supernatant and incubated for 1 hour at 37°C. After infection, cells were washed using cRPMI to remove excess unbound virus and were cultured in IL-2 medium for 8-12 days. At the peak of p24 antigen levels, as determined by p24 antigen capture (Coulter, Miami, FL), virus stocks were frozen in 1 ml aliquots and stored at -70°C for subsequent use. The same normal donor PBMC were used for each virus titration. Primary isolates were not passaged through neoplastic cell lines and the viruses used throughout were uncloned.

Virus stock titrations

Viral stocks were titered in fivefold dilutions using five replicate wells per virus dilution. One hundred microliters of diluted (1:6.7)
normal human plasma (NHP), 1 × 10^6 PHA-PBMC in 100 µl of IL-2 medium and 100 µl of viral stock (prepared at three times the desired final dilution) were mixed in 15 ml tubes (one tube per virus dilution). The final virus dilutions were from 5^3 to 5^7; cells were infected overnight (16-18 hours). The next morning, the cells were washed two times in 10 ml of wash medium (rRPMI with 2% FCS). After the second wash, cells were resuspended in 1.0 ml of IL-2 medium and 200 µl were plated in five replicate wells of a 96-well U-bottom culture plate (Costar, Corning, NY). At day 4, 100 µl of supernatant were removed and 100 µl of fresh IL-2 medium were added. At day 8, the supernatant was removed for p24 antigen capture. The dilution of virus required to infect 50% of replicate wells (TCID₅₀) was calculated by the Spearman-Karber method.³⁵

**Growth kinetics quantitated by HIV-1 p24 antigen capture (ELISA) and intracellular p24 detection (flow cytometry)**

Depending on the virus used, pretreated virus stock was diluted appropriately to 100-500 TCID₅₀ during the infection period. Cultures including 4971-infected, 9667-infected and uninfected cells were set up in 15-ml tubes for the infection period if extracellular p24 detection was required (cells washed after infection step) or were set up in 24-well plates (Costar, Corning, NY) directly if extracellular p24 detection was not required (cells not washed after infection step). PHA-PBMC and CD8-enriched PBMC were added at 1 × 10^6 cells per tube or well in 100 µl of IL-2 medium to appropriate tubes or wells. A 200-µl aliquot of diluted pretreated virus stock at the desired TCID₅₀ was added to infect cultures for overnight. The final volume was adjusted to 400 µl per well or tube using IL-2 medium. After overnight infection, cells used for quantitation of p24 antigen in supernatants were washed with 10 ml of wash medium. Cells that were assayed only for intracellular (IC) p24 were incubated with virus for the entire culture period of 10 days in a volume of 1.5 ml. 1.5 ml of IL-2 medium was added to each tube, and contents were transferred to a 24-well plate. At days 4, 7 and 10, a 450 µl aliquot of supernatant plus 50 µl of lyse buffer (Coulter, Miami, FL) were mixed for p24 antigen capture by ELISA. Cells were subcultured 1:2 (0.5 ml), counted and resuspended at 0.5 × 10^5 cells/tube for intracellular p24 detection by flow cytometry. Fresh IL-2 medium was added to each well to maintain the volume at 1.5 ml per well.

**p24 antigen capture ELISA**

HIV-1 p24 antigen in samples was quantitated using a p24 antigen capture ELISA kit (Coulter, Miami, FL), according to manufacturer’s instructions.

**Flow cytometric analyses**

Flow cytometric determination of intracellular HIV-1 p24 antigen was performed using the method described by Darden et al.³² Briefly, cells were counted and 0.5 × 10^6 cells were aliquoted to 12 × 75-mm polystyrene tubes. Cells were washed once by adding 3 ml of PBS with 2% heat-inactivated FCS (PBS-FCS) and centrifuging. After washing, the supernatant was decanted and cells were vortexed gently. Surface staining was performed first using an isotyping matched control, CD3-fluorescein isothiocyanate (FITC) mAb alone, CD4-PE mAb alone or CD4-PE and CD8-PerCP mAbs together. Cells were incubated at 4°C for 30 minutes in the dark, washed once, and resuspended in 100 µl of fixation medium (Fix and Perm kit, reagent A, Caltag Laboratories, Burlingame, CA) for 15 minutes at room temperature (RT) in the dark. After fixation, cells were washed once and resuspended in 100 µl of permeabilization medium (reagent B, Caltag). At this step, the anti-p24-FITC (KC57; Coulter, Miami, FL) mAb was added. Cells were stained for 20 minutes at RT in the dark, washed, and resuspended in 500 µl of 1% buffered paraformaldehyde. Samples were stored at least half an hour or overnight at 4°C and then analyzed by flow cytometry for total p24+ HIV-1-infected cells. A FACSsort (Becton Dickinson) and the Cell Quest software (Becton Dickinson) were used and instrument calibration was setup using CaliBRITE beads and FACSCOMP software (Becton Dickinson). Dead cells and debris were excluded from the lymphocyte gate and 10,000-50,000 cells were collected and analyzed for each sample. The percent of CD4+/CD8+, CD4+/p24+, CD8+/p24+, CD4-/p24+ and total p24+ cells in the lymphocyte gate were determined from gated two-color dot plots of p24-FITC versus CD4-PE, p24-FITC versus CD8-PerCP and CD4-PE versus CD8-PerCP. Uninfected cells were stained as controls. Compensation controls (CD3-FITC alone and CD4-PE alone) were used to set the appropriate quadrant markers (less than 0.5% of the cells were p24+ in the uninfected cultures).
RESULTS

As shown in Table 1, undpleted and CD8-enriched PBMC were infected overnight with either 9667 or 4971 at the indicated TCID₉₀. The kinetics of HIV-1 infection for CD4+ and CD8+ T lymphocytes peaked at day 7 or 10 post-infection (pi). The percent p24+/CD8+ T lymphocytes when analyzed by flow cytometry was found to be from 1% to 2% in CD8-enriched PBMC and in undpleted PBMC (Table 1). Infected CD8+ T lymphocytes were found at both day 7 and 10 pi in undpleted PBMC whereas in CD8-enriched PBMC, infected CD8+ T lymphocytes were found only at day 10 pi. Results from the p24 antigen quantitation showed that p24 antigen levels were proportional to the magnitude of the TCID₉₀ used to infect cells, and that the levels of p24 antigen in the supernatants of infected undpleted PBMC were significantly higher than that of infected CD8-enriched PBMC.

The flow cytometry data at day 10 pi showed that the % double positive CD4+/CD8+ T lymphocytes (upper right quadrant) of CD8 PerCP and CD4 PE panels was 1% (Fig. 1C, F, I) to 2% and 4% (Fig. 2C, F, I) in undpleted PBMC but 0% in CD8-enriched PBMC (Fig. 3B, D, F). When a back gate was created (gate R2), the double positive CD4+/CD8+ T lymphocytes (black dots) were found both in the upper right quadrant (infected lymphocytes) and upper left quadrant (uninfected lymphocytes) of the p24-FITC and CD4-PE (Fig. 1A, D, G and Fig. 2A, D, G) as well as p24-FITC and CD8-PerCP (Fig. 1B, E, H, Fig. 2B, E, H and Fig. 3A, C, E) panels. In the upper right quadrant of these two panels, we found both single positive T lymphocytes (gray dots of CD4+ and CD8+ T lymphocytes) as well as double positive CD4+/CD8+ T lymphocytes (black dots) infected with 4971 and 9667 virus. The existence of these double positive T lymphocytes required the presence of both CD4+ and CD8+ T lymphocytes in the PBMC. Cell populations lacking either CD4+ (Fig. 3) or CD8+ (data not shown) T lymphocytes contained very few or no double positive CD4+/CD8+ T lymphocytes (Fig. 3A, B, and Fig. 3D, respectively). Furthermore, we found p24+/CD4- T lymphocytes in the lower right quadrant of the p24-FITC and CD4-PE panels (Fig. 1D, G and Fig. 2D, G). The majority of p24+/CD8-/CD8- T lymphocytes were HIV-1 infected CD4 down-modulated PBMC.

DISCUSSION

In this report, we have described the susceptibility of CD8+ T

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Table 1 Virus kinetics and HIV-1 infection of CD8+ lymphocytes in Thai donor cells using intracellular p24 (flow cytometry) and extracellular (supernatant) p24 antigen capture (ELISA). Infection was determined by measuring HIV-1 p24 antigen in culture supernatants and in the cells on days 0, 4, 7 and 10 following inoculation of healthy donor cells with primary HIV-1 isolates. Samples were pooled from 2 to 4 culture wells and then were processed, as previously described in Materials and Methods.
Fig. 1 HIV-1 infection of CD4+ and CD8+ T lymphocytes as well as percent CD4+/CD8+ T lymphocytes in a Thai donor at day 10 after overnight infection with 4971 virus (NSI/B) and 9667 virus (NSI/E) at 100 TCID$_{50}$. The percent of cells in each quadrant is indicated and 10,000 cells were collected.
Fig. 2  HIV-1 infection of CD4+ and CD8+ T lymphocytes as well as percent CD4+/CD8+ T lymphocytes in a Thai donor at day 10 after overnight infection with 9667 virus (NS1/E) at 100 and 500 TCID₅₀. The percent of cells in each quadrant is indicated and 50,000 cells were collected.
Fig. 3  HIV-1 9667 virus infection of CD8-enriched cells in a Thai donor at day 10 pi. Cells were infected overnight at 100 and 500 TCID$_{50}$. The percent of cells in each quadrant is indicated and 50,000 cells were collected. Using 500 TCID$_{50}$, 1% of the total cells are p24+/CD8+, as indicated.
lymphocytes to HIV-1 infection in vitro. Our results showed that both CD8+/CD4- and CD4+CD8- T lymphocytes were infected by HIV-1, and that HIV-1 down-modulated CD4 receptors on the lymphocyte cell membranes. In contrast to our findings, De Maria showed that in vitro HIV-1 infection of CD3+/CD8+/CD4- T lymphocytes required the presence of infected CD4+ T lymphocytes and that infected CD8+ T lymphocytes were able to spread the infection to CD4+ T lymphocytes in turn. However, several studies have shown that CD8+/CD4- T lymphocytes may serve as in vivo reservoirs for the AIDS virus. Tsutoba et al. showed that CD4+/CD4- T lymphocyte lines generated from PBMC of HIV-1 infected persons were able to harbor replicating HIV-1. CD8+ T lymphocytes in the lung of AIDS patients who had AIDS-related chronic infection of the lower respiratory tract, which was characterized by a massive influx of HIV-1-specific CTL, also had and expressed HIV-1. HIV-1 proviral DNA has also been detected in CD4+ T lymphocytes, CD8+ T lymphocytes, dendritic cells and monocytes from the PBMC of AIDS patients. In AIDS patients with CD4 counts less than 200 cells/μl, a large percent of infected cells in PBMC were either dendritic cells or CD8+ T lymphocytes. Additionally, strong inverse correlation between the total CD8 count and the frequency of CD8+ T lymphocyte infection was found.

CD4 molecules and several chemokine receptors play important roles in the entry of HIV. Apart from CD4, monocytes and peripheral blood dendritic cells also express CD4 molecules, as well as CCR5, which is expressed on both CD4+ T lymphocytes and monocytes/macrophages. While CXCR4 is predominantly expressed on lymphocytes, resting and activated primary CD8+ T lymphocytes also express CXCR4 and CCR5. Stimulation of CD8+ T lymphocytes lead to de novo CD4 expression at the cell membrane, which resulted in susceptibility of these CD8+ T lymphocytes to HIV infection. In addition, Kitchen et al. also found that infection of immature CD4+/CD8+ T lymphocytes, which subsequently differentiate into mature CD8+ T lymphocytes, resulted in productively infected CD4- T lymphocytes. From the above four research articles, no molecular data showing the presence of HIV-1 proviral DNA in CD8+ T lymphocytes was reported, and it was assumed that these infected CD8+ T lymphocytes were generated from infection of immature, dual-positive CD4+/CD8+ T lymphocytes that serve as precursors for both single positive CD4+/CD8- and CD4-/CD8+ T lymphocytes. In contrast to these published results, we and Saha et al. found that HIV-1 can infect single positive CD8+/CD4- T lymphocytes directly without the presence of the CD4 molecule on the cell membrane.

Many reports have shown that HIV-1 can infect cells through a non-CD4-dependent mechanism of virus attachment and entry. A small number of HIV-1 isolates can infect cells using other coreceptors in addition to CD4 or even infect directly via CCR5 or CXCR4 without any requirement for CD4. Saha et al. characterized two new primary isolates of HIV-1 from immortalized, HIV-1 infected CD8+/CD4- T lymphocytes of an AIDS patient. These two viruses were able to replicate in CD4+ T lymphocytes and highly purified CD8+ T lymphocytes that contained no trace of CD4 mRNA. These viruses used CD4 receptors and CXCR4 coreceptors to infect CD4+ cells, while they used CD8 receptors to infect cells without the CXCR4 and CCR5 coreceptors. It was suggested that these viruses may use an unidentified coreceptor during the infection step. Although the Thai primary isolates 4971 (B) and 9667 (E) that we used were uncloned and were not passaged through neoplastic cell lines, our two viruses were able to replicate in CD4+ T lymphocytes and highly purified CD8+ T lymphocytes. We found that single positive CD4+/CD8- and CD8+/CD4- lymphocytes, as well as double positive CD4+/CD8+ lymphocytes, became infected with these two viruses.

Our virus culture method may select for variants that can replicate in certain cell types such as CD8+ T lymphocytes in which a different cellular receptor is used. The decline of CD4+ T lymphocytes in late stage AIDS patients may partly reflect the development of viral variants able to attack cells, such as CTL, which are not targeted at earlier stages. One of the factors contributing to the high frequency of CD8+ T lymphocytes occurring only in late-stage disease is the loss of CD4+ T lymphocytes from circulation. A substantial decrease in HIV-1 infection of CD8+ T lymphocytes upon disease progression may be related to a phenomenon by which HIV spreads to non-lymphoid tissues during later stages of disease, with concomitant loss of immune control. The decline in CD8+ T lymphocytes may be a direct consequence of the cytopathic
effect of HIV on this subset; HIV may therefore have a broader cellular tropism than previously described. These findings have important implications for understanding of the pathogenesis of HIV and for the design of therapeutic agents.

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REFERENCES

29. Kitchen SG, Luttigebuag CH, Zack JA. Mechanism of human immunodeficiency virus type 1 localization in CD4-negative thymocytes: differentiation from


