JOURNAL OF VIROLOGY, Sept. 1997, p. 6671–6676 Vol. 71, No. 9
0022-538X/97/$04.00+0

Human Immunodeficiency Virus Type 1 Infection of Mature $CD3^{hi}CD8^+$ Thymocytes

SHIRLEY LEE,[1]* HARRIS GOLDSTEIN,[2] MICHAEL BASELER,[3] JOE ADELSBERGER,[3] AND HANA GOLDING[1]

Laboratory of Retrovirus Research, Division of Viral Products, CBER, Food and Drug Administration, Bethesda, Maryland 20892[1]; Albert Einstein College of Medicine, Bronx, New York 10461[2]; and SAIC, Frederick, Maryland 21702[3]

Received 25 September 1996/Accepted 30 May 1997

Although $CD4^+$ cells are the primary targets of human immunodeficiency virus type 1 (HIV-1) infection, earlier reports have suggested that intrathymic infection of $CD8^+$ cells may occur. However, it was unclear whether HIV-1-infected $CD8^+$ thymocytes were truly mature single-positive (SP) cells. In the present study, SCID mice implanted with human fetal thymus and liver tissues (SCID-hu mice) were infected with three primary isolates of HIV-1 and infected thymocytes were analyzed to assess maturational status. After intra-implant or intraperitoneal injection with HIV-1, thymocytes were sorted by three-color flow cytometric analysis into mature populations of $CD3^{hi}CD4^+$ and $CD3^{hi}CD8^+$ SP cells of >99% purity (<0.3% CD4-containing cells in the $CD8^+$ population). The presence of HIV-1 provirus in the sorted thymocyte populations was determined by quantitative PCR. A fraction of mature $CD3^{hi}CD8^+$ thymocytes contained HIV-1 proviral DNA, and evidence of viral mRNA transcription in these cells was demonstrated by in situ hybridization. In contrast, when uninfected $CD3^{hi}CD8^+$ thymocytes were cocultured with HIV-1-infected $CD4^+$ thymocytes, no evidence of productive HIV-1 infection was detected. Thus, HIV-1 infection of $CD8^+$ thymocytes in the SCID-hu mouse does not occur by direct contact with the virus. Rather, cell surface CD4 is required; therefore, precursor cells are the likely primary target of HIV-1 infection in the thymus. During ontogeny, some of these infected cells continue their differentiation into mature $CD8^+$ SP thymocytes that contain proviral DNA and express viral RNA.

The function of $CD8^+$ T cells in controlling human immunodeficiency virus type 1 (HIV-1) infection through either cytotoxic activity or blocking viral replication in $CD4^+$ cells by the secretion of inhibitory factors is an important component of the host defense against HIV-1 (3, 18). A decrease in plasma viremia within weeks of HIV-1 infection correlates with the appearance in the peripheral blood of anti-HIV major histocompatibility complex class 1-restricted $CD8^+$ cytotoxic T cells (CTL) (7). The increased number of HIV-1-specific $CD8^+$ T cells initially observed in the peripheral blood samples of HIV-1-infected individuals decreases later in the disease course (13). This decline in HIV-1-specific CTL responses during clinical latency coincides with increasing viral load and may be a factor in the eventual collapse of the immune system and development of AIDS (10, 16). A role for HIV-1-specific $CD8^+$ T cells is indicated by the observation that long-term survivors of HIV-1 infection maintain normal $CD8^+$ T-cell numbers and persistent HIV-1-specific CTL responses but that individuals with progressive disease do not (16, 31). $CD8^+$ cells are also important in protection against infections by other pathogens, e.g., cytomegalovirus (20). Since HIV-1 does not infect mature $CD8^+$ T cells, a mechanism for $CD8^+$ T-cell depletion may be related to the decline in the naive $CD8^+$ T-cell population observed in the peripheral blood samples of HIV-1-infected children and adults (21, 22). Therefore, factors which influence $CD8^+$ T-cell number and function may be crucial in understanding regulation of the immune response in HIV-1 infection (14).

The SCID-hu mouse model has previously been used to characterize thymic abnormalities after HIV-1 infection (2, 8, 9, 27). In this system, HIV-1 proviral DNA has been detected not only in CD4-expressing thymocyte subsets (precursor $CD3^-CD4^{lo}CD8^-$, $CD3^-CD4^+CD8^+$ double positive [DP], and $CD4^+$ single positive [SP]) but also in the $CD8^+$ SP subset (1, 27, 28). Since peripheral blood $CD8^+$ thymocytes are not permissive for HIV-1 infection (19, 25), we questioned whether infected $CD8^+$ thymocytes are truly mature SP cells or rather DP thymocytes that express low levels of CD4, i.e., $CD4^{lo}CD8^+$, which have downmodulated their CD4 expression subsequent to viral infection. We therefore utilized the SCID-hu mouse model and flow cytometric sorting to determine whether a mature subset of $CD8^+$ SP thymocytes defined as $CD3^{hi}CD8^+$ were infected after intrathymic and intraperitoneal (i.p.) injections with primary isolates of HIV-1.

In this report, we provide direct evidence that the most mature $CD8^+$ thymocytes contain a population infected with HIV-1 wherein viral mRNA transcription occurs. Because $CD3^{hi}CD8^+$ thymocytes from an uninfected SCID-hu thymus were not infectable by coculture with HIV-1-infected $CD4^+$ thymocytes, this study suggests that infection of $CD8^+$ thymocytes occurs during earlier stages of intrathymic T-cell development.

MATERIALS AND METHODS

Infection of SCID-hu mice. SCID-hu mice were made as previously described by both a traditional approach (27) and a modified method (11, 12). By the traditional approach, 1-mm^3 pieces from a second-trimester human fetal thymus and liver were implanted under a single renal capsule of C.B-17 *scid*/*scid* mice. These SCID-hu mice were used within 7 months of implantation of human tissues for infection by the highly cytopathic SM primary isolate. Mice were anesthetized, and a flank incision was made to expose the thymic implant. HIV-1 was injected directly into the thymus at 400 to 4,000 50% tissue culture infective doses in a volume of ≤0.1 ml, and the flank incision was closed. Mice were sacrificed 2 to 3 weeks after intrathymic infection by lethal anesthetic inhalation, and the thymic implant was removed.

Modified SCID-hu mice were constructed by implanting second-trimester fetal

* Corresponding author. Phone: (301) 827-0739. Fax: (301) 496-1810.

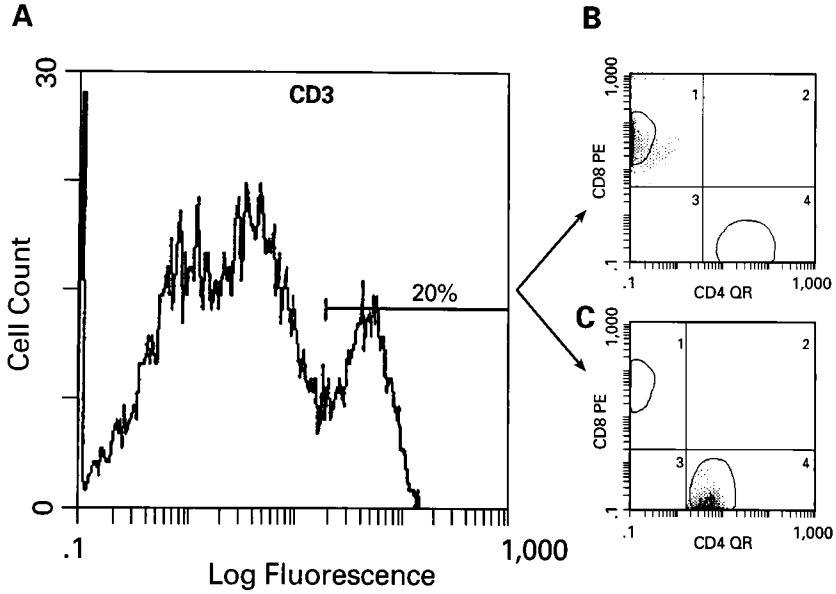

FIG. 1. Sorting of mature $CD8^+$ and $CD4^+$ SCID-hu thymocytes. SCID-hu thymocytes were stained with FITC–anti-CD3, QR–anti-CD4, and PE–anti-CD8 antibodies. Only cells that stained brightest for CD3 expression (A) were sorted into $CD8^+CD4^-$ cells (B) and $CD4^+CD8^-$ thymocytes (C).

tissues under both kidney capsules and increasing the quantities of human thymic and liver tissues implanted as previously described (11). These modifications result in larger grafts, increased graft survival, seeding of human lymphocytes in the peripheral lymphoid organs, and dissemination of HIV-1 infection after intrathymic or i.p. injection (11). The primary patient isolates of HIV-1 used in experiments with these mice, HIV-1_{28} and HIV-1_{59}, were isolated after coculture of peripheral blood mononuclear cells from a 15- and 17-month-old HIV-1-infected child, respectively, with phytohemagglutinin (PHA)-activated donor peripheral blood mononuclear cells as previously described (12). SCID-hu mice were infected by i.p. injection of 8,000 50% tissue culture infective doses in a volume of 0.8 ml. These SCID-hu mice were sacrificed by lethal ether inhalation 3 to 6 months after infection. Thymic implants and spleens were removed, and single-cell suspensions were prepared by mechanical dissociation.

Thymocytes used as negative controls for each experiment were from SCID-hu mice implanted with thymic and liver tissues from the same fetal donor but not injected with HIV-1.

Fluorescence-activated cell sorting (FACS) and analysis. Thymocytes were stained with anti-CD3–fluorescein isothiocyanate (FITC) (Becton Dickinson, Mountain View, Calif.), anti-CD4–quantum red (QR) (Sigma, St. Louis, Mo.), and anti-CD8–phycoerythrin (PE) (Becton Dickinson) according to each manufacturer's recommendations. Only thymocytes that expressed the highest densities of $CD3^+$ (approximately 20% [see Fig. 1]) were further sorted into $CD8^+$ and $CD4^+$ SP populations. All sorting was conducted in a negative-pressure room with an Epics Elite flow cytometer (Coulter Corp., Hialeah, Fla.) exclusively used for sorting HIV^+ cells and equipped with a biohazard containment chamber and a 15-nm argon laser with a 488-nm excitation wavelength. The purity of each of the sorted populations, $CD3^{hi}CD4^+CD8^-$ and $CD3^{hi}CD8^+CD4^-$ thymocytes, used in our study was >99%. Sorted cells were washed and either stored as pellets at −70°C until HIV-1 DNA PCR analysis or placed immediately in coculture.

Spleen cells were stained with FITC–anti-HLA class I monoclonal antibody (MAb) (Pharmingen, San Diego, Calif.), anti-CD8–PE, and anti-CD4–QR. For background controls, cells were stained with nonreactive isotype-matched MAbs. Spleen cells containing the total human cell population (HLA+) and sorted $HLA^+CD8^+CD4^-$ cells were used in HIV-1 DNA PCR analysis.

Detection of HIV-1 DNA by quantitative PCR. DNA PCR analysis to measure HIV-1 proviral DNA was performed by the method of Graziosi et al. (6). Briefly, cell pellets were lysed in 0.5 ml of buffer containing 0.001% Triton X-100, 0.0001% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Reaction mixtures (100 μl) contained 50 μl of DNA lysate, 50 pmol (each) of a primer pair (SK38 and SK39) specific for the *gag* gene, 200 μM (each) the four deoxynucleotide triphosphates, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 50 mM KCl, and 2 U of *Taq* polymerase. HIV-1 DNA was amplified for 25 cycles of denaturation (94°C), annealing (55°C), and extension (72°C). Amplified products were detected by liquid hybridization to [^{32}P]ATP-end-labeled SK19 probe, subjected to electrophoresis on 10% polyacrylamide gels, and exposed to film (Eastman Kodak Co., Rochester, N.Y.) for various lengths of time. For quantitation of the viral burden, serial 10-fold dilutions of 10^5 cells from HIV-1-infected animals were mixed with a constant number of uninfected Jurkat cells (10^6) prior to cell lysis and DNA extraction. Alternatively, serial dilutions of DNA from 10^5 cells were added to DNA from 10^6 Jurkat cells and amplified as described above. Similar results were obtained by both methods. The signals on autoradiographs were visually compared with those from simultaneously amplified DNAs from serially diluted ACH-2 T cells containing one provirus per cell. Video images of the films were obtained with a GDS 5000 system (UVP Inc., San Gabriel, Calif.), and densitometry was performed with SW5000 software (UVP Inc., Cambridge, England) as previously described (32). The signals obtained with thymocyte subpopulations were compared to standard curves generated with serially diluted ACH-2 cells.

Cell cocultures. Sorted HIV-1-infected $CD3^{hi}CD4^+CD8^-$ SP thymocytes (100% purity) were cocultured with uninfected $CD3^{hi}CD8^+CD4^-$ thymocytes (99.8% purity) at a 1:1 ratio (~2×10^6 [each]). Thymocytes were activated with 1 μg of PHA (Sigma Chemical Co.) per ml in AIM-V medium (Life Technologies, Inc., Grand Island, N.Y.) supplemented with 10% AB human serum, 2 mM L-glutamine, antibiotics, and interleukin-2 (IL-2) (20 U/ml) in a 1-ml final volume. On day 4 of coculture, cells were sorted again into >99% pure $CD3^{hi}$ $CD4^+CD8^-$ and $CD3^{hi}CD8^+CD4^-$ subsets. Sorted cells were pelleted and analyzed for the presence of HIV-1 DNA by PCR.

Detection of HIV-1 mRNA by in situ hybridization. In situ hybridization was performed by Molecular Histology, Inc. (Gaithersburg, Md.) as previously described (5). Sorted $CD3^{hi}CD4^+$ and $CD3^{hi}CD8^+$ SP thymocytes (10^6 cells) from infected and uninfected SCID-hu mice were suspended in 1 ml of plasma. Topical thrombin (150 μl; Gentrac, Inc., St. Louis, Mo.) was added at room temperature, resulting in plasma clot formation, and fixative was added to clots. Cells that contained clots were embedded in paraffin, and sections were cut and placed on silanized slides. Slides were deparaffinized, acetylated, and hybridized with an antisense HIV-1 probe (or control sense probe) representing the full-length HIV-1 genome. After hybridization, slides were exhaustively washed, digested with RNase, and coated with Kodak NTB3 emulsion. After a 4-day exposure, slides were developed and stained with hematoxylin.

RESULTS

HIV-1 proviral DNA is found in mature $CD8^+$ thymocytes. Only thymocytes that have undergone positive selection and differentiated into mature SP T cells express high levels of T-cell receptor (TCR)–CD3 on their surfaces (4, 15, 24). Therefore, to determine whether mature $CD8^+$ thymocytes could be infected after intrathymic or i.p. injection of SCID-hu mice with HIV-1, we analyzed the most mature population of $CD8^+$ SP thymocytes, those that stained the brightest with anti-CD3 MAb (Fig. 1). To verify the purities of $CD3^{hi}CD8^+$ and $CD3^{hi}CD4^+$ thymocyte populations, portions of sorted cells were routinely subjected to a second FACS analysis. Only

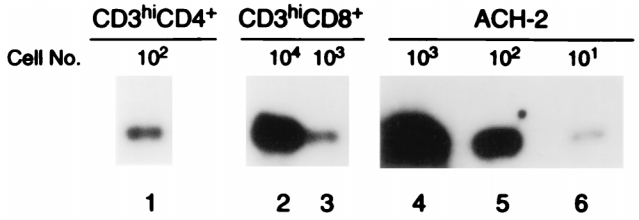

FIG. 2. Detection of HIV-1 DNA in mature SP thymocytes. Thymocytes were harvested from SCID-hu mice ~2 weeks after intrathymic injection with the HIV-1 SM isolate, sorted into CD3^{hi}CD4^{+} and CD3^{hi}CD8^{+} SP subsets, and analyzed after serial dilutions by DNA PCR for the presence of HIV-1 DNA with *gag* primers, as described in Materials and Methods.

those populations found to be >99% pure (Fig. 1B) were used in experiments. The CD3^{hi}CD8^{+} thymocytes used in these experiments contained ≤0.3% CD4^{+} cells. The CD3^{hi}CD8^{+} thymocyte population also expressed high levels of CD69, another marker characteristic of mature SP thymocytes (data not shown).

Intrathymic injection of SCID-hu mice with the primary HIV-1 isolate SM results in rapid depletion of the DP CD4^{+}CD8^{+} population in the thymic implant by 6 weeks after infection (2). Thus, mice were sacrificed within 2 to 3 weeks of infection. In all the mice examined, reduced thymic cellularity (40 to 50% of uninfected controls) was observed, with the main reduction observed in the DP population. At these time points, proviral DNA was detected in both CD3^{hi}CD4^{+} and CD3^{hi}CD8^{+} SP mature subsets, as shown by DNA PCR analysis (Fig. 2). By using ACH-2 titration as a reference, 51% of CD4^{+} SP thymocytes from thymuses infected with this highly cytopathic strain contained proviral DNA. In comparison, approximately 1% of CD3^{hi}CD8^{+} SP thymocytes from the same thymus contained HIV-1 proviral DNA. Since each HIV-infected cell may contain more than one copy of proviral DNA, these approximations represent upper limits of the percentages of infected cells.

Since the HIV-1 SM isolate establishes an acute infection which is highly cytopathic in SCID-hu mice, causing complete thymocyte depletion and thymic involution within 2 months, it was important to study SCID-hu mice infected with less cytopathic strains that do not lead to rapid depletion of the DP cell pool. Infection with the primary isolates HIV-1_{28} and HIV-1_{59} (and others) in a modified SCID-hu mouse model was shown to result in minimal effects on thymopoiesis up to 6 months after infection (12). This model may be more representative of most in vivo infections and allowed us to determine the presence of proviral DNA in SP populations at two time points, 3 and 6 months. As shown in Fig. 3, HIV-1 infection of both CD3^{hi}CD4^{+} (37%) and CD3^{hi}CD8^{+} (1.0%) thymocytes was detected by DNA PCR 3 months after i.p. injection of SCID-hu mice with patient isolate HIV-1_{28}.

In another set of experiments, we examined CD3^{hi}CD8^{+} thymocytes after i.p. injection with patient isolate HIV-1_{59}. Interestingly, CD3^{hi}CD8^{+} SP thymocytes contained more proviral DNA (10% of cells infected) than did CD3^{hi}CD4^{+} SP thymocytes (0.5% of cells infected) in two separate experiments. The results at 6 months postinfection with HIV-1_{59} are shown in Fig. 4. Similar results were obtained at 3 months postinfection. At that time, infected thymuses contained 81.9% ± 1.7% DP CD4^{+}CD8^{+} cells, 9.9% ± 1.2% SP CD4^{+} cells, and 7.0% ± 0.9% SP CD8^{+} cells. Thus, infection with this isolate did not induce significant depletion of the DP and CD4^{+} SP populations. The presence of a higher percentage of SP CD8^{+} cells that contain proviral DNA may represent a stochastic event, representing the normal maturation of HIV-1-infected DP cells into SP CD8^{+} and CD4^{+} cells. It is not clear whether direct infection of CD4 SP thymocytes occurred in these mice. In light of the relatively high percentage of CD8^{+} SP cells that contained proviral DNA, it was of interest to determine whether such infected cells seeded the periphery. Due to the number of cells required for sorting, the spleen was chosen as the peripheral lymphoid tissue to investigate. Spleen cells were sorted after three-color staining with anti-HLA class I, anti-CD8, and anti-CD4 into total human cells (HLA class I^{+}) and CD8^{+}CD4^{-} human cells and were analyzed by PCR for the presence of HIV-1 proviral DNA. As shown in Fig. 4, although the total human spleen cell population contained proviral DNA, a pure subpopulation of CD8^{+} cells did not. Since the mice were infected via the i.p. route, it is equally possible that infection of the human cells in the SCID-hu spleen occurred extrathymically and was restricted to peripheral CD4^{+} cells. Since mature SP CD8^{+} cells are not susceptible to HIV-1 infection (see below), any peripheral CD8^{+} cells that contained proviral DNA were likely to be recent emigrants from the thymus. At this point, we cannot exclude the possibility that low numbers of provirus-containing CD8^{+} cells seeded other compartments of the peripheral lymphatics not tested in our assay.

Mature SP CD8^{+} thymocytes cannot be infected by coculture with HIV-1-infected CD4^{+} thymocytes. It may have been that mature CD8^{+} SP thymocytes became infected with HIV-1 at an earlier stage of development when CD4 was expressed or were infected by cell-free or cell-associated virus. To determine whether mature CD8^{+} thymocytes can be infected by cell contact with HIV-1-infected CD4^{+} cells, we set up cocultures

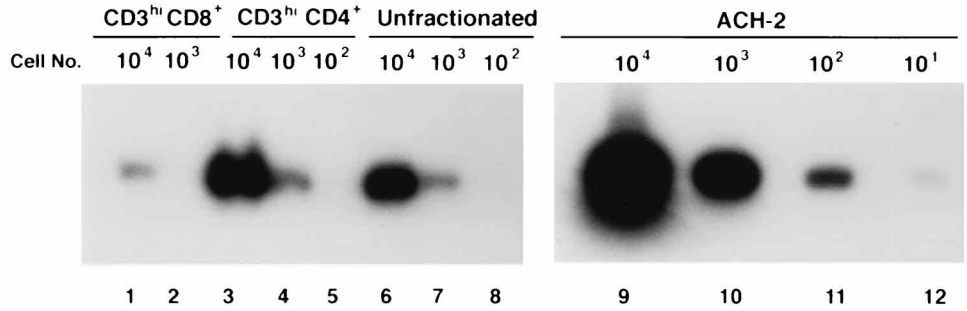

FIG. 3. Detection of HIV-1 DNA in SP thymocytes 3 months after infection of SCID-hu mice. Thymocytes were harvested from modified SCID-hu mice 3 months after i.p. injection with HIV-1_{28}. The CD3^{hi} population was sorted into CD3^{hi}CD4^{+} and CD3^{hi}CD8^{+} SP thymocytes and analyzed by DNA PCR for the presence of HIV-1 DNA as described in the legend to Fig. 2.

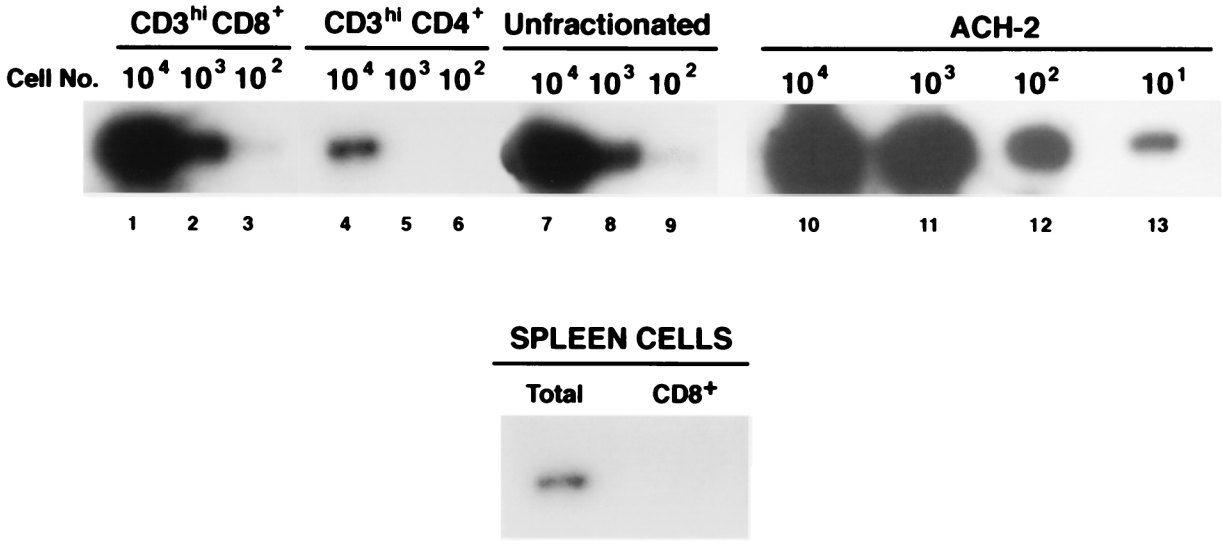

FIG. 4. Detection of HIV-1 DNA in $CD3^{hi}CD8^+$ SP thymocytes but not $CD8^+$ splenocytes from chronically infected SCID-hu mice. Six months after i.p. injection of HIV-1_{59}, thymocytes and spleen cells were harvested from infected SCID-hu mice. (Top) Unfractionated thymocytes and sorted $CD3^{hi}CD8^+$ and $CD3^{hi}CD4^+$ SP thymocytes were assayed. (Bottom) Human cells were isolated from infected SCID-hu spleens by sorting HLA class I-positive cells (total) or further sorting $CD8^+$ human T cells (5×10^3; >99% pure).

of $CD3^{hi}CD4^+$ thymocytes from a SCID-hu mouse infected with HIV-1_{28} with $CD3^{hi}CD8^+$ uninfected thymocytes (Fig. 5A) and $CD8^+$ thymocytes that were not further selected based on their CD3 surface expression (Fig. 5B). Both populations of SP $CD8^+$ cells were ≥99% pure by FACS analysis. Cultures were activated with PHA and IL-2. On day 4 of coculture, thymocytes were sorted again into $CD4^+$ and $CD8^+$ subsets and were analyzed by DNA PCR with HIV-1 *gag*-specific primers. Proviral DNA was detected only in $CD4^+$ thymocytes, not in mature $CD3^{hi}CD8^+$ thymocytes (Fig. 5A). The results shown are those for two coculture experiments that yielded the same results. In contrast, similar coculture experiments with the total population of $CD8^+$ thymocytes (i.e., not $CD3^{hi}$) demonstrated that these cells were infected by contact with HIV-1-infected $CD4^+$ thymocytes within 3 days (Fig. 5B). These data strongly suggest that at least a fraction of $CD8^+$ cells were in fact $CD3^{med}CD4^{lo}CD8^+$ cells (transitional cells), expressing low levels of CD4 that were not detected during FACS sorting and subsequent FACS analysis. The results of this experiment further demonstrate that similar to peripheral $CD8^+$ T cells, truly mature $CD8^+$ thymocytes cannot be infected directly by HIV-1. Therefore, the more likely explanation for the finding of proviral-DNA-containing $CD3^{hi}CD8^+$ thymocytes is that they became infected as immature DP thymocytes and continued their maturation into SP $CD8^+$ cells.

Both $CD3^{hi}CD4^+$ and $CD3^{hi}CD8^+$ thymocytes from HIV-1-infected SCID-hu mice express HIV-1 mRNA. The detection of HIV-1 DNA provirus in $CD3^{hi}CD8^+$ thymocytes may indicate latent or defective infection. Therefore, after determination that mature $CD3^{hi}CD8^+$ SP thymocytes contained integrated copies of HIV-1 proviral DNA, we investigated whether they also transcribe HIV-1 mRNA. An analysis of thymocytes by in situ hybridization demonstrated that both $CD3^{hi}CD4^+$ and $CD3^{hi}CD8^+$ thymocytes contained HIV-1 mRNA (Fig. 6). Thus, it is likely that HIV-1 infection of thymocytes which have completed differentiation into the mature $CD8^+$ SP subset is a productive infection.

DISCUSSION

The goal of the present study was to investigate whether intrathymic infection with HIV-1 results in the infection of mature $CD8^+$ thymocytes destined to exit the thymus. Our findings suggest that a small fraction of SP $CD3^{hi}CD8^+$ thymocytes (0.5 to 10%) are infected, but they are most likely eliminated before they exit the thymus. Infection of $CD8^+$ SP thymocytes may be another mechanism that contributes to the pathogenesis of HIV-1 infection, even in cases where no overt thymic destruction is observed. Infection of $CD8^+$ thymocytes may explain the decline in the numbers of peripheral blood naive $CD8^+$ cells in HIV-1-infected individuals (21, 22) and the gradual loss of protection against opportunistic infections.

We focused on $CD3^{hi}$ thymic populations in order to discriminate between the mature $CD8^+$ SP population and cells at earlier stages of intrathymic development that still express low levels of CD4. By using three HIV-1 primary isolates in this study, we found that $CD3^{hi}CD8^+$ thymocytes do contain HIV-1 proviral DNA, albeit at lower frequencies compared to those of $CD4^+$ SP cells for two of the three primary isolates studied. HIV-1 mRNA was also detected by in situ hybridization in both SP populations, suggesting productive infections of both. Our in vivo infection system is different from that of Uittenbogaart et al. (29), who found subsets of $CD4^-CD8^+$ TCR^{hi} and $CD4^-CD8^-TCR^{hi}$ thymocytes that contained proviral DNA in late stages (2 weeks) of in vitro HIV-1 infection. Since their in vitro system most likely did not allow for normal differentiation of DP thymocytes into SP mature subsets, the most likely explanation for their results was that these infected subsets represented $CD4^+$ populations that downmodulated their surface CD4 expression after viral infection. In our in vivo studies with less cytopathic viruses, it was previously shown that thymic cellularity and intrathymic differentiation were not significantly altered. Thus, most of the $CD3^{hi}CD8^+$ thymocytes sorted were probably truly mature SP cells. However, we cannot exclude the possibility that some DP thymocytes became infected at a transitional stage, namely, after they became

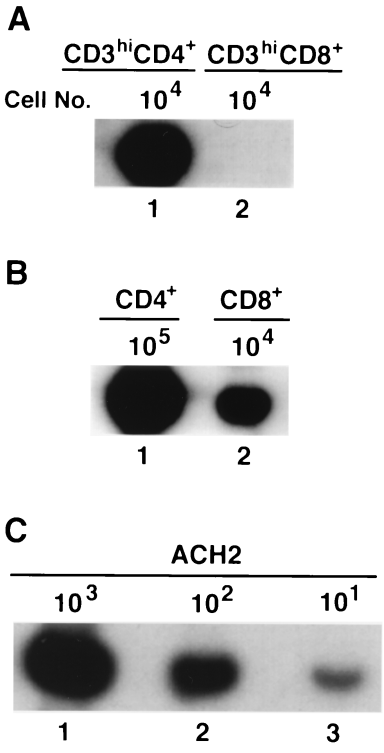

FIG. 5. Mature CD3^{hi}CD8^{+} SP thymocytes are not infected by cell contact with HIV-1-infected CD4^{+} thymocytes. (A) Sorted uninfected CD3^{hi}CD8^{+} SP thymocytes were stimulated with PHA (2 μg/ml) and cocultured in the presence of IL-2 (20 U/ml) with HIV-1-infected CD3^{hi}CD4^{+} SP thymocytes. On day 4 of coculture, cells were resorted and analyzed by DNA PCR with primers and ^{32}P-labeled probe specific for the HIV-1 *gag* gene. (B) Similar experiments were performed with CD8^{+} thymocytes from an uninfected SCID-hu mouse. (C) ACH-2 cells.

committed to the CD8 lineage and upregulated their CD3 levels but before they lost their surface CD4 expression. After infection, such cells could have lost their surface CD4 expression as part of their maturation process or due to virus-induced downmodulation.

Since we could not infect sorted CD3^{hi}CD8^{+} thymocytes in vitro either by contact with free virus in culture (unpublished data) or by cell-to-cell transmission in coculture with HIV-1-infected CD4^{+} thymocytes (Fig. 5), HIV-1 infection most likely occurs prior to the final maturation of CD8^{+} SP thymocytes. A reservoir of HIV-1-infected CD4^{+}CD8^{+} DP thymocytes which have escaped cell death could transmit the HIV-1 genome to progeny CD4^{+} and CD8^{+} SP cells during intrathymic maturation.

Surprisingly, one viral isolate, HIV-1$_{59}$, was associated with a greater viral load in CD8^{+} thymocytes compared to that in CD4^{+} thymocytes isolated from the same thymus. Since no preferential loss of CD4^{+} SP thymocytes was seen in the animals examined, it may represent one end of the spectrum of intrathymic maturation of infected DP thymocytes into SP subsets.

Finally, when we examined both thymocytes and spleen cells after infection with HIV-1$_{59}$, the observation of HIV-1-infected CD8^{+} cells was restricted to the thymus. At 6 months after infection, we were unable to detect infected CD8^{+} cells in the spleen, suggesting that infected CD8^{+} thymocytes did not emigrate to peripheral lymphoid organs. Due to the number of cells required for FACS sorting, we were unable to isolate CD8^{+} human lymphocytes from peripheral blood or lymphatic tissues other than the spleen. Thus, we cannot exclude the possibility that HIV-1-infected CD8^{+} thymocytes did exit the thymus but were undetected by our assay or were eliminated extrathymically. Our data are in agreement with those of Sleasman et al. (26), who studied peripheral blood cell subsets from 10 HIV-1-infected neonates and detected no CD8^{+} T cells that contained proviral DNA. Intrathymic depletion of CD8^{+} thymocytes may result from cell death by a direct or indirect cytopathic effect of HIV-1 (28). Thymic organs from some HIV-1-infected individuals have shown parenchymal damage, involution, and destruction (17, 23). However, these changes may occur late in HIV-1 disease. The naive CD8^{+} subset begins to decline early in HIV-1-infected individuals and continues to decrease throughout the course of disease (21, 22). Our finding of HIV-1 infection of CD8^{+} thymocytes with possible intrathymic destruction may explain the gradual decline in the number of peripheral blood naive CD8^{+} cells observed in HIV-1-infected humans. This may be especially true in congenitally infected infants that display rapid onset of immunodeficiency (30).

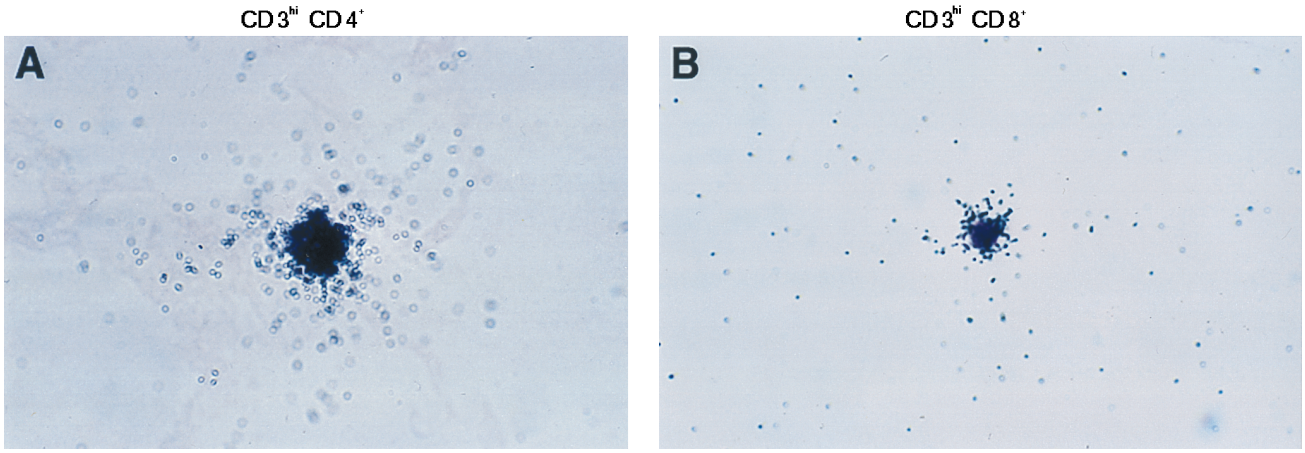

FIG. 6. Both CD3^{hi}CD4^{+}CD8^{-} (A) and CD3^{hi}CD8^{+}CD4^{-} (B) thymocytes express HIV-1 mRNA. SP thymocyte subsets from HIV-1$_{28}$-infected and uninfected SCID-hu mice were analyzed for HIV-1 mRNA expression by in situ hybridization as described in Materials and Methods. No positive signal was found in thymocytes derived from uninfected thymuses.

A reduction in the number of naive $CD8^+$ cells that seed the periphery is likely to deplete the peripheral immune organs of naive $CD8^+$ cells capable of responding to new infectious agents, which may lead to opportunistic infections. A decline in the naive $CD8^+$ T cells that seed the periphery, along with programmed cell death and destruction of activated peripheral $CD8^+$ cells, may contribute to the eventual reduction in the total $CD8^+$ T-cell population documented in late-stage disease.

ACKNOWLEDGMENTS

We thank Anthony Fauci and Sharilyn Stanley for providing SCID-hu mice and the HIV-1 SM isolate. Our thanks go to Basil Golding and Stuart Shapiro for critical review of the manuscript.

REFERENCES

1. **Aldrovandi, G. M., G. Feuer, L. Gao, B. Jamieson, M. Kristeva, I. S. Y. Chen, and J. A. Zack.** 1993. The SCID-hu mouse as a model for HIV-1 infection. Nature (London) **363:**732–736.
2. **Bonyhadi, M. L., L. Rabin, S. Salimi, D. A. Brown, J. Kosek, J. M. McCune, and H. Kaneshima.** 1993. HIV induces thymocyte depletion in vivo. Nature (London) **363:**728–732.
3. **Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso.** 1995. Identification of RANTES, MIP-1α, and MIP-1 as the major HIV-suppressive factors produced by CD8+ T cells. Science **270:**1811–1815.
4. **Ernst, B., C. D. Surh, and J. Sprent.** 1995. Thymic selection and cell division. J. Exp. Med. **182:**961–972.
5. **Fox, C. H., and M. Cottler-Fox.** 1993. In situ hybridization for detection of HIV in cells and tissues. Curr. Protocols Immunol. Suppl. **6:**12.8.1.
6. **Graziosi, C., G. Pantaleo, and A. S. Fauci.** 1992. Detection of HIV DNA and RNA by PCR. Curr. Protocols Immunol. Suppl. **5:**12.6.5.
7. **Haynes, B. F., G. Pantaleo, and A. S. Fauci.** 1996. Toward an understanding of the correlates of protective immunity to HIV infection. Science **271:**324–328.
8. **Jamieson, B. D., S. Pang, G. M. Aldrovandi, J. Zha, and J. A. Zack.** 1995. In vivo pathogenic properties of two clonal human immunodeficiency virus type 1 isolates. J. Virol. **69:**6259–6264.
9. **Kaneshima, H., L. Su, M. L. Bonyhadi, R. I. Connor, D. D. Ho, and J. M. McCune.** 1994. Rapid-high syncytium-inducing isolates of human immunodeficiency virus type 1 induce cytopathicity in the human thymus of the SCID-hu mouse. J. Virol. **68:**8188–8192.
10. **Klein, M. R., C. A. Van Baalen, A. M. Holwerda, S. R. Kerhof Garde, R. J. Bende, I. P. M. Keet, J. M. Eeftinck-Schattenkerk, A. D. M. E. Osterhaus, H. Schuitemaker, and F. Miedema.** 1995. Kinetics of gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. J. Exp. Med. **181:**1365–1372.
11. **Kollmann, T. R., M. Pettoello-Mantovani, X. Zhuang, A. Kim, M. Hachamovitch, P. Smarnworawong, A. Rubinstein, and H. Goldstein.** 1994. Disseminated human immunodeficiency virus 1 infection in SCID-hu mice after peripheral inoculation with HIV-1. J. Exp. Med. **179:**513–522.
12. **Kollmann, T. R., A. Kim, M. Pettoello-Mantovani, M. Hachamovitch, A. Rubinstein, M. M. Goldstein, and H. Goldstein.** 1995. Divergent effects of chronic HIV-1 infection on human thymocyte maturation in SCID-hu mice. J. Immunol. **154:**907–921.
13. **Levy, J. A.** 1994. HIV and the pathogenesis of AIDS, p. 169–171. ASM Press, Washington, D.C.
14. **Lewis, D. E., D. S. Ng Tang, A. Adu-Oppong, W. Schober, and J. R. Rodgers.** 1994. Anergy and apoptosis in CD8+ T cells from HIV-infected persons. J. Immunol. **153:**412–420.
15. **Lucas, B., F. Vasseur, and C. Penit.** 1994. Production, selection, and maturation of thymocytes with high surface density of TCR. J. Immunol. **153:**53–62.
16. **Miedema, F., and M. R. Klein.** 1996. AIDS pathogenesis: a finite immune response to blame. Science **272:**505–506.
17. **Papiernik, M., Y. Brossard, N. Mulliez, J. Roume, C. Brechot, F. Barin, A. Goudeau, J. F. Bach, C. Griscelli, R. Henrion, and R. Vazeux.** 1992. Thymic abnormalities in fetuses aborted from human immunodeficiency virus type 1 seropositive women. Pediatrics **89:**297–301.
18. **Paxton, W. A., S. R. Martin, D. Tse, T. R. O'Brien, J. Skurnick, N. L. VanDevanter, N. Padian, J. F. Braun, D. P. Kotler, S. M. Wolinsky, and R. A. Koup.** 1996. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposures. Nat. Med. **2:**412–417.
19. **Psallidopoulos, M. C., S. M. Schnittman, L. M. Thompson III, M. Baseler, A. S. Fauci, H. C. Lane, and N. P. Salzman.** 1989. Integrated proviral human immunodeficiency virus type 1 is present in $CD4^+$ peripheral lymphocytes in healthy seropositive individuals. J. Virol. **63:**4626–4631.
20. **Quinnan, G. V., N. Kirmani, A. H. Rook, J. F. Manishewitz, L. Jackson, G. Moreschi, G. W. Santos, R. Saral, and W. Burns.** 1982. Cytotoxic T cells in cytomegalovirus infection. N. Engl. J. Med. **307:**6–13.
21. **Rabin, R. L., M. Roederer, Y. Maldonado, A. Petru, L. A. Herzenberg, and L. A. Herzenberg.** 1995. Altered representation of naive and memory CD8 T cell subsets in HIV-infected children. J. Clin. Invest. **95:**2054–2060.
22. **Roederer, M., J. G. Dubs, M. T. Anderson, P. A. Raju, L. A. Herzenberg, and L. A. Herzenberg.** 1995. CD8 naive T cell counts decrease progressively in HIV-infected adults. J. Clin. Invest. **95:**2061–2066.
23. **Rosenzweig, M., D. P. Clark, and G. N. Gaulton.** 1993. Selective thymocyte depletion in neonatal HIV-1 thymic infection. AIDS **7:**1601–1605.
24. **Salomon, D. R., C. F. Mojcik, A. C. Chang, S. Wadsworth, D. H. Adams, J. E. Coligan, and E. M. Shevach.** 1994. Constitutive activation of integrin α4 1 defines a unique stage of human thymocyte development. J. Exp. Med. **179:**1573–1584.
25. **Schnittman, S. M., M. C. Psallidopoulos, H. C. Lane, L. Thompson, M. Baseler, F. Massari, C. H. Fox, N. P. Salzman, and A. S. Fauci.** 1989. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. Science **245:**305–308.
26. **Sleasman, J. W., L. F. Alexio, A. Morton, S. Skoda-Smith, and M. M. Goodnow.** 1996. CD4+ memory T cells are the predominant population of HIV-1-infected lymphocytes in neonates and children. AIDS **10:**1477–1484.
27. **Stanley, S. K., J. M. McCune, H. Kaneshima, J. S. Justement, M. Sullivan, E. Boone, M. Baseler, J. Adelsberger, M. Bonyhadi, J. Orenstein, C. H. Fox, and A. S. Fauci.** 1993. Human immunodeficiency virus infection of the human thymus and disruption of the thymic microenvironment in the SCID-hu mouse. J. Exp. Med. **178:**1151–1163.
28. **Su, L., H. Kaneshima, M. Bonyhadi, S. Salimi, D. Kraft, L. Rabin, and J. M. McCune.** 1995. HIV-1 induced thymocyte depletion is associated with indirect cytopathicity and infection of progenitor cells in vivo. Immunity **2:**25–36.
29. **Uittenbogaart, C. H., D. J. Anisman, B. D. Jamieson, S. Kitchen, I. Schmid, J. A. Zack, and E. F. Hays.** 1996. Differential tropism of HIV-1 isolates for distinct thymocyte subsets in vitro. AIDS **10:**F9–F16.
30. **Wilfert, C. M., C. Wilson, K. Luzuriaga, and L. Epstein.** 1994. Pathogenesis of pediatric human immunodeficiency virus type 1 infection. J. Infect. Dis. **170:**286–292.
31. **Wolinsky, S. M., B. T. M. Korber, A. U. Neumann, M. Daniels, K. J. Kunstman, A. J. Whetsell, M. R. Furtado, Y. Cao, D. D. Ho, J. T. Safrit, and R. A. Koup.** 1996. Adaptive evolution of human immunodeficiency virus-type I during the natural course of infection. Science **272:**537–542.
32. **Zaitseva, M., H. Golding, M. Betts, A. Yamauchi, E. Bloom, L. Butler, L. Stevan, and B. Golding.** 1995. Human peripheral blood $CD4^+$ and $CD8^+$ T cells express Th1-like cytokine mRNA and proteins following in vitro stimulation with heat-inactivated *Brucella abortus*. Infect. Immun. **63:**2720–2728.