Limited Evolution of Human Immunodeficiency Virus Type 1 in the Thymus of a Perinatally Infected Child

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Background. Involvement of the thymus during human immunodeficiency virus (HIV) infection may impair production of naive lymphocytes leading to more rapid depletion, but the characteristics of primary strains in the thymus are not well studied because of the unavailability of tissue in living individuals.

Methods. We studied the characteristics of HIV type 1 (HIV-1) in a 5-year old perinatally infected child with thymitis and compared the genomic sequences of the HIV-1 C2–V5 region of the env gene in the thymic tissue and peripheral blood.

Results. The thymus harbored predominantly viral sequences close to the founder HIV-1 variant that circulated in the blood at 2 and 3 months of age, whereas the peripheral blood virus at 5 years of age had evolved extensively. Viral sequences from circulating CD8+ T cells at 5 years of age phylogenetically clustered with those from the thymic tissue.

Conclusions. These results indicate the existence of a distinct thymic viral reservoir and suggest that circulating CD8+ T cells were infected in the thymus, presumably at the CD4+CD8+ thymocyte stage. They also demonstrate that not all thymic HIV infections will necessarily lead to severe thymic dysfunction. The characteristics of the virus strain seeding the thymus may dictate the rate of disease progression.

The thymus is where hematopoietic stem cells differentiate into CD4+ or CD8+ “naive” T cells, which then circulate in the blood and populate the lymphoid organs [1]. Infection of the thymus with human immunodeficiency virus type 1 (HIV-1) can disrupt the maturation process of thymocytes into lymphocytes and significantly compromise the immune system of the infected individual [2]. Histologic examination of postmortem thymic tissue of patients with AIDS, including fetuses and newborns, has indicated a number of pathologic changes [3–6]. Infection experiments of human thymic explants in the SCID-hu mouse model or of in vitro thymic cultures have demonstrated that some, but not all, laboratory strains of HIV can infect thymocytes or thymic epithelial cells and cause a variety of histopathologic effects [7, 8]. Several in vitro studies have further shown that different strains of HIV have dissimilar effects on the thymus, with certain syncytium-inducing, CXCR4-binding viral strains more likely to cause thymic disruption [7–10]. We have previously reported that ~15% of infants perinatally infected with HIV exhibited flow cytometric profiles indicative of thymic dysfunction, with depletion of both CD4+ and CD8+ T cells, as well as CD5+ B cells [11, 12]. It remains unclear whether infected infants without this profile did not have thymic involvement or had thymic infection with non–thymus-pathogenic strains. Despite the potential impact on disease progression in children, most available data on thymic HIV infection have been limited to laboratory models with HIV strains originally isolated from peripheral blood. There are very limited data on primary strains of HIV recovered directly from thymic tissue, because of the rare availability of fresh clinical specimens. In an analysis of
3 autopsy specimens of adults with AIDS by direct population sequencing of polymerase chain reaction (PCR) amplicons [13], it was found that thymus and lymph nodes harbored closely related envelope quasispecies; in another study of autopsies from 4 children, significant differences were found between blood and thymic variants [14].

In this study, we describe the genotypic characteristics of HIV-1 quasispecies in a child with perinatally acquired infection who had multicystic thymic enlargement at 5 years of age. Full-blood and thymic variants [14].

From 4 children, significant differences were found between related envelope quasispecies; in another study of autopsies 3 autopsy specimens of adults with AIDS by direct population

METHODS

Study subject. The patient was a 5-year-old African American girl with perinatally acquired HIV infection. At the age of 5 years, a mediastinal mass was seen on routine chest radiograph. A computerized tomographic study of the chest demonstrated an anterior mediastinal thymic multicystic mass. There were no symptoms attributed to this finding. Her clinical course had been characterized by the absence of opportunistic infections, mild developmental delay, failure to thrive that had responded to nasogastric feedings, and several bacterial infections such as pneumococcal pneumonia, shigellosis, and recurrent ear infections. She had been on zidovudine since infancy, and lamivudine was added to her regimen at 3 years of age (before the availability of highly active antiretroviral combinations). At the time of the chest radiographic finding, she had a CD4+ cell count of 400 cells/mm³, a CD8+ cell count of 1600 cells/mm³, and a virus load of 7000 copies/mL. The patient underwent a diagnostic open-wedge biopsy of the mediastinal mass in February 1998. The thymic tissue that was excised consisted of a lymphoepithelial multiloculated cyst lined by a flat epithelium and a rim of lymphoid tissue with germinal follicles. Recognizable thymic tissue was seen in the external layers.

Thymocyte preparation. After the thymic capsule, blood vessels, and connective tissue were carefully removed, the thymic tissue was dissected into individual lobules and rinsed extensively in sterile phosphate buffered saline (pH, 7.4). The thymocytes, together with interspersed thymic epithelial cells, were released into single-cell suspension through a cell strainer (70 µm; Falcon; BD Labware) in cold phosphate buffered saline. The cells were pelleted by centrifugation (250 g for 10 min).

Isolation of PBMCs. Heparinized blood was diluted 1:1 (volume) in Hanks’ buffered salt solution supplemented with 1% fetal calf serum. PBMCs were isolated by gradient centrifugation in Histopaque (Sigma Chemicals) at 400 g for 20 min at room temperature. The cells at the interface were collected, washed, and pelleted.

Separation of CD8+ T cells. PBMCs were first depleted of CD4+ cells by 2 treatment cycles of adsorption with macromagnetic beads coated with CD4-specific monoclonal antibodies (Dynal AS), according to the manufacturer’s instructions. The unattached cells were positively selected for CD8+ cells using CD8 antibody-coated micromagnetic beads (Miltenyi Biotec). Flow cytometry analysis of the isolated subset showed >99% purity.

DNA extraction. DNA from PBMCs as well as from thymic cells was extracted using the QIAamp Blood Kit (Qiagen), according to the manufacturer’s instructions.

Primers for PCR amplification. HIV-1 env gene amplification was performed by nested PCR analysis, as described in Delwart et al [15]. An aliquot of the extracted DNA was used for the first round of amplification using the primers ED5 and ED12 and the conditions described in Delwart et al [15]. Primers ES7 and ES8 were then used for the second round of amplification on an aliquot of the first reaction. The amplification reactions were carried out in a thermocycler for 40 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) and a final extension at 72°C for 10 min.

Molecular cloning. For cloning, 100 µL of each PCR reaction product was electrophoretically separated on a 1% agarose gel. The specific band of interest (size, ~700 bp) was excised from the gel, purified, and ligated into pCRII plasmid (Invitrogen). After transformation in Escherichia coli, at least 10 colonies from each sample were expanded. Plasmid DNA was screened on a 1% agarose gel after Eco RI digestion to confirm the correct size.

DNA sequencing and phylogenetic analysis. All DNA sequences of the clones were determined using the ABI Prism DNA sequencing kit (dye-terminator cycle sequencing ready reaction) on an ABI model 373 automated sequencer. M13 forward and reverse primers as well as the ES7 and ES8 primers were used for sequencing. Overlapping regions were identified, and sequences were edited by using the MacVector software program (Oxford Molecular). Sequences were aligned using the CLUSTAL function of the Megalign part of the Lasergene software package (DNASTAR). Phylogenetic relationships were constructed using neighbor-joining algorithms. Similar phylogenetic relationships were found in 1000 bootstrap replicates of neighbor-joining trees (Clustal W program).
RESULTS

Variability of HIV-1 env gene in peripheral blood and thymic tissue at 5 years of age. Eighteen HIV env clones spanning the C2–V5 region, generated from DNA of PBMCs (clones P1–P9) or thymic tissue (clones T1–T9), were sequenced (Figure 1). A high degree of sequence homology was found in the V3 region of all the clones, irrespective of the tissue origin. An A-to-T transversion in the PBMC-derived clones P3, P4, and P8 yielded an amino acid change (tyr328 to phe328) at the base of the V3 loop. At the deduced amino acid level, only 2 V3 variants were identified in PBMCs, one with the tyrosine phenotype (6 clones) and the other with the phenylalanine phenotype (3 clones). Among the thymus-derived clones, T5, T8, and T9 had a T-to-G transversion mutation at the same position. This mutation was silent. Therefore, all of the thymus clones exhibited a deduced V3 amino acid sequence with the tyrosine phenotype. The predicted tropism and phenotype of the clones were determined from the nucleotide sequences. All the thymic and PBMC genotypes isolated from our patient had V3 sequences suggestive of CCR5 usage [16], and of a non–syncytium-inducing phenotype, based on the low net charge (3.01 at pH, 7) due to acidic or neutral amino acid substitutions at positions 11 and 25 of the V3 region [17].

The V4 region sequence showed a more marked variability.

Figure 1. Alignment of the HIV-1 C2–V5 region of env-deduced amino acid sequences. Clones 2m and 3m are from peripheral blood mononuclear cells (PBMCs) collected at 2 and 3 months of age, respectively. P1–P9 are clones from amplified product from PBMCs sampled at the age of 5 years. CD8+1 and CD8+2 are clones from CD8+ T cells purified from the same aliquot of PBMCs used for P1–P9. T1–T9 are from thymus biopsy tissue collected at the same time as the PBMCs at 5 years of age.
in both composition and length of amino acid sequence between the conserved flanking regions. Higher variation was observed in thymus-derived clones, whereas PBMC-derived clones were generally more homogeneous. Additionally, unique V4 variants were observed in the thymus strains. A pattern of variable in-frame nucleotide insertion was characteristic of the thymus-derived clones. Three clones, T5, T8, and T9 (which had identical nucleotide sequences), had the longest V4 region when compared with the common V4 region length of PBMC-derived clones. The in-frame insertion of nucleotides was associated with the formation of a new potential N-glycosylation site. Six consensus potential sites for N-linked glycosylation were identified in most of the clones from both PBMCs and the thymus. An extra site was present for clones T5, T8, and T9. Clones P2, T2, T4, and T6 exhibited the loss of 1 potential N-linked glycosylation site. These high numbers of potential N-linked glycosylation sites have been recognized as a feature more common among chronically infected individuals [18]. The PBMC clone P2 showed 100% sequence identity with the thymic T4 clone and was just 1–2 nucleotides divergent (in silent mutations) from the T6 and T2 clones. This may suggest trafficking of infected cells from the thymus compartment to the peripheral blood.

**Sequence of HIV env gene in PBMCs obtained shortly after birth.** Cryopreserved PBMCs that had been obtained from the same patient at 2 months and 3 months of age, respectively, were available for analysis. The HIV sequences (designated 2m and 3m) most likely represented that of the founder species that was originally transmitted from the mother to the infant. The C2–V5 sequences were completely identical between the 2m and 3m species, as might have been expected. Their V3 region also presented 100% homology to all of the thymus-derived clones (T1–T9) at 5 years of age and differed from 6 of the 9 PBMC-derived clones by 1–2 amino acid substitutions. When the V4 region was compared with that of the founder species, the thymic and PBMC clones by 5 years of age had generally accumulated more mutations. Nevertheless, the PBMC clone P2 and the thymic clones T2 and T4 were identical to the founder sequence of 2m and 3m, whereas T6 diverged by only 2 amino acids. The in-frame insertion found in some thymic clones was absent in the founder species. The similarity between the various clones was further analyzed phylogenetically as described below.

**HIV env gene in CD8+ T cells.** HIV-1 infection of CD8+ T cells remains a controversial issue [19]. We examined the possible explanation that the occasional presence of HIV genome in peripheral blood CD8+ cells is the result of maturation of infected CD4+CD8+ thymocytes into single positive T cells. Highly purified peripheral blood CD8+ cells were analyzed for the HIV env DNA sequence. Because of the small amount of genomic CD8+ T cell DNA isolated, and because we would expect a small percentage of infected CD8+ cells, if at all, the number of PCR cycles was increased to 40 cycles both in the first and in the second PCR. An amplified product was detected and cloned in a PCR vector plasmid. Two clones (CD8+1 and CD8+2) were sequenced (Figure 1). On the basis of the C2–V5 sequences, a distinct HIV-1 variant was found in the CD8+ cells when compared with the population found in the unseparated PBMCs. Interestingly, their V4 region carried the in-frame insertion that was also found in the thymic clones.

**Phylogenetic analysis of HIV variants in different compartments.** Figure 1 shows the aligned predicted amino acid sequences of all clones analyzed. Phylogenetic relationships were constructed using the neighbor-joining algorithms and is shown in Figure 2. Similar phylogenetic relationships were found in 1000 bootstrap replicates of neighbor-joining trees. The phylogenetic tree indicated that the founder species (2m, 3m) were related more closely to the thymic species, whereas 8 of the 9 PBMC clones formed a separate and distinct cluster. The only exception is clone P2 from PBMCs, which was placed among the thymic clones and close to the founder species. The
sequences of the 2 clones from peripheral blood CD8+ cells revealed a close relationship with several of the thymic clones and were at a greater distance from all the PBMC clones.

DISCUSSION

Sequence analysis of HIV-1 variants isolated from infected individuals, including children, have revealed a highly homogeneous population within the HIV-1 env gene early following transmission [20–23]. This homogeneity extends to other tissue compartments [23]. In chronically infected individuals, on the other hand, heterogeneous and distinct viral populations emerge in different organs. Compartmentalization of HIV in the central nervous system, kidney, spleen, cutaneous tissue, and genital secretions has been described elsewhere [24–32]. It has been argued that this reflects a gradual diversification of a common dominant viral variant [33]. It is possible that different selection pressures in the different compartments, induced by factors such as immune escape, antiretroviral therapy, or specificity of target cells in different tissues, may play a role in this differential evolution.

HIV infection of the thymus among children raises several unique perspectives. Unlike any other organs involved, the thymus is central to the production of T lymphocytes, which are the prime targets, as well as major defenders, against HIV. Furthermore, antigens presented in the thymic microenvironment can potentially induce immune tolerance [34, 35], which may impact the local or systemic immunological pressure that is believed to be the main driving force for the diversification of the viral envelope gene. Moreover, there is a selective restriction of mature T cells to reenter the thymus [35, 36]. Its effect on the possible redistribution of virus quasispecies (through infected cells) between the peripheral blood and thymic compartments is unknown; there is some recent evidence of HIV-1 gene flow from thymus to lymphoid tissues but not vice versa [14].

Phylogenetic analysis of thymus-derived and PBMC sequences in our 5-year-old perinatally infected patient showed evidence of tissue-specific viral evolution. The thymus-derived sequences formed a tissue-specific cluster separate from PBMC-derived viral sequences. Five years after the transmission event, extensive viral evolution had taken place in the periphery, and the “founder” HIV species (the major variant found in the PBMCs at 2 and 3 months after infection) became a minor variant. In contrast, the virus population obtained from the thymic tissue was closer to the founder strain than the clones derived from the current PBMCs. This may indicate less immunologic pressure in this organ. The presence of the founder HIV-1 variant (clone P2, which is identical to thymic clones T2 and T4) in peripheral blood 5 years after transmission suggests that the thymus may act as a reservoir that continued to export virus to the periphery. Alternatively, the clone P2 may represent a virus in long-lived, latently infected peripheral lymphocytes (early archive). The presence of phylogenetically distinct clusters between thymic and PBMC clones suggests that peripherally infected lymphocytes do not freely move between blood and thymus.

Although CD4+ T cells are considered to be the primary target of HIV-1, a number of studies have shown in vivo infection of CD8+ T cells [37–41]. Several non–mutually exclusive mechanisms have been proposed to explain the presence of HIV proviral DNA in CD8+ T cells: (1) the CD8 molecule acting as HIV-1 receptor [41]; (2) HIV-1 transmission through cell-to-cell contact between persistently infected CD4+ and CD8+ lymphocytes [42]; (3) HIV-1 infection of peripheral blood CD8+ T cells through the CD4 molecule, for which gene expression is induced following activation through the T cell receptor complex [37, 39, 40]; and (4) HIV-1 infection of CD8+ lymphocytes during their precursor stage when they coexpress CD4 and CD8 in the thymus. Cluster analysis in our study showed that HIV-1 variants in peripheral CD8+ T cells were closely related to thymic HIV-1 variants, which favors the hypothesis that CD8+ T cells were likely infected in the thymus during T cell lymphopoiesis. This hypothesis is consistent with results obtained in previous studies with the SCID-hu mouse [7, 43]. Molecular studies of virus from the circulating CD8+ T cell subset could potentially provide indication of a thymic HIV reservoir, without requiring thymic tissue that is seldom available.

We have previously introduced the concept of “thymic dysfunction” to identify a population of perinatally HIV-1–infected infants who display a peripheral blood immunophenotypic profile resembling that of patients with severe DiGeorge congenital thymic anomaly [11, 12]. Almost all HIV-infected infants with this profile had rapid progression to AIDS/HIV-related death. The results of our current study demonstrated that not all thymic HIV infection will necessarily lead to severe thymic dysfunction, because the patient in this case presented with relatively slow disease progression. The difference may depend on the HIV variant involved. A previous report identified a lymphocytotropic, syncytium-inducing HIV-1 viral isolate in the thymus of a rapidly progressing HIV-infected adult patient [10]. In contrast, the viral sequences that we isolated from both the PBMCs and thymic tissue of this child predict monocyteotropic and non–syncytium-inducing characteristics. The patient’s relatively preserved CD4+ and CD8+ T cell counts were indeed consistent with a virus that does not induce extensive thymic destruction. It might be intriguing to speculate that the low rate of divergence of the thymic variant might be related to slow disease progression in this child [44]. Even though technical factors such as long-term storage of the specimens might have impaired our ability to detect heterogeneity of clones, our results suggest that, even for a patient without the “thymic
dysfunction” profile, a thymic viral reservoir can be established early after infection, replicating locally without causing extensive depletion of thymocytes. As we and others have shown, besides thymocytes, the thymic epithelium, macrophages, and dendritic cells in the thymic stroma can be a substrate of HIV replication [45–48]. We have previously demonstrated that the thymic virus isolated from this patient was able to infect thymic epithelia in culture [45]. The characteristics of the strain seeding the infant thymus may thus dictate the thymic pathology, which in turn impacts the rate of disease progression.

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References