Differential effects of R5 and X4 human immunodeficiency virus type 1 infection on CD4+ cell proliferation and activation

Christopher P. Locher,† Stephanie A. Witt, Rachel Kassel, Noah L. Dowell, Sue Fujimura and Jay A. Levy

INTRODUCTION

Most human immunodeficiency virus (HIV) infections contain a mixed population of viruses (sometimes referred to as quasispecies) that have diverse molecular genotypes and biological phenotypes. This diversity of HIV in an infected person contributes to virus evasion of the immune response and to the emergence of drug resistance (Nowak, 1995; Saag et al., 1996; Distler et al., 1991, 1992). In many cases of HIV infection, the virus isolated just before or during the development of AIDS has increased replicative capacity in T-cell lines, is resistant to the suppressive effects of β-chemokines and displays increased giant-cell formation (syncytium-inducing or SI) (Cheng-Mayer et al., 1988; Fenyo et al., 1988; Tersmette et al., 1988, 1989). These viruses differ from less cytopathic, non-syncytium-inducing (NSI) isolates that are usually found during the early state of infection. These latter viruses do not replicate in T-cell lines, but replicate well in macrophages and are sensitive to the effects of β-chemokines (Cheng-Mayer et al., 1990; Cocchi et al., 1995; Mackewicz et al., 1997; Schuitemaker et al., 1991; van’t Wout et al., 1994).

NSI and SI viruses differ in their use of chemokine co-receptors for entrance into host cells. NSI viruses utilize the β-chemokine cell receptor 5 (CCR5) and are called R5 isolates, whereas SI viruses utilize the CXCR4 coreceptor (Berger, 1997) and are called X4 isolates (Berkowitz et al., 1998). Mutations in variable regions 2 and 3 (V2 and V3) of the viral gp120 envelope appear to correlate with syncytium induction in T-cell lines, chemokine coreceptor usage and the R5 and X4 tropisms in macrophages and T-cell lines, respectively (Cheng-Mayer et al., 1988; Choe et al., 1996; Distler et al., 1995; Shioda et al., 1991, 1992). The mutations contained within the V3 region of the envelope impart a net (positive) charge that may facilitate a stronger interaction with the chemokine coreceptor.

Human immunodeficiency virus type 1 (HIV-1) isolates can be distinguished by their chemokine coreceptor usage. Non-syncytium-inducing (NSI), macrophage-tropic viruses utilize CCR5 and are called R5 viruses; syncytium-inducing (SI) isolates use CXCR4 and are known as X4 viruses. R5 and X4 HIV isolates are both transmitted but, in most cases, R5 viruses predominate in the blood prior to the development of AIDS-related pathogenesis. The reason for the selective growth of the R5 strain is not known, but could reflect a replication advantage of R5 viruses over X4 viruses in CD4+ cells. To explore this possibility, eight phenotypically distinct viruses were used to infect CD4+ cells and cellular proliferation and activation were evaluated. In unstimulated CD4+ cells, R5 virus isolates increased the level of cell activation compared with X4 virus isolates and uninfected control cells. In CD4+ cells that were stimulated with interleukin 2, both R5 and X4 viruses were found to decrease the level of cell proliferation and reduce the majority of the activation markers studied when compared with uninfected control CD4+ cells from the same donors. However, although equal amounts of CD4+ cells were infected, R5 virus-infected CD4+ cells showed a two- to fourfold increase in cellular proliferation over X4 viruses, as measured by [3H]thymidine incorporation (P=0.001) and nuclear expression of Ki67 (P=0.001). In addition, a larger proportion of CD4+ T cells infected with R5 viruses had significantly higher levels of activation-marker expression (e.g. CD25, CD71 and HLA-DR) than CD4+ T lymphocytes infected with X4 viruses (P<0.02). Taken together, these results indicate that CD4+ cells infected with R5 virus isolates may have a selective advantage over X4 virus-infected CD4+ T cells for survival and, hence, virus spread.

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CXCR4 (Berger, 1997; Distler et al., 1995; Korber et al., 1994).

Although both R5 and X4 HIV strains can be transmitted, R5 viruses predominate in the infected person shortly after infection and during the asymptomatic phase (Cornelissen et al., 1995; Wade et al., 1998; Zhu et al., 1993). The reason for this dominance of R5 viruses is not known. Recent studies have indicated that dendritic cells are infected productively with R5 isolates to a greater extent than with X4 isolates (Vanhon et al., 2000a, b) and may mediate HIV transmission through the mucosal lining (Meng et al., 2002). However, other studies suggested that dendritic cells may transmit HIV-1 independently of virion coreceptor usage (Hladik et al., 1999).

Previous studies in our laboratory showed that R5 viruses induce greater proliferation of CD4+ cells and CD71 expression than X4 viruses (Greco et al., 1999). The present studies were conducted to extend these findings and to further explore the effect of R5 and X4 HIV infection on proliferation and activation of CD4+ T lymphocytes. Additional phenotypic markers that are important in T-cell activation were studied, including CD25 [interleukin 2 (IL2) ß-chain receptor, a lymphocyte marker of activation and T-cell memory-cell marker], CD69 (an early indicator of lymphocyte activation), CD71 (transferrin receptor, a later indicator of lymphocyte activation and proliferation) and HLA-DR (a class II antigen and late marker of lymphocyte activation and proliferation). Together with [3H]thymidine incorporation and Ki67, which are indicators of DNA synthesis and T-cell proliferation, these activation markers were used to characterize the changes in T cells in response to HIV-1 infection in vitro.

**METHODS**

**Viruses isolates.** HIV-1SF122A and HIV-1SF162 were isolated from the central nervous system of AIDS patients and have a R5 phenotype (Cheng-Mayer & Levy, 1988; Liu et al., 1990). HIV-1SF13 and HIV-1SF33 were isolated from the peripheral blood of AIDS patients and have an X4 phenotype (Cheng-Mayer et al., 1988, 1990; Shioda et al., 1991, 1992). The K-1 and D-1 viruses were isolated from clinically healthy, HIV-1-infected subjects and have a R5 phenotype. The K-2 and D-2 viruses were isolated from the same subjects, respectively, at later time points and have an X4 phenotype. The phenotypes of the virus isolates were determined by using the MT-2 T-cell line assay (Koot et al., 1992). Bulk preparations of all viruses were made in cultured peripheral blood mononuclear cells (PBMCs) (Castro et al., 1988). Virus replication was monitored by particle-associated reverse transcriptase (RT) activity in the culture fluids (Hoffman et al., 1985). The infectivity of each HIV-1 viral stock was determined by standard TCID50 assays (McDougal et al., 1985).

**Molecular characterization of viruses.** Viral RNA was isolated from frozen aliquots of a bulk virus culture by using TRIzol/RNeasy according to the manufacturer's instructions (Qiagen). RT-PCR was used to amplify the envelope region encoding the V3 region of HIV-1 by using the primers CCTCAGCATTACAGGAAATGTG-CCAAG for the forward reaction and CCTCAGTGCGTCACTCCTAATGGTTCA for the reverse direction [a gift from Dr Eric Delwart, University of California, San Francisco (UCSF), CA, USA].

Cycling was performed according to the following protocol: 96°C for 90 s; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; 72°C for 7 min; 4°C thereafter. DNA sequencing was performed by the Biomolecular Resource Center, UCSF, and chromatograms were analysed by using ABI Prism (Applied Biosystems) and Macvector (Macworld) software programs.

**Virus cultures.** PBMCs were recovered from whole blood by gradient centrifugation using Ficoll-Hypaque (Sigma) (Castro et al., 1988). CD4+ T cells were then isolated immediately by using CD4+ immunomagnetic beads, according to the manufacturer's instructions (Dynal) (Mackewicz et al., 1991). The purified CD4+ cells were treated with 10 µg polybrene ml−1 (hexamethlbromide; Sigma) for 30 min prior to inoculation with 100 TCID50 virus per 105 CD4+ cells (an m.o.i. of 10−4). We found that purified CD4+ cells showed the most substantial differences between R5 and X4 viruses and the effect of the viruses on host-cell activation and proliferation. In contrast, results using PBMCs were difficult to interpret because the percentage changes were smaller and not significant.

Following 1 h incubation, the CD4+ cells were washed twice and resuspended at 2 × 106 cells ml−1 in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum, 100 U recombinant human IL2 ml−1 (Boehringer Mannheim), 2 mM L-glutamine, 100 U penicillin ml−1 and 100 µg streptomycin ml−1. Virus replication was monitored by using the RT assay (Hoffman et al., 1985). From many years of experience using this RT procedure, we have found that values of <5000 c.p.m. are negative (Mackewicz et al., 1995). Uninfected and unstimulated CD4+ T cells were used as negative controls; phytohaemagglutinin (3 µg ml−1) was used for positive controls of cellular activation. Cell cultures were passaged in fresh RPMI 1640 medium every 48 h and cell viability was determined by trypsin blue exclusion. Cell cultures with <70% cell viability were discarded. The control and HIV-infected cell cultures were maintained for up to 2 weeks and CD4+ cells from the same donor were used for each parallel experiment, using subject-matched or laboratory strains of R5 and X4 viruses.

**Cell proliferation.** To determine the degree of cellular proliferation, CD4+ cells from control and HIV-infected cultures were resuspended in complete medium that did not contain IL2 and plated in triplicate at 105 cells per well in 96-well plates. [3H]Thymidine [10 µCi (370 kBq); NEN Sciences] was then added to each well and the tissue-culture plates were incubated for 8 h. The plates were then harvested and the cells were obtained by using an MBI cell harvester (Skatron/Molecular Devices) and the incorporation of [3H]thymidine was measured on a β-scintillation counter (LKB 1205; Wallac/Perkin-Elmer). [3H]Thymidine incorporation (c.p.m. × 1000) was determined for each sample.

**Flow cytometry.** To characterize the differential expression of proliferation and activation markers on HIV-infected CD4+ T cells, two-colour flow cytometry was employed by using a FACSort with CellQuest software (BD Biosciences). Briefly, a monoclonal antibody (mAb) specific for intracellular Ki67 (Beckman Coulter) was used to measure cellular proliferation following permeabilization and fixation (BioErgonomics). Permea-Sure was used as a positive control to measure cellular proliferation (BioErgonomics). Cell-surface antigens were characterized by using phycocerythrin-conjugated mAbs specific for CD25, CD69, CD71 and HLA-DR (Becton Dickinson/Pharmingen). Acquisition of events was selected only on viable cells. To control for HIV-induced CD4 downmodulation, the proportion of cells expressing the cell-surface activation markers was defined as percentage positive in both CD4+ and CD4− cell populations. The percentage of positive CD4+ cells and mean fluorescence intensity were used to enumerate the mean number of molecules expressed in a given cell population.
Table 1. HIV-1 isolates, biological phenotype and amino acid sequences of viruses used in this study

Phenotype was determined by using the MT-2 cell assay as described in Methods. Sequences were determined from clones derived from RT-PCR of viral stocks used to infect primary CD4+ cells in this study.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>V3 amino acid sequence</th>
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<tbody>
<tr>
<td>North American consensus</td>
<td>CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
</tr>
<tr>
<td>Laboratory isolates</td>
<td></td>
</tr>
<tr>
<td>HIV-1SF128</td>
<td>NSI  CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
</tr>
<tr>
<td>HIV-1SF162</td>
<td>NSI  CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
</tr>
<tr>
<td>HIV-1SF13</td>
<td>SI   CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
</tr>
<tr>
<td>HIV-1SF33</td>
<td>SI   CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
</tr>
<tr>
<td>Primary isolates</td>
<td></td>
</tr>
<tr>
<td>K-1 early</td>
<td>NSI  CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
</tr>
<tr>
<td>K-2 late</td>
<td>SI   CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
</tr>
<tr>
<td>D-1 early</td>
<td>NSI  CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
</tr>
<tr>
<td>D-2 late</td>
<td>SI   CTRPNNTRKSIIHGPRAFYTTGGEIIGDIRQAH'C</td>
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**Statistical analysis.** The data from the cellular proliferation were analysed by using Student’s t-test. The results of the immunophenotypic markers were analysed by using non-parametric methods, as the distribution of the data was non-Gaussian; the significance of differences between these two groups was calculated with the Mann-Whitney U-test by using the StatView software program (Abacus Concepts).

**RESULTS**

**Effect of R5 and X4 viruses on CD4+ cell proliferation**

Two pairs of isolates that were passaged for several years in the laboratory using PBMCs (HIV-1SF128A and HIV-1SF162 as R5 viruses and HIV-1SF13 and HIV-1SF33 as X4 viruses) and two pairs of primary virus isolates from two subjects (K-1 and D-1 as R5 viruses and K-2 and D-2 as X4 viruses) were used. Each R5/X4 virus pair was cultured in bulk using PBMCs from the same donor and their biological R5/X4 phenotype was confirmed as described in Methods. The V3 envelope regions of these isolates were also sequenced to verify the genotype changes associated with the X4 biological phenotype and that each viral isolate was unique. In addition, for the X4 viruses, the amino acid changes revealed consistently increased net positive-charge mutations that were reported previously with the X4 phenotype (see Table 1).

In the first series of experiments, when CD4+ cells were not exposed to IL2, only X4 viruses could replicate at low levels, as measured by RT activity (Table 2). In these unstimulated CD4+ cells, the R5 virus isolates induced cell activation (Fig. 1). The addition of exogenous IL2 (100 U ml⁻¹) maintained active virus replication for both R5 and X4 virus isolates. IL2 also facilitated optimal virus production for both viral phenotypes between 6 and 8 days, whilst maintaining cell viability at >80%. Under these culture conditions, X4-infected CD4+ cells were eliminated more rapidly in the infected cell cultures (<70% cell viability) than the R5-infected CD4+ cells: between 8 and 10 days post-inoculation for X4 cell cultures and between 10 and 12 days post-inoculation for R5 cell cultures. For this reason, the HIV-infected cell cultures were evaluated for cell proliferation and the expression of activation markers 7 days post-virus inoculation. At that time, cell viability in the cultures is similar for R5 and X4

<table>
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<th>Not stimulated</th>
<th>IL2-stimulated</th>
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<tr>
<td></td>
<td>RT</td>
</tr>
<tr>
<td>Uninfected CD4+ cells</td>
<td>&lt;1·0</td>
</tr>
<tr>
<td>K-1 early</td>
<td>12·2 ± 3·1</td>
</tr>
<tr>
<td>K-2 late</td>
<td>251·0 ± 17·8</td>
</tr>
<tr>
<td>D-1 early</td>
<td>3·7 ± 1·2</td>
</tr>
<tr>
<td>D-2 late</td>
<td>16·7 ± 0·8</td>
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infected cultures, whereas at later times (day 10 post-inoculation), the cytopathic effect (CPE) was greater and cell viability was reduced to a substantially greater extent in X4 virus-infected cultures. In these studies, we found that R5 viruses did not decrease the ability of CD4+ cells to proliferate in IL2-stimulated cultures as much as X4 viruses, which induced a substantial decrease in the DNA-synthesis and cell-proliferation capacity of IL2-stimulated CD4+ cells (Fig. 1).

**Effect of R5 and X4 viruses on cell-proliferation markers**

We further studied the differential effects of R5 and X4 virus infection with purified CD4+ cells that were cultured with IL2 (100 U ml⁻¹) and infected with equal amounts of HIV (100 TCID₅₀ virus per 10⁶ CD4+ cells). All subsequent experiments used these identical culture conditions to measure the differential effects of R5 and X4 virus isolates on CD4+ cells. By using this strategy, we found that, after 7 days in culture, the CD4+ cells showed similar kinetics and levels of HIV-1 replication, as measured by intracellular staining of p24 (see below) and equal amounts of RT activity in the culture fluids (Fig. 2, Table 1). As CD4 is an important signalling molecule in the early activation step of this lymphocyte subpopulation, the lack of proliferation and reduced CD4 expression may be intrinsically related.

To determine whether this CD4 downregulation was a result of direct HIV infection, we determined the levels of HIV p24 expression within the CD4+ and CD4-downmodulated populations by using intracellular staining and fluorescence-associated cell-sorting analysis. After 7 days in culture, approximately 50% of the CD4+ cell population showed infection by R5 and X4 HIV isolates and 95% of the cells with CD4 downmodulation were infected (Fig. 4). These results, observed in several experiments, did not show any substantial difference in the percentage of CD4+ cells infected by R5 and X4 viruses.
Effect of R5 and X4 viruses on activation markers

We next compared the differences between R5 and X4 virus infection on expression of immune-activation markers in both the CD4⁺ and CD4⁻ populations. Specific immunophenotypic markers associated with cellular activation were studied: CD25 (IL2α-chain receptor), CD69 (an early marker of cellular activation), CD71 (transferrin receptor) and HLA-DR (a class II antigen and late marker of lymphocyte activation). Consistent with [³H]thymidine incorporation, Ki67 expression and CD4 downmodulation, we found that CD4⁺ cells infected with the X4 virus isolate were more likely than R5 virus-infected cells to have substantial downmodulation of markers of cellular activation. Compared with uninfected CD4⁺ cells, R5 virus isolates showed less downmodulation of phenotype markers on the infected CD4⁺ cells than the same CD4⁺ donor cells infected with X4 virus isolates.

To ascertain the expression of these immunophenotypic markers in multiple CD4⁺ cell cultures, the results were compiled from three independent experiments using different donor CD4⁺ cells. As HIV infection downmodulates CD4 expression, we combined the data from CD4⁺ and CD4⁻ lymphocytes to assess the total percentage change of phenotypic-marker expression. We found that the cell cultures infected with X4 viruses had significantly greater downmodulation of CD25 (P=0.016), CD71 (P=0.009) and HLA-DR (P=0.009), but not CD69, than the cell cultures infected with R5 viruses (Table 3). With the exception of CD69 and HLA-DR, R5 and X4 virus-infected CD4⁺ cell cultures downmodulated each of these markers compared with uninfected CD4⁺ cell cultures. Therefore, CD4⁺ cells infected by R5 viruses maintained higher levels of threshold cell proliferation and activation than CD4⁺ cells infected by X4 viruses.

DISCUSSION

The present studies demonstrate that HIV infection of IL2-stimulated CD4⁺ cells by both R5 and X4 viruses causes decreased cellular proliferation and activation in vitro, compared with uninfected CD4⁺ cells from the same donor. However, this decrease is more substantial for CD4⁺ cells infected with X4 viruses than with R5 viruses. This finding was demonstrated by a two-
fourfold greater CD4+ cell proliferation in R5 virus-infected cell cultures, as measured by nuclear expression of Ki67 and [3H]thymidine incorporation, compared with CD4+ cells infected with X4 viruses (Figs 3c, d and 5; Table 3). It is noteworthy that these differences were not related to increased virus replication in CD4+ cells (Table 2; Figs 2, 4) or a substantial reduction in cell viability by X4 virus isolates (Table 2). This decreased expression of activation markers and the reduction in cell proliferation were found consistently in cultures infected with primary X4 viruses isolated from the same subjects at later time points, as well as the defined HIV-1 X4 laboratory isolates. The higher level of cell proliferation and activation of CD4+ lymphocytes infected with R5 viruses compared with X4 viruses in vitro may help to explain the emergence of R5 viruses during the asymptomatic phase of HIV infection in vivo.

Several studies indicate that activation of memory CD4+ lymphocytes that co-express CCR5 may help to increase HIV replication and facilitate a predominance of HIV bearing an R5 phenotype (Annunziato et al., 2000; Greco et al., 1999; Kreisberg et al., 2001; Vicenzi et al., 1999). R5 virus preferentially infects CD62L+ CD4+ lymphocytes bearing CD25 and the memory marker CD45RO and can remain latent until the infected cell is stimulated with IL2 or tumour necrosis factor alpha (Blaak et al., 2000; Gondois-Rey et al., 2002; Poli & Fauci, 1993). In addition, the triggering of cellular activation by binding to the CCR5 coreceptor by R5 viruses may enhance kinase activity of secondary messengers that increase transcription-factor binding to the HIV-1 promoter in the long terminal-repeate region (Cicala et al., 1999; Popik & Pitha, 2000a, b). Moreover, binding of inactivated X4 HIV-1 causes increased apoptosis and upregulation of FasL/Fas, but not CD25 or CD69 (Esser et al., 2001; Lawson et al., 2004). Thus, the inherent affinity of R5 HIV-1 for resting memory CD4+ lymphocytes that co-express CCR5 may be a mechanism for enhancing R5 viral fitness and facilitating depletion of the memory subset of CD4+ lymphocytes. Our findings suggest that R5 virus isolates may persist longer in culture by enabling the infected CD4+ lymphocyte to maintain a higher threshold of activation and proliferation that promotes HIV-1 replication.

Activation of CD4+ lymphocytes by R5 viruses may also help to explain different effects of antiviral drugs on this lymphocyte subpopulation. For example, zidovudine appears to be preferentially phosphorylated into its antiviral form in activated lymphocytes (Gao et al., 1993, 1994; Shirasaka et al., 1995) and is most effective against R5 variants (Koot et al., 1993; van’t Wout et al., 1996). In contrast, didanosine is equally effective against both R5 and X4 variants and is biologically active in both resting and activated CD4+ T lymphocytes (van’t Wout et al., 1997).

In summary, we have found that infection of CD4+ T lymphocytes by R5 and X4 viruses is associated with decreased cellular proliferation and cell-surface expression of phenotypic markers of activation. Importantly, diminished cellular proliferation and activation were more substantial with CD4+ cells infected with X4 virus isolates.
Table 3. Diminished immunophenotypic cell-surface markers of activation in HIV-1-infected lymphocytes

The median percentage change in surface-marker expression was observed as an overall increase (+) or decrease (−) in HIV-infected CD4+ cell cultures. Viable CD4+ and CD4− cells were used in the flow-cytometric two-colour fluorescence analysis (as acquisition gates by forward and side scatter). All samples were analysed 7 days post-infection with patient-matched primary (early and late virus isolates, K-1, D-1, G1; and K-2, D-2, G2, respectively; n=6) or laboratory (NSI, HIV-12RA and HIV-1SF162; SI, HIV-1SF13 and HIV-1SF33; n=4) HIV isolates in vitro. Data are compiled from three independent experiments using CD4+ cells from three different HIV-seronegative donors. Statistics were performed by using the Mann–Whitney U-test. NS, Not statistically significant.

<table>
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<tr>
<th>Surface marker</th>
<th>Median expression in uninfected cells (%)</th>
<th>Median change in total cell population (%)</th>
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<tbody>
<tr>
<td></td>
<td>NSI viruses</td>
<td>SI viruses</td>
</tr>
<tr>
<td>CD25</td>
<td>53-5</td>
<td>−17-7</td>
</tr>
<tr>
<td>CD69</td>
<td>19-1</td>
<td>−16-3</td>
</tr>
<tr>
<td>CD71</td>
<td>32-2</td>
<td>−16-2</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>32-8</td>
<td>−9-5</td>
</tr>
</tbody>
</table>
than with R5 virus isolates. Therefore, long-term growth of X4 viruses in vitro is reduced. These differences in cellular proliferation and activation may be initiated by signal-transduction pathways that are activated via chemokine coreceptor binding (e.g. CCR5) by R5 virus isolates. In addition, other domains of the HIV-1 envelope may contribute to these differential effects of R5 and X4 viral subtypes. Some of these characteristics may result in the survival of R5-infected CD4+ T lymphocytes, to produce more R5 viral progeny than X4-infected CD4+ T lymphocytes. Our findings could also provide some explanation for the preferred replication of R5 viruses after virus transmission.

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