Virus and host determinants of HIV-1 infection and AIDS pathogenesis
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Chapter 4

'Adaptation to promiscuous usage of chemokine receptors is not a prerequisite for HIV-1 disease progression'

A.M. de Roda Husman, R.P. van Rij, H. Blaak, S. Broersen, H. Schuitemaker

Chapter A

Application to preliminary synthesis of enzyme inhibitors as not a prerequisite for HIV-1 protease inhibition.

Adaptation to promiscuous usage of chemokine receptors is not a prerequisite for HIV-1 disease progression

Fifty percent of human immunodeficiency virus type 1 (HIV-1)-infected individuals progress to AIDS in the presence of only non-syncytium-inducing (NSI) virus variants. These NSI variants are rapidly replicating and associated with a high viral load. Here, we studied whether disease progression in the absence of syncytium-inducing (SI) HIV-1 variants is associated with an expansion of the coreceptor repertoire of NSI HIV-1 variants. Biological HIV-1 clones isolated early and late in infection from progressors and LTS with wild-type or mutant CCR5 and CCR2b genotypes, were analyzed for their capacity to use CCR1, CCR2b, CCR3, CCR5, and CXCR4 on U87 cells co-expressing CD4. All HIV-1 clones were restricted to the use of CCR5. Lack of replication of all HIV-1 clones in peripheral blood mononuclear cells (PBMC) from a CCR5 A32 homozygous blood donor confirmed this result. These findings indicate that an expanded coreceptor repertoire of HIV-1 is not a prerequisite for a progressive clinical course of HIV-1 infection.

Recently, it has been shown that in addition to CD4, syncytium-inducing (SI) human immunodeficiency virus type 1 (HIV-1) variants mainly use the CXC chemokine receptor 4 (CXCR4). Some SI HIV-1 variants additionally use CC chemokine receptor 2b (CCR2b), CCR3 and/or CCR5. Non-syncytium-inducing (NSI) HIV-1 variants use CC chemokine receptor 5 (CCR5) for entry into their target cell. In the asymptomatic phase of infection, macrophage-tropic NSI HIV-1 variants predominate. In about fifty percent of HIV-1-infected individuals, SI T-cell-line-tropic HIV-1 variants emerge during the course of infection, preceding an accelerated CD4* T cell decline and a more rapid disease progression. In agreement with these observations, a transition from CCR5 usage to multiple coreceptor usage has been correlated with disease progression. However, the majority of individuals who do not develop SI variants also progress to AIDS, some of them even rapidly. This was even observed for individuals who were heterozygous for a 32-bp deletion in the CCR5 gene or individuals with a valine to isoleucine substitution in CCR2b, two genotypes that in general have been associated with a delayed disease progression.

NSI HIV-1 variants isolated from individuals with a progressive disease course have higher replicative capacity in vitro and are associated with a higher viral load in vivo compared to NSI HIV-1 from asymptomatic individuals. In the present study, we analyzed whether these NSI HIV-1 variants have an expanded coreceptor repertoire and/or whether such a phenomenon may also explain the rapid disease progression in some of the CCR5 A32 heterozygotes and CCR2b-64I carriers who lacked SI HIV-1 variants.

MATERIALS AND METHODS

Subjects. Sixteen participants of the Amsterdam Cohort Studies on AIDS in homosexual men were analyzed. Eight of these individuals were classified as long-term survivors (LTS: ACH 16, 68, 78, 337, 434, 441, 583, 750), since they had an asymptomatic follow-up of at least 9 years (mean follow-up 144 months after seroconversion; range, 124 to 152 months) with stable CD4* T cell counts (>400/mm³) in the absence of anti-retroviral therapy. These individuals harbored solely NSI variants. In addition, we studied four rapid progressors (AIDS diagnosis at 25 to 76 months after seroconversion: ACH 53, 172, 424, 638), three typical progressors (AIDS diagnosis at 99 to 109 months after seroconversion: ACH 19, 38, 142), and one slow progressor (AIDS diagnosis at 136 months after seroconversion, after a 10-year period of high and stable CD4* T cell counts: ACH 617). All progressors also lacked SI HIV-1 variants.

Peripheral blood mononuclear cell (PBMC) samples from at least two time points were analyzed. One sample was chosen as early as possible after seroconversion or entry in the cohort studies (mean: 21 and 19 months for LTS and progressors, respectively). The other sample was chosen as late as possible after seroconversion or entry.
in the cohort studies (mean: 111 months for LTS) or at time of AIDS diagnosis (mean: 74 months for progressors) (Fig 1).

**CCR5 and CCR2b genotyping.** Genomic DNA was isolated from cryopreserved PBMC (Qiagen blood kit; Westburg, Germany). CCR5 genotyping was performed by PCR analysis using primers flanking the 32-bp deletion in CCR5[393]. CCR2b genotyping was performed by restriction fragment length polymorphism PCR analysis as described previously[432].

**Analysis of CD4+ T cell counts.** T lymphocyte immunophenotyping for the CD4* T cells was carried out at 3-month intervals by flow cytometry. PBMC were stained with CD4 monoclonal antibody according to standard procedures for cell cytometric analysis.

**Quantification of RNA in serum.** HIV-1 RNA was quantified in serum by using a nucleic acid sequence based amplification assay (HIV-1 RNA QT; Organon Teknika, Boxtel, The Netherlands) as described previously[333,337].

**Virus isolation, SI phenotyping and determination of infectious cellular load.** Viruses were isolated under limiting dilution conditions as previously described[394,203]. Participant PBMC (0.5 to 4.0 x 10^6 cells/well in 32, 48 or 96 wells) were cocultivated with phytohemagglutinin (PHA)-stimulated healthy donor peripheral blood lymphocytes (PBL: 10^6/7 well) in 96-well microtiter plates. Every week, one-third of the culture supernatant was tested for p24 antigen by an in-house p24 antigen-capture ELISA[394]. At the same time, half of the cells was transferred to new 96-well plates, and 10^6 fresh PHA-stimulated healthy donor PBL were added to propagate the culture. The proportion of productively infected CD4* T cells was calculated with the formula for Poisson distribution, \( F = -\ln(F_D) \), where \( F_D \) is the fraction of negative cultures.

PBMC from wells tested positive were transferred to 25-ml culture flasks containing 5 x 10^6 fresh PHA-stimulated PBL in 5 ml of medium to grow virus stocks. From these cultures, the cell-free supernatant was stored at -70°C until use. To the remaining PBMC, MT2 cells (10^6) were added to analyze syncytium-inducing capacity of the virus clones[395]. The titer of the virus stocks was quantified by determination of the 50% tissue culture infection doses (TCID50) in PHA-stimulated PBL derived from a healthy blood donor with the common CCR5 genotype.

**Cell lines.** Human astroglialoma U87 cell lines stably expressing CD4 and either co-expressing CCR1, CCR2b, CCR3, CCR5 or CXCR4 were grown in Iscove's medium supplemented with 10% FCS, 5 μg/ml polybrene, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml puromycin. The U87-CD4 control cell line was grown in the same medium without puromycin.

**CCR3, CCR5 and CXCR4 expression on the corresponding U87 cell lines was monitored by flow cytometry. Staining for CCR3 (7B11; obtained from Leukocyte, Inc., via the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH[104,437]) and CCR5 (D27; Pharmingen, La Jolla, CA) was performed using unlabeled monoclonal antibodies, followed by FITC-conjugated goat anti-mouse IgG (CLB, Amsterdam, the Netherlands), blocking with normal mouse sera and staining with PE-conjugated anti-CD4 monoclonal antibody (CLB, Amsterdam, the Netherlands). For staining of the U87-CXCR4 cell line, PE-conjugated anti-CXCR4 monoclonal antibody (2D5; Pharmingen, La Jolla, CA) and TF-conjugated anti-CD4 (Caltag, Burlingame, CA) were used. Markers were set using isotype matched control antibodies.

CCR1 and CCR2 expression was monitored by RT-PCR. RNA was extracted from U87 cells using TRIzol reagent (Gibco-BRL, Gaithersburg, MD) and reverse transcription (RT) was performed using random primers with SuperScript Reverse Transcriptase (Gibco-BRL) according to the recommendations of the manufacturer. Before the RT reaction, RNA preparations were subjected to DNase treatment in the presence of 5 mM MgCl₂ and subsequent inactivation of the DNase for 10 min at 65°C in the presence of 5mM EDTA. PCR was performed on a DNA thermal cycler 480 (Perkin Elmer, Foster City, CA) with Taq polymerase (Promega, Madison, WI) using primers CCR1 F (AACTCCTGTCGCAAGAAGGTGAACG), CCR1 AS (TCCACTTCTGTA666CTTCTGTA66), CCR2F (TCTGTCACATCTGGTCTCAGG) and CCR2 AS (CCCTATGCTGTTTCTCTTCTTCTGTTTCTCG) in the presence of 3 mM 1 MgCl₂. Conditions of PCR were 5 min of denaturation at 94°C, 25 cycles of 1 min at 94°C, 1.5 min at 60°C, 1.5 min at 72°C; and 10 min elongation at 72°C. Chromosomal DNA isolated from the MT2 cell line was used as a positive control for PCR reactions. Primers for hypoxanthine phosphoribosyl transferase (HPRT)[438] were used as a positive control for RNA preparations. PCR was performed on RNA, without a prior RT reaction, to check for contamination of the RNA preparations with chromosomal DNA. PCR products were analyzed on an ethidium bromide stained 0.8 % agarose gel.

**Analysis of HIV coreceptor usage.** From each individual, 2 to 9 biological virus clones per time point were analyzed for their coreceptor use, with on average 5 virus clones from early and 6 clones from late time points. Selection of virus clones was such that clones with different time points of first detection during clonal isolation were included. To be able to study a sufficient number of clones from progressor ACH 172, who had a low cellular infectious load also at later time points, biological HIV-1 clones obtained from two late time points were used for analysis. In addition, HIV-1 biological clones from multiple time points from progressors ACH 617 and ACH 638 were analyzed since this allowed a more detailed analysis of the association between viral load and coreceptor
Fig. 1. Longitudinal analysis of CD4+ T cell counts and virus load of LTS (a) and progressors (b). Patient numbers are indicated in the upper left corner of each graph. CD4+ T cell numbers are indicated by open triangles (left y-axis), infectious cellular load by filled circles and viral RNA load by open circles (right y-axis). Filled arrowheads on the x-axis indicate time points of clonal isolation of NSI HIV-1 variants that were analyzed for coreceptor use. Open arrowheads indicate the time point of AIDS diagnosis.

RESULTS

LTS in general have a low viral load consisting of only NSI HIV-1 variants. However, 50% of AIDS patients also carry only NSI HIV-1 variants but these generally have a high viral load. Here we studied whether differences in the evolution of coreceptor usage between NSI variants from LTS and progressors could account for the differences in changes in the in vivo viral load and in the clinical course of HIV-1 infection. Biological virus clones were isolated from PBMC obtained early and late in infection from two LTS (ACH 441 and 583) who continuously had a low viral load, and from one LTS (ACH 68), two rapid progressors (ACH 53, 424), and one typical progressor (ACH 142) with increasing viral load (Fig. 1). At the late time point of virus isolation, the progressors had a high viral load. All individuals had the common

use.

Two primary SI HIV-1 clones, ACH39.28.H5 and ACH208.13.B1, with a known ability to use CCR3 and CXCR4 or CCR5 and CXCR4, respectively, and the HIV-1 variant IIIB, a kind gift of Dr. R. Gallo, were used as controls. For all 3 control viruses, an inoculum of 10^5 TCID50/ml was shown to be sufficient to establish infection in the U87 cell lines expressing the appropriate coreceptors.

To determine coreceptor use of biological virus clones, 10^4 cells of each U87 cell line were seeded in 96-well plates and after 24 hours these were inoculated with 0.2 ml of the different HIV-1 clones. The range of the inoculum size of the different virus clones obtained early or late in infection from the different individuals is given in Tables 2 and 3. The U87 cells were incubated with virus for another 24 hours, washed with phosphate-buffered saline (PBS) and 0.2 ml of fresh medium was added. At day 7, cells were detached by trypsinization and transferred to 24-well plates. To analyze the use of coreceptors other than CCR5 on primary cells, inoculation of PHA-stimulated PBMC derived from a healthy blood donor who had a CCR5 Δ32 homozygous mutant genotype was performed with the biological HIV-1 clones under study.

Supernatants harvested at days 7, 14 and 21 were analyzed for the presence of virus in an in-house p24 antigen-capture ELISA.

LTS in general have a low viral load consisting of only NSI HIV-1 variants. However, 50% of AIDS patients also carry only NSI HIV-1 variants but these generally have a high viral load. Here we studied whether differences in the evolution of coreceptor usage between NSI variants from LTS and progressors could account for the differences in changes in the in vivo viral load and in the clinical course of HIV-1 infection. Biological virus clones were isolated from PBMC obtained early and late in infection from two LTS (ACH 441 and 583) who continuously had a low viral load, and from one LTS (ACH 68), two rapid progressors (ACH 53, 424), and one typical progressor (ACH 142) with increasing viral load (Fig. 1). At the late time point of virus isolation, the progressors had a high viral load. All individuals had the common
CCR5 and CCR2b genotype. Coreceptor use was studied by inoculation of the U87 cell lines expressing CD4 alone or co-expressing CD4 and CCR1, CCR2b, CCR3, CCR5 or CXCR4. Expression of the coreceptors was monitored by FACS analyses for CCR3, CCR5 and CXCR4 (Fig. 2a) and by RT-PCR for CCR1 and CCR2 (Fig. 2b). Moreover, functioning of CCR3, CCR5 and CXCR4 was demonstrated by the use of control viruses with a known coreceptor usage (Table 1).

All NSI HIV-1 clones were able to productively infect the CD4 and CCR5 co-expressing U87 cell line (Table 2). However, irrespective the moment of isolation or the clinical course of the patient from whom the clones were isolated, none of the NSI HIV-1 clones was able to replicate in the U87 cell lines expressing CD4 and one of the other coreceptors.

It could be envisaged that besides the currently known receptors expressed on the U87 cell lines, other chemokine receptors expressed on PBMC could function as additional coreceptors for these NSI HIV-1 clones. To study this possibility and to exclude an in vitro effect exerted by the use of U87 cell lines, PHA-stimulated PBL obtained from a healthy blood donor with a CCR5 A32 homozygous genotype, were infected with the biological HIV-1 clones under study. In parallel, PHA-stimulated PBL derived from a CCR5 wild-type donor were inoculated. FACS analysis revealed the expression of CD4 and CXCR4 on cells derived from both donors, but CCR5 expression was only detected on cells of donor with the common CCR5 genotype (data not shown). Also, the presence of mRNA for CCR1, CCR2b, CCR3, CCR4, CCR5, and CXCR4 was confirmed for cells of both donors by RT-PCR (data not shown).

In agreement with our observations with the U87 cell lines, none of the NSI virus clones was able to establish infection in the homozygous CCR5 Δ32 cells. This observation excluded the capacity to use coreceptors that are expressed on CD4+ T cells other than the ones tested on the U87 cell lines. The three control viruses with the SI phenotype did establish a productive infection in the PBL derived from the CCR5 Δ32 homozygous blood donor as well as in PBL derived from the blood donor with the common CCR5 genotype (Table 1).

CCR5 Δ32 and CCR2b-64I have both been associated with a delayed HIV-1 disease progression. However, among individuals with a rapid clinical course, CCR5 Δ32 heterozygotes and CCR2b-64I carriers have been identified. It has been argued that disease progression in these individuals is mediated by SI HIV-1 variants that can by-pass CCR5 genetic defects. In our cohort however, some CCR5 Δ32 heterozygotes and individuals with the CCR2b-64I mutation showed a rapid disease progression in the presence of only NSI HIV-1 variants. Therefore, we analyzed the coreceptor use of NSI variants isolated early and late in the course of infection from three LTS with a CCR5 Δ32 heterozygous genotype (ACH 16, 78, 434), one LTS with a CCR2b-64I heterozygous genotype (ACH 750), and one LTS with a CCR5 Δ32/CCR2b-64I heterozygous genotype (ACH 337). This was compared with the coreceptor repertoire of NSI

<p>| Table 1. Coreceptor use of T-cell-line-adapted virus IIIIB and 2 primary SI HIV-1 isolates |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Isolate</th>
<th>CCR1</th>
<th>CCR2</th>
<th>CCR3</th>
<th>CCR5</th>
<th>CXCR4</th>
<th>control</th>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>39.28.H5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>208.13.B1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 2. Expression of HIV-1 coreceptors on U87 cell lines. (a) Flow cytometric analyses of the expression of CD4 and CCR3, CCR5, and CXCR4, respectively, on the indicated cell lines; (b) Transcripts of CCR1 and CCR2 in the indicated cell lines, analyzed by RT-PCR. Chromosomal DNA from the MT2 cell line was used as a positive control for PCR reactions. HPRT primers were used as a positive control for RNA preparations. PCR was performed on RNA, without a prior RT reaction (-RT), to check for contamination of the RNA preparations with chromosomal DNA.
HIV-1 variants isolated from two rapid progressors (ACH 172, 638), a typical progressor (ACH 38), and a slow progressor (ACH 617) with a CCR5 Δ32 heterozygous genotype, and from a typical progressor (ACH 19) who was heterozygous for CCR2b 641. The late time point of virus isolation coincided with an increased viral load in the progressors but also to some extent in LTS (Table 3). All NSI HIV-1 biological clones, irrespective the time point of isolation in the clinical course of infection, were restricted to the use of CCR5. None of these NSI HIV-1 were able to replicate in cells expressing CD4 either or not in combination with CCR1, CCR2b, CCR3 or CXCR4 (Table 3). In agreement with these observations, none of the virus clones was able to productively infect the PBMC from the CCR5 Δ32 heterozygous blood donor (Table 3).

**DISCUSSION**

Recently, several groups have described a correlation between changes in viral coreceptor use and disease progression in HIV-1-infected individuals.[23,92,103,131,432] In these studies, HIV-1 evolution of coreceptor use was found to be associated with conversion of the NSI to the SI HIV-1 phenotype. However, approximately 50% of HIV-1-infected individuals progress to AIDS in the absence of SI HIV-1 variants.[202,206] The NSI variants isolated from all these individuals generally show increased replication kinetics as compared with NSI HIV-1 isolated from LTS, giving rise to an increased cellular infectious load.[28,438] Rapid disease progression in the absence of SI HIV-1 variants can even be observed for HIV-1-infected individuals with a CCR5 Δ32 heterozygous genotype or a CCR2b 641 genotype.[356,432] These observations prompted us to study whether in those individuals that do not develop SI HIV-1 variants, development of NSI HIV-1 that are able to use multiple coreceptors is associated with disease progression.

Restricted CCR5 use of NSI HIV-1 clones that were isolated early and late in infection from LTS was observed which appeared to be in good agreement with the absence of disease progression in these individuals. However, biological NSI HIV-1 clones obtained early and late in infection from individuals with a progressive disease course could also only use CCR5. This excluded evolution of coreceptor use as an important determinant of disease progression of individuals harboring solely NSI HIV-1 variants. Also in individuals with a CCR5 Δ32 heterozygous genotype, in whom CCR5 functioning is considered to be suboptimal,[405] HIV-1 evolution was not driven towards alternative coreceptor use. It is feasible that NSI HIV-1 variants associated with a progressive disease course may use other, yet unidentified, coreceptors. Indeed, CCR8, GPR15, and STRL33, which are expressed on CD4+ T cells, have been described as a fusion cofactor for HIV-1.[89,443] However, none of the
Table 3. Coreceptor use of NSI HIV-1 clones isolated from LTS and progressors with the CCR5 Δ32 and/or CCR2 64I heterozygous genotype.

<table>
<thead>
<tr>
<th>Patient</th>
<th>CCR5 genotype</th>
<th>CCR2 genotype</th>
<th>Diagnosis by end of follow-up (months after seroconversion)</th>
<th>Time of coreceptor analysis (months after seroconversion)</th>
<th>Infectious cellular load (TCID/10^6 CD4+ T cells)</th>
<th>RNA load in serum (log copies/ml)</th>
<th>Number of clones analyzed (range of log virus titers)</th>
<th>Number of HIV-1 clones able to infect U87-CD4 cells co-expressing CCR2b, CCR3, CCR5, CXCR4, PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Δ32/+</td>
<td>A32/ as (143)</td>
<td>22 3.7</td>
<td>141 3.7</td>
<td>4 (3.2-4.1)</td>
<td>0</td>
<td>0 0 4 0 0 0</td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>Δ32/+</td>
<td>A32/ as (124)</td>
<td>14 3.8</td>
<td>114 3.8</td>
<td>4 (2.7-3.9)</td>
<td>0</td>
<td>0 0 4 0 0 0</td>
<td></td>
</tr>
<tr>
<td>434</td>
<td>Δ32/ +/</td>
<td>A32/ as (140)</td>
<td>115 3.0</td>
<td>13 3.0</td>
<td>5 (3.0-4.6)</td>
<td>0</td>
<td>0 0 5 0 0 0</td>
<td></td>
</tr>
<tr>
<td>337</td>
<td>Δ32/ +/</td>
<td>A32/ A32/ as (142)</td>
<td>122 4.2</td>
<td>143 4.2</td>
<td>5 (2.8-3.9)</td>
<td>0</td>
<td>0 0 5 0 0 0</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>Δ32/ +/</td>
<td>A32/ as (148)</td>
<td>112 4.3</td>
<td>16 4.3</td>
<td>5 (3.5-4.4)</td>
<td>0</td>
<td>0 0 5 0 0 0</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Δ32/ +/</td>
<td>Kaposi's sarcoma, NHL (101)</td>
<td>102 4.5</td>
<td>44 4.5</td>
<td>5 (3.9-4.4)</td>
<td>0</td>
<td>0 0 4 0 0 0</td>
<td></td>
</tr>
<tr>
<td>172</td>
<td>Δ32/ +/</td>
<td>Kaposi's sarcoma, NHL (25)</td>
<td>102 4.0</td>
<td>125 4.0</td>
<td>5 (3.9-4.4)</td>
<td>0</td>
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<td></td>
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<tr>
<td>617</td>
<td>Δ32/ +/</td>
<td>NHL (136)</td>
<td>112 4.5</td>
<td>16 4.5</td>
<td>5 (3.8-4.4)</td>
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<tr>
<td>638</td>
<td>Δ32/ +/</td>
<td>NHL (59)</td>
<td>102 4.6</td>
<td>44 4.6</td>
<td>5 (3.9-4.4)</td>
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<tr>
<td>19</td>
<td>Δ32/ +/</td>
<td>CM (99)</td>
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<td>125 4.6</td>
<td>5 (3.8-4.4)</td>
<td>0</td>
<td>0 0 4 0 0 0</td>
<td></td>
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</tbody>
</table>

*Δ32/+, CCR5 Δ32 heterozygote; +/+, CCR5 or CCR2 wild-type genotype; 64I/+, CCR2b 64I heterozygote

Our findings are in accordance with the restricted CCR5 use found by other groups for primary NSI bulk isolates or NSI HIV-1 clones. Use of CCR2b, CCR3, and CXCR4 has been reported but only for HIV-1 luciferase reporter viruses pseudotyped with NSI envelopes; however, even these viruses mainly used CCR5 for entry and their use of CCR2b, CCR3, and CXCR4 coreceptors may be due to the artificial system.

Although the identification of novel HIV-1 coreceptors continues, in this study we have shown that NSI variants, even those associated with disease progression, seem to be restricted to the use of CCR5. Others have shown that the majority of SI variants are CCR5- and/or CXCR4-restricted. These findings indicate that the number of coreceptors that will have to be targeted in vivo to control HIV-1 infection may be limited. Therefore, in addition to interfering with reverse transcription by use of reverse transcriptase inhibitors and the processing of immature proteins with protease inhibitors, targeting HIV-1 entry remains a promising additional strategy to delay progression to AIDS.

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Written informed consent was obtained from all participants. In the conduct of clinical research, human experimentation guidelines of the author's institution were followed.