Adaption to promiscuous usage of chemokine receptors is not a prerequisite for human immunodeficiency virus type 1 disease progression

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

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Adaptation to Promiscuous Usage of Chemokine Receptors Is Not a Prerequisite for Human Immunodeficiency Virus Type 1 Disease Progression

Ana-Maria de Roda Husman, Ronald P. van Rij, Hetty Blaak, Silvia Broersen, and Hanneke Schuitemaker

Fifty percent of individuals infected with human immunodeficiency virus type 1 (HIV-1) variants use the CXC chemokine receptor 4 (CXCR4). In addition, some SI HIV-1 variants use CC chemokine receptor 2b (CCR2b), CCR3, and/or CCR5 [1–6]. Non–syncytium-inducing (NSI) HIV-1 variants use CC chemokine receptor 5 (CCR5) for entry in their target cell [6–10]. In the asymptomatic phase of infection, macrophage-tropic NSI HIV-1 variants predominate [11–17]. In about 50% of HIV-1 infected individuals, SI T cell line–tropic HIV-1 variants emerge during the course of infection, preceding an accelerated CD4 cell decline and a more rapid disease progression [12, 14, 18–21]. In agreement with these observations, a transition from CCR5 usage to multiple coreceptor usage has been correlated with disease progression [4, 5, 22, 23]. However, the majority of individuals who do not develop SI variants also progress to AIDS, some of them even rapidly [12, 18]. 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 [24, 25]. 2 genotypes that, in general, have been associated with a delayed disease progression [24–29].

NSI HIV-1 variants isolated from individuals with a progressive disease course replicate more rapidly in vitro and are associated with a higher viral load in vivo than NSI HIV-1 from asymptomatic individuals [18]. In the present study, we analyzed whether these NSI HIV-1 variants associated with a progressive disease course have an expanded coreceptor repertoire and/or whether such a phenomenon may also explain the rapid disease progression in some of the CCR5 Δ32 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 (LTSs; ACH 16, 68, 78, 337, 434, 441, 583, 750); they had an asymptomatic follow-up of at least 9 years (mean follow-up, 144 months after seroconversion; range, 124–152 months) with stable CD4+ T cell counts (>400/mm³) in the absence of antiretroviral therapy [30]. These individuals harbored solely NSI variants. In addition, we studied 4 rapid progressors (AIDS diagnosis at 25–76 months after seroconversion: ACH 53, 172, 424, 638), 3 typical progressors (AIDS diagnosis at 99–109 months after seroconversion: ACH 19, 38, 142), and 1 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 2 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 LTSs and progressors, respectively). The other sample was chosen as late as possible after seroconversion or entry in the cohort studies (mean, 111 months for LTSs) or at time of AIDS diagnosis (mean, 74 months for progressors; figure 1).

CCR5 and CCR2b genotyping. Genomic DNA was isolated from cryopreserved PBMC (Qiagen blood kit, Hilden, Germany). CCR5 genotyping was done by polymerase chain reaction (PCR) analysis by use of primers flanking the 32-bp deletion in CCR5 [24]. CCR2b genotyping was done by restriction fragment length polymorphism PCR analysis as described elsewhere [25].

Analysis of CD4⁺ T cell counts. T lymphocyte immunophenotyping for the CD4⁺ T cells was done 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 use of a nucleic acid sequence based amplification assay (HIV-1 RNA QT; Organon Teknika, Boxtel, The Netherlands) as described elsewhere [31, 32].

Viruses, isolation, SI phenotyping, and determination of infectious cellular load. Viruses were isolated under limiting dilution conditions as described elsewhere [12, 20]. Participant PBMC (0.5–4.0 x 10⁴ cells/well in 32, 48, or 96 wells) were cocultivated with phytohemagglutinin (PHA)-stimulated healthy donor peripheral blood lymphocytes (PB; 10⁷ well) in 96-well microtiter plates. Every week, one-third of the culture supernatants was tested for p24 antigen by an in-house p24 antigen capture ELISA [33]. At the same time, half of the cells was transferred to new 96-well plates, and 10³ 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(F₀), in which F₀ is the fraction of negative cultures.

PBMC from wells tested positive were transferred to 25-mL culture flasks containing 5 x 10⁴ 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 (1 x 10⁴) were added to analyze the syncytium-inducing capacity of the virus clones [34]. The titer of the virus stocks was quantified by determination of the TCID₅₀ in PHA-stimulated PBL derived from a healthy blood donor with the common CCR5 genotype.

Cell lines. Human astrocytoma U87 cell lines stably expressing CD4 and coexpressing CCR1, CCR2b, CCR3, CCR5, or CXCXR4 were grown in Iscove’s medium supplemented with 10% fetal calf serum, 5 μg/mL Polybrene, 100 μM 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 CXCXR4 expression on the corresponding U87 cell lines was monitored by flow cytometry. Staining for CCR3 and CCR5 (2D7; Becton Dickinson, Mountain View, CA) was performed by use of unlabeled monoclonal antibodies, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (CLB, Amsterdam, The Netherlands), blocking with normal mouse sera and staining with phosphatidylethanolamine (PE)-conjugated anti-CD4 monoclonal antibody (CLB). CCR3 monoclonal antibody (7B11) was obtained from Leukosite (Cambridge, MA), through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH [35, 36]. For staining of the U87-CXCXR4 cell line, PE-conjugated anti-CXCXR4 monoclonal antibody (12G5; Becton Dickinson) and Tri Color–conjugated anti-CD4 (CALTAG Laboratories, South San Francisco) were used. Markers were set by use of isotype matched control antibodies.

CCR1 and CCR2 expression was monitored by reverse transcription (RT)–PCR. RNA was extracted from U87 cells by use of TRIzol reagent (Bethesda Research Laboratories, Gaithersburg, MD), and RT was done by use of random primers with Superscript Reverse Transcriptase (Bethesda Research Laboratories) 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 5 mM EDTA. PCR was done on a DNA thermal cycler 480 (Perkin-Elmer, Foster City, CA) with Taq polymerase (Promega, Madison, WI) by use of primers CCR1 S (AACTCTCGTGCCAGAAGGTGAAGC), CCR1 AS (TCCACTCTCGTAGGGTTCGAGG), CCR2 S (TGCTGTCACCACATCTGCTTCG), and CCR2 AS (CCCTATGCCTCTTCTCCTTGGG) in the presence of 3 mM/L MgCl₂. Conditions of PCR were 5 min of denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min 30 s at 60°C, and 1 min 30 s 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 HPRT [37] were used as a positive control for RNA preparations. PCR was done 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, 2–9 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 also had a low cellular infectious load at later time points, biological HIV-1 clones obtained from 2 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: this allowed a more detailed analysis of the association between viral load and coreceptor use.

Two primary SI HIV-1 clones, ACH 39.28.H5 and ACH 208.13.B1, with a known ability to use CCR3 and CXCXR4 or CCR5 and CXCXR4, 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 10⁶ TCID₅₀/mL was shown to be sufficient to establish infection in the U87 cell lines expressing the appropriate coreceptors.

To determine coreceptor usage of biological virus clones, 10³ cells of each U87 cell line were seeded in 96-well plates, and after 24 h 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
Figure 1. Longitudinal analysis of CD4 T cell counts and virus load of progressors (A). Patient numbers are indicated in the upper left corner of each graph. Δ, number of CD4 T cells (left y-axis); ●, infectious cellular load; ○, viral RNA load (right y-axis). Filled arrowheads (on the x-axis), time of clonal isolation of non-syncytium-inducing human immunodeficiency virus type 1 variants that were analyzed for coreceptor use. Open arrowheads, time of AIDS diagnosis.
Figure 1. (Continued.) Longitudinal analysis of CD4 T cell counts and virus load of long-term survivors (LTSs; B). Patient numbers are indicated in the upper left corner of each graph. △, number of CD4 T cells (left y-axis); ●, infectious cellular load; ○, viral RNA load (right y-axis). Filled arrowheads (on the x-axis), time of clonal isolation of non-syncytium-inducing human immunodeficiency virus type 1 variants that were analyzed for coreceptor use. Open arrowheads, time of AIDS diagnosis.
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CCR5 genotype</th>
<th>CCR2 genotype</th>
<th>Diagnosisa</th>
<th>Time of coreceptor analysis (months after SC)</th>
<th>Infectious cellular load (TCID/10^6 CD4 T cells)</th>
<th>RNA load (log copies/mL serum)</th>
<th>No. clones analyzed (range of log virus titers)</th>
<th>No. of clones able to infect U87 CD4 cell lineb</th>
<th>PBMCsb</th>
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<td>1</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td>583</td>
<td>wt</td>
<td>wt</td>
<td>as (149)</td>
<td>111</td>
<td>11</td>
<td>3</td>
<td>5 (3.4–4.9)</td>
<td>0 0 0 5 0 0 0 0</td>
<td></td>
</tr>
<tr>
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<td>wt</td>
<td>wt</td>
<td>PCP (76)</td>
<td>97</td>
<td>169</td>
<td>4.8</td>
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</tr>
<tr>
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<td>wt</td>
<td>wt</td>
<td>KS (109)</td>
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<td>424</td>
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<td>wt</td>
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</tr>
</tbody>
</table>

NOTE. SC, seroconversion; as, asymptomatic; PCP, Pneumocystis carinii pneumonia; KS, Kaposi’s sarcoma; CO, esophageal Candidiasis; wt, CCR5 or CCR2b wild-type genotype; wt^2, CCR5 and CCR2b wild-type genotype.

^ Diagnosis by end of follow-up.

^b Astroglioma U87 cell lines that stably express CD4 and one or none of the following CC chemokines: CCR1, CCR2, CCR3, CCR5, or CXCR4.
early or late in infection from the different individuals is given in tables 1 and 2. The U87 cells were incubated with virus for another 24 h, washed with 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 with a CCR5 Δ32 homozygous mutant genotype was done with the biological HIV-1 clones under study. Supernatants harvested at day 7, 14, and 21 were analyzed for the presence of virus in an in-house p24 antigen-capture ELISA [33].

Results

LTSs 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 LTSs 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 2 LTSs (ACH 441 and 583) who continuously had a low viral load, and from 1 LTS (ACH 68), 2 rapid progressors (ACH 53, 424), and 1 typical progressor (ACH 142) with increasing viral load (figure 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 coexpressing CD4 and CCR1, CCR2b, CCR3, CCR5, or CXCR4. Expression of the coreceptors was monitored by flow cytometry analyses for CCR3, CCR5 and CXCR4 (figure 2A) and by RT-PCR for CCR1 and CCR2 (figure 2B). Moreover, functioning of CCR3, CCR5, and CXCR4 was shown 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 coexpressing U87 cell line (table 2). However, irrespective of 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 Δ32 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. Flow cytometry analysis revealed the expression of CD4 and CXCR4 on cells derived from both donors, but CCR5 expression was only detected on cells of the donor with the common CCR5 genotype (data not shown). Also, the presence of mRNA for CCR1, CCR2b, CCR3, CCR4, and CXCR4 was confirmed on cells of both donors by RT-PCR (data not shown). In agreement with our observations of the U87 cell lines, none of the NSI virus clones was able to establish infection of 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 3 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 [24–29]. 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 bypass 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 3 LTSs with a CCR5 Δ32 heterozygous genotype (ACH 16, 78, 434), 1 LTS with a CCR2b-64I heterozygous genotype (ACH 750), and 1 LTS with a CCR5 Δ32/CCR2b-64I heterozygous genotype (ACH 337). This was compared with the coreceptor repertoire of NSI HIV-1 variants isolated from 2 rapid progressors (ACH 172, 638), a typical progressor (ACH 38), and a slow progressor (ACH 617) with a CCR5 Δ32/CCR1 CCR2 CCR3 CCR5 CXCR4 CD4

<table>
<thead>
<tr>
<th>Isolate</th>
<th>CCR1</th>
<th>CCR2</th>
<th>CCR3</th>
<th>CCR5</th>
<th>CXCR4</th>
<th>CD4</th>
<th>CCR5 Δ32/Δ32</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIIIIB</td>
<td>−</td>
<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>
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<tr>
<td>208.13 B1</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

NOTE: +, infection; −, no infection; PBMC, peripheral blood mononuclear cells.

**Table 1.** Coreceptors used by isolate of T cell-adapted virus IIIB and 2 primary isolates of syncytium-inducing human immunodeficiency virus type 1 to infect various cell lines.

Astroglialoma U87 cell lines that stably express CD4 and one or none of the following CC chemokines: CCR1, CCR2, CCR3, CCR5, or CXCR4; cell line labelled CD4 is the control.
Figure 2. Expression of human immunodeficiency virus type 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 on the indicated U87 cell lines, analyzed by reverse transcriptase–polymerase chain reaction (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 done on RNA, without a prior RT reaction (−RT), to check for contamination of the RNA preparations with chromosomal DNA.

observations, none of the virus clones was able to productively infect the PBMC from the CCR5 Δ32 heterozygous blood donor.

Discussion

Recently, several groups have described a correlation between changes in viral coreceptor use and disease progression in HIV-1 infected individuals [4, 5, 22, 23]. In these studies, HIV-1 coreceptor use evolution was found to be associated with conversion of the NSI to the SI HIV-1 phenotype. However, ~50% of HIV-1 infected individuals progress to AIDS in the absence of SI HIV-1 variants [20, 38]. The NSI variants isolated from all these individuals in general show increased replication kinetics compared with NSI HIV-1 isolated from LTSs, giving rise to an increased cellular infectious load [18]. 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 64I genotype [24, 25]. These observations prompted us to study whether in those individuals that do not develop SI HIV-1 variants, development of NSI HIV-1 that is 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 LTSs 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 also could 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 [39], HIV-1 evolution was not driven toward alternative coreceptor use.

It is feasible that NSI HIV-1 variants associated with a pro-
Table 3. Coreceptors used by clones of non-syncytium-inducing human immunodeficiency virus type 1 that were isolated from long-term survivors and from progressors with the CCR5 Δ32 and/or CCR2 64I heterozygous genotype.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CCR5 genotype</th>
<th>CCR2 genotype</th>
<th>Diagnosisa</th>
<th>Time of coreceptor analysis (months after SC)</th>
<th>Infectious cellular load (TCID/10⁶ CD4 T cells)</th>
<th>RNA load (log copies/mL serum)</th>
<th>No. clones analyzed (range of log virus titers)</th>
<th>No. of clones able to infect U87 CD4 cell lineb PBMc, CCR1, CCR2, CCR3, CCR5, CXCR4, CCR5 Δ32</th>
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<tr>
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<td>wt</td>
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<td>wt</td>
<td>as (140)</td>
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<tr>
<td>337</td>
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<td>64I/wt</td>
<td>as (142)</td>
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<td>6</td>
<td>3 (3.2-4.2)</td>
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<tr>
<td>750</td>
<td>wt</td>
<td>64I/wt</td>
<td>as (148)</td>
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<td>16</td>
<td>5 (3.2-3.7)</td>
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</tr>
<tr>
<td>38</td>
<td>DΔ32/wt</td>
<td>wt</td>
<td>KS (101)</td>
<td>21</td>
<td>44</td>
<td>9 (3.0-4.8)</td>
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<tr>
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<tr>
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NOTE. SC, seroconversion; as, asymptomatic; KS, Kaposi's sarcoma; NHL, non-Hodgkin's lymphoma; CM, Cryptococcosis meningitis; DΔ32/wt, CCR5 Δ32 heterozygote; wt, CCR5 or CCR2 wild-type genotype; 64I hz, CCR2b 64I heterozygote.

a Diagnosis by end of follow-up.

b Astroglioma U87 cell lines that stably express CD4 and one or none of the following CC chemokines: CCR1, CCR2, CCR3, CCR5, or CXCR4.
gressive disease course may use other, as 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 [40–44]. However, none of the 178 tested NSI HIV-1 variants isolated from 16 different individuals was able to productively infect PBMCs derived from a healthy blood donor with the CCR5 Δ32 homozygous mutant genotype or the U87-CD4 cell line. This makes it unlikely that any of the coreceptors expressed on PBMC will play a major role in NSI HIV-1 infection, although a defect in yet-unidentified coreceptors in conjunction with the CCR5 mutations cannot be excluded. Alternatively, NSI HIV-1 variants in individuals with a progressive clinical course may evolve to viruses with a higher affinity for CCR5 and/or CD4. This could contribute to the increased cellular infectious load also associated with NSI HIV-1 disease progression [18].

Our findings are in accordance with the restricted CCR5 use found by other groups for primary NSI bulk isolates [2, 4, 5, 22] or NSI HIV-1 clones [22]. CCR2b, CCR3, and CXCR4 use has been reported, but only for HIV-1 luciferase reporter viruses pseudotyped with NSI envelopes. However, even these viruses mainly used CCR5 for entry [4, 22], and their use of CCR2b, CCR3, and CXCR4 coreceptors may be caused by the artificial system.

Although the identification of novel HIV-1 coreceptors continues [44–49], 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 [2–4, 50]. These findings indicate that the number of coreceptors that will have to be targeted in vivo to control HIV-1 infection may still be limited. Therefore, in addition to interfering with RT 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.

Acknowledgments

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References


