Unexpected Coreceptor Usage of Primary Human Immunodeficiency Virus Type 1 Isolates from Viremic Patients under Highly Active Antiretroviral Therapy

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Recently, combinations of antiretroviral drugs (highly active antiretroviral therapy [HAART]) have led to a dramatic reduction of human immunodeficiency virus type 1 (HIV-1)-related clinical symptoms. Success of treatment is defined as almost complete suppression of plasma viremia, although in a sizable fraction of patients this goal is not achieved. We characterized primary HIV-1 isolates from 2 cohorts of patients in which HAART failed in terms of viral suppression. One cohort showed clinical benefit and stable or increasing CD4+ T cell numbers despite high viral load. The second viremic cohort had no CD4+ T cell recovery and exhibited typical AIDS-related symptoms. Primary isolates from HAART patients with minor clinical symptoms used CXCR4 as the most relevant receptor on primary cells. Thus, for the first time, it is shown that patients improving clinically under HAART harbor relatively high viral loads with viruses preferring CXCR4 as coreceptor.

Human immunodeficiency virus type 1 (HIV-1) establishes a persistent infection in CD4+ cells, including T cells and macrophages [1, 2]. Viral entry into cells is initiated by binding of the viral surface glycoprotein gp120 to the CD4 molecule [3–5]. Subsequently, the CD4/gp120 complex interacts, most likely via the third variable region (V3) loop, with an appropriate secondary coreceptor [6, 7], which leads to the activation of the fusion domain located on the viral transmembrane protein gp41. Chemokine receptors, a 7-transmembrane G-protein-coupled receptor family, serve as coreceptors for HIV-1, HIV-2, and simian immunodeficiency virus (SIV) [reviewed in 8–11]. Two members of this family, CCR5 and CXCR4, have been identified as the most physiologically relevant chemokine receptors for diverse strains of primary isolates [12]. Virus isolates from freshly infected or asymptomatic patients are usually macrophage tropic and use CCR5 [13–19], whereas isolates from symptomatic patients are often T cell tropic and preferentially use CXCR4 as coreceptor [20]. CCR5 coreceptor usage seems to be essential in the initial phase of transmission to establish a productive infection, because homozygous carriers of an inactivating CCR5 deletion, which has a frequency of ~1% in the white population, are highly resistant to HIV infection [21–24]. During the symptomatic phase of HIV infection, virus isolates are often able to use CXCR4 either in place of or in addition to CCR5, sometimes paired with the use of additional coreceptors such as CCR1, CCR2, CCR3, CCR4, BOB, Bonzo, CCR8, V28, or APJ [25–28]. In addition, Penn et al. [29] reported that the evolution of CXCR4-dependent viruses is associated with a rapid decline in CD4+ T cells ex vivo.

On the molecular level, it is well known that the V3 region of gp120 contains major determinants for T cell and macrophage tropism [30–32] and therefore plays a pivotal role in coreceptor usage [33–36]. Both cellular host range and usage of a specific coreceptor are primarily determined by the amino acid sequence and charge of the V3 loop, possibly influenced by the V1/2 region of the envelope protein [37–40].

Kaufmann et al. [41] reported a cohort of patients who remained viremic under HAART despite stable or increasing CD4+ cell counts, and Perrin and Telenti [42] report that >50% of patients being given HAART have increasing or stable CD4+ T cell counts, despite high and/or persistent plasma viremia. In these patients, the major goal of antiretroviral treatment—namely, reduction of viral plasma load—was not achieved. Therefore, we characterized primary HIV-1 isolates from patients in which HAART failed in terms of viral suppression. One of our cohorts showed, in addition to stable or increasing CD4+ T cell counts, a clinical benefit of antiretroviral treatment. This observation suggests that, in some cases, an improved or stabilized CD4+ T cell count while receiving HAART, and not the reduction of viremia, is the relevant factor for the observed clinical benefit. To determine whether there is evidence for a somewhat “benign” viremia in these patients, we studied primary virus isolates from viremic patients who were being given HAART with minor (mean CD4+ T cell count, 197 cells/μL) and major (mean CD4+ T cell count, 76 cells/μL) clinical symp-
toms in terms of cellular host range (T cell/macrophage tropism), coreceptor preferences, and analyzed V3 loop sequences and the viral clade.

Material and Methods

**Patient recruitment and virus isolation.** HIV-1–positive patients were recruited from the University Hospital, Frankfurt, Germany. Patients who showed a plasma viremia >60,000 copies HIV-RNA per milliliter of serum despite being under HAART were selected. For virus isolation, plasma from 15 mL of blood was diluted between 2-fold and 200,000-fold in CD8−-depleted donor peripheral blood mononuclear cells (PBMC) prestimulated for 2 days with phytohemagglutinin (PHA) and cultivated at a density of 3 × 10⁶ cells/mL in the presence of interleukin (IL)−2. Cultures were split every 3–4 days, and fresh prestimulated cells from a healthy donor were added weekly. Reverse-transcriptase (RT) activity in the supernatants was determined 3 times per week (Lenti-RT Activity Assy; Cavidi Tech, Uppsala, Sweden). These zero passages were maintained for up to 30 days. Virus stocks were produced by a further passage of supernatants from the zero passage.

**Macrophage preparation.** CD14+ cells were separated from PBMC by incubation with CD14-MicroBeads and subsequent magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14+ cells were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum and 100 units granulocyte-macrophage colony-stimulating factor per milliliter (Calbiochem, Bad Soden, Germany). Cells were seeded in 48-well plates (COSTAR, Cornying, NY) at 2.5 × 10⁶ cells/well in 0.4 mL RPMI 1640. Infections were performed in duplicates with an equivalent of 500 pg RT per isolate on 7-day-old macrophages in 0.5 mL overnight. Culture supernatants were collected until day 13. A final immunoperoxidase assay was performed.

**PBMC isolation and CCR5 typing.** PBMC were separated from heparinized blood with Ficoll-Histopaque 1077 (Sigma, Taufkirchen, Germany) density gradient centrifugation. Cells were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 20% fetal calf serum, 100 U/mL IL-2, and 5 μg/mL PHA for 3 days. A concentration of 10⁵ PBMC was lysed with 20 μL lysis buffer (1% polymerase chain reaction [PCR] buffer, 0.5 μM sodium dodecyl sulfate, and 5 μg/mL protease K) at 50°C for 3 h and 95°C for 10 min. Subsequently, the CCR5 genotype was determined by PCR. The primers 5′-CTTCAATTACACCTGCAGCTCT-3′ and 5′-ACCAGGCCCTTGCTCCTCTT-3′ flanked the 32-bp deletion. Samples from donors with the CCR5 wild-type gene (CCR5±/) generated PCR fragments of 183 bp, whereas those with the homozygous deletion (CCR5−/−) resulted in a 151-bp product. Donors who generated both products were considered to be heterozygous for the CCR5 deletion (CCR5±/−).

**Inhibition of HIV-1 replication by stromal derived factor (SDF)−1β on PBMC derived from CCR5±/− individuals.** PHA-activated PBMC derived from a CCR5−/− donor were preincubated at 37°C in the presence or absence of 2 μg/mL SDF-1β (R&D Systems, Minneapolis, MN) for 60 min. As control, PBMC derived from a CCR5±/− donor were used. A concentration of 1.2 × 10⁶ PBMC was then infected for 90 min with an equivalent of 500 pg RT per isolate. After infection, cells were washed, and SDF-1β was added in fresh medium containing IL-2. A concentration of 3 × 10⁶ cells/well in 0.2 mL was seeded in 96-well round-bottom microtiter plates (Greiner, Frickenhausen, Germany) in quadruplicate. Every 2–3 days 0.1 mL medium, with or without SDF-1β 2 μg/mL, was replaced. Culture supernatants from each well were assayed on days 4, 6, 8, and 10 for the presence of RT activity.

**Sequence analysis of V3 region.** Viral RNA was prepared from cell-free culture supernatants with the QiAamp viral RNA kit (Qiagen, Hilden, Germany). For PCR amplification of the V3 loop, primers 5′-ACAGTAGGAAAATTCCCCCTC-3′ and 5′-AAATGGTATGCTACGASAAG-3′ were used. PCR products were ligated into the pGEM-T vector (Promega, Mannheim, Germany), and different clones were sequenced with dye-labeled terminators and analyzed on an ABI 377 sequencer (PE Applied Biosystems, Foster City, CA).

**Phylogenetic analysis of primary isolates.** Phylogenetic analysis was performed by PCR amplification of the C2-V5 region of the envelope gene. Primers 5′-CCAATTTGCTCCTATCATCTCTCCCTC-3′ and 5′-AAATGTACGACATGAATGCAC-3′ were used for amplification. Similar to the V3 loop, PCR products were cloned and sequenced. To determine viral clades, we used 44 amino acids of the C2 region, the whole V3 region, and the connecting 22 amino acids. For creation of the phylogenetic tree, Pocket Phylip 3.5 Packet from Felsenstein (Prodist/Fitch) was employed.

**Neutralization assay with anti-CD4 antibodies.** For inhibition of virus infection via the CD4 molecule, the following monoclonal anti-human CD4 antibodies were used: anti-human CD4 antibody (Pharmingen, San Diego), Leu3a (Becton Dickinson, San Jose, CA), and 13B8.2 (Immunootech, Marseille, France). Inhibition of virus replication was detectable in a range of 0.2–4 μg antibody per milliliter of cell culture medium.

**Infection of GHOST cells coexpressing CD4 and various chemokine receptors.** GHOST indicator cells (originally produced by D. Littman and K. W. Remieni) were obtained from the MRC AIDS Reagent Project (ARP 074−082). GHOST cells are derived from human osteosarcoma cells expressing human CD4 and 1 of various coreceptors introduced via infection with the pBABEpuuro retroviral vector. GHOST cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 1% glutamine, 500 μg/mL G418, and 1 μg/mL puromycin (not parental). GHOST cells were seeded in 24-well plates (Falcon 3047, Becton Dickinson Labware, Lincoln Park, NJ) at 2 × 10⁴ cells/well in 0.4 mL. The next day, GHOST cells were infected with an HIV-1 equivalent of 500 pg RT in a volume of 0.1 mL. The following day, virus-containing medium was removed, and cells were washed twice with phosphate-buffered saline. Subsequently, 0.5 mL of complete medium, as described earlier, was added to each well. The day of virus addition was considered to be day 0. Supernatants from each well were collected on days 4, 6, and 8 after infection. On day 8, a final immunoperoxidase assay was performed. Supernatants were analyzed for RT activity (Lenti-RT Activity Assy, Cavidi Tech, Uppsala, Sweden).

**Analysis of GHOST cells by RT-PCR.** Each GHOST cell type was examined for coreceptor RNA expression (CCR1, CCR2, CCR3, CCR4, CCR5, BOB, Bonzo, and CXCR4) by RT-PCR. RNA isolation was performed with RNasy (Qiagen). For cDNA synthesis, the First-Strand—cDNA Synthesis kit (Pharmacia Biotech, Freiburg, Germany) was used.
**Immunoperoxidase assay.** Cells were fixed with methanol for 10 min at −20°C. After blocking for 20 min with 2% milk powder/PBS, cells were incubated for 1 h with HIV-1 positive sera from infected humans, diluted 1 : 2000 in 2% milk powder/PBS. After 3 washes with PBS, cells were incubated for 1 h with anti-human IgG horseradish peroxidase conjugate (Sigma, St. Louis), diluted 1 : 500 in 2% milk powder/PBS. Excess conjugate was removed by 3 washes with PBS. Thereafter, infected cells were visualized by means of sodium acetate buffer, pH 5, containing 0.02% 3-amin-9-ethylcarbazole and 0.02% H2O2.

**Results**

**Characterization of primary isolates from HAART-treated patients.** Primary virus isolates from viremic symptomatic HAART patients (P34-S, P35-S, P51-S, P59-S, and P64-S), viremic HAART patients with minor clinical symptoms (P53-A, P58-A, P61-A, P62-A, and P63-A), and untreated (naive) patients (P32-N, P65-N, P66-N, and P67-N) were tested for coreceptor usage to investigate a potential link with the clinical status of patients under therapy (table 1). Phylogenetic analysis of the gp120 C2-V3 region of the envelope gene showed all isolates to belong to clade B (data not shown). Patients were examined for the CCR5 32-bp deletion. With the exception of patient 65, who is heterozygous for the CCR5 32-bp deletion, all patients carry the CCR5 wild-type gene. With the exception of P61-A, all isolates tested (P32-N, P66-N, P67-N, P34-S, P35-S, P51-S, P59-S, P53-A, P58-A, P61-A, P62-A, and P63-A) were able to replicate on macrophages (data not shown).

**Coreceptor preferences of primary isolates from patients under HAART.** GHOST cells expressing CD4 and 1 of the following coreceptors, CCR1, CCR2b, CCR3, CCR4, CCR5, CXCR4, BOB (GPR15), or Bonzo (STRL33), were used for infection studies (table 1). To control expression and functionality of coreceptors on GHOST cells, the T cell adoptively transferred virus strain HIVIIIIB, the molecular HIV-1 clones SF2 and SF162, and cloned SIV from the African green monkey (SIVagm3), for which the coreceptor usage is known, were used for infection studies (table 1). As expected, SF162 infected GHOST-CCR5 cells, and HIVIIIIB mainly infected GHOST-CXCR4 cells, confirming the susceptibility of these cells and the rationale of the test system.

**Table 1.** Clinical characteristics and viral coreceptor preferences of virus primary isolates from patients under highly active antiretroviral therapy (HAART).

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD4 cells/mm³</th>
<th>Viral load (viral RNA copies/mL of plasma)</th>
<th>CDC stage</th>
<th>Coreceptor usage</th>
<th>HIV-positive since at least</th>
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<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>34-S</td>
<td>24</td>
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<td>C3</td>
<td>R1, R3, X4, BOB</td>
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<td>R5, X4, BOB</td>
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<td>51-S</td>
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<td>C3</td>
<td>R1, X4, BOB</td>
<td>1/1993</td>
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<td>10</td>
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<td>C3</td>
<td>R1, R3-5, X4, BOB</td>
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<td>52</td>
<td>600</td>
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<td>R4, R5</td>
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</tr>
<tr>
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<td>C3</td>
<td>R1, R3, BOB</td>
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<tr>
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<td>C3</td>
<td>X4</td>
<td>1/1985</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>65-N</td>
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<td>A2</td>
<td>R5, BOB</td>
<td>1/1997</td>
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<td>R5</td>
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<td>565,000</td>
<td>A2</td>
<td>R5, BOB</td>
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**NOTE.** A, patients under HAART with AIDS-related symptoms; patient 64 exhibited low virus load because of recent change of therapy; patients from this group are assigned “S” (symptomatic). B, patients under HAART with minor clinical symptoms; patients from this group are assigned “A” (asymptomatic). C, patients without antiretroviral treatment; patients from this group are assigned “N” (naive). HIV, human immunodeficiency virus. 

* CCR5 genotype was determined by a polymerase chain reaction (PCR)-based assay. Except for patient 65, who is heterozygous for the 32-bp deletion (CCR5Δ32/Δ32), all patients are homozygous for the wild-type CCR5 gene (CCR5Δ32/Δ32).

* Clinical and immunologic status of patient according to criteria of the Centers for Disease Control and Prevention (CDC) categories from 1993 revised CDC classification of HIV type 1 infection. Even if clinical and immunologic status of patients under therapy is improving, CDC stages, per definition, cannot be staged up.

* Coreceptor usage was determined by use of coreceptor expressing GHOST cells. Coreceptor designations based on recent nomenclature (1180) [27], where, by use of viruses, CCR5 are called R5 viruses and CXCR4, X4 viruses. P67-N did not replicate on GHOST cells, but coreceptor preferences could be determined on CCR5Δ32/Δ32 and CCR5Δ32/Δ32 peripheral blood mononuclear cells.
Figure 1. Growth kinetics of primary isolates from patients being given highly active antiretroviral therapy with stromal derived factor (SDF)-1 (▲) and without SDF-1 (□). CD8⁺-depleted peripheral blood mononuclear cells from a CCR5⁻⁻ donor were infected after preincubation with SDF-1. Virus replication was assessed by measuring reverse-transcriptase (RT) activity on days 4, 6, 8, and 10.

diagnosis and the numbers of coreceptors used. A wide variation of the extent of virus replication could be observed in GHOST cells expressing different coreceptors. Syncytia formation and cytopathic effects were preferentially seen with fast replicating viruses on GHOST cells, regardless of the coreceptor used (data not shown).

We next determined the coreceptors used for entry of PBMC from normal blood donors and from carriers homozygous for the CCR5 deletion in vitro. As expected, isolates that mainly used CCR5 on GHOST cells, like P32-N, P35-S, P65-N, P66-N, P67-N, and SF162, were not able to infect CCR5-negative PBMC (figure 1). In contrast, viruses that use CXCR4 or other coreceptors in place of CCR5 were shown to be replication competent on these PBMC. The natural CXCR4 ligand SDF-
1 completely inhibited P58-A, P62-A, and P63-A at a concentration of 2 μg/mL. P51-S, P53-A, and P61-A showed less, but significant, inhibition by SDF-1, indicating that these isolates were able to use CXCR4 on PBMC at least to some extent. The degree of inhibition by SDF-1 seen on CCR5-deficient PBMC was more effective than on wild-type PBMC, most likely because of CCR5 use (data not shown). P59-S was the only isolate that was not at all inhibited by SDF-1, indicating that neither CCR5 nor CXCR4 is necessary for this isolate to infect PBMC. Accordingly, P59-S exhibited a broad coreceptor spectrum on the GHOST cell series and could productively infect macrophages. All virus isolates from mildly symptomatic patients under HAART were able to replicate on CCR5+/− PBMC and could be inhibited by SDF-1, indicating that CXCR4 is their main coreceptor. In contrast, only 2 of 5 virus isolates from patients with symptoms being given HAART replicated on CCR5−/− PBMC and were not (P59-S) inhibited, or rarely (P51-S) inhibited, in the presence of SDF-1.

**Neutralization assay with anti-CD4 antibodies.** To test the CD4 dependency of viral entry, we determined the capacity of anti-CD4 antibodies to inhibit virus replication on different GHOST cell lines. A strong inhibition was observed with all tested viruses (P35-S, P59-S, P62-A, SF2, and SF162) in presence of anti-CD4 antibodies, indicating that the CD4 molecule is necessary for infection and that cell entry is promoted by both the CD4 molecule and 1 of the coreceptors (data not shown).

**Sequence analysis of the envelope V3 region.** It has been suggested that specific amino acid sequences in the V3 region of HIV-1 correlate with certain coreceptor usage [36]. To determine a consensus amino-acid motif that correlates with specific coreceptor use, the V3 regions of all studied primary isolates were sequenced. Alignment of the V3 sequences of different clones obtained from each isolate showed, with the exception of P51-S, sequence polymorphism (figure 2). No correlation was seen between the time point of first HIV diagnosis and the polymorphism of the V3 loop. Nearly all clones of primary isolates that could use 1 or 2 of the given coreceptors (P32-N, P63-A, P64-S, P65-N, and P66-N) had net charges of +4 and +5 within the V3 loop. Most isolates that could use ≥3 coreceptors had increased V3 loop charges between +6 and +8. Therefore, we confirm that an increased net positive charge of the entire V3 loop seems to be associated with an expanded or altered coreceptor usage [26]. P51-S and P59-S V3 loop charges from plasma and PBMC virus isolates were identical, with the exception of 1 of 11. V3 loops from the untreated patient group had an average positive charge of +5.00, whereas HAART patients with clinical symptoms and with minor clinical symptoms had charges of +6.54 and +6.00, respectively.

**Discussion**

Substantial success has been achieved over the last few years in the treatment of HIV-1–infected individuals with combinations of antiretroviral drugs. The main goal of treatment is to suppress plasma viremia, thereby diminishing AIDS-related morbidity and mortality. However, not all HIV patients benefit from HAART. In 1 study, 49% of HAART patients of the Swiss HIV Cohort did not show viral suppression [41], and similar data have been reported from others [45].

Primary HIV-1 isolates from 2 cohorts of patients in which HAART failed were characterized. One cohort showed minor clinical symptoms, no signs of disease progression, and stable or increasing CD4+ T cell numbers, despite a mean viral load of ~240,000 RNA copies/mL plasma, whereas the other cohort (mean viral load of 775,000 RNA copies/mL plasma) exhibited typical AIDS-related symptoms and no CD4+ T cell recovery. As a control group (mean viral load of 388,000 RNA copies/mL plasma), isolates from untreated patients in CDC stage A2 were taken.

It is generally accepted that V3 of gp120 is the major determinant for cellular tropism and coreceptor choice. Therefore, we determined the amino acid sequence of the V3 loops. We could not identify a consensus motif in the V3 loop that predicts special coreceptor usage. Four of 14 isolates (P35-S, P32-N, P65-N, and P67-N) encoded the consensus motif S/GXXXXGGPXXXXXXE/D proposed by Xiao et al. [27] to predict CCR5 receptor use. Indeed, in our hands, HIV-1 isolates that displayed the consensus sequence replicated, with the exception of P67-N, on GHOST-CCR5 cells. P67-N could replicate on CCR5+/− PBMC, was not inhibited by SDF-1, and was not replication competent on CCR5+/− PBMC, indicating an absolute requirement for CCR5 (data not shown). In addition, P35-S, P32-N, and P65-N were not able to grow on CCR5−/− PBMC. Here the results achieved with GHOST cells and primary cells fitted together.

Primary isolates with V3 sequences different from the published consensus motif, such as P66-N, P64-S, P62-A, and P59-S, could also infected GHOST cells via CCR5, indicating that other determinants, possibly in the V1/V2 region of the envelope, influence coreceptor binding [37–40]. Accordingly, P66-N and P64-S were also not able to replicate on CCR5-deficient PBMC. In contrast, P62-A and P59-S infected CCR5+/− PBMC, indicating the use of other coreceptors.

As reported by Scarlatti et al. [26], we found a correlation between the overall net charge of the V3 loop and the abundance of coreceptor usage, although the net charge is not linked to macrophage or T cell tropism of our isolates. Usually, macrophages are infected via CCR5 and, to a lesser extent, via CXCR4 [46, 47]. In our hands, all tested isolates except P61-A grew, at least to some extent, on macrophages. This is in disagreement with published results, where high positive V3 loop charges had been linked to T cell tropism and low charges to macrophage tropism [30, 48]. We found differences between the average V3 loop charge of untreated patients (+5.00), treated patients with clinical symptoms (+6.54), and treated patients with minor clinical symptoms (+6.06), indicating that
Figure 2. Alignment of V-3 loops from primary isolates and their quasispecies. Sequences of different isolates and quasispecies were manually aligned. Dashes denote an amino acid that is identical to amino acid in first sequence of an isolate. Sequence polymorphism is indicated by assigning >1 amino acids to same position. Total amino acid charge of V3 loop was calculated by subtracting no. of negatively charged amino acids from no. of positively charged amino acids. H, K, and R are positively charged amino acids; D and E are negatively charged amino acids. P, virus isolated from plasma; C, virus isolated from peripheral blood mononuclear cell cultures.

<table>
<thead>
<tr>
<th>Aminoacid sequence of the V3 domain</th>
<th>Primary Isolate</th>
<th>Net charge</th>
<th>No. of clones/total tested</th>
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<tr>
<td>CTRPNNHIS KRISIGPGRAFTMRY IGDIRKHC</td>
<td>P34-S (P)</td>
<td>+6</td>
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<tr>
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<td>P35-S (P)</td>
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<td>CTRPNNHIS KRISIGPGRAFTMRY IGDIRKHC</td>
<td>P51-S (C)</td>
<td>+8</td>
<td>8/8</td>
</tr>
<tr>
<td>CTRPNNHIS KRISIGPGRAFTMRY IGDIRKHC</td>
<td>P51-S (P)</td>
<td>+8</td>
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<td>P53-A (P)</td>
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<td>+7</td>
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<td>+7</td>
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</table>
HAART does not limit the development of viruses exhibiting a broader coreceptor usage.

To further analyze virus isolates from these patients, we performed coreceptor usage studies on GHOST cells expressing CD4 and 1 of 8 coreceptors. Most of the virus isolates tested could use ≥1 of the chemokine receptors offered. However, if only 1 coreceptor could be used, it was either CCR5 or CXCR4. For plasma and PBMC-derived isolates, no significant differences in coreceptor usage (data not shown) and in the V3 loop sequences and charges were found, as shown for isolates P51-S and P59-S (figure 2).

To test coreceptor preferences in an assay that is more closely related to the situation in vivo, coreceptor usage studies were performed on CCR5-positive and CCR5-negative PBMC. In addition, blocking experiments with SDF-1 were done to evaluate the importance of CXCR4 as coreceptor.

Comparison of the results obtained with the GHOST series and with the CCR5-negative PBMC seems to be difficult. One obvious limitation of the GHOST cell system is caused by the complex balance of CD4 expression level, the expression of the coreceptor of interest, and the background expression of other known and unknown coreceptors. Usage of a special coreceptor on GHOST cells does not predict that the same virus can efficiently use this coreceptor to infect primary cells. Furthermore, the replication capacities of primary isolates observed on PBMC and GHOST cells differed widely (data not shown). However, isolates exhibiting a more extended coreceptor usage on the GHOST cells as P51-S, P53-A, P58-A, P59-S, P61-A, and P62-A replicated better on CCR5-negative PBMC than isolates with a more restricted coreceptor spectrum, namely, those isolated from the untreated patients, which could not replicate on these PBMC at all.

All isolates from patients with minor clinical symptoms were sensitive to inhibition by SDF-1. Therefore, the most relevant receptor for these isolates is CXCR4. Inhibition by SDF-1 was stronger on CCR5-negative PBMC than on wild-type PBMC, most likely because viruses are dualtropic and therefore can still replicate in the presence of SDF-1 by use of CCR5 on wild-type PBMC. Replication of P58-A was blocked completely on both types of PBMC, indicating that CXCR4 is the only coreceptor that can be used on PBMC by this virus. Isolates from patients with symptoms who were being given HAART-infected primary cells almost independently of CXCR4, as tested on PBMC from CCR5+/− donors with and without SDF-1 (data not shown). Most clearly, this is the case for isolate P51-S and P59-S. Both isolates were shown to use, in addition to CCR5 and/or CXCR4, several other coreceptors on GHOST cells. These results were confirmed on CCR5-deficient PBMC on which both isolates could replicate almost unaffected by SDF-1. P34-S, P35-S, and P64-S showed no replication on CCR5-deficient PBMC. This stands in contrast to the results on the GHOST series, where all 3 isolates were able to use other coreceptors in addition to CCR5.

It has been hypothesized that broadening of HIV-1 coreceptor usage contributes to the in vivo pathogenicity by increasing the number of potential target cells [25, 26]. In contrast, for HIV-2, disease progression in infected individuals is significantly slower than that for HIV-1 infected patients [49], despite the broad coreceptor usage of HIV-2 isolates in vitro [44]. However, our results indicate that even for HIV-1, a wide coreceptor tropism in vitro does not determine pathogenicity alone. Other characteristics, like combined polymorphism in host genes, and viral factors (i.e., mutations in the nef or pol gene or reduced immunogenicity and cytotoxicity) may also contribute to pathogenicity. For P59-S and P51-S, which seemed to use a wide coreceptor spectrum on primary cells, a correlation to pathogenicity and T cell decline was observed. In contrast, isolates P34-S, P35-S, and P64-S from the symptomatic group of patients exhibited pathogenic potential, despite exclusive usage of CCR5 on PBMC.

In conclusion, we could show that CXCR4 is the most relevant coreceptor for primary isolates from viremic patients with minor clinical symptoms and stable or increasing CD4+ T cell counts being given HAART. Three of 5 isolates from patients with major clinical symptoms were dependent on CCR5. All isolates from this patient group could infect cells independent of CXCR4. This is in disagreement with former findings, where CXCR4 usage was linked to disease progression in the natural course of HIV-1 infection [25, 26] and to a rapid decline in CD4+ T cells ex vivo [29]. In addition, the V3 loop charges are lower in isolates from patients with minor clinical symptoms as compared to patients with major clinical symptoms. Further studies with sequential isolates from these patients should determine the influence of HAART and HAART failure on evolution and tropism of viral populations.

Acknowledgments

We thank J. Dürr, G. Winskovsky, and S. Raupp for their excellent technical assistance; M. Chudy for helping us to determine viral clades; M. Baiar for helpful discussions; S. Norley for critical reading of the manuscript; and D. Littman, V. Kewal Ramani, and the AIDS Reagent Projekt for providing us the GHOST indicator cells.

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