Differential Use of CCR5 by HIV-1 Clinical Isolates Resistant to Small-Molecule CCR5 Antagonists

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How HIV-1 resistant to small-molecule CCR5 antagonists uses the coreceptor for entry has been studied in a limited number of isolates. We characterized dependence on the N terminus (NT) and the second extracellular loop (ECL2) of CCR5 of three vircivirus (VCV)-resistant clinical isolates broadly cross-resistant to other CCR5 antagonists. Pseudoviruses were constructed to assess CCR5 use by VCV-sensitive and -resistant envelopes of subtype B and C viruses. We determined the extent of entry inhibition by monoclonal antibodies (MAbs) directed against the NT and ECL2 in the presence and absence of VCV and the capacity of these pseudoviruses to use CCR5 mutants that contained scanning alanine substitutions in the CCR5 NT and ECL2 domains. Sensitive and resistant viruses were completely and competitively inhibited by the ECL2-specific MAb 2D7, whereas the NT-specific MAb CTC5 led to partial noncompetitive inhibition. VCV-resistant clones showed greater sensitivity to 2D7 than VCV-sensitive clones, but in the presence of saturating VCV concentrations, the 2D7 susceptibilities of two VCV-resistant viruses were similar to that of VCV-sensitive virus. The entry of VCV-sensitive and -resistant isolates was impaired to differing degrees by alanine mutations in CCR5; substitutions in NT had the greatest effect on viral entry. HIV-1 clinical isolates broadly resistant to CCR5 antagonists demonstrated significant heterogeneity in their use of CCR5. This heterogeneity makes it difficult to draw general conclusions about the relationship between patterns of CCR5 antagonist resistance and the use of specific CCR5 domains for entry.

Mocaviro (MVC) and vicaviro (VCV) are allosteric noncompetitive antagonists that bind to CCR5 and prevent its interaction with the HIV envelope glycoprotein gp120 (24). The bridging sheet and base of the third hypervariable loop (V3) of gp120 interact with the N terminus (NT) of CCR5 on CD4+ cells; a second region near the tip of V3 interacts with the second extracellular loop (ECL2) of CCR5 (3, 4, 8, 9). HIV-1 isolates resistant to small-molecule CCR5 antagonists have been described in vitro and in vivo; these resistant viruses have adapted to use drug-bound CCR5 for entry (1, 2, 11, 13, 15, 17–23, 25, 26, 28).

We previously identified and described full-length env sequences of one subtype C and two subtype B clinical isolates of HIV-1 that developed resistance to VCV and are cross-resistant to MVC and the investigational CCR5 antagonist TAK-779 (7, 20, 25). Five to seven mutations distributed on either side of the V3 stem-loop emerged in viruses from VCV-treated patients over a period ranging from 24 to 144 weeks (7, 20, 25). Different V3 mutations were present in each isolate, with the exception of a proline substitution at position 306, which was common to all three VCV-resistant viruses (20). The accumulation of mutations conferred progressively higher levels of resistance and increased viral infectivity in the presence of drug, although the shared proline substitution at position 306 did not confer resistance when inserted individually into the pretreatment envelope sequence (7, 20).

Earlier studies demonstrated that HIV-1 isolates resistant to VCV or MVC have an increased dependency on the CCR5 NT and an impaired interaction with ECL2 (2, 18, 21). A clinical isolate resistant to the investigational CCR5 antagonist aplaviro and broadly cross-resistant to other antagonists was critically dependent on the NT in the presence of drug, whereas an MVC-resistant virus with a narrower resistance profile remained dependent on both the NT and ECL2 for entry (19, 23). Characterization of a broader range of clinical isolates is needed to understand more fully how development of antagonist resistance influences HIV-1 entry and coreceptor usage. To test the generalizability of these prior findings and to investigate viral entry in a larger pool of patients, we characterized the CCR5 NT and ECL2 dependence of clinical isolates of HIV-1 subtypes B and C with broad CCR5 antagonist resistance that emerged during VCV therapy.

MATERIALS AND METHODS

Pseudovirus construction and sensitivity to monoclonal antibodies directed toward CCR5. Pseudoviruses incorporating a luciferase reporter gene in the nef region of HIV-1 and full-length clonal envelopes from VCV-sensitive and -resistant viruses obtained from participants in AIDS Clinical Trials Group (ACTG) A5211 (subjects 07 [subtype C] and 57 and 85 [subtype B]) were constructed using previously described methods (6, 10, 12, 27). Informed consent was obtained from all subjects enrolled in the A5211 study (6). The monoclonal antibodies (MAbs) CTC5 (R&D Systems, Minneapolis, MN) and 2D7 (BD Biosciences, Franklin Lakes, NJ), which bind selectively to the NT and ECL2 domains of CCR5, respectively, were used to assess the dependence of mutant viruses on these domains for entry. Binding of these antibodies to CCR5 is not altered significantly in the presence of VCV (23). Two-fold serial dilutions of MAb were added to the wells of a 96-well plate (volume of 50 μl), followed...
Henrich et al.

A N-Terminus
Wild-type MDVQVSAPYDINVTSTSEPQKINVK
Y10A -------- A ----------
Y14A -------- A ----------
Y10A/Y14A A--A---------
D11A A--A---------

B ECL2
Wild-type RSKQKELGHYCTSHHFPFYSYQFWKNF
K17A -------- A ----------
H18A -------- A ----------
F182A -------- A ----------
Q186A -------- A ----------
Y187A --- A  --- A-----
YSQ(184-6)A ------ A------

FIG 1 Alanine scanning mutations in the N terminus (NT) and ECL2 of CCR5. The first 26 residues of the NT, which correspond to CCR5 amino acid positions 1 to 26 (A), and the first 26 residues of ECL2, which correspond to CCR5 amino acid positions 168 to 193 (B), are shown along with the locations of the alanine substitutions in mutant plasmids used to express full-length CCR5 in U87-CD4 cells. Dashed lines represent conserved residues. Alanine-substituted sulfated tyrosine moieties in the NT are located at positions 10 and 14.

by the addition of 2.0 × 10^4 U87-CD4-R5 cells suspended in 50 µl of Dulbecco’s modified Eagle medium (DMEM) with 15% fetal bovine serum (FBS) and penicillin-streptomycin. After a 1-h incubation, each well was inoculated in the presence of Polybrene (final concentration of 8 µg/ml) with an amount of pseudovirus sufficient to produce approximately 100,000 relative light units (RLU) of luciferase activity based on titration assays. After 72 h of incubation at 37°C, cells were lysed and luciferase activity was measured as described previously (12). Experiments were performed in triplicate wells, and each assay was performed at least twice. Pseudoviruses incorporating resistant envelope sequences were also assayed in the presence of saturating levels of VCV (250 nM). The maximum percent inhibition (MPI), mean 50% inhibitory concentration (IC50), and 95% confidence intervals were calculated from best-fit values using regression models using GraphPad Prism 5 (La Jolla, CA).

CCR5 alanine substitutions and viral entry. A panel of mutants carrying alanine substitutions in the CCR5 NT or ECL2 domains kindly provided by Tanya Dragic (Albert Einstein College of Medicine, Bronx, NY) (2) was used to explore the dependence of VCV-sensitive and -resistant viruses on these domains for entry (Fig. 1). Plasmids expressing wild-type or mutant CCR5 were transfected into U87-CD4 cells. One day after transfection, the cells were washed twice with DMEM with 15% FBS, Dulbecco’s modified Eagle medium (DMEM) with 15% fetal bovine serum (FBS) and penicillin-streptomycin. After a 1-h incubation, each well was inoculated in the presence of Polybrene (final concentration of 8 µg/ml) with an amount of pseudovirus sufficient to produce approximately 100,000 relative light units (RLU) of luciferase activity based on titration assays. After 72 h of incubation at 37°C, cells were lysed and luciferase activity was measured as described previously (12). Experiments were performed in triplicate wells, and each assay was performed at least twice. Pseudoviruses incorporating resistant envelope sequences were also assayed in the presence of saturating levels of VCV (250 nM). The maximum percent inhibition (MPI), mean 50% inhibitory concentration (IC50), and 95% confidence intervals were calculated from best-fit values using regression models using GraphPad Prism 5 (La Jolla, CA).

Hankenson et al.

RSLGKELGHYCTSHHFPFYSYQFWKNF
K17A -------- A ----------
H18A -------- A ----------
F182A -------- A ----------
Q186A -------- A ----------
Y187A --- A  --- A-----
YSQ(184-6)A ------ A------

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RESULTS

Viral inhibition by CCR5 MAbs. Vicriviroc-resistant viruses were identified in plasma samples from three A5211 subjects (subject 07 [Sub07], Sub57, and Sub58) with virologic failure at study weeks 28, 103, and 138, respectively. All VCV-sensitive (sens) and resistant (res) viruses were inhibited by the 2D7 MAb, which binds the ECL2 domain of CCR5; VCV-resistant viruses were more sensitive to 2D7 than VCV-susceptible viruses. In the presence of saturating concentrations of VCV, the IC50 of 2D7 for Sub07res and Sub57res increased, returning to wild-type levels for Sub07res. In contrast, the addition of VCV had no measurable effect on the IC50 of 2D7 for Sub85res (Table 1 and Fig. 2A to C).

Inhibition of Sub07sens, Sub57sens, and Sub85sens entry by CTC5 MAb, which binds the NT domain of CCR5, was modest, with MPIs of 13 to 23% (Fig. 2D to F). Sub07res and Sub85res showed greater inhibition by CTC5 than Sub07sens and Sub85sens (33.4 ± 5.7% [standard error of the mean] versus 12.7 ± 1.7% and 49.6 ± 2.0% versus 16.2 ± 4.5%, respectively), whereas Sub57res was inhibited to an extent similar to that of Sub57sens (34.8 ± 6.7% versus 22.8 ± 9.2%); IC50 could not be calculated. Sub57res and Sub85res demonstrated greater inhibition by CTC5 in the presence of VCV than in the absence of drug.

Impact of CCR5 alanine substitutions on viral entry. Figure 3 shows the relative entry of VCV-susceptible and -resistant viruses on cells expressing a panel of alanine scanning mutations in the NT and ECL2 domains of CCR5 compared to that of wild-type CCR5 cells. Individual NT mutations at positions 10, 11, 14 or the combination of mutations at positions 10 and 14 reduced entry of Sub07sens and Sub85sens by 59 to 100% compared to entry using wild-type CCR5, but entry of Sub57sens was relatively unaffected by these mutations. In contrast, entry of Sub57res was markedly reduced by each NT mutant tested. The addition of VCV had a minimal effect on capacity of VCV-resistant viruses to use the mutant CCR5s for entry.

Mutations in ECL2 had a variable effect on virus entry; the greatest reductions in entry were observed with the F182A and Q186A mutants of both CCR5 antagonist-sensitive and -resistant virus. Substitutions in ECL2 had minimal effects on the entry of VCV-sensitive and -resistant viruses from the same patients, with the exception of Y187A and the YSQ triple mutant.

TABLE 1 Sensitivity of pseudoviruses incorporating VCV-sensitive (sens) and VCV-resistant (res) envelope sequences to CCR5 ECL2-specific MAb 2D7

<table>
<thead>
<tr>
<th>Subject</th>
<th>Without VCV</th>
<th>With VCV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (µg/ml)</td>
<td>95% confidence interval</td>
</tr>
<tr>
<td>Sub07sens</td>
<td>0.38</td>
<td>0.25–0.67</td>
</tr>
<tr>
<td>Sub07res</td>
<td>0.08</td>
<td>0.05–0.11</td>
</tr>
<tr>
<td>Sub57sens</td>
<td>0.36</td>
<td>0.17–0.74</td>
</tr>
<tr>
<td>Sub57res</td>
<td>0.02</td>
<td>0.00–0.10</td>
</tr>
<tr>
<td>Sub85sens</td>
<td>0.42</td>
<td>0.32–0.57</td>
</tr>
<tr>
<td>Sub85res</td>
<td>0.08</td>
<td>0.06–0.09</td>
</tr>
</tbody>
</table>

*IC50 of 2D7 for resistant isolates was measured in the presence of saturating levels of VCV (250 nM). ND, not determined. IC50 and confidence intervals were calculated from a minimum of two independent experiments.
The Y187A mutant significantly reduced entry of Sub57res in the presence of VCV and reduced entry of Sub85res in the presence and absence of VCV. The YSQ triple mutant significantly reduced entry of Sub85res in the presence of VCV.

**DISCUSSION**

The three VCV-resistant HIV-1 clinical isolates we characterized had varied interactions with wild-type and mutant CCR5, demonstrating differences in the capacity of these viruses to use the NT and ECL2 domains for entry. Reduced entry of VCV-susceptible and -resistant viruses on cells expressing CCR5 with NT mutations provided evidence for strong reliance on this domain. In contrast, pseudoviruses expressing VCV-susceptible or -resistant envelopes were able to utilize CCR5 carrying a range of mutations in ECL2, suggesting greater plasticity of the gp120 interaction with this domain. Interestingly, two of the VCV-sensitive isolates we tested were heavily dependent on the NT for entry prior to the development of resistance, whereas one sensitive isolate tolerated NT mutations.

It has been proposed that HIV-1 with broad cross-resistance to CCR5 antagonists has adapted to be critically reliant on the NT for entry, which is less affected by drug binding than the ECLs, and that resistant viruses with a narrower cross-resistance profile remain reliant on both the NT and ECLs of CCR5 in the presence of drug (19, 21, 23). This conclusion, however, is based on data from a very small number of isolates, including only a single clinical isolate with a narrow cross-resistance profile (23). All three of the clinical isolates we studied were broadly cross-resistant to MVC, VCV, and TAK-779 (MPIs all >63% on U87-CD4-R5 cells and <14% on TZM-bl cells) (7, 20), but results of our experiments revealed heterogeneity in the interactions of different viruses with the NT and ECL2 domains. For example, the Y10A and Y14A substitutions in NT substantially reduced entry of Sub07sens and Sub85sens but had only a modest effect on entry of Sub57sens. A previous study of an MVC-resistant clinical isolate found that the resistant virus became critically reliant on Y14 and Y15 residues in the NT and H181 in ECL2 in the presence of saturating levels of the antagonist (21). In contrast, the H181A mutant had little or no effect on entry of the VCV-sensitive and -resistant viruses we studied. Addition of VCV did not produce a significant change in entry of VCV-resistant isolates into cells expressing the H181A CCR5 mutant. The varied phenotypes of the different VCV-resistant isolates on the series of CCR5 NT and ECL2 mutants most likely reflect the heterogeneity in HIV-1 gp120.

Although mutations in ECL2 had a more modest effect on viral entry compared with mutations in the NT, the VCV-resistant isolates we studied were highly susceptible to inhibition by MAb 2D7. These observations suggest that ECL2 plays a role in the entry of at least some VCV-resistant viruses and is able to accommodate changes in this region better than NT. We observed an increased susceptibility of resistant isolates to 2D7 inhibition that corrects, in some cases, with the addition of drug, whereas CTC5 resulted in greater MPI for resistant viruses than for sensitive viruses with the exception of Sub07. It is possible that inhibition by 2D7 reflects more general steric hindrance of gp120-CCR5 interactions rather than a specific blockade of the VCV-resistant gp120 with ECL2. Despite this potential limitation, CCR5 MAbs have been useful in probing viral interactions with the coreceptor (2, 17, 18). Our finding that the addition of VCV increased the extent of inhibition by 2D7 of Sub07res and Sub85res suggests that the V3 loop of the resistant viruses does indeed interact directly with ECL2. Interestingly, the shapes of the inhibition curves generated by MAbs directed against the NT and ECL2 suggest that 2D7 blocks entry by a competitive mechanism, whereas CTC5 appears to be a non-competitive inhibitive inhibitor.

Our study has a number of potential limitations. Only three VCV-resistant isolates were available for study. It is possible that more consistent patterns would have emerged if more CCR5 antagonist-resistant viruses were studied. In addition, the levels of CCR5 expressed on the U87 human glioblastoma cells used in our experiments do not completely mimic CCR5 expression on primary lymphocytes. Host factors such as polymorphisms in CCR5...
and related promoter regions result in interindividually different CCR5 expression and may have contributed to the selection of different viral solutions to the development of VCV resistance (5, 14, 16). Although beyond the scope of this study, analysis of these host factors is an important area for future investigation.

The development of in vivo resistance to small-molecule CCR5 antagonists is relatively rare, and as a result the interactions of resistant viruses with CCR5 have been characterized in a relatively small number of clinical isolates (2, 15, 17–19, 21–23). When considered in the context of other studies of CCR5 antagonist-resistant HIV-1 isolates, our findings suggest that each resistant variant relies on the CCR5 NT or ECL2 to a different degree, regardless of the extent of cross-resistance. Whereas all CCR5 antagonist-resistant HIV-1 must meet the constraint of being able to bind the drug-bound form of CCR5, the heterogeneity of env appears to permit a variety of solutions to this challenge within the context of different env backbones. Characterization of a larger number of resistant isolates from a variety of HIV-1 subtypes may eventually allow identification of common motifs and provide a better understanding of the structural requirements for the interaction of HIV-1 gp120 with CCR5.

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REFERENCES


