Epitope Switching as a Novel Escape Mechanism of HIV to CCR5 Monoclonal Antibodies

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In passing experiments, we isolated HIV strains resistant to MAb3952, a chemokine (C-C motif) receptor 5 (CCR5) monoclonal antibody (MAb) that binds to the second extracellular domain (extracellular loop 2 [ECL-2]) of CCR5. MAb3952-resistant viruses remain CCR5-tropic and are cross-resistant to a second ECL-2-specific antibody. Surprisingly, MAb3952-resistant viruses were more susceptible to RoAb13, a CCR5 antibody binding to the N terminus of CCR5. Using CCR5 receptor mutants, we show that MAb3952-resistant virus strains preferentially use the N terminus of CCR5, while the wild-type viruses preferentially use ECL-2. We propose this switch in the CCR5 binding site as a novel mechanism of HIV resistance.

HIV infects host cells by attaching to the CD4 receptor and subsequently binding to one of two major coreceptors, chemokine (C-C motif) receptor 5 (CCR5) or chemokine (C-X-C motif) receptor 4 (CXCR4). Both coreceptors are members of the superfamily of G-protein-coupled receptors, which are characterized by seven transmembrane helices, three extracellular loops, an amino-terminal domain (NTD), and an intracellular domain (2, 3). In CCR5, the two major binding regions for the viral gp120 envelope protein (Env) are the NTD and cellular domain (2, 3). In CCR5, the two major binding regions for the viral gp120 envelope protein (Env) are the NTD and cellular domain (2, 3).

Individuals who are homozygous for the CCR5 Δ32 deletion have a natural resistance to HIV-1 infection, while heterozygous individuals have a reduced rate of infection; homozygous CCR5 Δ32 individuals are healthy and immunocompetent, implying that normal CCR5 may be dispensable (7, 19, 27). This makes CCR5 an attractive target for antiretroviral drug development. The U.S. Food and Drug Administration approved maraviroc (Selzentry) in 2007 as the first small-molecule CCR5 inhibitor (20). Other CCR5 inhibitors, both small molecules (vicriviroc, aplaviroc, INC9B47, and others) and anti-CCR5 monoclonal antibodies (CCR5 MAb) (HGS004 and Pro-140) are or have been in clinical development (11, 16, 17, 22; E. Erickson-Viitanen, K. Abremski, K. Solomon, R. Levy, E. Lam, J. Whitcomb, R. Lloyd, R. Mathis, J. Reeves, and D. Burns, presented at the 15th Conference on Retroviruses and Opportunistic Infections, Boston, MA).

We recently discovered two highly potent mouse anti-CCR5 MAbs: RoAb13, whose epitope lies in the NTD of CCR5 and RoAb14, which binds to extracellular loop 2 (ECL-2) (13, 29). RoAb14 was chosen for further development due to its better antiviral potency and its broad activity against HIV strains from all clades, resulting in the deimmunized human antibody MAb3952.

Resistance to antiretroviral drugs is a common challenge in the treatment of HIV/AIDS patients. HIV can use several escape routes to develop resistance to CCR5 inhibitors: (i) increased affinity of the virus to the free receptor (25), (ii) virus binding to inhibitor-bound receptors consistent with an allosteric mechanism of inhibition (25, 28), and (iii) switch in coreceptor usage from CCR5 to CXCR4. The aim of this study was to select HIV variants resistant to our lead anti-CCR5 MAB using in vitro passaging experiments and to determine the mechanism of resistance. We identified two virus strains that are resistant to CCR5 MAB3952 but have increased sensitivity to RoAb13. This phenomenon can be explained by a change in the binding behavior from ECL-2 to the N terminus of the CCR5 receptor.

MATERIALS AND METHODS

Cells and virus strains. JC53BL (TZM-BL) and MAGI cells were obtained from the NIH AIDS Research and Reference Reagent program. JC53BL cells were cultured in Dulbecco modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% glutamine. MAGI cells were cultivated in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Human peripheral blood mononuclear cells (PBMC) were obtained from AllCells (Emeryville, CA), stimulated for 1 day in PBMC medium (RPMI 1640 medium containing 10% FBS, 1% penicillin-streptomycin, 2 mM t-glutamine, 1 mM sodium pyruvate, 0.1 mM minimal essential medium [MEM] nonessential amino acids) supplemented with 2 μg/ml phytohemagglutinin and maintained in PBMC medium containing 5 units/ml human interleukin 2 (IL-2) (Roche Applied Science, Indianapolis, IN). Unless noted, all cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

HIV-1 strain Bal was purchased from ABI (Colima, MD). CC1/85 was a gift from C. Stoddard (The J. David Gladstone Institutes, San Francisco, CA).

In vitro resistance development. The CCR5-tropic HIV-1 isolates CC1/85 and Bal were passaged in CD8-depleted PBMC pooled from four or more donors in the presence of increasing concentrations of the anti-CCR5 antibody MAb3952. CD8-positive cells were depleted using human CD8 microbeads and LD columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. For resistance selection, 2 × 107 CD8-depleted PBMC were infected at a multiplicity of infection of 1 with Bal or CC1/85 virus for 10 to 12 days. The initial MAb3952 concentration was 100 ng/ml, which is below the 50% inhibitory concentration...
FIG. 1. CCR5 MAb dose-response curves of MAb3952-resistant and wild-type Bal (A) and CC1/85 (B) HIV-1 strains in a PBMC-based assay. A total of 100,000 PBMC were infected with 800 pg p24 of the indicated virus strain in the presence of diluted CCR5 MAb MAb3952 (red), RoAb13 (blue), or 2D7 (black). After incubation for 6 days, virus production was determined using a p24 ELISA. NDC cultures are indicated by filled symbols and continuous lines, and MAb3952-resistant (MAb3952res) virus strains are indicated by open symbols and broken lines. The means ± standard deviations (error bars) of one representative experiment of three independent experiments are shown.

(IC50) for both virus strains. No-drug control (NDC) cultures treated with phosphate-buffered saline (PBS) volumes equivalent to the MAb3952 volume were performed in parallel. Viral replication was monitored every 3 days by infecting the reporter cell line JC53BL with 50 pg/ml, which is 400-fold lower than the prepassaging IC50.

PBMC-based antiviral assay. In a 96-well round bottom plate, 1 × 105 PBMC were infected with 800 pg p24 of the indicated HIV-1 strain in the presence of serially diluted inhibitor. Cells were incubated for 6 days at 37°C. Virus production was measured using a p24 enzyme-linked immunosorbent assay (ELISA) kit (PerkinElmer, Waltham, MA) according to the manufacturer’s instruction. IC50 was determined using the sigmoidal dose-response model with one binding site.

Cloning and sequencing of envelope genes. Envelope genes were amplified via reverse transcriptase PCR amplification as follows. RNA from cell culture supernatants was isolated with the ZR Whol-Body RNA MiniPrep kit (Zymo Research, Orange, CA) and reverse transcribed using SuperScriptIII (Invitrogen) and primer GAG TGA ATT AGC CCT TCC AGT CCC CC, followed by PCR amplification using the high-fidelity PCR system (Roche Applied Sciences) and primers 5'-CAC CAA GCT TTA GGC ATC TCC TAT GGC AGG AAG AAG and 3'-AGC TGG ATC GT CTC GAG ATA CTG CTC CCC CCA CCC. The PCR program consisted of the following: (i) an initial denaturation step of 2 min at 94°C; (ii) 35 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 62°C, and 2.5 min at 72°C; and (iii) an extension step of 10 min at 72°C. PCR products were cloned into pCDA/V5/GW/D-Topo (Invitrogen), and at least 10 individual clones were sequenced. All primers were obtained from Integrated DNA Technologies (San Diego, CA).

Site-directed mutagenesis was performed using the QuikChangeXL kit (Stratagene, La Jolla, CA). All clones were resequenced to confirm the mutation.

Single-cycle entry assay. To generate pseudotyped HIV-1 particles, 29T cells were cotransfected with pNL4-3/env and expressed envelope gene. Cell culture supernatants containing pseudotyped viral particles were harvested and filtered, and the titers of the virus on JC53BL cells were determined.

RESULTS

In vitro resistance selection. Virus strains resistant to MAb3952 were selected by passaging the R5-tropic viruses Bal and CC1/85 in human peripheral blood mononuclear cells (PBMC) in the presence of increasing concentrations of MAb3952. No-drug control (NDC) cultures were maintained in parallel. Bal and CC1/85 virus variants that were able to replicate at MAb3952 concentrations of 100 µg/ml were isolated after 11 and 14 passages, respectively. MAb3952-resistant virus strains (Bal_3952res and CC1/85_3952res) are not inhibited in a PBMC drug sensitivity assay with MAb3952 concentrations up to 20 µg/ml; however, the no-drug control viruses (Bal_NDC and CC1/85_NDC) were fully inhibited with 50% inhibitory concentrations (IC50) of 0.9 ± 0.4 µg/ml and 1.8 ± 0.6 µg/ml, respectively (Fig. 1 and Table 1). We also tested the

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Antiviral activity (IC50 [µg/ml]) of the following CCR5 MAB:</th>
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<tbody>
<tr>
<td>Bal_NDC</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Bal_3952res</td>
<td>&gt;20</td>
</tr>
<tr>
<td>CC1/85_NDC</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>CC1/85_3952res</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

a Strains Bal_3952 and CC1/85_3952res are MAb3952-resistant HIV strains, and Bal_NDC and CC1/85_NDC are no-drug controls (NDC).

b The means ± standard deviations of three independent experiments performed on four replicate samples are shown.
susceptibility of Bal\textsubscript{3952res} and CC1/85\textsubscript{3952res} to another ECL-2-binding antibody (2D7) and a NTD-binding antibody (RoAb13) (29). The MAb3952-resistant viruses are cross-resistant to the CCR5 MAb 2D7, while both NDC virus strains remain sensitive to 2D7 (Fig. 1 and Table 1). Surprisingly, Bal\textsubscript{3952res} is 60-fold more susceptible to RoAb13 than to Bal\textsubscript{NDC}. Even more striking is the increased susceptibility of CC1/85\textsubscript{3952res} virus to RoAb13; CC1/85\textsubscript{NDC} is not inhibited by RoAb13, while CC1/85\textsubscript{3952} is highly sensitive to this antibody (Fig. 1B and Table 1). All four viruses were insensitive to the CXCR4 small-molecule inhibitor AMD3100 (data not shown), suggesting that CXCR4 is not used as a coreceptor (6). The sensitivity to both MAb3952 and RoAb13 combined with a lack of sensitivity to AMD3100 indicates that these viruses remained R5-tropic after passaging.

Mutations associated with MAb3952 resistance. We next sought to determine specific amino acids in the viral envelopes that contribute to resistance against MAb3952. To this end, we amplified, cloned, and sequenced at least 10 individual clones for each virus. Consensus envelope sequences of MAb3952-resistant and NDC_Bal and CC1/85 viruses are shown in Fig. 2A and B, respectively.
resistance was associated with multiple mutations throughout gp160. The E84K and L85M mutations in conserved region 1, K163N in variable loop 2, and S531A in gp41 were detected in all MAb3952-resistant Bal clones, but not in the Bal_NDC or in the prepassaging Bal clones. Several additional mutations were found in some, but not all, MAb3952-resistant Bal clones (Fig. 2A). A mutation equivalent to S531A in Bal was also detected in all MAb3952 CC1/85 clones (S535A), but not in any of the CC1/85_NDC clones or in prepassaging CC1/85 clones (Fig. 2B). In addition, the R164I mutation in variable loop 2, V272I and D277N in conserved region 2, T315A and V322I in variable loop 3, and A613T, A779V, and Y834C in gp41 were present in all resistant CC1/85 clones, but not in any of the control clones (Fig. 2B). We next determined the individual contribution of several resistance mutations to overall MAb3952-resistance. Resistance mutations were introduced into envelope clones most closely resembling the consensus sequence. The E84K L85M mutation pair in a Bal_NDC envelope clone increased the MAb3952 IC_{50} 13.8-fold (Table 2). Similarly, a 12-fold increase was detected after introduction of the K163N mutation. We were unable to introduce the S531A mutation per site-directed mutagenesis into Bal_NDC clones and therefore decided to change alanine 531 back to the original serine in three independent MAb3952-resistant Bal clones. Depending on the particular clone, changing S531A resulted in a small, 0.3- to 0.9-fold change in the MAb3952 IC_{50} compared

FIG. 2—Continued.
to the parental Bal3952res clones (Table 2). Consistently, removal of the same mutation in two independent MAb3952-resistant CC1/85 clones also had only a minor effect on MAb3952 sensitivity, namely, a 1.2-fold increase for one clone and a 2.0-fold decrease for the other clone. Minor changes (1.9- and 1.6-fold, respectively) were also detected with the R164I mutation or the V272I D277N double mutation. Mutating Thr 315 to Ala increases MAb3952 sensitivity 6.7-fold; however, this was partially reversed by the T315A V322I double mutation which caused only a minor 2.0-fold increase in MAb3952 IC50 (Table 2). Taken together, we identified several mutations in the MAb3952-resistant Bal and CC1/85 envelopes that contribute to overall MAb3952 resistance.

Epitope switching as a novel mechanism of resistance of CCR5 antibodies. We hypothesized that resistance to the ECL-2-binding antibodies MAb3952 and 2D7 and the simultaneous increase in susceptibility to the NTD antibody RoAb13 could be explained by a change in the binding of the viral envelopes to CCR5. The NDC virus envelopes bind predominately to the ECL-2 of CCR5, and the resistant virus envelopes may have evolved to bind to the N terminus of CCR5, making them more susceptible to NTD antibodies but no longer sensitive to ECL-2 antibodies. To test this hypothesis, we performed infection experiments on MAGI cells expressing wild-type CCR5 or mutant versions of CCR5. Env clones resembling the consensus sequence of MAb3952-resistant and NDC Bal and CC1/85 viruses were chosen for pseudotyping. First, we confirmed that the pseudotyped virus-like particles reflect the CCR5 MAb resistance patterns of the respective passaging viruses. Pseudoviruses with Bal_NDC Env and CC1/85_NDC Env are sensitive to MAb3952 and to a lesser extent to 2D7 and RoAb13 (Fig. 3 and Table 3). Bal_3952res and CC1/85_3952res virus particles are resistant to both ECL-2 antibodies 2D7 and MAb3952. However, Bal_3952res and CC1/85_3952res pseudoviruses are more susceptible to RoAb13 than their respective NDC virus strains (Fig. 3 and Table 3). The different potencies of antibodies against specific virus strains in the PBMC assay compared to the MAGI assay can be explained by the lower genetic diversity of pseudovirus compared to the virus swarms used in the PBMC assay; however, the overall resistance pattern matches the results of the PBMC assay.

Next, we used MAGI cells expressing CXCR4 and wild-type (WT) CCR5 or two different mutant forms of CCR5 to determine how MAb3952-resistant and NDC viruses utilize the coreceptor. The cell lines we used included MAGI CXCR4 cells and MAGI CCR5 MAb MAb3952 (red), RoAb13 (blue), or 2D7 (black). Three days later, infection levels were determined using a luciferase reporter readout. NDC viruses are indicated by filled symbols and continuous lines, and MAb3952-resistant (MAb3952res) virus strains are indicated by open symbols and broken lines. The means ± standard deviations (error bars) of one representative experiment of three independent experiments are shown.

TABLE 2. Effects of individual mutations on MAb3952 susceptibility in a single-cycle entry assay

<table>
<thead>
<tr>
<th>HIV-1 strain and mutation(s)</th>
<th>IC50 (ng/ml)a</th>
<th>Original clone</th>
<th>Mutated clone</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bal E84K L85 M</td>
<td>161.1 ± 7.8</td>
<td>2,225.8 ± 1,670.4</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>K163N</td>
<td>161.1 ± 7.8</td>
<td>1,934.2 ± 1,241.7</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>S531A</td>
<td>2,218.0 ± 1,084.4</td>
<td>592.4 ± 227.0</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,695.0 ± 1,286.7</td>
<td>2,424.8 ± 1,413.2</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,170.2 ± 24.8</td>
<td>313.4 ± 56.9</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>CC1/85 R164I</td>
<td>342.5 ± 127.8</td>
<td>663.0 ± 169.6</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>V272I D277N</td>
<td>342.5 ± 127.8</td>
<td>556.6 ± 173.7</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>T315A</td>
<td>342.5 ± 127.8</td>
<td>2,285.6 ± 1,753.0</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>T315A V322I</td>
<td>342.5 ± 127.8</td>
<td>689.6 ± 168.3</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>S535A</td>
<td>917.5 ± 166.8</td>
<td>1,113.1 ± 152.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4,485.2 ± 728.0</td>
<td>2,211.3 ± 65.6</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

a The means ± standard deviations of three independent experiments performed on four replicate samples are shown.

b More than one MAb3952-resistant clone was mutated and analyzed. Three S531A clones and two S535A clones were examined.

FIG. 3. Dose-response curves of MAb3952-resistant and wild-type Bal (A) and CC1/85 (B) pseudotyped HIV-1 particles in the Magi cell entry assay. A total of 25,000 Magi CCR5 cells were infected with equal amounts of pseudotyped virus particles in the presence of diluted CCR5 MAb MAb3952 (red), RoAb13 (blue), or 2D7 (black). Three days later, infection levels were determined using a luciferase reporter readout. NDC viruses are indicated by filled symbols and continuous lines, and MAb3952-resistant (MAb3952res) virus strains are indicated by open symbols and broken lines. The means ± standard deviations (error bars) of one representative experiment of three independent experiments are shown.

TABLE 3. Antiviral activity of CCR5 MAbs against MAb3952-resistant and wild-type pseudotyped particles in the Magi cell entry assay

<table>
<thead>
<tr>
<th>HIV-1 strain</th>
<th>Antiviral activity (IC50 [µg/ml])a of the following CCR5 MAb:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAb3952</td>
</tr>
<tr>
<td>Bal_NDC</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>Bal_3952res</td>
<td>&gt;20</td>
</tr>
<tr>
<td>CC1/85_NDC</td>
<td>0.2 ± 0.09</td>
</tr>
<tr>
<td>CC1/85_3952res</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

a The means ± standard deviations of three independent experiments performed on four replicate samples are shown.
three independent experiments are shown. Luciferase readout. The means and standard deviations (error bars) of relative light units were measured 2 days after infection by flow cytometry. Infection levels were determined 3 days postinfection using a luciferase readout. The means ± standard deviations (error bars) of three independent experiments are shown.

FIG. 4. MAb3952-resistant pseudotyped HIV-1 particles preferentially use the N terminus of CCR5, while wild-type pseudotyped particles preferentially use ECL-2. Magi CXCR4 cells were transfected with DNA expressing an ECL-2 (CCR5 KE) or N-terminal mutant form of CCR5 (CCR5 N8) and infected with virus-like particles pseudotyped with envelopes from MAb3952-resistant and wild-type virus strains. Mock-transfected Magi CXCR4 served as a negative control, and stably expressing CCR5 wild-type cells served as a positive control. The percentage of CCR5-expressing cells and CCR5 expression levels of CCR5+ cells as determined by the mean fluorescence intensity (MFI) were measured 2 days after infection by flow cytometry. Infection levels were determined 3 days postinfection using a luciferase readout. The means ± standard deviations (error bars) of three independent experiments are shown.

FIG. 5. Schematic diagram of the binding sites of the wild-type and MAb3952 HIV-1 envelopes and the CCR5 antibodies MAb3952 and RoAb13. (A) The CC1/85 and Bal wild-type (WT) envelopes used in this study predominately bind to the ECL-2 epitope on the CCR5 surface (dark blue). This binding is not affected by the NTD antibody RoAb13. (B) The ECL-2 antibodies MAb3952 and 2D7 can effectively block binding of the wild-type viruses due to their shared binding epitope in ECL-2. (C) During resistance development with MAb3952, virus variants (dark red) that shifted their binding from ECL-2 to the NTD of CCR5 were selected. (D) MAb3952-resistant virus strains are now sensitive to the NTD antibody RoAb13. The two amino acids Lys171 and Glu172 which have been mutated to Ala in the experiments in Fig. 4 are shown as the circled letters K and E, while the N-terminal eight amino acids deleted in the same experiment are depicted as a dark red bar. Please note that this is a simplified diagram and does not reflect the actual size of the different components.

as a negative control, MAGI CCR5 wild-type cells, and MAGI CXCR4 cells transfected with two mutants of CCR5. CCR5 N8 has a deletion of the eight N-terminal amino acids removing the N-terminal binding site of HIV- and NTD-specific antibodies (8, 29). In the CCR5 KE mutant, Lys171 and Glu172 are mutated to Ala, abolishing the ECL-2 binding site (8, 29). Flow cytometry experiments on these cells with the CCR5 C-terminal-specific antibody C-20 revealed that the majority (89% ± 12%) of the MAGI CCR5 WT cells expressed CCR5, while the N8 and KE mutants were expressed at lower levels (37% ± 6% and 49% ± 3%, respectively) (Fig. 4). CCR5 expression on MAGI CXCR4 cells was detected in 4% ± 1% of the cells, only slightly higher than in the isotype control (3% ± 1% [data not shown]). Expression levels as measured by mean fluorescence intensity (MFI) on the CCR5-positive cells were comparable between the N8 and KE mutants but approximately 4-fold lower than the wild-type CCR5 (Fig. 4).

Infection of the CCR5-deficient MAGI CXCR4 cells with the MAb3952-resistant or the NDC pseudovirus strains was at background levels. Furthermore, these viruses were not susceptible to the CXCR4 inhibitor AMD3100, indicating that all viruses are R5-tropic (Fig. 4 and data not shown). Infection with both NDC pseudoviruses was higher in CCR5 N8-expressing cells than in CCR5 KE-expressing cells, implying that these viruses preferentially use the ECL-2 binding site of CCR5 to infect cells (Fig. 4). In contrast, infection with the MAb3952-resistant viruses was higher in the MAGI CCR5 KE cells than in the CCR5 N8 cells, suggesting that the MAb3952-resistant virus strains preferentially use the N terminus of CCR5 for infection (Fig. 4). Infection was reduced in both CCR5 mutant cell lines, due to reduced surface expression compared to the wild-type CCR5 cell line (Fig. 4). Furthermore, while mutants of CCR5 are able to support infection, the efficiency of virus binding to CCR5 may be impaired, also contributing to a reduction in infection. The preferential usage by the MAb3952-resistant viruses of the N terminus of CCR5 for infection is consistent with the increased susceptibility to the N-terminal antibody RoAb13. These data support the hypothesis that a change in the binding site of HIV Env to CCR5 is the underlying mechanism for the resistance to CCR5 MAb MAb3952.

DISCUSSION

The goal of this study was to select for CCR5 MAb3952-resistant HIV-1 strains and to understand the underlying mechanism of action. Resistance to CCR5 inhibitors can be the consequence of a change in tropism, the use of an inhibitor-occupied CCR5 receptor, or an increase in affinity of the resistant viral envelope to the free receptor.

Here, we provide data that HIV can develop resistance against anti-CCR5 MAbs by a fourth mechanism, namely, by shifting binding from ECL-2 to the N terminus of CCR5 (schematically summarized in Fig. 5). The preferential binding of wild-type Bal and CC1/85 virus envelope to the ECL-2 of CCR5 is not affected or only minimally affected by the NTD-binding antibody RoAb13 (Fig. 5A) as seen by the poor inhibition in Fig. 1 and Table 1. In contrast, the two ECL-2-specific antibodies MAb3952 and 2D7 prevent the wild-type viruses...
from attaching to the ECL-2 domain of CCR5, resulting in high antiviral activity (Fig. 5B). We hypothesize that during the development of resistance, virus variants were selected that shifted their binding from ECL-2 to the NTD (Fig. 5C) and that the resulting virus is now resistant to the ECL-2 antibodies MAb3952 and 2D7 (Fig. 5C) but sensitive to the NTD-binding antibody RoAb13 (Fig. 5D). Interestingly, a similar observation of epitope switching has been reported recently by Nolan et al. (21). In this study, a deletion of four amino acids on the N-terminal side of the V3 stem of HIV Env changed the tropism of the originally dual-tropic virus to an R5-tropic virus. Infection with this virus was also more dependent on the CCR5 NTD in a CCR5/CCR2 chimera panel and could be inhibited with NTD-specific, but not ECL-2-specific, antibodies (21).

Mechanistically, epitope switching has parallels to two established resistance mechanisms, the change in coreceptor utilization and the use of ligand-bound receptor. However, in contrast to a complete change from one coreceptor to another, it is a more subtle change of different regions on the same coreceptor. The use of ligand-bound receptor has been reported as a resistance mechanism for small-molecule CCR5 antagonists, such as maraviroc and vicriviroc, and is consistent with an allosteric mechanism of inhibition (25, 28). In this case as well as with coreceptor epitope switching, the virus adapted to utilize the CCR5 receptor bound to the inhibitor, either in the form of a small molecule or an antibody. Resistance through use of the ligand-bound receptor is characterized by a reduced maximal inhibition rather than a shift of the dose-response curve to the right (25, 28). The MAb3952-resistant pseudoviruses used in Fig. 3 are only minimally inhibited at the top assay concentration of 20 μg/ml, and it is difficult to determine whether these virus clones would be fully inhibited at higher antibody concentration or if they would rather plateau at levels below 100%. However, in contrast to dose-response curves obtained with allosteric small-molecule inhibitors, like maraviroc and vicriviroc (25, 28), the dose-response curves in Fig. 3 clearly shift to the right, which is indicative of competitive inhibition.

Conceptually, a reciprocal escape mechanism is possible for antibodies that bind to the N terminus of CCR5. Based on this concept, multiple domain binding CCR5 antibodies, such as PRO 140 (formerly known as PA14) (23) or 45549 (18) or bispecific antibodies, which have been proposed for cancer therapy (4), should be more resilient to this resistance mechanism. Alternatively, antibodies that are coupled to a second, independent pharmacophore, such as a fusion inhibitor (12, 14, 15), can also provide a higher barrier to resistance.

Resistance to MAb3952 and increased susceptibility to RoAb13 was associated with multiple mutations throughout Env. A similar, diffuse accumulation of mutations has been described after in vitro resistance selection with the ECL-2-specific antibody 2D7 (1). Aarons et al. excluded the use of other coreceptors, such as CXCR4, CCR3, and CCR1, by the resistant viruses but did not further elucidate the underlying mechanism of escape to 2D7 (1). We defined the contribution of several individual amino acids in gp160 for resistance to MAb3952 and identified several mutations: the E84K L85M double mutation and K163N in V1 and V2 of Bal, respectively, and the T315A mutation in V3 of CC1/85. Introduction of these mutations increased the IC50 for MAb3952 severalfold. Several amino acids (R298, N301, T303, I322, D324, I325, and R326 in the stem of the V3 loop as well as R419, I420, K421, Q422, W427, and R444) have previously been implicated as important contributors to gp120 binding to the N terminus of CCR5 (5). Many of these amino acids interact with sulfotyrosine 14 in the N terminus of CCR5 (10). However, the increased use of the CCR5 NTD by the MAb3952-resistant viruses described here did not coincide with a mutation of any of these amino acids. Similarly, MAb3952 resistance and increased RoAb13 susceptibility was not associated with a deletion of amino acids 9 to 12 on the N-terminal side of the V3 stem as recently described (21). Finally, we did not detect a loss of N-glycan at position N403, which has been shown to increase binding of both the NTD and ECL-2 of CCR5 (24).

Surprisingly, we observed two mutations in the tip of the V3 loop of the CC1/85 NDC virus that confer resistance to maraviroc, 315T and 322V (28). During passaging with MAb3952, both mutations reverted to 315A and 322V in CC1/85 3952res. We therefore expect that the MAb3952-resistant CC1/85 virus is sensitive to maraviroco; however, experiments to formally prove this hypothesis have not yet been performed.

In conclusion, this study proposes a novel mechanism by which HIV can develop resistance to CCR5 antibodies by using alternative binding sites on CCR5. This novel escape route emphasizes the need of combination therapy even with new classes of antiretroviral drugs, such as CCR5 antibodies, and the ongoing, urgent need to develop new and highly active antivirals with nonoverlapping resistance profiles.

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