Reduced fitness of HIV-1 resistant to CXCR4 antagonists

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HIV-1 strains with a syncytium-inducing phenotype that use CXCR4 (X4 strains) have been associated with faster disease progression and AIDS. Antiviral agents designed to block CXCR4 may prevent the emergence of X4 HIV strains but resistant strains that maintain the X4 phenotype can be raised by sequential passage in cell cultures. We have demonstrated that a laboratory adapted strain (NL4-3) and a cloned clinical isolate (CI-1) of HIV-1 cultured in the presence of the CXCR4 antagonist, AMD3100, became resistant to the compound without a change in co-receptor use. These strains became resistant through divergence with respect to the wild-type virus. Conversely, a clinical isolate made resistant to AMD3100 switched co-receptor use from X4 to R5 through a change in diversity from the original virus population. When dual infection competition/heteroduplex tracking assays were performed, all AMD3100-resistant strains, regardless of co-receptor use showed a significantly diminished fitness compared with the wild-type virus. Single virus infections, at a similar multiplicity of infection, also indicated that the wild-type strains possess better replicative ability than their corresponding resistant strains. Thus, viral resistance development to a CXCR4 antagonist such as AMD3100 is associated with reduced viral fitness.

Introduction

Human immunodeficiency virus type 1 (HIV-1) strains isolated from recently infected individuals are predominantly macrophage-tropic and non-syncytium-inducing (NSI), and require CC-chemokine receptors such as CCR5 as entry cofactors in combination with CD4 [1,2] (R5 HIV strains [3]). T-tropic are rapidly replicating, syncytium-inducing (SI) strains that use the CXCR4 receptor (X4 strains); they appear much later after the primary infection and their emergence is associated with a rapid decline of CD4 T cells that heralds the breakdown of the immune system and the onset of AIDS [2,4–8].

A major strategy in the fight against AIDS may be based on the prevention of the emergence of the more pathogenic CXCR4-using strains of HIV. AM D3100 is a potent anti-HIV agent that is targeted at the CXCR4 receptor [9,10]. AMD3100 blocks the intracellular signal induced by SDF-1 but does not induce a signal by itself; thus, it can be considered as an antagonist of CXCR4. We have shown that from a culture containing a heterogeneous population of HIV, composed of laboratory adapted X4 (NL4-3) and R5 (BaL) strains, AM D3100 leads to the selection of R5 over the X4 strains [11]. Moreover, addition of AM D3100 to peripheral blood mononuclear cells (PBMC) from infected individuals displaying the syncytium-inducing (SI) phenotype resulted in a complete inhibition of X4 virus [11].

We have generated resistant virus to the chemokine SDF-1 through passaging of the virus in the presence of increasing concentrations of SDF-1α. The SDF-1α-resistant virus was about 10-fold less sensitive than the wild-type virus to the bicyclam AM D3100 and was not able to switch to CCR5 co-receptor use [12,13]. Here, we have extended the observations made with the SDF-1α-resistant HIV-1 to AM D3100-resistant strains with regard to co-receptor use. We have evaluated the replicative ability of the AM D3100-resistant viruses as compared to wild-type strains and found that development of resistance to the CXCR4 antagonist is associated with reduced fitness consequently to the alterations in the gp120 envelope glycoprotein.
Materials and methods

Compounds, viruses and cells

The bicyclam AM D 3100 was synthesized as described previously [14]. The CCR5 antagonist TAK-779 was received from the NIH AIDS Reagent Program. SDF-1α was purchased from Peprotech (London, UK). Azidothymidine (AZT) was purchased from Sigma (St Louis, M o., USA). The HIV-1 strains NL4-3 and BaL, and the CD4 lymphoid cell lines SUP-T1, HUT 78, MT-4 and MT-2 were obtained through the MRC AIDS Reagent Program. U87-CD4 cells expressing either CCR5 or CXCR4 were obtained from the NIH AIDS Research and Reference Reagent Program.

Co-receptor use by different HIV-1 isolates

U87-CD4 cells expressing either CCR5 or CXCR4 (5x10⁴) were infected with 10 ng of p24 antigen of the corresponding virus strain and incubated for 24 h. Then, the cells were washed twice with phosphate-buffered saline (PBS) and fresh DMEM medium was added. Cells were incubated for a further 5 days and p24 antigen in the culture supernatant was measured by a commercial ELISA test (Innogenetics, Barcelona, Spain).

Determination of viral fitness by replication competition of defined mixtures of viruses

MT-4 cells were infected with wild-type, AM D 3100-resistant strains or a mixture of both viruses (the percentage of each strain being 0, 40, 60 or 100% of the total p24 count) at low multiplicity of infection (0.003) defined as the number of virus CCID₅₀ used per cell as determined by the MTT method [15]. Cell supernatant was harvested at different time intervals and HIV-1 p24 antigen production was quantified by ELISA. After 5 day incubation at 37°C, cell supernatant was used to infect fresh uninfected MT-4 cells. After three to four passages, DNA was isolated from infected cells for DNA sequencing.

Heteroduplex tracking assay of two HIV-1 env fragments

Dual infection/competition experiments were performed with PBMC from one HIV-seronegative blood donor as previously described [16]. Briefly, six HIV-1 isolates (Figure 1, Table 1) were added to growth competition experiments along with each of four control primary HIV-1 isolates (SI/X4 strains, A-92UG029 and E-CM U06, and NSI/R5 strains, A-92RW009 and C-92BR025). Competition between each control viral isolate and a test HIV-1 strain as determined by the MTT method [15]. Cell supernatant was incubated with 1x10⁶ cells for 2 h at 37°C, 5% CO₂, then, the cells were washed three times with PBS and resuspended in complete medium. Cells were harvested at day 15 and proviral DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Valencia, Calif., USA). HIV-1 env fragments were PCR-amplified and analysed using the heteroduplex tracking analysis (HTA), as previously described [16]. For each HIV-1 competition experiment, the final ratio of the two viruses produced from each of the three dual infections was determined by HTA. A relative fitness (W) value for each virus was obtained from the average of the three independent dual infections. The ratio of relative fitness values of each HIV-1 variant in the competition (average of three dual infections) is a measure of the fitness difference (WD) between both HIV-1 strains (WD=WM/WL), where WM and WL correspond to the relative fitness of the more and less fit virus, respectively. Total relative fitness values correspond with the average of relative fitness values, derived from a competition between a test HIV-1 strain and either A-92UG029, E-CM U06, A-92RW009 or C-92BR025 [16].

DNA sequence analysis

The gp120 proviral genome was isolated by PCR amplification of total cellular DNA purified from infected cells. For sequencing of the V3 loop, preparative PCR was performed with 5–20 μg of total DNA, purified by the QIAamp DNA Blood Kit, and 0.1 μg of each of the primers TACAATGTACACATGGAATTGCTCTCCCTGGTCCCCTCTGG were used for the PCR amplification. For sequencing of the V2 loop, primers AATTAACCCCACTCTGTGTTAGTTTA and TCTGGGTCCCTGAGGA. For sequencing of the V2 loop, primers TCTGGGTCCCTGAGGA. For sequencing of the V3 loop, primers TACAATGTACACATGGAATTGCTCTCCCTGGTCCCCTCTGG were used for the PCR amplification.

Table 1. Amino acid sequence alignment of the V3 loop region of HIV-1

<table>
<thead>
<tr>
<th>HIV-1 isolate</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HxB2</td>
<td>CTPRNNTKRIRIRQGPRGRAFVTIGK-GMRRQAC</td>
</tr>
<tr>
<td>Cl-1wt</td>
<td>CTPRNNTKRIRIRQGPRGRAFVTIGKIGDIRQAC</td>
</tr>
<tr>
<td>Cl-1res</td>
<td>CTPRNNTKRIRIRQGPRGRAFVTIGKIGDIRQAC</td>
</tr>
<tr>
<td>Cl-2wt</td>
<td>CTPRNNTKRIRIRQGPRGRAFVTIGKIGDIRQAC</td>
</tr>
<tr>
<td>Cl-2res</td>
<td>CTPRNNTKRIRIRQGPRGRAFVTIGKIGDIRQAC</td>
</tr>
</tbody>
</table>

Positions are shown for wild-type (wt) and AM D 3100-resistant (res) strains, relative to the sequence of HIV-1 HxB2 corresponding to gp160 position numbers 296–331. a [13], b [11].
first PCR reaction, and primers AATTAACCC-
CACTCTGTGTTAGTTTA and
TGATACTACTGGCCTGATTCCA for a second
preparative PCR. DNA sequencing was performed
directly on the purified PCR product following the
protocol provided by the ABI PRISM
cycle sequencing kit and analysed by an ABI PRISM
377 genetic sequencer. The Navigator and Factura DNA analysis
software (Perkin Elmer) were used to identify and quan-
tify ambiguous regions of the DNA sequence that are
produced when mixture of two sequences are detected.

Results

Resistance to AMD3100 is accompanied by
mutations in the V3 loop
Figure 1 shows the mutations found in the V3 loop of the
previously reported AM D3100-resistant, NL4-3-derived
strain [13], and of two clinical isolates that have been
made resistant to AM D3100 after infection of M T-4 cells
(CI-1 strain) or PBMC (CI-2 strain) [11] in the presence
of AM D3100. As previously shown [13,17], HIV-1 resis-
tance to the CXCR4 antagonist is associated with the
emergence of mutations in the gp120 sequence and, in
particular, in the V3 loop region. Notably, resistance to
AM D3100 could be developed through different, and
probably unrelated, patterns of mutations depending on
the parental wild-type virus used for selection.

Infection of cells with wild-type and
AMD3100-resistant strains
As shown in Table 1, both wild-type NL4-3 and
AM D3100-resistant viruses replicated in U87-CD4-
CXCR4 cells. No p24 antigen was detected in
U87-CD4-CXCR5 cells infected with either virus. Both
the wild-type clinical isolate 1 (CI-1) and the virus made
resistant to AM D3100 after sequential passage in M T-4
cells could replicate in U87-CD4-CXCR4 and U87-
CD4-CXCR5 strains. Thus, wild-type CI-1 and
AM D3100-resistant CI-1 could be defined as dualtropic
(R5X4) strains. Clinical isolate 2 (CI-2) could grow in
CXCR4-expressing cells, but after passage in PBMC in
the presence of AM D3100, it gained CCR5 use and lost
CXCR4 use in U87-CD4 cells. The HIV-1 BaL strain
could only replicate in U87-CD4-CXCR5 cells (Table 1).

Growth kinetics of wild-type and AMD3100-resistant
strains
To assess the replicative capacity of virus resistant to
CXCR4 antagonists, M T-4 cells were infected at a
similar moi (0.003) of wild-type CI-1, NL4-3 or
AM D3100-resistant CI-1 and NL4-3, or a mixture of
wild-type and its corresponding resistant virus. Cell
culture supernatants were evaluated for p24 antigen
production every 24 h for a 5-day period. As seen in Figure 2, the
wild-type virus of both the CI-1 and NL4-3 showed
higher virus production than the corresponding resistant
virus variants.

Determination of viral fitness by replication
competition of defined mixtures of viruses
The nucleic acid sequence of a fragment of the HIV-1 V3
region of gp120 from proviral DNA isolated from cells
infected with either wild-type or AM D3100-resistant
virus, or with mixtures of two virus strains was deter-
mmed. Proviral DNA sequence determination may serve
as a marker of the viral fitness of each strain [11]. As
expected, the DNA sequence corresponding to either

Table 1. Replication of different HIV-1 strains in U87-CD4 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Wild-type</th>
<th>AMD3100-res</th>
<th>Wild-type</th>
<th>AMD3100-res</th>
<th>Wild-type</th>
<th>AMD3100-res</th>
<th>BaL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NL4-3</td>
<td>CI-1</td>
<td>NL4-3</td>
<td>CI-1</td>
<td>NL4-3</td>
<td>CI-1</td>
<td></td>
</tr>
<tr>
<td>U87-CD4-CXCR4</td>
<td>&gt;60 000</td>
<td>&gt;60 000</td>
<td>&gt;60 000</td>
<td>&gt;60 000</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>U87-CD4-CCR5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&gt;60 000</td>
<td>&gt;60 000</td>
<td>&lt;5</td>
<td>&gt;10 000</td>
<td>44 900</td>
</tr>
<tr>
<td>Sensitivity to AMD3100 (EC50 µg/ml)†</td>
<td>0.005</td>
<td>0.1</td>
<td>0.005</td>
<td>0.5</td>
<td>0.01</td>
<td>&gt;5</td>
<td>&gt;5</td>
</tr>
</tbody>
</table>

*U87-CD4 cells (5x10⁵) expressing CXCR4 or CCR5 were infected with 10 ng of p24 antigen of the corresponding virus. At 24 h post-infection, cells were washed
twice in PBS and resuspended in fresh medium. Five days after infection, p24 antigen in the cell supernatant was measured by a commercial test (Innogenetics, Barcelona, Spain).

† EC50 or the concentration required to inhibit by 50% the replication of the corresponding HIV-1 strain as measured by the MT-4 - MTT method [15] for HIV-1
strains CI-1 and NL4-3 and the corresponding AMD3100-resistant strains, or evaluation of p24 antigen production of infected PBMC for HIV-1 BaL, CI-2 and the
corresponding AMD3100-resistant strain, as described [11].
wild-type CI-1 or AM D3100-resistant CI-1 virus was found if the cells were infected for 100% with wild-type or AM D3100-resistant strain, respectively (Figure 3). However, in a mixed virus infection and after 15 days of culture in MT-4 cells, only the DNA sequence corresponding to the wild-type strain could be recovered. Similar results were obtained when the experiment was done with the NL4-3 virus and the corresponding AM D3100-resistant virus (data not shown).

Detection and quantification of both HIV-1 strains in a dual infection/competition by HTA
To better characterize the fitness of wild-type and AM D3100-resistant virus, we used a modified HTA to detect and quantify both HIV-1 isolates in the dual infections. Dual exposure and infection of host cells provides the best in vitro measure of relative HIV-1 fitness.

For these growth-competition experiments, we performed three dual infections at different moi of both viruses (see 'Materials and methods') in PBM C. This technique to evaluate viral fitness has been validated before [16] with a number of control HIV-1 isolates of different subtypes that were selected based on moderate but different fitness values in pairwise competition experiments. Based on HTA results, we calculated relative fitness values for both isolates in each competition experiment (that is, three dual infections). The relative fitness of each HIV-1 isolate was calculated as the average production in the three dual infections divided by the initial inoculum. As can be seen in Figure 4a, the wild-type strains of CI-1, NL4-3 and CI-2 showed greater fitness than the corresponding AM D3100-resistant strains in dual infection competition experiments in PBM C.

Fitness difference in a competition between two HIV-1 isolates was obtained as described in 'Materials and methods'. Competition between each control strain and wild-type or AM D3100-resistant HIV-1 isolates involved the same three dual infections and fitness calculation as described above. Figure 4b displays the fitness difference relative to four HIV-1 control strains. Even though the equation for WD (WD = WM/WL) always produces a positive value, WD was plotted as negative when the control strain was more fit than the test isolate, and vice versa for a positive WD. The wild-type isolates generally out-competed the control strains in growth competition experiments. However, AM D3100-resistant CI-1, NL4-3 and CI-2 were less fit than the subtype E control strain (CMU06) and had, in general terms, less or similar fitness than the subtypes A strains (92UG029 and 92RW009) and C (92BR025) strain.

Discussion
Initial studies with AM D3100 showed that after 63 passages with increasing doses, the mutant virus had 15 amino-acid changes scattered throughout gp120, indicating that escape from AM D3100 is not easy. A similar experiment with SDF-1 showed that the escape mutant still used CXCR4, but in an AM D3100-insensitive manner. The AM D3100-resistant virus could still grow in CXCR4+, CCR5-, MT-4 cells, suggesting that it still used CXCR4 as co-receptor. However, alternative co-receptor use has not been formally addressed. Here, we show that the X4 N L4-3 AM D3100-resistant strain became resistant through mutations in the gp120 without gain of CCR5 use and that the dual tropic CI-
1 retained both CXCR4 and CCR5 usage (Table 1). These strains did not gain alternative co-receptor use when tested in HOS cells expressing CCR2b, CCR3 and CCR4 (data not shown).

HIV-1 resistance to AMD3100 may arise by two different mechanisms. In a heterogeneous population of HIV-1 strains that use either CCR5 or CXCR4 to enter CD4 cells, HIV quasispecies that are naturally
resistant to blockade of CXCR4 (R5 strains) rapidly outgrow the CXCR4-using strains [11]. In many clinical isolates of X4 phenotype, R5/AMD3100-resistant strains are maintained at low-level replication but are clearly less fit. Resistance to AMD3100 occurs through a change in diversity of the infecting population, that is, a shift from CXCR4- to CCR5-using virus strains [11].

The AMD3100-resistant viruses selected in CXCR4+, CCR5– cells were derived from clonal parental viruses (NL4-3 or CI-1) that are highly homogeneous. We have shown that the NL4-3-derived AMD3100-resistant virus is cross-resistant to agents that block gp120 function [18] and other CXCR4-blocking agents [12,19], and that the accumulation of mutations leads to the emergence of the resistant phenotype [20]. Therefore, the emergence of resistance to CXCR4 antagonists, without a change in co-receptor use, may occur by a significant change in divergence from the parental strain.

It is intriguing how HIV-1 overcame anti-HIV activity of AMD3100 but retained CXCR4 use. Co-receptor switching for the AM D3100-resistant CI-1 (Table 1) and the AM D3100-resistant NL4-3 [13] was not possible since a CCR5-negative cell line (M T-4) was used for development of resistance. There may be more than one way for gp120 to interact with CXCR4, while still permitting fusion, since different HIV-1 strains have different requirements for their interaction with CXCR4 [21,22]. It is obvious that in the presence of AM D3100, and without an alternative co-receptor, HIV will have to evolve so as to use CXCR4 differently. Changes in the envelope glycoproteins have been associated with increased pathogenicity of the resulting virus [23,24]. Env-associated cell death could be mediated by membrane fusion-dependent processes [25]. We have shown that cells expressing HIV-1 IIIB-env may induce apoptosis [26] through a signal that is independent of CXCR4-G-protein-coupled signalling [26], but can be inhibited by AM D3100 [27]. CXCR4 may induce intracellular signals through at least two described pathways [28,29] that, in turn, may have a distinct effect on cell activation and viability. Differential use of CXCR4 by gp120 could lead to the onset of intracellular signals that induce T-cell death before virus replication occurs or alternatively, that directly hinder or block HIV replication. In single virus infections, AM D3100-resistant X4 strains showed reduced replication capacity compared with the corresponding wild-type virus (Figure 2). These experiments were done by infecting M T-4 cells at the same moi defined as the number of CCID50 used per cell. That is, at an infectious dose that allowed for similar virus-dependent cytopathicity but that generated increased replication by the wild-type virus, suggesting that AM D3100-resistant X4 strains may be more cytopathic. We also show that the virus strain that was selected after treatment with AM D3100 had a lower fitness than the corresponding wild-type strain (Figure 4). In the case of the clinical isolate (CI-2), the strains that maintained a lower replication ability were rapidly selected. In the case of the X4 strains (NL4-3 and CI-1), selection was slow and also led to lower fitness.
Differential use of CXCR4 induced under AMD 3100-selective pressure, for a virus that is already adapted for CXCR4 use, appears to be a disadvantage. Evolution should favour HIV strains that have a functional envelope glycoprotein that allows for sufficient virus-chemokine receptor interaction to continue productive viral replication.

Taken together, the significant number of mutations that are necessary for resistance without a change in co-receptor use, the lower viral fitness that is associated with an altered affinity for CXCR4 and the availability of alternative co-receptors to evade the blockade of CXCR4, suggest that CXCR4-using strains that are resistant to CXCR4 antagonists are unlikely, though not excluded, to emerge in vivo. Finally, our results support the hypothesis that virus-cell fusion is an important determinant of HIV-induced cell death.

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