CAPÍTULO 5

EFECTO DEL BLOQUEO DEL RECEPTOR DE QUIMIOCINAS CXCR4

5.1.- T-CELL-LINE-TROPIC HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 THAT IS MADE RESISTANT TO STROMAL CELL-DERIVED FACTOR 1α CONTAINS MUTATIONS IN THE ENVELOPE gp120 BUT DOES NOT SHOW A SWITCH IN CORECEPTOR USE

5.2.- SHIFT OF CLINICAL HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ISOLATES FROM X4 TO R5 AND PREVENTION OF EMERGENCE OF THE SYNCYTIUM-INDUCING PHENOTYPE BY BLOCKADE OF CXCR4

T-CELL-LINE-TROPIC HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 THAT IS MADE RESISTANT TO STROMAL CELL-DERIVED FACTOR 1α CONTAINS MUTATIONS IN THE ENVELOPE gp120 BUT DOES NOT SHOW A SWITCH IN CORECEPTOR USE

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ABSTRACT

The NL4.3 T-cell-line-tropic human immunodeficiency virus type 1 strain is sensitive to the CXC chemokine stromal cell-derived factor 1a (SDF-1α), the natural ligand for CXC chemokine receptor 4 (CXCR4); the 50% inhibitory concentration (IC_{50}) in MT-4 cells is 130 ng/ml. We generated resistant virus through passaging of the virus in the presence of increasing concentrations of SDF-1α. After 24 passages, the virus was no longer sensitive to SDF-1α (SDF-$1\alpha^{res}$ virus) (IC_{50} ,>2μg/ml) and became resistant to SDF-1β (IC_{50} ,>2μg/ml) and to a specific CXCR4 monoclonal antibody (IC_{50} , >20 μg/ml). The SDF-$1\alpha^{res}$ virus was about 10-fold less sensitive than the wild-type virus to the bicyclam AMD3100, a specific CXCR4 antagonist. The SDF-$1\alpha^{res}$ virus contained the following mutations in the gp120 molecule: N106K in the V1 loop; S134N and F145L in the V2 loop; F245I in the C2 loop; K269E, Q278H, I288V, and N293D in the V3 loop; a deletion of 5 amino acids (FNSTW) at positions 364 to 368 in the V4 loop; and R378T in the CD4 binding domain. Replication of the NL4.3 wild-type virus and the SDF-$1\alpha^{res}$ virus was demonstrated in U87 cells that coexpressed CD4 and CXCR4 (U87.CD4.CXCR4) but not in U87.CD4.CCR5 cells. Thus, the resistant virus was not able to switch to the CC chemokine receptor 5 (CCR5) coreceptor (the main coreceptor for macrophage-tropic viruses). The SDF-$1\alpha^{res}$ virus replicated in HOS.CD4 cells expressing CCR1, CCR2b, CCR3, CCR4, CCR5, and CXCR4 but also in HOS.CD4.pBABE cells. However, all HOS transfectant cells expressed a low level of CXCR4. Neither of the two virus strains was able to infect HOS.CXCR4 or HOS.CCR5 transfectants, demonstrating the ne-cessity of the CD4 receptor. The T-cell-line-tropic SDF-$1\alpha^{res}$ virus was thus able to overcome the inhibitory effect of SDF-1α through mutations in gp120 but still needed CXCR4 to enter the cells.

JOURNAL OF VIROLOGY
Volumen 72, Number 5, 1998, p: 4032-4037

INTRODUCTION

CXC chemokine receptor 4 (CXCR4) was recently shown to be a coreceptor used by T-cell-line-tropic (T-tropic) human immunodeficiency virus (HIV) strains to enter target cells (5, 27), whereas CC chemokine receptor 5 (CCR5) allows the entry of macrophage-tropic (M-tropic) HIV strains (2, 9, 16, 20, 21). The CXC chemokine stromal cell-derived factor 1α (SDF-1α), the natural ligand for CXCR4, has been shown to inhibit T-tropic (such as the NL4.3 strain) but not M-tropic viruses and to inhibit primary HIV isolates (6, 33). Also, CXCR4 is used by HIV type 2 (HIV-2) strains to enter cells, even in the absence of the CD4 receptor (23). Monoclonal antibody (MAb) 12G5 specifically binds to CXCR4 and inhibits infection with T-tropic HIV type 1 (HIV-1) strains, dual-tropic HIV-1 strains, and HIV-2 strains (32), although variation in its antiviral activity has been described, depending on the viral strain and the target cells used in the assays (32, 42). A change in coreceptor use from predominantly CCR5 toward CXCR4 is correlated in HIV-1-infected patients with progression to AIDS (40), and this change is also associated with a switch from the non-syncytium-inducing to the syncytium-inducing phenotype (41) and a decrease in $CD4^+$ T-cell counts. During disease progression in patients, the virus expands its coreceptor use to CCR5, CCR3, CCR2b, and CXCR4. The use of these coreceptors is dependent on the sequence of the V3 loop of viral gp120 (10, 44, 45). However, many virus strains are capable of using more than one coreceptor. Typically T-tropic syncytium-inducing viruses not only use CXCR4 to infect cells but also can use other coreceptors, such as CCR5 (8, 39). Recently, several new coreceptors were identified: Bonzo/ STRL33 (3, 17, 31), BOB/GPR15 (17, 26), GPR1 (26), and US28 (35); these can also be used by immunodeficiency viruses to enter cells.

We previously reported the development of HIV-1 resistance to the polyanion dextran sulfate (DS) (25), the oligonucleotide AR177 (24), and the bicyclam derivative AMD3100 (18), which are all potent inhibitors of HIV-1 and HIV-2 replication (4, 14, 15, 34). The first two compounds inhibit binding and fusion of the virus (4, 38); the bicyclam does not inhibit virus binding but acts as a specific CXCR4 antagonist and therefore inhibits entry of the virus into the cells (19, 36, 40). For all of the NL4.3 virus strains that were made resistant to DS (DS^{res} virus) (25), AR177 ($AR177^{res}$ virus) (24), or AMD3100 ($AMD3100^{res}$ virus) (18), mutations were always situated in *env* glycoprotein 120 (gp120).

Here we describe a T-tropic NL4.3 virus strain that was made resistant to the CXC chemokine SDF-1α (the SDF-1α^{res} virus strain). We investigated the pattern of cross resistance of the virus to other inhibitors of virus binding and fusion. The resistant phenotype of the SDF-1α^{res} virus could be attributed to a number of mutations in gp120. The SDF-1α^{res} mutant did not change its coreceptor use.

MATERIALS AND METHODS

Virus stocks and cell lines. The HIV-1 T-tropic molecular clone NL4.3 (1) was obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program. Human osteosarcoma HOS.CD4 cells, which express human CD4 and the chemokine receptors CCR1, CCR2b, CCR3, CCR4, CCR5, and CXCR4 or pBABE, and HOS cells, which express CCR5 or CXCR4 (12, 17), were obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program. Astroglioma U87.CD4 cells transfected with CXCR4 or CCR5 were kindly provided by Nathaniel R. Landau. The transformed MT-4 T-cell line has been described elsewhere (28). The AMD3100-resistant NL4.3 virus was generated as described previously (18, 19). Cells were infected with different concentrations of virus, and the supernatant was collected 5 to 10 days after infection and stored at -20°C (17, 25). HIV-1 core antigen in the culture supernatant was analyzed with the p24 antigen enzyme-linked immunosorbent assay kit from DuPont (Brussels, Belgium).

Compounds and chemokines. DS (molecular weight, 5,000), a sulfated polysaccharide, was purchased from Sigma Chemie (Deisenhofen, Germany). The bicyclam derivatives AMD2763 and AMD3100 were synthesized as described previously (7) and kindly provided by Geoffrey Henson (AnorMed, Langley, Canada). Oligonucleotide AR177, also called T30177 or Zintevir, was provided by Robert F. Rando (Aronex Pharmaceuticals, The Woodlands, Tex.). 3'-Azido-3'-deoxythymidine (AZT) was obtained from Wellcome (Beckenham, United Kingdom). The CXC chemokine SDF 1α, SDF-1β, and the anti-CXCR4 MAb 12G5 were obtained from R &D Systems Europe Ltd., Oxon, United Kingdom.

MAbs and flow cytometric analyses. The anti-gp120 MAb NEA9305 (DuPont), specifically recognizing the V3 loop epitope RIQRGPGRAFVTGK of HIV-1, was used. The anti-CD4 MAb Leu-3a and isotype-matched control MAbs were purchased from Becton Dickinson (Erembodegem, Belgium). The staining protocols

were described in detail elsewhere (37, 38). Cells were analyzed with a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) flow cytometer. Data were acquired and analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems) on an Apple Macintosh computer.

Selection of HIV-1 NL4.3 mutant strains. MT-4 cells were infected with HIV-1 NL4.3 in medium containing SDF-1α at 100 ng/ml. Cultures were incubated at 37°C until an extensive cytopathic effect (CPE) was observed (4 to 5 days). The culture supernatant was used for further passage of virus in MT-4 cells in the presence of increasing concentrations of SDF-1α up to 2 μg/ml.

DNA sequence analysis of gp120. MT-4 cells were infected with wild-type virus or SDF-1α^{res} virus and incubated for 4 days at 37°C. The cells were washed in phosphate-buffered saline, and total DNA was extracted with a QIAamp blood kit (Qiagen, Westburg, The Netherlands). PCR amplification was performed with ULTMA DNA polymerase with proofreading capacity (Perkin-Elmer Cetus, Norwalk, Conn.) according to De Vreese et al. (18). The PCR product was electrophoresed in an agarose gel, and the relevant band was excised and purified with a QIAquick purification kit. DNA sequencing was performed as described in detail by Esté et al. (25), and sequences were analyzed with DNA Navigator software (Perkin-Elmer).

RESULTS

Selection of the SDF-1α^{res} strain. HIV-1 NL4.3 was passaged in MT-4 cells in the presence of SDF-1α at a starting concentration corresponding to the 50% inhibitory concentration (IC_{50}) (100 ng/ml). Virus replication was monitored microscopically by the appearance of CPE. Every 4 or 5 days, the replicating virus was passaged in fresh uninfected cells in the presence of SDF-1α at the same concentration as in the previous passage or at a twofold higher concentration, depending on the CPE observed. After 24 passages (100 days), virus that was fully able to replicate in MT-4 cells in the presence of 2 μg of SDF-1α per ml was recovered. Virus from passage 20 could grow in the presence of 2 μg of SDF-1α per ml, and the induced CPE was comparable to that of the wild-type virus.

To demonstrate the gradual decrease in the antiviral activity of SDF-1α, MT-4 cells were infected with 100 50% cell culture infective doses ($CCID_{50}$) of the HIV-1 NL4.3 wild type or NL4.3 SDF-1α^{res} from passage 10 or passage 24, and SDF-1α was

added to the cells at different concentrations up to 1 μg/ml. At 5 days after infection, the cells were analyzed for CD4 expression because productive infection of MT-4 cells by T-tropic viruses is accompanied by the disappearance of CD4 from the T-cell surface (13). The uninfected MT-4 cells were 96% $CD4^+$ (Fig. 1, top panel) (37), whereas only 2, 4, and 1% of cells infected with the NL4-3 wild type, NL4-3 SDF-1α^{res} (passage 10),

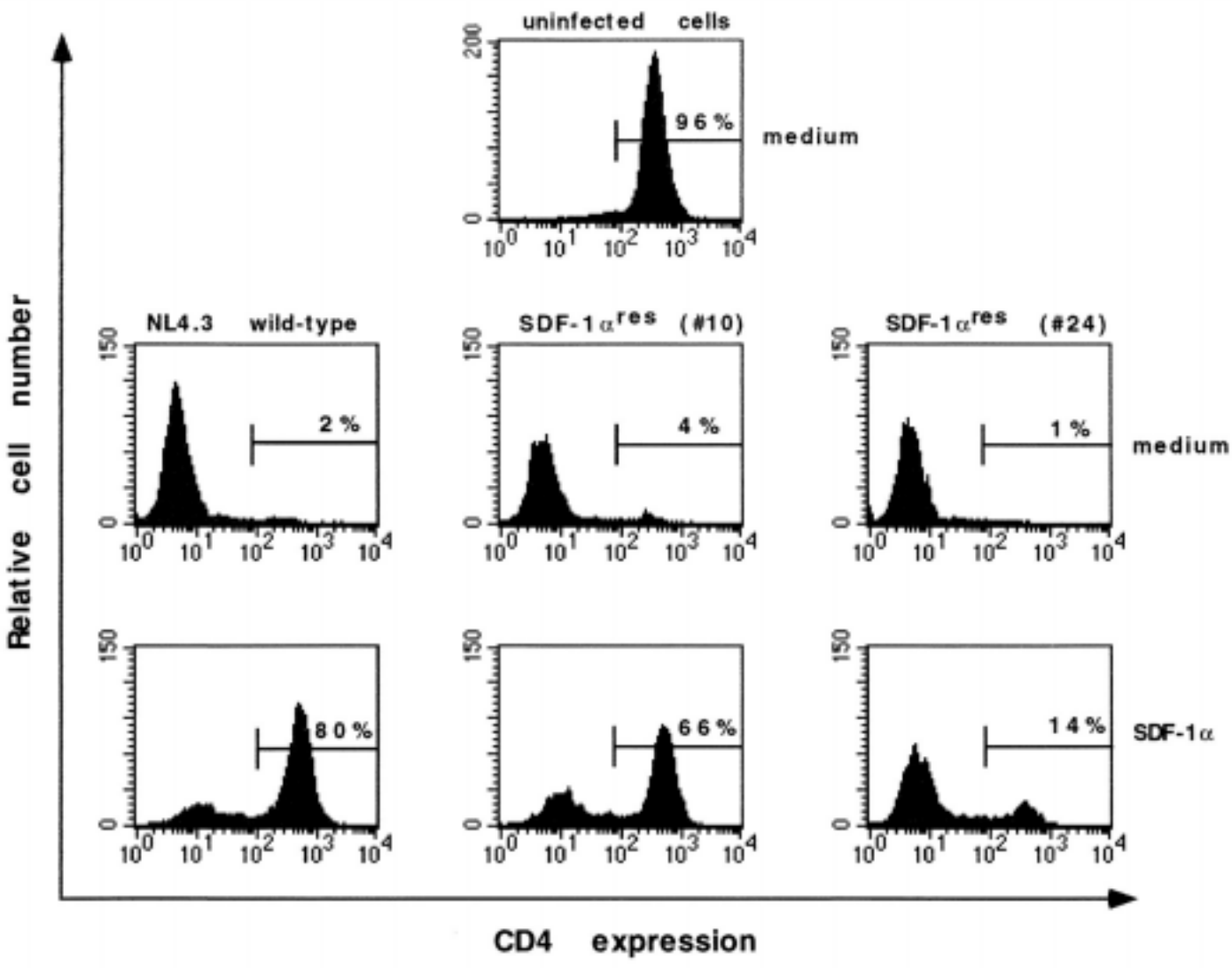


Figure 1. Effect of SDF-1α (1 μg/ml) on NL4.3 wild-type, SDF-1α^{res} (passage 10), and SDF-1α^{res} (passage 24) HIV-1 replication in MT-4 cells, as monitored by CD4 expression. Cells were infected with the virus strains at 100 $CCID_{50}$ in the presence or absence of SDF-1α and stained 5 days after infection with MAb Leu-3a directly labeled with phycoerythrin. As a control, uninfected cells also were stained with MAb Leu-3a. The percentage of $CD4^+$ cells (uninfected cells) is indicated in each histogram.

and NL4-3 SDF-1α^{res} (passage 24), respectively, expressed CD4 (Fig. 1, middle panels). As can be seen in the lower panels of Fig. 1, SDF-1α was active against the NL4.3 wild type (80% of the cells still expressed CD4), less active against NL4.3 SDF-1α^{res} (passage 10) (66% CD4^{+}), and virtually inactive against NL4.3 SDF-1α^{res} (passage 24) (14% CD4 1). The IC_{50} s of SDF-1α, as calculated from CD4 expression in these cultures, were 150 ng/ml for the wild type, 800 ng/ml for SDF-1α^{res} (passage 10), and >1 μg/ml for SDF-1α^{res} (passage 24). These IC_{50} s were comparable to the IC_{50} s calculated from the p24 antigen contents of these cultures. In all of the further experiments, NL4.3 SDF-1α^{res} virus (passage 24) was used and referred to as SDF-1α^{res}.

In Fig. 2, MT-4 cells were infected with the wild-type virus and the SDF-1α^{res} virus and analyzed for gp120 expression with an anti-gp120 MAb (NEA9305) 5 days after infection. The expression of gp120 in SDF-1α^{res} virus-infected cells (94%) (Fig. 2E) was comparable to that in wild-type virus-infected cells (91%) (Fig. 2B). SDF-1α at 1 μg/ml was highly protective against the wild-type virus (only 19% of the cells expressed gp120) (Fig. 2C) and inactive against the SDF-1α^{res} virus (93% gp120-positive cells) (Fig. 2F). The IC_{50} s of SDF-1α, as calculated from gp120 expression in these cultures, were 90 ng/ml for the wild-type virus and >1 μg/ml for the SDF-1α^{res} virus. Again, these IC_{50} s were comparable to those calculated from the p24 antigen contents of these cultures.

Cross-resistance to other compounds. The wild-type virus that had been grown in MT-4 cells in parallel with the SDF-1α^{res} virus but in the absence of SDF-1α was as sensitive as the original virus stock to SDF-1α (IC_{50} , 130 ng/ml) (Table 1). SDF-1β, which differs from SDF-1α only in four carboxyterminal amino acids (6, 43), was inactive against the SDF-1α^{res} virus, and it was somewhat less active against the wild-type virus than SDF-1α (IC_{50} , 200 ng/ml). We also examined the effect of the anti-CXCR4 MAb 12G5 on the replication of the wild-type virus and the SDF-1α^{res} virus. MAb 12G5 inhibited the replication of the NL4.3 wild-type by 50% at 8 μg/ml; however, the anti-CXCR4 MAb had no effect whatsoever on the replication of the SDF-1α^{res} virus up to a concentration of 20 μg/ml (Table 1).

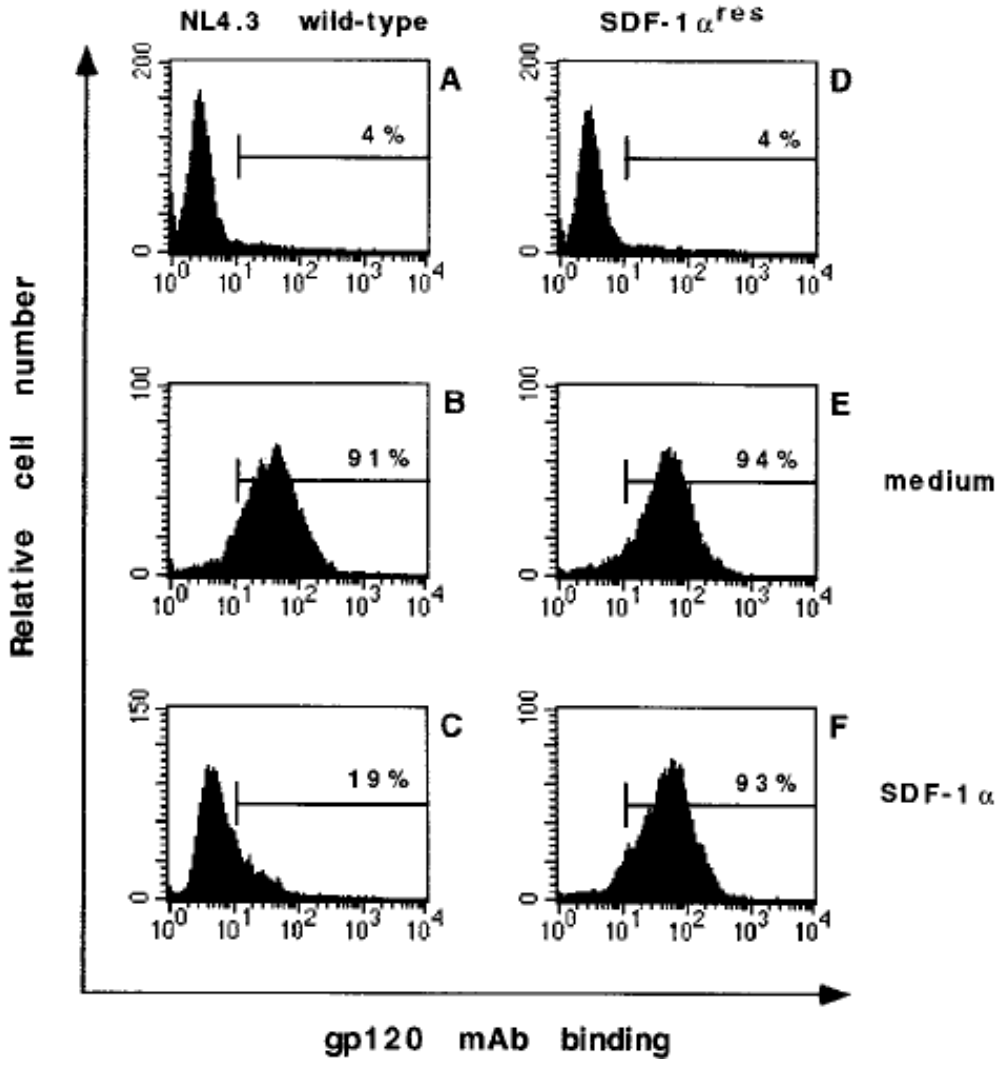


Figure 2. Effect of SDF-1α (1 μg/ml) on NL4.3 wild-type (A, B, and C) and SDF-1α^{res} (D, E, and F) HIV-1 replication in MT-4 cells, as monitored by anti gp120 MAb binding. Cells were infected with the virus strains at 100 $CCID_{50}$ and stained 4 days after infection with MAb NEA9305. The percentage of gp120-positive cells (HIV-1-infected cells) is indicated in each histogram. In panels A and D, cells were stained with the secondary antibody only.

We recently demonstrated that the bicyclams are specific CXCR4 antagonists (36, 40). Therefore, two prototypes, AMD2763 and AMD3100, were tested for their antiviral activity against the SDF-1α^{res} virus. The SDF-1α^{res} virus proved partially cross-resistant to AMD2763 and AMD3100 (10-fold decrease in sensitivity) (Table 1). The SDF-1α^{res} virus was not cross-resistant to the HIV binding or fusion inhibitors AR177 (Zintevir) and DS (Table 1) and the reverse transcriptase inhibitor AZT (Table 1). SDF-1α and AMD2763 were completely inactive against the AMD3100-resistant

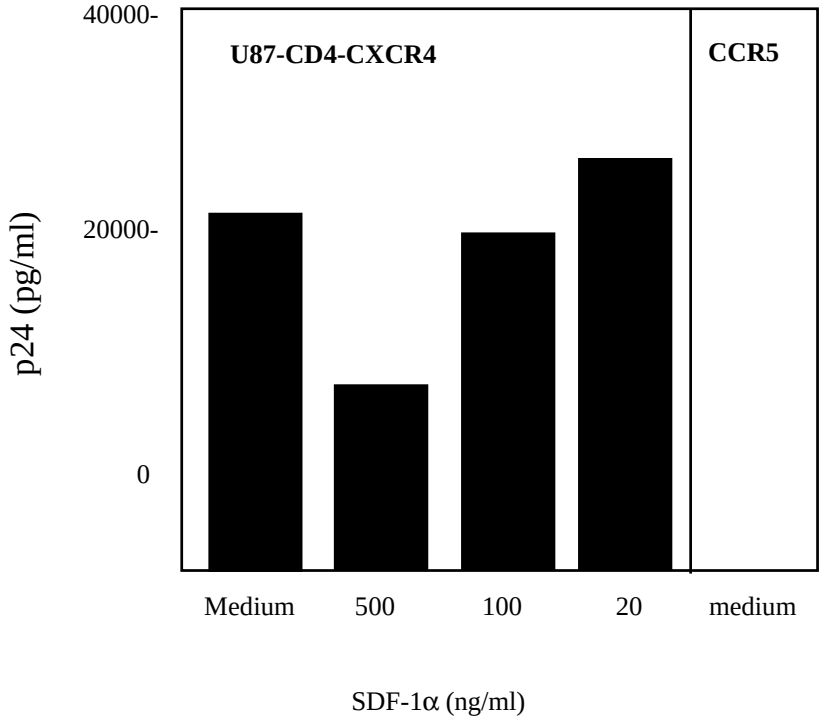
virus (18, 36) (Table 1), the CXC chemokine SDF-1β and the anti-CXCR4 MAb had no activity against the AMD3100^{res} virus (Table 1), but the AMD3100^{res} virus still retained marked sensitivity to AMD3100 (18) (Table 1).

Table 1. Anti-HIV activity of SDF-1α, SDF-1β, MAb 12G5, and other compounds against wild-type, SDF-1α^{res}, and AMD3100^{res} viruses in MT-4 cells [a]

Anti-HIV agent	IC_{50} (μg/ml) for:		
	Wild-type Virus	SDF-1α^{res} virus	AMD3100^{res} virus
SDF-1α	0.130	> 2	>2
SDF-1β	0.200	>2	>2
MAb 12G5	8	>20	>20
AMD3100	0.006	0.065	0.689
AMD2763	0.3	2.5	>25
DS	0.2	0.2	>5
AR177	1.1	1.5	>5
AZT	0.0006	0.0008	0.0005

[a] Virus yield was monitored in the cell-free supernatant from MT-4 cells 4 to 5 days after infection by a viral p24 antigen enzyme-linked immunosorbent assay. Results represent mean values from three to eight separate experiments.

U87.CD4 transfectants. To determine whether the SDF-1α^{res} virus might use a different coreceptor in MT-4 cells, the replication of the SDF-1α^{res} virus was tested in the astroglioma cell line U87 stably expressing CD4 and CXCR4 or CD4 and CCR5 (17). Cells were incubated with 10^3 pg of p24 from either wild-type or SDF-1α^{res} virus per ml, and the p24 concentrations were measured 6 to 10 days later. Both virus strains were able to infect U87.CD4.CXCR4 at comparable levels (Fig. 3). SDF-1α was active against the wild-type virus in these transfected cells, although to a somewhat lesser extent than in MT-4 cells (Table 1), other $CD4^+$ T-cell lines (data not shown), or peripheral blood mononuclear cells (36). SDF-1α had no significant activity against the SDF-1α^{res} virus (Fig. 3B). The T-tropic NL4.3 wild-type virus was, as expected, not able to infect U87.CD4.CCR5 cells, and the SDF-1α^{res} virus was not able to replicate in these cells either (less than 5 pg of p24 per ml; under the detection limit) (Fig. 3).



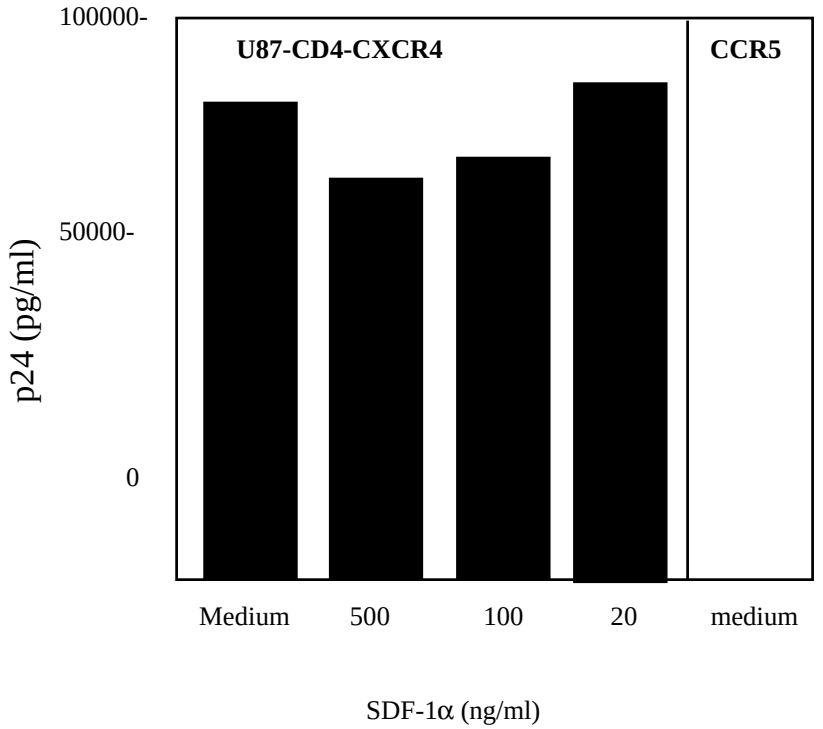


Figure 3. Replication of SDF-1α^{res} virus in U87 cells. U87.CD4.CXCR4 and U87.CD4.CCR5 cells were infected with NL4-3 wild-type (A) and SDF-1α^{res} (B) HIV-1 in the presence of different concentrations of SDF-1α (500, 100, and 20 ng/ml). p24 antigen levels were measured 8 days after infection.

As controls, the M-tropic HIV-1 BaL strain and simian immunodeficiency virus strain MAC251, known to use CCR5 to enter cells (2, 22), were found to replicate in U87.CD4.CCR5 cells (data not shown). When U87.CD4.CCR5 cells were incubated with 10^4 pg of p24 from either wild-type or SDF-1α^{res} virus per ml, the concentrations of p24 measured 6 to 10 days later were still below the detection limit. These results suggest that both the wild-type and the SDF-1α^{res} virus strains use CXCR4 for entry and infection and that the SDF-1α^{res} virus cannot switch to CCR5 as a coreceptor for entry.

HOS transfectants. The SDF-1α^{res} virus replicated in HOS. CD4.CCR1, HOS.CD4.CCR2b, HOS.CD4.CCR3, HOS.CD4. CCR4, HOS.CD4.CCR5, and HOS.CD4.CXCR4 cells equally well, whereas the NL4.3 wild-type virus replicated preferentially in HOS.CD4.CXCR4 cells, although viral replication could be measured in the other HOS.CD4 transfectants. In addition, HOS.CD4.pBABE cells were also infected with the SDF-1α^{res} virus. However, all HOS transfectant cells express low amounts of CXCR4 (35a). This finding demonstrates that the SDF-1α^{res} virus still uses CXCR4, although this virus could use a smaller amount of CXCR4 receptors to enter the cells. Neither virus strain, even at 10 ng of p24, was able to replicate in HOS cells expressing CXCR4 or CCR5 (data not shown). Thus, the SDF-1^a res virus, like the wild-type virus, needs the CD4 receptor together with the CXCR4 coreceptor to enter target cells.

DNA sequence analysis of the *env* gene of SDF-1αres. We identified several mutations in the gp120 gene sequence of the SDF-1α^{res} virus strain that were not present in the wild-type virus strain (Table 2). Four mutations were clustered in the V3 loop region: K269E, Q278H, I288V, and N293D. Other mutations were found in the V1 (N106K), V2 (S134N and F145L), C2 (F245I), and V4 (R378T) regions of the SDF-1α^{res} virus. Remarkably, a deletion of 5 amino acids (FNSTW) at positions 364 to 368 in the V4 loop was found. The F245I mutation in C2, all four mutations in the V3 loop, and the deletion of 5 amino acids were also found in the AMD3100 res virus (18).

Table 2. Mutations in gp120 of the SDF-1α^{res} virus

Amino acid position (region)	Wild-type virus		SDF-1α^{res} virus	
	Codon	Amino acid	Codon	Amino acid
106 (V1)	AAT	N	AAG	K
134 (V2)	AGC	S	AAC	N
145 (V2)[a]	TTC	F	TTA	L
245 (C2)	TTC	F	ATC	I
269 (V3)[a]	AAC/AAA	N (K)	GAA	E
278 (V3)[a]	CAG	Q	CAT	H
288 (V3)[a]	ATA	I	GTC	V
293 (V3)[a]	AAT	N	GAT	D
Δ364-368[a]	TTT AAT AGT ACT TGG	FNSTW	Deletion	Deletion
387 (V4)	AGA	R	ACA	T

[a] Mutations that were also present in the AMD3100^{res} virus (18).

DISCUSSION

A factor allowing the entry of T-tropic HIV-1 strains was identified by genetic complementation of murine CD4$^+$ cells and was named fusin (27). A few months later, this factor was shown to be the receptor for the CXC chemokine SDF-1α, and fusin was renamed CXCR4 (6, 33). This receptor is used by HIV-1 and HIV-2 strains to enter cells (6, 23, 33). It does not allow infection by M-tropic HIV strains, which instead use CCR5 (2, 9, 16, 20, 21). The V3 domain of gp120 was found to be necessary, although other domains of gp120 were also found to play a role in the interaction with CCR5 (44, 45). The role of the V3 domain in the interaction of gp120 with CXCR4 has not been directly demonstrated, but a complete V3 loop substitution of a T-tropic strain with an M-tropic strain resulted in a switch from CXCR4 to CCR5 (11).

It also has been shown by immunoprecipitation that in the presence of CD4, gp120 forms a complex with CXCR4, suggesting that both CXCR4 and CD4 interact directly with the viral envelope (30). Further support for a direct interaction between CXCR4 and gp120 is given by the mutations observed in gp120 of the SDF-1α^{res} virus. Four of the nine mutations in gp120 are located in the V3 domain. Also, mutations were found in other domains of gp120, and one was also present in the CD4 binding domain. Therefore, the CXCR4 binding site is probably not limited to the V3 loop alone.

The SDF-1α^{res} virus is no longer sensitive to the chemokines SDF-1α and SDF-1β, which are the natural ligands for the CXCR4 receptor. The anti-CXCR4 MAb 12G5 is reported to inhibit HIV-1 and HIV-2 infection at 1 to 20 μg/ml, although the ability of this MAb to block infection by T-tropic isolates of HIV-1 is highly dependent on the viral isolate and the target cell (32); MAb 12G5 is even inactive against certain T-tropic viruses, such as the IIIB strain (42). This fact suggests that other cofactors may be involved or that some viruses may use a different epitope of CXCR4 that is not blocked by MAb 12G5. We obtained an IC_{50} of 8 μg/ml for the NL4.3 strain in MT-4 cells. This MAb also was more active against dual-tropic viruses in MT-4 cells (data not shown), a result which corresponds to what has already been described by other investigators using other T-cell lines (42) and which suggests the usage of different epitopes by dual- and T-tropic viruses. MAb 12G5 completely lost its activity against the SDF-1α^{res} virus, even at a concentration of 20 μg/ml (Table 1).

The two bicyclams, AMD2763 and AMD3100, were only about 10-fold less inhibitory to the SDF-1α^{res} virus than to the wild-type virus. The antiviral activity profile of AMD3100 suggests that it directly interacts with the CXCR4 receptor: AMD3100 inhibits the binding of an anti-CXCR4 MAb to its receptor, blocks infection by T-tropic viruses but not M-tropic viruses, and inhibits intracellular SDF-1α signaling in a concentration-dependent fashion (36, 40). It is therefore reasonable to speculate that the SDF-1α^{res} virus has adapted to use a different binding site on the CXCR4 coreceptor. Of the nine mutations detected in gp120 of the SDF-1α^{res} NL4.3 virus strain, four were located in the V3 domain and all four were also detected in the AMD3100^{res} virus (18).

The SDF-1α^{res} virus was not able to switch to the CCR5 coreceptor. The data obtained with U87.CD4 cells demonstrated this result clearly. U87.CD4 cells are negative for MAb 12G5 staining (23; unpublished data) and do not express CXCR4

mRNA (27). The results obtained with the HOS transfectants were more confusing, due to the low expression of CXCR4 on the parental cells. HOS cells are positive for CXCR4 mRNA (29) and weakly positive when stained with MAb 12G5 (35a). With a higher virus input (10^4 pg of p24 per ml), the NL4.3 virus was also able to replicate in all of the HOS.CD4 cell lines. Thus, although CXCR4 is expressed in HOS cells, the wild-type virus was not able to use it as avidly as the SDF-1α^{res} virus. The presence of CD4 on the cell membrane was still necessary for the SDF-1α^{res} virus (as for the wild-type virus) to enter the target cells, because HOS cells transfected with only CXCR4 could not be infected (data not shown). However, AMD3100 was still active against the SDF-1α^{res} virus when tested in all HOS.CD4 cell lines at an IC_{50} comparable to that obtained in MT-4 cells. This finding also demonstrates that the SDF-1α^{res} virus uses CXCR4 as a coreceptor for entry into cells. The results obtained with the transfected cells in the presence of AMD3100 also demonstrated that the SDF-1α^{res} virus does not use CCR1, CCR2b, CCR3, or CCR4 to enter cells. Also, the SDF-1α^{res} virus is not capable of using two newly described chemokine receptors, Bonzo and BOB (17). Because U87.CD4 cells are positive for Bonzo/ STRL33 (17), the SDF-1α^{res} virus is able to infect these cells only when CXCR4 is expressed (Fig. 3). The SDF-1α^{res} virus also does not use BOB/GPR15, because although CEMX174 cells are positive for this receptor (17) (but also positive for CXCR4) and the SDF-1α^{res} virus is able to infect CEMX174 cells, the CXCR4 antagonist AMD3100 is able to inhibit SDF-1α^{res} virus infection at an IC_{50} of 70 ng/ml in these cells (35a).

It took 24 passages in cell cultures (100 days) for the NL4.3 strain to become resistant to SDF-1α (>20-fold resistance). In comparison, it took 17 passages in cell cultures (100 days) for the NL4.3 strain to become resistant to DS (IC_{50}, >125 μg/ml) (>900-fold resistance) (25), 33 passages (182 days) for >200-fold resistance to AR177 (IC_{50}, >125 μg/ml) (24), 25 passages (120 days) for >172-fold resistance to AMD2763 (IC50, >250 μg/ml) (19), and 63 passages (almost 1 year) for 300-fold resistance to AMD3100 (IC_{50}, 546 ng/ml) (18). We never obtained complete resistance against AMD3100 with the NL4.3 strain. The SDF-1α^{res} virus was still sensitive to AMD3100, showing that this compound has a much stronger interaction with CXCR4 than the CXC chemokine itself, a finding which is also reflected in the larger number of mutations present in gp120 of the AMD3100 ^{res} virus than in gp120 of the SDF-1α^{res} virus (18).

In conclusion, the NL4.3 SDF-1α^{res} virus overcomes the inhibitory effects of SDF-1α by mutations in gp120 but is not able to switch to another coreceptor.

ACKNOWLEDGMENTS

We thank Sandra Claes and Erik Fonteyn for excellent technical assistance. We are grateful to Nathaniel R. Landau for kindly providing the U87.CD4 transfectant cell lines.

This work was supported by grants from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen, the Belgian Geconcerteerde Onderzoekacties, the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek, the Janssen Research Foundation, and the Fundació IRSI-CAIXA.

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SHIFT OF CLINICAL HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ISOLATES FROM X4 TO R5 AND PREVENTION OF THE EMERGENCE OF THE SYNCYTIUM-INDUCING PHENOTYPE BY BLOCKADE OF CXCR4

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ABSTRACT

The emergence of X4 human immunodeficiency virus type 1 (HIV-1) strains in HIV-1 infected individuals has been associated with $CD4^+$ T-cell depletion, HIV-mediated $CD8^+$ cell apoptosis, and an impaired humoral response. The bicyclam AMD3100, a selective antagonist of CXCR4, selected for the outgrowth of R5 virus after cultivation of mixtures of the laboratory-adapted R5 (BaL) and X4 (NL4-3) HIV strains in the presence of the compound. The addition of AMD3100 to peripheral blood mononuclear cells infected with X4 or R5X4 clinical HIV isolates displaying the syncytium-inducing phenotype resulted in a complete suppression of X4 variants and a concomitant genotypic change in the V2 and V3 loops of the envelope gp120 glycoprotein. The recovered viruses corresponded genotypically and phenotypically to R5 variants in that they could no longer use CXCR4 as coreceptor or induce syncytium formation in MT-2 cells. Furthermore, the phenotype and genotype of a cloned R5 HIV-1 virus converted to those of the R5X4 virus after prolonged culture in lymphoid cells. However, these changes did not occur when the infected cells were cultured in the presence of AMD3100, despite low levels of virus replication. Our findings indicate that selective blockade of the CXCR4 receptor prevents the switch from the less pathogenic R5 HIV to the more pathogenic X4 HIV strains, a process that heralds the onset of AIDS. In this article, we show that it could be possible to redirect the evolution of HIV so as to impede the emergence of X4 strains or to change the phenotype of already-existing X4 isolates to R5 phenotype.

JOURNAL OF VIROLOGY
Volumen 73, Number 7, 1999, p: 5577-5585

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) strains isolated from newly infected individuals are predominantly macrophage tropic (MT) and non-syncytium-inducing (NSI), and require CC-chemokine receptors such as CCR5 as entry cofactors in combination with CD4 (1, 15) (referred to as R5 HIV strains [2]). T-tropic (TT) strains are rapidly replicating, syncytium-inducing (SI) strains that use the CXCR4 receptor (referred to as X4 strains [2]); 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 the AIDS (9, 15, 19, 32, 33, 35). SI X4 viruses appear to exert their deleterious effect on the immune system not only by direct cytopathic effects on $CD4^+$ T cells but also by indirect killing of $CD8^+$ T cells that is mediated by CXCR4 (22). Furthermore, it has also been shown that lymphoid cells infected with R5 strains retain their immunocompetence but that, conversely, infection by X4 strains blocks the immune response to specific antigens (20). This implies that the immunodeficiency hallmarking the progression of AIDS is due, at least in part, to the emergence of the more pathogenic SI X4 strains (3). Therefore, it can be inferred that strategies directed to prevent the emergence of X4 strains would be beneficial to HIV-infected individuals.

It has been recently shown that the bicyclam AMD3100 is a highly potent inhibitor of X4 HIV strains, and its mode of action resides in a selective antagonism of CXCR4 (15, 28), the receptor for the CXC-chemokine stromal cell-derived factor-1 (SDF-1) (5). AMD3100 competes with the binding of SDF-1, to its receptor, shuts off the intracellular Ca^{2+} mobilization induced by SDF-1 and does not trigger an intracellular signal by itself.

In this article, we show that the evolution of HIV-1 can be directed so as to prevent the emergence of the more pathogenic X4 strains over the less pathogenic R5 strains by blockade of the CXCR4 receptor.

MATERIALS AND METHODS

Compounds, viruses and cells. The bicyclam AMD3100 [1,1'-(1,4-phenylenebis(methylene))-bis(1,4,8,11-tetrazacyclotetradecane) octahydrochloride dihydrate] was synthesized at Johnson Matthey as described previously (6). SDF-1α was purchased from R&D Systems (London, UK). Zidovudine (AZT) was purchased from Sigma (St. Louis, MO). The HIV-1 strains NL4-3 and BaL and the $CD4^+$

lymphocytic cell lines SUP-T1 and MT-2 were obtained through the Medical Research Council (MRC) AIDS reagent program. U87-CD4 cells expressing either CCR5 or CXCR4 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD).

Determination of viral fitness by replication competition of defined mixtures of viruses. Phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) (1 x 10^6 in 1 ml volumes), were infected with 25 ng of a mixture of the HIV strains NL4-3 and BaL (the percentage of each strain being 0, 20, 40, 60, 80 or 100% of the total p24 count) in the presence of AMD3100. The cells were incubated for 24 h and then washed twice in phosphate-buffered saline (PBS) and resuspended in medium containing AMD3100 (1 µg/ml). After 5 days incubation at 37°C, DNA was isolated from infected cells for DNA sequencing. In similar experiments, PHA-stimulated PBMC infected with a predetermined mixture of 99% NL4-3 and 1% BaL in the absence and presence of AMD3100 (1 µg/ml) were cultured and passaged every 7 days in uninfected PHA-stimulated PBMC. After 28 days in culture, p24 antigen was measured in the culture supernatant and DNA was isolated from infected cells for DNA sequencing.

Virus growth in the presence of AMD3100. PHA-stimulated PBMC were infected with low-passage clinical HIV-1 isolates in the presence of AMD3100. HIV replication was measured (every 7 to 8 days) by a p24 antigen detection method (Coulter). The p24 antigen-positive supernatant was further passaged in fresh PBMC. After 4 passages (28 days) in the presence of the drug, recovered virus was used for the phenotype assay in MT-2 cells, and DNA was isolated for PCR amplification, DNA sequencing and cloning.

***In vitro* emergence of the SI phenotype.** The third variable region (the V3 loop) of the envelope of HIV contains a major neutralization epitope and determinants of cell tropism (23), SI capacity, replication rate (11), and coreceptor use (8). The recombination of a V3 loop DNA sequence corresponding to a R5 strain into the DNA sequence of a X4 strain is sufficient to modified the coreceptor use of the resulting virus from X4 to R5 (8). Furthermore, the phenotype of NSI, slow replicating HIV-1 converts to SI fast-replicating strains after prolonged culture in SUP-T1 cells. Mutations within the V3 loop have been shown to be responsible for the conversion into the SI phenotype (11, 12). Therefore, the evolution of HIV strains from R5 phenotype into X4 or R5X4 phenotype can also be monitored by genotypic changes that lead to amino acid changes

in the V3 loop. The viral clone 168.1 (11, 12, 24) of the NSI slow replicating phenotype was cultured in SUP-T1 cells in the absence or presence of AMD3100 (1 µg/ml). Every 5 or 6 days, the number of syncytia in the cultured cells were scored, and cells were passaged in fresh medium with or without compound. Once syncytia were scored positive in the untreated sample, the AMD3100 culture was continued for 55 more passages (i.e., until 405 days after the initial infection). DNA was isolated from infected cells for DNA sequencing.

Cloning and phylogenetic analysis of HIV-1 *env*. PCR fragments of the *env* gene from proviral DNA were cloned in the pCR-Script SK(+) cloning vector (Stratagene, La Jolla, CA) by following the manufacturer's instructions and the procedure described elsewhere (18). Clones were isolated for DNA sequencing, and phylogenetic analysis was done with the neighbour-joining method using the Clustal X (34) software. Bootstrap resampling was used to assess the strength of support for each branch of the phylogenetic trees.

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 to 20 µg of total DNA purified by the QUIAGEN blood kit and 0.1 µg of each of the primers TACAATGTACACATGGAATT and ATTACAGTAGAAAATTCC. Then, a second preparative PCR, reaction which amplifies the V3 loop region of gp120, was done with primers TGGCAGTCTAGCAGAAGAAG and TCTGGGTCCCCTCCTGAGGA. For sequencing of the V2 loop, primers AATTAACCCCACTCTGTGTTAGTTTA and GCTCTCCCTGGTCCCCTCTGG were used for the first PCR reaction and primers AATTAACCCCACTCTGTGTTAGTTTA and TGATACTACTGGCCTGATTCCA were used 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 sequences were analysed by an ABI PRISM genetic sequencer. The Navigator and Factura DNA analysis software packages (Perkin Elmer) were used to identify and quantify ambiguous regions of the DNA sequence that are produced when a mixture of two sequences is detected.

Determination of virus phenotype (MT-2 assay). MT-2 cells were infected with different HIV-1 isolates. Cell cultures were monitored for syncytium formation for up to 14 day post infection.

Coreceptor use by different clinical isolates. U87-CD4 cells expressing either CCR1, CCR2b, CCR3, CCR5 or CXCR4 (5×10^3) were infected with 10 ng of p24 antigen of the corresponding virus strain and incubated for 24 hours. Cells were then washed twice with PBS, and fresh Dulbecco's modified Eagle's medium was added. Cells were incubated for four more days, and p24 antigen in the culture supernatant was measured.

RESULTS

Viral fitness determined by replication competition with defined X4-R5 virus mixtures. The effect of AMD3100 on the replication of mixtures of two laboratory-adapted HIV isolates, the X4 isolate NL4-3 and the R5 isolate BaL, was evaluated based on the sequence of the V3 region of gp120 from proviral DNA isolated from PBMC that had been infected with these virus mixtures. The nucleic acid sequence of a fragment of the HIV-1 V3 region of gp120 from proviral DNA isolated from cells infected with either NL4-3 or BaL or from mixtures of these two virus strains was determined. Proviral DNA sequence determination may serve as a marker of the viral fitness of each strain (21). As expected, the DNA sequence corresponding to either NL4-3 or BaL was found if the cells were infected for solely with the NL4-3 or BaL strain, respectively. When the cells were infected with a mixture of these strains, DNA sequence analysis showed that the proviral DNA sequence could not be aligned with either the NL4-3 or the BaL sequences but rather corresponded to a mixture of both sequences (data not shown). Six sample sequencing chromatograms are shown in Fig. 1. DNA sequences (positions 50 to 54) correspond to amino acids I and R/N (amino acids 16 and 17) in the V3 loop of gp120. This region is located before the insertion QR in the V3 loop of NL4-3 and could be aligned in all sequences. As expected, the chromatograms indicated the gradual replacement of BaL (sequence AAAT) by NL4-3 (sequence CCGT) when the NL4-3 level in the input virus was increased. Even at the lowest NL4-3 level tested (20% NL4-3 to 80% BaL), the NL4-3 sequence could be detected. However, in the presence of AMD3100, only the BaL strain was detected in the proviral DNA even at highest NL4-3/BaL ratio in the infecting virus mixture (80% NL4-3 to 20% BaL) (Fig. 1).

Effect of AMD3100 on the outgrowth of X4 and R5 from X4-R5 virus mixtures. The results presented above indicated that the replication of the X4 strain NL4-3 is suppressed in the presence of AMD3100. To further assess the influence of AMD3100 on the replicative ability of X4 and R5 virus strains in X4-R5 virus mixtures,

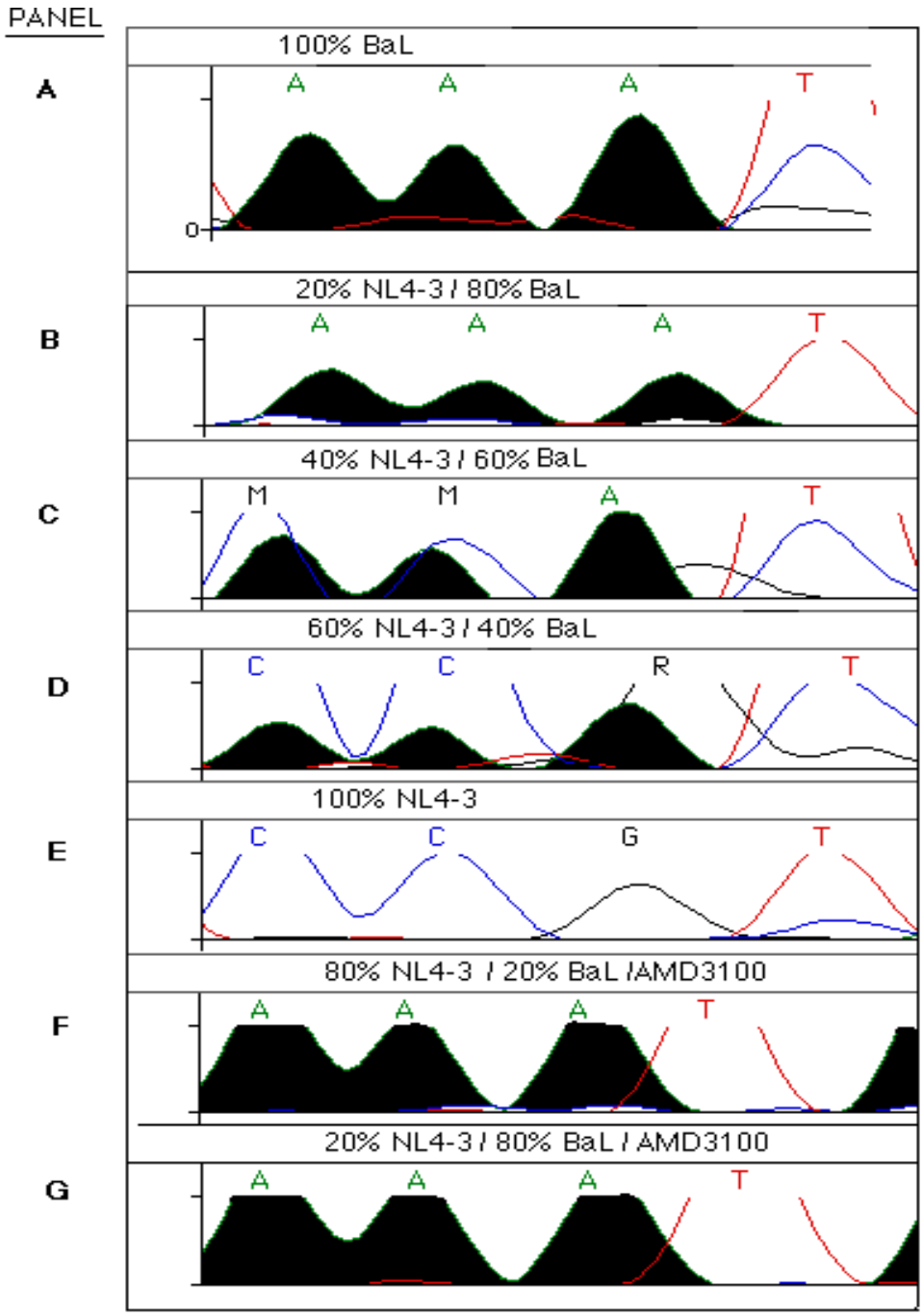

Figure 1. Determination of viral fitness in a mixed virus population. PHA-stimulated PBMC were infected with either HIV-1 BaL, HIV-1 NL4-3 or a mixture of BaL and NL4-3. At 7 days postinfection, proviral DNA was amplified from infected cells and the DNA sequence from the V3 loop coding region was obtained and aligned with NL4-3 and BaL proviral sequences. The sample sequencing chromatograms of positions (7144 to 7148 relative to the HXB_2 sequence) in the V3-loop DNA coding region indicate the displacement of BaL proviral DNA by NL4-3 proviral DNA in the infected PBMC. The sequence AAAT (Panel A) corresponds to the BaL proviral sequence. Replacement of BaL by NL4-3 can be monitored by the appearance of the CCGT sequence, as indicated by relative increase in the size of the empty peaks, depending on the ratio of NL4-3 in the infecting virus population (Panel B to E). In AMD3100-treated cells only the BaL sequence emerged regardless of the proportion of BaL in the infecting virus population (Panels F and G). M and R represent the presence of multiple bases in a 50%-50% proportion, at a given position (M indicates the presence of A or C; R indicates the presence of A or G). The scale of the electropherograms has been reduced as to increase sensitivity in the detection of ambiguous (mixture) sequences.

a mixture composed of 99% NL4-3 and 1% BaL was used to infect PHA-stimulated PBMC that were then cultured for 28 days (four passages) in the presence or absence of AMD3100. NL4-3 virus replication was inhibited by AMD3100, and NL4-3 proviral DNA became undetectable after 21 days in culture (data not shown). Both NL4-3 and BaL weredetectable in the virus progeny at 28 days of an initial virus mixture containing 99% and 1% BaL (as demonstrated by sequencing the V3 loop region of the proviral DNA recovered after 28 days in culture). However when, this virus mixture was cultured for 28 days in the presence of AMD3100, only the BaL strain could be recovered (Fig. 2).

Determination of phenotype of clinical HIV isolates grown in the presence of AMD3100. *In vivo* HIV infection is characterized by the existence of marked heterogeneity in viral populations (25). In order to better reproduce these conditions, we studied the effect of CXCR4 blockade on the replication of six clinical isolates, three that were defined as SI and three that were defined as NSI in the MT-2 syncytium phenotype assay. PBMC from these six HIV-infected individuals were cocultured with PHA-stimulated PBMC from healthy donors in the presence or absence of AMD3100 (1 μg/ml). After 28 days (four passages) of culture, supernatants were recovered, their viral phenotypes were analyzed by the MT-2 syncytium phenotype assay, and their susceptibility to AMD3100 and AZT were evaluated. Results are summarized in Table 1. All the NSI strains, grown in the presence or absence of AMD3100, were resistant to AMD3100 but sensitive to AZT. Conversely, the SI strains from untreated cultures showed sensitivity to AMD3100 and AZT, but, after growing in the presence of AMD3100, they became insensitive to AMD3100 (50% effective concentration > 1 μg/ml) while remaining sensitive to AZT. Syncytia were observed in MT-2 cells early as 3 days postinfection when the cells had been inoculated with the SI strains from untreated cultures. However, virus recovered from the cells grown in the presence of AMD3100 did not induce syncytia in MT-2 cells even after 14 days of culture. Similarly, NSI strains also did not induce syncytia. The reference strain NL4-3 scored positive for syncytia in the MT-2 test as early as 3 days post infection, while the BaL strain remained negative for up to 14 days (data not shown).

Determination of genotype of clinical HIV isolates grown in the presence of AMD3100. Proviral DNA of cells infected with clinical HIV isolates for up to 28 days in the presence or absence of AMD3100 was amplified, and fragments of the *env* gene, corresponding to the V2 and V3 loops, were sequenced. No significant changes in the

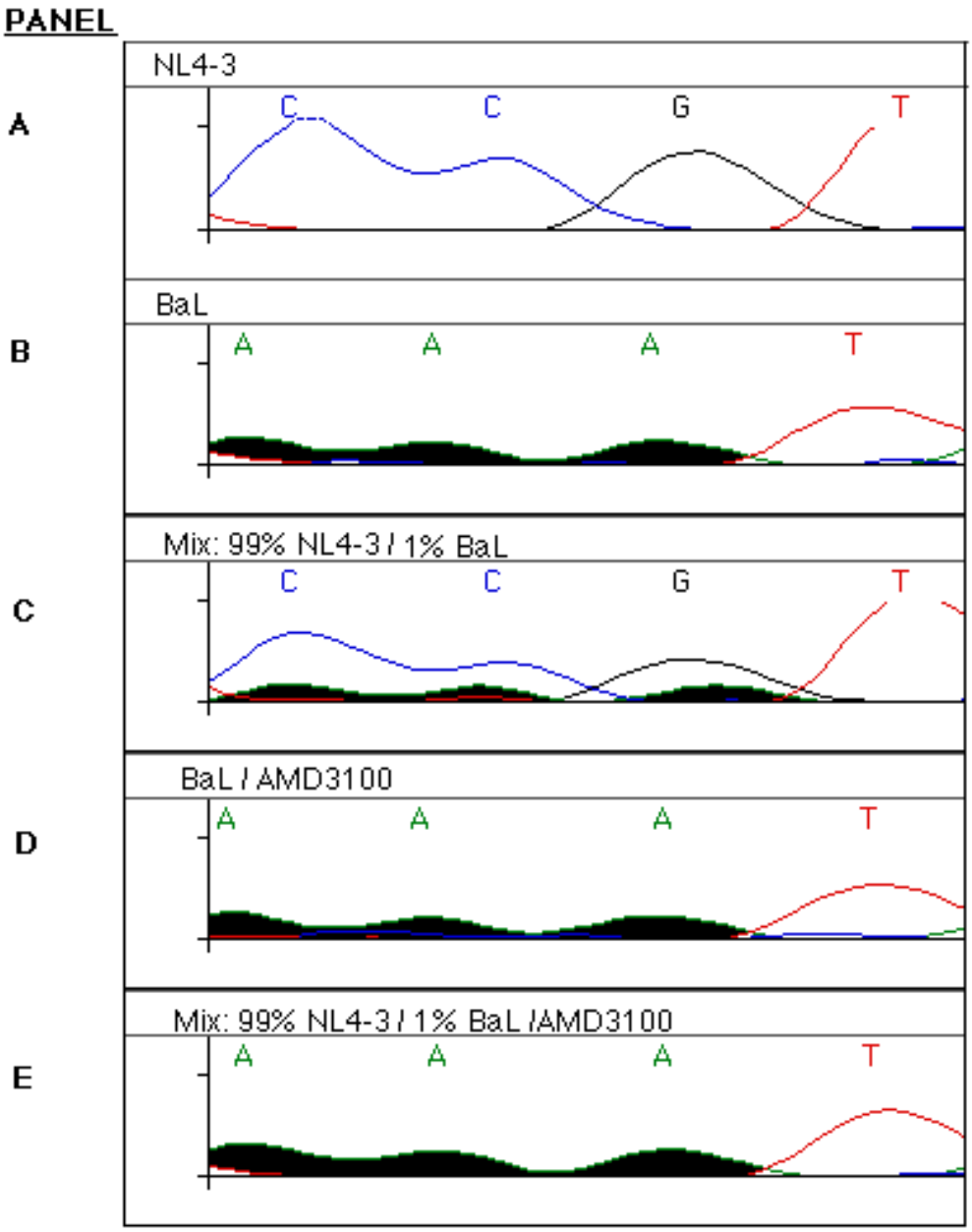

Figure 2. Selection of the R5 virus after sequential passage of a mixed R5-X4virus population in the presence of AMD3100. Stimulated PBMC were infected with either HIV-1 NL4-3 or BaL or a mixture thereof comprising 99% NL4-3 and 1% BaL. At 28 days postinfection, proviral DNA was amplified and sequenced. The dominating virus population can be determined by the proportion of the peaks corresponding to the NL4-3 sequence CCG (empty peaks; Panel A) or the BaL sequence AAA (filled peaks; Panel B and D) in the sample sequence chromatograms. The scale of the electropherograms has been reduced as to increase sensitivity in the detection of ambiguous (mixture) sequences. The NL4-3 strains is the dominating virus in untreated cells infected with the 99% NL4-3 1% BaL mixture (Panel C). However, the BaL strain becomes the dominating virus if this virus mixture was exposed to AMD3100 (Panel E).

NSI clinical isolates before and after 28 days of virus replication in the presence of AMD3100 were observed compared to the untreated virus DNA sequences. However, several mutations in the SI strains cultured in AMD3100-treated cells that were not present in the untreated samples were noted. Amino acid changes were found in those V3 loop region (isolates AOM and CST) and V2 loop region (isolates AOM and FCP), that have been associated with SI and NSI phenotype and HIV tropism (Fig. 3) (23, 29). PCR products corresponding to the V3 loop sequence were also cloned, and individual

Table 1. Low-passage clinical isolates of HIV-1 that were cultured in the presence of AMD3100: phenotype in MT-2 cells and sensitivity to AMD3100 and AZT.

	EC_{50}[b] (µg/ml) following treatment with:		
Clinical Isolate [a]	AMD3100	AZT	Phenotype in MT-2 cells[c]
NSI strains			
MDM (-)	>1	0.005	-
MDM (+)	>1	0.005	-
MCS (-)	>1	0.004	-
MCS (+)	>1	0.004	-
JGA (-)	>1	0.005	-
JGA (+)	>1	0.02	-
SI strains			
CST (-)	0.13	0.013	+++
CST (+)	>1	0.01	-
AOM (-)	0.11	0.005	+++
AOM (+)	>1	0.006	-
FCP (-)	0.14	0.005	+++
FCP (+)	>1	0.005	-

[a]Clinical isolates or supernatant recovered from PHA-stimulated PBMC of HIV infected persons with PBMC from negative donors that scored positive for p24 antigen no later than 14 days after the beginning of cocultivation. The cell supernatants were then used for determination of the SI phenotype in MT-2 cells and selection in the presence of AMD3100. The (-) and (+) labels undicate strains that were cultured for 28 days in the absence or presence of AMD3100 (1 µg/ml) respectively.
[b]EC50, concentration required to inhibit by 50% the p24 antigen production by HIV-1 infected PBMC: $1x10^6$ cells were infected with 10 ng of p24 from each of the clinical isolates in the presence of varying concentrations of the corresponding drug. Four days after infection of cells, one half (0.5 ml) of the total volumen of cell supernatant was removed and an equal volume of fresh medium containing the appropriate drug concentration was added. At day 7, p24 antigen in the supernatant was determined.
[c]MT-2 cells were infected with the corresponding virus isolate. Syncytia were scored microscopically at 14 days postinfection. Scores are expressed as percentage of the wild-type NL4-3 level as follow: 76 to 100% (++++), 51 to 75% (+++), 25 to 50% (++), 1 to 24% (+).

clones were sequenced. The consensus sequence derived from the alignment of clone sequences from each virus (data not shown) was identical to the proviral sequence that was determined by sequencing of the amplified pDNA shown in Fig. 3. Figure 4 shows the phylogenetic analyses of the V3 loop amino sequences from two patients' isolates of the SI phenotype that showed changes in the V3 amino acid composition after treatment with AMD3100. Cloned sequences corresponding to the untreated AOM or CST isolates and treated AOM or CST isolates were clustered in separate parts of the tree, indicating a clear shift in the composition of the viral population after treatment with AMD3100. Two clones of the untreated CST isolate clustered together with the treated CST clones, suggesting that the emerging population, although in a minor proportion, was already present in the untreated clinical isolate. Furthermore, sequences from the AMD3100-treated virus clustered together and closer to the V3 sequence of the R5 strain BaL but more distant from the V3 sequence of the X4 strain NL4-3.

Coreceptor use of clinical isolates after culture in AMD3100. As seen in Fig. 5, U87-CD4 cells expressing CCR5 supported the replication of all HIV clinical isolates of the NSI phenotype, as evaluated by p24 antigen production after 5 days postinfection. However, no virus replication was detected in the CXCR4-transfected cells. Conversely, the SI strains were able to infect the CXCR4-transfected cells, and one SI isolate (CST) was able to replicate in both the CXCR4- and CCR5-transfected cells. After being cultured for 28 days in the presence of AMD3100, all the recovered virus strains replicated in CCR5-transfected cells but not in CXCR4-transfected cells regardless of the coreceptor used by the original virus isolate. The replication of the untreated or treated clinical isolates was marginal (<10% of the principal coreceptor used) in U87-CD4 cells expressing CCR1, CCR2b, or CCR3 (data not shown).

Blockade of CXCR4 prevents the emergence of the SI phenotype. Simulating what happen during the course of infection (that is, that R5 strains evolve in some individuals into X4 or R5X4 [dual tropic] HIV strains) the phenotype of NSI slow-replicating HIV-1 converts to SI fast-replicating strains after prolonged culture in SUP-T1 cells (24, 35). Upon prolonged propagation in SUP-T1 cells, the NSI virus 168.1 tended to give rise to virus mutants with an SI phenotype and high replicative capacity. The viral clone 168.1 with an NSI slow-replicating phenotype was cultured in SUP-T1 cells in the absence or presence of AMD3100 (1 μg/ml). Every 4 or 5 days, the number of syncytia in the cultured cells were scored, and cells were passaged in fresh medium with or without compound. In the untreated cells, syncytia were first detected after 100

Clinical Isolate-phenotype in MT-2 cells	V2 loop sequences
CST(-)/SI :	CSFNISTSIRGKVQKEYAFFYKLDIIPIDNDTTSYKLTSC
CST(+)/NSI:	-----T-G– -D-----R-L– -R– -LV----E-N– -RII--
AOM(-)/SI :	CSFNITKNIGNKKHTEYSLFYKLDVVPIKNDNQSYTLINC
AOM(+)/NSI:	------TS----------E-------
FCP(-)/SI :	CSFNITTNIRDKVKREHALFYSLDIVKIEDKNETTSYGFNTSYRLRSC
FCP(+)/NSI:	----------M-K-Q----------EN-------L---------
MDM(-)/NSI:	CSFNISTSIRGKVQKEYAFFYKLDIIPIDNDTTSYKLTSC
MDM(+)/NSI:	----------
MCS(-)/NSI:	CSFNVTTSIRDKVQKEYALFYKLDVVPIDNDNNNSYRLINC
MCS(+)/NSI:	----------E--.-------
JGA(-)/NSI:	CSFNITTNLKDKVRNSALFYSLDVVPIENSTSNTSYRLRSC
JGA(+)/NSI:	----------

	V3 loop sequences
CST(-)/SI :	CTRHSHKTRRRIHRGPGRAFYTTGDIIGDMRKAHC
CST(+)/NSI:	---L-NN---S-NI----------I-Q---
AOM(-)/SI :	CTRPNKNTRKRIHIGPGRAFYATGDIIGDIRQAHC
AOM(+)/NSI:	-I---N---RG--M----T---G.--V--------
FCP(-)/SI :	CTRPNNNTRKGIHIGPGRTFYATGEIIGDIRKAHC
FCP(+)/NSI:	----------S----------
MDM(-)/NSI:	CTRPNNNTRKGIHMGPGKSFYVTDIIGDIRQAHC
MDM(+)/NSI:	----------
MCS(-)/NSI:	CTRPNNNTRKSINIGPGRAFYATGDIIGDIR Q/K AYC
MCS(+)/NSI:	---------R---------- K ---
JGA(-)/NSI:	CCTRPNNNTIKSIHMGSGKAFYLTGQVVGDIRQAHC
JGA(+)/NSI:	----------

Figure 3. Amino acid sequence of the V2 and V3 loop regions of HIV-1 clinical isolates cultured in the presence or absence of AMD3100. Low-passage clinical HIV isolates belonging to the SI phenotype (isolates CST, AOM and FCP) or the NSI phenotype (isolates MDM, MCS and JGA) were cultured in PHA-stimulated PBMC for 28 days in the presence (+) or absence (-) of 1 µg/ml AMD3100. Proviral DNA was isolated from the infected cells and submitted to PCR amplification and DNA sequencing of the V2 and V3 coding regions.

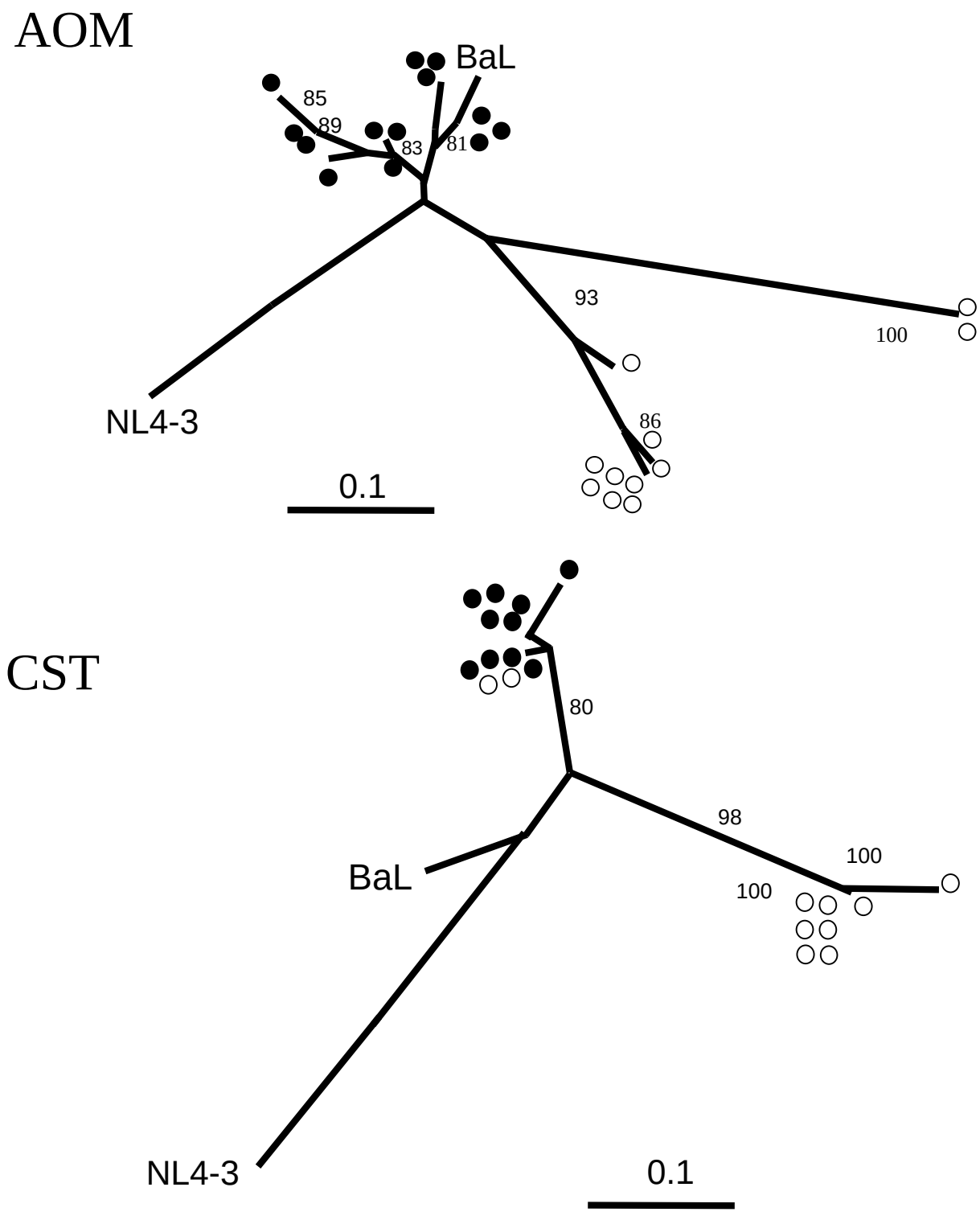

Figure 4. Unrooted phylogenetic tree analysis of V3 sequence clustering from two HIV-1 isolates of SI phenotype (AOM and CST isolates) cultured in the presence or absence of AMD3100 for 28 days. Phylogenetic analysis of the amino acid sequences were done with the neighbor-joining method WITH the Clustal X software (34). Clones corresponding to the samples from untreated and AMD3100-treated cultures are labeled with empty and filled circles, respectively. The V3 sequences of HIV-1 BaL and NL4-3 were included for comparison. At least 10 clones of each virus were used to construct the phylogenetic trees.

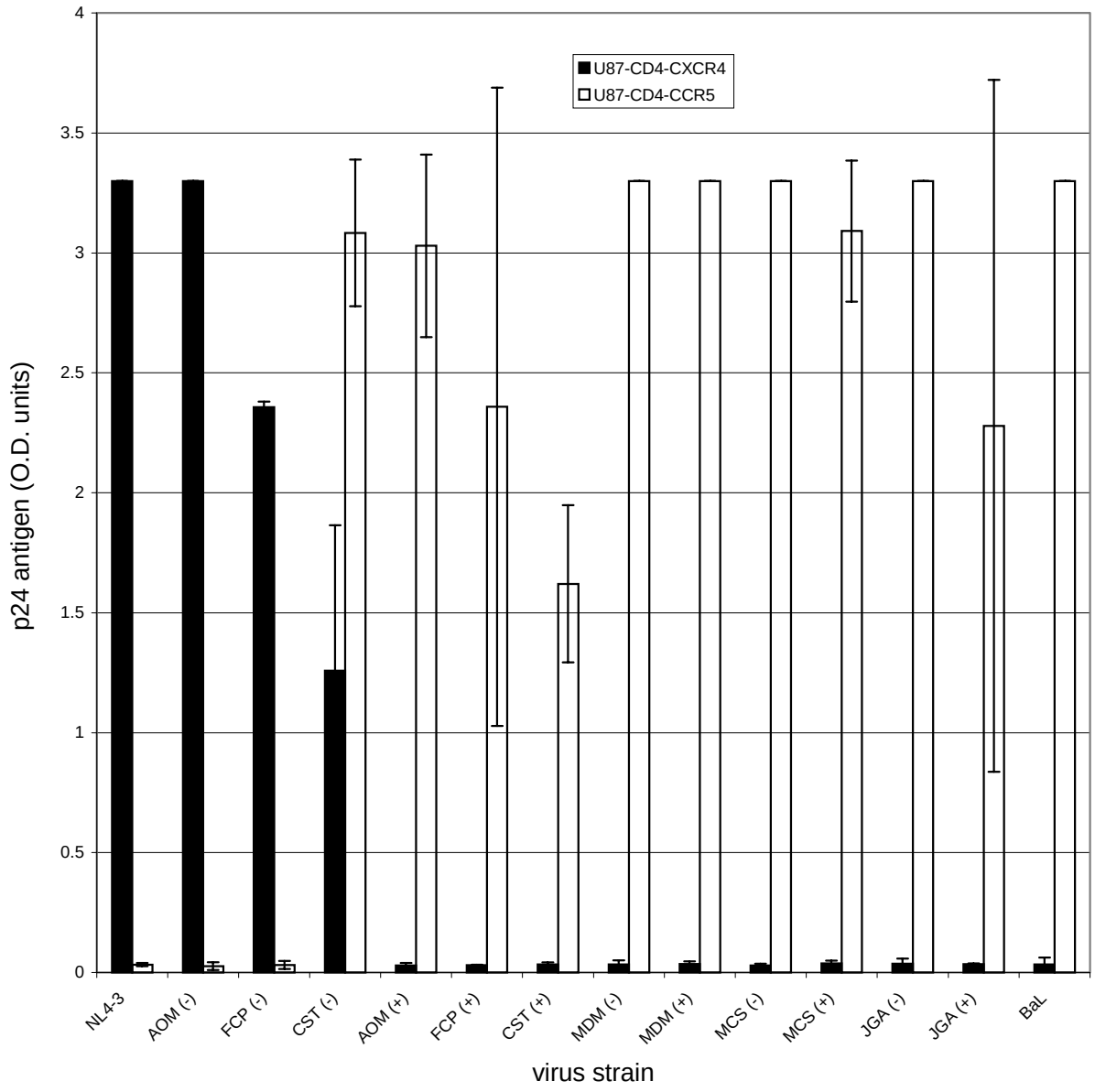

Figure 5. Coreceptor use of clinical HIV strains after treatment with AMD3100. U87-CD4 cells (5 x 10^3 cells) expressing CCR5 (empty bars) or CXCR4 (filled bars) were infected with 10 ng of p24 antigen of the HIV-1 strains that were cultured in PBMC for 28 days in the presence (+) or absence (-) of AMD3100 (1 µg/ml). Cells were incubated for 24 h, washed twice in PBS, and resuspended in fresh medium. At 5 days postinfection, p24 antigen was detected in the cell-free supernatant. The phenotype of the parental HIV-1 strains was determined in MT-2 cells (see table 1): SI for isolates CST, AOM and FCP and NSI for isolates MDM, MCS and JGA. The HIV-1 NL4-3 and BaL strains are included for comparison.

days in culture. At 200 days post infection, clear cytopathic effect (CPE) and formation of syncytia were noted in the untreated culture. Conversely, no CPE or syncytium formation was detected in the AMD3100 treated cells even after 305 days after the first

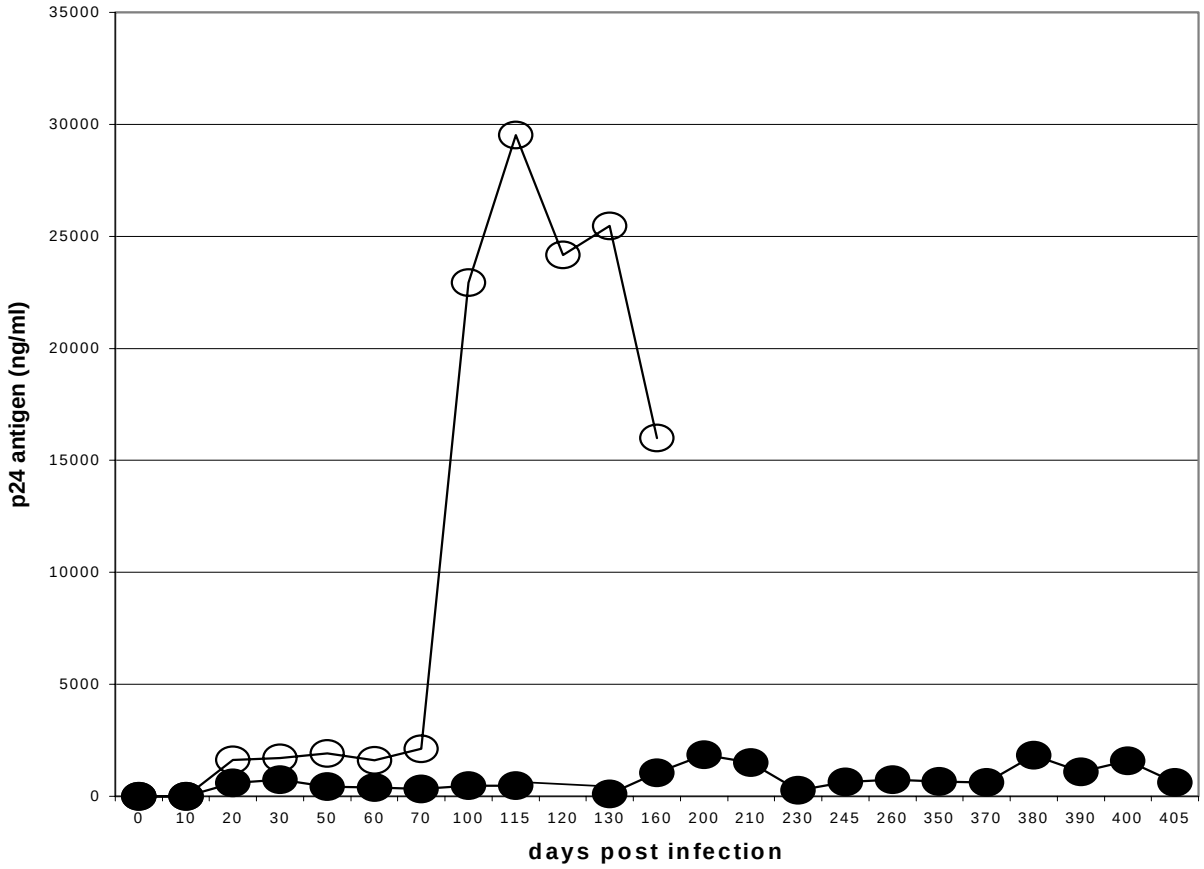

Figure 6. Replication of NSI strains in SUP-T1 cells in the presence of AMD3100. SUP-T1 cells permit the growth of the HIV-1 strain 168.1 Replication of HIV-1 168.1 was sustained for up to 200 days without AMD3100 (empty circles) or 405 days in the presence of 1 µg/ml AMD3100 (filled circles). Syncytia were noted in the untreated sample after 100 days of culture but were not detected in the treated sample for 405 days.

detection of syncytia in the untreated culture (405 days postinfection), despite low but continuous virus replication (Fig. 6).

To test if genotypic changes paralleled the change from NSI phenotype to SI phenotype, the gp120 proviral genome was isolated by PCR amplification of total cellular DNA purified from infected cells for sequencing of the V3 loop coding region. DNA sequence analysis of proviral DNA isolated from untreated cells where syncytia were observed, detected the emergence of mutations in the V3 loop that have been shown to predict the SI phenotype (11, 12, 23) (Fig. 7). Amplified DNA from the untreated cells at day 100 postinfection, when the first syncytia were noted, showed the presence of a mixture of two amino acids, serine (S) or arginine (R), at position 11 of

the V3 loop (data not shown). Proviral DNA amplified and sequenced from day 200 postinfection (100 days after the first detection of syncitia), revealed the emergence of mutations at position 6 of the V3 loop, from asparagine (N) to lysine (K); at position 11 from serine (S) to arginine (R); and from glycine (G) to arginine (R) at position 28 of the V3 loop (Fig, 7). Furthermore, there was a net increase in the overall charge of the V3 loop from 3+ to 5+. However, in the culture that was treated with AMD3100, no changes were noted in the V3 region of the recovered virus even 305 days after the first detection of syncytia in the untreated culture (405 days postinfection).

HIV-1 strain	V3 genotype	Charge
168,1/NSI phenotype:	CTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHC	3+
168,1/SI phenotype:	-----K----R----------------R-.-----	5+
168,1/AMD3100:	----------	3+
168,10:	----------R----------T--Q---N------	6+

Figure 7. V3 loop amino acid sequence of the parental NSI strain (168.1/NSI phenotype) and that obtained from proviral DNA isolated from cells at 200 days (168.1/SI phenotype) and 260 days (168.1/AMD3100) after initiation of the experiment from untreated and AMD3100-treated cells respectively. The amino acid sequence of HIV-1 168.10 strain (11) of SI phenotype is included for comparison. "-" represents homology, "." represents an amino acid deletion.

DISCUSSION

HIV-1 strains isolated from infected individuals are predominantly MT and NSI and mainly use CCR5 as coreceptor for entry into $CD4^+$ T-cells (R5 strains). Over the course of the infection TT SI variants that use CXCR4 coreceptor appear (X4 strains). Their emergence has been implicated with $CD4^+$ T-cell decline, $CD8^+$ T-cell apoptosis (22), specific irreversible effects on B cell activity (19), and the onset of AIDS and disease progression (9, 19, 30, 36).

A major strategy in the fight against AIDS may consist in the prevention of the emergence of the more pathogenic CXCR4-using strains of HIV. AMD3100 is a potent anti-HIV agent that is targeted at the CXCR4 receptor (15, 28). 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. Its great potency against TT HIV variants (the ratio between the 50% cytotoxic concentration and the 50% effective concentration is > 100,000) makes it an ideal candidate to prevent the emergence of X4 strains.

Here we have shown in this article that cultivation of a heterogeneous population of HIV, composed of laboratory-adapted, TT (NL4-3) strain and a MT (BaL) strain, in the presence of AMD3100 leads to the selection of the MT over the TT strain (Fig. 1). Even when the initial virus population consisted of only 1% BaL (and 99% NL4-3), BaL completely took over the population after 21 days of subcultivation in PHA-stimulated PBMC. HIV-infected individuals harbor a swarm of closely related viruses, the so-called HIV quasispecies, in which R5 and X4 strains may coexist. From our results it can be surmised that under selective pressure against CXCR4 receptor, only MT strains will continue to replicate. That is, in a heterogeneous population, as is the case of a viral population in an infected individual, the fitness of MT quasiespecies will be greater than that of TT ones in the presence of AMD3100.

Addition of AMD3100 to PBMC from individuals infected with virus displaying the SI phenotype resulted in a complete block of the SI viruses. Recovered viruses showed reduced sensitivity to AMD3100 and could no longer induce syncytium formation in MT-2 cells. These viruses replicated in CCR5-transfected cells but not in CXCR4-transfected cells. Conversely, the NSI strains remained insensitive to AMD3100 and continued to replicate solely in CCR5-transfected cells. Phylogenetic analysis revealed a drastic change in the viral population upon AMD3100 treatment as predicted from the selection of MT R5 quasispecies. Surprisingly, the clinical isolate FCP (an SI strain) did not show significant changes in the V3 loop region after incubation with AMD3100. However, there were notable changes in the V2 loop coding region which led us to suspect that these changes are responsible for the phenotypic changes observed. The V2 loop genotype has also been associated with SI-NSI phenotype and HIV tropism (23, 29). Nevertheless, our results clearly show genotypic and phenotypic changes in all treated clinical isolates.

The bicyclam AMD3100 is not active against MT strains of HIV-1 (28). Conversely, AMD3100 was equally active against dual-tropic viruses (which use CCR3,

CCR5, CCR8 and CXCR4) (27) in PBMC. More recently, Zhang and Moore (37) have also reported that inhibition of a dual-tropic virus (R5-X4) was inhibited (although only partially) by AMD3100. These results suggest that selection with AMD3100 will favor the emergence of R5 strains over dual-tropic variants. Our results show that only those quasispecies that use CCR5 are allowed to survive while both X4 or R5-X4 strains are selected out. That is, AMD3100 exerts selective pressure over both X4 (i.e., AOM and FCP isolates) and R5-X4 (i.e., CST isolate) strains. Furthermore, the clinical isolate CST represents a viral population comprising quasispecies that may use CCR5, CXCR4 or both. After selection with AMD3100, the CST isolate seems to replicate less efficiently in CCR5 cells. The replication of both R5 and R5-X4 quasispecies may account for the relatively high p24 antigen production in U87-CD4-cells of the untreated CST isolate; however, after treatment with AMD3100, p24 antigen production reflects only the replication of the selected R5 quasispecies.

De Vreese et al.(14) have developed a partially AMD3100-resistant HIV-1 strain that continued to replicate in MT-4 ($CXCR4^+$) cells. This AMD3100-resistant strain was selected from a highly adapted laboratory strain (NL4-3) that deviates from the consensus sequence of primary clinical isolates. Furthermore, the AMD3100-resistant strain was selected in the lymphoid cell line (MT-4) that does not allow replication of MT strains. By slowly increasing the concentration of AMD3100 after subsequent passages, the parental NL4-3 strain accumulated an increasing number of mutations, that finally rendered the virus resistant to AMD3100. Conversely, the present results indicate that in a system in which R5 strains are able to replicate at the expense of the X4 strains, the R5 strains take over the population, while the X4 strains vanish. Our results suggest that AMD3100 favored the selection of preexisting quasispecies without the need for ongoing mutations. Under the conditions used, passage of NL4-3 in PHA-stimulated PBMC in the presence of 1 µg/ml of AMD3100 resulted in the “knockout” of the NL4-3 virus and proviral DNA-negative cells at 21 days after infection. We postulate that the treatment of AIDS patients with a CXCR4 antagonist may revert the SI-X4-TT phenotype to a less pathogenic phenotype. Suboptimal concentrations of AMD3100 would allow SI-X4 variants to escape the inhibitory activity of AMD3100; nevertheless, many mutations accumulating in the gp120 gene of AMD3100-resistant virus could indicate that resistance may not be easily acquired *in vivo* (14).

Mirroring what happen during the course of infection (that is, that R5 strains evolve in some individuals into X4 or R5X4 [dual tropic] HIV strains [36]), the

phenotype of NSI, slow replicating HIV-1 converts to SI fast-replicating strains after prolonged culture in SUP-T1 cells (11, 12, 24). These viruses are able to efficiently replicate in transformed T-cell lines and to form syncytia when grown in MT-2 cells. HIV-1 isolates 168.1 (NSI) and 168.10 (SI) are sequential isolates obtained from the same asymptomatic individual by coculture of his peripheral blood lymphocytes (PBL) with healthy donor PBL (11, 24). In the T-cell line SUP-T1, the syncytium-inducing capacity of a chimeric HXB-2 virus containing only the V3 region from 168.1 or 168.10 accorded with the phenotype of HIV-1 isolates 168.1 (NSI) and 168.10 (SI) (12). Upon prolonged propagation in SUP-T1 cells, the NSI virus 168.1 tended to give rise to virus mutants with a SI and high replicative capacity. We have confirmed, by detection of mRNA by reverse transcriptasePCR (data not shown), that SUP-T1 cells express low but detectable levels of chemokine receptor CCR5 and high levels of CXCR4 (13), explaining why R5 strains such as 168.1, can infect this cell line, albeit a low rate of virus replication. In contrast, X4 strains easily infect and propagate in SUP-T1 cells. In this model, AMD3100, prevented the emergence of the SI phenotype and genotype that is observed in untreated infected cells despite slow but continuos viral replication. No CPE or syncytium formation was detected in the AMD3100-treated cells even after 305 days of the first detection of syncytia (405 days postinfection).

These results further support the notion that CXCR4 antagonism maintains the replication of NSI slowly replicating R5 strains while suppressing the replication of SI rapidly replicating X4 strains. We postulate that treatment of an HIV-positive asymptomatic individual with a CXCR4 antagonist would prolong the asymptomatic phase of its viral infection.

Recent studies by Tachibana *et al.* (31) and Zhou *et al.* (38) have revealed that mice lacking CXCR4 or SDF-1 expression exhibit haematopoietic and cardiac defects, suggesting that CXCR4 and SDF-1 may play an important role in embryonic development and could have nonredundant functions in adults, thus raising some concerns about the use of CXCR4 antagonists as therapeutic agents against HIV. Furthermore, CXCR4-dependent migration to SDF-1 appears to be essential for human stem cell function in NOD-SCID mice (26). No toxicity was observed after administration of AMD3100 (10 mg/kgof body weight/day b.i.d.) to SCID-hu Thy/Liv mice for 28 days in spite of a significant decrease in HIV viral load in the infected mice (10). Low concentrations of a CXCR4 antagonist could be sufficient to prevent or delay X4 strain emergence without inducing an unwanted effect. Alternatively, other

strategies such as intrakine blockade of CXCR4 on targeted cells (7) or CD4-chemokine receptor pseudotypes (17) could selectively block the use of CXCR4 in T lymphocytes. Nevertheless, ongoing clinical trials with AMD3100 will have to demonstrate both its safety and efficacy as a chemotherapeutic agent against HIV and AIDS. CXCR4 antagonists could be intended as deterrents for the emergence of X4 strains, more than to decrease viral load levels which can be effectively achieved by triple drug combinations of recerse transcriptase inhibitors and protease inhibitors (4). The concurrent observations that we have made with both laboratory HIV strains and clinical HIV isolates point to the potential usefulness of CXCR4 antagonists in preventing the switch from R5 to X4 that is generally considered as a hallmark for the onset of AIDS and/or progression of the disease. Our findings also suggest that CCR5-blocking agents might speed the evolution and outgrowth of more pathogenic HIV-1 variants that use CXCR4, thereby accelerating the course of disease. The ability of different HIV-1 strains to use coreceptors in addition to CCR5 or CXCR4 *in vitro* appears to be irrelevant to their drug sensitivity in primary cells (37). Combinations of both CCR5 and CXCR4-blocking agents could effectively inhibit HIV replication and prevent selection of X4 variants.

ACKNOWLEDGMENTS

We thank Miguel Angel Martinez de la Sierra for help with the phylogenetic analysis.

This work was supported by grants from the Spanish Fondo de Investigación Sanitaria (FIS 98/0868), and the Fundació irsiCaixa, the Belgian Geoncerteerde Onderzoeksacties (95/5) and the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (G.0104.98). J.B. is the recipient of a "*Ajut per a la Reincorporació de Doctors*" (RED) fellowship from the Generalitat de Catalunya.

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CAPÍTULO 6

DISCUSIÓN GENERAL Y PERSPECTIVAS

Recientes progresos en el conocimiento de la patogénesis de la enfermedad causada por el virus de la inmunodeficiencia humana junto con el desarrollo de potentes agentes antiretrovirales han resultado en una abundancia de opciones de tratamiento para los individuos infectados por el VIH. La rápida tasa de replicación viral unida a con la alta tasa de mutación, permiten una alta probabilidad de evolución de mutantes resistentes a los fármacos, si la replicación no es completamente suprimida por los regímenes de droga antivirales suministrados (1).

La aparición de la terapia antiretroviral altamente activa (*highly active antiretroviral therapy* -HAART-), consistentes en combinaciones de inhibidores de la proteasa y de la transcriptasa reversa, proporcionó un método potente y clínicamente efectivo de supresión de la carga viral en individuos infectados por el VIH. Sin embargo, aunque inicialmente muy exitosa, una experiencia clínica más amplia ha revelado limitaciones en este régimen terapéutico, con el cual hasta un 40% de los individuos tratados no pueden mantener un control sobre la replicación viral. Avances en el conocimiento del proceso por el cual el VIH-1 entra en la célula huésped, han señalado este punto como una diana para el desarrollo de nuevas drogas que añadir al actual armamento terapéutico.

El primer paso en el proceso de fusión viral implica la unión del complejo de la envuelta viral al antígeno de superficie celular CD4 a través de la glucoproteína gp120. Este paso es vulnerable para agentes que se unan tanto a la molécula gp120 como al antígeno CD4. En la primera parte de este trabajo se evaluaron dos clases de

compuestos diferentes dirigidos al bloqueo de este primer paso del ciclo de replicación viral: el oligonucleótido AR177 (Zintevir) y las albúminas cargadas negativamente. Ambos tipos de compuestos mostraron una potente actividad anti-VIH, inhibiendo cepas de VIH-1 *in vitro* a muy bajas concentraciones. En el estudio del mecanismo de acción, mostramos evidencias directas de que ambos compuestos, AR177 y las albúminas cargadas negativamente, interaccionan con la molécula gp120. Esto nos permite sugerir un modo de acción común para ambas clases de compuestos: la unión de dichas moléculas a la glucoproteína gp120, acción que previene una asociación estable entre el VIH-1 y las células $CD4^+$.

La capacidad de los polianiones de inhibir la replicación viral, fue descrita ya en el año 1968 (2), siendo años más tarde mostrados también como potentes inhibidores de la replicación del virus de la inmunodeficiencia humana (3, 4) ;sin embargo su fuerte actividad anticoagulante y su elevada toxicidad *in vivo* habían detenido su uso como agentes terapéuticos (5, 6). Las albúminas cargadas negativamente (ACN), cuyos prototipos son las albúminas de suero humano succiniladas (Suc-ASH) y las albúminas de suero humano aconitiladas (Aco-ASH), habían sido identificadas como potentes inhibidores de la replicación de VIH-1 *in vitro*, no mostrando ningún efecto sobre la coagulación sanguínea a diferencia de lo que ocurría con otros polianiones como el dextran sulfato o la heparina (7, 8).

El mecanismo de resistencia a Suc-ASH y a Aco-ASH fue analizado mediante la caracterización de cepas resistentes seleccionadas en cultivo celular. Como resultado del análisis de la secuenciación de la proteína Env de estas cepas resistentes, observamos que surgieron mutaciones en la molécula gp120. Estos resultados nos permiten concluir que la diana molecular de estas albúminas cargadas negativamente es la proteína de la envuelta viral gp120.

Contrariamente a lo que en un principio se podría suponer, el patrón de mutaciones presente en ambas cepas resistentes a las ACN, en la cepa resistente a AR177 o incluso en la previamente publicada cepa resistente a dextrán sulfato (9), no es idéntico. A pesar de que todos estos compuestos estarían interactuando con la misma proteína viral, y de que las cepas son resistentes y muestran cros-resintencia entre los diferentes compuestos inhibidores de la unión viral, estas cepas presentan una aparición de mutaciones diferente. Esto sugiere que la resistencia puede venir determinada no por unos cambios de aminoácidos determinados, sino más bien puede depender del cambio global en la molécula gp120, es decir, en la adquisición de resistencia a estos

compuestos no sería tan importante dónde están localizadas las mutaciones, sino cómo estas mutaciones están afectando a la conformación global de la molécula gp120 y a su exposición sobre la superficie de la membrana viral. Esto explicaría por qué diferentes mutaciones en la molécula gp120 nos dan un mismo fenotipo viral: la resistencia a los inhibidores de unión. Apoyando esta explicación también estaría lo mostrado por Kuipers et al (10), los cuales muestran que péptidos linearizados de V3 tienen una afinidad más débil para las ACN que la que presentan en una conformación más nativa, sugiriendo así mismo, que la conformación espacial de la glucoproteína de la envuelta, además de la distribución de la carga, pueden influenciar la unión a estos inhibidores de la unión viral.

Ambas cepas resistentes a las ACN mostraron mutaciones en la región de DNA que codifica para la glucoproteína gp41. Estudios anteriores habían mostrado que se podía dar una interacción entre la molécula gp41 y las albúminas de suero humano (11). Esta observación, junto con la aparición de estas mutaciones en las cepas que han desarrollado resistencia a las albúminas modificadas, nos permite sugerir que la unión a la glucoproteína gp41 podría jugar un papel adicional en el bloqueo de la infección por VIH *in vitro*. Esta aparición de mutaciones en gp41 no ha sido descrita para ninguna de las cepas resistentes a los inhibidores de unión analizados hasta el momento. Los cambios observados en las cepas resistentes a ANC, nos indican que estas albúminas podrían tener un mecanismo de acción doble, porque además de interaccionar con gp120 también podrían estar modificando la acción de gp41. La posible interacción de las ACN con gp41 le aporta, actualmente, un mayor interés al estudio de estos agentes, ya que los inhibidores de fusión que se unen a gp41 están mostrando ser unos potentes inhibidores de VIH y están teniendo un gran éxito en estudios clínicos en fase II (12-14). El estudio de esta interacción nos puede aportar un mayor conocimiento del mecanismo de acción de gp41, así como información para el desarrollo de otros agentes que bloqueen la función de esta proteína y que no sean péptidos derivados del dominio N-terminal de gp41. Por otro lado, esta interacción albúmina-péptido, debido a los altos niveles de albúmina que encontramos en el suero humano, podrían influenciar la eficacia de esas terapias anti-VIH-1 basadas en el uso de péptidos de gp41 (11).

Nosotros mostramos que los prototipos de ACN, Suc-ASH y Aco-ASH, actúan en el primer paso del ciclo de replicación viral a través de una unión directa a la molécula viral gp120. Este mecanismo de acción es compatible con los resultados que Kuipers et al mostraron posteriormente, y en los cuales se presenta la interacción de las

albúminas cargadas negativamente con la tercera región variable (V3) de la glucoproteína gp120. En este trabajo, los autores muestran además, que es posible la interacción de las ACN con V3 de aislados NSI (10), concordando con otro estudio en el cual se demuestra que aislados primarios NSI son inhibidos de igual forma por estas albúminas cargadas negativamente que los aislados SI (15). En relación a estos datos, nosotros mostramos que estas proteínas modificadas no interactúan con los correceptores de VIH-1 CXCR4 o CCR5, confirmando así la no inhibición preferencial de una u otra variante viral y siendo el efecto antiviral independiente de la capacidad de inducir sincitios de los aislados.

Estudios farmacocinéticos y toxicológicos de estas albúminas modificadas tanto en monos, como en ratas o ratones mostraron que las concentraciones necesarias para inhibir la replicación de VIH en plasma pueden ser logradas por administración intravenosa sin efectos adversos ni cambios histológicos en diversos órganos (8, 16). Además, la observación de que estos compuestos se redistribuyen rápidamente al sistema linfático (en el cual las concentraciones antivirales necesarias son alcanzadas) es muy prometedora considerando la importante función reservorio que este compartimento del cuerpo juega en la patogénesis por VIH-1 (17). A pesar de todos estos prometedores resultados *in vitro* o en animales *in vivo*, es poco probable que en humanos puedan llegar a ser administradas estas ACN en su actual composición, al igual que ocurre con otras proteínas de alto peso molecular. Esto no descarta que, ahora que conocemos que presentan actividad antiviral y cuál es su mecanismo de acción, puedan ser nuevamente modificadas de tal forma que se aumente su biodisponibilidad y perfil farmacológico.

Estudios previos habían puesto de manifiesto que el oligonucleótido compuesto de desoxiguanosina y timidina AR177, era un potente inhibidor de la replicación de VIH-1, proponiéndose que la inhibición de la integrasa de VIH-1 podría contribuir a la actividad antiviral de dicho compuesto (18-20). Nosotros indicamos que es la proteína gp120 de la envuelta de VIH-1 y no la integrasa la principal diana de AR177.

Un método muy útil de identificar las proteínas virales diana de un compuesto antiviral es a través del desarrollo de resistencia a ese compuesto *in vitro*. Igual que previamente ocurrió con dextrán sulfato o AMD3100, una cepa resistente al inhibidor de unión AR177 puede aparecer tras un largo y secuencial pase del virus en presencia de concentraciones crecientes de dicho compuesto. La secuenciación de la cepa NL4-3

AR177 resistente, mostró la aparición de mutaciones en las regiones V2, V3 y V4 de la molécula gp120, encontrándose sólo dos mutaciones silentes en el gen de la integrasa (19). Estas mutaciones en la integrasa no pueden explicar por qué esta cepa se vuelve resistente a la acción inhibitoria de AR177, siendo probablemente, las mutaciones observadas en la glucoproteína de la envuelta viral gp120, el motivo de la resistencia observada. En conclusión, estos datos de resistencia indican que la principal acción antiviral de AR177 en cultivo celular está localizada sobre la molécula gp120, aunque una actividad antiviral secundaria sobre la integrasa del VIH-1 no puede descartarse completamente.

Los oligonucleótidos, usados durante mucho tiempo como sondas en biología molecular, están siendo considerados, actualmente, como potenciales armas terapéuticas para el tratamiento de una gran variedad de enfermedades humanas. Teóricamente, conociendo la diana molecular que está implicada tanto en la causa como en el efecto de cualquier tipo de desorden clínico humano, éste puede ser tratado con oligonucleótidos. Las aplicaciones clínicas de compuestos oligonucleotídicos han sido bastante variadas hasta la fecha: cáncer e infecciones virales así como procesos inflamatorios, son algunos de los desórdenes para los cuales oligonucleótidos están siendo usados ahora en humanos (21). Fomivirsen (ISIS 2922), un oligonucleótido antisentido usado para el tratamiento de la retinitis por citomegalovirus (CMV) en pacientes con SIDA, ha sido el primer oligonucleótido en recibir aprobación de comercialización por la US Food and Drug Administration (FDA).

Algunos estudios han indicado que el perfil de toxicidad de los oligonucleótidos puede ser tanto específico de secuencia como específico de especie. En el caso concreto de AR177, este oligonucleótido está compuesto exclusivamente por desoxiguanosinas y timidinas, lo que le proporciona una estructura tetraédrica compacta. Esta estructura se cree que es la responsable de su potente inhibición de VIH e inusual estabilidad bioquímica (22), además de dotarle de una baja toxicidad en primates (23, 24). En monos, parece tener dos tipos de toxicidades que serían preocupantes: toxicidad hemodinámica, manifestada como alteraciones en la presión de la sangre, y toxicidad hematológica, manifestada como alteraciones en las células blancas de la sangre y aumento en el tiempo de coagulación. No obstante, algunos estudios han mostrado que el grado de toxicidad hemodinámica y/o hematológica en primates está directamente relacionada a la concentración de droga alcanzada en plasma (24, 25), sugiriendo que la toxicidad puede ser minimizada por una disminución en el tiempo de infusión

intravenosa de la droga. Asimismo, AR177 tiene una larga vida media en plasma, sangre y tejidos y es ampliamente distribuido, alcanzando en ratas altas concentraciones en tejidos linfoides y no linfoides (26).

A pesar de todo ello, Zintevir fue evaluado en un ensayo clínico en fase I/II y no se obtuvieron resultados esperanzadores. Aunque *in vitro* este compuesto presenta actividad anti-VIH, su actual composición lo convierte en un pobre agente terapéutico *in vivo*. Los resultados obtenidos hasta el momento con AR177 nos pueden ayudar en el diseño de futuros agentes antivirales, que, compartiendo su mismo mecanismo de acción, mejoren sus características de biodisponibilidad y actividad *in vivo*.

Después de que en el primer paso del ciclo de replicación del VIH, el virus se ha unido a la molécula CD4, en el segundo paso de este ciclo, la unión a CD4 induce una serie de cambios conformacionales en la glucoproteína de la envuelta, cuyo resultado es la exposición de una región de gp120 previamente oculta y que permite que la molécula gp120 ahora se una a los correceptores virales (27, 28). El hallazgo de que los ligandos naturales de estos correceptores, las quimiocinas, interrumpen el proceso de entrada viral bloqueando efectivamente la replicación viral, junto con la observación de que la ausencia del receptor CCR5 no parece tener ningún efecto deletéreo en humanos (29, 30), han dado muchas esperanzas de que se puedan desarrollar agentes que imiten el efecto protector de la delección del receptor CCR5, volviendo a los correceptores parcial o completamente inaccesibles a las interacciones de la envuelta del virus con la superficie celular. Esto se puede conseguir mediante la inducción de la dowregulación del correceptor de la superficie celular o bloqueando los lugares de unión de gp120 con inhibidores competitivos. Como ya se ha comentado anteriormente, las estrategias antivirales que interrumpen las interacciones de la superficie celular con el VIH tienen una ventaja sobre las terapias actualmente usadas. Estas nuevas terapias, prevendrían la entrada del virus en lugar de inhibir la replicación o la maduración viral, volviendo así a las células “inmunes” a la infección, es decir, pueden parar la diseminación de provirus integrados los cuales son imposibles de erradicar sin la destrucción de la célula hospedadora. Tales estrategias son atractivas también porque tienen como diana proteínas celulares, las cuales son relativamente invariantes en comparación a los altamente variables antígenos virales que tienen la capacidad de adquirir rápidamente resistencia a las drogas.

Hasta la fecha han sido presentadas varias pequeñas moléculas inhibidoras de la entrada del VIH a través de la interacción con CXCR4 o CCR5, siendo todas moléculas antagonistas de los receptores: los inhibidores de CXCR4 –AMD3100, T22 y ALX40-(31-35), y los inhibidores de CCR5 – TAK-779 o Schering C - (36) (37). Las diferencias entre ambas clases de inhibidores, probablemente refleja la diferente distribución de la carga en la superficie de ambos correceptores: mientras la superficie del receptor CXCR4 es fuertemente aniónica, CCR5 presenta una superficie casi neutra. Estas diferentes propiedades de los dos correceptores determinan el tipo de compuestos que han sido identificados como inhibidores.

Los biciclanes, cuyo prototipo es AMD3100, son una clase de compuestos antivirales que actúan como potentes y selectivos inhibidores de la replicación del VIH-1 y VIH-2 (31, 38, 39). La fuerte correlación aquí mostrada entre la actividad anti-VIH de estos compuestos y la interacción de CXCR4, además de la ausencia de inhibición de cepas de VIH-1 de fenotipo R5, indican que el bloqueo del correceptor CXCR4 es el mecanismo de acción de los biciclanes. La restricción mostrada en el número y disposición de grupos amino en la estructura del biciclán sugiere que para una fuerte interacción con CXCR4 se requiere una disposición determinada de cargas positivas. Esto fue confirmado cuando evaluamos el mecanismo de acción de conjugados de antibióticos aminoglicósidos con L-argininas. Estos compuestos, que ya habían sido descritos anteriormente como potentes inhibidores *in vitro* de la interacción Tat-TAR (40, 41), se parecen en sus propiedades químicas a péptidos oligocatiónicos como ALX-40. Los aminoglicósidos conjugados con argininas interactúan con CXCR4, presumiblemente a causa de su naturaleza catiónica. Paralelamente a lo ocurrido con los biciclanes, cambios en la estructura química de éstos antibióticos modificados en cuanto a la distribución de la carga positiva, también redujo su actividad inhibitoria; el número y la posición de los residuos de nitrógeno en la molécula afecta a la actividad antiviral de estos compuestos. En conclusión, el conjunto de resultados descritos, sugiere que la naturaleza catiónica de los biciclanes, los aminoglicosidos conjugados a L-argininas y los demás antagonistas de CXCR4 conocidos hasta el momento, conduce a interacciones electrostáticas con los residuos cargados negativamente de CXCR4 (42), impidiendo de este modo la interacción de la glucoproteína de la envuelta de VIH con el correceptor CXCR4.

Cuando alguno de estos inhibidores de la entrada viral sea usado *in vivo*, ¿cuál será la evolución del virus bajo esa presión selectiva? En un modelo experimental que

trataría de reflejar lo que ocurriría *in vivo*, el bloqueo selectivo del receptor de CXCR4 mediante el antagonista AMD3100, nos muestra que ejerciendo presión selectiva contra el receptor CXCR4 podemos eliminar totalmente las variantes virales, altamente patogénicas, de tipo X4 o tipo X4/R5, conduciendo a una selección de cepas que exhiben un genotipo y fenotipo del tipo R5. El estudio aquí realizado con SDF-1α ,o el mostrado anteriormente por De Vresse et al con AMD3100 (43), indican que con el cultivo continuado de la cepa X4 NL4-3 en presencia de concentraciones crecientes del ligando natural de CXCR4 o un antagonista de dicho receptor se da la generación de mutantes resistentes, los cuales exhiben un gran número de mutaciones diseminadas a lo largo de la glucoproteína gp120. Este sistema de cultivo *in vitro*, podría clasificarse como un sistema favorecedor de la aparición de cepas mutantes resistentes de tipo X4, ya que el virus crece en presencia de dosis subóptimas y crecientes de los compuestos, además de utilizarse una línea celular que carece del receptor CCR5, no permitiendo así el crecimiento de cepas del tipo R5. La generación de esta cepa resistente a SDF-1α nos indica que la aparición de resistencia a un antagonista de CXCR4 no es un proceso sencillo y no sería fácilmente adquirida *in vivo*. Se necesitan un gran número de mutaciones en la proteína de la envuelta gp120 para permitir al virus crecer en presencia de compuestos que interaccionan con CXCR4. Consecuentemente, en un sistema *in vitro* que imita más fielmente la situación que se daría *in vivo*, y con una concentración óptima del antagonista de CXCR4 capaz de inhibir totalmente la entrada viral, observamos el bloqueo total de la entrada de una cepa de tipo X4. No detectamos la presencia de DNA proviral, aún después de un largo tiempo en cultivo, con lo cual no se dará la oportunidad de la generación de mutantes que escapen a la presión selectiva del fármaco. En conclusión, nuestros resultados sugieren que el tratamiento de pacientes VIH-positivos con un antagonista de CXCR4 puede revertir el fenotipo viral de tipo X4 a un fenotipo menos patogénico de tipo R5 prolongándose así la fase asintomática de la enfermedad.

Aunque los antagonistas de CXCR4 parecen ser una gran promesa terapéutica, todavía queda por evaluar si: (1) estos análogos no presentan toxicidad; (2) exhiben un buen perfil farmacológico y (3) son antiviralmente activos en humanos. Compuestos altamente polares tales como T22, ALX40 y AMD3100 a menudo presentan una biodisponibilidad limitada que restringe su potencial como fármacos. Este problema se verá aumentado en el caso de ALX40 y T22 (y sus derivados T134 y T140) debido a su naturaleza peptídica (32, 33, 35).

En ensayos clínicos en fase I, AMD3100 fue bien tolerado por voluntarios sanos, siendo ampliamente distribuido por todo el cuerpo y aunque presentó una baja biodisponibilidad oral, cuando se administró intravenosamente se alcanzaron niveles en sangre por encima de la concentración previamente descrita para mostrar un efecto antiviral (44). Actualmente se está llevando a cabo un estudio clínico en fase 2, que ha sido ampliado a seis lugares diferentes en USA.

Los datos mostrados por el ensayo clínico en individuos sanos son de especial relevancia dado que se había reportado que ratones transgénicos deficientes en SDF-1α o en CXCR4 presentaban graves malformaciones durante el desarrollo embrionario (45-47). Mas recientemente Onai et al (48) mostraron que ratones adultos transducidos con una intrakina de SDF-1, exhibían una linfopoyesis y mielopoyesis alterada, sugiriendo un papel esencial de CXCR4 y SDF-1 en la hematopoyesis en adulto. Estas observaciones cuestionan el uso de una terapia basada en los antagonistas de CXCR4. Sin embargo, estas alteraciones predichas en adultos por este trabajo no son observadas ni en humanos en el ensayo clínico llevado a cabo con AMD3100, ni concuerdan con los datos obtenidos en un modelo animal en el cual ratones transgénicos BALB/c tratados con AMD3100 durante 28 días no mostraron ningún tipo de efecto adverso, lo cual parece indicar que el antagonismo a CXCR4 no es tóxico (49). Esto podría explicarse porque se ha encontrado la existencia de una mutación en la región promotora del gen SDF-1, posiblemente involucrada en un aumento de la producción de la quimiocina. El significado de esta mutación es un tema controvertido (50) pero su existencia nos indica que variantes genéticas en el sistema CXCR4/SDF-1 y variaciones en el nivel de expresión tanto de CXCR4 como de SDF pueden ser soportadas por individuos sanos. Además, la recién descrita existencia de un receptor alternativo de SDF-1 podría, parcialmente, compensar el bloqueo de CXCR4 mediante AMD3100 (51). La presencia de este receptor alternativo podría permitir que los mecanismos desencadenados por SDF-1 se continuasen dando en el individuo en presencia de un antagonista de CXCR4, no observándose por tanto efectos deletéreos.

En conjunto, nuestros resultados muestran que el bloqueo del complejo proceso de entrada del virus de la inmunodeficiencia humana nos puede ofrecer una nueva oferta de fármacos antivirales que no sólo mejorarían las terapias anti-VIH actualmente existentes, sino que nos pueden ayudar a aumentar el conocimiento del proceso de infección del VIH, además de ayudarnos a diseñar nuevas drogas quizá capaces de llegar a la erradicación de la infección por el virus de la inmunodeficiencia humana.

Además, los datos aquí presentados sugieren que es necesario seguir evaluando nuevos compuestos para poder llegar a descubrir nuevos y más potentes agentes antivirales.

Por todo esto, las observaciones que hacemos en la última parte de este trabajo, tanto con cepas de laboratorio como con aislados virales primarios, apuntan a la potencial utilidad de antagonistas de CXCR4 en la prevención del cambio de fenotipo de R5 a X4 que generalmente es considerado el marco de la aparición del SIDA y /o la progresión de la enfermedad. Dos de las cuestiones, con relación a estos y otros hallazgos, que hoy día aún permanecen sin respuesta son: ¿qué factores provocan este cambio de fenotipo de cepas de tipo X4 a cepas de tipo R5 a lo largo de la progresión de la enfermedad? y ¿qué determina la diferente citopatogenicidad observada entre las cepas T-trópicas X4 y las cepas M-trópicas R5?

El cambio de fenotipo viral podría venir determinado por factores virales y/o por factores presentes en el individuo. Desconocemos si existe algún factor viral que predestine a la progresión hacia cepas de tipo X4, si existe algún gen viral implicado en una posterior progresión. En cuanto a los factores presentes en el individuo, aún hoy día se desconoce qué papel están jugando las quimiocinas y los receptores de quimiocinas en este cambio de fenotipo viral. Los receptores de quimiocinas y su expresión, así como los niveles de expresión de algunas quimiocinas en los individuos, podrían estar determinando o participando en la propensión a la evolución de la enfermedad. Estudios de los niveles de CC quimiocinas y su correlación con la progresión no han ofrecido resultados concluyentes, y aún no se he determinado si la expresión del receptor CXCR4 o los niveles de SDF-1 podrían estar relacionados con el cambio de fenotipo. Tanto los niveles de CXCR4 como los niveles de SDF-1 varían de individuo a individuo. Elevados niveles de CXCR4 y/o bajos niveles de SDF-1 en plasma o en tejido linfoide, donde está ocurriendo la replicación viral, podrían favorecer la diseminación de cepas de tipo X4, altamente patogénicas. Si esto fuese así, la determinación de estos niveles de expresión nos darían información sobre la posible evolución de la enfermedad, pudiendo ser utilizados como un marcador de progresión. Además, otro punto interesante a estudiar con relación a la quimiocina SDF-1, es qué efecto presenta la variante genética SDF-1 3’A, si la variante está correlacionada con un aumento en el nivel de la quimiocina en plasma o en algún otro compartimento celular y si esto se puede relacionar con los diferentes niveles de SDF-1 encontrados en los individuos, pudiendo existir una correlación con la progresión de la enfermedad o

pudiendo estar implicada en la prevención del cambio de fenotipo viral que se observa en algunos individuos VIH-positivos.

Un mayor conocimiento de los factores que controlan el cambio de fenotipo viral nos podría ayudar en la prevención de la aparición de cepas de tipo X4. Los efectos en los individuos infectados de la aparición de cepas de tipo X4 son múltiples, no conociéndose aún cómo estas cepas causan dichos efectos. La aparición de cepas X4 está correlacionada con una rápida caída en el número de células $CD4^+$, así como con la apoptosis de células $CD4^+$ y de células $CD8^+$. Aunque se sabe que este efecto inductor de apoptosis podría ser resultado del contacto Env-CXCR4, todavía queda por descubrir que determinantes de la proteína Env son los responsables y si todos los efectos causados por este tipo de cepas X4 única y exclusivamente son debidos a la proteína de la envuelta Env o existen otros factores, como una mayor capacidad replicativa o alguna modificación en algún otro gen viral, que también están influyendo. Además de estar implicadas en una pérdida masiva de células, estas cepas X4 también provocan disfunción celular: el tejido linfoide en presencia de cepas de tipo X4 se vuelve inmunosuprimido. Esta inmunosupresión parece incluso ocurrir en ausencia de replicación viral, lo que apuntaría a un efecto de la proteína de la envuelta, que generando quizá algún tipo de señalización aberrante da como resultado la disfunción del tejido, aunque cuál es el verdadero mecanismo por el que esto ocurre es, de momento, un enigma.

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CAPÍTULO 7

CONCLUSIONES

De nuestro estudio se puede concluir que:

- ✓ Los diferentes agentes evaluados mostraron actividad antiviral *in vitro* frente a diferentes cepas de VIH-1.
- ✓ Se ha determinado que:
  - ✓ El mecanismo de acción de los compuestos AR177 y de las albúminas cargadas negativamente, es la interacción con la glucoproteína de la envuelta viral gp120.
  - ✓ El mecanismo de acción de los biciclanes y de los aminoglicósidos conjugados con argininas, es la interacción con el receptor de quimiocinas CXCR4.

  Por tanto, todos estos agentes están dirigidos contra las etapas tempranas del ciclo de replicación del VIH-1.
- ✓ Mostramos la creación de cepas de VIH-1 resistentes, como método útil para determinar el mecanismo de acción de fármacos dirigidos a las etapas tempranas del ciclo de replicación viral.

- ✓ El VIH-1, frente al bloqueo del receptor de quimiocinas CXCR4, evoluciona de dos formas muy diferentes:
    - ✓ Frente a concentraciones subóptimas del antagonista, y sin la posibilidad de utilizar otro correceptor, el virus adquiere un gran número de mutaciones en la glucoproteína de la envuelta que le permite crecer sin cambiar de correceptor.
    - ✓ Frente a concentraciones óptimas del antagonista, y en presencia del receptor CCR5, se da un cambio de fenotipo de tipo X4 a fenotipo de tipo R5 y se previene el cambio de fenotipo de tipo R5 a tipo X4.

- ✓ En conjunto, el bloqueo del proceso de entrada nos puede ofrecer una nueva oferta de fármacos antivirales que mejoren la terapia anti-VIH existente, además de ayudarnos a aumentar el conocimiento del proceso de infección.

AGRADECIMIENTOS

La presente memoria representa el fruto de cuatro años de trabajo, recogido gracias a la ayuda de muchas personas que de un modo u otro me han apoyado día a día. A todos ellos quisiera agradecerles muy sinceramente su incondicional apoyo.

En primer lugar quisiera dar las gracias al Dr. José A. Esté por haberme ayudado a aprender casi todo lo que hoy se del VIH, aunque lo más importante es que me ha enseñado a ser independiente en el trabajo. Fue duro pero valió la pena. Gracias. Y a Julià, gracias por las largas discusiones frente al café nuestro de cada día, porque esos momentos me han ayudado a crecer científicamente.

Gracias al Dr. Bonaventura Clotet y a Lidia Ruiz por apoyarme y confiar siempre en mí, y por brindarme la oportunidad de empezar a trabajar en este campo, para mí tan apasionante, de la investigación del virus de la inmunodeficiencia humana.

Además, esta tesis no hubiese sido posible sin la ayuda de todas las personas que forman la Fundació irsi-Caixa: gracias a Arantxa porque esta tesis también es fruto de su trabajo; a Silvia por además de ser compañera ser mi mejor amiga dentro y fuera del laboratorio, a Marta por poder compartir con ella mis tensiones, miedos y frustraciones; a Cecilia por comprender mis lágrimas en los momentos difíciles; a Teresa por ayudarme a sobrellevar la veteranía; a mis compañeros Jordi y Anuska con los que peleé contra la apoptosis, las quimiocinas, los X4, los R5...,y al resto de las personas de irsi: Lourdes, el Dr. Miguel Angel Martínez y Mariona; el Dr. Javier Martínez y Ana, Rafi,

Esther, Albert. Y a la nueva generación: Mercedes, Julia, Kristina y Natalia. También quisiera agradecer toda la ayuda, siempre tan amablemente prestada, del equipo de la Unidad Clínica del SIDA y de la Fundació Lluita Contra la SIDA.

Quisiera dar las gracias a todas las personas del Departamento de Inmunología del Hospital Universitario Germans Trias i Pujol, porque todas ellas han sido siempre de gran ayuda y una fuente inagotable de suministros en momentos de urgencia.

I want to give thanks to Dr. Leonid Margolis for allowing me to finish this work, as well as to give me the opportunity to begin a new scientific stage in this laboratory. And to Dr. Jean-Charles Grivel to encourage me to conclude my doctoral work and to continue in the world of the scientific research in a moment in which the vocation had failed me.

Un enorme gracias a mis padres, hermanos y amigos, especialmente a Nacho, que aunque no entendiendo mis largas ausencias por mis interminables jornadas en el laboratorio, siempre me han apoyado incondicionalmente.

Con especial cariño quiero mencionar a mi hermana Nuria. Son tantas las cosas a agradecer ...,muchas gracias por estar siempre a mi lado.

La memoria me falla y por ello me imagino que me dejo algunos nombres en el tintero que se merecen estar aquí presentes. Espero que no me lo tengáis en cuenta. Y espero que comprendáis que después de tanto fenol, mercaptoetanol..., y en plena época de las vacas locas, el cerebro ya no es lo que era.