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Altered sensitivity of an R5X4 HIV-1 strain 89.6 to coreceptor inhibitors by a single amino acid substitution in the V3 region of gp120

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Abstract

The replication of several R5X4 strains is blocked by single CXCR4 inhibitors such as AMD3100 or T140 although the target cells express both CXCR4 and CCR5 *in vitro*. To identify which region(s) of the Env are involved in the increased sensitivity to CXCR4 inhibitors, we isolated a T140-escape mutant using R5X4 HIV-1 strain 89.6. An isolated mutant harbored a single amino acid substitution in the V3 region of the Env (arginine 308 to serine R308S). Luciferase-reporter HIV-1 pseudotyped with the mutant Env showed that the substitution conferred total resistance to CXCR4 antagonists but increased sensitivity to a CCR5 antagonist TAK-779 in the infection of the cells expressing both CCR5 and CXCR4. Analyses using the cells expressing a single coreceptor showed that the mutant Env predominantly and efficiently utilized CCR5 rather than CXCR4 while retaining R5X4 phenotype. These results indicated that the sensitivities of the R5X4 strain to coreceptor inhibitors were altered by a single amino acid substitution in the V3 region of gp120.

Keywords: HIV; Coreceptor antagonist; CXCR4 usage; Envelope; V3 region

1. Introduction

The entry of human immunodeficiency virus type 1 (HIV-1) is initiated by the binding of viral envelope glycoprotein gp120 to CD4 followed by interactions with cellular coreceptor, either CCR5 or CXCR4. In general, CCR5-using viruses (called R5 viruses) are predominant throughout the course of infection, and are associated with transmission between humans. In contrast, CXCR4-using viruses emerge at late stage of infection in some cases, and are thought to be associated with accelerated disease progression (Connor et al., 1997; Scarlatti et al., 1997). In these individuals, most of CXCR4-using strains still utilize CCR5 (called R5X4 viruses), while several strains exclusively utilize CXCR4 alone (called X4 viruses). Although R5X4 viruses are able to utilize both coreceptors, the replication of several R5X4 strains was inhibited by CXCR4 inhibitors alone in the infection of cells expressing both coreceptors (Ghezzi et al., 2001; Glushakova et al., 1999; Stalmeijer et al., 2004). However, it still remains to be identified which region(s) of gp120 of these R5X4 viruses are associated with the increased sensitivity to CXCR4 inhibitors. In the present study, we isolated an escape mutant from a CXCR4 inhibitor T140 using R5X4 HIV-1 strain 89.6. The T140-escape mutant harbored a single amino acid substitution from arginine to serine (R308S) in the V3 region of gp120. The substitution conferred total resistance to CXCR4 antagonists and increased sensitivity to a CCR5 antagonist TAK-779, indicating that the mutant Env preferentially utilized CCR5 over CXCR4. These results suggest that the sensitivities of the R5X4 strain to coreceptor inhibitors were altered by a single amino acid substitution in the V3 region.

2. Materials and methods

2.1. Cells and culture conditions

A HeLa-CD4/LTR-β-gal cell line (MAGI) (Kimpton and Emerman, 1992) was provided by M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and maintained in Dulbecco's modified Eagle medium (DMEM) (ICN, Costa Mesa, CA) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD), 0.2 mg/ml of G418, 0.1 mg/ml of hygromycin, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. A CCR5-expressing HeLa-CD4/LTR-β-gal cell line (MAGI/CCR5) was established

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and maintained as previously described (Maeda et al., 2000). The 293T cell line was maintained in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The $CD4^+$ T cell line PM1/CCR5, which expresses high level of CCR5, was established using a retrovirus vector as previously described (Maeda et al., 2000; Yusa et al., 2005), and maintained in RPMI 1640 (Gibco BRL, Grand Island, NY) medium supplemented with 10% FBS, 0.2 mg/ml of G418, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. A human CD4-expressing glioma cell line (NP2/CD4), and its derivative cell lines (NP2/CD4/CXCR4 and NP2/CD4/CCR5) were maintained in Eagle's minimum essential medium (MEM; Gibco BRL) supplemented with 10% FBS and appropriate antibiotics as previously described (Jinno et al., 1998; Maeda et al., 2000). NP2/CD4 cells expressing both CCR5 and CXCR4 were also established from NP2/CD4/CCR5 cells transduced with CXCR4 gene using a retroviral vector as previously described (Maeda et al., 2000). Peripheral blood mononuclear cells (PBMCs) from seronegative donors were isolated using lymphocyte separation medium (LSM) (ICN), stimulated with PHA (phytohemagglutin) (Sigma–Aldrich, St. Louis, MO) for 3 days, and used for viral infection in the presence of 10 U/ml of recombinant IL-2 (Sigma–Aldrich).

2.2. Viruses and reagents

The laboratory strains of HIV-1, Ba-L, YU-2, 89.6 (Collman et al., 1992), and LAI were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. The R5 laboratory strains of HIV-1, JR-FL and JR-CSF were kindly provided by Y. Koyanagi (Kyoto University). The primary isolates of HIV-1 were obtained from S. Matsushita (Kumamoto University, Center for AIDS Research). The peptide CXCR4 antagonist T140 (Tamamura et al., 1998) was a gift from H. Tamamura and N. Fujii (Kyoto University). The small-molecule nonpeptide CXCR4 antagonist AMD3100 (Schols et al., 1997a,b) was supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. A CCR5 antagonist TAK-779 (Baba et al., 1999) was obtained from Takeda Chemical Industries (Osaka, Japan). Azidothymidine (AZT) was purchased from Sigma–Aldrich.

2.3. Determination of the virus sensitivity to coreceptor antagonists

The sensitivity of viruses to coreceptor antagonists was determined using MAGI/CCR5 as previously described (Maeda et al., 2000). Briefly, MAGI/CCR5 cells (2×10^4 per well) were seeded in 48-well flat-bottomed plates (Iwaki, Chiba, Japan). The following day, the cells were incubated with various concentrations of coreceptor antagonists for 1 h, and the virus was added to all wells at a concentration that would result in 200 blue cells per well in the control. Forty-eight hours after the virus exposure, cells were fixed and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The blue cells in each well were counted under a microscope. All experiments were done in triplicate. The sensitivity of viruses to coreceptor antagonists was expressed as 50% effective concentration (EC_{50}), the concentration of the drug which reduced the infection levels by 50% in comparison with the infected, drug-free control.

2.4. Construction of mutant Env expression vectors

NL4-3 and JF-FL Env expression vectors (pCXN-NL*env* and pCXN-FL*env*) were prepared as described previously (Maeda et al., 2000). The 89.6 *env* gene was supplied by T. Kimura (Kumamoto University) and re-cloned into pCXN2 designated pCXN-89.6*env*. For the substitution of the V3 region, the AflII and NheI sites were introduced into both pCXN-89.6*env* and pCXN-FL*env* by PCR-mediated site-directed mutagenesis to give pCXN-FLAN*env* and pCXN-89.6AN*env*, respectively. Incorporation of an AflII site led to no amino acid substitutions in JR-FL *env* (CTG AAA (Leu Lys) → CTT AAG (Leu Lys)), and one amino acid substitution in 89.6 *env* (CTA AAT (Leu Asn) → CTT AAG (Leu Lys)). Incorporation of an NheI site led to two amino acid substitutions (GTT ATA (Val Ile) in JR-FL *env* and 89.6 *env* → GCT AGC (Ala Ser)). Each recombinant *env* was verified by restriction enzyme mapping and sequence analysis. To obtain the V3 region containing the mutation R308S, a cloned plasmid carrying R308S was amplified by PCR using primers containing AflII and NheI as follows: 5′-CAGCTTAAGGAATCTGTAGTAATTAA-3′, and 5′-TTGCTAGCTATCTGTTGTAAAGTGT-3′ (underlined are the AflII and NheI restriction enzyme sites, respectively). The amplified fragment was then cloned into pCR-TOPO (Invitrogen, Carlsbad, CA), and verified using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA). The AflII–NheI fragment carrying R308S was finally ligated into pCXN-89.6AN*env* to generate pCXN-89.6AN*env* R308S.

2.5. Isolation of a T140-escape mutant from an R5X4 HIV-1 strain 89.6

For the isolation of a T140-escape mutant from the 89.6 strain, PM1/CCR5 cells were treated with increasing amounts of T140, and then infected with the 89.6 strain. The viral replication was monitored by observation of the cytopathic effect in PM1/CCR5 cells. After 20 passages of the virus in PM1/CCR5 cells at a final T140 concentration of 10 μM, T140 was removed from the virus-infected cells, and then the virus was recovered from the cell culture supernatant. The sensitivity of the escape mutant to CXCR4 antagonists was determined using MAGI/CCR5 cells as described above. The virus was also passaged in the absence of T140 to exclude the effect of long-term culture. The DNA was extracted from virus-infected cells using the urea method, and subjected to PCR using *Taq* DNA polymerase (Promega, Madison, WI). The V1–2 region sequences were amplified using primers: 5′-ACATGGTAGATCAGATGCATGA-3′ and 5′-TGGCTGAAAGGATACCTTTGGA-3′. The V3 region sequences were amplified using primers: 5′-CAGCTTAAGGAATCTGTAGTAATTAA-3′, and 5′-TTGCTAGCTATCTGTTGTAAAGTGT-3′. The amplified

products were cloned into pCR-TOPO (Invitrogen), and the *env* regions of the virus were then sequenced using an ABI PRISM 377 automated sequencer (Applied Biosystems).

2.6. Determination of the coreceptor antagonist sensitivity

Recombinant luciferase-reporter virus stocks pseudotyped with various HIV-1 Envs were generated by co-transfection of 293T cells with 20 μg of HIV-1NLLucΔBgl, and 10 μg of pCXN2 plasmids expressing Env using the calcium phosphate method (Maeda et al., 2000). At 2 days post-transfection, the cell culture supernatant was filtered (0.45 μm pore-size) and used as a luciferase-reporter virus. To determine the sensitivity to coreceptor antagonists in NP2/CD4 cells expressing different coreceptors, the cells (1×10^4 per well) in 48-well plates were first incubated with various concentrations of coreceptor antagonists for 1 h at 37 °C, and then infected with the above luciferase-reporter viruses. For the PBMCs, the cells (5×10^5 per well) were infected with the above luciferase-reporter viruses in the presence of each coreceptor antagonist at a concentration of 1 μM. Forty-eight hours after the infection, the cells were lysed with 100 μl of luciferase assay buffer (Promega). Luciferase activity was measured by adding 100 μl of luciferase assay substrate (Promega) to 20 μl of lysate and reading the light activity in a luminometer, Lumat LB 9501/16 (EG&G Berthold, Bad Wildbad, Germany). The light activity is reported as relative light units (RLU). The sensitivity of the virus to coreceptor antagonists in NP2/CD4 cells was expressed as EC_{50}, i.e. the concentration of the drug which reduced the infection levels by 50% in comparison with the infected, drug-free control in triplicate experiments. In the case of PBMCs, antiviral effect of each coreceptor antagonist was expressed as the % infectivity compared with drug-free control in triplicate experiments.

2.7. Coreceptor usage by HIV-1 using a single-round replication assay

For the determination of coreceptor usage by luciferase-reporter viruses, NP2/CD4 cells expressing either CCR5 or CXCR4 (Maeda et al., 2000) were infected with the same amount (2 ng of p24 antigen) of each virus. Forty-eight hours after infection, luciferase activity was determined as mentioned above.

The ability to use low levels of CCR5 by various Envs was determined to infect NP2/CD4 clones expressing high and low levels of CCR5 with luciferase-reporter virus stocks. Both high and low levels of CCR5-expressing cells were infected with four different titers of the virus stocks which were prepared by threefold serial dilutions, and luciferase activity was determined after 48 h post-infection. The ratio (low CCR5/high CCR5) for each different virus titer was calculated in triplicate experiments, and the means and standard deviations of ratios obtained from four different titers of the viral stocks were calculated.

2.8. Assay of drug sensitivity using PM1/CCR5

Viral titration was performed using PM1/CCR5 cells by the standard limiting dilution method in round-bottom 96-well microtiter plates (BD Biosciences Falcon, Franklin Lakes, NJ). Fifty percent of the cell culture infectious dose ($CCID_{50}$) value of each virus preparation was determined in PM1/CCR5 cells. For antiviral assay, PM1/CCR5 cells (10^4 cells/ml) were infected with HIV-1 for 1 h at 37 °C at a titer of 50 $CCID_{50}$, and 100 μl of the cell suspension was distributed into 96 round-bottom plates containing several dilutions of the compounds. After 5 days incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Pauwels et al., 1988). For entry inhibitors, PM1/CCR5 cells were preincubated with each concentration of coreceptor antagonists for 1 h before infection, and antiviral assays were performed as described above. The results of drug sensitivity were expressed as EC_{50} defined as the concentration of drug that protected virus-induced cytopathic effect by 50% compared to the infected drug-free control in a triplicate experiment. Cytotoxicity of the compounds was always evaluated in parallel with their antiviral activity.

3. Results

3.1. The R5X4 strain 89.6 is sensitive to T140 in the cells expressing both CCR5 and CXCR4

We first determined the antiviral effect of coreceptor antagonists, T140 (Tamamura et al., 1998) and TAK-779 (Baba et al., 1999) against various strains of HIV-1 with different coreceptor usage (Table 1). Infection of MAGI/CCR5 cells was performed in the presence of coreceptor antagonists at concentrations ranging from 1 to 1000 nM for T140, 1 to 10,000 nM for TAK-779, respectively. Antiviral effects were determined by a standard X-gal staining method, and expressed as the 50%

Table 1
Sensitivity of various strains of HIV-1 to coreceptor antagonists

Virus	Phenotype	MAGI/CCR5[a]		PM1/CCR5[b]	
		TAK-779	T140	TAK-779	T140
JR-FL	R5	3.2 ± 0.2	>1000	140 ± 20	>1000
Ba-L	R5	6.6 ± 0.4	>1000	120 ± 60	>1000
YU-2	R5	1.1 ± 0.1	>1000	22 ± 9.0	>1000
MOK	R5	7.2 ± 0.9	>1000	180 ± 9.0	>1000
YKI	R5	1.7 ± 0.4	>1000	63 ± 10	>1000
LAI	X4	>10,000	1.6 ± 0.3	>10,000	5.2 ± 0.6
TKA	X4	>10,000	17 ± 5.0	>10,000	880 ± 280
YHI	X4	>10,000	20 ± 2.0	>10,000	360 ± 190
89.6	R5X4	>10,000	5.4 ± 0.2	>10,000	18 ± 0.2
KMT	R5X4	>10,000	24 ± 6.0	>10,000	>1000
TIK	R5X4	>10,000	10 ± 0.2	>10,000	>1000

[a] Infection of MAGI/CCR5 cells was performed in the presence of TAK-779 or T140, and X-gal positive cells were counted. The sensitivities of the virus to coreceptor antagonists were expressed as the 50% effective concentration (EC_{50}). Data shown represent mean EC_{50} values (nM) with standard deviations from experiments performed in triplicate.

[b] Infection of PM1/CCR5 cells was performed in the presence of TAK-779 or T140, and the numbers of viable cells were counted by MTT assay 5 days post-infection as described in Section 2. The sensitivities of the virus to coreceptor antagonists were expressed as the EC_{50}. Data shown represent mean EC_{50} values (nM) with standard deviations from experiments performed in triplicate.

effective concentration (EC_{50}). As previously shown by others (Tamamura et al., 1998) and ourselves (Harada et al., 2004a,b; Maeda et al., 2003), T140 blocked the replication of X4 but not R5 strains (Table 1), while TAK-779 blocked the replication of R5 but not X4 strains. In agreement with published data (Ghezzi et al., 2001; Glushakova et al., 1999; Stalmeijer et al., 2004; Yi et al., 2005), the replication of all R5X4 strains tested here was also blocked by T140 alone, although the cells express both CCR5 and CXCR4. The EC_{50} values, ranging from 5 to 24 nM, of R5X4 strains were comparable to those of X4 strains. In contrast, TAK-779 was not able to inhibit the replication of R5X4 strains in the cells expressing both coreceptors. To confirm the antiviral effect of T140 against R5X4 strains, PM1/CCR5 was chosen as a target since this $CD4^+$ T cell line also supports the replication of CCR5- and/or CXCR4-using strains. This cell line was used in a colorimetric MTT assay to determine the antiviral effect of T140 and TAK-779. Infection by various strains of HIV-1 was performed in the presence of T140 or TAK-779, and the antiviral effect was determined using the 50% inhibitory concentration of cytotoxicity of the cells (Table 1). Similarly to the experiment using MAGI/CCR5 cells, all R5 strains were totally resistant to T140, and sensitive to TAK-779, whereas the X4 strain (LAI) was sensitive to T140, and totally resistant to TAK-779. Intriguingly, primary X4 strains (TKA and YHI) were insensitive to T140 in contrast with the laboratory strain (LAI). Among R5X4 strains tested here, the replication of the 89.6 strain was again inhibited by T140, comparable to LAI, whereas the other R5X4 strains (KMT and TIK) were markedly resistant. We finally confirmed the antiviral effect of T140 against the 89.6 strain using PBMCs which express both coreceptors. The replication of the 89.6 strain was also blocked by T140 but not by TAK-779 (data not shown). These results indicated that the 89.6 strain was quite sensitive to the CXCR4 inhibitor in spite of the ability to utilize both coreceptors in the established cell lines.

3.2. Selection of a T140-escape mutant using PM1/CCR5

In order to determine which region(s) of the 89.6 strain were associated with the increased sensitivity to T140, we isolated a T140-escape mutant using the PM1/CCR5 cell line since the cells with prominent ballooning are observed after infection by various strains of HIV-1 including R5 and R5X4 viruses. Every 3–4 days, the supernatant was transferred to fresh culture when massive syncytia were detected by microscopy. After 20 passages of the 89.6 strain in the presence of T140 at a final concentration of 10 μM in PM1/CCR5 cells, the sensitivity of the recovered virus to T140 was determined using MAGI/CCR5 cells. The escape mutant had fivefold greater resistance to T140 (Fig. 1) ($EC_{50} = 19.8 \pm 0.4$ nM) compared with the wild-type 89.6 strain ($EC_{50} = 3.3 \pm 0.8$ nM). In contrast, the passaged virus in the absence of T140 did not confer resistance to T140 ($EC_{50} = 3.2 \pm 0.6$ nM).

3.3. Amino acid sequences of the T140-escape mutant

To determine which region(s) were responsible for the reduced sensitivity of the T140-escape mutant, the V1–2, V3, V4, C3, and C4 region of the gp120 was sequenced after cloning the PCR product of each region using DNA from infected PM1/CCR5 cells as a template. Nine to twelve clones from each PCR product were isolated and sequenced. The analyses of *env* region revealed that both viruses selected in the presence of T140 and passaged in the absence of T140 had the same substitutions in the V2 region (proline to glutamine at codon 184 and glutamic acid to lysine at codon 186) (Fig. 2A). In contrast, only the escape mutant had an arginine to serine substitution at codon 308 (R308S) in the V3 region (8 out of 12 clones) (Fig. 2B). No consistent mutation was observed in the other regions of gp120 including V1, V4, C3, and C4 in the escape mutant (data not shown).

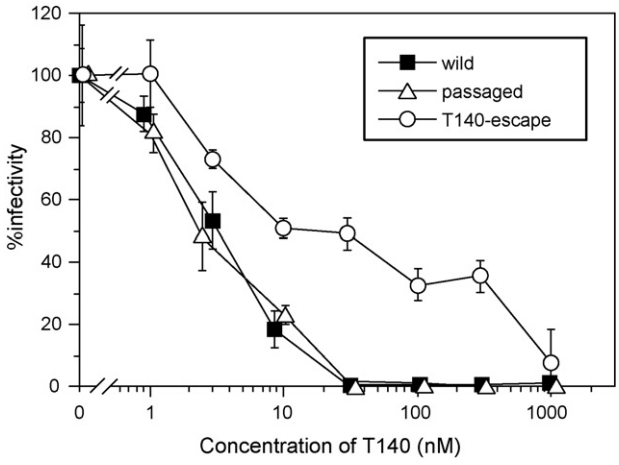

Fig. 1. Sensitivity of the T140-escape mutant selected in PM1/CCR5. MAGI/CCR5 cells were treated with various concentrations of T140, followed by inoculation with the wild-type 89.6 strain (closed square), the passaged virus in the absence of T140 (open triangle), and the escape mutant selected in the presence of T140 (open circle). Blue cells were counted 2 days after infection. The extent of virus replication is represented as a percentage of blue cells counted in the absence of T140. The data are expressed as means ± standard deviations from experiments performed in triplicate.

3.4. A single amino acid substitution in the V3 region conferred resistance to CXCR4 antagonists and increased sensitivity to a CCR5 antagonist

To confirm the role of the mutation R308S in the reduced sensitivity to T140, the luciferase-reporter HIV pseudotyped with wild-type Envs (89.6 wt and 89.6AN wt), or 89.6AN R308S Env was generated. In order to exclude the effect of minor coreceptors frequently utilized by R5X4 viruses, we used NP2/CD4 cells expressing both coreceptors as a target since this cell line does not express any coreceptors, except for CCR5 and CXCR4, to permit HIV-1 entry (Jinno et al., 1998). The cells were infected with above luciferase-reporter viruses in the presence of coreceptor antagonists, and the sensitivities were determined. The R308S substitution conferred total resistance to T140 (Fig. 3A, Table 2). The mutant Env was also cross-resistant to another CXCR4 antagonist AMD3100 (Fig. 3B, Table 2). However, the mutation conferred increased sensitivity to TAK-779 (Fig. 3C, Table 2). Finally, PBMCs were infected with luciferase-reporter HIV with

(A) V1-2 amino acid sequence

			Fraction
89.6	125	CVTLNCTNLNITKNTTNPTSSSWGMMEKGEIKNCSFYITTSIRNKVKKEYALFNRLDVVPIENTNNTKYRLISC 198	of clones
without T140		.....Q.K.....	7/12
		.....K.....	2/12
		.....	2/12
		.....D.....Q.K.....	1/12
with T140		.....Q.K.....	7/11
		.....	2/11
		.....K.....Q.K.....	1/11
		.....N.....	1/11

(B) V3 amino acid sequence

			Fraction
89.6	298	CTRPNNNTRRRLSIGPGRAFYARRNIIGDIRQAHC 332	of clones
without T140		.....	9/9
with T140		.....S.....	8/12
		.....	3/12
		.....S.....	1/12

Fig. 2. V1–2 (A) and V3 (B) amino acid sequences from 89.6 infected cells passaged in the absence and presence of T140. Amplified products from infected PM1/CCR5 cells in the absence and presence of T140 after 20 passages were cloned, and 9–12 clones from each sample were sequenced. The amino acid sequences of V1–2 and V3 regions of the wild-type 89.6 strain are shown in the top line. In each set of clones, the deduced amino acid sequence of the V2 and V3 regions was aligned by single amino acid code. The fraction of clones containing each unique Env sequence is indicated on the right. Identity with this sequence at individual amino acid positions is indicated by dots.

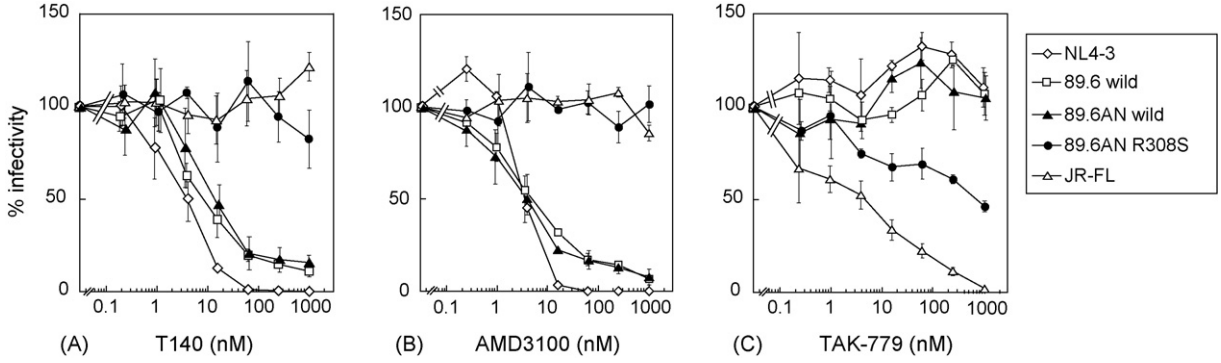

Fig. 3. Sensitivity of luciferase-reporter HIV-1 pseudotyped with various Envs to T140, AMD3100 or TAK-779 in NP2/CD4 cells expressing CCR5 and CXCR4. NP2/CD4/CCR5/CXCR4 cells were infected with luciferase-reporter viruses pseudotyped with Envs from NL4-3 (open diamond), 89.6 wild (open square), 89.6AN wild (closed triangle), 89.6AN R308S (closed circle), or JR-FL (open triangle) in the presence of T140 (A), AMD3100 (B) or TAK-779 (C). Luciferase activity was measured 2 days after infection. The extent of virus replication is represented as a percentage of luciferase activity produced in the absence of coreceptor antagonists. The data are expressed as means ± standard deviations from experiments performed in triplicate.

Table 2
The sensitivities of luciferase-reporter virus pseudotyped with various Envs to various inhibitors in double coreceptor expressing cells

Envelope (phenotype)	NP2/CD4/CCR5/CXCR4			
	T140	AMD3100	TAK-779	AZT
JR-FL (R5)	>1000	>1000	3.6 ± 1.9	49 ± 11
NL4-3 (X4)	3.0 ± 1.1	3.4 ± 0.3	>1000	63 ± 13
89.6 wild (R5X4)	6.4 ± 0.5	5.4 ± 1.4	>1000	99 ± 6
89.6AN wild (R5X4)	9.3 ± 1.5	4.2 ± 1.8	>1000	108 ± 8
89.6AN R308S (R5X4)	>1000	>1000	440 ± 77	85 ± 4

NP2/CD4/CCR5/CXCR4 cells were infected by luciferase-reporter HIVs with various Envs in the presence of T140, AMD3100, TAK-779, and AZT. The luciferase activities of infected cells were determined 48 h post-infection. Data shown represent mean EC_{50} values (nM) with standard deviations from experiments each performed in triplicate.

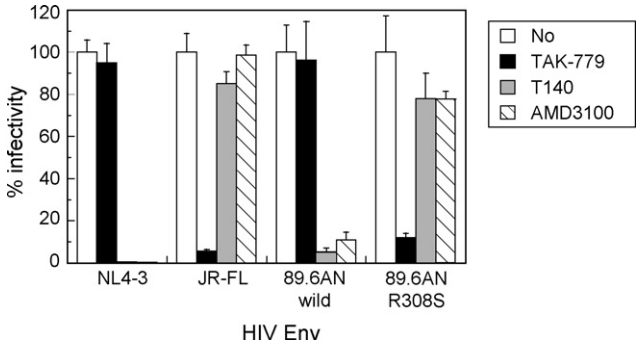

Fig. 4. Sensitivity of luciferase-reporter HIVs pseudotyped with various Envs to coreceptor antagonists in PBMCs. PHA-stimulated PBMCs were infected with luciferase-reporter HIVs with indicated Envs in the absence (open bar), or presence of TAK-779 (closed bar), T140 (shaded bar), and AMD3100 (diagonal bar) at a concentration of 1 μM each. Luciferase activity was measured 2 days after infection, and the extent of viral replication was expressed as a percentage of luciferase activity of infected cells in the absence of coreceptor antagonists. The representative data from three independent experiments are shown and are expressed as means ± standard deviations from experiments performed in triplicate.

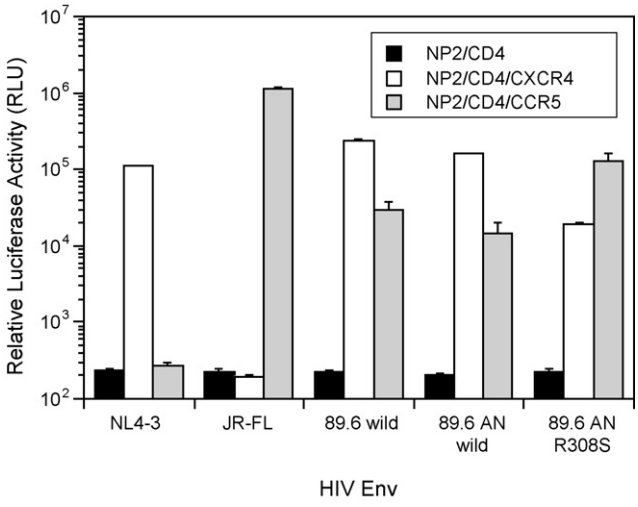

Fig. 5. Coreceptor usage of luciferase-reporter HIV-1s pseudotyped with various Envs. NP2/CD4 alone (filled bar), NP2/CD4 expressing CXCR4 (open bar) or CCR5 (shaded bar) were infected with the same amount (2 ng of p24 antigen) of luciferase-reporter viruses pseudotyped with various Envs. Luciferase activity was measured 2 days after infection. The representative data from three independent experiments are shown, and are expressed as geometric means ± standard deviations from experiments performed in triplicate.

R308S Env. At a concentration of 1 μM of each coreceptor antagonist, the replication of the virus carrying R308S Env was inhibited by TAK-779 alone, while the replication of the viruses with 89.6 wild-type Env (89.6AN wt) was inhibited by T140 and AMD3100, but not by TAK-779 (Fig. 4).

3.5. The mutation R308S did not confer reduced sensitivity to CXCR4 antagonists in terms of CXCR4-mediated entry

To check whether the virus with R308S Env also confers resistance to T140 with regard to CXCR4-mediated entry, NP2/CD4/CXCR4 cells were used as target cells. The EC_{50} values of R308S Env to T140 and AMD3100 were, however, slightly lower than those of wild-type Envs in NP2/CD4/CXCR4 cells (Table 3). These results showed that the mutation R308S in the 89.6 Env did not confer reduced sensitivity to CXCR4 antagonists in terms of CXCR4-mediated entry.

3.6. R308S Env efficiently utilized CCR5 than CXCR4

In order to check whether the virus carrying R308S Env changes coreceptor use, NP2/CD4 cells expressing a single coreceptor either CXCR4 or CCR5 were infected with the same amount of luciferase-reporter viruses as previously described (Maeda et al., 2000). The cells were infected with the same amount of luciferase-reporter viruses as previously described (Maeda et al., 2000). Luciferase activities after infection of cells showed that all Envs derived from the 89.6 strain were able to utilize both CXCR4 and CCR5 (Fig. 5). However, the luciferase activities of NP2/CD4/CXCR4 infected by the virus with wild-type Envs were ∼10-fold higher than that of NP2/CD4/CCR5. In contrast, the luciferase activity of NP2/CD4/CCR5 infected by the virus with R308S Env was 10-fold higher than that of NP2/CD4/CXCR4. These results indicated that the virus carrying R308S Env predominantly utilized CCR5 over CXCR4, and those with wild-type Envs had the opposite preference.

The higher efficiency of R308S Env to utilize CCR5 than wild-type Envs was also supported by the observation that R308S Env more readily entered NP2/CD4/CCR5 clones with lower levels of CCR5 expression (Fig. 6).

Since an efficient utilization of CCR5 by Env is reported to be correlated to a decreased sensitivity to CCR5 antagonists (Reeves et al., 2002), we then checked the sensitivity of the virus

Table 3
Sensitivity of luciferase-reporter virus pseudotyped with various Envs to coreceptor antagonists in single coreceptor expressing cells

Envelope (phenotype)	NP2/CD4/CXCR4			NP2/CD4/CCR5	
	T140	AMD3100	AZT	TAK-779	AZT
JR-FL (R5)	NA	NA	NA	17 ± 3.3	71 ± 1
NL4-3 (X4)	8.7 ± 2.4	3.0 ± 0.9	61 ± 13	NA	NA
89.6 wild (R5X4)	12.6 ± 2.3	2.5 ± 0.3	130 ± 13	0.7 ± 0.2	130 ± 44
89.6AN wild (R5X4)	13.7 ± 0.1	1.8 ± 1.1	130 ± 30	1.2 ± 0.1	160 ± 31
89.6AN R308S (R5X4)	2.9 ± 0.6	0.5 ± 0.1	85 ± 5	12 ± 0.2	100 ± 17

NP2/CD4/CXCR4 and NP2/CD4/CCR5 cells were infected with luciferase-reporter HIVs with various Envs in the presence of indicated inhibitors. The luciferase activities of infected cells were determined 48 h post-infection. Data shown represent mean EC_{50} values (nM) with standard deviations from experiments performed in triplicate. NA: not applicable.

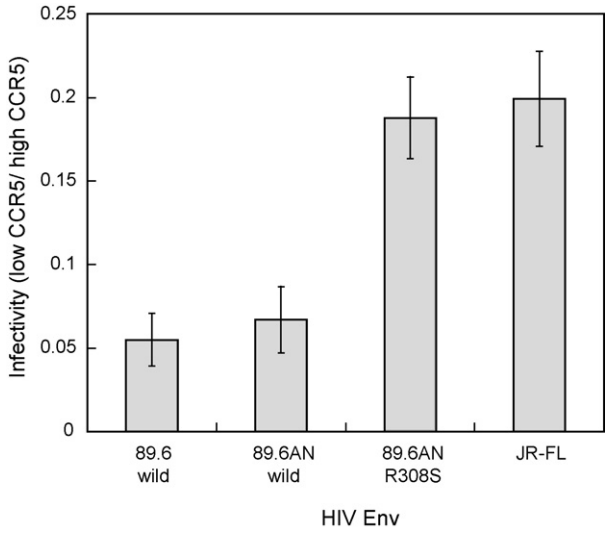

Fig. 6. Infectivity of luciferase-reporter HIVs pseudotyped with different Envs in the cells expressing low levels of CCR5. NP2/CD4 clones expressing high and low levels of CCR5 were infected with four different titers of the virus stocks, and luciferase activities were determined 2 days after infection. The ratio (low CCR5/high CCR5) in each different viral titer was calculated in triplicate experiments. The data are expressed as means ± standard deviations of ratios obtained from four different viral stocks.

carrying R308S Env to TAK-779 using NP2/CD4/CCR5. As shown in Table 3, R308S Env had ∼10-fold reduced sensitivity to TAK-779 compared to the wild-type Envs.

4. Discussion

It has been reported that the replication of several R5X4 strains is blocked by single CXCR4 inhibitors such as AMD3100 or T140 in $CD4^+$ primary T cells expressing both CXCR4 and CCR5 *in vitro* (Ghezzi et al., 2001; Stalmeijer et al., 2004) and lymphoid tissue *ex vivo* (Glushakova et al., 1999). In the present study, we also found that single CXCR4 antagonists inhibited the replication of R5X4 strains in the $CD4^+$ transformed cell line, MAGI/CCR5. Among these R5X4 strains, the replication of the 89.6 strain was further inhibited by CXCR4 antagonists in PM1/CCR5 cells and PBMCs. In order to determine which region(s) of gp120 are associated with the increased sensitivity to T140, we isolated a T140-escape mutant from an R5X4 strain 89.6 using PM1/CCR5 cells. The escape mutant harbored a single amino acid substitution (R308S) at the 11th position of V3 loop. The single mutation R308S in the 89.6 Env conferred total resistance to T140 and AMD3100 but increased sensitivity to TAK-779 in the infection of cells expressing both coreceptors (Figs. 3 and 4). In general, the conversion of amino acid from arginine to serine at position 308 (11th position in the V3 loop) indicates the loss of CXCR4 use according to an 11/25 rule: if the 11th or 25th positions of V3 loop are positively charged, viruses will use CXCR4; otherwise they use CCR5 (Resch et al., 2001). In addition, the mutation R308S combined with other substitutions in the V3 region was reported to alter the cellular tropism (De Jong et al., 1992). In our present study, the mutation R308S in the V3 region of the 89.6 strain also provided total resistance to CXCR4 inhibitors and partially increased sensitivity to CCR5 inhibitors although the mutant Env was retaining R5X4 phenotype (Figs. 5 and 6). These results indicated that the 89.6 strain carrying R308S may preferentially utilize CCR5 over CXCR4.

It still remains unclear why most R5X4 viruses were unable to utilize CCR5 on the cell surface when CXCR4 was masked by its antagonists in $CD4^+$ cells expressing both coreceptors. Mellado et al. (2001) previously showed that a monoclonal antibody against CCR2 blocked the replication of R5 and X4 viruses, although the viruses were unable to utilize CCR2 as a coreceptor. This finding was explained by the ability of this antibody to induce oligomerization of CCR2 with CCR5 and CXCR4. Although heterodimers between CCR5 and CXCR4 have not been observed (Babcock et al., 2003), it is possible that the binding of gp120 or coreceptor antagonists to $CD4^+$ cells might induce hetero-oligomerization of them. Since the assembly of approximately four to six coreceptor molecules is thought to be necessary for viral entry (Kuhmann et al., 2000), the binding of coreceptor antagonist to the hetero-oligomeric coreceptors complexes may alter their whole conformation, resulting in the incompetent fusion pore. In such circumstances, the ability of Env to interact with the lower amounts of free CCR5 molecules by changing the net charge of the V3 loop might be necessary for successful fusion. However, additional studies are required to confirm this hypothesis using techniques such as co-immunoprecipitation and fluorescence resonance energy transfer (FRET) analysis between CCR5 and CXCR4 (Rodríguez-Frade et al., 2004).

In summary, we have identified the single amino acid substitution in the V3 region involved in the increased sensitivity of the 89.6 strain to single CXCR4 inhibitors. Further studies will be required to confirm our studies using various backgrounds of R5X4 strains.

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