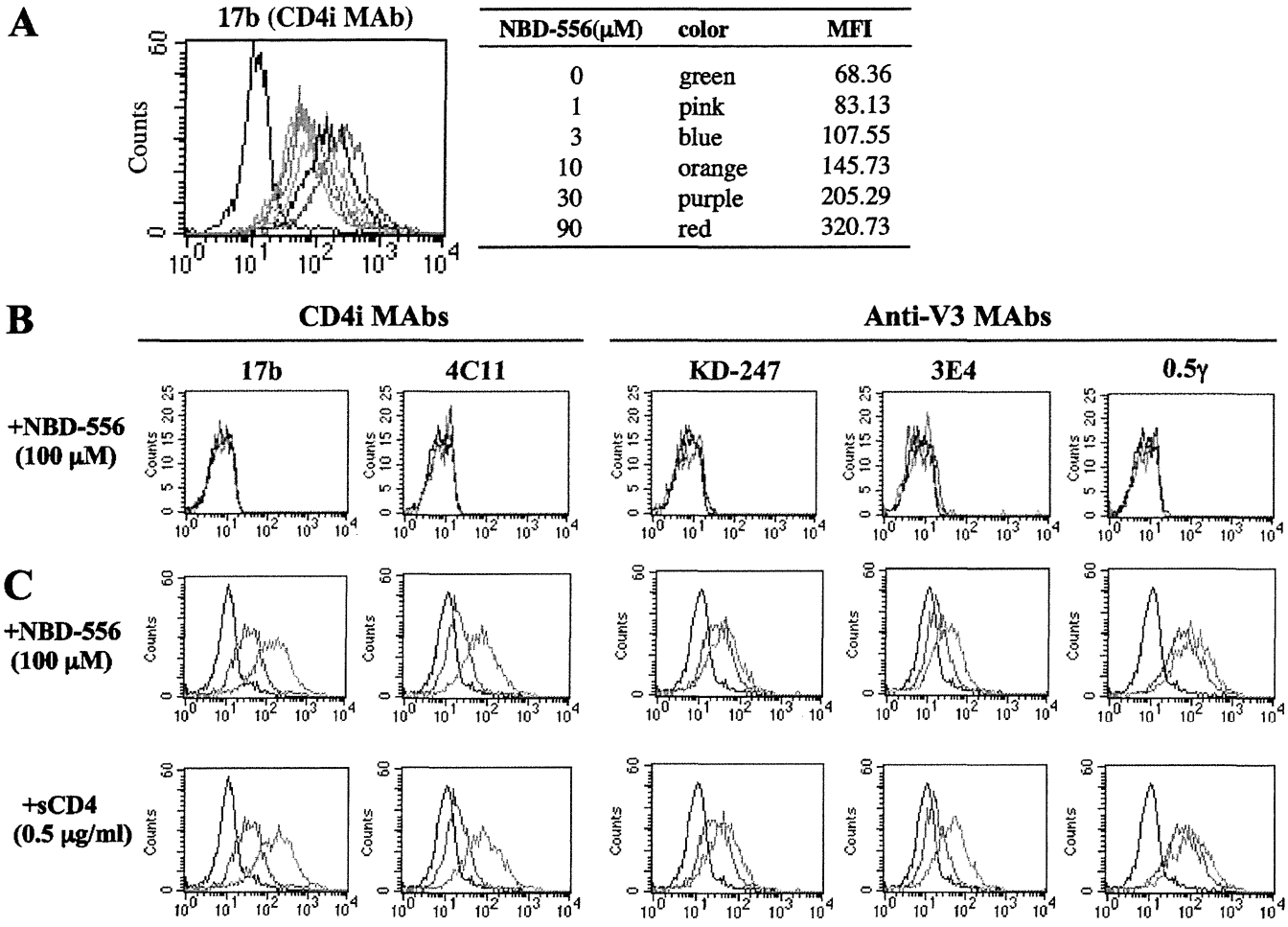

NBD-556(μM)	color	MFI
0	green	68.36
1	pink	83.13
3	blue	107.55
10	orange	145.73
30	purple	205.29
90	red	320.73

FIG. 2. Comparisons of MAb binding to cell surface-expressed gp120 with sCD4 and NBD-556. HIV-1_{JR-FL} chronically infected PM1 cells were preincubated with or without NBD-556 (A, 1 to 90 μM; C, 100 μM) or sCD4 (C, 0.5 μg/ml), and uninfected PM1 cells were also preincubated with or without NBD-556 (B, 100 μM) for 15 min, and then incubated with various anti-HIV-1 MAbs (17b, 4C11, KD-247, 3E4, and 0.5γ) at 4°C for 30 min. The cells were washed, and a fluorescein isothiocyanate-conjugated anti-human IgG was used for detection. (A) Color lines show the concentrations of NBD-556: green, 0 μM; pink, 1 μM; blue, 3 μM; orange, 10 μM; purple, 30 μM; and red, 90 μM. (B and C) Red line shows no preincubated with NBD-556 or sCD4. Blue line shows preincubated with NBD-556 or sCD4. Black line shows using a control IgG MAb.

isolates, HIV-$1_{Pt.1}$ and HIV-$1_{Pt.3}$, with IC_{50}s of 3.6 and 11.8 μM, respectively (Table 1). YYA-004 did not show significant anti-HIV activity against any of the strains tested up to a concentration of 100 μM. The *in vitro* cytotoxicities of NBD-556 and YYA-004 toward PM1/CCR5 cells used for the anti-HIV-1 infectivity studies were determined by using the MTT assay. The CC_{50} values of NBD-556 and YYA-004 toward PM1/CCR5 cells were 140 and 350 μM, respectively (Table 1).

Comparison of Ab binding to cell surface-expressed HIV-1_{JR-FL} Env with NBD-556 and sCD4. To compare the effect of NBD-556 with that of sCD4 with respect to the induction of conformational change in the trimeric gp120, the binding of CD4i MAbs (17b and 4C11) and anti-V3 MAbs (KD-247, 3E4, and 0.5γ) to cell surface-expressed Env proteins on HIV-1_{JR-FL} chronically infected PM1 cells was analyzed by fluorescence-activated cell sorting. Comparisons of the binding profiles of the Abs to the cell surfaces were carried out using the mean fluorescence intensity (MFI). The binding of CD4i MAb 17b increased gradually as the amount of the CD4-mimicking small compound NBD-556 increased from 0 to 90 μM (Fig. 2A, the MFI increased from 68.36 to 320.73). As shown in Fig. 2C, the binding of both CD4i MAbs 17b and 4C11 increased remarkably after pretreatment with 100 μM NBD-556 (the MFIs increased from 43.3 to 201.57 and from 24.43 to 96.06, respectively). Moreover, the binding of all of the anti-V3 MAbs—KD-247, 3E4, and 0.5γ—was enhanced by pretreatment with NBD-556 (the MFIs increased from 34.59 to 51.9, from 22.97 to 39.07, and from 86.61 to 145.08, respectively). sCD4 pretreatment of the Env-expressing cell surface also caused remarkable enhancement of the binding for not only the CD4i MAbs but also the three anti-V3 MAbs, similar to pretreatment with NBD-556. These results indicate that the CD4-mimicking compound NBD-556 can induce the conformational changes in gp120 that are caused by binding of sCD4.

Highly synergistic interactions of KD-247 combined with NBD-556. Both neutralizing anti-V3 MAb KD-247 and NBD-556 block the viral entry process, especially at the stage of the interaction between CD4 and gp120 (CD4-binding site). Each of these agents binds to either the V3 loop or the CD4 cavity. Furthermore, our previous observations suggested that neu-

TABLE 2. Combination indices for KD-247, 4C11, or 0.5δ and for sCD4 or NBD-556 against HIV-1_{JR-FL} and HIV-1_{IIIB}

Combination	Virus	CI values at different ICs[a]		
		IC_{50}	IC_{75}	IC_{90}
KD-247+sCD4	HIV-1_{JR-FL}	0.313	0.266	0.277
KD-247+NBD-556	HIV-1_{JR-FL}	0.174	0.043	0.011
4C11+NBD-556	HIV-1_{IIIB}	0.473	0.445	0.860
0.5δ+NBD-556	HIV-1_{IIIB}	47.8	20.1	8.56

[a] The multiple-drug effect analysis of Chou et al. (6) was used to analyze the effects of the drugs in combination. IC, inhibitory concentration. CI < 0.9, synergy; CI = 0.9 to 1.1, additivity; CI > 1.1, antagonism. The data shown are representative of two or three separate experiments.

TABLE 3. Inhibitory activities of NBD-556 and sCD4 toward infection of HIV-1_{IIIB} escape variants from NBD-556 and sCD4

Virus	IC_{50}[a]	
	NBD-556 (μM)	sCD4 (μg/ml)
HIV-1_{IIIB}	12	0.52
HIV-$1_{NBD-R(20)14p}$	>30	5.7
HIV-$1_{NBD-R(50)17p}$	>30	>10
HIV-$1_{sCD4-R(20)5p}$	>30	>10

[a] PM1/CCR5 cells (2×10^3) were exposed to 100 $TCID_{50}$ of each passaged virus and then cultured in the presence of various concentrations of sCD4 or NBD-556. The IC_{50}s were determined by using the MTT assay on day 7 of culture. All assays were conducted in duplicate. The data shown are representative of two or three separate experiments.

tralizing MAb KD-247 selects escape variants with greater sensitivities to sCD4 (33). Based on this notion, we examined the synergy of this MAb with sCD4 or the CD4-mimicking compound NBD-556 against wild-type HIV-1_{JR-FL}. The multiple-drug effect analysis of Chou et al. (6) was used to analyze the effects of combining KD-247 with sCD4 or NBD-556. As shown in Table 2, all of the CI values for KD-247 with the two CD4-gp120 interaction inhibitors (sCD4 and NBD-556) were <0.5 against HIV-1_{JR-FL} at all of the inhibitory concentrations tested. In particular, the CI values for the combinations of KD-247 with NBD-556 were <0.1 for IC_{75} and IC_{90}. These results suggest that combinations of KD-247 with the CD4-gp120 binding inhibitors sCD4 and NBD-556 produce very highly synergistic effects. We further examined the synergy of CD4i MAb 4C11 or anti-CD4bs MAb 0.5δ with NBD-556 against wild-type HIV-1_{IIIB}. The combination of 4C11 and NBD-556 showed synergy against HIV-1_{IIIB} for IC_{50} and IC_{75}. As expected, the IC values for NBD-556 and anti-CD4 binding site MAb, 0.5δ, which may compete with the CD4 mimetic for the CD4-binding site, were >5 against HIV-1_{IIIB} at all of the inhibitory concentrations tested. However, at lower concentrations, additive effects were observed between NBD-556 and anti-CD4bs MAb 0.5δ (data not shown). These results indicate that NBD-556 may bind within or near the epitope of the anti-CD4bs MAb and then induce the conformational changes in Env.

Selection of NBD-556 and sCD4 escape variants. To select NBD-556- and sCD4-resistant HIV-1 variants *in vitro*, we exposed PM1/CCR5 cells to HIV-1_{IIIB} and serially passaged the viruses in the presence of increasing concentrations of NBD-556 or sCD4. As a control, HIV-1_{IIIB} was passaged under the same conditions without the antiviral agents to allow us to monitor the spontaneous changes that occurred in the virus during prolonged PM1/CCR5 cell passages (designated the passage control). The selected viruses were initially propagated in the presence of 1 μM NBD-556 or 0.5 μg of sCD4/ml and, during the course of the selection procedure, the concentrations of the NBD-556 and sCD4 were increased to 50 μM and 20 μg/ml, respectively. At passages 14 and 17 for NBD-556 and passage 5 for sCD4, the supernatants containing the viruses, which were designated HIV-$1_{NBD-R(20)14p}$, HIV-$1_{NBD-R(50)17p}$, and HIV-$1_{sCD4-R(20)5p}$, respectively, were harvested, and the sensitivities of the viruses to NBD-556 and sCD4 were determined by the MTT assay (Table 3). The IC_{50}s for NBD-556 against HIV-1_{IIIB}, HIV-$1_{NBD-R(20)14p}$, and HIV-$1_{NBD-R(50)17p}$ were 12, >30, and >30 μM, respectively. The IC_{50}s of sCD4 against HIV-1_{IIIB} and HIV-$1_{sCD4-R(20)5p}$ were 0.52 and >10 μg/ml, respectively. HIV-$1_{NBD-R(20)14p}$, HIV-$1_{NBD-R(50)17p}$, and HIV-$1_{sCD4-R(20)5p}$ were also examined for their cross-resistance with one another. Each resistant variant was found to be cross-resistant to NBD-556 and sCD4 (Table 3). These results indicate that the HIV-1_{IIIB} virus acquired resistant phenotypes against NBD-556 and sCD4 during the distinct *in vitro* selection processes.

Sequences of the envelope region of the NBD-556 and sCD4 mutants. To determine the genetic basis of the resistance in the variant HIV-1_{IIIB} strains, the C1 to C4 region of the *env* gene was amplified from genomic DNA extracted from the infected cells and cloned, and the PCR-amplified products were sequenced (Fig. 3). At passage 8 for 6 μM NBD-556, five mutations (A281D, E370A, S375N, A433T, and A436T) were observed. At passage 21 in the culture where HIV-1_{IIIB} was propagating in the presence of 50 μM NBD-556, four amino acid substitutions of Ser to Asn at position 375 (S375N, 11 of 11 clones) in C3, Ala to Lys at position 342 (A432K, 1 of 11 clones) in C4, Ala to Thr at position 433 (A433T, 4 of 11 clones) in C4, and Ala to Thr at position 436 (A436T, 1 of 11 clones) in C4 were observed (Fig. 3A). These results did not contradict a previous study in which gp120 mutants (S375W, I424A, W427A, and M475A) with changes in residues that contacted the Phe43 cavity did not detectably bind NBD-556 by isothermal titration calorimetry (23). On the other hand, in the selection with sCD4, seven mutations (E211G, P212L, V255E, N280K, S375N, G380R, and G431E) appeared during the passages. At passage 5 in the culture where HIV-1_{IIIB} was propagating in the presence of sCD4 (20 μg/ml), four substitutions of E211G (1 of 10 clones), V255E (5 of 10 clones), G380R (1 of 10 clones), and G431E (2 of 10 clones) were detected for sCD4 at 20 μg/ml (Fig. 3B).

To compare the two mutation profiles obtained during the *in vitro* selection with NBD-556 and sCD4, molecular modeling of NBD-556 docked into gp120 was performed by docking simulations using the FlexSIS module of SYBYL 7.1 (Fig. 4). The atomic coordinates of the crystal structure of gp120 with sCD4 were retrieved from the PDB (entry 1RZJ). As shown in Fig. 4, almost all of the mutations lay along the inside of the CD4 cavity in the selection of NBD-556, with similar three-dimensional positions to the mutations induced by sCD4. These findings demonstrate that NBD-556 binds to the CD4 cavity or in the vicinity of the CD4-binding site.

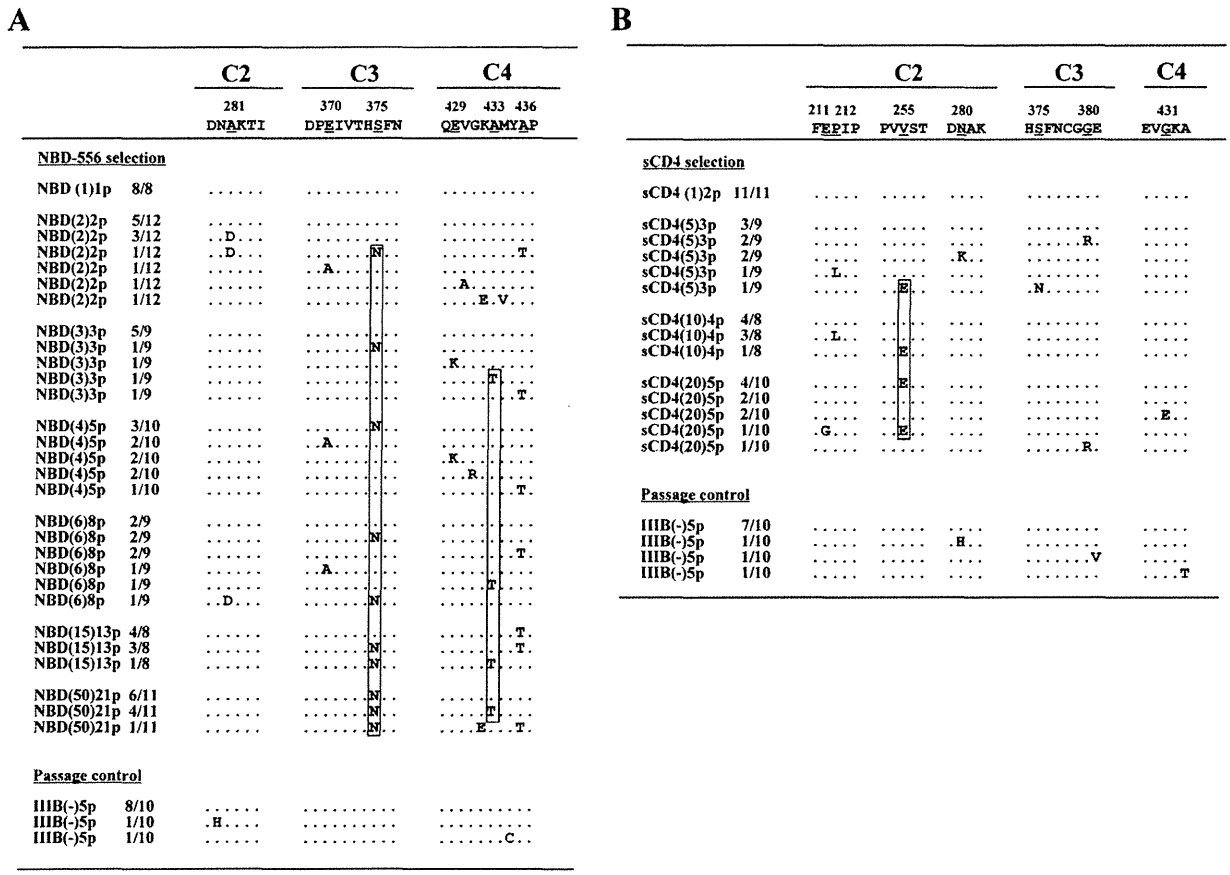

FIG. 3. Alignment of the gp120 amino acid sequences from the indicated passages in the NBD-556 and sCD4 escape processes. The amino acid sequences were deduced from the nucleotide sequences of the *env*-encoding regions of proviral DNA isolated from cells infected with the HIV-1_{IIIB} variants selected in the presence of NBD-556 (A) or sCD4 (B) and the passage control. The amino acid sequences of the envelope proteins of the baseline HIV-1_{IIIB} are shown at the top as a reference. The identity of the sequences at the individual amino acid positions is indicated by dots. The numbers of clones with the given amino acid substitutions among a total of 8 to 12 clones are listed. The number in parentheses denotes the concentrations of NBD-556 or sCD4. The major mutations of NBD-556 and sCD4-resistant variants at final passage are boxed.

Sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4- and NBD-556-resistant envelope mutations to NBD-556, sCD4, and MAbs. To confirm whether the mutations were responsible for the reduced sensitivities to NBD-556 and sCD4, a single-round replication assay was performed. The β-galactosidase reporter viruses were pseudotyped with wild-type Env (HIV-1_{WT}) or Env singly mutated with V255E in C2 (HIV-1_{V255E}), S375N in C3 (HIV-1_{S375N}), and A433T in C4 (HIV-1_{A433T}). The mutations that arose in the absence of NBD-556 (the passage control) are not related to resistance because the control passage did not show any significant increase in IC_{50} (data not shown). With respect to the mutations in the presence of NBD-556 three mutations, S375N, V255E, and A433T were consistently and increasingly observed during the process of selection. Additional mutations in "escape variants" other than S375N, V255E, and A433T were observed; however, these mutations were not consistently detected in passages and did not accumulate during selection. Thus, we considered the three mutations—S375N, V255E, and A433T—related to the development of resistance to both NBD-556 and sCD4, although some involvement of additional mutations in the development of a resistant phenotype is undeniable. As shown in Fig. 5A, all of the mutant clones were completely resistant to NBD-556 at concentrations of up to 20 μM. YYA-004 without the *p*-chlorophenyl group was unable to inhibit infection of all of the clones tested (Fig. 5B). The clone with V255E, which was induced by *in vitro* selection with sCD4, was highly resistant to sCD4 compared to the wild-type virus (114-fold-higher IC_{50}) (Fig. 5C). However, the other pseudotyped viruses, HIV-1_{S375N} and HIV-1_{A433T}, were slightly resistant compared to HIV-1_{WT} (4- and 2-fold-higher IC_{50}s, respectively). We also examined the sensitivities of the pseudotyped clones containing Env mutations to anti-gp120 glycan MAb 2G12, anti-CD4bs MAb b12, and anti-CD4 MAb RPA-T4 by a single-round replication assay (Fig. 5D to F). All of the mutant viruses showed almost the same neutralization sensitivities as the wild-type virus to the 2G12, b12, and RPA-T4 MAbs. These results indicate that the three mutations induced by *in vitro* selection with NBD-556 and sCD4 were responsible for the resistance to NBD-556, whereas the NBD-selected variants containing S375N in C3 and A433T in C4 of gp120 had moderately resistant phenotypes against sCD4, as shown by the sensitivities of the NBD-556-passaged viruses to sCD4 determined by the multiround assay (Table 3).

To examine whether the resistance mutations affected the sensitivity of a CD4i MAb against HIV-1, we determined the

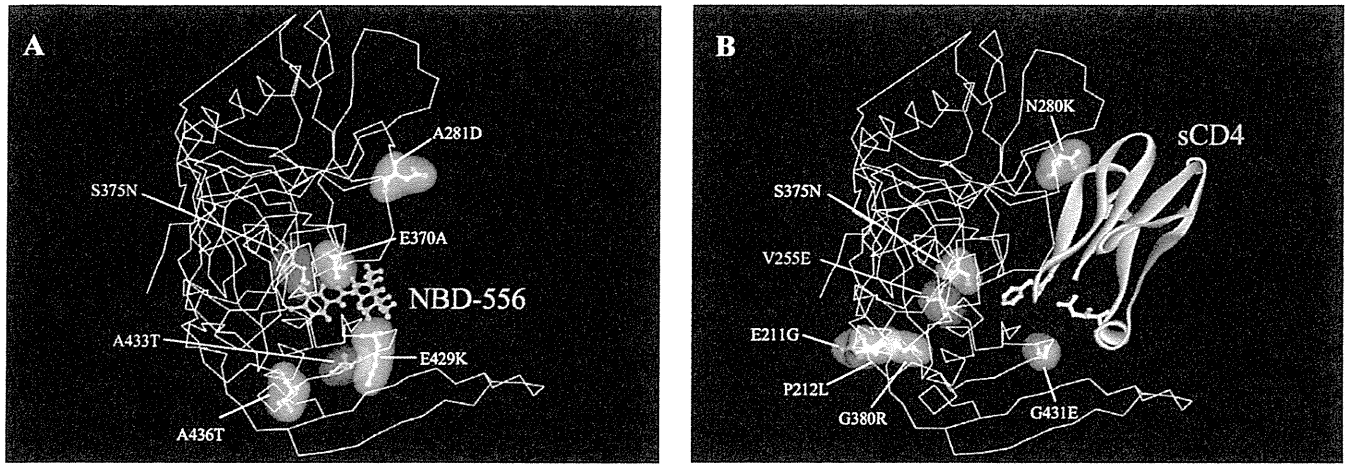

FIG. 4. Locations of substitutions in HIV-1_{IIIB} gp120 induced by *in vitro* selection with NBD-556 or sCD4. The side chains of the mutated residues that appeared during the *in vitro* selection with NBD-556 (A) or sCD4 (B) are shown in yellow and purple. The amino acid substitutions that confer resistance in HIV-1 are indicated in purple. The crystal structure of gp120 with sCD4 was retrieved from the PDB (entry 1RZJ). The structure of compound NBD-556 docked into gp120 was created by using the FlexSIS module of SYBYL 7.1.

sensitivities of HIV strains pseudotyped with the sCD4- and NBD-556-resistant envelope mutations to CD4i MAb 4C11 with or without the CD4-mimicking compound. As expected, NBD-556-pretreated HIV-1_{WT} was more sensitive to 4C11 than the untreated virus (IC_{50}s, 0.12 versus 0.72 μg/ml) (Fig. 6). On the other hand, all of the mutant viruses were completely resistant to 4C11 with or without NBD-556 pretreatment. These results suggest that the CD4 and NBD-556 resistance mutations in gp120 hide the epitope for a particular Ab against a CD4-induced epitope, similar to primary R5 viruses.

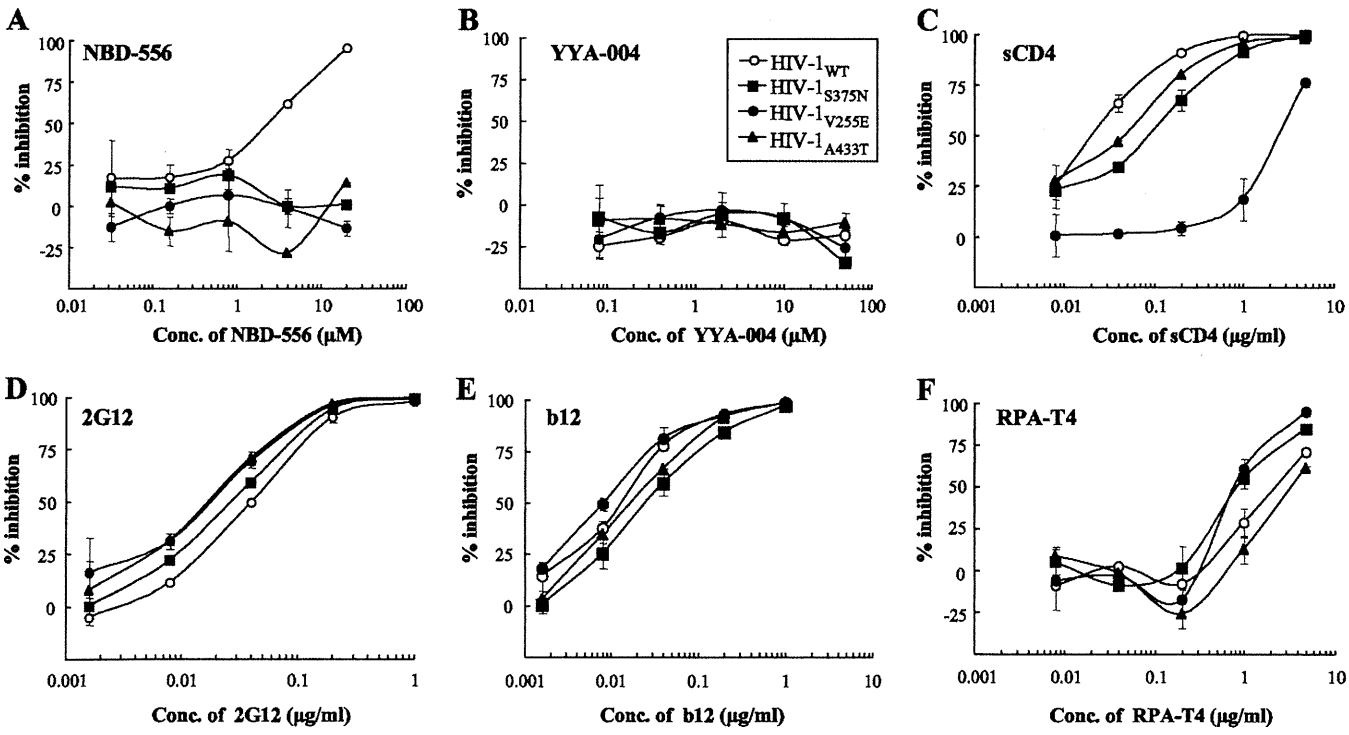

FIG. 5. Sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistance envelope mutations to NBD-556, YYA-004, sCD4, and MAbs. The sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistance envelope mutations to NBD-556 (A), YYA-004 (B), sCD4 (C), 2G12 (D), b12 (E), and RPA-T4 (F) are shown. NBD-556, YYA-004, sCD4, and MAbs at various concentrations and a pseudovirus suspension corresponding to 100 $TCID_{50}$ were preincubated for 15 min on ice and then added to the target cells (TZM-bl). The inhibitory effects were determined by measuring the β-galactosidase activities on day 2 of culture. All assays were conducted in triplicate, and the data shown represent the means ± the standard deviations (SD) derived from the results of two to three independent experiments.

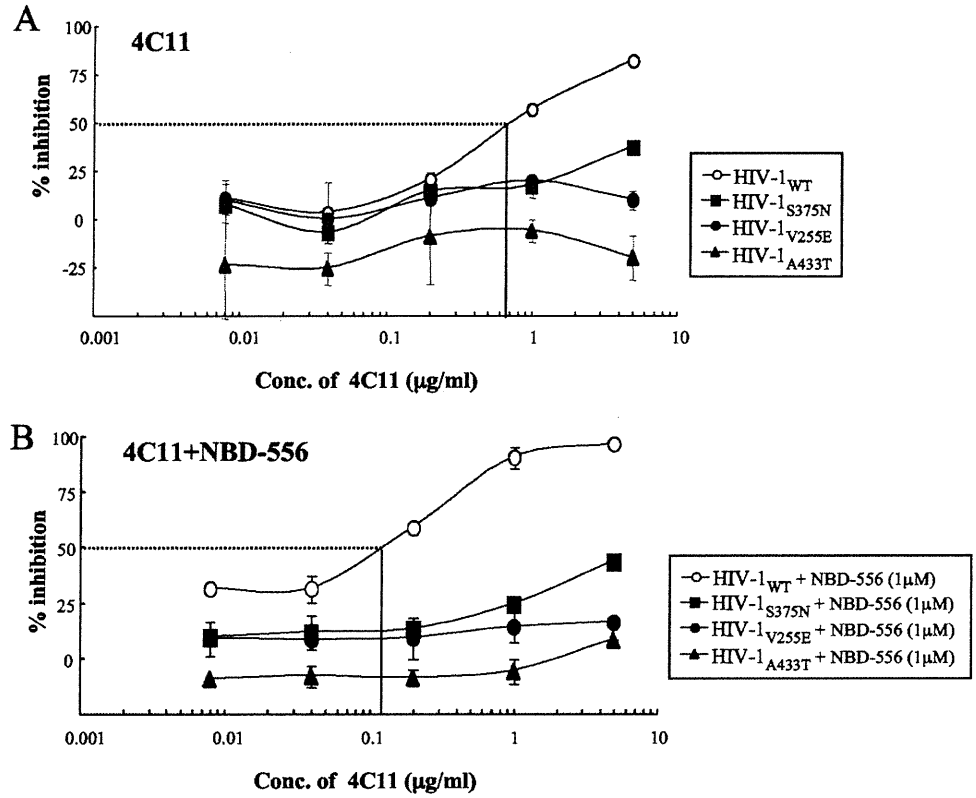

FIG. 6. Sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistance envelope mutations to CD4i MAb 4C11 with or without NBD-556. The sensitivities of β-galactosidase reporter HIV strains pseudotyped with the sCD4 and NBD-556 resistance envelope mutations to CD4i MAb 4C11 in the absence (A) or presence (B) of NBD-556 are shown. 4C11 at various concentrations and a pseudovirus suspension corresponding to 100 $TCID_{50}$ were preincubated with or without NBD-556 (1 μM) for 15 min on ice and then added to the target cells (TZM-bl). The inhibitory effects were determined by measuring the β-galactosidase activities on day 2 of culture. All assays were conducted in triplicate, and the data shown represent the means ± the SD derived from the results of two to three independent experiments.

NBD-556-mediated enhancement of the neutralization activities of plasma Abs against an autologous isolate. Neutralization escape has been documented in HIV-1 subtype B viruses, with contemporaneous viruses showing less sensitivity to autologous neutralization than earlier viruses (2). For one patient (patient 3 [Pt.3]) infected with a subtype B virus, the autologous neutralizing activities in plasma IgG obtained close to the time of the virus isolation were measured in the presence or absence of NBD-556 (0, 1, 2, 4, and 8 μM) by the MTT assay. As shown in Fig. 7A, the plasma IgG neutralizing activity was much less potent against the variant ($HIV\text{-}1_{Pt.3}$) from the same time point (IC_{50} of >200 μg/ml for IgG). However, $HIV\text{-}1_{Pt.3}$ pretreated with at least 1 μM NBD-556 became sensitive to the contemporaneous plasma IgG compared to the untreated virus. To examine which kinds of NAbs are enhanced by NBD-556, we determined the susceptibilities of $HIV\text{-}1_{Pt.3}$ to anti-V3 MAb KD-247 and CD4i MAb 4E9C with or without NBD-556. The virus was completely resistant to both MAbs (IC_{50}s of >100 μg/ml) in the absence of NBD-556, while NBD-556-pretreated $HIV\text{-}1_{Pt.3}$ became sensitive to KD-247 and 4E9C (IC_{50}s of 10.0 and 20.8 μg/ml, respectively) (Fig. 7B). These results indicate that CD4-mimicking small compounds such as NBDs have potent NAb-enhancing activities toward plasma Abs that cannot access the neutralizing epitopes hidden within the trimeric Env, such as CD4i and anti-V3 Abs.

DISCUSSION

In this study, we observed that NBD-556 could bind to a CD4-binding site, followed by the induction of conformational changes in gp120 similar to those observed upon sCD4 binding. Although we used a limited number of viruses and plasma IgG preparations obtained from an HIV-1-positive patient for testing the synergistic effects between NBD-556 and neutralizing antibody, we also found highly synergistic interactions between NBD-556 and not only CD4i MAbs but also anti-V3 MAbs. Moreover, our data indicated that small compounds such as NBDs can enhance the potency of NAbs in HIV-1-infected patients against the contemporaneous viruses, which are resistant to neutralization by Abs in the plasma.

We illustrated the sites of the mutations induced by NBD-556 on the structure of unliganded gp120 of SIV obtained from the PDB (entry 2BF1) to compare the sites before and after binding of the CD4-mimicking compound. As shown in Fig. 8, the mutations lay in front of the outer domain in gp120, which was near to or within the CD4-binding site. These findings indicate that NBD-556 attaches to the CD4-binding site or the

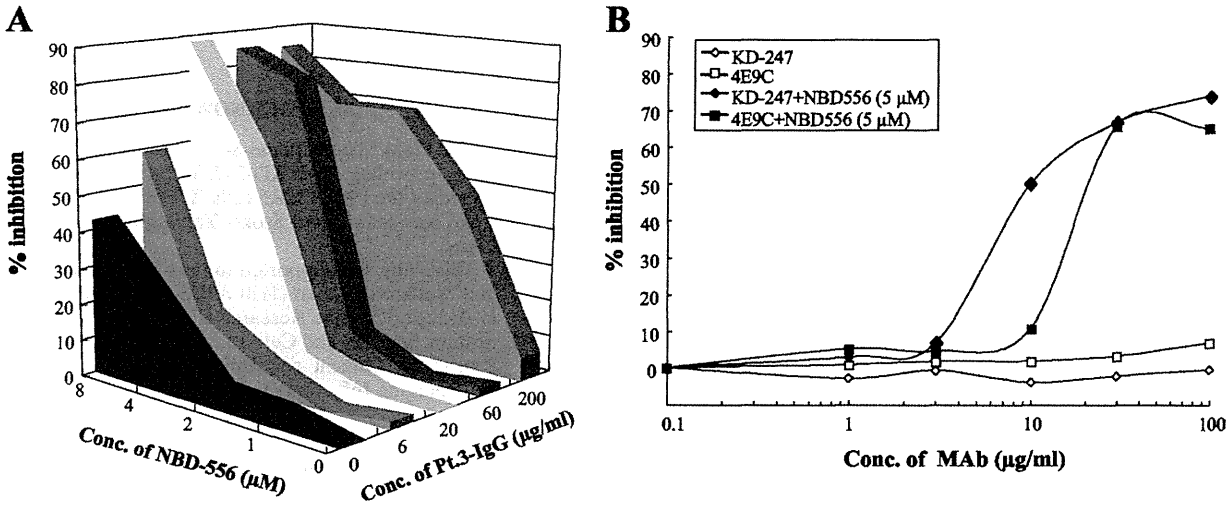

FIG. 7. NBD-556-mediated enhancement of the neutralization activity of plasma IgG against the autologous isolate. (A) The sensitivities of the HIV-$1_{Pt.3}$ primary isolate to the autologous plasma IgG (Pt.3-IgG) in the absence or presence of NBD-556 (1, 2, 4, and 8 μM) were determined by the MTT assay. (B) The sensitivities of the HIV-$1_{Pt.3}$ primary isolate to KD-247 (anti-V3 MAb; diamonds) and 4E9C (anti-CD4i MAb; squares) in the absence (open symbols) or presence (filled symbols) of 5 μM NBD-556 were determined by the same assay. The data shown are representative of two or three separate experiments.

surrounding residues in the unliganded form of gp120 and that, after the conformational changes of the envelope glycoproteins, probably the CD4-liganded form induced by the attack by NBD-556, the compound could penetrate and be held for a while in the CD4 cavity. In a recent study, Haim et al. (14) showed that sCD4-mimicking compounds have the ability to inactivate HIV-1 by prematurely triggering active but transient intermediate states of the envelope glycoproteins. In the transient intermediate states, several neutralizing epitopes in gp120 may be accessible to the neutralizing Abs. These data and our present results suggest that some NBD analogs, which bind to the cavity tightly and for a longer time, as well as cell surface CD4 inducing a more stable envelope glycoprotein intermediate state, show highly potent NAb-enhancing activities.

Madani et al. (23) reported that replacement of gp120 Ser375 with a glycine residue dramatically reduced the HIV-1 sensitivity to enhancement by any of the NBD-556 analogs, suggesting that a certain element of the Ser375 side chain contributes to the NBD-556 efficacy. They also reported that viruses bearing envelope glycoproteins with Ser375 mutated to alanine exhibited greater enhancement by NBD-556 and some NBD-556 analogs than the viruses with wild-type envelope glycoproteins, suggesting that the hydroxyl group of Ser375 is detrimental to the binding and/or activity of some NBD-556 analogs that contain large para-phenyl substituents. Mutations

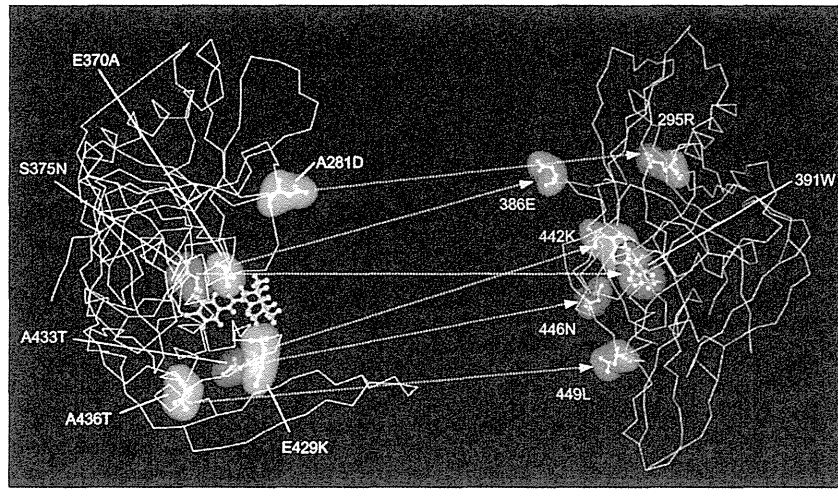

FIG. 8. Comparisons of the locations of the mutations induced by NBD-556 between the structures of unliganded and liganded gp120. The side chains of the mutated residues that appeared during *in vitro* selection with NBD-556 are shown in yellow, green and purple in the liganded (left) or unliganded (right) structures. The amino acid substitutions that confer resistance in HIV-1 are indicated in purple (S375N and A433T). The crystal structures of liganded and unliganded gp120 were retrieved from the PDB (entries 1RZJ and 2BF1, respectively). The corresponding sites of the NBD-resistant mutations are also shown on the unliganded gp120.

of other gp120 residues lining the Phe43 cavity or vestibule (Val255, Thr257, Glu429, and Val430) significantly decreased the enhancement of virus infection by the NBD-556 analogs. Our *in vitro* selection study showed that the key mutations for NBD-556 resistance were S375N and A433T and that minor mutations related to NBD-556 resistance were A281D, E370A, E429K, and A436T (Fig. 4). Thus, alterations to several gp120 residues, namely, S375N, A433T, and V255E, that line the Phe43 pocket or reside around and inside the cavity can negatively affect the entry inhibitory effect of NBD-556 on HIV-1 infection (Fig. 5).

Decker et al. (9) reported that the chemokine coreceptor binding sites of HIV-1 from subtypes A, B, C, D, F, G, and H and circulating recombinant form (CRF) 01, CRF02, and CRF11 elicit high titers of CD4i Abs during natural human infection and that these Abs bind and neutralize viruses as divergent as HIV-2 in the presence of sCD4. Recently, Davis et al. (7) showed that transplantation of HIV-1 V3 epitopes into an HIV-2 envelope scaffold provides a sensitive and specific means to detect and quantify HIV-1 V3 epitope-specific NAbs in human sera. They used this HIV-2/HIV-1 V3 scaffolding strategy to study the kinetics of the development and breadth of V3-specific NAbs in longitudinal sera from individuals acutely infected with subtype C or subtype B HIV-1. Their results indicated that high-titer broadly reactive V3-specific Abs are among the first to be elicited during acute and early HIV-1 infection, although these Abs lack neutralizing potency against primary HIV-1 viruses, which effectively shield V3 from Ab binding to the functional Env trimer (8). These observations strongly support the idea that the major problem facing the development of CD4i-based or V3-based immunogens is not sequence variation within the epitopes, but rather that access of most CD4i and anti-V3 Abs to their epitopes in functional Env complexes is blocked. As shown in Fig. 7A, plasma IgG from a seropositive patient exhibited strongly enhanced neutralizing activity against the contemporaneous virus after treatment with NBD-556. Therefore, we consider that small compounds such as NBDs can enhance the neutralizing activities of CD4i and certain anti-V3 Abs *in vivo* at the acute stage of HIV-1 infection or in combination with anti-V3 NAbs as a passive immunization.

In general, small molecules have certain advantages from a therapeutic standpoint because of their low propensity for immunogenicity, high metabolic stability, easy large-scale production, and relatively low cost. Small molecule Ab-enhancing therapeutics such as NBD compounds would have additional benefits over available treatment approaches to HIV. Since CD4i and anti-cryptic V3 Abs are already present in a large number of HIV-1-infected patients, no prevaccination would be necessary for the induction of NAbs. Moreover, the use of bifunctional small molecules, such as an entry inhibitor and a NAb enhancer, should be effective for passive immunization of the anti-HIV NAbs enhanced by the accessibility of epitopes after binding of sCD4, such as 17b (27) and KD-247 (11, 12). Elucidation of the molecular details governing the interactions between gp120 and NBD compounds will assist in optimization efforts, as well as in the evaluation of this strategy in more complex biological models for HIV infection. Consequently, we will continue to synthesize such NBD analogs to search for drugs with more potent power to change the tertiary structure of the envelope glycoproteins and lower toxicity toward the host cells.

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Human immunodeficiency virus type 1 evasion of a neutralizing anti-V3 antibody involves acquisition of a potential glycosylation site in V2

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It has been reported that the addition of a potential *N*-linked glycosylation site (PNGS) to the gp120 human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein provides protection against neutralizing antibodies (NAbs) by acting as a 'glycan shield'. In this study, we induced insertion of a PNGS into the V2 region of HIV-1_{BaL} with the KD-247 anti-V3 neutralizing monoclonal antibody. In the presence of KD-247 (200 μg ml^{-1}) at passage five, viruses with 3 aa mutations in the C2 (T240S and I283T) and V3 (T319A) regions expanded from pre-existing variants. After six passages with KD-247 (>300 μg ml^{-1}), a PNGS emerged in the V2 region in addition to C2 (T240S) and V3 mutations (R315K and F317L). A variant with a PNGS insertion in V2, but no V3 mutations was sensitive to KD-247, whereas a clone with a V2 PNGS insertion and mutations in V3 demonstrated a high level of resistance to KD-247. Replication kinetic analysis revealed that the F317L mutation in V3 played a compensatory role for fitness-loss caused by the PNGS insertion in V2. The evading HIV-1 variant did not revert back to the wild-type virus after 14 passages without KD-247. These findings demonstrate that the virus with fitness-loss mutations can replicate equally as well as the wild-type virus to acquire some key mutations in the V3 stem and the C2 region, and the compensated variants containing PNGS do not revert back to the ancestral virus even in the absence of NAb.

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INTRODUCTION

A neutralizing antibody (NAb) against human immunodeficiency virus type 1 (HIV-1) is an essential component of a protective vaccine. However, primary isolates of HIV-1 are relatively resistant to neutralization compared with variants selected for growth in permanent cell lines (Moore *et al.*, 1995; Pugach *et al.*, 2004). Studies addressing differences between neutralization-sensitive and -resistant variants have revealed several mechanisms that are responsible for neutralization resistance in primary isolates. These mechanisms include the occlusion of epitopes within the envelope glycoprotein (Env) oligomer and the extensive glycosylation and extension of variable loops from the surface of the complex leading to steric and conformational blocking of receptor-binding sites (Kwong *et al.*, 2002; McCaffrey *et al.*, 2004; Pinter *et al.*, 2004; Saunders *et al.*, 2005). The structural features of one envelope glycoprotein, Env gp120, mean that it can tolerate a vast array of mutations permitting the selection of neutralization evading variants, as has been previously demonstrated in culture assays, animal models and infected individuals (Johnson & Desrosiers, 2002).

Although there are ample data showing that NAbs can protect against HIV-1 infection *in vitro* and *in vivo*, their activity in infected humans remains controversial (Cao *et al.*, 1995; Deeks *et al.*, 2006; Montefiori *et al.*, 2001; Sullivan *et al.*, 1993). Passive transfer of a combination of broadly neutralizing monoclonal antibodies (mAbs) 2G12, 2F5 and 4E10 in patients during a structured treatment interruption resulted in a significant delay in viral rebound in some patients compared with viral rebound in the absence of these antibodies (Trkola *et al.*, 2005). This would indicate that viral suppression was due to the antiviral activity of the administered antibodies. Subsequent studies addressing the pharmacokinetics of each mAb (Joos *et al.*, 2006), neutralization-resistance mutations (Manrique *et al.*, 2007) and protective neutralization titres *in vivo* (Trkola *et al.*, 2008) using samples from the study further supported the protective effects of NAb *in vivo*.

Clinical studies examining NAbs in primary infections have suggested that the majority of recently infected individuals generate a vigorous antibody response against autologous virus. However, the rapid evolution of HIV in the presence

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are AB521136–AB521148.

A supplementary figure is available with the online version of this paper.

of NAb results in the emergence of evading mutants. As a consequence, at any time during the early stages of HIV infection, NAbs are more likely to recognize the earlier form of the viruses as opposed to the recent variants. Despite evidence of phenotypic resistance, the genetic basis of the mechanism allowing primary viruses to evade NAbs is poorly understood.

Wei *et al.* (2003) found that glycosylation of Env plays an important role in evading neutralization. The evolving 'glycan shield' can sterically block antibody binding without mutation at the antibody-binding site (Wei *et al.*, 2003). Also, insertion of potential *N*-linked glycosylation sites (PNGSs) along with other mutations has been associated with viral evasion of NAbs (Bunnik *et al.*, 2008; Wei *et al.*, 2003). Conversely, Frost *et al.* (2005) reported that viral evasion of NAb correlates to the rate of amino acid substitution rather than changes in glycosylation and insertions or deletions in Env (Frost *et al.*, 2005). This would suggest that the individual contribution of PNGSs to the neutralization sensitivity of HIV-1 depends on the presence of other mutations in the Env sequence. However, the relationship between PNGSs and mutations of NAb resistance has not been investigated because of technical difficulties, resulting from the polyclonal nature of NAbs and the primary isolates used in previous clinical studies. To clarify the genetic mechanisms responsible for evading neutralization, it is important to analyse individual mutations, resulting from neutralization evasion of NAbs in an *in vitro* culture system.

Neutralization evasion from anti-V3 mAbs has been reported and associated with amino acid substitution within the epitope of the V3 loop and outside V3 (Gorny *et al.*, 2004; Masuda *et al.*, 1990; Park *et al.*, 1998; Pinter *et al.*, 2004; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006; Zolla-Pazner, 2004). However, the role(s) of PNGSs in resistance to neutralization is not clear because the induction of PNGSs under neutralizing mAbs pressure *in vitro* has not been reported.

In this study, we obtained evasion mutants harbouring PNGSs in the V2 region and mutations in the C2 and V3 regions, during induction of neutralization evasion mutants from anti-V3 mAb KD-247 in HIV-1_{BaL}. KD-247 is a humanized mAb that demonstrates cross-neutralizing activity against HIV-1 isolates in clade B. The epitope of KD-247 was mapped to 6 aa, IGPGRA, at the tip of the V3 loop (Eda *et al.*, 2006). A series of analyses using viral clones that have corresponding mutations present in evading viruses revealed a mutant that has both a PNGS-insertion in V2 and mutations in V3 along with a highly resistant phenotype to the NAb. However, the mutant requires further mutation to compensate for reduced replication ability. Studies to elucidate replication kinetics indicated that the F317L mutation in V3 and the T240S mutation in C2 play a key role in maintaining resistant mutations in V2 and V3, which were related to the fitness-loss. Our study partially explains the complex nature of the development of neutralization resistance observed in previous clinical studies.

RESULTS

Selection of anti-V3 mAb KD-247 evasion mutants from HIV-1_{BaL}

To select an HIV-1 variant that is able to evade neutralization by KD-247 *in vitro*, we exposed PM1/CCR5 cells to HIV-1_{BaL} and serially passaged the virus in the presence of increasing concentrations of KD-247. PM1/CCR5 cells were highly sensitive to both X4 and R5 HIV-1 infection, displaying prominent syncytia (Yusa *et al.*, 2005). As a control, HIV-1_{BaL} was passaged under the same conditions without mAb to monitor spontaneous changes that occurred in the virus during prolonged PM1/CCR5 cell passage (denoted as passage control). Because HIV-1_{BaL} was sensitive to neutralization by KD-247 with an IC_{50} of 3.8 μg ml^{-1} as determined by an MTT assay (data not shown), the selected virus was initially propagated in the presence of 5 μg KD-247 ml^{-1}. During the course of the selection procedure, the mAb concentration was increased to 2000 μg ml^{-1}. Following five rounds of passage (p5), a viral variant designated HIV-1_{BaL} (200) p5 arose that replicated in the presence of 200 μg KD-247 ml^{-1}. After passage 16, a viral variant designated HIV-1_{BaL} (2000) p16 arose that infected PM1/CCR5 cells efficiently in the presence of 2000 μg KD-247 ml^{-1}. We harvested viruses at six passages (p2, p5, p6, p7, p10 and p16) as well as a baseline virus, HIV-1_{BaL} (0) p0, and a passage control designated HIV-1_{BaL} (0) p10. These viruses were evaluated for their sensitivity to KD-247 by using TZM-bl as target cells (Table 1). The IC_{50} values of KD-247 against HIV-1_{BaL} (0) p0, HIV-1_{BaL} (200) p5, HIV-1_{BaL} (300) p6 and HIV-1_{BaL} (2000) p16 were 0.32 ± 0.2, 5.68 ± 1.48, >100 and >100 μg ml^{-1}, respectively, indicating that HIV-1_{BaL} acquired a resistant phenotype against KD-247 during *in vitro* selection. At passage 5, HIV-1_{BaL}

Table 1. Neutralization sensitivities of passaged variants to KD-247

	Passage no.	KD-247 conc. (μg ml^{-1})	IC_{50} (μg ml^{-1})*
Baseline virus	p0	0	0.32 ± 0.20
Passage control	p10	0	0.09 ± 0.04
KD-247 selection	p2	10	0.54 ± 0.19
	p5	200	5.68 ± 1.48
	p6	300	>100
	p7	400	>100
	p10	1000	>100
	p16	2000	>100

*TZM-bl cells (2×10^4 cells per well) were exposed to 300 $TCID_{50}$ of passage control (p10) or KD-247 selected variants (p2, p5, p6, p7, p10 and p16) in the presence of various concentrations of KD-247 in 96-well flat-bottom microculture plates and incubated for 48 h. The IC_{50} values were determined by using a chemiluminescent assay for β-galactosidase detection. Data shown represent the means ± SD from the results of three independent experiments.

acquired a moderately resistant phenotype and after passage 6 the virus had developed a highly resistant phenotype.

DNA sequence of the envelope region of evasion mutants

To determine the genetic basis of resistance in the variant HIV-1_{BaL} strains, the C1–C4 region of the env gene was amplified from genomic DNA extracted from the infected cells, cloned and sequenced (Fig. 1).

At passage 5, moderately resistant variants with T240S, I283T and T319A mutations were in the majority. However, the proportion of variants decreased gradually as the KD-247 concentration was increased (Fig. 1b and Fig. 2e). This observation suggests that at low concentrations of KD-247, the variants with moderate resistance to the anti-V3 mAb are selected from the pre-existing variants.

Insertion of a PNGS in the V2 region and an amino acid substitution at the V3 tip (R315K) were observed at passages 5 and 6, respectively. Both of these alterations were not evident in the passage control but became dominant at later passages in the presence of higher concentrations of KD-247 (Figs 1 and 2). To examine whether the insertion of a PNGS in the V2 region existed among the baseline HIV-1_{BaL} variants, we sequenced a total of 61 env clones from HIV-1_{BaL} before selection. No PNGS insertion was observed in the V2 region among the baseline clones (Fig. 1). This result indicates that the virus with the PNGS insertion in V2 either did not exist or existed at a very low level within the baseline variants.

Neutralization sensitivities of pseudoviruses that have a mutated env gene

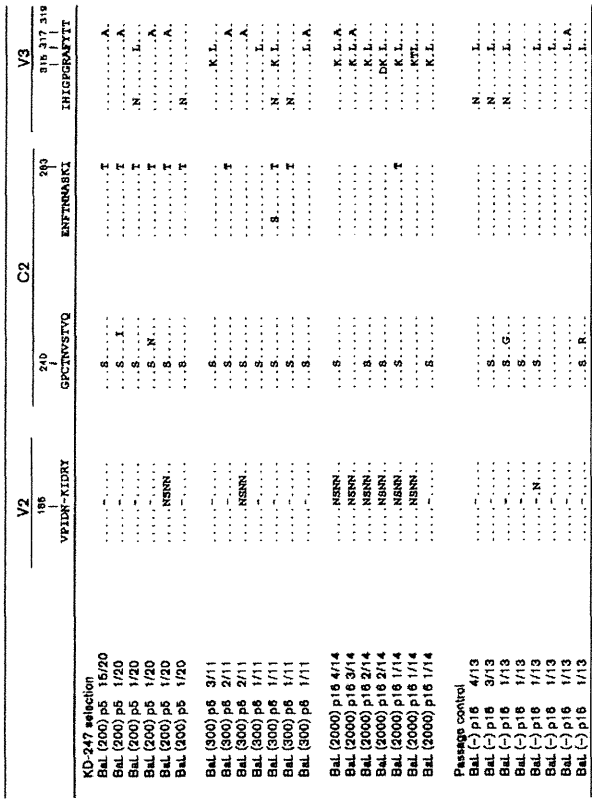
To determine which substitutions were responsible for KD-247 resistance, we constructed chimeric viruses, which contained the representative envelopes of HIV-1_{BaL} (200) p5 and HIV-1_{BaL} (1000) p13 and were designated BaL-STA and BaL-PNGS/SKL, respectively (Fig. 3). Chimeric envelopes were constructed by replacing wild-type sequences with mutated envelope-encoding sequences for V2, C2 and V3 in the HIV-1_{BaL} wild-type (BaL-WT) virus with the resulting viruses designated BaL-PNGS and BaL-SKL. Sensitivity was compared between the BaL-WT and mutant viruses using a single-round neutralization assay. As shown in Fig. 4(a), the V3 mutated pseudoviruses, BaL-SKL and BaL-PNGS/SKL were highly resistant to KD-247 (>2500- and >5500-fold, respectively) compared with wild-type virus, whereas the C2 and V3 mutated virus at passage 5, BaL-STA, was partially resistant (25-fold). The susceptibility of the clone with the PNGS insertion in the V2 region alone (BaL-PNGS) to KD-247 was highly comparable to that for BaL-WT, indicating that this variant is sensitive to KD-247. In contrast, the clone with both the PNGS-insertion in V2 and mutations in V3 had a highly resistant phenotype to the mAb. The IC_{50} value for BaL-PNGS/SKL was slightly higher than for BaL-SKL in three independent experiments, although there was no significant difference between these two clones.

To determine the effect of these KD-247-induced mutations in gp120 to other entry inhibitors, we examined the sensitivities of these chimeric pseudotyped viruses to rsCD4, 2D7 and maraviroc, a CCR5 inhibitor. The IC_{50} values for rsCD4, 2D7 and maraviroc to the chimeric viruses were comparable to those of wild-type virus (Fig. 4b–d).

Analyses of replication kinetics of infectious molecular clones with mutant env

In order to clarify the role of the PNGS insertion during the process of neutralization evasion, we constructed replication-competent viruses with the PNGS in the V2 region and/or mutations in C2 and V3 of gp120 by using pWT/BaL proviral plasmid (Fig. 3; they were designated HX-BaL-X). Using these competent viruses with Env mutations we compared the replication kinetics in the absence of KD-247. As shown in Fig. 5(a), HX-BaL-PNGS containing a PNGS in V2 had low levels of p24 antigen compared with the wild-type clone (HX-BaL-WT), whereas HX-BaL-PNGS/SKL containing a PNGS and mutations in the C2 and V3 regions exhibited a replication rate equivalent to the wild-type and HX-BaL-SKL. We also compared their replication kinetics in the presence of low, moderate and high concentrations of KD-247 (Fig. 5b). High concentrations of KD-247 (2000 $\mu g\ ml^{-1}$) resulted in HX-BaL-PNGS/SKL replicating slightly faster than HX-BaL-SKL. HX-BaL-PNGS demonstrated slightly more efficient replication kinetics than the wild-type in the presence of a low concentration of KD-247 (5 $\mu g\ ml^{-1}$), while there was no difference between the two viruses when KD-247 was used at 50 $\mu g\ ml^{-1}$. These results indicated that viruses harbouring a PNGS in V2 were selected for at low concentrations of KD-247 but at high concentrations of the mAb, variants harbouring the additional V3 mutations outgrew the former variants.

To elucidate which amino acid mutation would compensate for the fitness-loss induced by insertion of a PNGS, we compared the replication kinetics of the infectious clones with individual mutations in addition to the PNGS insertion (Fig. 5c). The variants with the T240S, R315K and F317L mutations (HX-BaL-PNGS/SKL) and F317L mutation (HX-BaL-PNGS/L) exhibited a higher replication rate than HX-BaL-PNGS. On the other hand, viruses containing the T240S or R315K mutation in addition to the PNGS insertion (HX-BaL-PNGS/S or HX-BaL-PNGS/K, respectively) replicated as well as or less efficiently than HX-BaL-PNGS. These results suggest that the replication deficiency of the HIV-1_{BaL} variant with the PNGS in the V2 region was compensated by the F317L mutation in the V3 region. An improvement in replication capacity of HX-BaL-PNGS/K was observed in variants with the additional T240S mutation (Fig. 5c).



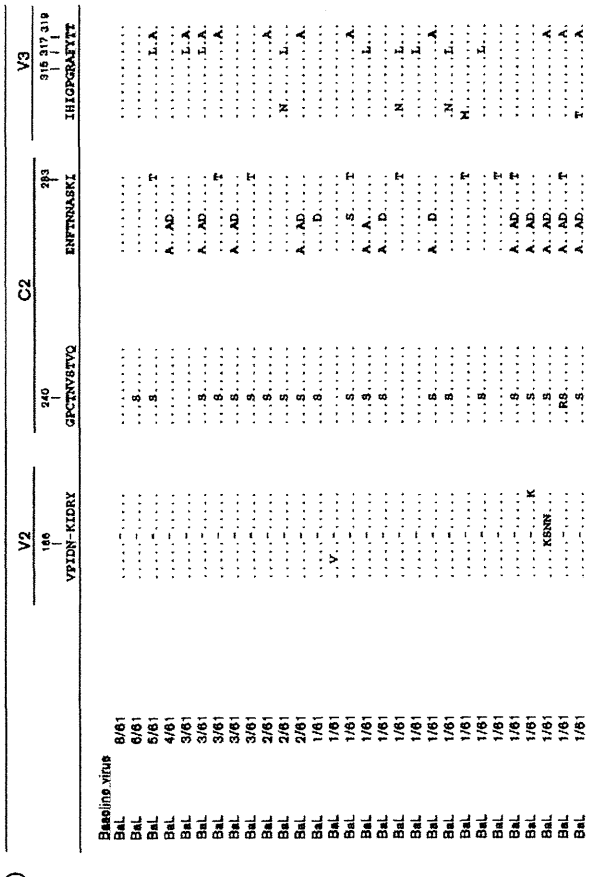


Fig. 1. Amino acid sequences deduced from the nucleotide sequences of env-encoding regions of pDNA isolated at the baseline (a) and indicated passages (p5, p6 and p16) from HIV-1_{BaL} variants selected in the presence of KD-247, and (b) the passage control (p16).The amino acid sequences of the envelope proteins of a clone of HIV-1_{BaL} at baseline are shown at the top as a reference. Identity to the sequence at individual amino acid positions is indicated by dots. The numbers of clones with the given amino acid substitutions among a total of 61 clones (a) and 11–20 clones (b) are listed.

Both the T240S (41/61 clones, 67.2 %) and F317L (18/61, 29.5 %) substitutions were observed in the HIV-1_{BaL} sequence of the baseline viruses. The ratio of threonines and serines at position 240 in gp120 of the passage control remained unchanged at passage 16 (8/13, 61.5 %). The F317L variant in the passage control increased gradually and became more prevalent in the viral population at passage 16 (12/13, 92.3 %) (Fig. 2). These findings suggest that the serine substitution at position 240 in the C2 region of gp120 was required to compensate for inefficient replication rates caused by the R315K and T319A substitutions in the V3 region. Furthermore, the F317L substitution in V3 might play a role in adaptations to survive in PM1/CCR5 cells *in vitro* and also compensate for fitness-loss by the PNGS-insertion in the V2 region induced by KD-247 selection.

Sensitivity of infectious molecular clones containing mutated Env protein to KD-247 and a CCR5 inhibitor

To compare the neutralization sensitivity of the infectious molecular clones, the IC_{50} values were determined for KD-247 by using TZM-bl cells (Table 2). The neutralization sensitivities of clones containing only the PNGS in V2, T240S in C2 and F317L in V3 were virtually the same as the wild-type, whereas the HX-BaL-PNGS/K demonstrated a high level of resistance to KD-247 when compared with HX-BaL-K containing the R315K mutation alone (P=0.006). We constructed a mutant containing an amino acid insertion in V2 without the addition of a glycosylation site and with the R315K mutation (designated HX-BaL-Q/K). The sensitivity of this mutant was comparable to that of the HX-BaL-K mutant (P=0.89). These results taken together suggest that the variant with the PNGS in addition to the R315K mutation in V3 had the highest resistance phenotype to KD-247. We also evaluated sensitivities of these clones to the CCR5 inhibitor, maraviroc. No significant difference was detected for the IC_{50} values of the wild-type and mutant variants to maraviroc.

We then investigated whether the mutations that conferred KD-247 resistance, which were related to a loss in replication efficiency, reverted back to the baseline after several passages in the absence of the anti-V3 mAb. Two mutations, F317L and R315K in the V3 region, the PNGS in the V2 and the T240S mutation in C2 remained dominant in the popu-

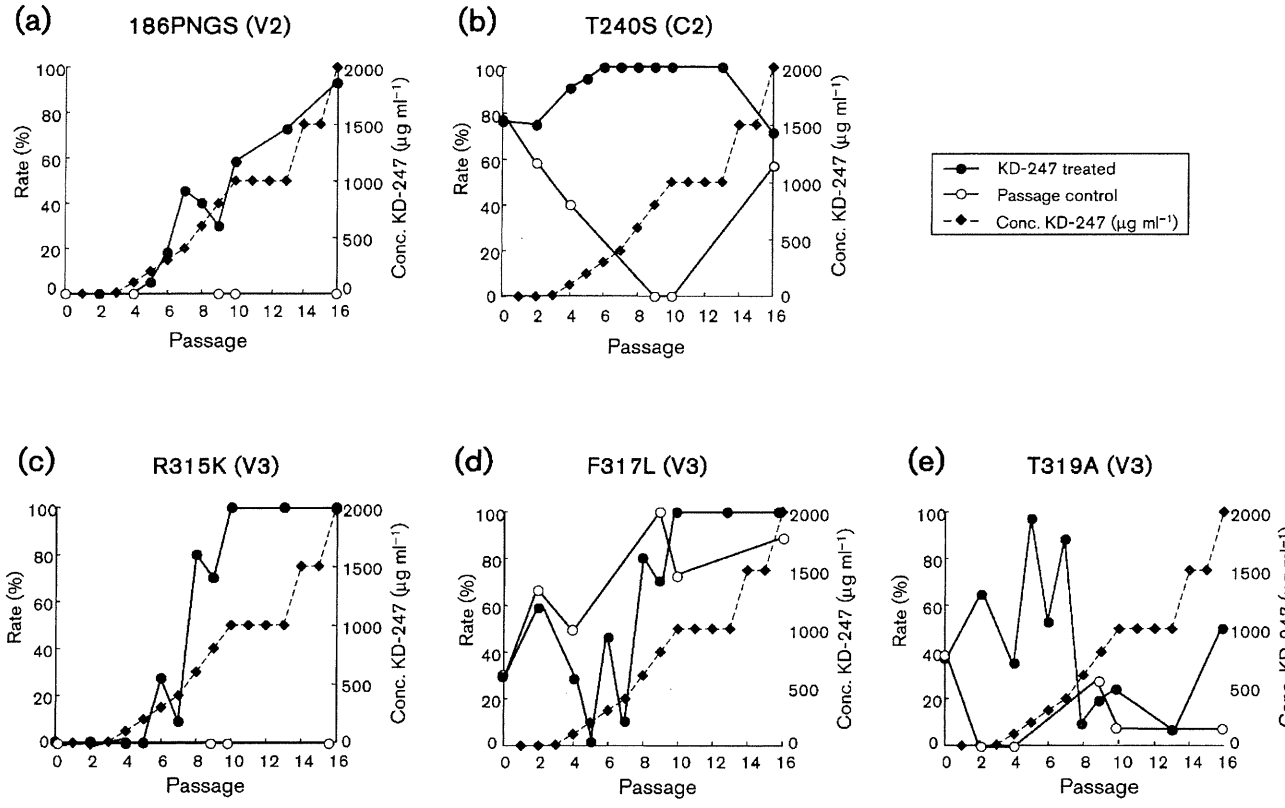


Fig. 2. The gp120 mutation profile of HIV-1_{BaL} evasion variants from KD-247 *in vitro*. The ratio of the PNGS insertion in the V2 region and mutations in the C2 and V3 regions in gp120 of HIV-1_{BaL} variants were plotted for each passage. The *y*-axis indicates the percentage of PNGS insertions or mutations in the tested clones and the *x*-axis shows the concentration of KD-247 (μg ml^{-1}).

lation of viruses that were adept at evasion after culturing for 16 passages in the presence of KD-247 and an additional 14 passages without KD-247 (Fig. 6). The T319A mutation was observed in 90% of clones sequenced at passage 19. However, the frequency of this mutation gradually decreased thereafter (Fig. 6e). These findings show that after acquisition of the mutations in order to compensate for fitness-loss, the HIV-1 variants did not revert back to the wild-type after 14 passages without KD-247.

DISCUSSION

HIV-1 evolution in relation to evasion of humoral immunity has been observed in the early stages of HIV-1

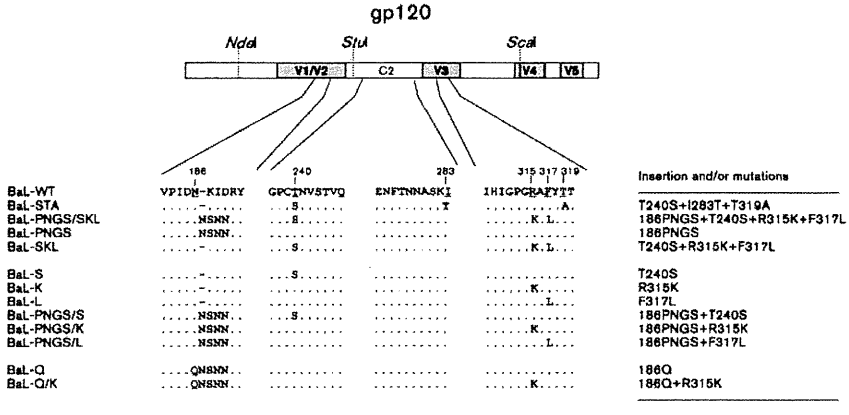


Fig. 3. Schematic representation of recombinant HIV-1_{BaL} env genes used for analysis of the genetic basis for resistance to KD-247. Mutated env genes were amplified from passaged HIV-1_{BaL} virus-infected PM1/CCR5 cells in the absence or presence of KD-247. The recombinant env genes were constructed by replacing each region of passaged control with corresponding sequence of escaped variant of HIV-1_{BaL} or by site-directed mutagenesis. The locations and numbers of specific amino acids, based on the HXB2 sequence, are shown above the reference HIV-1_{BaL} sequence.

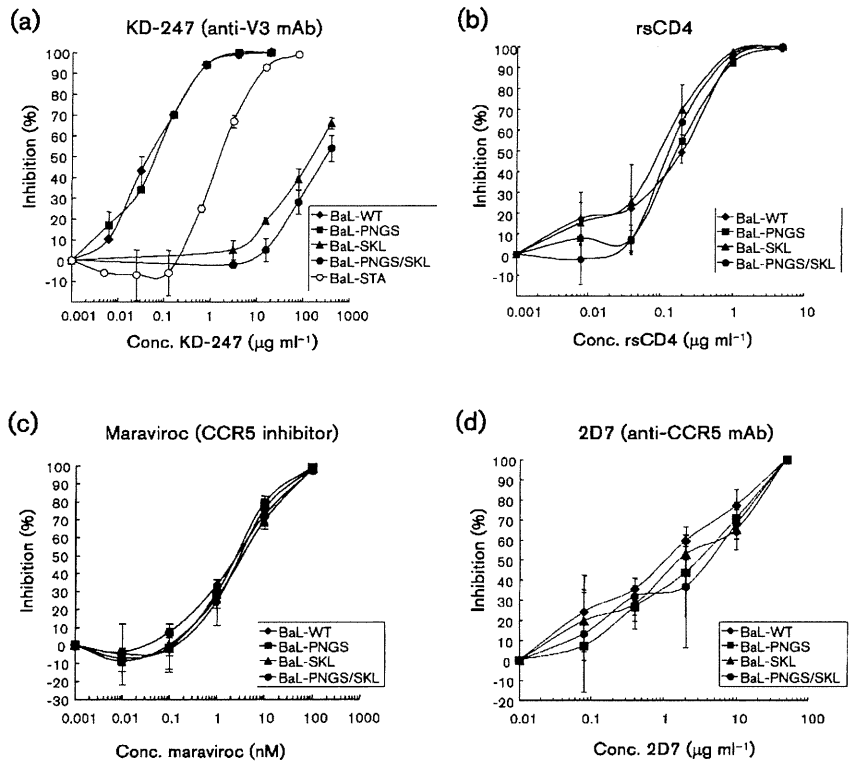


Fig. 4. Sensitivities of HIV-1 strains pseudotyped with recombinant HIV-1_{BaL} env genes to KD-247, 2D7, rsCD4 and CCR5 inhibitor. KD-247, 2D7 (anti-CCR5 mAb), rsCD4 and maraviroc (CCR5 inhibitor) were pre-incubated with 300 $TCID_{50}$ of each HIV-1_{BaL} pseudotype virus for 30 min, then added to TZM-bl target cells. Inhibitory effects were determined by measuring β-galactosidase activity on day 2 of culture.

infection and has been suggested to act as a driving force for the establishment of viral quasispecies *in vivo* (Bunnik *et al.*, 2008; Frost *et al.*, 2005; Mahalanabis *et al.*, 2009; Richman *et al.*, 2003; Wei *et al.*, 2003). In response to NAb pressure, the numbers and/or positions of surface-expressed carbohydrates can evolve to create a continuously changing glycan shield on the surface of the Env protein (Wei *et al.*, 2003). Large sequence variation in the variable loops, including large insertions and deletions, and changes in the number of PNGS in these regions have also been associated with evasion of NAbs (Sagar *et al.*, 2006; Saunders *et al.*, 2005). An especially strong influence of a mutation in the V1/V2 domain on neutralization activity has been reported by a number of investigators (Krachmarov *et al.*, 2005; Pinter *et al.*, 2005; Shibata *et al.*, 2007). However, insertion of a PNGS in the V2 region of the R5 virus during *in vitro* selection using an anti-V3 mAb has not previously been reported.

In this study, we obtained neutralization evading mutants using HIV-1_{BaL} by *in vitro* selection with the anti-V3 mAb, KD-247, and analysed the functional role of the mutations *in vitro*. In the presence of low concentrations of KD-247, viruses with 3 aa mutations in C2 (T240S and I283T) and V3 (T319A) expanded from pre-existing variants in the baseline population. In the presence of high concentrations of KD-247, an increase in the number of mutants that had a PNGS in the V2 region and containing C2 (T240S) and V3 (R315K and F317L) mutations was observed. To identify how each of these mutations affects resistance to KD-247 and the replication kinetics of the virus, we constructed pseudoviruses and infectious clones containing each mutation and combinations of these mutations and compared the IC_{50} and replication kinetics with the wild-type virus. Our results showed that the HIV-1_{BaL} variant with the PNGS in the V2 region and no V3 mutations was sensitive to KD-247. The clone with both the V2 PNGS and mutations in V3 had a high level of resistance to KD-247 and was more resistant than variants with the V3 mutation alone (Table 2). In replication kinetic analyses, the F317L mutation in V3 and the T240S mutation in C2 played a compensatory role for a fitness-loss caused by the V2 PNGS-insertion and R315K mutation in the V3-tip. Our data indicate that the virus with fitness-loss mutations can replicate as well as the wild-type virus to acquire some key mutations in the V3 stem and the C2 region of gp120 with or without exposure to KD-247.

In order to estimate the mechanism of neutralization resistance conferred by the T319A mutation observed at passage 5, we simulated structures of the V3 region using Swiss-PdbViewer software (Supplementary Fig. S1, available in JGV Online) (Guex *et al.*, 1999). According to the simulation data the side chain of arginine at position 315 on the V3 loop in the 315R, 317F and 319A variant at passage 5 was bent to the C-terminal side of the protein compared with its position in the 315R, 317F and 319T variant. It is possible that alanine at position 319 may contribute to KD-247 resistance by altering the three dimensional conformation of the V3 loop.

Our initial analysis using pseudoviruses suggested that the variant with the PNGS alone was sensitive to KD-247

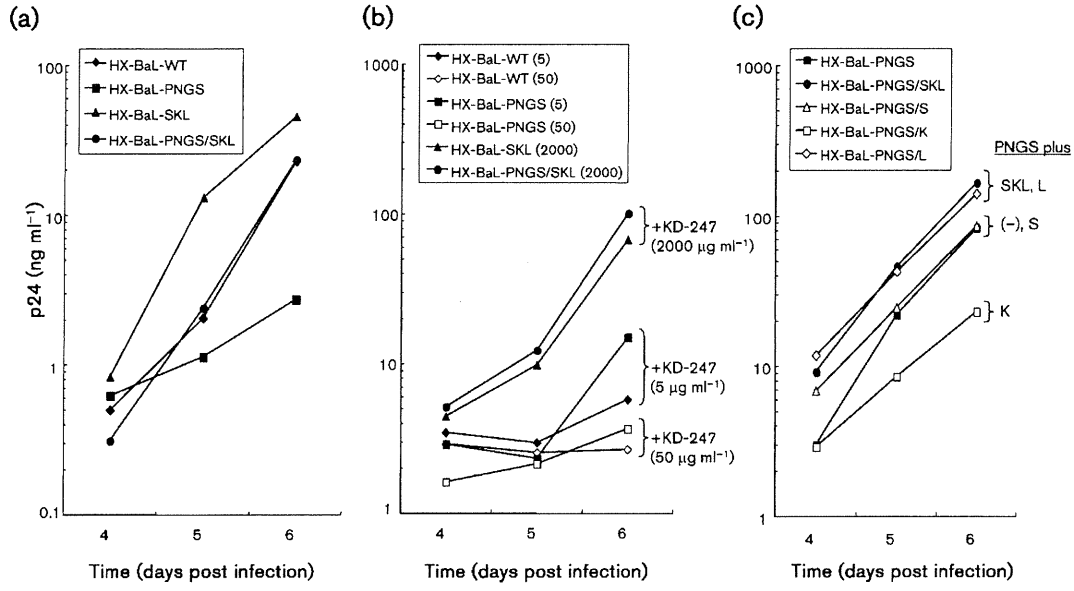


Fig. 5. Viral infectivity of HIV-1 infectious clones with recombinant HIV-1_{BaL} mutant env genes. HIV-1 infectious clones with the env gene sequences listed in Fig. 3 were prepared as described in Methods. PM1/CCR5 cells were exposed to the infectious clones [input p24 amount; (a) 2 ng and (b, c) 10 ng] and cultured for 6 days in the presence or absence of KD-247. The replication of the infectious clones with mutant Env was monitored by measuring the amounts of p24 Gag protein produced in the culture supernatants in the absence (a) or presence (b) of KD-247. The clones with a PNGS and various other mutations were also monitored in the absence of KD-247 (c).

(Fig. 4a). Moreover, the difference in neutralization sensitivity between the viruses with the V3 mutations alone or those with the V2 modifications was not clear in this single round assay (Fig. 4a). We then constructed a panel of replication competent viruses to compare resistance. As shown in Table 2 HX-BaL-PNGS/K containing the R315K mutation in V3 and the PNGS demonstrated a high level of resistance to KD-247 when compared with HX-BaL-K containing the R315K mutation alone. These data suggest that the contribution of the PNGS in neutralization resistance was moderate in a single cycle of viral replication but played a significant role in multiple rounds of infection. It is also possible that the existence of the other mutations such as T240S and F317L in the pseudovirus might have some influence on neutralization sensitivity.

Table 2. Anti-HIV-1 activities of KD-247 and the CCR5 inhibitor, maraviroc

Mutant virus	$IC_{50} \pm$ SD of maraviroc (nM)*	$IC_{50} \pm$ SD of KD-247 (μg ml^{-1})*
HX-BaL-WT	2.0±0.72	0.092±0.028
HX-BaL-PNGS	1.2±0.28	0.047±0.028
HX-BaL-Q	1.9±1.3	0.12±0.047
HX-BaL-S	1.8±0.72	0.087±0.021
HX-BaL-L	2.6±0.33	0.036±0.012
HX-BaL-STA	2.5±1.7	4.6±0.71
HX-BaL-PNGS/SKL	2.4±0.29	214±84
HX-BaL-K	1.6±0.35	285±76†‡‖
HX-BaL-PNGS/K	2.7±0.52	582±59†‡§
HX-BaL-Q/K	1.6±0.32	276±31†§‖

*TZM-bl cells (2×10^4 cells per well) were exposed to 300 $TCID_{50}$ of the infectious clones with wild-type or mutant Env in the presence of various concentrations of maraviroc or KD-247, and incubated for 48 h. IC_{50} values were determined by using a chemiluminescent assay for β-galactosidase detection. All assays were conducted in duplicate or triplicate and the data shown represent means ± SD from the results of three independent experiments.

†*P*-values <0.05 were considered statistically significant (Student's *t*-test). ‡P=0.006, §P=0.007, ‖P=0.89.

Although we did not examine whether the PNGS in V2 at position 186 was actually glycosylated or not, previous studies describing the assignment of glycosylation sites for IIIB and SF2 gp120 show that it is glycosylated (Cutalo *et al.*, 2004; Zhu *et al.*, 2000). The difference in neutralization resistance of HX-BaL-PNGS/K which has the PNGS-insertion in V2 with the counterpart HX-BaL-Q/K of amino acid insertion without glycosylation further supports glycosylation of the site in HIV-1_{BaL} Env (Table 2). HX-BaL-PNGS, which contained the PNGS alone, demonstrated low levels of p24 production compared with the wild-type (Fig. 5a). Moreover, the variant with the R315K mutation in addition to the PNGS further hindered the replication capacity of the variant (Fig. 5c). Interestingly, HX-PNGS-SKL, which contained the additional T240S mutation in the C2 and F317L in the V3 regions, could replicate at the same level as the wild-type clone. These

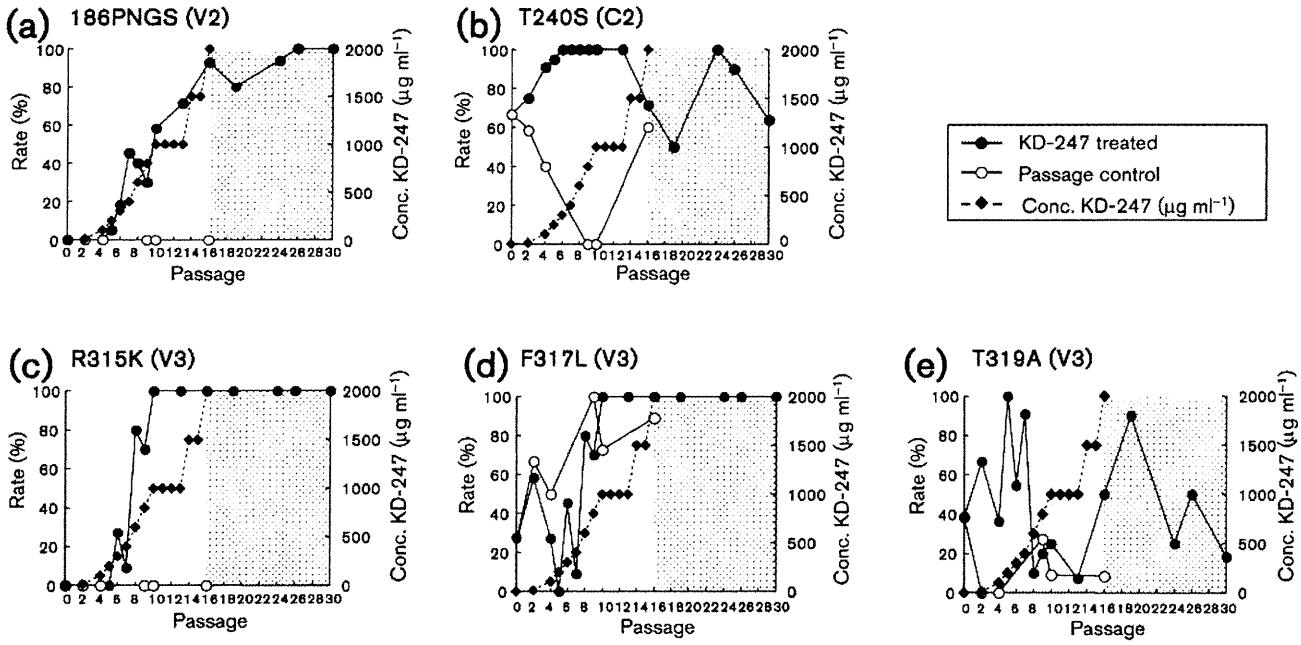


Fig. 6. The gp120 mutation profile of KD-247 HIV-1_{BaL} evasion variants for 14 additional passages without KD-247. The ratio of the PNGS insertion in the V2 region and mutations in the C2 and V3 region of HIV-1_{BaL} evasion variants in gp120 were plotted for 16 passages in the presence of KD-247 and an additional 14 passages in the absence of the mAb. The *y*-axis shows the percentage of PNGS insertions or mutations in the tested clones. The *x*-axis shows the concentration of KD-247.

observations indicate the role of the PNGS and the V3 mutation in neutralization resistance, together with the contribution of two other mutations which compensate for loss in replication ability induced by the first mutations in the process of neutralization evasion.

The interaction of V1/V2 with V3, including the influence on V2 glycosylation, has been reported not only for the neutralization sensitivity but also coreceptor usage (Bontjer *et al.*, 2009; Nabatov *et al.*, 2004). Additionally, mutations associated with resistance to CCR5 antagonists have been mapped to the V3 loop of gp120 and in some cases, to the outside of V3 including the N terminus of gp41 (Anastassopoulou *et al.*, 2009; Baba *et al.*, 2007; Berro *et al.*, 2009; Kuhmann *et al.*, 2004; Marozsan *et al.*, 2005; Ogert *et al.*, 2008; Westby *et al.*, 2007). We then evaluated pseudoviruses and infectious clones derived from the evasion mutants for their sensitivities to maraviroc and a mAb to CCR5 (2D7). As shown in Fig. 4(c, d) and Table 2, no change in co-receptor usage or sensitivity to maraviroc was observed in the evasion mutants.

To elucidate the stability of these evasion variants, we cultured HIV-1_{BaL} (2000) p16 viruses in the absence of KD-247 (Fig. 6). Despite culturing these viruses for 14 passages, no decrease in the proportion of the population containing the PNGS or the R315K, T240S and F317L mutations was observed. The T319A mutation appeared to subside at passage 30. Bunnik *et al.* (2008) reported that reversion of NAb-induced changes in amino acid usage in Env was observed at a late stage of infection in the face of declining neutralizing immunity, suggesting a negative effect of these changes on viral fitness (Bunnik *et al.*, 2008). In contrast, our results suggest that the addition of compensatory amino acid changes can stabilize the replication capacity of the evasion variants with the PNGS and R315K mutation.

In conclusion, we induced highly resistant viral variants against anti-V3 mAb, KD-247, harbouring a PNGS in the V2 region and a V3 mutation, together with mutations that compensate for replication deficiencies induced by the resistant mutations. The mutations associated with viral evasion may play a role in multiple different mechanisms which contribute towards evasion of antibody neutralization. The elucidation of the interplay between these mutations which results in neutralization evasion of the virus has important implications for the development of effective vaccines against HIV-1.

METHODS

Cells, culture conditions, reagents and viruses. PM1/CCR5 cells (Yusa *et al.*, 2005) were maintained in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone), 50 U penicillin ml^{-1}, 50 mg streptomycin ml^{-1} and 100 μg G418 (Nacalai) ml^{-1}. TZM-bl cells (Platt *et al.*, 1998; Wei *et al.*, 2002) obtained from the AIDS Research and Reference Reagent Program (ARRRP) and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FCS. KD-247 was provided by the Chemo-Sero-Therapeutic Research

Institute (Eda *et al.*, 2006). The mAb, 2D7, was purchased from BD Biosciences Pharmingen. Human recombinant soluble CD4 (rsCD4) was purchased from R&D Systems. Maraviroc, a CCR5 inhibitor, was a gift from Pfizer Inc. The R5 isolate, HIV-1_{BaL} (Gartner *et al.*, 1986), was passaged in PM1/CCR5 cells and the culture supernatant was stored at −150 °C prior to use.

Isolation of a KD-247-resistant mutant from HIV-1_{BaL} *in vitro*. The selection of KD-247 evasion variants from HIV-1_{BaL} was performed as described previously (Yoshimura *et al.*, 2006). Briefly, PM1/CCR5 cells (4×10^4) were exposed to 500 times $TCID_{50}$ of HIV-1_{BaL} pre-incubated with KD-247 for 30 min at 37 °C. After incubation for 5 h, cells were centrifuged, resuspended in RPMI 1640 medium supplemented with 10 % FCS without KD-247. The culture supernatant was harvested on day 6 and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of increasing concentrations of KD-247. When the virus began to propagate rapidly in the presence of KD-247, the mAb concentration was further increased. After the virus had been passaged in the presence of up to 2000 μg KD-247 ml^{-1}, the KD-247-resistant virus, HIV-1_{BaL} (2000) p16, was recovered from the cell culture supernatant. After 16 passages with KD-247, we continued culturing the virus for a further 14 passages without KD-247. HIV-1_{BaL} virus was also passaged for the same period in PM1/CCR5 cells in the absence of KD-247, and the resulting virus was designated HIV-1_{BaL} (−) p16. Proviral DNA (pDNA) from infected cells at various passages was subjected to DNA sequencing.

Amplification of pDNA and nucleotide sequencing. pDNA was extracted and nested PCR was performed to amplify the gp120 C1–C4 coding region as described previously (Wang *et al.*, 2002). The primers used were as follows: for the first-step PCR, 1B (5′-AGAAAGAGCAGAAGACAGTGGCAATGA-3′) and H (5′-TAGTGCTTCCTGCTGCTCCCAAGAACCC-3′); for the second-step PCR, 2B (5′-AGCAGAAGACAGTGGCAATGAGAGTGA-3′) and F (5′-ATATAATTCACTTCTCCAATTGTCCCTCAT-3′). The PCR products were inserted into a TA vector (Invitrogen) and sequenced.

Neutralization-sensitivity assay. The neutralization-sensitivity of each passaged HIV-1_{BaL} virus to KD-247 was determined by using TZM-bl cells. Briefly, a virus concentration of 300 $TCID_{50}$ was incubated with various dilutions of KD-247 in duplicate for 30 min at 37 °C in a 96-well flat-bottom culture plate (Corning-Costar). Freshly trypsinized cells (2×10^4 cells in 50 μl of 10 % FCS/DMEM containing 10 μg DEAE-dextran ml^{-1}) were added to each well. After incubation for 2 days at 37 °C, β-galactosidase activity in each well was measured by using Galacto-Star substrate (Applied Biosystems).

Construction of mutant envelope expression vectors. pDNA isolated from the infected cells at various passages was cloned into envelope expression vectors as described previously (Li *et al.*, 2005; Shibata *et al.*, 2007). Briefly, we amplified the full-length gp160 regions from the most frequent clones at the baseline, passage 5 and passage 13 by using LA *Taq* (Takara) with primers ENVA (5′-GGCTTAGGCATCTCCTATGGCAGGAAGAA-3′) and ENVN (5′-CTGCCAATCAGGGAAGTAGCCTTGTGT-3′), and the PCR products were inserted into the pCR-XL-TOPO vector (Invitrogen) and designated pCR-XL-BaL-WT, pCR-XL-BaL-p5 and pCR-XL-BaL-p13, respectively. Chimeric vectors were generated based on the pCR-XL-BaL-WT by replacing the fragments from pCR-XL-BaL-p5 and pCR-XL-BaL-p13 digested at the restriction enzyme sites indicated below. The *NdeI–ScaI* fragment for the pCR-XL-BaL-p5 env gene was subcloned into pCR-XL-BaL-WT, designated pCR-XL-BaL-STA. The *NdeI–ScaI*, *NdeI–StuI* and *StuI–ScaI* fragments for the pCR-XL-BaL-p13 env gene were subcloned into the pCR-XL-BaL-WT, designated pCR-XL-BaL-PNGS/SKL, pCR-XL-BaL-PNGS and pCR-XL-BaL-SKL, respectively. Each *EcoRI* fragment of these vectors was ligated into pCXN2 to give pCXN-BaL-WT, pCXN-BaL-STA, pCXN-BaL-PNGS/SKL, pCXN-BaL-PNGS and pCXN-BaL-SKL.

Pseudovirus preparation. Approximately 5 μg $pSG3^{\Delta env}$ (Wei *et al.*, 2002) and 0.5 μg pRSV-Rev (Hope *et al.*, 1990), supplied by the ARRRP and 4.5 μg HIV-1_{BaL} env-expressing $pCXN_2$ were co-transfected into 293T cells. At 24 h after transfection, the pseudovirus-containing supernatants were harvested, filtered and stored at −150 °C.

A single-round assay for measuring neutralization of the pseudoviruses. A single-round infectivity assay was used to measure the neutralization of HIV-1_{BaL} pseudoviruses as described previously (Li *et al.*, 2005). Briefly, reagents including an entry inhibitor, mAbs or rsCD4 at various concentrations and a pseudovirus suspension corresponding to 300 $TCID_{50}$ were pre-incubated for 30 min at 37 °C. The virus-compound mixtures were added to TZM-bl cells in a 96-well plate (2×10^4 cells per well). After incubation for 2 days at 37 °C, the β-galactosidase activity in each well was measured as described above. The reduction in infectivity was determined by comparing the relative light units in the presence and absence of each compound and was expressed as the percentage of neutralization.

Construction of chimeric pWT10/BaL env proviruses. Chimeric proviruses were constructed from the pWT/BaL proviral plasmid (from the ARRRP) (Hwang *et al.*, 1991) by replacing the region encoding the envelope gp160. Briefly, the env genes obtained from escaped HIV-1_{BaL} variants or induced by site-directed mutagenesis were substituted into the pWT/BaL vectors after digestion at the restriction enzyme sites *SalI* and *BamHI*. The resulting replication-competent viruses were designated HX-BaL-X (e.g. HX-BaL-WT, HX-BaL-PNGS/SKL etc.).

Preparation of infectious clones and viral replication assays in PM1/CCR5 cells. Approximately, 5 μg of the plasmids from the env mutants were transfected into 293T cells by using the Effectene transfection reagent (Qiagen). At 48 h after transfection, the virus-containing supernatants were harvested, filtered and frozen in aliquots at −150 °C. Viral yields were quantified by using the HIV-1 p24 antigen ELISA (ZeptoMetrix). PM1/CCR5 cells (3×10^4) were exposed to pWT/BaL env chimeric viruses corresponding to 2 ng or 10 ng of p24 for 4 h at 37 °C. Following incubation, cells were centrifuged and resuspended in RPMI 1640 medium supplemented with 10 % FCS and cultured for 6 days. Viral replication was monitored by measuring the concentration of p24 antigen in culture supernatants.

Statistical analysis. Statistical correlations were analysed by using the Student's *t*-test. *P*-values <0.05 were considered statistically significant.

Nucleotide sequence accession numbers. The sequence data of env expression vectors from passaged samples have been deposited under the GenBank accession numbers AB521136–AB521148.

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Review Article Open Access

In vitro and *In vivo* Resistance to Human Immunodeficiency Virus Type 1 Entry Inhibitors

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Abstract

Viral entry is one of the most important targets for the efficient treatment of Human immunodeficiency virus type 1 (HIV-1)-infected patients. The entry process consists of multiple molecular steps: attachment of viral gp120 to CD4, interaction of gp120 with CCR5 or CXCR4 co-receptors, and gp41-mediated fusion of the viral and cellular membranes. Understanding the sequential steps of the entry process has enabled the production of various antiviral drugs to block each of these steps. Currently, the CCR5 inhibitor, maraviroc, and the fusion inhibitor, enfuvirtide, are clinically available. However, the emergence of HIV-1 strains resistant to entry inhibitors, as commonly observed for other classes of antiviral agents, is a serious problem. In this review, we describe a variety of entry inhibitors targeting different steps of viral entry and escape variants that are generated *in vitro* and *in vivo*.

Keywords: CD4-gp120 binding inhibitor; CCR5 antagonist; CXCR4 antagonist; Fusion inhibitor; Resistance; HIV-1

Introduction

The development of chemotherapy with antiretroviral agents has reduced the morbidity and mortality of Human immunodeficiency virus type 1 (HIV-1)-infected individuals. Successful treatment of HIV-1-infected patients using chemotherapy is partly due to a combination of different classes of antiviral agents against the viral protease or reverse transcriptase. However, successful eradication of the virus from infected individuals has not been achieved by antiviral treatment, and is often limited by the emergence of drug-resistant HIV-1 strains [1-3]. These problems highlight the need to develop novel anti-HIV-1 drugs that target different steps of the viral replication process. Viral entry is currently one of the most attractive targets for the development of new drugs to control HIV-1 infection. Viral entry proceeds through Env (gp120, gp41)-mediated membrane fusion, and consists of sequential steps: (i) attachment of viral gp120 to the CD4 receptor; (ii) binding of gp120 to CCR5 or CXCR4 co-receptors; and (iii) fusion of the viral and cellular membranes (Figure 1). A large number of inhibitors targeting different steps of the viral entry process have been developed, including peptides/peptide mimics, small molecules, and monoclonal antibodies (MAb).

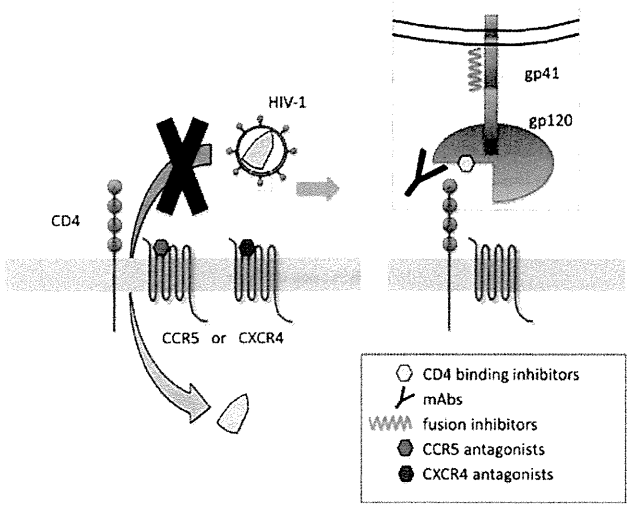

Figure 1: Molecular targets of inhibitors of HIV-1 entry into the target cell.

Enfuvirtide (also known as T-20) was the first of a new class of drugs known as fusion inhibitors, which was approved by the U.S. Food and Drug Administration (FDA) in 2003. Approval was given for the use of this drug in combination with other anti-HIV-1 medications to treat advanced HIV-1 infection in adults and children aged six years and older. The drug is an antiviral peptide that prevents HIV-1 entry by blocking gp41-mediated fusion [4-6]. Small compounds that can bind to the pockets of the extracellular loops of a coreceptor are expected to be potent antiviral agents. Several small-molecule CCR5 inhibitors have progressed through clinical development [7]. Maraviroc [8,9], a CCR5 antagonist, is the second entry inhibitor approved by the FDA in 2007 for treatment-experienced patients infected with a CCR5-tropic (R5-tropic) virus. Extensive research is currently underway to develop the next generation of entry inhibitors, however, the emergence of viral strains resistant to entry inhibitors, as well as other classes of antiviral

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