Increased HIV-1 sensitivity to neutralizing antibodies by mutations in the Env V3-coding region for resistance to CXCR4 antagonists

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HIV-1 passage in cell culture in the presence of chemokine receptor antagonists can result in selection of viruses with env mutations that confer resistance to these inhibitors. In the present study, we examined the effect of HIV-1 env mutations that confer resistance to CXCR4 antagonists on envelope (Env) sensitivity to neutralizing antibodies (NAbs). Serial passage of CXCR4-tropic HIV-1 NL4-3 in PM1/CCR5 cells under CXCR4 antagonists KRH-3955, AMD3100 and AMD070 yielded two KRH-3955-resistant, one AMD3100-resistant and one AMD070-resistant viruses. These viruses had multiple env mutations including the Env gp120 V3 region. The majority of viruses having these CXCR4 antagonist-resistant Envs showed higher sensitivity to NAbs 447-52D, b12 and 2F5 targeting the V3 region, the gp120 CD4-binding site and the gp41 membrane proximal region, respectively, compared to NL4-3 WT virus. Recombinant NL4-3 viruses with the V3-coding region replaced with those derived from the CXCR4 antagonist-resistant viruses showed increased sensitivity to NAbs b12, 2F5 and 447-52D. Molecular dynamics simulations of Env gp120 outer domains predicted that the V3 mutations increased levels of fluctuations at the tip and stem of the V3 loop. These results indicate that mutations in the V3-coding region that result in loss of viral sensitivity to CXCR4 antagonists increase viral sensitivity to NAbs, providing insights into our understanding of the interplay of viral Env accessibility to chemokine receptors and sensitivity to NAbs.

INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) entry into the host cell is a multistep process. The viral envelope glycoprotein (Env) is composed of a trimer of heterodimer subunits of gp120 and gp41. HIV-1 entry is triggered by interaction of gp120 with CD4 and chemokine receptors (CKRs), such as CCR5 and CXCR4. This interaction induces conformational changes in gp120 and gp41, leading to fusion of viral and cellular membranes (Wilen et al., 2012; Wyatt & Sodroski, 1998).

A variety of inhibitors have been developed against the interaction between gp120 and CKRs, of which the CCR5 entry inhibitor maraviroc (MVC) is approved for clinical use (Gulick et al., 2008). Other CCR5 antagonists not
approved for clinical use include AD101 and vicriviroc (VVC) (Strizki et al., 2001, 2005; Tsamis et al., 2003). These antagonists bind to CCR5 and suppress replication of CCR5-tropic (R5) viruses by allosterically inhibiting virus entry into the cells. Several studies have reported CCR5 antagonist-resistant HIV-1 carrying multiple env mutations, selected in vitro and in vivo. CCR5 antagonist-resistant virus-derived Env s can bind to the inhibitor-bound CCR5 for virus entry (Kuhmann et al., 2004; Marozsan et al., 2005; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007). Interestingly, previous studies have shown increased sensitivity of CCR5 antagonist-resistant HIV-1 to some neutralizing antibodies (NAbs). It has been reported that AD101-resistant viruses have increased sensitivity to anti-gp120 CD4-induced epitope (CD4i), anti-gp120 V3 and anti-gp41 membrane proximal external region (MPER) antibodies, whereas VVC-resistant viruses have increased sensitivity to anti-gp120 glycan and anti-MPER antibodies (Berro et al., 2009; Pugach et al., 2008). MVC-resistant viruses have been shown to exhibit higher sensitivity to anti-gp120 CD4-binding site (CD4bs), anti-V3 and anti-CD4i NAbs (Yoshimura et al., 2014). These observations suggest that env mutations that confer CCR5 antagonist-resistance may result in loss of viral resistance to NAbs.

In contrast to CCR5 antagonists, no CXCR4 antagonists have been approved for clinical use to date, although multiple CXCR4 antagonists including T-22 and AMD3100 have been developed (De Clercq et al., 1994; Moyle et al., 2009; Murakami et al., 1997, 2002; Nakashima et al., 1992; Schols et al., 1997). These antagonists show antiviral activity against CXCR4-tropic (X4) HIV-1. We previously reported that KRH-3955 is a potent and selective CXCR4 antagonist inhibiting replication of X4 viruses. This compound is orally bioavailable and has higher anti-HIV-1 activity in vivo than AD101 (Murakami et al., 1997, 2002; Nakashima et al., 1992; Schols et al., 1997). To analyse the genetic basis of the loss of the sensitivity to CXCR4 antagonists, we selected mutant viruses less sensitive to CXCR4 antagonists and compared the sequences in their env region. As shown in Fig. 1, KRH-3955-resistant mutations accumulated mainly in the V3-coding region of HIV-1 env. KRH-3955-resistant virus A (KRH-3955R) had mutations in C1 (A48V), V3 (S306R, Q310H and N325D) and gp41 (L520F) regions, while KRH-3955-resistant virus B (KRH-3955R) had mutations in V3 (T303I, S306R, Q310H and N325D), C4 (E429K) and gp41 (L520F). However, AMD3100-resistant (AMD3100R) and AMD070-resistant (AMD070R) mutations were found in multiple regions of env. Amino acid substitutions were identified in V3 (S306R, A316T and N325D), C3 (T341I and E351K), C5 (R480K) and gp41 (D547G and M687I) for AMD3100 8 and in C1 (D78N and V85I), C2 (D269N), V3 (S306R, A316T and A329T), V4 (P417L), C5 (V489I) and gp41 (L520F, D547G, N624D and K808R) with additional deletions in V4 at positions 393–397 (STWFN) for AMD070R viruses. All of the CXCR4 antagonist-resistant viruses shared the S306R mutation in the V3 region. The passage control virus had mutations in multiple regions of env (Fig. 1), but not the V3-coding region and maintained sensitivity to the CXCR4 antagonists comparable to the parental NL4-3 WT (data not shown).

To evaluate the susceptibility of the CXCR4 antagonist-resistant viruses to CXCR4 entry inhibitors in more detail, we constructed env chimeric clones using each resistant virus and compared IC50 values of the chimeras to that of NL4-3 WT (Fig. 2, Table S1, available in the online Supplementary Material). All of the env chimeric viruses displayed similar levels of resistance to KRH-3955 (4.2–7.1-fold) (Fig. 2a). Similar results were obtained with AMD3100 (8.7–18-fold), AMD070 (10–18-fold) and T140 (2.8–4.5-fold) (Fig. 2b–d). These data indicate that the env chimeric clones displayed similar levels of resistance to CXCR4 antagonists regardless of the mutation patterns in env.

**RESULTS**

**Selection of mutant viruses less sensitive to CXCR4 antagonists in vitro**

To analyse the genetic basis of the loss of the sensitivity to CXCR4 antagonists, we selected mutant viruses less sensitive to CXCR4 antagonists and compared the sequences in their env region. As shown in Fig. 1, KRH-3955-resistant mutations accumulated mainly in the V3-coding region of HIV-1 env. KRH-3955-resistant virus A (KRH-3955R) had mutations in C1 (A48V), V3 (S306R, Q310H and N325D) and gp41 (L520F) regions, while KRH-3955-resistant virus B (KRH-3955R) had mutations in V3 (T303I, S306R, Q310H and N325D), C4 (E429K) and gp41 (L520F). However, AMD3100-resistant (AMD3100R) and AMD070-resistant (AMD070R) mutations were found in multiple regions of env. Amino acid substitutions were identified in V3 (S306R, A316T and N325D), C3 (T341I and E351K), C5 (R480K) and gp41 (D547G and M687I) for AMD3100 and in C1 (D78N and V85I), C2 (D269N), V3 (S306R, A316T and A329T), V4 (P417L), C5 (V489I) and gp41 (L520F, D547G, N624D and K808R) with additional deletions in V4 at positions 393–397 (STWFN) for AMD070R viruses. All of the CXCR4 antagonist-resistant viruses shared the S306R mutation in the V3 region. The passage control virus had mutations in multiple regions of env (Fig. 1), but not the V3-coding region and maintained sensitivity to the CXCR4 antagonists comparable to the parental NL4-3 WT (data not shown).

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**Susceptibility of the CXCR4 antagonist-resistant viruses to NAbs**

To assess the impact of the CXCR4 antagonist-resistant mutations on NAbs, we tested the susceptibility of the env chimeric clones to NAbs that bind different regions of Env, including gp120 V3 (447–52D), CD4bs (b12 and VRC01), in PM1/CCR5 cells in the presence of these CXCR4 antagonists. The resulting viruses had multiple mutations in env including the Env gp120 V3-coding region. Analyses using recombinant viruses indicated increased sensitivity to NAbs caused by mutations in the V3 region coding for less sensitivity to the CXCR4 antagonists tested.
CD4i (17b), the cluster of glycans in gp120 (2G12), V1/V2 (PG16) and gp41 MPER (2F5) regions using TZM-bl cells. Compared with NL4-3 WT, KRH-3955R A Env and KRH-3955R B Env became sensitive to 447-52D (58- and 94-fold), b12 (3.5- and 8.1-fold), 17b (4.2- and 4.5-fold) and 2F5 (3.3- and 4.0-fold). On the other hand, AMD3100R Env and AMD070R Env became sensitive to 447-52D (6.3- and 2.5-fold) and showed enhanced resistance to 17b relative to NL4-3 WT (Fig. 3, Table S2). Among the env chimeric clones, only AMD3100R Env became resistant to 2G12 and sensitive to PG16 (22-fold) (Fig. 3e, f). Changes in the sensitivity of the env chimeric clones to VRC01 were not observed (Fig. 3b). These results demonstrate that depending on their location in env, the CXCR4 antagonist-resistant mutations exhibited altered neutralization sensitivity.

Impact of mutations in the V3 region on CXCR4 antagonist and neutralization sensitivity

As previously reported (Kanbara et al., 2001; Moncunill et al., 2008; Schols et al., 1998; de Vreese et al., 1996), all of the mutant viruses had several mutations in V3-coding region. To examine whether the V3 mutations were responsible for the loss of viral sensitivity to CXCR4 antagonists, we generated V3 chimeric clones from each env chimeric clone. KRH-3955R A V3 and KRH-3955R B V3 displayed similar levels of resistance to each CXCR4 antagonist (Fig. 4). IC50 values of these V3 chimeric clones were similar to their parental env chimeric clones (Table S1). In contrast to KRH-3955R V3, IC50 values of AMD3100R V3 and AMD070R V3 were twofold higher than their parental env chimeric clones, although these V3 chimeric viruses were also resistant to each CXCR4 antagonist (Fig. 4, Table S1). These data suggest that mutations in the V3 region are the major determinant in the loss of viral sensitivity to CXCR4 antagonists. However, mutations outside of the V3-coding region may also be associated with resistance to CXCR4 antagonists.

We next examined whether the V3 mutations induced changes in the sensitivity of HIV-1 to a subset of NAbs (Fig. 5, Table S2). All of the V3 chimeric clones demonstrated increased sensitivity to 447-52D (33-441-fold), b12 (2.7-8.4-fold) and 2F5 (2.6-4.7-fold) relative to NL4-3 WT, suggesting that the V3 mutations of CXCR4 antagonist-resistant viruses alter accessibility to epitopes on the V3, CD4bs and gp41 MPER (Fig. 5a, c, g). Among the V3 chimeras, KRH-3955R A V3 did not show altered sensitivity to 17b and PG16 (Fig. 5d, f), indicating that the T303I mutation affects the sensitivity of HIV-1 to 447-52D, b12, 2F5, 17b and PG16. AMD3100R Env also showed increased sensitivity to 17b (sixfold) (Fig. 5d). Among the V3 chimeric viruses, only AMD3100R V3 showed the most enhancement in susceptibility to 447-52D (441-fold), b12 (8.4-fold), 17b (14-fold) and 2F5 (4.7-fold) relative to NL4-3 WT (Fig. 5, Table S2). However, KRH-3955R A V3 did not show altered sensitivity to 17b and PG16 (Fig. 5d, f), indicating that the T303I mutation affects the sensitivity of HIV-1 to 447-52D, b12, 2F5, 17b and PG16. AMD3100R V3 also showed increased sensitivity to 17b (sixfold) (Fig. 5d). Among the V3 chimeric viruses, only AMD3100R became resistant to 2G12 (fivefold relative to NL4-3 WT) as observed with AMD3100R Env, suggesting that the V3 mutations of AMD3100-resistant viruses reduce sensitivity to 2G12 NAb (Fig. 5e). Similar to the env chimeric clones,

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Fig. 1. Amino acid sequences of gp120 and gp41 in CXCR4 antagonist-resistant viruses. Amino acid substitutions and deletions in (a) gp120 and (b) gp41 are indicated in black. Amino acid numbering is based on HxB2 Env. Domain abbreviations: SP, signal peptide; C1–C5, conserved domains 1 to 5; V1–V5, variable domains 1 to 5; FP, fusion peptide; HR1, heptad repeat 1; HR2, heptad repeat 2; TM, transmembrane domain; CD, cytoplasmic domain.
no changes in neutralization sensitivity of the V3 chimeric clones were observed with VRC01 (Fig. 5b). These data demonstrate that the V3 loop modification for loss of viral sensitivity to CXCR4 antagonists was associated with altered neutralization sensitivity.

**S306R mutation increases sensitivity to 447-52D and b12 NAbs**

To determine which mutation in the V3-coding region contributes to loss of viral sensitivity to CXCR4 antagonists and increased neutralization sensitivity to 447-52D and b12, we constructed single- and double-point mutants bearing the S306R mutation, which was shared among all of the CXCR4 antagonist-resistant viruses. We failed to produce T303I and T303I/S306R mutants as infectious particles due to gp120 shedding and loss of infectivity (data not shown). All of the double-point mutants, but only the Q310H single-point mutant became approximately twofold more resistant to KRH-3955 compared to NL4-3 WT (Fig. 6). Similar trends were observed with AMD3100, AMD070 and T140 (data not shown). We also constructed V3 chimeric viruses reverting R to S at position 306 in V3, but these clones were not resistant to any of the CXCR4 antagonists (data not shown).

We subsequently tested the sensitivity of single-point mutants to 447-52D and b12. Only S306R increased sensitivity to 447-52D (30-fold) and b12 (10-fold) in the single-point mutants compared to NL4-3 WT (Fig. 7). These data suggest that the S306R, which is shared with all of the CXCR4 antagonist-resistant viruses, is crucial to the loss of viral sensitivity to CXCR4 antagonists and imply that the point mutation induces conformational changes not only in V3 but also in the gp120 core.

**Molecular dynamics (MD) simulations of the HIV-1 gp120 outer domain**

The above results suggest that the V3 mutations induced changes in the binding ability to CXCR4 and NAbs. To examine how the V3 mutations that altered sensitivity to CXCR4 antagonists and NAbs affect the fluctuations of the gp120 outer domain, gp120 outer domains of NL4-3 WT and its V3 mutants (KRH-3955R V3, AMD3100R V3 and AMD070R V3) were subjected to MD simulations. As previously reported for CCR5-tropic HIV-1 and SIV gp120s (Kuwata et al., 2013; Naganawa et al., 2008; Yokoyama et al., 2012, 2016; Yuan et al., 2013), CXCR4-tropic gp120s reached a state of thermodynamic equilibrium in solution after 20 ns of the MD simulations. We examined atomic fluctuations of individual amino acid residues in this equilibrated state by calculating root mean square fluctuation (RMSF) of the Cα atoms (Case et al., 2010) using 12 500 snapshots of structures from 25 to 50 ns of each MD simulation. The RMSFs were maximal at the V3 tip in NL4-3 and its V3 mutants, suggesting a general physical property...
Fig. 3. Sensitivity of CXCR4 antagonist-resistant env chimeric HIV-1 viruses to anti-Env neutralizing antibodies. The sensitivity of env chimeric viruses derived from CXCR4 antagonist-resistant viruses to (a) 447-52D, (b) VRC01, (c) b12, (d) 17b, (e) 2G12, (f) PG16 and (g) 2F5 is shown. The data points in all panels are mean values±SEM of at least three independent experiments.
of the coreceptor-binding site (Fig. 8a). Notably, a marked increase in the RMSF value was detected in the V3 regions (Fig. 8a), indicating changes in fluctuation profiles of amino acid residues in the V3 loop. First, V3 mutations increased the levels of fluctuations at the V3 tip and stem (Fig. 8b). An additional peak of RMSF value was observed in the V3 tip for KRH-3955R B. Second, the mutations also increased the levels of fluctuations in the N-terminal portion of the V3 base.

DISCUSSION

Two hypotheses have been proposed for HIV-1 acquiring resistance to CXCR4 antagonists: (1) changing the mode of using coreceptors and (2) switching coreceptor usage, such as from CXCR4 to CCR5. Prior studies have only considered the first possibility because T-cell lines that express low levels of CCR5, such as MT-4, were used (Kanbara et al., 2001; Moncunill et al., 2008; Schols et al., 1998; de Vreese et al., 1996). In the current study, NL4-3 virus was passaged in the presence of CXCR4 antagonists using PM1/CCR5 cells which express high levels of CCR5 to examine the second possibility. We examined the coreceptor usage of each CXCR4 antagonist-resistant HIV-1; however, coreceptor usage was not altered (Table S3). At the 135 passages, all of the env chimeric viruses showed cross-resistance to CXCR4 antagonists with high concentrations completely inhibiting HIV-1 replication, although their infectivity was comparable to NL4-3 WT virus (Fig. 2). Previous studies showed that the CXCR4 interaction sites of KRH-3955 are different from those of AMD3100 (Gerlach et al., 2001; Murakami et al., 2009; Rosenkilde et al., 2007). Our and previous findings suggest that HIV-1 acquires resistance to CXCR4 antagonists in a competitive way without switching coreceptor usage, which appears different to the mechanisms of resistance to CCR5 antagonists (Kuhmann et al., 2004; Marozsan et al., 2005; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007). Yoshimura et al. (2014) showed that R5 HIV-1 acquired resistance to MVC by reductions in the maximal per cent inhibition (MPI) after IC50 shift, suggesting that X4 HIV-1 can also adapt to drug-bound CXCR4 after extended passages of the virus.

Several studies have been shown that N-glycosylation at 301, the residues 306, 321 and 322 and net charges of V3-coding region are associated with determining coreceptor usage (Cardozo et al., 2007; Fouchier et al., 1992; de Jong et al., 1992; Ogert et al., 2001). Xiang et al. (2013) showed that residues 310 and 311 were essential for coreceptor usage in the context of HXBc2 Env. In the current study, although the net charges of V3-coding region in the CXCR4 antagonist-resistant viruses were not increased compared with NL4-3 WT virus, some of these essential
Fig. 5. Sensitivity of CXCR4 antagonist-resistant V3 chimeric HIV-1 to anti-Env neutralizing antibodies. The sensitivity of env chimeric viruses derived from CXCR4 antagonist-resistant viruses to (a) 447-52D, (b) VRC01, (c) b12, (d) 17b, (e) 2G12, (f) PG16 and (g) 2F5 are shown. The data points in all panels are mean values±SEM of at least three independent experiments.
residues for coreceptor usages were substituted. T303I mutation, which is located within the glycosylation motif, was observed in the KRH-3955R resistant virus, not in the other resistant and passage control viruses (Fig. 1). However, we failed to produce the infectious clones with T303I single and T303I/S306R double mutations. These results suggest that the T303I mutation may contribute to resistance of KRH-3955, but also be associated with a severe fitness cost without other mutations in the Env. We showed that the single Q310H mutation is highly potent and the S306R mutation synergizes with other mutation(s) to induce resistance to CXCR4 inhibitors (Fig. 6), suggesting that these mutations contribute to acquiring resistance to CXCR4 antagonists by increasing the affinity between V3 loop and CXCR4.

Our MD simulations of gp120 outer domain provide structural bases of viral resistance against CXCR4 antagonists. The study revealed that the V3 mutations for the CXCR4 antagonists could increase the levels of fluctuations of the potential coreceptor-binding sites of virus, i.e. V3 tip and base (Huang et al., 2007). Because structural fluctuations of the interaction surface in solution play key roles in molecular interactions (Dodson et al., 2008; Karplus & Kuriyan, 2005; Ode et al., 2012), it is possible that the physical changes in V3 result in changes in the binding affinity of gp120 to the drug-unbound CXCR4, which in turn could decrease inhibitory effects of CXCR4 antagonists. Alternatively, the changes in V3 fluctuations might confer HIV-1 ability to bind to the drug-bound CXCR4, as suggested with the resistant mutations against the CCR5 antagonist.
Further structural study is necessary to address each of the issues.

As previous reports for other entry inhibitors (Berro et al., 2009; Pugach et al., 2008; Reeves et al., 2005; Yoshimura et al., 2014), we showed that CXCR4 inhibitor-resistant Env altered neutralization sensitivity to some NAbs (Fig. 3, Table S2). We showed that the clones with KRH-3955\textsuperscript{R}A and B Env became sensitive to CD4bs Ab b12, but not VRC01 (Fig. 3, Table S2). Previously, Li et al. (2011) tested the VRC01 interaction with the functional viral spike using Ala scanning Env pseudovirus mutants to assess the neutralizing potency of VRC01 in comparison to that of b12. The authors showed that several clones with mutation in V3 tip and stem regions became highly sensitive to b12; however, these mutated residues had no impact on the neutralizing potency of VRC01. In our results of the MD simulation, the RMSF of the resistant Env regions increased in the V3 tip and stem compared to that of WT Env (Fig. 8). Our and

![Fig. 7. Sensitivity of single-point mutants in V3 to 447-52D and b12 neutralizing antibodies. The sensitivities of point mutants in V3 to (a) 447-52D and (b) b12 are shown. The data points in all panels are mean values±SEM of at least three independent experiments.](image)

![Fig. 8. MD simulations of the HIV-1 gp120 outer domain. Molecular modelling and MD simulation of HIV-1 gp120 outer domain were done with modules in MOE (Chemical Computing Group) and in the AMBER 11 program package (Case et al., 2010). RMSF values of the Ca atoms representing atomic fluctuations of the main chains of individual amino acid residues during MD simulations were calculated with 12 500 snapshots from 25 to 50 ns of each MD simulation using the ptraj module in AMBER (Case et al., 2010). Distributions of RMSF values in the gp120 outer domain (a) and V3 region (b) are shown. Asterisks indicate amino acid residues with markedly increased RMSF values.](image)
previous findings suggest that VRC01 targets its epitope on the functional spike in a highly precise manner, overcoming the change of fluctuation and steric constraints that restrict the binding of many Env ligands, but not b12.

Comparing the KRH-3955R A V3 to that of KRH-3955R B, the T303I mutation has a large impact on neutralizing sensitivity (Fig. 5, Table S2). Some studies reported that the loss of glycosylation in V3 affects the neutralization sensitivity of some HIV-1 strains (Koch et al., 2003; Li et al., 2008; Polzer et al., 2009; Wang et al., 2013). Recently, Zolla-Pazner et al. (2015) proposed that the glycan on N301 restricts the mobility of the V3 loop. Based on this report, it is likely that the V3 loop of KRH-3955R B is more exposed than that of KRH-3955R A, leading to increased sensitivity to 447-52D Ab. Further structural analysis studies are needed to investigate whether the T303I mutation occurs in KRH-3955-resistant viruses due to the specific interaction between gp120 and CXCR4 in the presence of KRH-3955.

Moreover, our MD simulations also provide structural bases for changes in viral neutralization sensitivities against 447-52D and 17b monoclonal antibodies. 447-52D preferentially binds the GPR motif in the V3 tip (Hioe et al., 2010), whereas CD4-induced (CD4i) antibody binds to CD4i epitope containing V3 base (Huang et al., 2007). Therefore, it is possible that the changes in the fluctuations at the V3 tip and base lead to an increase in the binding affinity of gp120 not only to CXCR4 but also to 447-52D and 17b antibodies. Thus, our present and previous (Yuan et al., 2013) MD simulation studies predict that acquisition of resistance against HIV-1 entry inhibitors can simultaneously result in changes in sensitivity to V3 and CD4-induced antibodies. Further structural study is necessary to address each of the issues.

Our data suggest that mutations in the V3-coding region that result in CXCR4 antagonist-resistance increase viral sensitivity to some NAbs. These findings suggest that the CXCR4 antagonist-resistant variants may become neutralization-sensitive and not survive in vivo when the patients who harbour X4 virus are treated with CXCR4 antagonists. For this reason, it is possible that CXCR4 antagonists, such as KRH-3955, suppress HIV replication, especially in patients with high levels of circulating anti-Env NAbs.

We have a limitation for explanation of the enhancement of neutralization sensitivity of the CXCR4 antagonist-resistant viruses because we used only laboratory-adapted virus NL4-3 in this study. Our study could be more significant and complete if we could include neutralizing resistant primary isolates in addition to NL4-3.

**METHODS**

**Compounds and neutralizing antibodies.** KRH-3955 and AMD070 were synthesized at Kureha Corporation (Murakami et al., 2009). T140 was a gift from Dr Tamamura (Tamamura et al., 1998). AMD3100 was purchased from Sigma Aldrich. 447-52D (Gorny et al., 1992) was purchased from Polymun Scientific Immunobiologische Forschung. b12 (Barbas et al., 1992; Burton et al., 1991, 1994; Roben et al., 1994), VRC01 (Wu et al., 2010), 17b (Sullivan et al., 1998; Thali et al., 1993; Trkola et al., 1996; Wyatt et al., 1995, 1998), 2G12 (Buchacher et al., 1994; Crawford et al., 1999; Etemad-Moghadam et al., 1999; Mascola et al., 1999; Trkola et al., 1996), PG16 (Walker et al., 2009) and 2F5 (Buchacher et al., 1994; Purtscher et al., 1994, 1996) were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

**Cells.** HEK293T and TZM-bl cells (Platt et al., 1998, 2009; Takeuchi et al., 2008; Wei et al., 2002) were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS. PM1/CCR5 cells (Maeda et al., 2008) were maintained in RPMI-1640 containing 10% FBS. U87.CD4, U87.CD4.CXCR4 and U87.CD4.CXCR5 cells (Björndal et al., 1997) were maintained in DMEM containing 15% FBS, 1 µg ml−1 puromycin and 300 µg ml−1 G418.

**Preparation of viral stocks and infectivity assay.** Viral stocks were prepared as previously described (Murakami et al., 2009) with minor modifications. Briefly, HEK293T cells were plated (2.3 × 106 cells) on a T-25 cm2 flask in 5 ml of medium. One day later, 3 µg of pNL-4.3 (Adachi et al., 1986), pNL(AD8) (Freed et al., 1995) or mutant plasmids was transfected into the cells by the calcium phosphate precipitation methods. Six hours after transfection, the supernatant was replaced with fresh medium. Two days after transfection, the culture supernatant was harvested and clarified through a 0.45 µm pore filter (Millipore). Aliquots of viral stocks were stored at −80 °C. The p24 antigen content was measured using HIV-1 p24 antigen ELISA kit according to the manufacturer’s instructions (ZeptoMetrix).

Virus infectivity was measured using TZM-bl cells. Cells were plated in 96-well plates at 2.0 × 104 cells per well in a volume of 100 µl a day prior to infection. Viral stocks were inoculated and incubated at 37 °C in 5% CO2. Viral stocks were assayed at twofold dilutions ranging from 8 to 0.03 ng of p24 antigen. Twenty-four hours later, luciferase activity was measured using the Steady-Glo Luciferase Assay system (Promega). Luminescence was detected using a Veritas Microplate Luminometer (Promega).

**Selection of mutant viruses less sensitive to CXCR4 antagonists in vitro.** Mutant viruses less sensitive to CXCR4 antagonists were isolated by serial passages of X4 NL4-3-infected PM1/CCR5 cells with increasing concentrations of KRH-3955, AMD3100 or AMD070. Starting concentrations corresponded to IC50 of the antagonists (KRH-3955: 2.5 nM, AMD3100: 100 nM and AMD070: 50 nM). These concentrations did not affect the viability of mock-infected PM1/ CCR5 cells (data not shown). The culture supernatant was harvested on days 4–6 and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of the same or increasing concentrations of the CXCR4 antagonists. Dose escalation was performed when cytopathic effect was observed. NL4-3 was passaged in the absence of CXCR4 antagonists in PM1/CCR5 cells as a control.

**Amplification of env regions of proviral DNA and nucleotide sequencing.** At the 135 passages, proviral HIV-1 DNA was extracted from lysates of infected cells when the drug concentrations were 15 nM for KRH-3955 and 3000 nM for both AMD3100 and AMD070. Proviral DNA was subjected to PCR amplification with KOD-plus-Neo (Toyobo) using the primers NLenv 5’-tagacagtcatatatgaaacttg3’ and NLenv 5’-tcgtaaacaccctctctcctc3’ following dA addition. The PCR products were purified and cloned into pCR-XL TOPO vector using TOPO XL PCR Cloning kit (Invitrogen). The whole env region of the passaged viruses was sequenced using an ABI3130 Genetic Analyzer (Applied Biosystems). We sequenced 10–15 clones of each passaged virus and selected a major clone for constructing env chimeric clones used in this study.
Sequences of the env genes were compared between NL4-3 WT and the CXCR4 antagonist-resistant viruses.

Construction of env chimeric proviruses. Env chimeric proviruses from CXCR4 antagonist-resistant variants were constructed as follows. Both pNL4-3 and the pCR-XL TOPO vector which contains the whole env region of each passaged virus were digested with EcoRI and XhoI. The EcoRI–XhoI fragment (3.1 kb) of pNL4-3 was replaced with that of the pCR-XL TOPO vector. These plasmids were designated KRH-3955<sup>E</sup> AEnv, KRH-3955<sup>B</sup> BEnv, AMD3100<sup>E</sup> Env and AMD700<sup>B</sup> Env.

Determination of coreceptor usage. U87.CD4 (or U87.CD4, CXCR4 or U87.CD4.CXCR5) cells were infected with Env chimeric clone viruses at 37 °C for 18 h. The infected cells were washed three times with the culture medium (DMEM containing 10% FBS) and incubated in the culture medium at 37 °C in 5% CO<sub>2</sub>. Five days after infection, the p24 antigen content of the culture supernatants was measured using the HIV-1 p24 antigen ELISA kit as described above. NL4-3 and NL (AD8) were used as a positive control for X4 and R5 HIV-1, respectively.

Construction of V3 chimeric proviruses. V3 chimeric proviruses from the env chimeric viruses were constructed as follows. Briefly, pNL4-3 was digested with BamHI and EcoRI, BamHI–EcoRI fragments (2.7 kb) were cloned into the BamHI and EcoRI sites of pUC19 and designated pUC-NC. The env chimeric viruses were then digested with Stul and Nhel, and the Stul–Nhel fragments (0.4 kb) were cloned into Stul and Nhel sites of pUC-NC. Finally, plasmids were digested with EcoRI and Nhel, and cloned into EcoRI and Nhel sites of pNL4-3. These plasmids were designated KRH-3955<sup>E</sup> A V3, KRH-3955<sup>B</sup> B V3, AMD3100<sup>E</sup> V3 and AMD700<sup>B</sup> V3.

Site-directed mutagenesis. Mutagenesis procedures were performed according to overlapping extension PCR methods (Ho et al., 1989). To construct single-point mutants, pUC-NC was used as a template for amplification of DNA. The following complementary oligonucleotide primers were designed with QuickChange Primer Design Program (http://www.genomics.agilent.com/primerDesignProgram.jsp): Env T303I (5′-TAATTGTGACAAGCCCAACAACATAAGAAGAGATGCTGATTCCAGGGG-3′), Env S306R (5′-CCAAACACAATAAGAGGAGAAGATCTGATACCCGTTACCCGAGG-3′), Env Q319H (5′-CACCTTGCCCGTGCTGGGACCCACTTGCTTCTTCTGTG-3′), Env A316T (5′-TCTCTATTGTAACACCTGTCCTTTCTGGCCTC-3′), Env N325D (5′-CAATGTGAATTGTACAAGACCCAACAACAATATAAGAAAGATGCTGATTCCAGGGG-3′), Env A329T (5′-CAATGTGAATTGTACAAGACCCAACAACAATATAAGAAAGATGCTGATTCCAGGGG-3′), Env N325D (5′-CAATGTGAATTGTACAAGACCCAACAACAATATAAGAAAGATGCTGATTCCAGGGG-3′), Env T303I/S306R (5′-CAATGTGAATTGTACAAGACCCAACAACAATATAAGAAAGATGCTGATTCCAGGGG-3′), Env N325D (5′-CAATGTGAATTGTACAAGACCCAACAACAATATAAGAAAGATGCTGATTCCAGGGG-3′). The PCR procedures were performed using PrimeSTAR MAX DNA polymerase according to the manufacturer’s instructions (Takara). The amplified DNA was digested with EcoRI and Nhel, and cloned into EcoRI and Nhel sites of pNL4-3. The double-point mutants were constructed in the same way as the single-point mutants. The S306R single-point mutant was used as a template for the double-point mutants.

Drug susceptibility assay. Anti-HIV-1 activity was determined by measurement of the protection against HIV-1-induced cytopathogenicity in PM1/CCR5 cells. Briefly, 1.6 × 10<sup>5</sup> PM1/CCR5 cells were infected with virus at 37 °C for 2 h. The inoculum was adjusted to the equivalent infectivity level of NL4-3 WT in TZM-bl cells (20 ng p24 antigen). The infected cells were plated in 96-well plates at 2.0 × 10<sup>4</sup> cells per well in a volume of 100 µl. Serially diluted test compounds (KRH-3955, AMD3100, AMD070 and T140) were added to HIV-1-infected PM1/CCR5 and incubated at 37 °C in 5% CO<sub>2</sub>. KRH-3955 was assayed at twofold dilutions ranging from 25 to 0.2 nM. The other compounds were assayed at twofold dilutions ranging from 1000 to 31 nM. Five days after infection, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega). IC<sub>50</sub> was defined as the concentration corresponding to 50% protection from HIV-1-induced cytopathogenicity in PM1/CCR5 cells. Fold differences were calculated based on the IC<sub>50</sub> values of WT and each chimeric clone.

Neutralization assay. TZM-bl cells were plated in 96-well plates at 2.0 × 10<sup>4</sup> cells per well in a volume of 100 µl 1 day before infection. Test viruses were mixed with serially diluted NAbs in a volume of 100 µl and incubated at 37 °C for 40 min, followed by inoculation of TZM-bl cells with the virus–NAb mixture. b12 was diluted at fivefold dilutions ranging from 1 to 0.02 µg ml<sup>−1</sup>. 447-52D was assayed at fivefold dilutions ranging from 20 to 0.03 µg ml<sup>−1</sup> for WT and from 10 to 0.02 µg ml<sup>−1</sup> for the other viruses. The other NAbs were assayed at fivefold dilutions ranging from 10 to 0.02 µg ml<sup>−1</sup>. Virus doses were adjusted to the equivalent infectivity levels of WT (NL4-3) containing 5 ng of p24 antigens in TZM-bl. One day after infection, luciferase activity was measured using the Steady-Glo Luciferase assay system as described above. The IC<sub>50</sub> was defined as the NAb concentration that yielded a 50% reduction in luciferase activity compared with the control wells after subtracting background signal. Fold differences were calculated based on IC<sub>50</sub> values of WT and each chimeric clone.

Molecular dynamics (MD) simulation of HIV-1 gp120 outer domain. HIV-1 gp120 outer domain structures with various V3 mutations were constructed by the homology modelling method with Molecular Operating Environment (MOE) (Chemical Computing Group) as described previously (Naganawa et al., 2008; Yokoyama et al., 2012; Yuan et al., 2013). The crystal structure of HIV-1 gp120 containing the entire V3 region at a resolution of 3.30 Å (1 Å = 0.1 nm) (PDB code: 2QAD) (Huang et al., 2005) was used as the template structure. MD simulations were performed to analyse changes in structural dynamics of protein interaction surface in solution as described previously (Naganawa et al., 2008; Yokoyama et al., 2012, 2016; Yuan et al., 2013; Kuwata et al., 2013). The simulations were done by the pmdem module in the AMBER 11 program package (Case et al., 2010) with the AMBER ff99SB-ILDN force field (Lindorff-Larsen et al., 2010), and the TIP3P water model for simulations of aqueous solutions (Jorgensen et al., 1983). A non-bonded cut-off of 10 Å was used. Bond lengths involving hydrogen were constrained with SHAKE, a constraint algorithm to satisfy a Newtonian motion (Ryckaert et al., 1977), and the time for all MD simulations was set to 2 fs. After heating calculations for 20 ps until 310 K using the NVT ensemble, simulations were executed using the NPT ensemble at 1 atm, at 310 K and in 150 mM NaCl for 50 ns.

Calculation of root mean square fluctuation (RMSF). RMSF was calculated as previously described (Naganawa et al., 2008; Yokoyama et al., 2012, 2016; Yuan et al., 2013; Kuwata et al., 2013) to quantify structural dynamics of molecules in the MD simulations. RMSFs of the Ca atoms were calculated to obtain information on atomic fluctuations of individual amino acid residues during MD simulations (Case et al., 2010). The 12 500 snapshots obtained from MD simulations of 25–50 ns were used to calculate the RMSF. The average structures were used as a reference for the RMSF calculation. The RMSF, which quantifies the differences between the average values and those obtained at given times of
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