proteins fused to the fragments. In our present study, by using BiFC, homo-oligomeric forms of CCR5 were detected to some extent without natural ligands, and further enhanced by CCR5 antagonists. In addition, susceptibility of sorted CCR5 oligomers-enriched cell fraction was found to be less susceptible compared to monomer-enriched fraction, indicating the preferential recognition of CCR5 monomer by R5 HIV-1.

Results

Detection of oligomeric forms of CCR5 without ligands

It has been shown that CCR5 exists as homo-oligomer without natural ligands such as CCL3, CCL4, and CCL5 (Benkirane et al., 1997; El-Asmar et al., 2005; Hammad et al., 2010; Issafras et al., 2002; Mellado et al., 2001; Sohy et al., 2009). However, it still remains to be determined whether oligomeric forms of CCR5 are structurally different from CCR5 monomer. To this end, BiFC assay was employed to detect oligomeric forms of CCR5. The CCR5 expression vectors fused to the N- and C-terminal fragments of green fluorescence protein (Kusabira-Green: KG) were constructed, and co-expressed in 293T cells. When the both proteins are expressed and close together, refolded KG protein results in KG signal. This fluorescent signal can be easily detected by flow cytometry or fluorescence microscopy. To analyze the structural differences between monomeric and oligomeric forms of CCR5, the cells were further stained with anti-CCR5 monoclonal antibodies (mAbs) recognizing different epitopes of CCR5 such as N-terminal (clones CTC8, 3A9), second extracellular domain (ECL2) (clones 2D7, 45531), or multiple conformation (clone 45549). As shown in the upper panel of Fig. 1, we were able to detect fluorescent (KG) signal using flow cytometry when both CCR5-KGN and CCR5-KGC were co-expressed in 293T cells, indicating the oligomerization of CCR5 without ligands. We also noticed that proportions of CCR5+KG+ subset were almost equal (24–26%) in all anti-CCR5 mAb clones (CTC8, 3A9, 2D7, and 45531) except the clone 45549. In contrast, CCR5+KG− subset was differentially stained by anti-CCR5 mAbs (Fig. 1, upper panel). The proportions of CCR5+KG− subset were high in the clones 2D7 and CTC8 (62% and 55%, respectively), intermediate in the clone 45531 (43%), and low in the clone 3A9 (26%). These results suggested that monomeric forms of CCR5 were structurally different from the oligomeric forms.

Enhancement of CCR5 oligomerization by CCR5 antagonists

Although natural ligands such as CCL3, CCL4, and CCL5 have been showing to induce oligomerization of CCR5 (Chelli and Alizon, 2002; Hernanz-Falcon et al., 2004; Rodriguez-Frade et al., 1999; Vila-Coro et al., 2000), it has not been determined how CCR5 antagonists such as TAK-779 or MVC affect the oligomerization of CCR5. Therefore, we also applied BiFC technique to check the effects of CCR5 antagonists on the oligomerization status of CCR5. After co-expressing CCR5-KGN and -KGC in 293T cells in the presence of MVC, the cells were stained with above-mentioned anti-CCR5 mAbs. The proportions of CCR5+KG+ subset were largely increased in all anti-CCR5 mAb clones (Fig. 1, lower panel) compared to those of the same fraction in the absence of ligands (Fig. 1, upper panel). Notably, the proportion of CCR5+KG+ subset was increased in the clone 45549 though its reactivity was quite low in the absence of ligands, confirming that conformational changes of CCR5 were indeed induced by MVC. To verify the enhancement of CCR5 oligomerization by CCR5 antagonists, we then checked whether another CCR5 antagonist TAK-779 enhanced oligomerization of CCR5. The CCR5-KG-expressing 293T cells were stained with 2D7 mAb that was able to equally detect both CCR5+KG− and CCR5+KG+ subsets as shown in Fig. 1, and KG-positive percentages in 2D7-positive population were determined by flow cytometry. We found that TAK-779 also enhanced the oligomerization of CCR5, while a CXCR4 antagonist AMD3100 had no effect (Fig. 2A). In particular, MVC had higher activity to enhance CCR5 oligomerization than TAK-779 in 293T cells. Western blot analysis using 293T cells expressing FLAG-tagged CCR5 with cross-linker indicated that CCR5 largely existed as monomer but also as dimer in the absence of ligands though lesser extent (Fig. 2B). It was also shown that MVC was able to induce expression of not only dimer but also more than dimer forms of CCR5. Notably, the level of CCR5 expression was up-regulated by MVC though the reason was uncertain. Native-PAGE analyses also revealed the similar results (Supplementary Fig. S1). The enhancement by CCR5 antagonists was also observed in different cell types such as HeLa, and NP2 cell lines though both had comparable activities in these cell lines (Supplementary Fig. S2). Dose-escalating study revealed that the concentrations

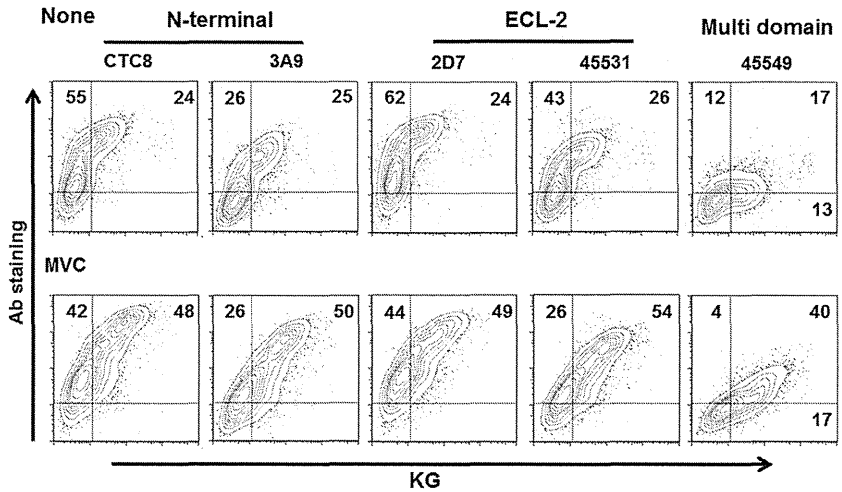

Fig. 1. Flow cytometry analyses of CCR5-KG-expressing 293T cells by anti-CCR5 mAbs in the presence or absence of MVC. The 293T cells were transfected with both CCR5-KG expression vectors, and incubated at 37 °C for 48 h in the absence of ligands (upper panel) or the presence of MVC at 2 μM (lower panel). Anti-CCR5 mAbs recognizing N-terminus (clones CTC8 and 3A9), ECL-2 (clone 2D7, 45531), and multiple domains (clone 45549) were used for the detection of CCR5, and analyzed by flow cytometry. The *y*-, and *x*-axes show the mean fluorescence intensity of CCR5 and KG, respectively. The number of each column shows the percent positive in each region.

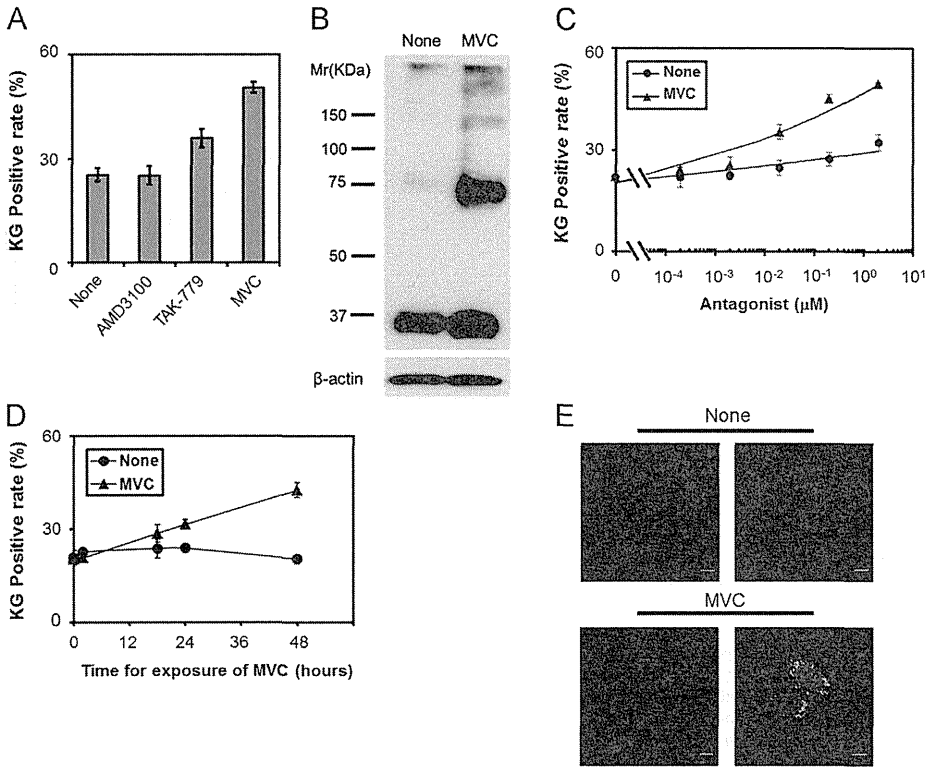

Fig. 2. Enhanced homo-oligomerization of CCR5 by CCR5 antagonists. (A) The 293T cells were transfected with expression vectors of CCR5-KG, and incubated in the presence or absence of AMD3100, TAK-779 or MVC at 2 μM each. The cells were stained with anti-CCR5 mAb 2D7, and analyzed by flow cytometry. The data shown represent the mean values of the percentage of KG-positive in 2D7-positive cell fraction ± standard deviations of three independent experiments. (B) The 293T cells were transfected with pCCR5-FLAG in the presence or absence of MVC. The cells were cross-linked by DSP, lysed, and analyzed by Western blot using anti-FLAG mAb. (C) Transfected cells were treated with increasing concentrations of TAK-779 or MVC ranging from 0.2 nM to 2 μM, and incubated at 37 °C for 48 h. The data shown represent the mean values of percent positive of KG ± standard deviations of three independent experiments. (D) The 293T cells expressing CCR5-KG were incubated at 37 °C for the indicated time of period in the presence of MVC (1 μM). Results are mean values of CCR5-KG positive rates ± standard deviations from experiments performed in triplicate. (E) Transfected HeLa cells with CCR5-KG were incubated in the absence (upper panel) or presence of MVC (lower panels) at 37 °C for 48 h, and fixed with 4% paraformaldehyde. Representative images in the middle sections of the cells are shown. Nuclear staining by DAPI is shown in blue. Scale bars correspond to 10 μm.

for enhanced oligomerization of CCR5 by TAK-779 or MVC were indeed corresponded to the inhibitory concentrations against HIV-1 infection (Fig. 2C). For example, in the case of MVC, the EC_{50} value of inhibitory activity against R5 HIV-1 (JR-FL) was 3.7 ± 1.4 nM (data not shown), while the EC_{50} value of activity to enhance oligomerization of CCR5 was 7.4 ± 5.1 nM, indicating that CCR5 could be oligomerized at enough concentrations for inhibiting R5 HIV-1 infection.

It has been shown that oligomerization of CCR5 was induced shortly after the addition of natural ligands as previously described (Chelli and Alizon, 2002; Hernanz-Falcon et al., 2004; Rodriguez-Frade et al., 1999; Vila-Coro et al., 2000). However, a time-course experiment showed that more than 24 h were necessary to enhance CCR5 oligomerization by MVC (Fig. 2D). Confocal laser scanning microscopy also showed that the CCR5-KG signals were located not only at the plasma membrane but also in the cytoplasm without ligands, and were further augmented by MVC (Fig. 2E). These results suggested that oligomerization of CCR5 needed de novo synthesis of CCR5 and occurred in the intracellular compartments before expressed on the cell surface.

Infection of KG-positive and -negative cell fractions with R5 HIV-1

Since the structures of oligomeric forms of CCR5 were possibly different from monomeric CCR5 as shown in Fig. 1, we next analyzed the abilities of R5 HIV-1 to recognize monomeric and oligomeric forms of CCR5. To this end, we first stained CD4-positive 293T cells expressing CCR5-KGN and -KGC with anti-CCR5 mAb CTC8, which was able to recognize both monomeric and oligomeric forms of CCR5, and had no neutralizing activity against CCR5-using HIV-1 (data not shown). The KG-positive and -negative subsets having the same CCR5 fluorescent intensity were then collected by fluorescent-activated cell sorter. The mean fluorescence intensities of KG in KG-positive and -negative cell fractions after sorting were 30.2 and 4.1, respectively, while mean fluorescence intensities of CCR5 were comparable (72.3 and 61.1 respectively) (Fig. 3A). The mean fluorescence intensity of CD4 was also confirmed to be comparable in KG-positive and -negative cell fractions (183 and 178, respectively). The sorted each cell fraction was then infected with HIV-1 pseudotyped with various strains of R5 Envs including JR-FL, YU-2, and Ba-L. Since the transfection of CD4-293T cells with KG-expressing vectors was possible to influence the cell condition, each cell fraction was also infected with HIV-1 pseudotyped with vesicular stomatitis virus G protein (VSV-G), which utilizes the ubiquitously expressing molecule(s) although the receptor for VSV-G remains to be confirmed (Coil and Miller, 2004; Schlegel et al., 1983). To normalize the entry efficiency of R5 HIV-1 in each fraction, we divided luciferase activities infected with R5 pseudotyped HIV-1 by those infected with VSV-G pseudotyped HIV-1. The susceptibility of CCR5+KG+ subset to R5 HIV-1 was then compared with that of CCR5+KG− subset. Although each single cell of CCR5+KG− or CCR5+KG+ subset was supposed to have oligomeric and monomeric forms of CCR5 to some extent, respectively, we found that entry efficiencies of R5 HIV-1 in CCR5+KG+ subset were always lower than those in

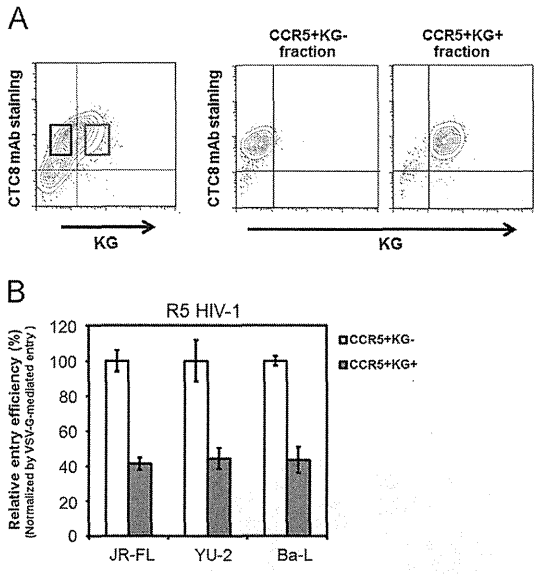

Fig. 3. Sorting of CCR5+KG− and CCR5+KG+ fractions and infection with luciferase-reporter HIV-1 pseudotyped with R5 Envs. (A) CD4-positive 293T cells expressing CCR5-KG were stained with anti-CCR5 mAb CTC8 (shown in the left panel). Representative data of flow cytometric analysis is shown. The KG− (shown in black rectangle) and KG+ (shown in gray rectangle) fractions with the same mean fluorescence intensities of CCR5 were gated, and sorted by fluorescent-activated cell sorter. Each sorted fraction was analyzed using flow cytometry (shown in the right panel). (B) Each sorted fraction was infected with luciferase-reporter HIV-1 pseudotyped with R5 Envs or VSV-G, and luciferase activities of infected cells were determined 24 h post-infection. Entry efficiency of each R5 Env in each fraction was normalized by that of VSV-G. Relative entry efficiency of CCR5+KG+ fraction (shown in gray bar) was expressed as the percentage of that of CCR5+KG− fraction (shown in white bar). The data are expressed as means ± standard deviations in triplicate experiments.

CCR5+KG− subset (Fig. 3B). These results indicated that R5 Envs preferentially recognized monomeric forms of CCR5 rather than its oligomeric forms.

Infection of KG-positive and -negative cell fractions with R5X4 HIV-1

We next checked the susceptibilities of CCR5+KG− and CCR5+KG+ subsets to another CCR5-using HIV-1, R5X4. As we mentioned earlier, there were several phenotypes in the strains of R5X4 HIV-1 such as dual-R5 and dual-X4 (Symons et al., 2011; Toma et al., 2010). We then selected 89.6 as dual-X4, KMT, TIK, and 89.6R308S as dual-R5 as previously described (Maeda et al., 2008). Similar to R5 HIV-1, dual-R5 preferentially infected CCR5+KG− fraction compared to CCR5+KG+ fraction (Fig. 4). Notably, single mutation in 11th position of the V3 loop in 89.6 (89.6R308S), which changed viral phenotype from dual-X4 to dual-R5 (Maeda et al., 2008), also significantly infected CCR5+KG− fraction than CCR5+KG+ fraction. In contrast, wild type 89.6 (dual-X4) comparably infected both CCR5+KG− and CCR5+KG+ fractions. These results indicated that dual-R5 but not dual-X4 HIV-1 also preferentially recognized monomeric CCR5 for the entry.

Infection of CCR5-KG-positive and -negative cell fractions with MVC-resistant HIV-1

Since the CCR5 antagonist MVC strongly enhanced CCR5 oligomerization in 293T cells as shown in Figs. 1 and 2, MVC-resistant HIV-1 seemed to evolve to use oligomeric forms of CCR5 for the entry. In general, MVC-resistant HIV-1s were shown to recognize MVC-bound form of CCR5 to reduce sensitivity to MVC as previously described by others and us (Kuhmann et al., 2004; Maeda et al., 2011; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007; Yuan et al., 2011). We therefore sought to infect CCR5+KG− and CCR5+KG+ fractions with MVC-resistant HIV-1 in the absence or presence of 2 μM MVC, respectively. Similar to general R5 HIV-1s, MVC-resistant HIV-1 also preferentially infected CCR5+KG− fraction in both the absence and presence of MVC compared with CCR5+KG+ fraction (Fig. 5A). We further infected both fractions at various concentrations of MVC ranging from 100 nM to 10 μM in order to check whether MVC-resistant HIV-1 recognizes MVC-bound forms of CCR5. Both fractions were also infected with MVC-sensitive HIV-1 carrying JR-FL Env to check whether sensitivity of general R5 HIV-1 to MVC is different between them. We found that MVC-sensitive HIV-1 had reduced sensitivity to MVC in CCR5+KG− fraction compared with CCR5+KG+ fraction (Fig. 5B), supporting the preferential recognition of CCR5 monomer by CCR5-using Env. We further observed reduced maximal inhibition of MVC-resistant HIV-1 in CCR5+KG− fraction compared with CCR5+KG+ fraction (Fig. 5B). These results indicated that MVC-resistant HIV-1 was likely to use MVC-bound forms of CCR5 monomer though MVC augmented CCR5 oligomerization.

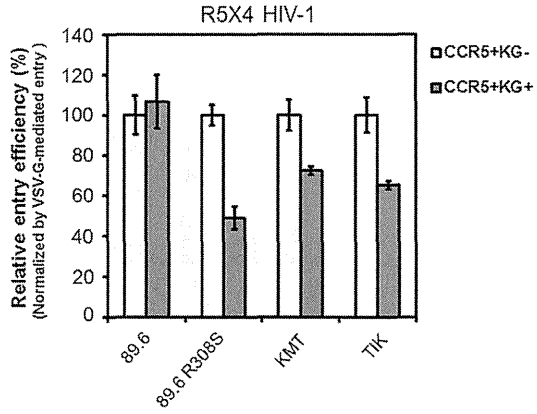

Fig. 4. Infection of CCR5+KG− and CCR5+KG+ fractions with luciferase-reporter HIV-1 pseudotyped with R5X4 Envs. Sorted CCR5+KG− and CCR5+KG+ fractions were infected with luciferase-reporter HIV-1 pseudotyped with R5X4 Envs or VSV-G. The luciferase activities of infected cells were determined 24 h post-infection. Entry efficiency of each R5X4 Env in each fraction was normalized by that of VSV-G. Relative entry efficiency of CCR5+KG+ fraction (shown in gray bar) was expressed as the percentage of that of CCR5+KG− fraction (shown in white bar). The data are expressed as means ± standard deviations in triplicate experiments.

Discussion

HIV-1 coreceptors CCR5 and CXCR4 are members of the seven transmembrane (7-TM) G protein-coupled receptors (GPCRs) superfamily. Recent data have shown that many GPCRs including chemokine receptors function as dimers or higher-order oligomers. To assess the formation of dimerization/oligomerization of GPCRs, fluorescent- or bioluminescent-based techniques have been applied such as BiFC, fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) assay (reviewed in (Vidi et al., 2011)). In the case of BiFC, the expression vectors for BiFC are generally comprised of two fragments of non-functional fluorescent protein split by N- and C-terminus (KGN and KGC of Kusabira–Green: KG in our case). When GPCRs fused to KGN and KGC are brought in close proximity, fluorescent signal can be detected by refolding of the fluorescent protein, KG. It should be noted that KG-signal could be only detected when KGN and KGC are brought together but not the same pairs such as KGN–KGN or KGC–KGC. Nonetheless, we were able to show KG-positive cells in both CCR5-KGN and CCR5-KGC expressing cells

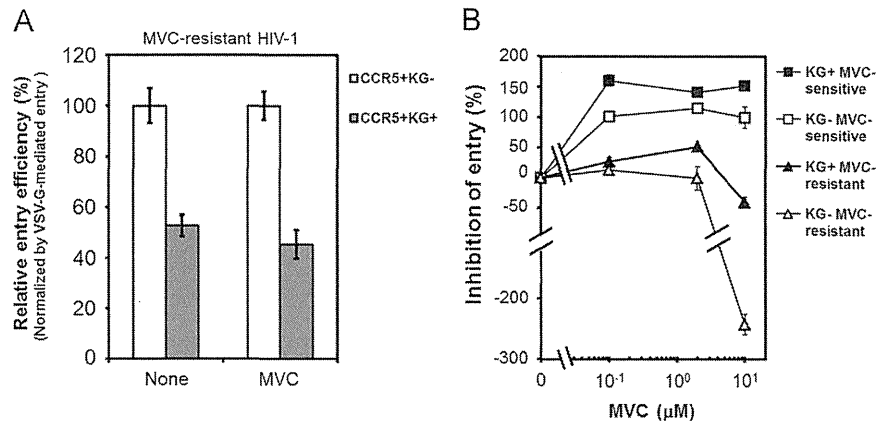

Fig. 5. Infection of CCR5+KG− and CCR5+KG+ fractions with luciferase-reporter HIV-1 pseudotyped with JR-FL and MVC-resistant Envs. (A) Sorted CCR5+KG− and CCR5+KG+ fractions were infected with luciferase-reporter HIV-1 pseudotyped with MVC-resistant Env or VSV-G in the absence or presence of 2 μM MVC. The luciferase activities of infected cells were determined 24 h post-infection. Entry efficiencies of MVC-resistant Env in CCR5+KG− and CCR5+KG+ fractions in the presence or absence of MVC were normalized by those of VSV-G, respectively. Relative entry efficiency of CCR5+KG+ fraction (shown in gray bar) was expressed as the percentage of that of CCR5+KG− fraction (shown in white bar). (B) Percentages of inhibition of MVC-sensitive (JR-FL) and MVC-resistant HIV-1s are expressed as relative values, with that of MVC-sensitive HIV-1 in CCR5+KG− fractions at 10 μM MVC being 100%. The data are expressed as means ± standard deviations in triplicate experiments.

(Fig. 1) without ligands, which were further increased by the CCR5 antagonists but not by the CXCR4 antagonist (Figs. 1 and 2). These results indicated that CCR5 was able to form dimer/oligomers. It is well known that CXCR4 exists as constitutive higher order oligomers without natural ligands (Supplementary Fig. S1) (Babcock et al., 2003; Hamatake et al., 2009; Issafras et al., 2002; Percherancier et al., 2005; Toth et al., 2004; Wu et al., 2010). CCR5 could also exist as dimer or higher order oligomers as recently described (Babcock et al., 2003; Benkirane et al., 1997; Issafras et al., 2002), although to a lesser extent than CXCR4. On the present study, we further showed not only the existence of CCR5 monomer/dimer forms without its ligands but also the enhanced oligomerization by the antagonists (Fig. 2 and Fig. S1). It has been shown that natural ligands for CCR5 such as CCL5 (RANTES) or CCL4 (MIP-1β) have been shown to induce homo-oligomerization of CCR5 (Chelli and Alizon, 2002; Hernanz-Falcon et al., 2004; Rodriguez-Frade et al., 1999; Vila-Coro et al., 2000), though its physiological role remains to be determined. Similarly, CXCR4's natural ligand SDF-1 also induced homo-dimerization of CXCR4 as previously described (Percherancier et al., 2005; Toth et al., 2004; Vila-Coro et al., 1999). In contrast, oligomerization of chemokine receptors by their antagonists has not been described to date though another GPCR melatonin receptor was reported to form dimer by both agonists and antagonists (Ayoub et al., 2002). The dimerization of melatonin receptor by both agonist and antagonists was explained by the stabilization of its conformations. Interestingly, in the presence of MVC, CCR5+KG+ subset became well detected by the anti-CCR5 mAb recognizing the conformational epitope (clone 45549) (Fig. 1), indicating that conformations of CCR5 induced by MVC might be also structurally stable. In our flow cytometric analyses without addition of ligands, CCR5+KG+ subsets were equally detected by most anti-CCR5 mAbs except the clone 45549 (Fig. 1). Given that several antigenic conformations of CCR5 existed on the cell surface (Berro et al., 2011; Lee et al., 1999), oligomer forms of CCR5 might have the similar antigenic conformations while monomeric forms had different antigenic conformations.

Previous reports have shown that several GPCRs were homo- or hetero-oligomerized in endoplasmic reticulum (ER) (Herrick-Davis et al., 2006; Issafras et al., 2002; Milligan, 2010; Salahpour et al., 2004; Vischer et al., 2011). In our BiFC assay using confocal laser scanning microscopy, CCR5-KG signals were also detected not only at plasma membrane but also in intracellular compartments, both of which were further enhanced by the addition of MVC (Fig. 2E). Time-course experiments also showed that more than 24 h were needed for the enhanced oligomerization of CCR5 by MVC (Fig. 2D). These findings suggested that oligomerization of CCR5 were formed during early biosynthesis and protein maturation in the ER, and that MVC may further enhance CCR5 oligomerization by the binding of intracellular CCR5. Thus, it is possible that MVC could penetrate into the cell membrane and act before the expression of CCR5 on the cell surface. It is of note that the concentration to induce oligomerization of CCR5 was sufficiently low similar to the concentration that is able to inhibit R5 HIV-1 replication (Fig. 2C), indicating the concentrations of MVC, which would be achieved in HIV-1-infected individuals treated with MVC, seems to induce oligomerization of CCR5 to some extent *in vivo*, although the pharmacological and pathological roles of MVC-induced CCR5 oligomerization in primary T cells and macrophages still remains to be determined.

As we mentioned above, it is possible that CCR5 monomer may have multiple forms, whereas the oligomers may have relatively fixed forms. Since R5 HIV-1 is supposed to recognize specific forms of CCR5 (Berro et al., 2011, 2013), we attempted to check which form of CCR5, monomer or oligomer, is used by R5 HIV-1. To this end, CCR5+KG− and CCR5+KG+ subsets expressed in CD4-positive 293T cells were fractionated by fluorescent-activated cell sorter, and infected with pseudotyped R5 HIV-1. The CCR5+KG− and CCR5+KG+ subsets could have relatively lower and higher amount of oligomeric forms, respectively, while both CCR5+KG− and CCR5+KG+ subsets are supposed to have monomeric and oligomeric forms of CCR5 in flow cytometry analysis. Nevertheless, we were able to show that CCR5+KG+fraction was less susceptible to R5 and dual-R5 HIV-1 than CCR5+KG− fraction (Fig. 3 and 4). It is thus likely that R5 and dual-R5 Envs preferentially recognized monomeric forms of CCR5. The dimerization induced by the monoclonal antibody CCR5-02 was previously reported to cause blocking of HIV-1 entry (Vila-Coro et al., 2000). Our present study further clarified that the oligomerization of CCR5 without ligands also affected the susceptibility to R5 and dual-R5 HIV-1.

Although the susceptibility of KG-negative cell fraction to R5 and dual-R5 HIV-1 was significantly high, dual-X4 HIV-1 89.6 equally infected both CCR5+KG− and CCR5+KG+ fraction (Fig. 4). It is therefore possible that dual-X4 lost the preferential recognition of monomeric forms of CCR5, and may commence recognizing homo-oligomeric forms of CCR5. Intriguingly, the single mutation in 89.6 from arginine to serine at 11th position of the V3 loop (89.6R308S), which changed the tropism from dual-X4 to dual-R5 (Maeda et al.,

2008), also reverted to recognize monomeric forms of CCR5. Hence, the single amino acid substitution was sufficient to lose preferential recognition of monomer forms of CCR5 for CCR5-using HIV-1.

As described by others and us, MVC-resistant HIV-1 recognized MVC-bound and -unbound forms of CCR5 (Kuhmann et al., 2004; Maeda et al., 2011; Pugach et al., 2007; Trkola et al., 2002; Westby et al., 2007; Yuan et al., 2011). Since MVC was found to enhance CCR5 oligomerization in our present study, we then sought to check whether MVC-resistant HIV-1 recognizes MVC-bound forms of CCR5 oligomers. However, similar to R5 HIV-1, we found that MVC-resistant HIV-1 recognized both MVC-bound and -unbound forms of CCR5 monomer (Fig. 5). Since numbers of MVC-bound forms of CCR5 monomer would be dependent on the surface expression levels of CCR5 and cell types, our findings may partly explain why susceptibility to CCR5 antagonists was dependent on the cell types as previously described (Berro et al., 2011). Taken together, it is likely that R5 HIV-1 including MVC-resistant HIV-1 constrained to use monomeric forms of CCR5 for the entry.

In conclusion, we were able to show that oligomeric forms of CCR5 were less susceptible to R5 HIV-1 than the monomeric forms. However, our findings were obtained from the cells expressing high levels of CCR5 *in vitro*. Therefore, it is quite important to understand the role of CCR5 oligomerization in primary T cells and macrophages for HIV-1 entry *in vivo*. The methods to detect native forms of homo- and hetero-oligomerized CCR5 and their susceptibilities to HIV-1 in primary cells should be established and analyzed to elucidate the role of CCR5 oligomerization for HIV-1 infection *in vivo*.

Materials and methods

Cells and culture conditions

The 293T and HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 U/ml of penicillin and 100 μg/ml of streptomycin. A human CD4-expressing glioma cell line (NP2/CD4) was maintained in Eagle's minimum essential medium (MEM; Gibco BRL) supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Jinno et al., 1998).

Coreceptor antagonists

A CXCR4 antagonist AMD3100 (Schols et al., 1997a, 1997b) and a CCR5 antagonist maraviroc (MVC) (Dorr et al., 2005) were supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. A CCR5 antagonist TAK-779 (Baba et al., 1999) was kindly obtained from Takeda Chemical Industries (Osaka, Japan).

Construction of retrovirus vector and transduction of 293T cells with the CD4 gene

The cDNA encoding human CD4 was obtained by PCR using human lymphocyte cDNA as the template. The primers used were as follows: 5′-CTCGAGTCGCCACCATGAACCGGGGAGTCCCTTTTAGC-3′ and 5′-TCAAATGGGGCTACATGTCTTCTGAAACCG-3′ (underlined are *Xho*I site). The amplified product was cloned into pCR-TOPO (Invitrogen), and the sequence was verified using 3130 Genetic Analyzer (Applied Biosystems). A CCR5 carrying *Xho*I-*Eco*RI fragments was ligated into pMSCVpuro (Clothech) to generate pMSCVpuro-CD4. Retrovirus vector was produced according to the manufacturer's instructions, and 293T cells were then transduced and selected by puromycin (Sigma-Aldrich). The CD4 expression of the transduced 293T cells was verified by anti-CD4 monoclonal antibody (RPA-T4, eBioscience).

Expression vectors

CCR5 expression vectors for BiFC was constructed using phmKGN-MN and phmKGC-MN (MBL, Japan) according to the manufacture's instructions. Briefly, human CCR5 gene was amplified using pCR2-CCR5 as a template (Maeda et al., 2000). Primers used were: 5′-CTCGAGGAACAAGATGGATTATCAAGTG-3′ and 5′-GTCTAGATTACT TGTCGTCATCGTCTTTGTAGTCCAAGCCCACAGATA-3′ (underlined are *Xho*I and *Xba*I restriction enzyme sites, respectively). The amplified product was cloned into pCR-TOPO, and the sequence was verified using 3130 Genetic Analyzer. The *Xho*I-*Xba*I fragment carrying CCR5 gene was then ligated into both phmKGN-MN and phmKGC-MN using *Xho*I and *Xba*I sites to generate pCCR5-KGN and pCCR5-KGC respectively. To construct an expression vector of FLAG-tagged CCR5, CCR5 sequence was amplified using primer: 5′-CTCGAGGAACAAGATGGAT-TATCAAGTG-3′ and 5′-GTCTAGATTACTTGTCGTCATCGTCTTTGTAGTC-CAAGCCCACAGATAT-3′ (underlined are the *Xho*I and *Xba*I restriction enzyme sites, respectively). The amplified product was cloned into pCR-TOPO, and the sequence was then verified using a 3130 Genetic Analyzer. The amplified fragment was finally ligated into phmKGC-MN expression vector (In this vector, split fluorescence protein, Kusabira–Green: KG, was replaced with FLAG-tag by digestion of the *Xho*I and *Xba*I restriction enzyme sites). Expression vectors for JR-FL, 89.6, 89.6R308S, KMT and TIK Envs were prepared as previously described (Maeda et al., 2000, 2008). Expression vectors for Ba-L and YU-2 Envs were kindly supplied by K. Yoshimura (National Institute of Infectious diseases, Tokyo). An expression vector for MVC-resistant Env was prepared as previously described (Yuan et al., 2011, 2013).

Production of recombinant luciferase-reporter virus

Recombinant luciferase-reporter virus of pseudotyped with various HIV-1 Envs or VSV-G were produced by transfection of 293T cells using the calcium phosphate method (ProFection Mammalian Transfection System, Promega) as previously described (Maeda et al., 2000, 2008). The cells culture supernatant was collected 48 h post-transfection, filtered with 0.45 μm pore-size, and stocked at −80 °C until use. The p24 Gag in the culture supernatant was measured using HIV-1 p24 Ag ELISA kit (Zeptometrix) according to manufacture's instructions.

Detection of the CCR5 expression in KG-positive and -negative cell population

The 293T cells were transfected with CCR5-KG expression vectors, pCCR5-KGN and pCCR5-KGC, using calcium phosphate method, and incubated for 48 h or indicated time of period at 37 °C in the presence or absence of 2 μM of AMD3100, TAK-779 or MVC. In a dose-escalating study, transfected cells were treated with various concentrations of MVC (ranging from 0.0002 μM to 2 μM) for 48 h. To detect the CCR5 in CCR5-KG-transfected cells, the cells were first incubated with anti-CCR5 mAbs, 3A9, CTC8, 45531, 45549 (R&D Systems), or 2D7 (BD Pharmingen) for 30 min at 4 °C. The cells were then stained with β-phycoerythrin-conjugated anti-mouse IgG antibody (Jackson Immuno Research). For direct detection of the CCR5 expression in CCR5-KG-transfected cells, the cells were stained with anti-human CCR5 mAb 2D7 conjugated with Alexa Fluor 647 (BioLegend) for 30 min at 4 °C. The cells were analyzed by FACScan or FACSCalibur fluorescent-activated cell sorter (Becton Dickinson).

Detection of monomeric and oligomeric forms of CCR5 by Western blot

The transfected 293T cells with pCCR5-FLAG expression vector were incubated at 37 °C for 48 h with or without MVC. The cells were treated with DSP (dithiobis[succinimidylpropionate]) cross-linker according to the manufacture's instructions (Thermo Scientific), and solubilized using 1% Brij O10 (Sigma-Aldrich) lysis buffer (1% Brij O10, 20 mM Tris–HCl pH 8.2, 0.15 M NaCl, 5 mM iodoacetamide) including protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were then separated by SDS-PAGE, blotted onto PVDF (polyvinylidene fluoride, Immobilon-P, Millipore) membrane. The membranes were incubated with anti-FLAG mAb (Wako) or anti-β-actin mAb (Sigma-Aldrich) for 90 min, followed by staining with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson Immuno Research). The signals were detected using Chemi-Lumi One (Nacalai Tesque).

Confocal laser scanning microscopy

The HeLa cells were plated to collagen (Atelo Cell)-coated 8-well glass slides (Lab-Tek). The cells were transfected with both pCCR5-KGN and pCCR5-KGC using Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. Transfected cells were incubated at 37 °C for 48 h in the presence or absence of 1 μM MVC. The cells were fixed with 4% paraformaldehyde (Wako) for 15 min, and analyzed using LSM-700-ZEN confocal laser scanning microscopy (Carl Zeiss) with a 60X objective lens. The images were processed using LSM Imaging Browser (Carl Zeiss).

Fluorescence-activated cell sorting of CCR5-KG-positive and -negative cell fraction and infection with pseudotyped HIV-1

The CD4-293T cells were transfected with pCCR5-KGN and pCCR5-KGC using calcium phosphate method. After 48 h cultures at 37 °C, cells were stained with anti-CCR5 mAb CTC8, followed by staining with APC-conjugated anti-mouse IgG. The cells were then sorted into CCR5+KG− and CCR5+KG+ fractions with the same expression levels of CCR5 by using FACS AriaII (Becton Dickinson) according to the manufacture's instructions. Sorted each fraction was then incubated with the same amount (40 ng of p24Ag) of luciferase-reporter HIV-1 pseudotyped with various HIV-1 Envs including R5 (JR-FL, YU-2, Ba-L), R5X4 (89.6 wt, 89.6 R308S (Maeda et al., 2008), KMT, and TIK), MVC-resistant Env (T199K/T275M/V3-M5) (Yuan et al., 2011, 2013) or VSV-G at 37 °C for 30 min to allow adsorption of the virus. The cells were washed to remove unadsorbed virus, seeded into a 96-well plate, and cultured at 37 °C for 24 h. Luciferase activity was measured using a luminometer, Lumat LB 9501/16 (EG&G Berthold, Bad Wildbad). The entry efficiency of HIV-1 infected by HIV-1 Envs in each cell fraction was normalized by the luciferase activity of the same fraction infected by VSV-G.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.12.034.

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PLOS ONE

V3-Independent Competitive Resistance of a Dual-X4 HIV-1 to the CXCR4 Inhibitor AMD3100

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Abstract

A CXCR4 inhibitor-resistant HIV-1 was isolated from a dual-X4 HIV-1 *in vitro*. The resistant variant displayed competitive resistance to the CXCR4 inhibitor AMD3100, indicating that the resistant variant had a higher affinity for CXCR4 than that of the wild-type HIV-1. Amino acid sequence analyses revealed that the resistant variant harbored amino acid substitutions in the V2, C2, and C4 regions, but no remarkable changes in the V3 loop. Site-directed mutagenesis confirmed that the changes in the C2 and C4 regions were principally involved in the reduced sensitivity to AMD3100. Furthermore, the change in the C4 region was associated with increased sensitivity to soluble CD4, and profoundly enhanced the entry efficiency of the virus. Therefore, it is likely that the resistant variant acquired the higher affinity for CD4/CXCR4 by the changes in non-V3 regions. Taken together, a CXCR4 inhibitor-resistant HIV-1 can evolve using a non-V3 pathway.

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Introduction

The entry of human immunodeficiency virus type 1 (HIV-1) is initiated by an interaction of viral envelope glycoprotein gp120 with the principal receptor CD4 and one of the coreceptors, either CCR5 or CXCR4, expressed in the target cells. HIV-1 is classified into three phenotypes based on its ability to use CCR5 (R5), CXCR4 (X4), or both (R5X4 or dual-tropic). Certain dual-tropic viruses are further classified into those that prefer CCR5 (dual-R5) or CXCR4 (dual-X4) [1,2]. It has been shown that the coreceptor usage of HIV-1 is mainly determined by the third variable region of gp120 (V3 loop) [3,4,5] that is composed of ~35 amino acids. In particular, the number and position of positively charged amino acids in the V3 loop are important for coreceptor selectivity such as the 11/25 rule. If the 11th or 25th positions of the V3 loop are positively charged, viruses will use CXCR4. Otherwise, they will use CCR5 [6]. Lack of an N-linked glycan at the 6th position of the V3 loop is also involved in CXCR4 usage [7,8,9]. In general, R5 viruses are predominant in the early stage of infection, whereas CXCR4-using viruses (dual-tropic and X4 viruses) emerge at the late stage of infection and are associated with disease progression in half of HIV-1-infected individuals [10,11,12]. It has been postulated that coreceptor inhibitors or natural ligands of CCR5 or CXCR4 might induce the coreceptor shift of HIV-1 between CCR5 and CXCR4. However, *in vitro* studies have shown that these escape variants acquired resistance using the same coreceptor. For example, MIP-1α (a natural ligand for CCR5)-induced escape variants of R5 HIV-1 and selected viruses exhibit substitutions in the V2 region and V3 loop without changing CCR5 usage of the virus [13]. CCR5 inhibitors such as maraviroc (MVC) and vicriviroc also do not change coreceptor usage from CCR5 to CXCR4, and induce resistance in R5 HIV-1 that harbors several substitutions in the V3 loop and non-V3 regions. In general, the resistant viruses are able to recognize the CCR5 inhibitor-bound form of CCR5 called as non-competitive resistance [14,15,16,17,18,19,20,21] if there are no pre-existing X4 variants [22] though a maraviroc-resistant HIV-1 through competitive resistance mechanisms has been reported *in vivo* [23]. SDF-1α (a natural ligand for CXCR4) and CXCR4 inhibitors such as AMD3100 and T134 also induce selection of inhibitor-resistant variants among X4 viruses without changing coreceptor usage [24,25,26,27,28,29]. Although these resistant variants contain various mutations in multiple regions of gp120, the majority of mutations accumulate in the V3 loop, and some of these mutations are shared in different resistant variants. These observations indicate that the V3 loop is a crucial region for the acquisition of CXCR4-inhibitor resistance. Thus, the V3 loop is also the principal determinant for resistance to natural ligands and coreceptor inhibitors. Conversely, we have previously induced reversion of HIV-1 from dual-X4 to dual-R5 [30] using the CXCR4 inhibitor T140. The reversion is indeed associated with substitution in the 11th position of the V3 loop from arginine to serine [30], which is consistent with the 11/25th rule. Nevertheless, it remains elusive how coreceptor inhibitors induce evolution of HIV-1 to use different coreceptors or acquire resistance. Here, we selected AMD3100-escape variants from a dual-X4 HIV-1

carrying the V3 loop from CRF01_AE, which has no positively charged amino acids at the 11th or 25th positions and lacks an N-linked glycan in the V3 loop, to elucidate HIV-1 evolution for escape from CXCR4 inhibitors.

Materials and Methods

Ethics statement

The study protocol was approved as a part of "the study of immunological and virological analysis in HIV-1 infection (#540) " by the ethics committee for epidemiology and general study in the Faculty of Life Sciences in Kumamoto University and the National Center for Global Health and Medicine. Written informed consent was obtained from all studied individuals according to the Declaration of Helsinki.

Reagents and cells

The CXCR4 antagonist AMD3100 [29,31], CCR5 antagonist MVC [32], and recombinant human sCD4 were supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (Bethesda, MD, USA). Another CXCR4 inhibitor, T134, was kindly provided by Dr. Hirokazu Tamamura, Tokyo Medical and Dental University, Tokyo, Japan.

The TZM-bl cell line [33] was provided by Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker). The human embryonic kidney 293T cell line was obtained from American Type Culture Collection (ATCC), and maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The human CD4+ T cell line SupT1 was obtained from ATCC, and its derivative cell line SupT1/CCR5, which expressed high levels of CCR5, was established using a retroviral vector as described previously [13,21], and maintained in RPMI 1640 (Sigma) medium supplemented with 10% FBS, 0.2 mg/mL G418, 100 U/mL penicillin, and 100 μg/mL streptomycin. The CD4-expressing glioma cell line (NP2/CD4) [34,35] was provided by Dr. Hoshino (Gunma University), and its derivative cell lines, NP2/CD4/CXCR4, NP2/CD4/CCR5, and NP2/CD4/CXCR4/CCR5, were established as described previously [13] and maintained in Eagle's minimum essential medium (Sigma) supplemented with 10% FBS and appropriate antibiotics.

Construction of an Env expression vector and infectious molecular clone carrying the V3 loop from CRF01_AE HIV-1

cDNAs of viral RNA from CRF01_AE-infected individuals were prepared as previously described [36]. The *env* region was first amplified using the following primers: 5′-GGTAGAGCAGATGCAGGATG-3′ and 5′-GTGGGTGCTATTCCTAGTGGTTC-3′. Nested PCR was performed using primers carrying *Afl*II and *Nhe*I restriction enzyme sites: 5′-GCACCTTAAGAAATCTGTAGAAATCAATTG-3′ and 5′-GCTAGCTACCTGTTTTAAAGCTTTATACC-3′ (underlines denote *Afl*II and *Nhe*I sites, respectively). The amplified product was then cloned into a pCR-TOPO vector (Invitrogen) and sequenced using an ABI PRISM 3771 automated sequencer (Applied Biosystems). For construction of the Env expression vector carrying the V3 loop from CRF01_AE, the *Afl*II-*Nhe*I fragment of cloned V3 regions was introduced into the *Afl*II-*Nhe*I cloning site of pCXN-JR-FLan [19,20,21]. To construct the infectious molecular clone, the *Afl*II-*Nhe*I fragment was similarly introduced into pJR-FLan [19,20,21] as described previously, resulting in pJR-FLan carrying the V3 loop from CRF01_AE.

Construction of infectious molecular clones and Env expression vectors with mutations

Env expression vectors with single or multiple mutations were constructed using *Dra*III, *Eco*RV, *Nhe*I, and *Bsa*BI sites in KI812.7 *env*. Each PCR fragment carrying a mutation was substituted with wild-type *env*, resulting in an Env expression vector with a single mutation. Env expression vectors with different combinations of mutations were constructed by swapping the restriction fragments. Similarly, infectious molecular clones with mutations were constructed as described previously [19,20,21].

Virus preparation

A pseudotyped virus carrying the luciferase gene was prepared as previously described [13]. Briefly, 293T (3×10^6 cells) were transfected with 20 μg pNL-LucΔBglII and 10 μg pCXN-Env vectors. To produce infectious viral clones, 293T cells were transfected with 30 μg infectious HIV-1 clones. Virus-containing culture supernatants were recovered at 48 h post-transfection, filtered through a 0.22-μm filter (Millipore), and then stored at −80°C until use. The p24 Gag in the supernatant was measured using a p24 Ag ELISA (Zeptometrix) according to the manufacturer's protocol.

Isolation of AMD3100-escape variants from HIV-$1_{JR\text{-}FLan/KI812.7}$

To isolate AMD3100-escape variants from HIV-$1_{JR\text{-}FLan/KI812.7}$, the virus was passaged in SupT1/CCR5 cells with increasing concentrations of AMD3100. Viral replication was monitored by observing the cytopathic effect on SupT1/CCR5 cells. After 21 passages of the virus in SupT1/CCR5 cells at a final AMD3100 concentration of 4 μM, AMD3100 was removed from the virus-infected cell cultures, and the virus was recovered from the culture supernatant. The sensitivity of the escape variant to coreceptor antagonists was determined using TZM-bl cells. DNA was extracted from virus-infected cells using a QIAamp DNA Blood kit (Qiagen) and then subjected to PCR using *Taq* DNA polymerase (Promega). The V3 region sequences were amplified using the following primers: 5′-GCACCTTAAGAAATCTGTAGAAATCAATTG-3′ and 5′-GCTAGCTACCTGTTTTAAAGCTTTATACC-3′. The amplified products were cloned into pCR-TOPO (Invitrogen), and then the *env* regions of the virus were sequenced using the ABI PRISM 3130 automated sequencer.

Determination of drug sensitivity of replication-competent viruses

The sensitivity of replication-competent viruses to coreceptor inhibitors was determined using TZM-bl or SupT1/CCR5 cells. For TZM-bl cells, the cells were infected with viruses at 37°C for 2 days in the presence of various concentrations of coreceptor inhibitors. Luciferase activities of the cells were measured using a luminometer (Lumat LB 9501/16; Berthold). The sensitivity of the virus to coreceptor inhibitors was expressed as the 50% effective concentration (EC_{50}), which was the drug concentration that reduced infection levels by 50% compared with that in the infected, drug-free control of triplicate experiments. For SupT1/CCR5 cells, 5×10^3 cells in U-bottom 96-well microplates were infected with the same amount of virus (100 $TCID_{50}$) in the presence of various AMD3100 concentrations, and then cultured

A CRF01_AE consensus: CTRPSNNTRT SITIGPGQVF YRTGDIIGDI RKAYC
KI812.7: ....YRKI.. .FR..... .K..G.L..P K....

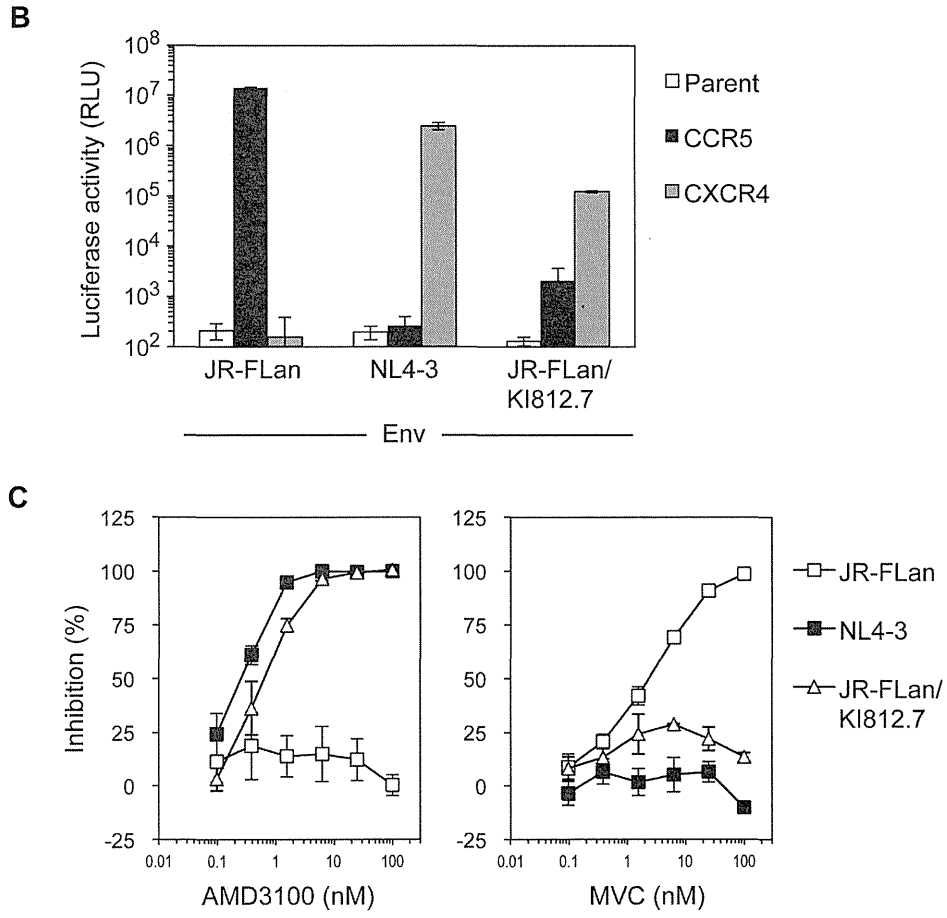

Figure 1. Virological characterization of HIV-1 carrying the V3 loop from CRF01_AE KI812.7. (A) V3 loop amino acid sequences of CRF01_AE consensus and KI812.7. Dots denote sequence identity. (B) Coreceptor usage of JR-FLan carrying the V3 loop from KI812.7. NP2/CD4 cells and NP2/CD4 cells expressing CCR5 or CXCR4 were infected with the same amount of luciferase-reporter pseudotyped virus (10 ng p24Ag). Luciferase activities were measured at 48 h post-infection. Data are the geometric means ± SD of triplicate experiments. (C) Susceptibility of replication-competent HIV-$1_{JR\text{-}FLan/KI812.7}$. TZM-bl cells were infected with replication-competent virus in the presence of AMD3100 or maraviroc (MVC), and then the luciferase activities of the infected cells were measured at 48 h post-infection. Data represent the extent of inhibition of replication relative to that in the absence of AMD3100 or MVC.
doi:10.1371/journal.pone.0089515.g001

for 6 days. The cytopathic effect was determined using an MTT assay as described previously [37].

Determination of drug sensitivity and coreceptor usage of pseudotyped viruses

To determine the coreceptor inhibitor sensitivity of pseudotyped viruses carrying the luciferase gene, NP2/CD4 cells expressing both CCR5 and CXCR4 were used as target cells. Briefly, the target cells (1.5×10^4 cells) were seeded in 48-well culture plates. The following day, the cells were incubated in the presence or absence of various concentrations of coreceptor inhibitors at 37°C for 30 min. The virus (50 ng p24 Ag) was then added to the cells and incubated at 37°C for 48 h. Luciferase activities of the cells were measured using the luminometer. The sensitivity of the virus to coreceptor inhibitors was expressed as the EC_{50}. To examine the coreceptor usage of the virus, NP2/CD4 cells expressing either CCR5 or CXCR4 were infected with pseudotyped viruses carrying the luciferase gene. Luciferase activities were measured after 48 h of infection in triplicate experiments using the luminometer.

Determination of entry efficiency of the virus

Entry efficiency of the virus was determined using a single-round replication assay. Briefly, NP2/CD4/CXCR4/CCR5 cells were infected with the same amount (10 ng p24 Ag) of pseudotyped HIV-1 carrying the luciferase gene. Luciferase activity was measured at 48 h post-infection using the luminometer.

Results

Coreceptor usage of a CRF01_AE-derived HIV-1 and its sensitivity to coreceptor inhibitors

We previously isolated a CXCR4 inhibitor-escape variant from dual-X4 HIV-1 89.6, which has a substitution at the 11th position of the V3 loop [30]. This change does not confer reduced sensitivity to CXCR4 inhibitors, but induces reversion of dual-X4 to dual-R5. However, it remains to be determined how

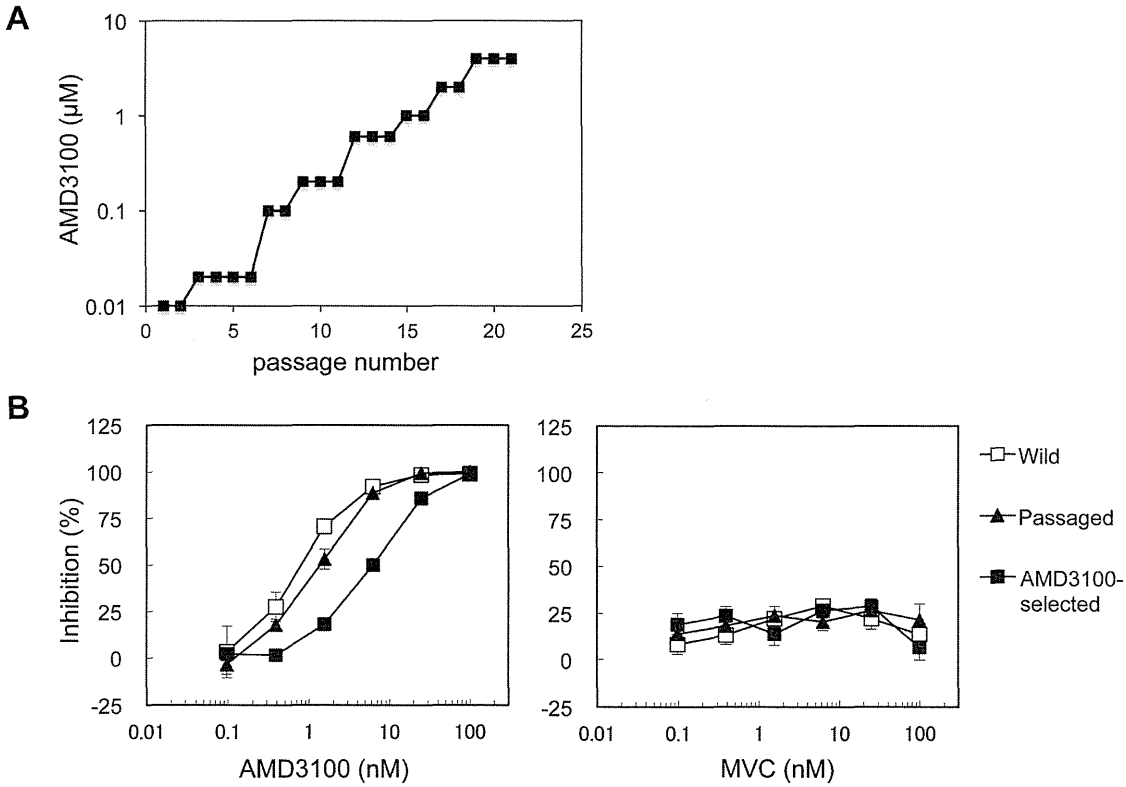

Figure 2. Selection of AMD3100-escape variants from HIV-$1_{JR\text{-}FLan/KI812.7}$. (A) Induction of AMD3100-resistant variants from HIV-$1_{JR\text{-}FLan/KI812.7}$. Replication-competent HIV-$1_{JR\text{-}FLan/KI812.7}$ was passaged using SupT1/CCR5 cells in increasing concentrations of AMD3100 in the range of 20 nM to 4 μM. (B) Susceptibilities of AMD3100-selected variants to AMD3100 and MVC. TZM-bl cells were treated with various concentrations of AMD3100 or MVC, and infected with wild-type HIV-$1_{JR\text{-}FLan/KI812.7}$, the virus passaged in the absence of AMD3100, or the selected virus in the presence of 4 μM AMD3100. Luciferase activities of TZM-bl cells were measured at 48 h post-infection. Data represent the extent of inhibition of replication relative to that in the absence of AMD3100 or MVC.
doi:10.1371/journal.pone.0089515.g002

CXCR4-using HIV-1 without a positively charged amino acid at the 11th position of the V3 loop escapes from CXCR4 inhibitors. Since higher prevalence of CXCR4-using HIV-1 in CRF01_AE compared to subtype B has been reported [38], we first cloned and sequenced the *env* regions of HIV-1s from 21 CRF01_AE-infected individuals in a Japanese cohort to find CXCR4-using HIV-1 lacking positively charged amino acids at the 11th and 25th positions of the V3 loop. Among them, two out of five clones isolated from individual KI812 had a unique amino acid sequence (KI812.7) as shown in Fig. 1A. Although the 11th and 25th positions of the V3 loop did not contain charged amino acids, the net charge of the V3 loop was +7. Furthermore, there was no putative N-linked glycosylation site at the 6th position. Geno2-pheno coreceptor algorithms [39] (http://coreceptor.bioinf.mpi-inf.mpg.de/) predicted that the virus was capable of using CXCR4 as a coreceptor (false positive rate: 0.1%). To confirm the coreceptor usage of the virus, an Env expression vector and an infectious molecular clone carrying the V3 loop derived from KI812.7 were constructed using pJR-FL as a backbone, which were designated as pCXN-FLan/KI812.7 and pJR-FLan/KI812.7, respectively. As we reported previously, the virus pseudotyped with JR-FLan and NL4-3 Env exclusively infected NP2/CD4 cells expressing CCR5 and CXCR4, respectively (Fig. 1B). In contrast, luciferase activity of CXCR4-expressing cells infected with virus carrying FLan/KI812.7 Env was ~100-fold higher than that of CCR5-expressing cells, indicating that FLan/KI812.7 Env preferentially used CXCR4 over CCR5. These results confirmed that substitution of the V3 loop with KI812.7 changed coreceptor usage from R5 to X4 (Fig. 1B). Furthermore, an infectious clone, HIV-$1_{JR\text{-}FLan/KI812.7}$, was sensitive to the CXCR4 inhibitor AMD3100 (EC_{50} value: 0.62±0.21 nM) as well as X4 HIV NL4-3 (EC_{50} value: 0.26±0.04 nM), but resistant to the CCR5 inhibitor MVC in both CCR5- and CXCR4-expressing TZM-bl cells (Fig. 1C). Taken together, the virus carrying JR-FLan/KI812.7 Env was a dual-X4 HIV-1.

Selection of AMD3100-resistant variants from HIV-$1_{JR\text{-}FLan/KI812.7}$

To elucidate how CXCR4-using HIV-1 escapes from the CXCR4 inhibitor AMD3100, we isolated AMD3100-escape variants from HIV-$1_{JR\text{-}FLan/KI812.7}$ using a SupT1 cell line expressing high levels of CCR5. This cell line was able to support both CXCR4- and CCR5-using HIV-1 replication, thereby permitting both resistance to AMD3100 and coreceptor switching of the virus. To select AMD3100-escape variants, SupT1/CCR5 cells were passaged in increasing concentrations of AMD3100. The virus was also passaged in the absence of AMD3100 to exclude the effect of long-term culture. After 21 passages of the virus in the presence of 4 μM AMD3100 (Fig. 2A), the virus was recovered and its sensitivity to AMD3100 was determined using TZM-bl cells. As a result, the selected virus displayed reduced sensitivity (4-fold) to AMD3100 compared with that of the passaged virus in the absence of AMD3100 and the wild-type virus (Fig 2B). The EC_{50} value of the selected virus was 62 nM,

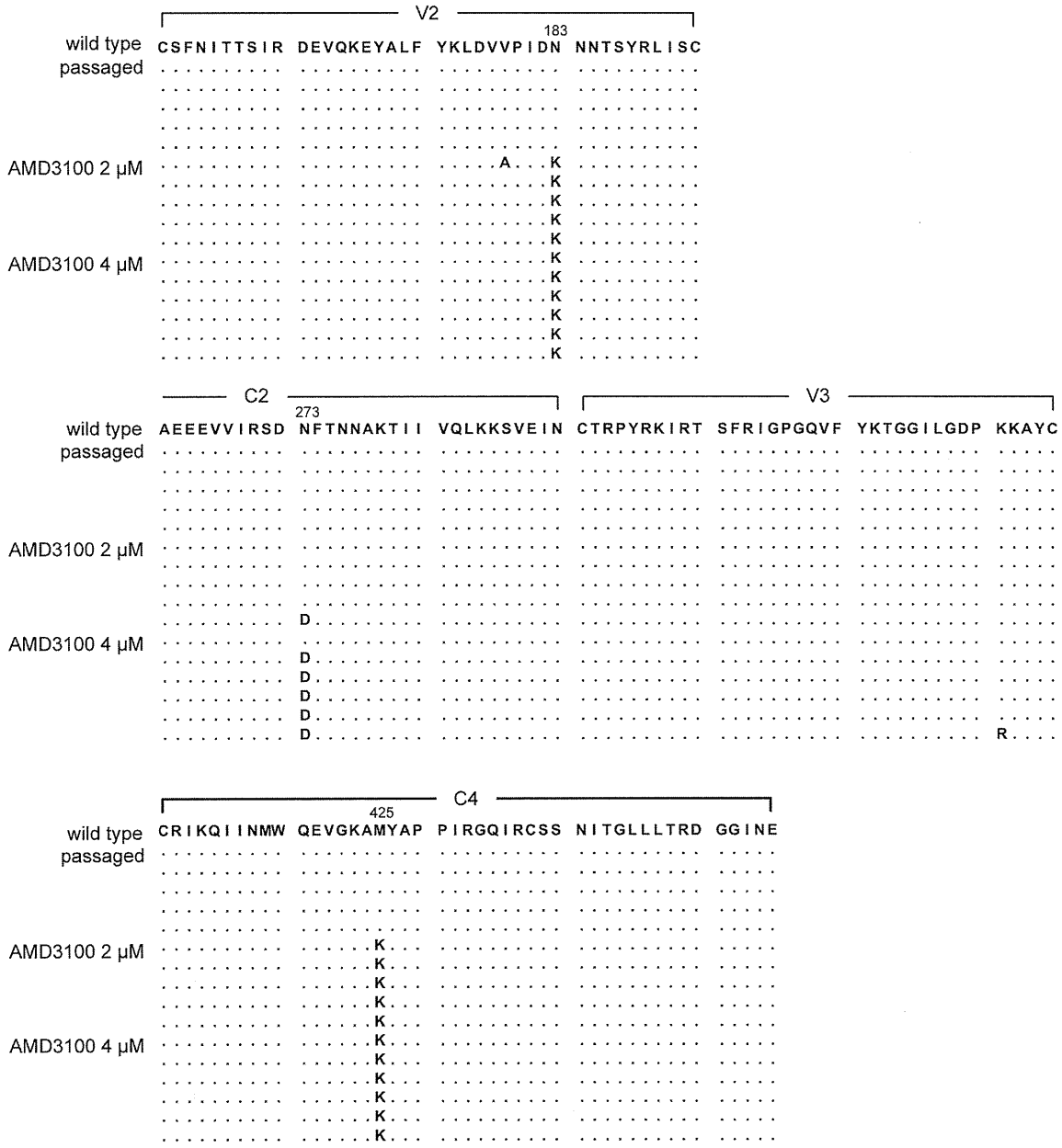

Figure 3. Amino acid sequences of the AMD3100-escape variant. Amplified products from infected SupT1/CCR5 cells in the absence or presence of AMD3100 were cloned, and five to six clones from each sample were sequenced. The amino acid sequences of V2, C2, and C4 of the wild-type HIV-$1_{JR\text{-}FLan/KI812.7}$ are shown in the top line. In each set of clones, the deduced amino acid sequence was aligned by the single amino acid code. Identity with this sequence at individual amino acid positions is indicated by dots.
doi:10.1371/journal.pone.0089515.g003

whereas that of the passaged virus was 14 nM. Furthermore, entry of the selected virus was completely inhibited by high concentrations of AMD3100, and the virus was completely resistant to MVC in TZM-bl cells. These results suggested an absence of coreceptors switching from CXCR4 to CCR5 and a competitive resistance profile of the virus to AMD3100.

Amino acid sequences of the AMD3100-resistant HIV-1

To determine which regions were responsible for the reduced sensitivity of the escape variant to AMD3100, the V1–C4 regions of the envelope gene were sequenced using DNA amplified from infected cells as a template. In the selected virus at 2 μM AMD3100, the virus harbored an N138K substitution in the V2 region and a M425K substitution in the C4 region. Furthermore, the escape variant acquired an N273D substitution in the C2 region at 4 μM AMD3100 (Fig. 3). Most clones passaged in the presence of AMD3100 did not have substitutions in the V3 loop (one clone had a K to R substitution at the 31th position of the V3 loop). In contrast, no remarkable changes were observed in the passaged virus in the absence of AMD3100 (Fig. 3).

Non-V3 regions are involved in the reduced sensitivity to AMD3100

To examine which substitutions were responsible for the reduced sensitivity to AMD3100, we constructed and produced

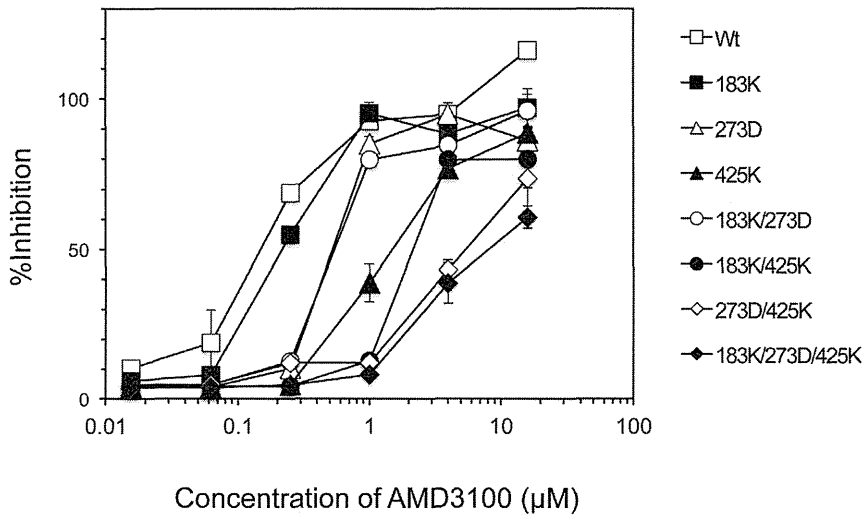

Figure 4. Susceptibilities of replication-competent recombinant viruses. SupT1/CCR5 cells were infected with the same amount of replication-competent recombinant viruses carrying mutations (100 $TCID_{50}$) in the presence of various concentrations of AMD3100, and then cultured for 6 days. Cytopathic effects were determined by an MTT assay. Data are the means ± SD of triplicate experiments.
doi:10.1371/journal.pone.0089515.g004

infectious molecular clones carrying single or multiple mutations. The sensitivity of each mutant was then determined by an MTT assay using SupT1/CCR5 cells (Fig. 4, Table 1). In single mutants, M425K and N273D conferred 10-fold and 3-fold reduced sensitivities to AMD3100, respectively, whereas N183K was almost dispensable. Furthermore, mutants carrying both N273D and M425K (N273D/M425 and N183K/N273D/M425K) conferred a more than 40-fold reduced sensitivity to AMD3100. To confirm the sensitivity of mutants to AMD3100 using a single-round entry assay, we constructed Env expression vectors carrying single or multiple mutations. Pseudotyped viruses carrying the luciferase gene were produced by cotransfection of 293T cells with these vectors and an *env*-lacking luciferase-reporter HIV-1 construct. The sensitivity of each mutant was then determined using NP2/CD4 cells expressing both CXCR4 and CCR5 (Table 2). In single mutants, N273D and M425K substitutions conferred reduced sensitivity to AMD3100 (4.1-fold and 2.6-fold, respectively), whereas N183K had a minor effect (1.5-fold) as shown in Table 2. The N293D mutation combined with M425K (273D/425K) conferred increased resistance to AMD3100 (10-fold). In contrast, addition of N183K had a minor effect on the reduced sensitivity to AMD3100 in combination with N273D/M425K (13-fold). These results indicated that both N273D and M425K were mainly involved in the reduced sensitivity to AMD3100. The reduced sensitivity to AMD3100 was thus independent of the V3 loop.

We next determined whether viruses carrying these mutations were cross-resistant to another CXCR4 inhibitor, T134 [24] (Table 2). We found that a single M425K mutation and combinations with M425K were cross-resistant to T134 (3-fold).

Table 1. Susceptibility of recombinant viruses to AMD3100 determined by MTT assays.

virus	EC_{50} (μM)[a]
	AMD3100
wild type	0.15±0.02[b](1.0)
183K	0.21±0.01 (1.5)
273D	0.52±0.02 (3.6)
425K	1.5±0.23(10)
183K/273D	0.54±0.01 (3.7)
183K/425K	2.2±0.05(14)
273D/425K	5.9±1.4(40)
183K/273D/425K	10.3±2.5(70)

[a]SupT1/CCR5 cells (5×10^3) were infected with 100$TCID_{50}$ recombinant viruses, and then the cytotoxicity induced by HIV-1 was measured at day 6 post-infection by an MTT assay to determine the effective concentration of 50% inhibition (EC_{50}).
[b]Mean ± SD (n = 3). Numbers in parenthesis represent fold changes of EC_{50} values compared with that of the wild type.
doi:10.1371/journal.pone.0089515.t001

Table 2. Susceptibilities of recombinant pseudotyped viruses to CXCR4 inhibitors determined by single-round entry assays.

virus	EC_{50} (μM)[a]			
	AMD3100		**T134**	
wild type	0.012±0.0047[b]	(1.0)	0.033±0.0086	(1.0)
183K	0.018±0.0015	(1.5)	0.023±0.0064	(0.7)
273D	0.052±0.029	(4.1)	0.034±0.0010	(1.0)
425K	0.032±0.012	(2.6)	0.097±0.011	(2.9)
183K/273D	0.031±0.015	(2.4)	0.054±0.014	(1.6)
183K/425K	0.020±0.012	(1.6)	0.062±0.011	(1.9)
273D/425K	0.12±0.014	(10)	0.061±0.013	(1.8)
183K/273D/425K	0.16±0.031	(13)	0.099±0.013	(3.0)

[a]NP2/CD4/CXCR4/CCR5 cells (1.5×10^4) were infected with pseudotyped virus (50 ng p24Ag) in the presence of CXCR4 inhibitors, and then the luciferase activity was measured at 48 h post-infection to determine the effective concentration of 50% entry inhibition (EC_{50}).
[b]Mean ± SD (n = 3). Numbers in parenthesis represent fold changes of EC_{50} values compared with that of the wild type.
doi:10.1371/journal.pone.0089515.t002

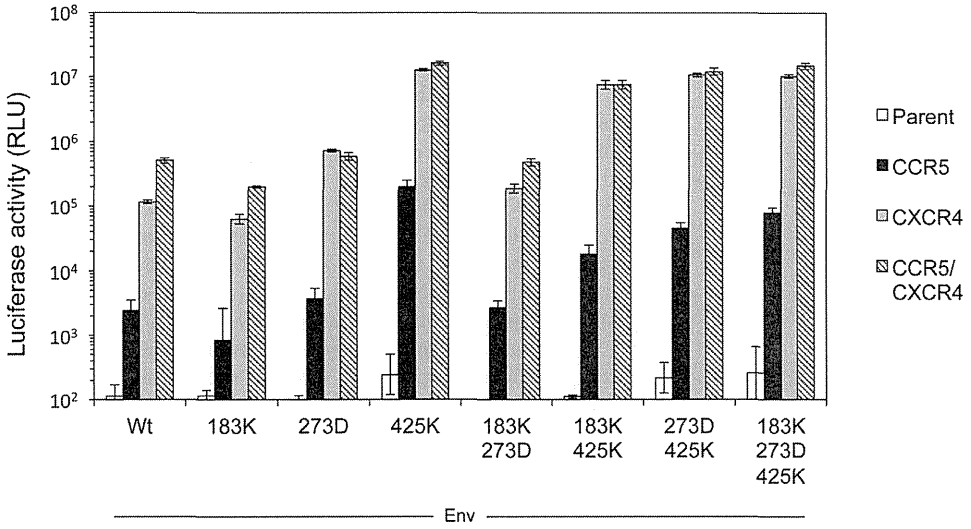

Figure 5. Coreceptor usage and entry efficiency of recombinant pseudotyped HIV. Coreceptor usage of the recombinant luciferase-reporter HIV was determined using NP2/CD4 cells expressing either CCR5 or CXCR4. Entry efficiencies of recombinant pseudotyped HIVs were determined using NP2/CD4 cells expressing both CXCR4 and CCR5. The cells were infected with the same amount of luciferase-reporter pseudotyped virus (10 ng p24Ag) with the indicated mutations. Luciferase activities were measured at 48 h post-infection. Data are the geometric means ± SD of triplicate experiments.
doi:10.1371/journal.pone.0089515.g005

However, similar to the wild-type, N273D was sensitive to T134.

Involvement of the C4 region in enhanced replication of AMD3100-resistant HIV-1

We next evaluated whether these mutations changed the coreceptor preference from CXCR4 to CCR5. To this end, NP2/CD4 cells expressing either CCR5 or CXCR4 were infected with the luciferase-reporter HIV-1 pseudotyped with single or multiple mutations (Fig. 5). After infection of CXCR4-expressing cells with all recombinant viruses derived from JR-FLan/KI812.7, luciferase activities were ~100-fold higher than those of CCR5-expressing cells. This result indicated that all Envs, including N183K, N273D, and M425K, did not change preferential use of CXCR4. We also determined the entry efficiencies of the mutants using NP2/CD4 cells expressing both CXCR4 and CCR5. Luciferase activities of the cells infected with the same amount of the viruses (10 ng p24 Ag) showed that the single M425K substitution, but not N183K and N273D, increased the entry efficiency compared with that of the wild-type virus (Fig. 5). Mutations combined with 425K (183K/425K, 273/425K, and 183K/273D/425K) also had similar infectivities to that of the single mutation (Fig. 5), indicating that M425K substitution was

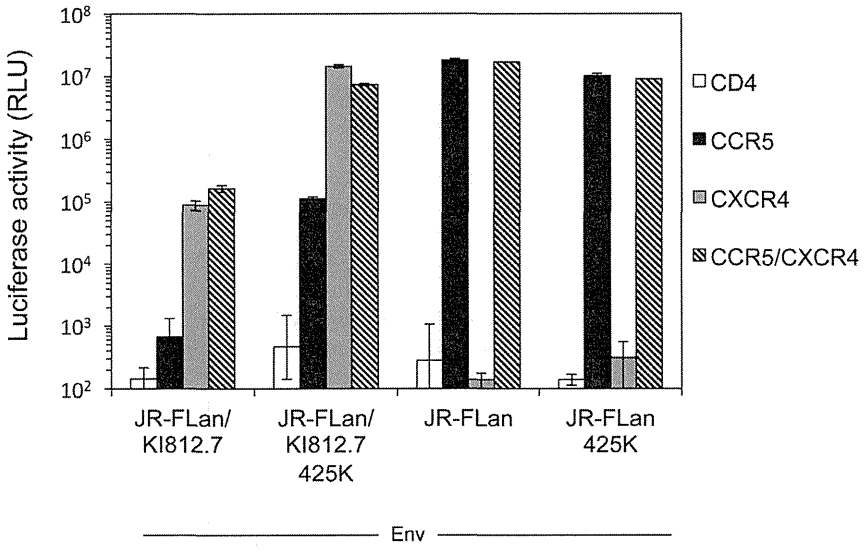

Figure 6. Effect of M425K substitution on JR-FLan Env. Coreceptor usage and entry efficiency of recombinant luciferase-reporter HIV pseudotyped with the indicated Envs were determined using NP2/CD4 cells expressing either CXCR4 or CCR5 and cells expressing both CXCR4 and CCR5, respectively. The cells were infected with the same amount of luciferase-reporter pseudotyped virus (10 ng p24Ag) with the indicated mutations. Luciferase activities were measured at 48 h post-infection. Data are the geometric means ± SD of triplicate experiments.
doi:10.1371/journal.pone.0089515.g006

Table 3. Susceptibility of recombinant pseudotyped viruses to sCD4 determined by single-round entry assays.

virus	EC_{50} (μg/mL)[a]
	sCD4
wild type	>10
183K	>10
273D	6.9±0.26[b]
425K	0.42±0.040
183K/273D	8.6±0.89
183K/425K	0.35±0.024
273D/425K	0.14±0.0033
183K/273D/425K	0.22±0.016

[a]NP2/CD4/CXCR4/CCR5 cells (1.5×10^4) were infected with pseudotyped virus (50 ng p24Ag), and then the luciferase activity was measured at 48 h post-infection to determine the effective concentration of 50% entry inhibition (EC_{50}).
[b]Mean ± SD (n = 3).
doi:10.1371/journal.pone.0089515.t003

essential for enhancement of viral infectivity. We next determined whether the M425K substitution also enhanced the entry efficiency of JR-FLan Env. However, M425K substitution in JR-FLan Env neither increased the luciferase activity in NP2/CD4/CCR5/CXCR4 (Fig. 6) nor changed coreceptor usage from CXCR4 to CCR5. These results indicated that the enhanced infectivity by M425K substitution was only observed in the context of Env carrying the V3 loop from KI812.7.

Involvement of C2 and C4 regions in the increased sensitivity to soluble CD4

Because the change in the C2 region (N273D) was located in loop D, which is associated with resistance to the monoclonal neutralizing antibody VRC01 and soluble (s)CD4 [40], we examined whether the mutations also affected the sensitivity to sCD4 (Table 3). We found that the wild-type virus was resistant to sCD4 (the EC_{50} value was more than 10 μg/ml). N183K mutation did not change the sensitivity, whereas N273D increased the sensitivity to sCD4 to some extent (EC_{50}: 6.9±0.26 μg/mL). In contrast, M425K largely increased the sensitivity to sCD4 (EC_{50}: 0.42±0.04 μg/mL). These results indicated that not only the C2 mutation but also the C4 region mutation affected the increased sensitivity to sCD4.

Discussion

Characterization of CXCR4 inhibitor-resistant HIV-1 is important to understand how the virus can escape from inhibitors targeting coreceptors although clinical application of CXCR4 inhibitors for treatment of HIV-1-infected individuals remains a matter of debate. In the present study, we successfully isolated an AMD3100-escape variant from dual-X4 HIV-1. Interestingly, the variants had substitutions in C2 and C4 regions (N273D and M425K, respectively), which were responsible for their resistance to AMD3100 based on site-directed mutagenesis experiments. In contrast, no remarkable changes were observed in the V3 loop. In general, the V3 loop is a crucial determinant for coreceptor selectivity and resistance to coreceptor inhibitors and natural ligands. CXCR4 inhibitor-resistant X4 viruses show numerous mutations in the V3 loop and other regions, although the responsible region(s) have mostly not been determined for the reduced sensitivity to CXCR4 inhibitors [24,25,26,27,28]. In our previous study, we also selected a CXCR4 inhibitor-escape variant from dual-X4 HIV-1, which had serine to arginine substitution at the 11th position of the V3 loop [30]. *In vitro* experiments have revealed that coreceptor selectivity of HIV-1 is determined by the amino acid sequence of gp120, particularly the number [41,42] and position of charged amino acids in the V3 loop such as the 11/25 rule. Thus, amino acid substitution in the V3 loop can predict the loss of CXCR4 usage. Indeed, mutational analysis confirmed reversion of dual-X4 to dual-R5 by substitution. Conversely, the dual-X4 virus used in this study did not have a positively charged amino acid at the 11th or 25th position of the V3 loop, such as arginine or lysine. However, the Geno2pheno coreceptor algorithm predicted CXCR4 use of this virus because of an increased net positive charge and lack of an N-linked glycan in the V3 loop. In fact, analyses of coreceptor usage revealed that the virus carrying the V3 loop from KI812.7 predominantly used CXCR4 as the coreceptor. Furthermore, the AMD3100-escape variant was found to predominantly use CXCR4 without reversion from CXCR4 to CCR5. Therefore, it is likely that viruses not carrying a charged amino acid at the 11th position of the V3 loop lose their ability to revert from CXCR4 to CCR5 use. To acquire resistance to CXCR4 inhibitors, such viruses may need to induce substitutions in the V3 loop or different regions of gp120, such as C2 and C4 regions.

It has been suggested that CXCR4 inhibitor-resistant viruses exhibit reduced fitness [25], probably because of lower affinity of gp120 for CXCR4. Notably, M425 is located in the β21 sheet of the gp120 bridging sheet that is thought to be important for coreceptor binding together with the stem of the V3 loop (V3 stem) [43,44,45]. Because there was no reduction in the maximum plateau inhibition [18] for AMD3100 in this escape variant, it is unlikely that the selected virus recognized the AMD3100-bound form of CXCR4 [46]. Instead, sufficient concentrations of AMD3100 reached~100% inhibition of the selected virus (right shift in the EC_{50} value), indicating competitive resistance. It is thus possible that the M425K substitution may alter the binding affinity for CXCR4 in the context of the V3 loop from KI812.7 [47,48,49] to retain the viral replication fitness. Indeed, the M425K substitution was cross-resistant to another CXCR4 inhibitor, T134, and dramatically enhanced the entry efficiency of the virus carrying the V3 loop from KI812.7 (~100-fold) but not from the JR-FLan background. Other studies have also shown that escape from the CCR5 inhibitor vicriviroc is not associated with a loss of fitness [50], which is not caused by changes in the V3 loop, similar to our variant, but rather substitutions in the fusion peptide domain of gp41 [51]. Taken together, it is possible that coreceptor inhibitor-resistant viruses need to retain or increase the affinity for their coreceptors by changing V3 or non-V3 regions, which are probably dependent on the configuration of the V3 loop.

In contrast, the N273D substitution was also shown to be an important determinant for reduced sensitivity to AMD3100, although the mutation did not significantly increase the entry efficiency of the virus. In fact, N273D is located in loop D of the C2 region and is associated with the loss of N-glycan, indicating alteration of the whole structure of gp120 via steric hindrance. It has been shown that N273A affects sensitivities to the broadly neutralizing monoclonal antibody VRC01 and sCD4 [40]. The structure of VRC01 in complex with the gp120 core revealed that the VRC01 heavy chain binds to the gp120 CD4bs in a manner similar to that of CD4 [52]. Indeed, our mutations, not only N273D but also M425K, conferred sensitivity to sCD4, suggesting that these substitutions affect the CD4 binding affinity. It has been reported that AMD3100 directly interacts with Asp^{171} and Asp^{262}

of CXCR4 [53], as well as ECL2 and TM4 [54]. However, inhibitory activities of AMD3100 in CXCR4 mutants at these positions are dependent on the strain of CXCR4-using HIV-1 [53]. Thus, different CXCR4-using HIV-1s vary in their dependence on residues in one or the other domains [55]. Taken together, it is possible that gp120 with N273D or M425K might recognize a different portion of the CD4/CXCR4 complex and alter their affinity. However, structural analysis of the gp120 core carrying these mutations with the V3 loop from KI812.7 is necessary to address these issues.

In conclusion, it is possible to induce a CXCR4 inhibitor-resistant virus from CXCR4-using HIV-1 without changing the V3 loop. The configuration of the V3 loop might be the major determinant for selection of such resistant viruses, which may also determine how the virus evolves for resistance or the coreceptor switch. Further structure-based analyses are necessary to elucidate these molecular mechanisms.

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Author Contributions

Conceived and designed the experiments: YM HT. Performed the experiments: HT YM. Analyzed the data: YM HT. Contributed reagents/materials/analysis tools: YN KM MT SO KY. Wrote the paper: YM SH.

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第2章 生物薬品の品質，安全性の向上に関する検討 ①

過去の事例に学ぶウイルス汚染の防止対策
～血漿分画製剤の感染事例とその対策～

The infection control policy of the biological products which employed the past incidents : The cases of blood plasma protein preparations

日本PDA製薬学会 バイオウイルス委員会 SALLY分科会

PDA Japan Chapter, Bio-Virus Safety Committee : SALLY Team

はじめに

バイオウイルス委員会では2003年より11年間の活動を通して，生物薬品(Biologics)製剤について，ウイルス安全性のレギュレーションや実際の試験デザインに関して，多方面から分析，調査し，年会での報告，論文投稿，書籍の執筆やセミナー開催などを通して，会員をはじめとする医薬品業界全体に有用な情報・提言を発信してきた。本稿では，委員会のSALLY分科会が，2011年から過去の感染事例を調査して得た知見のうち，副題の「血漿分画製剤の感染事例とその対策」についてまとめた。なお，分科会名であるSALLYはこの目的(温故知新)を意味する "Suggestion And Lesson Learned Yield a clue" の略称である。

血漿はヒト血液の血球以外の液体成分で，免疫制御因子，血液凝固制御因子などに代表されるような数多くの生理活性物質を含む。血漿分画製剤は，血漿から医薬品として有用な目的成分を単離・精製して製造する製剤で，**図1**に示すような特性を持っている。血友病に代表されるような特定のタンパク質の欠乏や分子異常が原因で発症するさまざまな疾患の治療と予防になくてはならない医薬品であるが，1バッチの原料として，数千から数万人分の血漿を使用することから，既知，未知を問わず，ウイルスなどの感染性因子が混入するリスクへの対策が必須である。

血漿分画製剤では，血液凝固因子製剤によって起きた

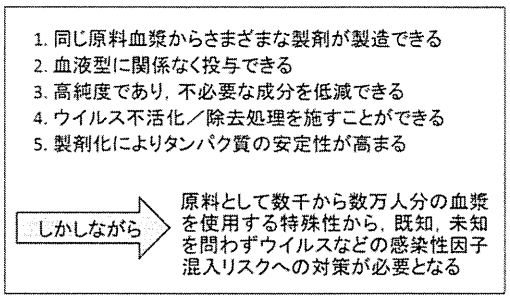


図1　血漿分画製剤の特性

HIV(ヒト免疫不全ウイルス，AIDS：後天性免疫不全症候群の原因ウイルス)やHCV(C型肝炎ウイルス)の感染が，いわゆる薬害としてよく知られている。また，混入の可能性が高いウイルスとしては，古くから輸血後肝炎対策として注目されてきたHBV(B型肝炎ウイルス)，現在でも多くの国民が感染既往を有するHPV-B19(ヒトパルボウイルスB19)がある。本稿ではこれら4つのウイルスの事例から学んだことが，どのように感染性因子対策に生かされてきたかを時系列的にレビューし，対策の現状と課題を報告する。

1．感染事例，ウイルス同定，検査と対策の変遷

4ウイルスの感染事例，ウイルスの発見，検査方法導入の変遷を時系列で示した(**図2**)。古くから輸血後肝炎やHPV-B19による伝染性紅斑〔リンゴ(ほっぺ)病と呼ば

れることもある]は知られていたが，当然のごとく，いずれもその原因ウイルスが同定・発見されて初めて検査方法が確立された。言い換えると感染被害拡大を抑制する重要なポイントは，感染事例の報告後いかに早く原因ウイルスを同定し，検査方法導入に至るまでの期間を短くすることができるか，である。現在までに，ウイルスクリアランス試験*1に関するガイドライン，感染症定期報告制度の導入，貯留保管の開始*2，遡及調査*3の開始を経て，感染対策は飛躍的に向上している。

個別のウイルス感染事例を述べる前に，原料血液と製造プロセスに対する対策の変遷を図3，図4に整理した。1960年代までは，ドナーの問診，血液比重検査などの検査を経た血液を原料に，輸血用血液製剤が製造されたが，血漿分画製剤の原料血漿は海外から輸入していた。また，製造工程のウイルス不活化，除去工程は，コーンの分画法と，一部の製剤で液状加熱処理が行われていた程度であった。1970年代，当時問題となっていた輸血後肝炎対策として血清学的検査が導入され，原料血液の肝炎に対する安全性確保が初めて具体化した。1980年代になって，血漿分画製剤の原料として国内献血由来のものが徐々に使われるようになってきた。またこの頃，一部製剤に乾燥加熱処理が導入された。さらに1990年代になると，ウイルスの同定を踏まえた核酸増幅検査（NAT：nucleic-acid amplification testing）が導入され，高い検出感度で原料を検査できるとともに，工程中へウイルス除去ろ過膜やS/D（有機溶媒・界面活性剤）処理を導入する企業が増えてきた。一方，原料血液に対しても，上述した貯留保管が導入されることで，検査をすり抜ける確率はさらに低下した。

*1血漿分画製剤の製造工程が，どの程度ウイルスを除去，不活化できるかをテストする

*2献血者が供血時点では感染しているがNATでも検出できない場合に備え，一定期間（6カ月間）保管することによって原料血漿の安全性を確保する。

*3感染性因子の存在が疑われたドナーの血液，あるいは輸血履歴をさかのぼり，それら原料から製造された血液製剤の情報を調査し，感染の可能性について科学的に分析評価する仕組み。感染の伝播を抑制することができる。

次に，これら一連の対策に至る過程で起きた各ウイルスの感染事例とその教訓を，ウイルスの発見，対策の進んだ順に関連情報を交えて報告する。

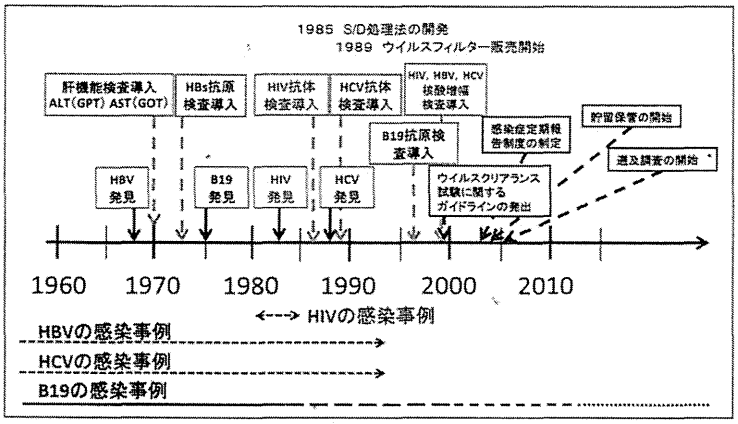


図2 感染事例，ウイルスの発見，検査の変遷

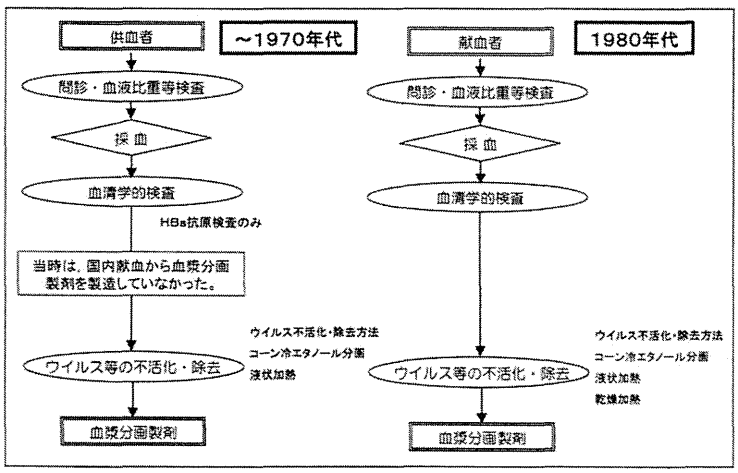


図3 製剤化までのウイルス汚染対策

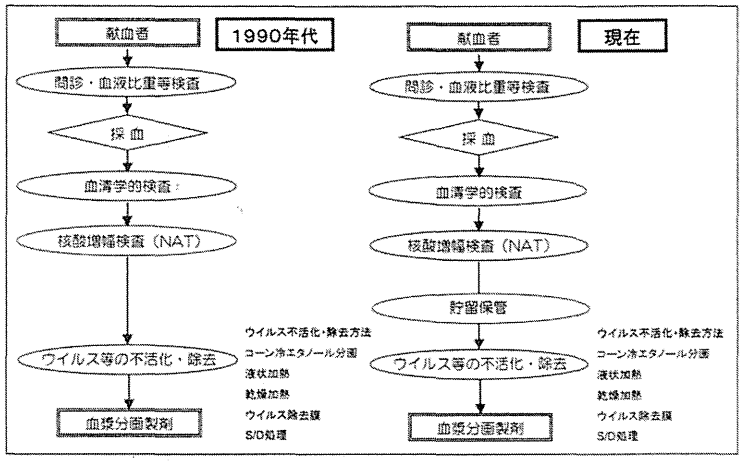


図4　製剤化までのウイルス汚染対策

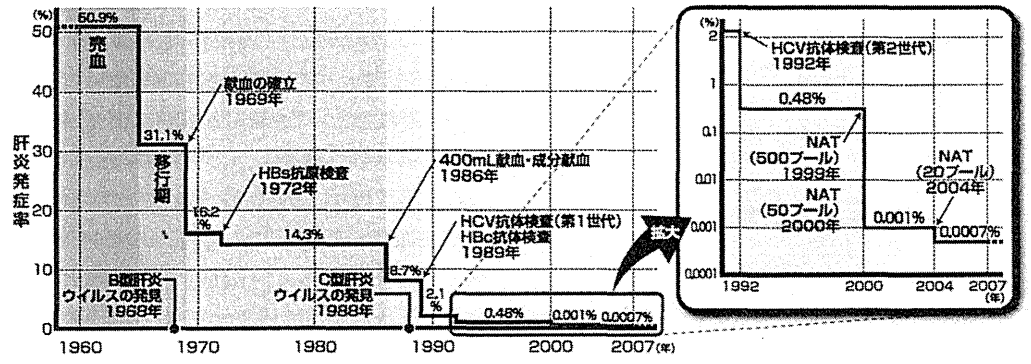
従来からHBVは，母子感染や輸血といった，「患者の血液や粘液に直接接触する行為」によって，感染が伝播すると考えられていたが，最近，保育園などでの集団感染をきっかけに調査が進み，現在は唾液，汗，尿，涙にもウイルスが検出され，父子感染があることもわかってきた（図6）。したがって，感染事例が激減したとはいえ，HBVによる製剤への混入リスクが今後高まる可能性は否定できないため，血液はもちろん，血漿分画製剤のスクリーニングを継続する意義は今後も高いと考えられる。

2．HBVの感染事例

1960年代は，輸血した患者の実に50％が輸血後肝炎を発症した。そのため，当時原因ウイルスと考えられたHBVは，輸血用血液の汚染対策の第一のターゲットであった。特に，後述するライシャワー事件を契機に献血制度が確立し，HBs抗原検査，成分献血，HBc抗体検査，さらにNAT導入を経て1990年代には0.48％まで感染事例は激減した（図5）。さらに原料血漿を50人から20人の小単位で（ミニ）プールし，NATを用いた全数検査をすることで，2000年代には実に0.0007％まで低下した。さらに，このミニプールによる全数検査は，上述の遡及調査を可能とした。

- これまでの常識
  - 感染経路：垂直感染（母子感染）
  - 水平感染：輸血，注射器の複数回使用，性行為，臓器移植など
  - 慢性化すると一部が肝硬変，肝細胞癌に至るが，寛解も多い
- 保育園でのB型肝炎集団発生事例（佐賀県）（2002年）
  - ①佐賀市を中心とした症例検索，②保育所関係者のスクリーニング，③感染源検査，④感染経路調査等を行った結果，合計25人（園児19人，職員6人）が当該保育所内でHBVに感染した疑い
  - 保育園のような体液（唾液，汗，尿，涙）の接触機会が多い場所での感染リスクの認識
- 認識の変化
  - 日常生活の中でも感染が起こり得る
  - 明確で予想可能な感染経路だけではない
  - 製造工程で作業者からの汚染可能性は「？」

図6　HBVによる感染



「日本赤十字社輸血後肝炎の防止に関する特定研究班」研究報告書を基に，日本赤十字社が輸血情報（0811-116）として作成

図5　日本における輸血後肝炎の推移