Resistance profile of a neutralizing anti-HIV monoclonal antibody, KD-247, that shows favourable synergism with anti-CCR5 inhibitors

Kazuhisa Yoshimura\textsuperscript{a}, Junji Shibata\textsuperscript{a}, Tetsuya Kimura\textsuperscript{a}, Akiko Honda\textsuperscript{a}, Yosuke Maeda\textsuperscript{b}, Atsushi Koito\textsuperscript{a}, Toshio Murakami\textsuperscript{c}, Hiroaki Mitsuya\textsuperscript{d} and Shuzo Matsushita\textsuperscript{a}

\textbf{Background:} The high-affinity humanized monoclonal antibody (MAb) KD-247 reacts with a tip region in gp120-V3 and cross-neutralizes primary isolates with a matching neutralization sequence motif.

\textbf{Methods:} We induced an HIV-1 variant that was resistant to KD-247 by exposing the JR-FL virus to increasing concentrations of KD-247 in PM1/CCR5 cells, which expressed high levels of CCR5 in vitro. We determined the amino acid sequence of the gp120-encoding region of the JR-FL escape mutant from KD-247. To confirm that this substitution was responsible for the KD-247-resistance, a single-round replication assay was performed. We further evaluated the anti-HIV-1 interactions between KD-247 and various CCR5 inhibitors in vitro.

\textbf{Results:} At passage 8 of the culture in the presence of 1000 \( \mu \text{g/ml} \) KD-247, one amino acid substitution, Gly to Glu at position 314 (G314E), was identified in the V3-tip of gp120. A pseudotyped virus with the G314E mutation was highly resistant to KD-247. Unexpectedly, this mutant virus was sensitive to CCR5 inhibitors, RANTES, recombinant human soluble CD4 (rsCD4) and an anti-CCR5 MAb, but resistant to an anti-CD4 MAb, compared with the wild-type virus. We also found that combinations of KD-247 and CCR5 inhibitors were highly synergistic.

\textbf{Conclusions:} The present data suggest that KD-247 has certain advantages for possible passive immunotherapy. They are: high concentrations of KD-247 are needed for viral acquisition of KD-247 resistance; the escape variants are more sensitive to CCR5 inhibitors and rsCD4; and there are high levels of synergism between KD-247 and CCR5 inhibitors at all concentrations tested.

\textit{AIDS} 2006, 20:2065–2073

\textbf{Keywords:} HIV-1, KD-247, anti-V3 monoclonal antibody, broadly neutralizing, CCR5 inhibitor, synergism
Introduction

In a recent paper, we described a cross-neutralizing anti-V3 antibody, KD-247, against primary isolates via sequential immunization with six peptides from V3 that contained a neutralizing epitope of HIV-1 [1]. The ability of KD-247 to neutralize HIV-1 may be dependent on site-specific binding to an epitope on the viral envelope glycoprotein. The complementarity determining regions of KD-247 were transferred from the mouse monoclonal antibody (MAb) C25, which was designed to have broad neutralization activity against HIV-1 clade B isolates. The recognition site of KD-247 was mapped to five or six amino acids around the PGR core sequence at the tip of the V3 region of gp120. The shortest reactive peptide recognized by KD-247 was determined to be IGPR, although the epitope was stabilized by the addition of one or more supplemental amino acids. The GPGR sequence in the V3 tip is highly conserved among HIV-1 strains [2]. In a recent study, we showed that the reshaped MAb KD-247 was suitable for use not only as an antibody for passive immunization for the prevention of HIV infection but also as an antibody for passive transfer immunotherapy for infected individuals [3].

In the present study, we induced HIV-1 variants that escaped from neutralization by KD-247 in vitro by continuously exposing the R5 virus JR-FL to increasing concentrations of KD-247 and defined the virological properties of a pseudotyped HIV-1 clone carrying the KD-247 escape-associated env gene mutation. We also evaluated the anti-HIV-1 interactions between KD-247 and various CCR5 inhibitors in vitro.

Materials and methods

Cells, culture conditions and reagents

The CD4-positive T-cell line PM1 was maintained in RPMI 1640 (Sigma, St. Louis, Missouri, USA) supplemented with 10% heat-inactivated foetal calf serum (Hyclone, Logan Utah, USA), 50 U/ml penicillin and 50 μg/ml streptomycin. PM1/CCR5 cells were generated by standard retrovirus-mediated transduction of PM1 cells with pBABE-CCR5 provided by the National Institutes of Health AIDS Research and Preference Reagent Program [4]. PM1 and PM1/CCR5 cells were analysed for their surface expressions of CD4, CCR5 and CXCR4 using a FACS Calibur (Becton Dickinson, Franklin Lakes, New Jersey, USA). 293T cells were maintained in Dulbecco’s modified Eagle medium (DMEM; Sigma) supplemented with 10% heat-inactivated FCS. The CD4 human osteogenic sarcoma cell line GHOST was maintained in DMEM supplemented with 10% FCS, 200 μg/ml G418 (Gibco BRL, Rockville, Maryland, USA) and 100 μg/ml hygromycin B (Sigma). The GHOST derivatives GHOST-hi5 and GHOST-CXCR4 stably expressed CCR5 and CXCR4, respectively, as described elsewhere [5], and were selected with 1 μg/ml puromycin (Sigma).

17b, a CD4-induced (CD4i) MAb, was a kind gift from J. Robinson (Department of Pediatrics, Tulane University Medical Center, New Orleans, Louisiana, USA). 447-52D, an anti-gp120 V3 MAb, was a kind gift from S. Zolla-Pazner (Department of Pathology, New York University School of Medicine, New York, USA). 2D7, an anti-CCR5 MAb, and RPA-T4, an anti-CD4 MAb, were purchased from BD Biosciences Pharmingen (San Jose, California, USA). 2’,3’-dideoxyinosine (ddI, didanosine) was from Calbiochem, San Diego, California, USA. 3’-thiacytidine (3TC, lamivudine) was a kind gift from R. F. Schinazi (Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, USA). Saquinavir (SQV) was kindly provided by Roche Products Ltd., Welwyn Garden City, UK. Amprenavir (APV) was a kind gift from GlaxoSmithKline, Middlesex, UK. Nelfinavir (NFV) and indinavir (IDV) were kindly provided by Japan Energy Inc., Tokyo, Japan. Recombinant human soluble CD4 (sCD4), MIP-1α, MIP-1β and RANTES were from R&D Systems Inc., Abingdon, UK. The CCR5 inhibitors TAK-779 [6] and SCH-351125 (SCH-C) [7] were synthesized as previously described. AK-602, a CCR5 inhibitor, was kindly provided by Ono Pharmaceutical Co. Ltd., Osaka, Japan [8].

Isolation of a KD-247-resistant mutant from JR-FL in vitro

For the selection of a KD-247 escape virus, JR-FL [9] was treated with various concentrations of KD-247 and then infected in PM1/CCR5 cells as previously described with minor modifications [10]. Viral replication was monitored by observation of any cytopathic effects in PM1/CCR5 cells. The culture supernatant was harvested on day 7 and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of increasing concentrations of KD-247. After the virus was passaged in the presence of up to 1000 μg/ml KD-247 in PM1/CCR5 cells, a KD-247-resistant virus, JR-FL(1000)8P, was recovered from the cell culture supernatant. JR-FL was also passaged for the same time periods in PM1/CCR5 cells in the absence of KD-247 to exclude any effects of the long-term culture of eight passages, and the resulting virus was designated JR-FL(−)8P.

The sensitivities of the passage 8 JR-FL viruses in the presence or absence of KD-247 to various drugs or MAb were determined as previously described with minor modifications [11]. Briefly, PM1-CCR5 cells (2 × 10^5 well) were exposed to 100 50% tissue culture inhibitory doses (TCID_{50}) of the JR-FL(1000)8P or JR-FL(−)8P in the presence of various concentrations of drugs or MAb in 96-well round-bottom plates. The 50% inhibitory concentration (IC_{50}) values were determined using the MTT {3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium}
bromide) (MTT) assay on day 7 of culture. All assays were performed in duplicate.

Viral RNA (0.5 µg) extracts from cell culture supernatants at several concentrations of KD-247 were reverse-transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA). The cDNA obtained were subjected to PCR amplification using Taq polymerase. After cloning the amplified products into pCR2.1, the Env regions in both the passaged and selected viruses were sequenced using an ABI PRISM 310 automated DNA sequencer (Applied Biosystems).

**Construction of mutant envelope expression vectors and production of pseudovirions**

For the construction of mutant envelope expression vectors, we used pCXN2, which contains a chicken actin promoter. Briefly, the JR-FL env region was cloned by PCR and ligated into pCR2.1, generating pCR2-FLwt. The EcoRI fragment of pCR2-FLwt containing the entire env region was ligated into pCXN2 to give pCXN-FLwt [9]. A mutant Env (G314E) expression vector was generated from pCXN-FLwt using a QuickChange site-directed mutagenesis kit (Stratagene, Chedar Creek, Texas, USA) and the primers JR-FLv3G/Env (5’–TACATA-TAGGACCCAGAGGAGCATTATAC–3’) and JR-FLv3G/Env (5’–GTATAAAAATGCTCTCTGGTCC TATATGTA–3’) according to the manufacturer’s protocol, and designated pCXN-FLG314E.

Plasmids pNL4-3.Luc.R.*E* and pRSV-Rev [12], supplied by the NIH AIDS Research and Reference Reagent Program, and plasmid pCXN2, expressing wild-type or G314E Env, were cotransfected into 293T cells using the Effectene Transfection Reagent (Qiagen, Valencia, California, USA). At 24 h after the transfection, the pseudovirus-containing supernatants were harvested, filtered through a 0.2-µm pore-size filter and stored at −150°C. For measurement of the pseudovirus activities, a luminescence assay with GHOST-hi5 cells was used as previously described [13].

**Neutralization assays**

A single-cycle infectivity assay was used to measure the neutralization of JR-FLwt or JR-FLGPER pseudovirions as described previously [13]. Briefly, MAb at various concentrations and a pseudovirus suspension corresponding to 200 TCID₅₀ were preincubated for 15 min on ice. The virus–antibody mixtures were added to GHOST-hi5 cells, which had been seeded in a 96-well plate (1.5 × 10⁴ cells/well) on the previous day. The cultures were incubated for 2 days at 37°C, washed with phosphate-buffered saline and lysed with lysis buffer (Luc PGC-50; PicaGene, Tokyo, Japan). Following transfer of the cell lysates to luminometer plates (Costar 3912), the luciferase activity (in relative light units) in each well was measured using Luciferase Substrate (100 µl/well; PicaGene) in a TR717 microplate luminometer (Applied Biosystems). The reduction in infectivity was determined by comparing the relative light units in the presence and absence of MAb and expressed as the percent neutralization. The same assay was repeated two to three times.

**In vitro binding assay to the JR-FLwt or JR-FLGPER envelope**

The JR-FL gp160 coding sequence was amplified from the infectious clone vector (pJR-FL) using the primers ENVAPHI-HindIII and pRSV-Rev [12], and subcloned into the corresponding sites in pDNR-JR-FL wt using a QuikChange site-directed mutagenesis kit and the primers JR-FLv3G/Env and JR-FLv3G/Env according to the manufacturer’s protocol. The wild-type and mutated env gene fragments were then subcloned into pLP-IRE2-EGFP (Clontech) using Cre-recombinase (Clontech) according to the manufacturer’s instructions, and designated pLP-EGFP-JR-FLwt and pLP-EGFP-JR-FLGPER, respectively.

293T cells were cotransfected with pRSV-Rev (0.5 µg) and pLP-IRE2-EGFP, pLP-EGFP-JR-FLwt or pLP-EGFP-JR-FLGPER (9.5 µg) using the Effectene Transfection Reagent (Qiagen). After 36 h, the cells were harvested, incubated with each anti-HIV-1 MAb with or without rsCD4 (0.5 µg/ml) in combination with biotin-conjugated anti-human IgG and peridinin chlorophyll-a protein-conjugated streptavidin (BD Biosciences Pharmingen), gated for the GFP-positive area and analyzed using a FACSCalibur.

**Data analysis and evaluation of synergy**

Analysis of the synergistic, additive or antagonist effects of the antiviral agents was first performed according to the median effect principle using the CalcuSyn version 2 computer program [14,15] to provide estimates of the IC₅₀ values of the antiviral reagents in different combinations. Combination indices (CI) were estimated from the data and reflected the nature of the interactions between KD-247 and the CCR5 inhibitors against JR-FL(–)8P in PM1/CCR5 cells, as evaluated using the MTT assay. Specifically, CI < 0.9 indicated synergy, 0.9 < CI < 1.1 indicated additive and CI > 1.1 indicated antagonism. The value of CI was directly proportional to the amount of synergy in the combination regimen. For example, values of CI < 0.5 represented a high degree of synergy, whereas values of CI > 1.5 represented significant antagonism. This approach has been widely used in analyses of antiviral interactions and was chosen to allow comparability with published literature.
**Statistical analysis**

Statistical correlations were analysed using Student’s t-test. \( P < 0.05 \) were considered statistically significant.

**Results**

**Selection of a KD-247 escape variant**

For the isolation of a KD-247 escape mutant from R5 HIV 

in vitro, PM1 cells expressing high levels of CCR5, designated PM1/CCR5 cells, which were highly sensitive to both X4 and R5 HIV infection and accompanied by prominent syncytia were used as the target cells. An R5 HIV strain, JR-FL, which uses CCR5 as its coreceptor was used for the selection of a KD-247 escape virus.

In order to select an HIV-1 variant that can escape from neutralization by KD-247 in vitro, we exposed PM1/CCR5 cells to JR-FL, and serially passaged the virus in the presence or absence of KD-247, designated PM1/CCR5 cells to JR-FL, and serially passaged the virus in CCR5 as its coreceptor was used for the selection of a KD-247 escape variant. For the isolation of a KD-247 escape mutant from R5 HIV strains, we exposed PM1/CCR5 cells (2 × 10^5) to 100 TCID50 of JR-FL(−)8P or JR-FL(1000)8P, and then cultured in the presence of various concentrations of MAb or inhibitors. The IC_{50} values were determined using the MTT assay on day 7 of culture. Data shown represent values derived from the results of two or three independent experiments conducted in duplicate.

**Sequencing of the envelope region of the KD-247 escape mutant**

To determine the region responsible for the reduced sensitivity of the escape mutant to KD-247, the C1–C4 regions of the envelope were sequenced after cloning of the PCR product of each region using cDNA synthesized from viral RNA obtained from the supernatants of infected cells as templates. A total of 12–16 clones for each PCR product were isolated and sequenced. Analyses of the env sequences of these products revealed that the selected virus had a Gly→Etu substitution at codon 314 (G314E) in the V3 region of the envelope at passage 7 (600 μg/ml; 10/12 clones) and passage 8 (1000 μg/ml; 12/16 clones) (Fig. 1). Some changes in the envelope sequence in other regions, including C1, V1, V2, C2, C3, V4, and C4 of the escape mutant were found as well as in V3 around the IGPGR sequence even at early time points in the presence of the selective pressure. It is possible that these mutations also confer resistance to KD-247 but lead to virus of decreased fitness and thus they did not expand in a next passage except for the G314E. On the other hand, the virus passaged in PM1/CCR5 cells was initially propagated in the presence of 1 μg/ml KD-247, and during the course of the selection procedure, the MAb concentration was increased to 1000 μg/ml. At passage 8, the supernatants containing the passaged viruses in the presence or absence of KD-247, designated JR-FL(1000)8P and JR-FL(−)8P, respectively, were harvested and titrated for their infectivities and sensitivities to KD-247, CCR5 inhibitors (TAK-779, SCH-C and AK-602), nucleoside reverse transcriptase inhibitors (SCH-C7, SCH-C, and AK-602), and anti-CD4 antibodies (RPA-T4) and inhibitors (TAK-779, SCH-C, and AK-602), as evaluated by the MTT assay and AK-602, designated PM1/CCR5 cells, which were highly sensitive to both X4 and R5 HIV infection and accompanied by prominent syncytia were used as the target cells. An R5 HIV strain, JR-FL, which uses CCR5 as its coreceptor was used for the selection of a KD-247 escape virus.

In order to select an HIV-1 variant that can escape from neutralization by KD-247 in vitro, we exposed PM1/CCR5 cells to JR-FL, and serially passaged the virus in the presence or absence of KD-247, designated PM1/CCR5 cells to JR-FL, and serially passaged the virus in CCR5 as its coreceptor was used for the selection of a KD-247 escape variant. For the isolation of a KD-247 escape mutant from R5 HIV strains, we exposed PM1/CCR5 cells (2 × 10^5) to 100 TCID50 of JR-FL(−)8P or JR-FL(1000)8P, and then cultured in the presence of various concentrations of MAb or inhibitors. The IC_{50} values were determined using the MTT assay on day 7 of culture. Data shown represent values derived from the results of two or three independent experiments conducted in duplicate.

### Table 1. Anti-HIV-1 activities of various MAb and inhibitors toward KD247-resistant JR-FL.

<table>
<thead>
<tr>
<th>Antibody or inhibitor</th>
<th>JR-FL(−)8P</th>
<th>JR-FL(1000)8P</th>
<th>Fold change</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>KD-247 (μg/ml)</td>
<td>6.3 ± 5.0</td>
<td>&gt; 100</td>
<td>&gt; 16</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>TAK-779 (nM)</td>
<td>217 ± 50.3</td>
<td>54.7 ± 29.5</td>
<td>0.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>SCH-C (nM)</td>
<td>27.5 ± 3.5</td>
<td>8.0 ± 1.4</td>
<td>0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>AK-602 (nM)</td>
<td>7.1 ± 4.5</td>
<td>0.15 ± 0.08</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Didanosine (μM)</td>
<td>1.0 ± 0.57</td>
<td>1.0 ± 0.27</td>
<td>1.0</td>
<td>0.98</td>
</tr>
<tr>
<td>Lamivudine (μM)</td>
<td>0.33 ± 0.01</td>
<td>0.29 ± 0.04</td>
<td>0.9</td>
<td>0.30</td>
</tr>
<tr>
<td>Nelfinavir (μM)</td>
<td>0.03 ± 0.001</td>
<td>0.036 ± 0.006</td>
<td>1.1</td>
<td>0.56</td>
</tr>
<tr>
<td>Indinavir (μM)</td>
<td>0.017 ± 0.004</td>
<td>0.016 ± 0.005</td>
<td>0.9</td>
<td>0.85</td>
</tr>
<tr>
<td>Amprenavir (μM)</td>
<td>0.022 ± 0.001</td>
<td>0.017 ± 0.008</td>
<td>0.8</td>
<td>0.47</td>
</tr>
<tr>
<td>Saquinavir (μM)</td>
<td>0.0038 ± 0.0004</td>
<td>0.0034 ± 0.0004</td>
<td>0.9</td>
<td>0.42</td>
</tr>
<tr>
<td>rsCD4 (μg/ml)</td>
<td>3.3 ± 0.07</td>
<td>0.57 ± 0.48</td>
<td>0.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Anti-CD4 MAb (RPA-T4) (μg/ml)</td>
<td>0.01 ± 0.004</td>
<td>0.03 ± 0.004</td>
<td>3.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Anti-CCR5 MAb (2D7) (μg/ml)</td>
<td>0.19 ± 0.03</td>
<td>0.066 ± 0.005</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>MIP-1α (μg/ml)</td>
<td>0.0086 ± 0.002</td>
<td>0.0029 ± 0.001</td>
<td>0.5</td>
<td>0.11</td>
</tr>
<tr>
<td>MIP-1β (μg/ml)</td>
<td>0.39 ± 0.08</td>
<td>0.23 ± 0.18</td>
<td>0.6</td>
<td>0.22</td>
</tr>
<tr>
<td>RANTES (μg/ml)</td>
<td>0.045 ± 0.0007</td>
<td>0.005 ± 0.001</td>
<td>0.1</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^a\)PM1/CCR5 cells (2 × 10^5) were exposed to 100 TCID50 of JR-FL(−)8P or JR-FL(1000)8P and then cultured in the presence of various concentrations of MAb or inhibitors. The IC_{50} values were determined using the MTT assay on day 7 of culture. Data shown represent values derived from the results of two or three independent experiments conducted in duplicate.

\(^b\)\( P < 0.05 \) were considered statistically significant (shown in bold type). IC, Inhibitory concentration.
without KD-247 did not show the G314E substitution at either passage 4 (0/12 clones) or passage 8 (0/16 clones) (Fig. 1).

**Susceptibilities of HIV-1 containing the KD-247-associated G314E substitution to MAb and drugs**

To confirm whether the G314E mutation was responsible for the reduced sensitivity to KD-247, a single-round replication assay was performed. Luciferase-reporter viruses were pseudotyped with wild-type JR-FL (JR-FLwt) or singly mutated with G314E (JR-FLG314E) in the V3 region. As shown in Fig. 2a, JR-FLG314E was completely resistant to KD-247 up to 100 µg/ml. We also examined the sensitivities of the pseudotyped clones to rsCD4, anti-CD4 MAb RPA-T4 and anti-CCR5 MAb 2D7 by a single-round replication assay (Fig. 2b–d). As expected, JR-FLG314E was more sensitive to rsCD4 and 2D7, but fourfold more resistant to RPA-T4, compared to JR-FLwt, similar to the results for the passaged viruses with or without KD-247.

Next, we determined the sensitivities of JR-FLG314E to three CCR5 inhibitors (TAK-779, SCH-C and AK-602). The IC₅₀ values of TAK-779, SCH-C and AK-602 against JR-FLG314E were 20-, 10- and 5-fold lower than the corresponding values against JR-FLwt, respectively (Fig. 2e–g). These results confirmed that the G314E mutation was associated with the observed reduction in the sensitivities of JR-FL(1000)P to KD-247 and RPA-T4, and also with the increased sensitivities to rsCD4, 2D7 and CCR5 inhibitors.

Next, we analysed the sensitivities of JR-FLwt and JR-FLG314E to another broad-specificity neutralizing anti-V3 MAb 447-52D and the CD4i MAb 17b (Fig. 2h and i). Interestingly, JR-FLG314E was more sensitive to both 17b (≤ 0.8-fold change in the IC₅₀) and 447-52D (0.1-fold change in the IC₅₀) than JR-FLwt (Fig. 2h and i). A similar result regarding neutralization sensitivity to 17b was reported when viruses were pretreated with rsCD4 [16]. In our result, JR-FLG314E was more sensitive to 17b.

---

**Fig. 1.** V3 amino acid sequences from the supernatants of JR-FL-infected PM1/CCR5 cells passaged in the presence or absence of KD-247. Viral RNA from the cell culture supernatants at several concentrations of KD-247 was reverse-transcribed. After subjecting the obtained cDNA to PCR amplification and cloning, the env regions in the viruses passaged in the presence or absence of KD-247 were sequenced. The wild-type JR-FL amino acid sequence of V3 is shown at the top. The numbers on the right show the numbers of clones with the listed sequence among the total number of clones tested. In each set of clones, the deduced amino acid sequence of the V3 region was aligned by the single amino acid code. Dots denote sequence identity. DS, Direct sequence.
than JR-FLwt preincubated with rsCD4 (1 µg/ml) (Fig. 2i).

Comparison of antibody binding to cell surface-expressed wild-type and GPER mutant Env
To elucidate the mechanism of the increased sensitivities of the escape virus with the G314E mutation in the V3-tip to 17b and 447-52D, wild-type or mutant Env-expressing 293T cells were established by transfecting each Env expression plasmid, and then stained with the MAb in the presence or absence of rsCD4 (0.5 µg/ml). Binding of a patient’s IgG, KD-247, 17b or 447-52D, to the surface-expressed Env proteins was assayed using a fluorescence-activated cell sorter analysis. As shown in Fig. 3, KD-247 bound to the wild-type JR-FL Env, but not the GPER mutant Env, while the other anti-V3 MAb, 447-52D, bound to both the Env proteins very well, especially the mutant Env. The mean fluorescence intensity (MFI) of 447-52D increased from 87.56 (wild-type Env) to 219.47 (GPER Env). Without rsCD4, the CD4i 17b MAb bound slightly to the wild-type Env (MFI, 33.21; Fig. 3) but failed to neutralize JR-FLwt (Fig. 2i). On the other hand, in the presence of rsCD4 (0.5 µg/ml), a shift in the MFI was observed with 17b binding to the surface of wild-type-Env-expressing cells. In contrast to these data for wild-type Env, 17b bound to the mutant Env efficiently in the absence of rsCD4 at a higher level than to the wild-type Env in the presence of rsCD4 (MFI, 97.33 for the mutant Env versus 56.61 for the wild-type Env; Fig. 3). 17b was also able to neutralize JR-FLGPER, even in the absence of rsCD4 (Fig. 2i). These results suggest that the G314E mutation in the V3-tip induces the expression of cryptic epitopes for antibodies against the CD4i epitope and V3 loop, such that the mutant virus is neutralized by the CD4i MAb without rsCD4 or by lower concentrations of the anti-V3 MAb compared with the wild-type virus.

Highly synergistic interactions of KD-247 combined with CCR5 inhibitors
Both neutralizing MAb and chemokine receptor inhibitors attack the viral entry process, especially at the stage of the chemokine receptor-gp120 (V3)
interaction. Each of them binds to either CCR5 or gp120. Furthermore, our present observations suggest that the neutralizing MAb KD-247 selects an escape variant with greater sensitivity to chemokine receptor inhibitors. Based on this notion, we attempted to test the synergy of this MAb with chemokine receptor inhibitors against wild-type JR-FL.

The multiple-drug-effect analysis of Chou and colleagues [14,15] was used to analyse the effects of combinations of KD-247 with CCR5 inhibitors against JR-FL(C08P) in PM1/CCR5 cells (Table 2). As shown in Table 2, all the CI values for KD-247 with the CCR5 inhibitors (TAK-779, AK-602 or SCH-C) were <0.5 at all the inhibitory concentrations tested. In particular, the CI values for the combinations of KD-247 with SCH-C and AK-602 were less than 0.1 for IC90. These results suggest that combination of KD-247 with any of the tested CCR5 inhibitors produces very highly synergistic interactions at not only high but also low inhibitory concentrations. We further evaluated the in vitro interactions between KD-247 and representatives of each class of currently available antiretroviral agents. Although KD-247 had favourable drug interactions with all of the agents (data not shown), the synergistic effects of KD-247 and CCR5 inhibitors were the most potent among all the combinations tested in this study.

Table 2. Combination indices (CI) for KD-247 and CCR5 inhibitors against virus JR-FL(C08P).

<table>
<thead>
<tr>
<th>CCR5 inhibitor used in combination with KD-247 (2.5–160 μg/ml)</th>
<th>IC50</th>
<th>IC25</th>
<th>IC90</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK-602 (0.3–20 nM)</td>
<td>0.21</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>TAK-779 (12.5–800 nM)</td>
<td>0.23</td>
<td>0.19</td>
<td>0.16</td>
</tr>
<tr>
<td>SCH-C (3–100 nM)</td>
<td>0.18</td>
<td>0.08</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*The multiple-drug-effect analysis of Chou and colleagues was used to analyse the effects of the drugs in combination [15]. IC, Inhibitory concentration. CI < 1, synergy; 0.9 < CI < 1.1, additivity; CI > 1.1, antagonism.

Discussion

Although KD-247 shows clinical promise as a passive immunization agent for suppressing viral spread in phenotype-matched HIV-infected individuals, we also know that HIV-1 always escapes from the selection pressure of any one inhibitor by obtaining mutation(s). Therefore, we induced an HIV-1 variant that could escape from neutralization by KD-247 in vitro by continuously exposing the R5 virus JR-FL to increasing concentrations of KD-247 and defined the virological...
properties and susceptibilities of this variant to other monoclonal antibodies (CD4i, anti-V3, anti-CD4 and anti-CCR5 MAb). The present data suggest that the KD-247 escape variant, which has a G314E mutation in the V3-tip, has not only a highly resistant phenotype against KD-247 but also greater sensitivities to CCR5 inhibitors and rsCD4, and needs higher concentration of anti-CD4 antibody for entry blocking compared with the corresponding control virus after eight passages in the absence of KD-247. These phenomena were confirmed using a pseudotyped virus containing the KD-247 escape-related G314E mutation by a single-round neutralizing assay. No previous studies have reported this G314E mutation in the V3-tip region of the R5 virus using in vitro selection by MAb. This mutation is also very rare in clinical isolates from HIV-1-infected patients [17]. Interestingly, this mutation in the V3-tip also influences the sensitivities to CCR5 inhibitors, rsCD4, anti-CD4 MAbs and CD4i MAbs 17b. It is not clear why KD-247 escape mutant became sensitive to rsCD4 and CCR5 inhibitors. It is conceivable that higher expression of CCR5 and CD4 on PM1/CCR5 cells may have some effect on the selection of such phenotype.

The ability to provide effective long-term antiretroviral therapy for HIV-1 infection has become a complex issue, since 40–50% of patients who initially achieve favourable viral suppression to undetectable levels subsequently experience treatment failure [18]. Moreover, a recent study reported that viruses with resistance to at least one drug were present in 1 of 10 antiretroviral-naive patients in Europe [19]. As more drug-resistant HIV-1 isolates emerge, new classes of potent antiretroviral agents targeting different steps of the HIV replicative cycle and new combinations of agents targeting different molecules, such as gp120 and CD4 or CCR5, are a welcome addition to the HIV arsenal. CCR5 inhibitors represent a new class of agents aimed at inhibiting viral entry. Following binding of gp120 to the CD4 receptor, CCR5 antagonists inhibit the interaction of gp120 with its coreceptor, an integral step in the fusion of HIV to the host cell [6–8]. As with other antiretroviral agents, resistance will likely prove to be a problem for CCR5 inhibitors [4,20]. Thus, the best strategy for preventing the occurrence of resistance is to use them in combination with other potent antiretroviral drugs. In the present study, we found that combinations of KD-247 with CCR5 inhibitors showed very strong synergistic interactions. When both antiviral reagents become available in the near future, these combinations will represent an efficient weapon against HIV-1. However, the benefit of these combinations to patients with HIV-1 infection needs to be further evaluated in clinical trials.

Taken together, the present data suggest that KD-247 has at least five advantages: (i) it exerts potent activity against a wide spectrum of subtype B HIV-1 variants, presumably due to its interaction with the IGPGR sequence in the gp120 V3 tip; (ii) viral acquisition of KD-247-resistance requires a very high concentration of KD-247 in vitro; (iii) at least some representatives of each class of currently available antiretroviral agents remain active against the virus variant selected in vitro with KD-247; (iv) the escape variant becomes more sensitive to CCR5 inhibitors and rsCD4, and is less dependent on CD4 binding for entry; and (v) combinations of KD-247 with CCR5 inhibitors show highly synergistic interactions at all inhibitory concentrations tested to date.

Acknowledgements

We thank J. Robinson for kindly providing the 17b, S. Zolla-Pazner for kindly providing the 447-52D, and Hirotomo Nakata, Kenji Maeda and Yasuhiro Kou for technical support. We also thank Yuki Azakami for excellent technical assistance.

This work was supported in part by the Ministry of Health, Labor and Welfare of Japan (H-16-AIDS-001 and -012), Grant-in-aid for Scientific Research (C-18591119) from the Ministry of Education, Science and Culture of Japan and the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Re-emerging Infectious Diseases.

References