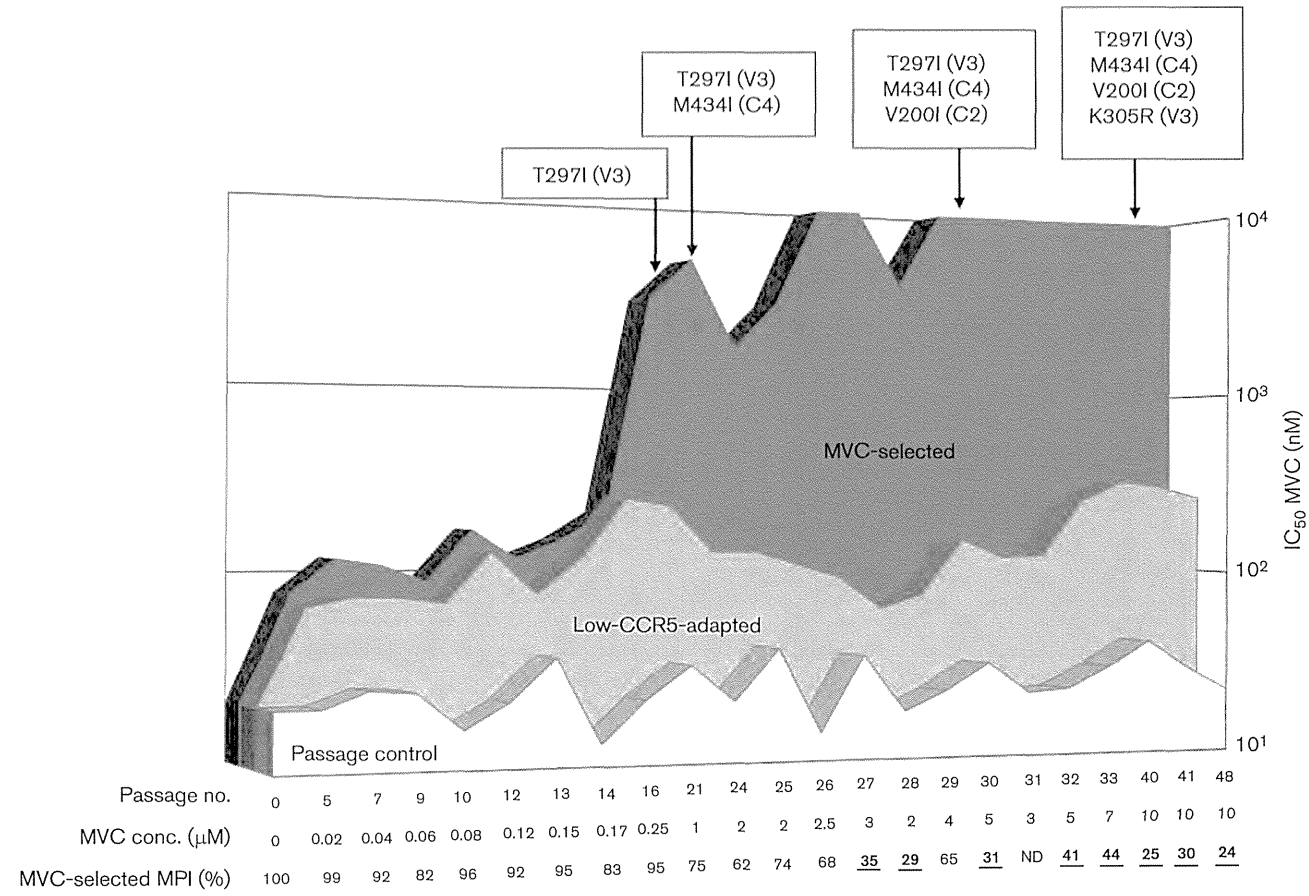

Fig. 2. Susceptibility of passaged variants to MVC. The sensitivity and MPI of each passaged variant to MVC was determined by a multi-round assay using the WST-8 assay as described in Methods. The *x*-axis shows the passage number, concentration of MVC (μM) and MPI values. The mutations observed in the highly MVC-resistant variants are shown above the graph.

1 to 14, the MVC-selected and low-CCR5-adapted variants had almost equal IC_{50} values and the MPIs were high. After 16 passages, the IC_{50} values of the MVC-selected variants continued to increase to >10 μM, whilst the MPIs decreased to 24 % at 48 passages, especially after 27 passages. The low-CCR5-adapted variants maintained an IC_{50} value of ~200 nM and high MPIs (90–100 %) until the end of the experiment (passage 48). Conversely, the passage control variants did not show remarkable changes in their IC_{50} values and MPIs throughout the passages (IC_{50} values of ~20 nM, MPI 95–100 %). The low-CCR5-adapted variant was also resistant to two other CCR5 inhibitors, APL and TAK-779 (data not shown).

These findings suggested that the phenotype of the MVC-selected variants under low concentrations of the drug corresponded with that of the low-CCR5-adapted variants until 14 passages; then, under high concentrations, the MVC-selected variants acquired additional mutations for high resistance to the CCR5 inhibitor.

Comparison of the Env region sequences of the MVC-selected and low-CCR5-adapted mutants

To determine the genetic basis of the resistance in the HIV-1_{KP-5} variants and compare the substitutions between the MVC-selected and low-CCR5-adapted variants, the Env genes were sequenced (Figs 3, 4 and S2). At 17 passages, all substitutions in both the MVC-selected and low-CCR5-adapted variants were selected from the baseline viruses. Five of these substitutions in gp120, i.e. K8R, C11W (signal peptide), D141N (V1), E321D (V3) and I463T (V5), were observed in both passaged variants. Conversely, at positions 137 (K or E), 148 (Q or K) and 187 (G or D), the amino acids differed between the MVC-selected and low-CCR5-adapted variants. After 16 passages, the MVC-selected variants acquired four additional mutations, i.e. T297I (V3), M434I (C4), V200I (C2) and K305R (V3), at passages 17, 21, 34 and 41, respectively, which were not observed in the low-CCR5-adapted variants (Figs 2–4 and S2). After acquisition of M434I in C4 (21 passages), the MPI of the MVC-selected variants decreased gradually

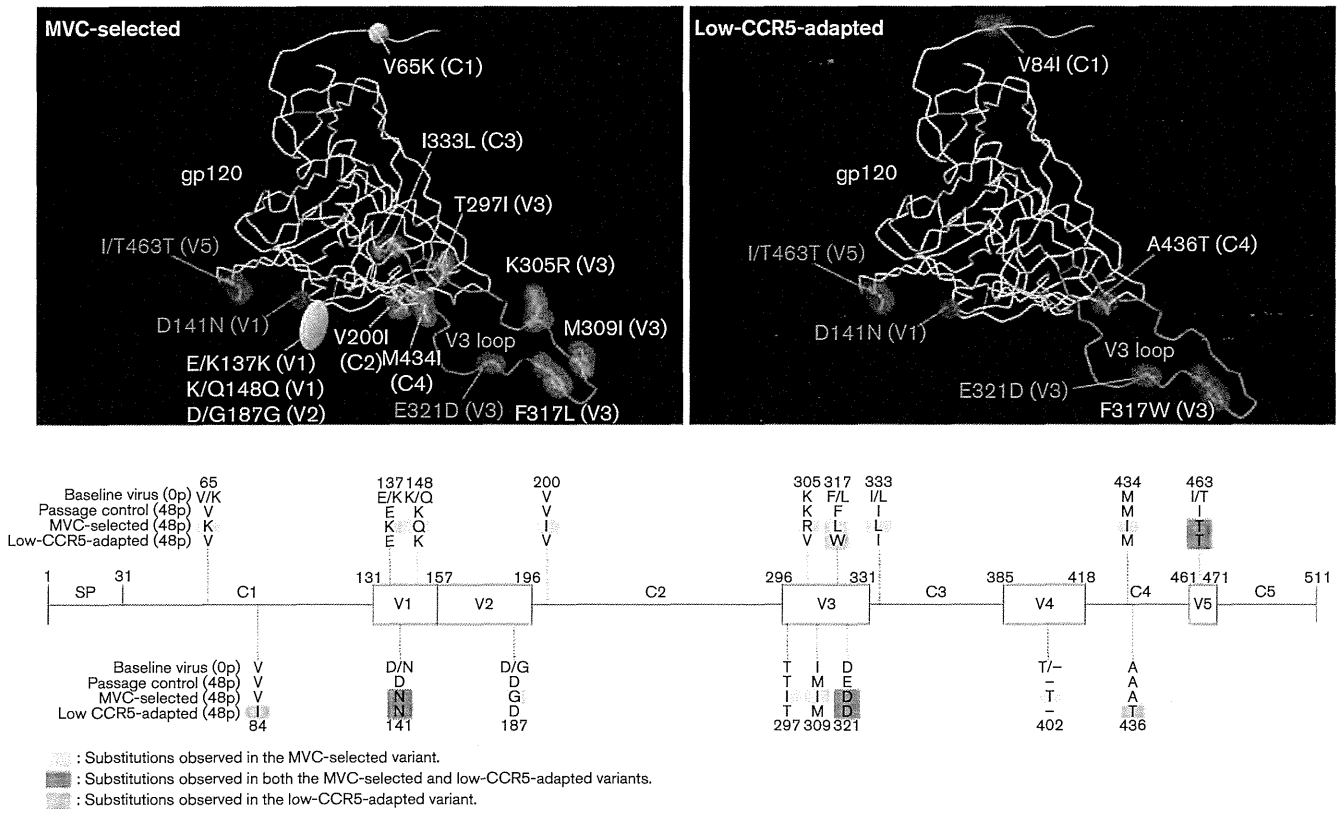

Fig. 3. Comparison of the locations of the mutations in the MVC-selected and low-CCR5-adapted gp120. The side chains of the mutated residues that appeared during the MVC selection (left) and low CCR5 adaptation (right) are shown in yellow (only MVC selection), pink (only low CCR5 adaptation) and green (both). A summary schema is also provided.

(from 74 to 24 %) (Fig. 2). The most important amino acid substitution for the reduction in the MPI might be K305R, because the MPI of the variant cultured without MVC after 48 passages increased by reverting from R to K at position 305 (data not shown). Three additional mutations, i.e. F317W (V3), V84I (C1) and A436T (C4), were observed in the low-CCR5-adapted variants at 17, 21 and 48 passages, respectively. These mutations might be compensatory for viral fitness following culture in the low-CCR5-expressing cells, because the MPIs of the variants with these three mutations did not differ from those of the variants prior to the acquisition of these mutations (>90 %).

These findings suggest that under low concentrations of MVC, the variants were selected from the baseline viruses similarly to the low-CCR5-adapted variants (IC_{50} shift and high MPI), whilst under high concentrations of the drug, the selected variants required additional mutations to use drug-bound coreceptors for entry into the target cells.

To compare the two mutation profiles obtained from the MVC-selected and low-CCR5-adapted variants at 48 passages, the crystal structure of gp120 was used (Figs 3 and 4). Comparison of the sequences of the two passaged variants based on the Protein Data Bank (PDB ID: 2B4C) crystal structure of gp120 showed that the MVC-selected variant harboured many substitutions within and around the V3 region, i.e. the CCR5 N-terminal-binding site, compared with the low-CCR5-adapted variant in the three-dimensional (3D) position. In a magnification of the CCR5 N-terminal-binding site (Fig. 4), three of four mutations, i.e. T297I, M434I and V200I, were concentrated around the V3 base and finally K305R appeared in the V3 stem region after 41 passages.

To determine the positions of MVC-selected mutations in the gp120 trimer form, we illustrated the sites of mutations on the structure of the BG505 SOSIP trimer obtained from the PDB (ID: 3J5M) (Fig. S3) (Lyumkis *et al.*, 2013). Almost all of the MVC-selected mutations occurred at the upper and outer side of the trimer. Several MVC-selected mutations, i.e. V65K, V200I, K305R, M309I, F317L and M434I, lay relatively close to the neighbouring gp120. These findings demonstrated that these mutations may affect trimer formation and expose neutralizing antibody epitopes.

Susceptibilities of the infectious clones with mutant Env to anti-Env mAbs

In a previous study, a CCR5 inhibitor (AD101)-resistant infectious clone was sensitive to neutralization via V3 and

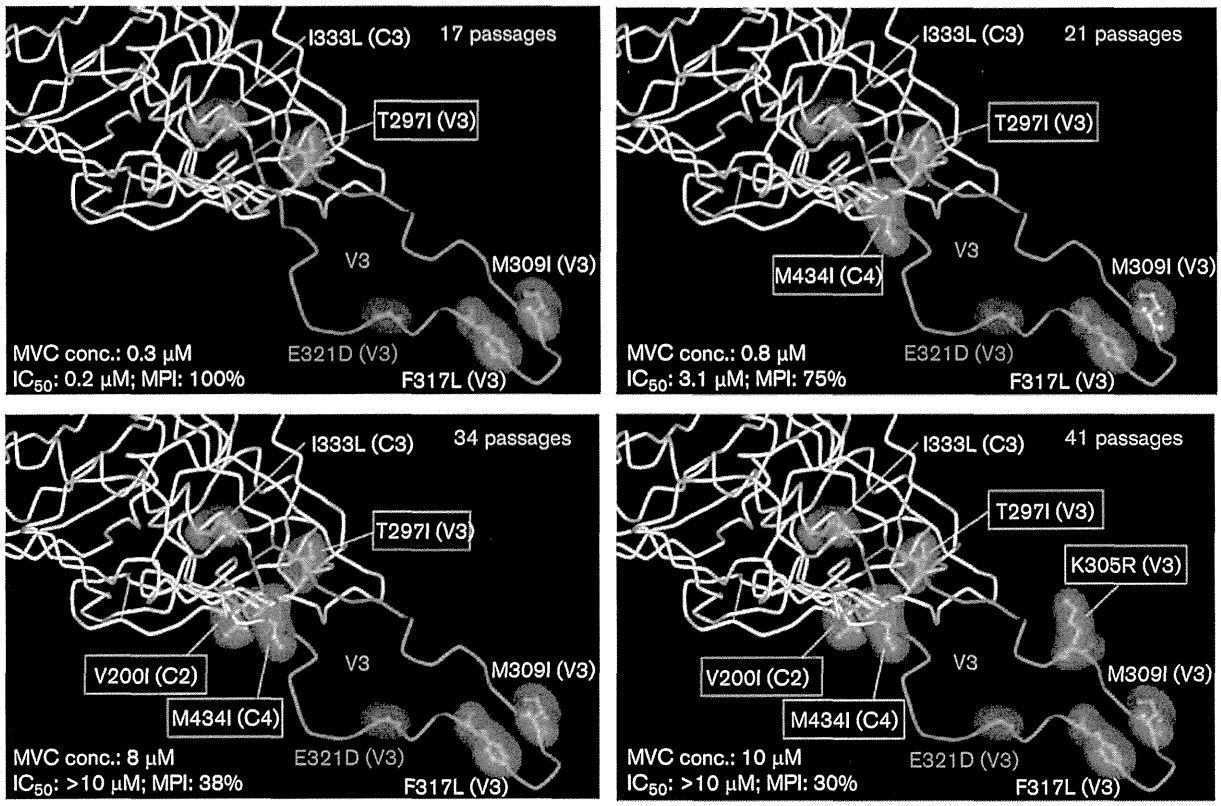

Fig. 4. Enlargement of the area of the CCR5 N-terminal-binding site and V3 loop in gp120. The side chains of the mutated residues that appeared during *in vitro* selection with MVC at 17 (upper left), 21 (upper right), 34 (lower left) and 41 passages (lower right) are shown. The crystal structure of gp120 was retrieved from the Protein Data Bank (PDB ID: 2B4C).

CD4i epitopes (Berro *et al.*, 2009). To examine whether our three passaged variants became sensitive to anti-Env mAbs, we constructed three infectious clones with each 48-passaged Env (Fig. 5). The clone with the Env of the MVC-selected variant showed a low MPI (56 %) under a high concentration of MVC, which was also seen with the passage control and low-CCR5-adapted clones (Fig. 5a). Using these infectious clones, we tested the susceptibilities to the anti-Env mAbs b12 [anti-CD4 binding site (anti-CD4bs)], 4E9C (anti-CD4i) and KD-247 (anti-V3). As shown in Fig. 5(b), the MVC-selected and low-CCR5-adapted clones showed higher sensitivity to b12 than the passage control clone, with IC_{50} values of 0.22, 0.31 and 0.86 μg ml^{-1}, respectively. The MVC-selected and low-CCR5-adapted clones became highly sensitive to 4E9C compared with the passage control clone (IC_{50} values of 0.08, 0.41 and >5 μg ml^{-1}, respectively) (Fig. 5c). Moreover, the clone with the MVC-selected Env was highly sensitive to anti-V3 mAb KD-247, while the low-CCR5-adapted and passage control clones were not (IC_{50} values of 0.04, >100 and >100 μg ml^{-1}, respectively) (Fig. 5d).

These findings indicated that the MVC-selected clone with its greater number of mutations might contribute to exposure of neutralizing epitopes for these three mAbs, whilst the low-CCR5-adapted mutations could change the conformation of Env to become sensitive to anti-CD4i and CD4bs mAbs, but not anti-V3 mAb.

Susceptibilities of the infectious clones with mutant Env to autologous plasma IgGs

We also examined whether the infectious clones with the passaged Env mutations were neutralized by autologous plasma IgGs. As shown in Fig. 6(a), none of the autologous plasma IgGs could neutralize the passage control clone at concentrations up to 100 μg ml^{-1}. In the low-CCR5-adapted clone, some of the plasma IgGs slightly inhibited the replication of the virus under high concentrations, but did not reach the 50 % inhibition level (Fig. 6b). Conversely, all seven plasma IgGs were able to completely neutralize the clone with the MVC-selected Env (IC_{50} 2.6–37 μg ml^{-1}, MPI 79–97 %) (Fig. 6c).

These findings show that the MVC-selected clone with the greater number of mutations also might contribute to exposure of neutralizing epitopes for autologous plasma IgGs.

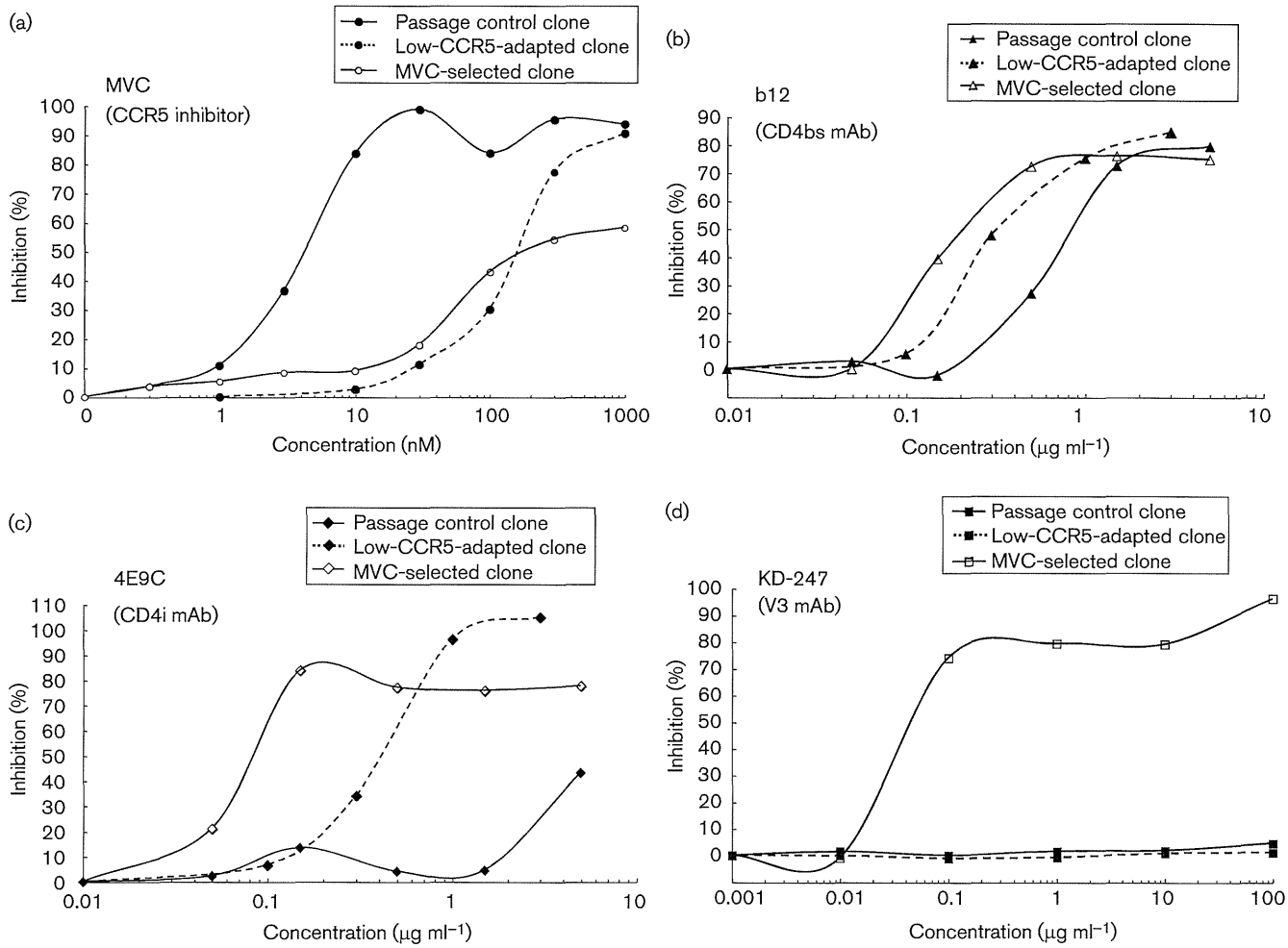

Fig. 5. Sensitivities of infectious clones with the passage control, low-CCR5-adapted and MVC-selected Env mutations to MVC and anti-Env mAbs. The sensitivities of the infectious clones with the passage control (filled symbols), low-CCR5-adapted (filled symbols and dotted lines) and MVC-selected (open symbols) Env mutations to (a) MVC, (b) b12, (c) 4E9C and (d) KD-247 are shown. The sensitivities of each infectious clone to MVC and mAbs were determined by the WST-8 assay as described in Methods. All assays were conducted in duplicate.

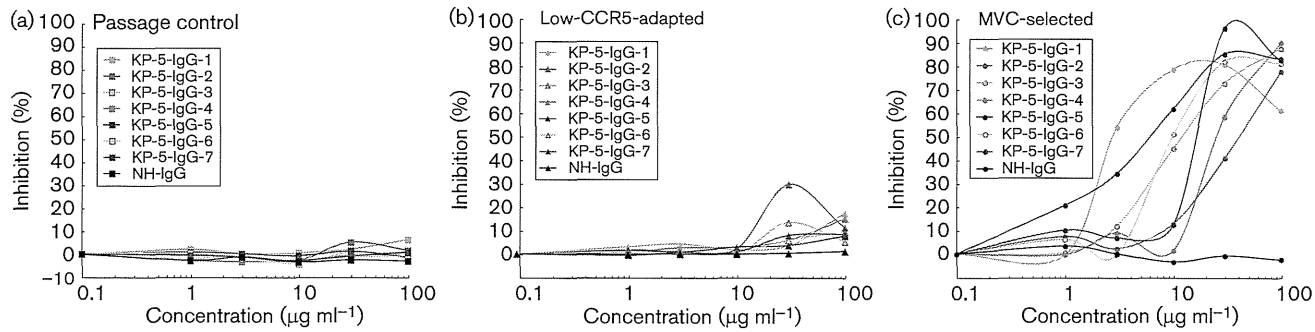

Fig. 6. Sensitivities of infectious clones with the passage control, low-CCR5-adapted and MVC-selected Env mutations to autologous plasma IgGs. The sensitivities of the infectious clones with the (a) passage control, (b) low-CCR5-adapted and (c) MVC-selected Env mutations to seven autologous plasma IgGs (KP-5-IgG-1 to KP-5-IgG-7; coloured symbols) and normal human plasma IgG (NH-IgG; black symbols) are shown. The sensitivity of each infectious clone to the plasma IgGs was determined by the WST-8 assay as described in Methods. All assays were conducted in duplicate.

DISCUSSION

The CCR5 inhibitors, MCV and VCV, are allosteric inhibitors of virus entry, hence resistance to these drugs is evidenced by a reduction in the plateau of virus inhibition curves rather than by increases in IC_{50} (Dragic *et al.*, 2000; Maeda *et al.*, 2006, 2008a; Roche *et al.*, 2011b; Tsamis *et al.*, 2003). One study reported that resistant mechanisms contribute to the altered recognition of drug-bound CCR5 by an MVC-resistant HIV-1 strain. This study demonstrated very efficient usage of drug-bound CCR5, characterized by increased dependence on the CCR5 N terminus (Tilton *et al.*, 2010). Another report demonstrated a similar yet distinct mechanism of escape from MVC by MVC-resistant Env, with comparatively less efficient usage of drug-bound CCR5 (Roche *et al.*, 2011b). In the absence of the drug, MVC-resistant Env maintains a highly efficient interaction with CCR5, similar to that of MVC-sensitive Env, and displays a relatively modest increase in dependence on the CCR5 N terminus (Roche *et al.*, 2011b). However, in the presence of the drug, MVC-resistant Env interacts much less efficiently with CCR5 and becomes critically dependent on the CCR5 N terminus. In the current study, we induced MVC-resistant HIV-1, which harboured many substitutions within and around the V3 region, i.e. the CCR5 N-terminal-binding site *in vitro*. In order to determine whether the resistant variant displayed an increased CCR5 N-terminal dependence, we determined the sensitivity of each variant to anti-CCR5 N-terminal mAb, CTC-5. All passaged variants were completely resistant to CTC-5; however, the MVC-selected variant became sensitive to CTC-5 when MVC (1 μM) was added in the assay, as reported previously (Berro *et al.*, 2009). These results suggest that the four mutations associated with the CCR5-binding site in the MVC-selected variant might create an increased dependency on interaction with the CCR5 N terminus.

In this study, we attempted to determine the difference between the MVC-selected and low-CCR5-adapted variants in parallel using an *in vitro* passage system. Under low concentrations of MVC, the MPI reduction was not observed in either the MVC-selected variant or the low-CCR5-adapted variant, although both passaged variants had common substitutions in the V1, V3 and V5 regions from quasi-species. Compared with the baseline viruses, under high concentrations of MVC, the resistant variants acquired mutations within the area of the CCR5-binding site in gp120 by evolution and/or selection from minor subsets. Following acquisition of the latter mutations, the variants with mutant Env showed a considerably reduced MPI (24 %). These results indicate that mutants arising from passage in low-CCR5-expressing cells can influence the IC_{50} shift, but not a reduction in the MPI.

One previous study showed that although numerous changes were observed in V3 and other regions of gp160, genotypic analysis of the cloned *env* sequences revealed no specific mutational pattern associated with reduced susceptibility to VCV in a phase 2 clinical trial (Pantophlet & Burton, 2006). Using the Los Alamos Database, we found that the frequencies of the four mutations occurred in <10 % of 1501 subtype B viruses (V200I, 5.1 %; T297I, 5.9 %; K305R, 9.5 %; M434I, 2.5 %). Assays for these mutations might provide useful clinical markers for determining the sensitivity of HIV-1 to MVC. One limitation of the present study was that only one primary isolate was used and a single *in vitro* passage series was used for variant selection. Further studies using multiple isolates and multiple passage cultures are required to determine if this MVC-susceptibility model applies to other HIV subtypes and cell systems. Ogert *et al.* (2009) and Anastassopoulou *et al.* (2009) reported that multistep resistance mutations during *in vitro* selection that reduced the MPI values to VCV were driven by the K305R substitution and the H308P substitution was related to a reduction in the MPI plateau level to VCV resistance. Henrich *et al.* (2010) also reported such mutations *in vivo*, as S306P was not detected in the baseline virus population, but was necessary for maximal resistance when incorporated into V3 backbones that included pre-existing VCV resistance mutations. Our *in vitro* study also showed that the K305R mutation contributed to maximal resistance to MVC when incorporated into V3 and CCR5 N-terminal-binding site backbones that included pre-existing MVC resistance mutations. Moreover, our MVC-selected variant with MVC (1 μM) became sensitive to the CCR5 N-terminal mAb (data not shown). Conversely, Roche *et al.* (2011a) reported that the MVC-resistant variants increased reliance on sulfated tyrosine residues in the CCR5 N terminus without common gp120 resistance mutations. One resistant clone (17-Res) harboured I317F, A322D and I323V substitutions in the V3 loop, whilst the other resistant clone (24-Res) had P308S and Ala inserted at the 313 position in the V3. In our MVC-resistant variant, we found some mutations at the same positions (305, 309, 317 and 321) in the V3 region as those of 17-Res and 24-Res clones (Roche *et al.*, 2011a). It is still not clear whether such mutations around the V3 loop stem region contribute to increased reliance on the CCR5 N terminus, and further studies are needed to determine the relationship between each mutation and CCR5 N terminus dependency.

HIV Env evades antibody recognition of conserved epitopes by several means, including decoration with a dense glycan shield, hypervariable loops that mask conserved features and high intrinsic conformational dynamics that render it a poorly defined antigen (Pantophlet & Burton, 2006). In the present study, the infectious clone with the Env of the low-CCR5-adapted virus became sensitive to anti-CD4i mAb, but not anti-V3 mAb and autologous plasma IgGs. Conversely, the clone with the highly MVC-resistant Env was neutralized by the anti-V3 mAb at low concentrations (<0.1 μg ml^{-1}) and also by the autologous plasma IgGs. These findings suggest that the low-CCR5-adapted mutations are related to accessibility of the anti-CD4i mAb to its epitopes, whilst the greater number of mutations in the MVC-selected virus may provide access to the epitopes of

not only anti-CD4bs and anti-CD4i mAbs, but also the anti-V3 mAb and autologous plasma IgGs. In preliminary data, we have confirmed the presence of such anti-CD4i and anti-V3 antibodies in plasma samples from the subject from whom HIV-1_{KP-5} was isolated (unpublished data). *In vivo*, where potent levels of Env neutralizing antibodies may be present, the MVC-selected variants may become neutralization-sensitive and not survive. For this reason, it is possible that CCR5 inhibitors, such as MVC, suppress HIV replication for long periods, especially in patients with high levels of circulating anti-Env neutralizing antibodies prior to treatment with MVC.

As some of the mutations in the MVC-selected variant are close to the epitope for KD-247, those mutations might influence the sensitivity and/or binding affinity to KD-247. Moreover, the mutations around and within the V3 loop may also affect the association with the V2 loop by opening of the trimer. Our study did not allow us to distinguish this possibility. Thus, further studies with single and combinations of mutations in Env to determine the binding affinity to the neutralizing antibodies by FACS and/or ELISA are ongoing.

Following CD4 binding, the CD4-binding site on gp120 becomes ordered and the bridging sheet subdomain forms, drawing the V1/V2 loops into a 'down' orientation and positioning them alongside CD4 (Guttman *et al.*, 2012). The MVC-selected variant in our study became highly sensitive to anti-CD4i and V3 neutralizing mAbs compared with the passage control virus. Further analysis of the effect of the CCR5 inhibitor-resistant Env to neutralizing antibodies would be of interest because, as reported in our previous work (Yoshimura *et al.*, 2006), the anti-V3 mAb KD-247-resistant variant became highly sensitive to CCR5 inhibitors.

METHODS

Viruses. Primary HIV-1 viruses were isolated from patients and passaged in PHA-activated PBMCs. Infected PBMCs were co-cultured for 5 days with PM1/CCR5 cells and the culture supernatants were stored at −150 °C until use (Yoshimura *et al.*, 2010). HIV-1_{KP-5} was isolated from a subject prior to MVC therapy but who has subsequently been taking combination antiretroviral therapy containing MVC since September 2009. The HIV-1_{KP-5} was isolated before starting the combination antiretroviral therapy.

Cells, culture conditions and reagents. The $CD4^+$ T-cell line PM1 (Lusso *et al.*, 1995) was obtained through the AIDS Research and Reference Reagent Program (ARRRP). The PM1/CCR5 cell line was a kind gift from Dr Yosuke Maeda (Kumamoto University, Kumamoto, Japan) (Maeda *et al.*, 2008b). The CCR5 inhibitor MVC was kindly provided by Pfizer (Groton, CT, USA).

Flow cytometric analysis. PM1 and PM1/CCR5 cells were analysed for surface expression of CCR5 and CXCR4. The cells (5×10^5) were incubated with phycoerythrin-labelled anti-CCR5 mAb 2D7, phycoerythrin-labelled anti-CXCR4 mAb 12G5 or isotype-matched control mAbs (BD Biosciences) and analysed using a FACSCalibur (Becton Dickinson).

***In vitro* selection of HIV-1 variants using anti-HIV drugs.** HIV-1_{KP-5} was infected into PM1/CCR5 cells and treated with various concentrations of MVC to induce the production of MVC-resistant variants as described previously (Harada *et al.*, 2013; Hatada *et al.*, 2010; Yoshimura *et al.*, 2006, 2010), with minor modifications. Briefly, PM1/CCR5 cells (4×10^4) were exposed to 500 $TCID_{50}$ HIV-1_{KP-5} and cultured in the presence of MVC. 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 MVC. We also passaged the virus in the absence of MVC in PM1/CCR5 cells and the parental cell line PM1. Proviral DNA was extracted from lysates of infected cells at different passages and subjected to nucleotide sequencing.

Amplification of proviral DNA and nucleotide sequencing. Proviral DNA was subjected to PCR amplification using PrimeSTAR GXL DNA polymerase and Ex-*Taq* polymerase (Takara) as described previously (Harada *et al.*, 2013; Hatada *et al.*, 2010; Yoshimura *et al.*, 2006, 2010). Primers 1B and H were used for the gp120 region (Harada *et al.*, 2013; Hatada *et al.*, 2010). The first-round PCR products were used directly in a second round of PCR using primers 2B and F for gp120 (Harada *et al.*, 2013; Hatada *et al.*, 2010). The second-round PCR products were purified and cloned into the pGEM-T Easy Vector (Promega), and the *env* region in each passaged virus was sequenced using a 3500xL Genetic Analyzer (Applied Biosystems).

Susceptibility assay. The sensitivities of the passaged viruses to various drugs were determined as described previously (Harada *et al.*, 2013; Hatada *et al.*, 2010; Yoshimura *et al.*, 2006, 2010), with minor modifications. Briefly, PM1/CCR5 cells were plated in 96-well round-bottom plates (2×10^3 cells per well), exposed to 100 $TCID_{50}$ of the viruses in the presence of various concentrations of drugs and incubated at 37 °C for 7 days. The IC_{50} values were then determined using a Cell Counting kit-8 (WST-8 assay; Dojindo Laboratories). All assays were performed in duplicate or triplicate.

PHA-activated PBMCs (1×10^6 cells ml^{-1}) were exposed to 100 $TCID_{50}$ of each HIV-1 strain and cultured in the presence or absence of various concentrations of drugs in 96-well microculture plates. The concentration of p24 antigen produced by the cells was determined on day 7 using a Lumipulse F system (Fujirebio) (Maeda *et al.*, 2001). IC_{50} values were determined by comparison with the p24 production level in drug-free control cell cultures (Shirasaka *et al.*, 1995). All assays were performed in triplicate.

Construction of chimeric NL4-3/KP-5 *env* proviruses. Chimeric proviruses were constructed from the pNL4-3 proviral plasmid (ARRRP) by overlapping PCR as described previously (Shibata *et al.*, 2007), with minor modifications. Briefly, the gp160 coding sequences were amplified from the cloning vectors using the primers EnvFv (5′-AGCAGAAGACAGTGGCAATGAGAGCGAAG-3′) and EnvR (5′-TTTTGACCACTTGCCACCCATCTTATAGC-3′). A portion of the NL4-3 provirus spanning nt 5284–6232 was amplified with primers NL(5284)F (5′-GGTCAGGGAGTCTCCATAGAATGGAGG-3′) and NL(6232)Rv (5′-CTTCGCTCTCATTGCCACTGTCTTCTGCT-3′). This fragment encompasses the unique *Eco*RI restriction site in pNL4-3. Another fragment from the NL4-3 provirus spanning nt 8779–9045 was amplified using the primers NL(8779)F (5-GCTATAAGATGGGTGGCAAGTGGTCAAAA-3) and NL(9045)R (5-GATCTACAGCTGCCTTGTAAGTCATTGGTC-3). This fragment includes the unique *Xho*I restriction site in pNL4-3. Overlapping PCR was used to join the gp160 coding sequence from the desired clone to the fragment encompassing nt 8779–9045 that had been amplified from pNL4-3. The resulting fragment was then similarly joined to the amplified fragment encompassing nt 5284–6232 from pNL4-3.

The sensitivities of the three infectious clones to KD-247 (anti-V3 mAb) (Eda *et al.*, 2006), b12 (anti-CD4bs mAb; kindly provided by

Dr Dennis Burton, Scripps Research Institute, La Jolla, CA) (Kessler *et al.*, 1997), 4E9C (anti-CD4i mAb) (Yoshimura *et al.*, 2010) and autologous plasma IgGs were also determined by the WST-8 assay. Plasma samples were collected from the patient seven times from January 2010 to April 2011 and purified using Protein A Sepharose Fast Flow (GE Healthcare) (Kimura *et al.*, 2002; Yoshimura *et al.*, 2010). The purified plasma IgGs were designated KP-5-IgG-1 to KP-5-IgG-7.

Crystal structure of gp120. To compare the sequences of the MVC-selected and low-CCR5-adapted variants in 3D space, the crystal structures of the gp120 monomer and trimer were obtained from the PDB (IDs: 2B4C and 3J5M). Figures were generated using ViewerLite version 5.0 (Accelrys).

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Passive transfer of neutralizing mAb KD-247 reduces plasma viral load in patients chronically infected with HIV-1

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Objective: Neutralizing antibodies against HIV-1 such as a humanized mAb KD-247 can mediate effector functions that attack infected cells *in vitro*. However, the clinical efficacy of neutralizing antibodies in infected individuals remains to be determined. We evaluated the safety, tolerability and pharmacokinetics of KD-247 infusion and its effect on plasma HIV-1 RNA load and $CD4^+$ T-cell count.

Design and methods: KD-1002 is a phase Ib, double-blind, placebo-controlled, dose-escalation study of KD-247 in asymptomatic HIV-1 seropositive individuals who did not need antiretroviral therapy. Individuals were randomized to 4, 8 or 16 mg/kg KD-247 or placebo, and received three infusions over a 2-week period.

Results: Patients were randomized to receive one of the three doses of KD-247 and the treatment was well tolerated. We observed a significant decrease in HIV RNA in the 8 and 16 mg/kg KD-247 cohorts, with two individuals who achieved more than 1 log reduction of HIV RNA. Two patients in the 16 mg/kg cohort had selections and/or mutations in the V3-tip region that suggested evasion of neutralization. Long-term suppression of viral load was observed in one patient despite a significant decrease in plasma concentration of KD-247, suggesting effects of the antibody other than neutralization or loss of fitness of the evading virus.

Conclusion: The results indicate that KD-247 reduces viral load in patients with chronic HIV-1 infection and further clinical trials are warranted.

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Introduction

Despite the significant reduction in morbidity and mortality following combination antiretroviral therapy (cART), there is emerging evidence that people with successfully treated HIV-1 infection age prematurely, leading to progressive multiorgan diseases referred to as comorbidity. The pathogenic process has been associated with long-term use of antiviral drugs, residual viral production and subsequent chronic inflammation [1]. In contrast to the current cART that only targets viral replication, neutralizing or nonneutralizing antibodies against HIV-1 can mediate effector functions that attack infected cells *in vitro* [2,3]. However, the clinical efficacy

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of neutralizing antibodies in infected individuals remains to be determined.

Previous studies revealed that human antibodies to HIV-1 can neutralize a broad range of viral isolates *in vitro* and protect nonhuman primates against infection [4–6]. Effective control of HIV-1 by combinations of broadly neutralizing antibody (bnAb) in chronically infected humanized mice and simian–human immunodeficiency virus (SHIV)-infected macaques have been reported [7–9]. However, previous human studies concluded that treatment with neutralizing antibodies had only limited effects against established HIV-1 infection [10,11].

Here, we report the results of a phase Ib dose-escalation study of a neutralizing mAb, KD-247 (international nonproprietary name: suvizumab) in asymptomatic HIV-1 seropositive individuals who did not at the time need cART. The epitope recognized by the mAb was mapped to IGPGR at the tip of the third variable loop of HIV-1 gp120 (V3-tip) that covers about half of HIV-1 in subtype B [12,13]. KD-247 belongs to the antibodies that have limited breadth and potency in standard neutralization assays as compared with bnAbs [14]. However, passive transfer of KD-247 may have an impact on patients infected with HIV-1 that matches for KD-247 binding.

The objectives of this phase Ib study were to evaluate the safety and tolerability of three infusions of KD-247 over 2 weeks in HIV-1 seropositive individuals, to determine the pharmacokinetic parameters and to assess the effect of KD-247 infusions on plasma HIV-1 RNA load and $CD4^+$ T-cell counts.

Materials and methods

Protein-based KD-247 binding test

The binding activity of KD-247 to recombinant proteins that expressed the V3 region was examined prior to the phase Ib study. The gene containing the V3 region in viruses extracted from patients' plasma or peripheral blood mononuclear cells (PBMCs) was amplified by a nested PCR method using first primers, 5′-ACACATG GAATTAGGCCAGT-3′ (OA-4) and 5′-AAATTCCC CTCCACAATTAA-3′ (OD-4), and second primers, 5′-GCCGGATCCTCAACTCAACTGCTGTTAAAT-3′ (EB-2) and 5′-GCTCTGCAGTCAAATTTCTGGGT CCCCTCCTGAGG-3′ (EC-2). After the purification of the amplified DNA, this segment was cleaved and inserted into a vector plasmid containing β-galactosidase (β-Gal). Separately, each cloned *Escherichia coli* with its gene sequence analysed was cultured, and the recombinant fusion protein derived from the V3 region and β-Gal (V3-Gal) was obtained. An ELISA was performed to normalize the V3-Gal concentration. The expressed V3-Gal or commercially available β-Gal (CN Bioscience, La Jolla, California, USA) as a reference standard was added to a 96-well ELISA plate immobilized with the β-Gal antibody (Chemicon International, Temecula, California, USA). Peroxidase-labelled β-Gal antibody (Rockland Immunochemicals, Limerick, Pennsylvania, USA) was used as a detecting antibody. To evaluate the reactivity of KD-247 to each V3-Gal protein, 200 ng/ml of V3-Gal was captured on a plate coated with the β-Gal antibody, followed by incubation with KD-247 (1 μg/ml) and the reactivity was detected by the peroxidase-labelled anti-human IgG antibody.

KD-1002 clinical trial

KD-1002 was a phase I, double-blind, placebo-controlled, dose-escalation, cohort study of KD-247 in asymptomatic HIV-1 seropositive individuals who did not currently need ART. The study was conducted by investigators (who enrolled patients) at 15 study centres in the USA. Eligible patients were randomized to one of three doses of KD-247 (4, 8 or 16 mg/kg) or placebo (physiological saline), and received three infusions over a 2-week period (days 1, 8 and 15). A minimum of six active and three placebo patients for each dose cohort had to complete 2 weeks of infusions. Patients in all three cohorts were followed for 12–16 weeks after the final infusion of study drug. Dose escalation could proceed only after review of the safety data up to Day 18 for all patients in the lower-dose cohort.

In addition to usual entry criteria, patients were considered eligible for participation in the study if they were asymptomatic HIV-1 seropositive individuals who at the time of study did not need ART; demonstrated an HIV-1 RNA copy number of 1000–100 000 and $CD4^+$ T-cell count more than 350 cells/μl; and who by genotyping had a sequence of the portion of the HIV envelope gene encoding the principal neutralizing determinant that is consistent with neutralization by KD-247. Plasma HIV-1 RNA samples were measured using the Roche Amplicor HIV-1 RNA assay (standard) with a dynamic range of 400–750 000 copies/ml and the Roche Amplicor Ultrasensitive plasma HIV-1 RNA assay (ultrasensitive) with a dynamic range of 50–100 000 copies/ml (Roche Diagnostic Systems, Branchburg, New Jersey, USA). The $CD4^+$ and $CD8^+$ cell counts were measured using a flow cytometer (BD FACSCanto II; BD Biosciences, Franklin Lakes, New Jersey, USA). Potential switches of coreceptor use were monitored for the 8 and 16 mg/kg cohorts by the Trofile assay (Monogram Biosciences, San Francisco, California, USA), which uses the complete gp160 coding region of the HIV-1 envelope protein.

Genotypic screening for the clinical study was performed by SRL Medisearch Inc. (Tokyo, Japan). The gene containing the V3 region in viruses extracted from patients' plasma was amplified by a nested PCR method using first primers (OA-4 and OD-4) and second primers

(EB-2 and EC-2). After purification of the amplified DNA, this segment was cloned and sequenced to determine the gene containing the V3 region. Patients were screened by V3-tip amino acid sequences deduced from the results of genotypic analyses and judged as suitable for KD-247 treatment if all 10 viral clones conformed to the reference sequences. Six sequences of V3-tip that had a GPGR sequence and high binding activity to KD-247 (XIGPGRAL, XIGPGRSF, XIGPGRTF, XIGPGRAI, XIGPGRAF and XVGPGRAL; X was not K, P and R) were selected as the reference sequences for the genotyping test (Fig. 1a and b).

The study protocol and all amendments, written study patient information, informed consent form and any other appropriate study-related information were reviewed and approved by an independent ethics committee or institutional review board at each study centre. The study was conducted in accordance with Good Clinical Practice as required by the International Conference on Harmonisation guidelines and in accordance with country-specific and/or local laws and regulations governing clinical studies of investigational products. Compliance with these requirements also constituted conformity with the ethical principles of the Declaration of Helsinki. Prior to initiation of any study procedures, an informed consent agreement explaining the procedures of the study, together with the potential risk, was read by and explained to all patients.

The trial was registered at the ClinicalTrial.gov (National Library of Medicine at the National Institutes of Health) database with the registration number NCT00917813.

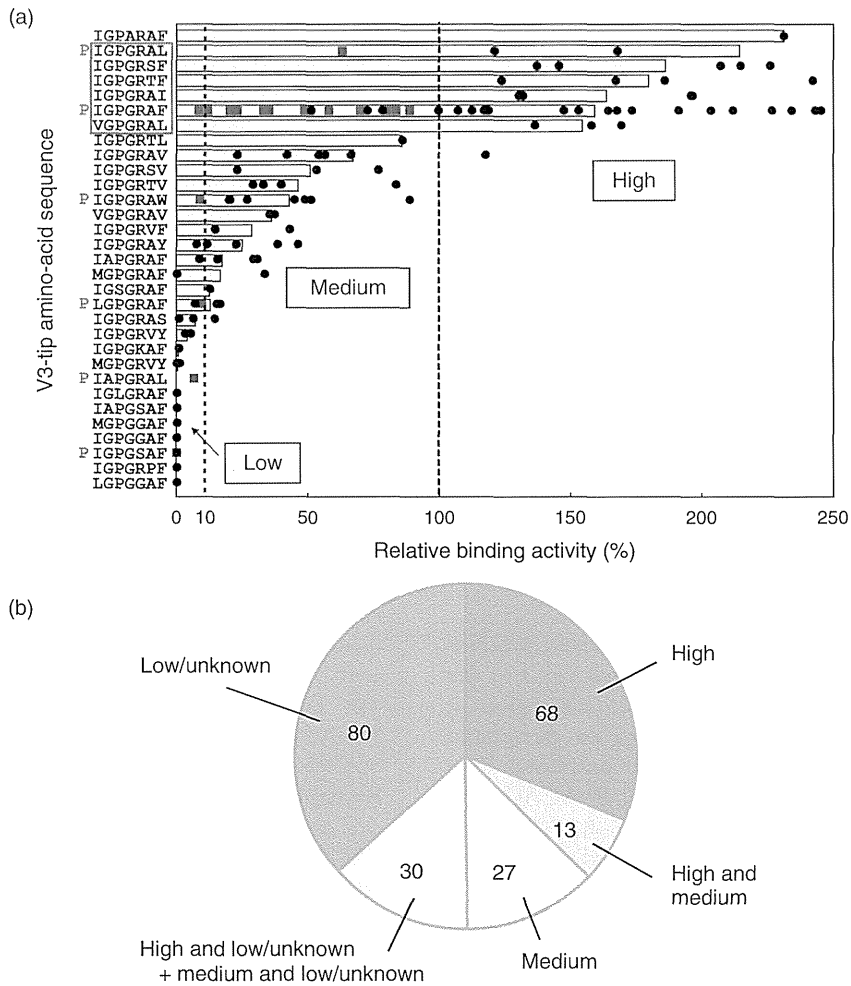

Fig. 1. Determination of reference sequences by a protein-based KD-247 binding test. (a) The binding of KD-247 to the recombinant fusion protein derived from the V3 region of HIV-1 gp120 and b galactosidase (V3-Gal) was indicated as relative activity of 100% in the case of the V3 sequence from the MN strain. The amino acid sequences of the V3-tip region in the viral clones whose mean relative KD-247 binding activity exceeded 100% were IGPARAF, IGPGRSF, IGPGRAL, IGPGRTF, IGPGRAI, VGPGRAL, and IGPGRAF. Above 100%, 10–100%, and <10% of binding activity was categorized as high, medium, and low, respectively. (b) Frequency of estimated KD-247 binding abilities using the 218 data from screened patients is shown. All 10 viral clones derived from 68 patients were estimated as high binding activity.

Pharmacokinetic analysis

Serum concentrations of KD-247 in pharmacokinetic samples were determined by ELISA using an antigen peptide [15]. The lower limit of quantitation for KD-247 was 0.2 μg/ml. Similarly, the two samples that were collected from the infusion bag were analysed for KD-247 concentration and reported as two individual concentrations. The average of the two concentrations was used for the calculation of actual total dose infused. Pharmacokinetic parameters for KD-247 were calculated from serum concentrations of the antibody by compartmental and noncompartmental methods. Actual sampling times were used for the computation of pharmacokinetic parameters. All pharmacokinetic calculations were performed using WinNonlin Professional version 5.2 (Pharsight Corp., Mountain View, California, USA) or SAS version 9.2 (SAS Institute, Cary, North Carolina, USA) or NONMEM version 6.0 (Certara USA, Inc., Princeton, New Jersey, USA).

Statistical analyses

Analyses of independent virological and immunological data were performed by two-tailed Mann–Whitney *U* tests. Statistical differences among groups were determined by performing one-way analysis of variance or the Kruskal–Wallis test with Dunn multiple comparison posthoc test. *P* value less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, California, USA).

Results

Determination of reference sequences

Prior to the phase Ib study, the binding activity of KD-247 to a protein that expressed the V3 region was evaluated, and patient selection criteria by genotyping were established (Fig. 1a). The binding of KD-247 to V3-Gal was indicated as relative activity of 100% in the case of the V3 sequence derived from a subtype B strain MN, which had the subtype B consensus sequence at the V3-tip. Relative binding activities of KD-247 and the amino acid sequence of the V3-tip of the recombinant V3-region expressing proteins were analysed using 122 HIV-1 clones derived from plasma or PBMCs. The amino acid sequences of the V3-tip region in the viral clones whose mean relative KD-247 binding activity exceeded 100% were IGPARAF, IGPGRSF, IGPGRAL, IGPGRTF, IGPGRAI, VGPGRAL and IGPGRAF, although the number of IGPARAF was only one. Most of the results using the recombinant V3 protein were in accord with the results using the short peptides by Pepscan [12]. However, a proline residue at the amino terminal of the V3-tip sequence markedly decreased the binding activity of KD-247 (Fig. 1a, red squares). The value bar of this graph indicates the mean binding activity, except for V3-Gal wherein there was a proline residue at the amino terminus. Considering the results of the examinations using V3-Gal and short peptides, and the consensus sequence of the subtype B virus, six sequences that had GPGR sequences and high binding activity to KD-247 (XIGPGRAL, XIGPGRSF, XIGPGRTF, XIGPGRAI, XIGPGRAF and XVGPGRAL; X was not K, P and R) were selected as the reference sequences of genotyping on the phase Ib clinical study.

Individual disposition

For the genotyping test for suitability, all 10 clones amplified from plasma RNA from 68 patients out of 218 matched the reference sequences corresponding to the high binding activity to KD-247 (Fig. 1b). A total of 295 patients were screened and 30 from the population with high binding activity were randomized and received study treatment, with seven receiving 4 mg/kg KD-247, six receiving 8 mg/kg KD-247, seven receiving 16 mg/kg KD-247 and 10 receiving placebo. Twenty-eight patients (93.3%) completed the study. There were no imbalances in the patient demographics that were expected to affect the data or interpretation of the results. Patient disposition and demographic and baseline information are summarized in Table 1 and Supplementary Table 1, http://links.lww.com/QAD/A629.

Safety and pharmacokinetic analysis

In general, KD-247 was well tolerated. There was no evidence of allergic or hypersensitivity reactions. Although the number of patients in each cohort was small, there was no evidence to support any hepatic, renal or cardiac toxicity. Supplementary Fig. 1, http://links.lww.com/QAD/A629 shows the phramacokinetic profiles of KD-247 for Day 1 following Infusion 1 and up to Day 99 following Infusion 3 on Day 15. All patients had concentrations above the lower limit of quantification (0.2 μg/ml) on Day 99. Dose proportionality across the dose range of 4–16 mg/kg for both Day 1 and Day 15 was observed. The systemic clearance varied from 18.3 to 22.9 ml/h, indicating that KD-247 was cleared slowly from the central compartment. Accumulation ratio as measured by Day 15/Day 1 exposure varied from 1.41 to 1.61 for C_{max} and from1.67 to 1.78 for $AUC_{(0-\tau)}$, indicating that there was some accumulation of KD-247 following three infusions.

Effect of KD-247 infusions on plasma HIV-1 RNA load and $CD4^+$ T-cell counts

There was a trend towards moderate increase in $CD4^+$ and $CD8^+$ counts across dose cohorts (Supplementary Fig. 2a and b, http://links.lww.com/QAD/A629). The counts were not significantly higher than those for placebo. The changes in log-transformed plasma HIV-1 RNA from baseline in each cohort after Infusion 1 (a, Day 1), 2 (b, Day 8) and 3 (c, Day 15) are shown in Fig. 2. The impact of Infusion 1 was not evident for the viral load

Table 1. Patient demographics and baseline information.

Characteristic	Statistics	Patient group				
		Placebo ($n=10$)	KD-247 4 mg/kg ($n=7$)	KD-247 8 mg/kg ($n=6$)	KD-247 16 mg/kg ($n=7$)	Total ($n=30$)
Sex						
Percentage male	*n* (%)	90.0%	100%	100%	85.7%	93.3%
Age (years)	Mean	36.7	38.6	35.8	43.4	38.5
	SD	11.9	8.8	8.1	13.0	10.7
Weight (kg)	Mean	81.45	86.29	88.40	74.33	82.31
	SD	12.72	19.19	14.44	11.79	14.77
BMI (kg/m^2)	Mean	26.156	28.097	28.932	24.837	26.856
	SD	2.330	6.725	3.668	1.762	4.052
HIV-1 RNA (copies/ml)	Mean	15093	20226	20797	49729	25513
	SD	14404	17020	25059	28603	24335
$CD4^+$ cell count (cells/μl)	Mean	690	405	426	424	509
	SD	293	155	51	151	232
$CD8^+$ cell count (cells/μl)	Mean	823	1113	932	848	918
	SD	277	436	290	338	339
Coreceptor use[a]	R5 cases/total	6/6	NT[b]	3/5	4/5	13/16

CCR5, chemokine CC receptor 5; SD, standard deviation.
[a]Coreceptor use of plasma viruses was monitored for the 8 and 16 mg/kg cohorts by Trofile assay and expressed numbers of CCR5 use (R5) in total cases.
[b]Not tested.

reduction, and changes in plasma RNA were comparable to those in the placebo cohort in many cases (Fig. 2a). However, a moderate decrease in HIV-1 RNA was observed in the 8 and 16 mg/kg cohorts after Infusion 2, and three of six cases in the 8 mg/kg cohort and four of seven cases in the 16 mg/kg cohort showed a reduction greater than the average ± standard deviation (SD) of the placebo cohort (Fig. 2b). There was a significant reduction in HIV-1 RNA in the 8 and 16 mg/kg cohorts after Infusion 3, and five of six cases in the 8 mg/kg cohort and five of six cases in the 16 mg/kg cohort showed a reduction greater than the average ± SD of the placebo cohort (Fig. 2c). Longitudinal follow-up of HIV-1 RNA loads and log-transformed changes from baseline for each patient in all cohorts are summarized in Supplementary Table 2, http://links.lww.com/QAD/A629. The time points at which we detected a significant reduction of HIV-1 RNA load over the placebo cohort are shown in Fig. 2d and summarized in Supplementary Table 3, http://links.lww.com/QAD/A629. The changes in log-transformed plasma HIV-1 RNA from baseline in each cohort throughout the trial are shown in Fig. 2e, with additional time points that had a significant reduction in viral load. The dose proportional reduction of viral load continued for nearly 29 days, and thereafter, the level of suppression decreased with the plasma concentration of KD-247 (Fig. 2e). Although the mean profiles showed moderate decreases in viral RNA, the impact of KD-247 on individual cases was noteworthy. Among these, Case #03017 on Day 8 and Case #12044 on Day 16 achieved more than 1 $\log_{10}$ copies/ml reduction in HIV-1 RNA. The emergence of neutralization escape mutation (R315K) was observed on Day 22 for both cases (Table 2).

To clarify the impact of KD-247 on the reduction of plasma viral load, we analysed longitudinal changes in plasma concentration of KD-247 with HIV-1 RNA for the 16 mg/kg cohort (Fig. 3). We observed a reduction of HIV-1 RNA and viral-load set points in four of six cases. Case #10012 (Fig. 3a) was a typical case in which the reduction of plasma viral load was not seen on Day 1. However, reduction of HIV-1 RNA was observed at the predose of Infusion 2 on Day 8, and a further decrease was observed at Day 15. The plasma viral load remained at a lower level than baseline. Escape mutants for KD-247 were not found in this case. In contrast, Case #03017 (Fig. 3b) followed a different clinical course. Plasma viral load suppression was observed immediately after Infusion 1 and the effect persisted on Days 8 and 15. However, viral rebound was detected owing to the emergence of the neutralization escape mutants with R315K on Day 22. Case #12044 was remarkable in that the suppression of plasma viral load was observed at the predose of Infusion 2 on Day 8, and continued on Day 99 when the plasma concentration of KD-247 decreased to the lower level (Fig. 3c). We detected emergence of R315K mutation at a low frequency (1 in 10 clones) on Day 22, but the mutant was not found on Day 99 (Table 2). These results suggest that a sustained level of KD-247 blood concentration may not be necessary to control the viral load in blood.

We observed moderate suppression of plasma viral load in Case #1037 (Fig. 3d), especially after Infusion 3 on Day 15. Although the effect was marginal, the suppression persisted long after the final infusion. Viral load reduction by KD-247 was not evident for Cases #15017 and #1034 (Fig. 3e and f, respectively), although temporal suppression was observed after Infusion 3 on Day 15. The data

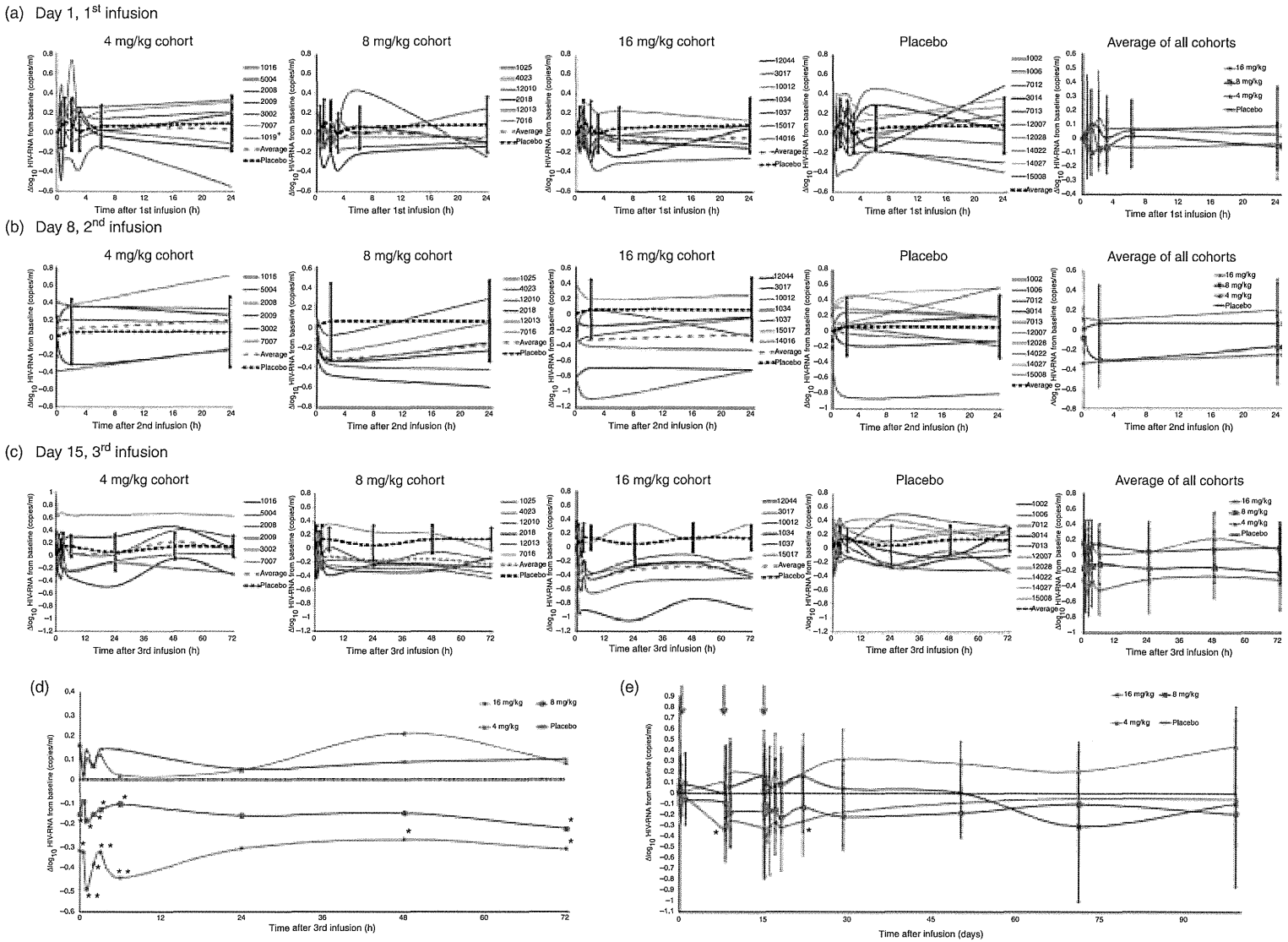

Fig. 2. Impact of three infusions of KD-247 on plasma HIV-RNA in each cohort. Changes in log-transformed plasma HIV-1 RNA from baseline in each cohort after Infusion 1 (a, Day 1), 2 (b, Day 8), and 3 (c, Day 15) of KD-247 are shown with average ± standard deviation (SD) of all cohorts. Each line represents a single patient. Black dotted line shows average ± SD of that observed for the placebo cohort, and red broken line shows the geometric mean changes for each treatment cohort. The average ± SD of the changes in plasma viral load in each cohort are shown with colors: red for 16 mg/kg, blue for 8 mg/kg, green for 4 mg/kg, and purple for placebo. The time points of significant reduction of HIV-1 RNA load are indicated in (d), which corresponds to the time points after Infusion 3, when most of the difference was observed. Changes in plasma HIV-1 RNA from baseline in each cohort throughout the trial are shown in (e). Black dotted line shows the zero level for changes in (e) and (d). Red arrows represent days of infusions. Significance was determined by two-tailed Mann–Whitney U tests. $*P < 0.05$, $**P < 0.01$.

Table 2. Emergence of KD-247 neutralization escape and mismatched variants.

Patient group	Cohort	Patient	Variations of the V3-tip sequence (number of clones)[a]		
			Screening	FU1 (Day 22)	FU5 (Day 99)
Placebo	3	12028	HIGPGRAF (10)	HIGPGRAF (10)	HIGPGRAF (9) HISPGRAF (1)
	3	15008	SIGPGRAF (10)	SIGPGRAF (10)	PIGPGRAF (10)
4mg/kg	1	2009	SIGPGRAF (10)	PIGPGRAF (6) TMGPGRVF (3) PIGPGIMQ (1)	TMGPGRVF (4) PIGPGRAF (3) SIGPGRAF (3)
	1	7007	HIGPGRAF (10)	HIGPGRAF (10)	HIGPGRAF (5) HIGPGRAV(5)
8mg/kg	2	7016	SIGPGRAF (10)	SIGPGRAF (3) TIGPGRAF (3) NIGPGRAF (2) PIGPGRAF (2)	TIGPGRAF (8) SIGPGRAF (1) PIGPGRAF (1)
16mg/kg	3	3017	TIGPGRAF (10)	NMGPGRAF (5) TIGPGKAF (5)	NMGPGRAF (8) TIGPGKAF (2)
	3	12044	HIGPGRAF (10)	HIGPGRAF (9) HIGPGKAF (1)	HIGPGRAF (8) PIGPGRAF (2)

[a]Red letters indicate mismatched variants including escape mutation of R315K.

suggested that the selection process of the patients appropriate for KD-247 infusion only by V3 genotyping was a limitation. Some primary isolates with reference V3 sequences have a resistance phenotype to KD-247; therefore, involvement of outside V3 for resistance to KD-247 may have accounted for the lack of response [16,17].

Emergence of resistant mutants or mismatched variants

The genotyping test performed for the follow-up samples revealed the emergence of resistant mutants or mismatched variants in seven patients, including two in the placebo cohort (Table 2). Potential switches of coreceptor use were monitored for the 8 and 16 mg/kg cohorts by the Trofile assay. Dual/mixed virus populations of HIV-1 isolates were found in three patients on Day 1. No tropism shifts were observed in the placebo group. Only two patients in the 8 mg/kg cohort had tropism shifts during the study. None of the patients with a tropism shift had significant antiviral response (Supplementary Table 4, http://links.lww.com/QAD/A629).

Discussion

In this phase Ib study, KD-247 was well tolerated and we observed significant decreases in HIV RNA in the 8 and 16 mg/kg KD-247 cohorts. We observed reduction of HIV-1 RNA and viral load set-point in four of six cases in the 16 mg/kg cohort, and long-term suppression of viral load in one patient, despite a significant decrease in plasma concentration of KD-247. It may be necessary to raise the blood concentration of KD-247 to a high level to achieve the initial suppressive effect on viral load. However, maintenance of blood concentration of the antibody may not be essential for a prolonged effect on viral load. In a previous animal model study, KD-247 was administered weekly eight or nine times to monkeys after challenge with pathogenic SHIV. The effects of KD-247 were observed in the lymph node compartment rather than the peripheral blood. The effects of KD-247 on $CD4^+$ T cells in the lymph nodes were observed in a monkey who was unable to maintain the blood concentration of KD-247 because of the emergence of antiidiotype antibody to KD-247 [15]. The observation may partly relate to the prolonged effect of KD-247.

Long-term suppression of viral load observed in one patient (Case #12044) despite a significant decrease in plasma concentration of KD-247 may have interesting implications. Recently, Barouch *et al.* [8] demonstrated the profound therapeutic efficacy of PGT121 and PGT121-containing mAb cocktails in rhesus monkeys chronically infected with SHIV-SF162P3. Virus rebounded in most animals when serum mAb titres declined to undetectable levels, although a subset of animals maintained long-term virological control in the absence of further mAb infusions. Direct antibody-mediated cytotoxic effects on cells chronically infected with HIV-1 were suggested in a humanized mouse model [18]. The phenomenon may be relevant to that observed for Patient #12044 in the present study. The prolonged impact of the neutralizing mAb may partly be owing to the effects other than neutralization, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated virus inhibition (ADCVI) that can attack virus-producing cells. The importance of nonneutralizing effector activities of bnAbs *in vivo* has been reported in mouse models [19]. Although it is difficult to evaluate ADCC and ADCVI *in vivo*, these

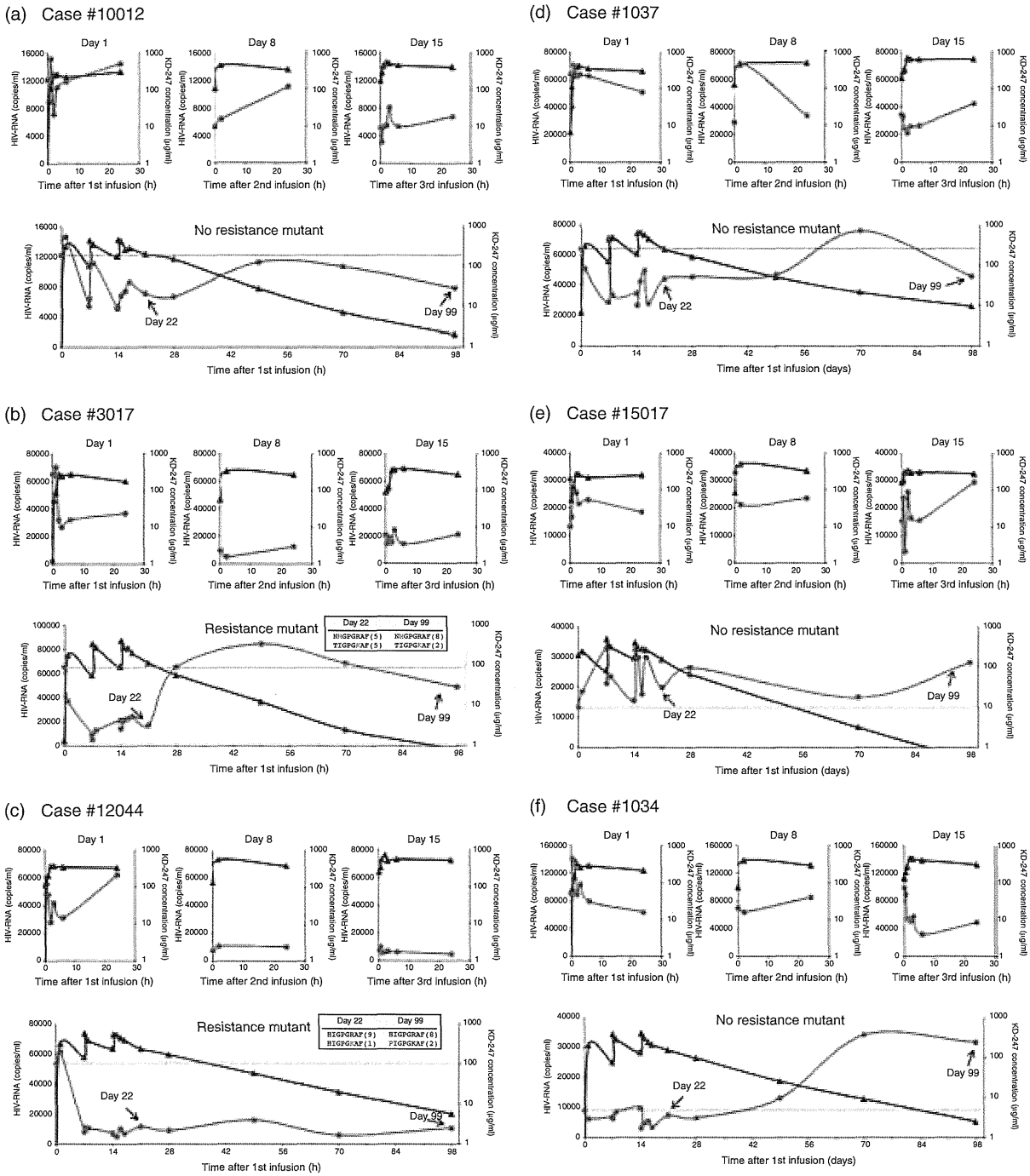

Fig. 3. Longitudinal analysis of HIV-1 RNA reduction and plasma concentration of KD-247 for 16 mg/kg cohort. Plasma concentration of KD-247 is shown as blue triangles with three peaks in an earlier period corresponding to the three infusions of antibody. Plasma HIV RNA is shown as red circles. Emergence of KD-247 resistance mutants with R315K was indicated on Days 22 and 99.

FC-receptor mediated functions were detected for KD-247 *in vitro* (Supplementary Fig. 3, http://links.lww.com/QAD/A629). In addition, secondary immunological responses following viral degradation in antigen-presenting cells by antibody-dependent phagocytosis may have an impact on host antiviral immune responses, including HIV-specific $CD8^+$ T cells, as suggested by the animal model [8]. The enhancement of cell-mediated immunity may in part account for the apparent reduction of viral load set-points after viral rebound from the baseline. We previously reported a KD-247 escape mutant with R315K that showed a less-fit phenotype as compared with the wild-type virus [20]. Although subsequent recovery of the fitness was observed with additional

mutations, fitness cost of the escape mutants may have contributed in part to the reduction of HIV-1 RNA in patients with R315K mutation.

The data in the present clinical trial together with recent studies in animal models [7–9,18] may have an implication for future combination therapy because passive transfer of KD-247 had a significant effect on HIV-1 replication in chronically infected patients. We reported that the neutralization escape mutants to KD-247 became sensitive to chemokine CC receptor (CCR)5 inhibitors [21]. Conversely, resistance mutants to a CCR5 inhibitor, maraviroc, became sensitive to several neutralizing mAbs including KD-247 [22]. Furthermore, a series of in-vitro experiments suggested synergistic effects of the combination of KD-247 and CCR5 antagonists including maraviroc ([21], unpublished observation by S.M. and K.Y.). In view of such a complementary nature of resistance, combination therapy using KD-247 and CCR5 inhibitors warrants future clinical trials. The current results may also imply limitations of monotherapy with conventional antibodies such as KD-247. New-generation bnAbs, especially used in combination, are better candidates for antibody-based treatment in terms of potency and breadth of action. KD-247 may have a role in patients infected with certain viruses that match for neutralization. Combination of KD-247 with certain $CD4^{+}$-mimetic compounds that markedly enhances the neutralization/binding activity of the antibody may also deserve further investigation [23,24].

Results of this clinical trial should be interpreted with caution because of the small sample size. However, current data and the results from recent primate models taken together warrant further clinical trials of neutralizing mAbs in several different settings with or without ART [25,26].

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S.M., K.Y. and T.M. designed the study. K.P.R. conducted some of the in-vitro assays for KD-247, including ADCC and ADCVI. J.P. and S.M. led the statistical analysis of the clinical trial. S.M. led the studies and wrote the article with all coauthors. In addition to the authors, the KD-1002 study group includes the following investigators and contributors to the design, conduct or analysis of the study: Principal investigators: E. DeJesus, M. Markowitz, G. Richmond, M. Thompson, R. Liporace, P. Ruane, C. Brinson, K. Staszkow, J. Gathe, Jr., A. Scribner, S. Shoham, H. Marcelin, R. Redfield, T. Sligh and A. Scarsella; and the KD-1002 Protocol Team of Quintiles: E. Vigdorth, J. Bush, C. Gibson G.. Breed, D. Despard, P. Ajiboye, B. Tedrow, P. Udeshi, J. Hoglind, R. Rao, R. Kalmadi and A. Armitage.

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Conflicts of interest

S.M., K.Y. and K.P.R. declare no competing financial interests. J.P. is an employee of Quintiles Inc. T.M. is an employee of the Chemo-Sero-Therapeutic Research Institute (Kaketsuken). Kaketsuken paid Quintiles to conduct the clinical trial and analyse the results of the study. The authors have no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this article.

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Complementary and synergistic activities of anti-V3, CD4bs and CD4i antibodies derived from a single individual can cover a wide range of HIV-1 strains

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ABSTRACT

Antibodies with modest neutralizing activity and narrow breadth are commonly elicited in HIV-1. Here, we evaluated the complementary and synergistic activities of a set of monoclonal antibodies (MAb) isolated from a single patient, directed to V3, CD4 binding site (CD4bs), and CD4 induced (CD4i) epitopes. Despite low somatic hypermutation percentages in the variable regions, these MAbs covered viral strains from subtypes B, C, A and CRF01_AE and transmitted/founder viruses in terms of binding, neutralizing and antibody-dependent cell-mediated cytotoxicity (ADCC) activities. In addition, a combination of the anti-V3 and CD4bs MAbs showed a synergistic effect over the neutralization of HIV-1_{JR-FL}. A humoral response from a single patient covered a wide range of viruses by complementary and synergistic activities of antibodies with different specificities. Inducing a set of narrow neutralizing antibodies, easier to induce than the broadly neutralizing antibodies, could be a strategy for developing an effective vaccine against HIV-1.

Introduction

Despite the great advances in the treatment of HIV-1 infection, there are still major obstacles to effective control of HIV-1 infection. Active replication persistence and immune activation under suppressive highly active antiretroviral therapy (HAART) (Buzon et al., 2010; Palmer et al., 2008; Richman et al., 2009), secondary effects of the drugs (Montessori et al., 2004; Reust, 2011), and the socio-economic burden of long-term treatment (Boyer, 2009; Naik et al., 2009) are still present; making the development of a protective vaccine desirable. Neutralizing antibodies are an important component of a protective vaccine-induced immune responses and much effort has been focused on the development of broadly neutralizing antibodies against conserved epitopes on the functional Env trimer of HIV-1 (Bonsignori et al., 2011; Corti et al., 2010; Walker et al., 2009).

Advances in antibody technology have uncovered broadly neutralizing Abs (bNAbs) (Marasco and Sui, 2007; Zhu et al., 2013; Zuo et al., 2011) and their efficacy has been proved in non-human animal models. Protection from infection by Simian immunodeficiency virus (SIV) was correlated with the humoral response produced after vaccination with Env and/or Gag and Pol of rhesus macaques (Barouch et al., 2013; Roederer et al., 2014). Protection was also observed in rhesus macaques vaccinated with Env derived peptides and challenged with chimeric simian-human immunodeficiency viruses (SHIV) $SHIV_{162P3}$ and $SHIV_{C2/1}$ (DeVico et al., 2007; Eda et al., 2006a). Passive administration of antibodies was also proved useful in protecting for and controlling SHIV and HIV-1 infection. In rhesus macaques chronically infected with $SHIV_{162P3}$, passive administration of bNAbs (PGT121, 3BNC117 and b12 combined or alone) reduced viral load and resulted in control of the infection (Barouch et al., 2013; Ng et al., 2010). Similar results were observed in humanized mice chronically infected with HIV-1_{YU2} after the passive administration of bNAbs 45–46^{G54W}, PG16, PGT128, 10-1074 and 3BC176 (Klein et al., 2012). Passive administration of MAbs PGT121, b12, 2G12, 2F5 and 4E10 also offered protection from infection with SHIV (Hessell et al., 2010, 2009a, 2009b; Mascola et al., 2000, 1999; Moldt et al., 2012; Parren et al., 2001) even when the antibody (b12) was administered topically (Veazey et al., 2003); or when the administered antibodies were purified IgG from infected chimpanzees (Shibata et al., 1999).

It has been proposed that an immunization strategy that could elicit such antibodies would be protective in humans (Stamatatos

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0042-6822/

et al., 2009); however, to date there is no vaccine that induces their production.

In naturally infected HIV-1 patients, bNAbs are not commonly produced; instead, antibodies are often directed against strain-specific or non-neutralizing sites in Env (Burton et al., 1991; Corti et al., 2010). Only 10 to 25% of HIV-1-infected individuals generate neutralizing antibodies, and a minority of these individuals, approximately around 1%, is considered elite neutralizers, besides, bNAbs appear late (1 to 3 years) after infection (Binley et al., 2008; Deeks et al., 2006; Dhillon et al., 2007; Doria-Rose et al., 2010, 2009; Gray et al., 2011; Sather et al., 2009; Simek et al., 2009) and frequently harbor uncommon characteristics which probably pose obstacles to their generation, including high levels of somatic mutations, long heavy-chain complementarity-determining regions 3 (CDRH3s), frequent insertions or deletions, and high levels of polyreactivity (Haynes et al., 2012b; Huber et al., 2010; Klein et al., 2013; Scheid et al., 2011; Sok et al., 2013; Xiao et al., 2009). Moreover, when the immunoglobulin sequences of bNAbs are experimentally reverted to their germline precursors, as they are found on naive B cells, binding to HIV-1 Env is often significantly diminished or even completely abrogated (Bonsignori et al., 2011; Haynes et al., 2012b; McGuire et al., 2014; Scheid et al., 2011; Wu et al., 2011; Xiao et al., 2009). This suggests difficulties in inducing bNAbs in HIV-1-infected patients and also by vaccination, because many rounds of affinity maturation are required which means that immunizations should be repeated many times as well.

Antibodies to the V3 loop, CD4bs and CD4i have been produced by HIV infection or vaccination, but neutralization by these antibodies is generally not broadly effective for preventing HIV-1 infection because of steric constraints blocking the access of these antibodies to the epitopes, and mutations in their epitopes that allow to escape from these antibodies. However, these modest neutralizing antibodies appear faster after infection (even as early as 2 weeks after sero-conversion) and are also capable of exert pressure over the virus (Bar et al., 2012; Haynes et al., 2012b; McGuire et al., 2014; Overbaugh and Morris, 2012; Pollara et al., 2014).

Besides neutralization, non-neutralizing responses, specifically the ADCC activity has been associated with protection from HIV. The most remarkable case is the RV144 trial result, which showed a 31.2% of vaccine efficacy (Rerks-Ngarm et al., 2009) and it has been proposed that the ADCC activity of V1/V2 antibodies induced by the vaccine may be the most important correlation for protection (Haynes et al., 2012a; Rerks-Ngarm et al., 2009; Wren et al., 2012). Vaccination in animal models has shown similar results (Hessell et al., 2007; Xiao et al., 2010) and it has also been reported that broader ADCC responses correlate with long-term control of HIV, slow progression of disease and lower viremia (Nag et al., 2004; Wren et al., 2013; Xiao et al., 2010).

It is certainly desirable for HIV-vaccines to induce antibodies that neutralize global isolates of diverse subtypes. However, in view of the difficulty in inducing bNabs in uninfected subjects, the induction of a complementary set of antibodies with limited neutralizing activity may be an attainable alternative approach. We have been following a single patient infected with HIV that has a cross-neutralizing activity to a variety of HIV-1 isolates including a panel of clinical isolates belonging to subtypes B, C, CRF01_AE and A. The patient is a hemophiliac who has been infected with HIV-1 for more than 25 years without any antiretroviral treatment. To elucidate the mechanism to control viruses in this patient we established a series of MAbs and demonstrated that a combination of antibodies to the V3 loop, CD4bs and CD4i covered effectively a wide range of viruses by their complementary and synergistic activities.

Results

Isolation and classification of monoclonal antibodies from an HIV-1 infected patient with long-term non-progressive disease

A total of 1718 B-cell clones were established by Epstein-Barr virus (EBV) transformation from the patient KTS376 who has had controlled HIV-1 infection for more than 25 years without any

Table 1
Subclass, target and genetic characteristics of human monoclonal antibodies against HIV-1 obtained from a patient with non-progressive disease.

No	Clone	Subclass	Target	Gene usage		Somatic mutation (%)		CDRH3 length
				VH	VL	VH	VL	
1	0.5γ (1C10)	IgG1κ	V3	VH3-30	VK2-28	10.8	4.1	18
2	1D9	IgG1κ	V3	VH3-30	VK2-28	12.8	3.5	16
3	5G2	IgG1κ	V3	VH3-30	VK2-28	12.8	6.5	16
4	16G6	IgG1λ	V3	VH5-51	VL3-19	4.9	6.4	7
5	717G2	IgG2κ	V3	VH3-30	VK2D-29	10.8	7.1	21
6	2F8	IgG1λ	V3	–	–	–	–	–
7	3E4	IgG1κ	V3	–	–	–	–	–
8	3G8	IgG1κ	V3	–	–	–	–	–
9	42F9	IgG1κ	CD4bs	VH3-20	VK1-39	2	2.5	19
10	49G2	IgG1λ	CD4bs	VH1-18	VL1-44	5.9	1.8	22
11	82D5	IgG1λ	CD4bs	VH1-18	VL1-44	6.2	1.7	22
12	0.5δ(3D6)	IgG1λ	CD4bs	–	–	–	–	–
13	4E3	IgG1κ	CD4bs	–	–	–	–	–
14	7B5	IgG2λ	CD4bs	–	–	–	–	–
15	4E9C	IgG1κ	CD4i	VH1-69	VK3-15	6.6	1.8	22
16	916B2	IgG1λ	CD4i	VH1-69	VL7-46	8.7	5.9	16
17	917B11	IgG1λ	CD4i	VH1-69	VL1-51	5.9	2.8	28
18	4C11	IgG1λ	CD4i	–	–	–	–	–
19	5D6S	IgG1κ	CD4i	VH1-69	VK3-20	9.3	9.6	26
20	7F11	IgG2κ	CD4i	–	–	–	–	–
21	5E8	IgG2κ	–	–	–	–	–	–
22	7B9N	IgG3κ	–	–	–	–	–	–
23	9F7	IgG1κ	–	–	–	–	–	–
24	39D5	IgG3κ	–	–	–	–	–	–
25	43D7	IgG2κ	–	–	–	–	–	–

VH: Variable heavy chain. VL: Variable light chain. CDRH3: Third complementary determinant region of the heavy chain. Not determine.