

1 **Introduction**

2 The envelope protein (Env) of human immunodeficiency virus type-1 (HIV-1)
3 presents on the virus surface as 'spikes' composed of trimers comprising three gp120-gp41
4 complexes (6, 32, 33). Among the regions that induce the neutralization antibody (NAb)
5 response, the third variable domain (V3 loop) of gp120 is considered one of the major
6 targets of the host immune response (23, 69). It has been estimated that as much as half the
7 antibody response against HIV-1 Env in patient sera is directed against the V3 region (43).
8 A recent crystallographic study revealed that the V3 loop contains features that are essential
9 for coreceptor binding and that the extended nature and antibody accessibility of V3 is
10 associated with its immunodominance (20).

11 HIV-1 primary isolates are relatively resistant to neutralization by NAb and
12 recombinant soluble CD4 (rsCD4) compared with variants selected for growth in
13 permanent cell lines (42, 52, 55). Studies addressing differences between
14 neutralization-sensitive and -resistant variants have revealed the involvement of several
15 mechanisms that underlie the neutralization resistance of primary isolates, including the
16 occlusion of epitopes within the oligomer, extensive glycosylation and extension of variable
17 loops from the surface of the complex, as well as steric and conformational blocking of
18 receptor binding sites (7, 12, 32, 38, 49, 50, 54, 62). The structural features of gp120
19 tolerate a vast array of mutations that permit the selection of neutralization escape variants,
20 as has been previously demonstrated in culture assays, animal models and infected
21 individuals (24).

22 Although there is ample data showing that NAb can protect against HIV-1
23 infection in vitro and in animal models in vivo, activity in infected humans remains

1 controversial (3, 4, 9, 14, 22, 40, 48, 58). Studies addressing NAb in primary infections
2 have suggested that most recently infected individuals mount a vigorous antibody response
3 against autologous viruses. However, the rapid evolution of HIV in the presence of NAb
4 results in the emergence of escape mutants. As a consequence, at any time during an early
5 stage of the HIV disease, NAb are more likely to recognize earlier autologous viruses than
6 contemporaneous ones. Despite evidence of phenotypic resistance, the genetic basis of the
7 mechanism allowing primary viruses to escape from NAb is poorly understood. Wei et al.
8 found that glycosylation in the envelope plays an important role in allowing escape from
9 neutralization (62). In contrast, in a recent study Frost et al. found that viral escape from
10 NAb is correlated with the rate of amino acid substitution rather than changes in
11 glycosylation or insertions or deletions in the envelope (14). Because of the polyclonal
12 nature of NAb in patient sera, it is difficult to clarify the genetic mechanism responsible for
13 neutralization escape.

14 Neutralization escape from anti-V3 monoclonal antibodies (MAbs) has been
15 induced in TCLA viruses in several experiments, and associated with amino acid
16 substitution within the epitope in the V3 loop (8, 37, 65). However, Park et al. showed that
17 human sera with neutralizing antibodies, which contained polyclonal antibodies directed at
18 the V3 region, induced neutralization-resistant variants without V3 amino acid substitution
19 (46). Neutralization studies using anti-V3 antibodies against primary isolates suggest that
20 the neutralization-resistant phenotype is associated with changes in the sequences outside
21 V3, rather than variation within the V3 epitope (29, 62). However, the contribution of each
22 change in the envelope to the emergence of escape mutants remains unclear because they
23 are not selected under neutralizing MAbs pressure.

1 Recently, we described a humanized MAb, KD-247 that displayed
2 cross-neutralizing activity against HIV-1 clade B isolates (11). The epitope of KD-247 was
3 mapped to six amino acids around the PGR core sequence at the tip of the V3 loop. The
4 shortest reactive peptide recognized by KD-247 was determined to be IGPGR, which is
5 shared by 49% of HIV-1 isolates in clade B (35). As well, complete protection from
6 challenge infection by a pathogenic strain of SHIV_{89.6} was observed when using a high
7 concentration of the antibody in an animal model (10). A molecularly cloned CCR5-tropic
8 HIV-1 strain, JR-FL, which is relatively resistant to neutralization (15, 50), was exposed to
9 KD-247 to obtain a neutralization-escape mutant (67). Induction of the
10 neutralization-resistant virus with a mutation in the V3-tip was observed in the presence of
11 a high concentration of KD-247 and the escape variant was found to be more sensitive to
12 CCR5 inhibitors and rsCD4 than the original strain (67).

13 The present study sought to understand how virus mutation impacts on the activity
14 of an anti-V3 MAb, KD-247. For this we subjected a primary R5 virus, MOKW, to
15 selection pressure by KD-247. The present data suggested that it is necessary to pass a
16 phased step so that the escape mutant against the anti-V3 antibody can emerge.
17 Neutralization escape variants with V2 mutations in gp120 could be selected at relatively
18 low KD-247 pressures, but high concentrations of KD-247 were required for induction of a
19 completely resistant variant with amino acid substitution in the epitope. Moreover, we
20 present evidence suggesting that some V2 mutations change the tertiary or quaternary
21 structure of the envelope trimers on the viral surface that are involved in the neutralization
22 resistance of the primary isolate. Clarification of the mechanisms responsible for this
23 neutralization resistance may provide important insight into possible methods for the

1 induction of potent and cross-neutralizing antibody responses capable of neutralizing
2 various primary isolates.

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1 **Materials & Methods**

2 **Cells, culture conditions, reagents and viruses.** PM1/CCR5 cells (68) were maintained in
3 RPMI-1640 (Sigma) supplemented with 10% heat-inactivated calf serum (FCS: HyClone
4 Laboratories, Logan, UT), 50 U/ml of penicillin, 50 mg/ml of streptomycin and 100 µg/ml
5 of G418 (Sigma). 293T cells were maintained in Dulbecco's modified Eagle medium
6 (DMEM; Sigma) supplemented with 10% heat-inactivated FCS. The CD4- and
7 CCR5-expressing human osteogenic sarcoma cell line GHOST-hi5 was maintained in
8 DMEM supplemented with 10% FCS, G418 (200 µg/ml), hygromycin B (100 µg/ml;
9 Sigma) and puromycin (1 µg/ml; Sigma).

10 KD-247, an anti-gp120-V3 antibody, was produced as previously described (11).
11 17b, a monoclonal antibody against the CD4-induced epitope and IgGb12, a monoclonal
12 antibody against the CD4-binding epitope were provided by the National Institutes of
13 Health AIDS Research and Reference Reagent Program. 447-52D, an anti-gp120 V3 MAb,
14 was gift from Dr. Suzan Zolla-Pazner (New York University School of Medicine). 2D7, an
15 anti-CCR5 MAb, and RPA-T4, an anti-CD4 MAb, were purchased from BD Biosciences
16 Pharmingen (San Jose, CA, USA). Recombinant human soluble CD4 (rsCD4) was
17 purchased from R&D systems, Inc. (Minneapolis, MN). TAK-779, a CCR5 inhibitor, was
18 kindly provided by Takeda Chemical Industries Ltd. (Osaka, Japan). AK-602, a CCR5
19 inhibitor, was gifted by Ono Pharmaceutical Co. Ltd., Osaka, Japan.

20 The R5 primary HIV-1, MOKW, was isolated from a drug-naïve Japanese patient
21 (36). This virus was passaged in phytohemagglutinin (PHA)-activated peripheral blood
22 mononuclear cells (PBMCs) and the culture supernatant stored at -80°C until use.

23

1 **Isolation of a KD-247-resistant mutant from MOKW in vitro.** The selection of KD-247
2 escape variants from MOKW was performed as previously described (67). Briefly, MOKW
3 was pre-incubated in the presence of KD-247 for 30 min at 37°C, and then PM1/CCR5 cells
4 (4×10^4) were exposed to 500 TCID₅₀ (50% tissue culture infective dose) of the
5 pre-incubated MOKW. After incubation for 5 h at 37°C, cells were pelleted down and
6 resuspended in RPMI-1640 supplemented with 10% FCS without KD-247. Viral
7 replication was monitored by observation of the cytopathic effects (CPEs) in PM1/CCR5
8 cells. The culture supernatant was harvested on day 7 and used to infect fresh PM1/CCR5
9 cells for the next round of culture in the presence of increasing concentrations of KD-247.
10 When the virus began to propagate rapidly in the presence of KD-247, the MAb
11 concentration was further increased. After the virus was passaged in the presence of up to
12 2000 µg/ml KD-247 in PM1/CCR5 cells, a KD-247-resistant virus, MOKW9p(2000), was
13 recovered from the cell culture supernatant. MOKW was also passaged for the same time
14 period in PM1/CCR5 cells in the absence of KD-247, and the resulting virus was
15 designated MOKW9p(-).

16

17 **Amplification of viral cDNA and nucleotide sequencing.** Viral RNA was extracted from
18 cell culture supernatants with several concentrations of KD-247 using a QIAamp viral RNA
19 kit (Qiagen). Viral RNAs were reverse-transcribed using a High Capacity cDNA Archive
20 Kit (Applied Biosystems) with specific antisense primer ENVN
21 (5'-CTGCCAATCAGGGAAGTAGCCTTGTGT-3'). Nested PCR was performed to
22 amplify the gp120 C1-C4 coding region as described previously (60). The primers used
23 were as follows: for the first-step PCR, 1B

1 (5'-AGAAAGAGCAGAAGACAGTGGCAATGA-3') and H
2 (5'-TAGTGCTTCCTGCTGCTCCCAAGAACCC-3'); for the second-step PCR, 2B
3 (5'-AGCAGAAGACAGTGGCAATGAGAGTGA-3') and F
4 (5'-ATATAATTCACCTTCTCCAATTGTCCCTCAT-3'). The products of the nested PCR
5 were inserted in the TA vector (Invitrogen) and sequenced using Big Dye Terminator
6 ver.1.1 (Applied Biosystems) in accordance with the manufacturer's instructions. The
7 sequence data from passaged samples were deposited in the DNA Data Bank of Japan
8 under accession numbers AB262847-AB262951.

9

10 **MTT assay.** The neutralization-sensitivities of the each passaged MOKW viruses to
11 KD-247 were determined as previously described (67). Briefly, PM1/CCR5 cells (2×10^3
12 cells/well) were exposed to 100 TCID₅₀ of each passaged virus in the presence of various
13 concentrations of KD-247 in 96-well round-bottom microculture plates and incubated at
14 37°C for 7 days. After removal of 100 µl of medium from each well, 10 µl of MTT solution
15 (7.5 mg/ml) in PBS was added to each well and the plate was incubated at 37°C for 3 h.
16 After incubation, 100 µl of acidified isopropanol containing 4% (vol/vol) Triton X-100 was
17 added to each well to dissolve the formazan crystals. The optical density (wavelength, 570
18 nm) was measured using a microplate reader. Assays were performed in duplicate or
19 triplicate.

20

21 **Construction of mutant envelope expression vectors.** Proviral DNA was extracted from
22 each batch of passaged MOKW-infected PM1/CCR5 cells using a QIAamp DNA blood
23 mini kit (Qiagen). For the construction of each passaged envelope expression vector, we

1 used pCXN2, which has a chicken-actin promoter. Briefly, we amplified MOKW gp160
2 regions using LA *Taq* (Takara) with primers ENVA
3 (5'-GGCTTAGGCATCTCCTATGGCAGGAAGAA-3') and ENVN (see above). The
4 products of the PCR were inserted into pCR-XL-TOPO (Invitrogen). The sequences of the
5 amplified *env* region of MOKW were confirmed using an ABI 377 automated DNA
6 sequencer. The *EcoRI* fragment of pCR-XL-MOKW containing the entire *env* region was
7 ligated into pCXN2 to give pCXN-MOKW-RDP, pCXN-MOKW-KNL/C3m and
8 pCXN-MOKW-KNL/V3m. The pCXN-MOKW-KNL vector was constructed by replacing
9 the *StuI-Bsu36I* fragment of pCXN-MOKW-KNL/C3m with a corresponding MOKW-RDP
10 fragment. The pCXN-MOKW-RDP/V3m and pCXN-MOKW-RDP/C3m vectors were
11 constructed by replacing the *StuI-Bsu36I* fragment of pCXN-MOKW-RDP with the
12 corresponding pCXN-MOKW-KNL/V3m or pCXN-MOKW-KNL/C3m fragments,
13 respectively. pCXN-MOKW-KNP, pCXN-MOKW-RDL, pCXN-MOKW-KDL and
14 pCXN-MOKW-RNL were generated by site-directed mutagenesis using the QuickChange
15 Site-Directed Mutagenesis Kit (Stratagene) in accordance with the manufacturer's
16 instructions. These sequence data have been deposited in the DNA Data Bank of Japan
17 under accession numbers AB262952-AB262961.

18

19 **Pseudovirus preparation.** Five μg of pNL4-3.luc.R'E' and 0.5 μg of pRSV-Rev (18),
20 supplied by the NIH AIDS Research and Reference Reagent Program, and 4.5 μg of the
21 MOKW Env-expressing pCXN2 were cotransfected into 293T cells using the Effectene
22 Transfection Reagent (Qiagen). At 24 h after the transfection, the pseudovirus-containing
23 supernatants were harvested, filtered through a 0.2- μm pore-size filter and stored at -80°C .

1 To measure the pseudovirus activity, a luminescence assay using GHOST-hi5 cells was
2 used as previously described (60).

3

4 **Neutralization assays.** A single-cycle infectivity assay was used to measure the
5 neutralization of MOKW pseudoviruses as described previously (60). Briefly, MAb at
6 various concentrations and a pseudovirus suspension corresponding to 100 TCID₅₀ were
7 preincubated on ice for 15 min. The virus-antibody mixtures were added to GHOST-hi5
8 cells, which on the preceding day had been seeded in a 96-well plate (1.5x10⁴ cells/well).
9 Cultures were incubated for 2 days at 37°C, washed with PBS and lysed with lysis buffer
10 (Luc PGC-50; PicaGene). Following transfer of the cell lysates to luminometer plates
11 (Coastar 3912), the luciferase activity (in relative light units) in each well was measured
12 using Luciferase Substrate (100 µl/well; PicaGene) in a TR717 microplate luminometer
13 (Applied Biosystems). The reduction in infectivity was determined by comparing the
14 relative light units in the presence and absence of MAb and was expressed as the
15 percentage of neutralization. The same assay was repeated 2-3 times.

16

17 **In vitro binding assay to the MOKW envelope-expressing cell surfaces.** In vitro binding
18 assays were performed as previously described (53, 67). *EcoRI* fragments of MOKW *env*
19 genes from pXL-MOKWs were subcloned into the corresponding sites in pDNR-1r
20 (Clontech). The vectors were sequenced to confirm the presence of the desired *env* gene
21 and the absence of other changes. The *env* gene fragments were then subcloned into
22 pLP-IRES2-EGFP (Clontech) using Cre-recombinase (Clontech) in accordance with the
23 manufacturer's instructions. 293T cells were cotransfected with pRSV-Rev (0.5 µg) and

1 pLP-IRES2-EGFP-MOKW (9.5 µg) using the Effectene Transfection Reagent. After 36 h,
2 the cells were harvested, incubated with each anti-HIV-1 MAb in combination with
3 biotin-conjugated anti-human IgG and PerCP-conjugated streptavidin (BD Pharmingen),
4 gated for the GFP-positive area, and analyzed using a FACSCalibur flow cytometry system.

5
6 **MAb-gp120 binding assay.** Culture supernatants containing the pseudotyped viruses were
7 treated with 1% nonionic Nonidet P-40 to provide a source of gp120. Binding assays for
8 MAbs to gp120 were then performed essentially as described elsewhere (41, 59). Briefly,
9 gp120s from transfected culture supernatants, diluted in Tris-buffered saline containing
10 10% FCS and 1% Nonidet P-40, were captured onto solid phase via their carboxyl termini
11 using sheep polyclonal antibody D7324 (Aalto Bioreagents, Dublin, Ireland). MAb was
12 added in PBS containing 10% FCS and 0.1% nonionic detergent Tween 20, and bound
13 MAb was detected with alkaline phosphatase-conjugated goat anti-human IgG (Sigma)
14 followed by addition of phosphatase substrate (Sigma). A_{405} measurements were taken
15 using a microplate reader.

16
17 **Construction of chimeric NL4-3/MOKW *env* proviruses.** Chimeric proviruses were
18 constructed from the pNL4-3 proviral plasmid (AIDS Research and Reference Reagent
19 Program, National Institute of Allergy and Infectious Diseases) by overlapping PCR as
20 previously described with minor modifications (31). Briefly, the gp160 coding sequences
21 were amplified from the cloning vectors using the primers EnvFv
22 (5'-AGCAGAAGACAGTGGCAATGAGAGCGAAG-3') and EnvR
23 (5'-TTTTGACCACTTGCCACCCATCTTATAGC-3'). A portion of the NL4-3 provirus

1 from nucleotides 5284 to 6232 was amplified with primers NL(5284)F
2 (5'-GGTCAGGGAGTCTCCATAGAATGGAGG-3') and NL(6232)Rv
3 (5'-CTTCGCTCTCATTGCCACTGTCTTCTGCT-3'). This fragment encompasses the
4 unique *EcoRI* restriction site in pNL4-3. Another fragment from the NL4-3 provirus
5 spanning nucleotides 8779 to 9045 was amplified using primers NL(8779)F
6 (5'-GCTATAAGATGGGTGGCAAGTGGTCAAAA-3') and NL(9045)R
7 (5'-GATCTACAGCTGCCTTGTAAGTCATTGGTC-3'). This fragment includes the
8 unique *XhoI* restriction site in pNL4-3. Overlapping PCR was used to join the
9 gp160-coding sequence from the desired clone to the fragment encompassing base 8779 to
10 9045 that had been amplified from pNL4-3. The resulting fragment was then similarly
11 joined to the amplified fragment encompassing bases 5284 to 6232 from pNL4-3. The
12 product was digested with *EcoRI* and *XhoI* and subcloned into the corresponding site in
13 pBluescript SK(+) (Stratagene) for sequencing and subsequent manipulation. The
14 *EcoRI-XhoI* fragment for each *env* gene was then subcloned back into pNL4-3. The end
15 results were proviral plasmids that differed from each other only in the *env* gene. The
16 resulting plasmids were designed pNL-MOKW-RDL and pNL-MOKW-KNL.

17

18 **Virus preparation and viral replication assay in PM1/CCR5 cells.** Three μg of
19 pNL-MOKW-RDL or pNL-MOKW-KNL was transfected into 293T cells using the
20 Effectene Transfection Reagent. At 24 h after transfection, the virus-containing
21 supernatants were harvested, filtered through a 0.2- μm pore-size filter and frozen in
22 aliquots at -150°C . Viral yields were quantified by a RETROtek HIV-1 p24 Antigen ELISA
23 kit (ZeptoMetrix). PM1/CCR5 cells (1×10^4) were exposed to NL4-3/MOKW *env* chimeric

1 viruses corresponding to 10ng of p24, and then pre-incubated for 4 h at 37°C. After
2 incubation, cells were pelleted down and resuspended in RPMI-1640 supplemented with
3 10% FCS. Viral replication was monitored by measuring of p24 kinetics in duplicate.

1 Results

2 **Anti-HIV-1 activity of KD-247 for the primary R5 isolate MOKW.** KD-247
3 recognized an epitope that contains the IGPGR sequence located at the tip of V3 and
4 neutralized primary HIV-1 in clade B with matching sequence motifs (11). To study how
5 bulked primary R5 virus can escape from anti-V3 antibody, we selected a genetically
6 heterogenous HIV-1 primary isolate, MOKW, rather than a molecular clone to allow escape
7 mutants to be selected from a quasi-species pool as well as generated de novo. MOKW was
8 isolated by standard PBMC culture from a drug-naïve Japanese patient infected with HIV-1
9 by heterosexual contact (36). The isolate was sensitive to neutralization by KD-247 with an
10 IC_{50} of 3.4 $\mu\text{g/ml}$, which is comparable to the IC_{50} values of Ba-L, JR-FL and 89.6 (data not
11 shown).

12
13 **Selection of KD-247 escape mutants from MOKW.** To select an HIV-1 variant
14 that could escape neutralization by KD-247 in vitro, we exposed PM1/CCR5 cells to
15 MOKW, and serially passaged the virus in the presence of increasing concentrations of
16 KD-247. PM1/CCR5 cells were highly sensitive to both X4 and R5 HIV infection and were
17 accompanied by prominent syncytia (68). As a control, MOKW was passaged under the
18 same conditions but without KD-247 to allow us to monitor spontaneous changes that
19 occurred in the virus during prolonged PM1/CCR5 cell passaging. The selected virus was
20 initially propagated in the presence of 10 $\mu\text{g/ml}$ KD-247, and during the course of the
21 selection procedure, the MAb concentration was increased to 2000 $\mu\text{g/ml}$ (Fig. 1A). After 5
22 rounds of passaging, a viral variant, designated MOKW5p(200), arose that replicated in the
23 presence of 200 μg of KD-247 per ml. Moreover, after 9 rounds of passaging, a viral

1 variant, designated MOKW9p(2000), arose that infected PM1/CCR5 cells efficiently in the
2 presence of 2000 $\mu\text{g/ml}$ KD-247 (Fig. 1A). We harvested each passaged virus and a
3 passaged control virus, designated MOKW9p(-), and evaluated their sensitivity to KD-247
4 using the MTT assay (Fig. 1B). The IC_{50} values of KD-247 against MOKW9p(-),
5 MOKW5p(200) and MOKW9p(2000) were 0.15 $\mu\text{g/ml}$, 16 $\mu\text{g/ml}$ and >100 $\mu\text{g/ml}$,
6 respectively, indicating that MOKW had acquired a resistant phenotype against KD-247
7 during the in vitro selection.

8

9 **Sequences of the envelope region of the KD-247 escape mutants.** To determine the
10 genetic basis underlying the resistance of the variant MOKW strains, the *env* gene was
11 amplified and sequenced. The C1-C4 regions of the envelope were sequenced after cloning
12 the PCR product for each region using cDNAs synthesized from viral RNAs obtained from
13 the supernatants of infected cells as previously described (67). A total of 6-9 clones for each
14 sample from PCR products from the passaged viruses were isolated and sequenced.

15 Before beginning selection by KD-247, the V2 regions of MOKW *env* had variable
16 amino acid sequences (Fig. 2). In the first passage, two amino acid (aa) mutations in the V2
17 region and two aa mutations in the C3 region (5 of 9 clones) appeared, and after the 5th
18 passage, the ratio of V2 and C3 mutated variants had further increased (7 of 8 clones).
19 However, after the 9th passage, the C3 mutations had completely disappeared, and a
20 Pro-to-Leu substitution (P313L) in the V3-tip emerged in addition to the mutations in the
21 V2 region (Fig. 2). The appearance of escape mutants with a V3-tip mutation was
22 anticipated, because prior studies on the profile of KD-247 binding to peptides suggested
23 that amino acid substitution at the V3-tip abrogates MAb binding (11). Some changes in the

1 envelope sequence in other regions, including C1, V1, C2, V4 and C4, of the escape mutant
2 were found even at early time points in the presence of the selective pressure. It is possible
3 that these mutations also confer resistance to KD-247, but lead to viruses with decreased
4 fitness, and thus they did not expand in the subsequent passage (Fig. 2; some data not
5 shown). The virus passaged in PM1/CCR5 cells without KD-247 did not show the P313L
6 substitution at passage 9 (0 of 9 clones) (Fig. 2). However, accumulation of a leucine to
7 proline mutation at position 175 (L175P) in the V2 region was observed in the culture
8 without KD-247. This mutation was not found in any passaged variants with KD-247.

9
10 **Neutralization sensitivities of mutated MOKW pseudoviruses.** To determine
11 which substitutions were responsible for KD-247 resistance, we constructed
12 luciferase-reporter viruses, which were pseudotyped with the representative envelopes of
13 MOKW5p(200), MOKW9p(2000) and passaged viruses without KD-247, MOKW9p(-),
14 and designated MOKW-KNL/C3m, MOKW-KNL/V3m and MOKW-RDP, respectively
15 (Fig. 3). Chimeric envelopes were constructed by replacing the mutated-region (V2, V3 or
16 C3) with a corresponding MOKW-RDP one (designed MOKW-KNL, MOKW-RDP/V3m,
17 MOKW-RDP/C3m, respectively), and then sensitivity was compared with that of the
18 passaged virus without KD-247. As shown in Fig. 4, the V3-tip mutated pseudoviruses,
19 MOKW-KNL/V3m and MOKW-RDP/V3m, were completely resistant to KD-247
20 (>25000-fold), whereas V2 mutated viruses, MOKW-KNL/C3m and MOKW-KNL, were
21 only partially resistant (125-fold and 500-fold respectively) (Fig. 4 and Table 1). The
22 involvement in neutralization resistance of mutation in the V1/V2 region has been reported
23 by number of researchers (12, 49, 50, 54). Our results show that the MOKW variants that

1 had V2 mutations and a resistant phenotype against KD-247 were selected under pressure
2 from relatively low concentrations of KD-247 (10-200 µg/ml), and that evolution of fully
3 resistant variants with mutation in the V3-tip was observed under pressure from high
4 concentrations of the antibody.

5 We then determined whether the KD-247 escape variants remained sensitive to
6 other neutralizing antibodies (447-52D and 17b), rsCD4, anti-CCR5 antibody (2D7),
7 anti-CD4 antibody (RPA-T4) and the small molecule CCR5 inhibitor (TAK-779) (Fig. 4
8 and Table 1). The KD-247 escape variants with the P313L mutation, MOKW-KNL/V3m
9 and MOKW-RDP/V3m, were also resistant to another anti-V3 MAb, 447-52D, and V2
10 mutated viruses without V3 mutation, MOKW-KNL/C3m and MOKW-KNL, were partially
11 resistant (the same as for KD-247). In contrast, the V2 mutated viruses, MOKW-KNL/C3m,
12 MOKW-KNL/V3m and MOKW-KNL, showed resistance to rsCD4 and 17b (a MAb to the
13 CD4-induced epitope; CD4i) compared with the pseudoviruses without V2 mutations,
14 MOKW-RDP, MOKW-RDP/C3m and MOKW-RDP/V3m. Moreover, the pseudoviruses
15 with V3-tip mutations, MOKW-KNL/V3m and MOKW-RDP/V3m, became significantly
16 more sensitive to TAK-779 and 2D7 as compared with pseudoviruses without the P313L
17 mutation (Fig. 4 and Table 1). No significant differences with respect to sensitivity to
18 RPA-T4 were observed between any pseudoviruses (Table 1). These data indicate that
19 V3-tip and V2 mutations confer neutralization resistance against anti-V3 antibodies, and
20 that these mutations affect viral sensitivity to neutralizing antibodies recognizing different
21 epitopes and anti-CCR5 antibody/agents.

22

23 **Binding affinity of neutralizing antibodies to MOKW Envs on the cell surface.**

1 To elucidate the mechanism by which escape virus variants with V3-tip and V2 mutations
2 become less sensitive to neutralizing antibodies, MOKW Env-expressing 293T cells were
3 established by transfection with each Env expression plasmid, and then stained with the
4 MAbs. Binding of KD-247, 447-52D and 17b to the surface-expressed Env proteins was
5 assayed using fluorescence-activated cell sorter analysis. As shown in Fig. 5A, the mean
6 fluorescence intensities (MFI) of KD-247 binding to the Envs without either V2 or V3
7 mutations, in the MOKW-RDP and MOKW-RDP/C3m cells, were 30.13 and 29.20
8 respectively. However, the corresponding values for the V3-tip mutated Env-expressing
9 cells, MOKW-KNL/V3m and MOKW-RDP/V3m, were almost the same as negative
10 controls (6.90 and 6.66, respectively). The MFI of the V2-mutated Env-expressing cells,
11 MOKW-KNL/C3m and MOKW-KNL, indicated a lower binding affinity (17.89 and 19.18,
12 respectively) than for Envs without V2 and V3 mutations. The binding pattern of 447-52D
13 to these Env-expressing cells was similar to that of KD-247 (Fig. 5B). However, reduction
14 in the binding of 17b was observed for strains with V2-mutated Envs, MOKW-KNL/C3m,
15 MOKW-KNL/V3m and MOKW-KNL, whereas no difference in 17b binding was noted for
16 the V3-mutants without V2 mutations (Fig. 5B). These findings are consistent with the
17 results of a single-round neutralization assay (Fig. 4). Taken together, these data suggest
18 that the mutations in V2 have a significant influence on access by antibodies to V3 as well
19 as to the CD4i epitope. This is because access by antibodies to the epitopes of the
20 functional envelope is related to neutralization sensitivity/resistance.

21

22 **Identification of the V2 region site responsible for the neutralization-resistant**
23 **phenotype by site-directed mutagenesis of specific residues.** Because KD-247

1 recognizes an epitope containing the IGPGR amino acid sequence in the V3-tip, the MAb
2 could not bind the V3-tip of mutated Envs. Consequently, KD-247 does not neutralize
3 V3-tip mutated virus strains (67). However, the mechanism of neutralization resistance
4 associated with V2 mutations is not known. To clarify the responsible mutation in the V2
5 region that confers the escape phenotype with respect to KD-247, we introduced V2 amino
6 acid changes individually and in combination into the MOKW-RDP Env expression vector
7 (Fig. 3), and measured the sensitivities of pseudoviruses with these envelopes to KD-247.
8 As shown in Fig. 7, the R166K/D167N double mutant, MOKW-KNP, showed almost the
9 same neutralization sensitivity as MOKW-RDP against KD-247. Surprisingly, a single aa
10 change (P175L in MOKW-RDL) was sufficient to confer >10000-fold resistance upon
11 MOKW-RDP, with an IC_{50} of >100 μ g/ml. R166K/P175L (MOKW-KDL) mutations also
12 conferred resistance. Both MOKW-RDL and MOKW-KDL were much more resistant than
13 the fully V2 mutated virus, MOKW-KNL (>100-fold and >10-fold resistance, respectively)
14 (Fig. 6). The D167N/P175L (MOKW-RNL) mutant was more resistant than MOKW-KNL
15 (10-fold), but less resistant than MOKW-RDL and MOKW-KDL. We also constructed a
16 V2-mutatnt of JR-FL and confirmed that JR-FL with aa substitution of Leu to Pro in 175th
17 residue became highly sensitive to KD-247 compared with JR-FL with Leu at 175th aa in
18 Env (data not shown). These results suggest that the 175th residue (Pro or Leu) is the
19 crucial amino acid for determining neutralization sensitivity against KD-247, and that the
20 phenotypic influence of the R166K and D167N changes is strictly context dependent,
21 requiring the presence of Leu at residue 175.

22 We then determined whether these pseudoviruses with various V2 mutations
23 remained sensitive to other neutralizing antibodies (447-52D and IgGb12), rsCD4, 2D7,

1 RPA-T4 and TAK-779 (Fig. 6). MOKW-RDL and MOKW-KDL were also resistant to
2 another anti-V3 MAb, 447-52D, CD4 binding site MAb, IgGb12, and rsCD4. MOKW-RNL
3 was partially resistant compared with MOKW-KNL, but less resistant than MOKW-RDL
4 and MOKW-KDL against 447-52D, IgGb12 and rsCD4. These results were similar to those
5 for KD-247. All V2 mutated clones were sensitive to TAK-779 and 2D7, as was
6 MOKW-RDP (Fig. 6 and not shown). However, the anti-CD4 MAb RPA-T4 neutralized
7 both MOKW-RDL and MOKW-KDL at an approximately 3-fold lower concentration
8 compared with other viruses (Fig. 6). These results suggest that the 166th and 167th amino
9 acids (RD and KN) may help compensate for any reduced fitness of viruses with Leu at the
10 175th aa. On the other hand, Pro at position 175 in MOKW-RDP might be accumulated
11 because it confers better fitness to replicate on PM1/CCR5 cells in the absence of KD-247
12 pressure.

13

14 **Binding affinity of MAbs against monomeric or cell-surface expressed gp120**
15 **with mutations in V2.** To determine the difference in binding of MAbs to monomeric
16 gp120 of MOKW Env with V2 mutations relative to that of MOKW-RDP, we performed
17 MAb binding assays. Monomeric gp120 was prepared from pseudoviruses that had a series
18 of V2 mutations and was captured on an ELISA plate followed by detection by MAbs. No
19 difference was noted in the binding activity of KD-247 or 447-52D to monomeric gp120
20 from V2-mutated and MOKW-RDP envelopes (Fig. 7). These results suggest that
21 V2-mutated Envs retain the neutralizing epitope at least in monomeric gp120.

22 In contrast to the monomeric form, gp120 expressed on the cell surface contained,
23 to a certain degree, functional envelope oligomers that were directly related to the

1 infectivity and neutralization sensitivity of the virus. To compare the binding activity of
2 MAbs for the surface-expressed Env with the results obtained for monomeric gp120, 293T
3 cells transfected with MOKW-RDP and MOKW-KNP, a V2-mutant strain, were subjected
4 to FACS analysis. As shown in Fig. 8A and 8B, the relative binding of KD-247, 447-52D
5 and IgGb12 to Env expressed on the cell surface was no different than for MOKW-RDP
6 and MOKW-KNP.

7 Consistent with the results of the single round neutralization assay shown in Fig. 6,
8 MOKW-RDL had the lowest binding affinity for all tested MAbs. To determine which
9 mutations (166K or 167N) further influence binding affinity, in addition to the
10 MOKW-RDL background, we constructed MOKW-KDL and MOKW-RNL Envs and
11 measured the binding affinity by FACS. The MOKW-KDL Env was found to have a
12 slightly greater binding affinity for KD-247, 447-52D and IgGb12 than MOKW-RDL. But
13 cell surface binding of all tested MAbs to MOKW-RNL was better than for MOKW-KDL.
14 The strain with fully V2-mutated Env, MOKW-KNL, had a binding profile that was
15 intermediate between single- or double-mutated Envs and non-mutated Env, but in the case
16 of IgGb12, the binding affinity of MAbs for MOKW-KNL was comparable to that for
17 MOKW-RDP. These data were consistent with the results obtained from the neutralizing
18 assay using a high concentration of each MAb (Fig. 6).

19

20 **Comparison of replication kinetics between NL-MOKW-RDL and**
21 **NL-MOKW-KNL.** Although the MOKW-RDL variant was much more resistant against
22 KD-247 than the MOKW-KNL variant (Fig. 6) and the RD sequence was more prevalent
23 than KN at positions 166 and 167 in the V2 region before selection (Fig. 2), the MOKW