

1 variants with the 166K/167N/175L were selected and outgrown under KD-247 pressure
2 (Fig. 2). It was possible that the KN sequences at positions 166 and 167 are necessary to
3 compensate for the fitness of the variants with 175L in PM1/CCR5 cells as shown in Fig. 6.
4 To clarify the role of KN at positions 166 and 167 in replication, we constructed replication
5 competent viruses with MOKW Env with RD or KN in addition to 175L (NL-MOKW-RDL
6 and NL-MOKW-KNL) and compared their replication kinetics. As shown in Fig. 9,
7 NL-MOKW-KNL replicated faster than NL-MOKW-RDL in PM1/CCR5 cells. These data
8 suggested that KN sequences at positions 166 and 167 with 175L variant confer replication
9 advantage in PM1/CCR5 cells. Therefore, the intermediate resistant variant MOKW with
10 the KNL sequence in the V2 region might replicate more rapidly than the highly resistant
11 variant MOKW with RDL against KD-247 in the course of selection.

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1 **Discussion**

2 Although an attack from the humoral immune response, especially anti-V3 NAb, is lasting
3 against HIV-1 in vivo, it is not clear why the V3-tip sequence is conserved in the course of
4 the infection. In the present study, by using a genetically heterogeneous HIV-1 primary R5
5 isolate, MOKW we found that V2- and C3-mutated virus expanded in conditions with a
6 relatively low concentration of KD-247. Further, we found that the V3-tip mutated virus
7 was induced only in conditions with a high concentration of MAb (more than 500 µg/ml).
8 Using region-swapping analysis, it was found that both V2 and V3-tip mutations can cause
9 an escape phenotype against anti-V3 antibody. Neutralization escape variants with V2
10 mutations could be selected from quasi-species existing in the primary isolate at relatively
11 low antibody pressures. On the other hand, highly resistant variants with amino acid
12 substitutions in the V3 epitope emerged via evolution of the virus in the presence of a high
13 concentration of the MAb.

14 The V1/V2 region of gp120 is highly diverse, not only in respect to virus subtypes
15 but also in respect to intra-species diversity in the same patient (16, 61, 66). The primary
16 isolate, MOKW, also displayed diversity in the V2 region (Fig. 2) and the 1st passaged
17 virus already harbored mutations in the V2 region. Many researchers have reported that the
18 V1/V2 domain strongly influences neutralization of the anti-V3 MAbs, MAbs to the other
19 epitopes and rsCD4 (12, 13, 25, 27, 3044, 49, 50). Moreover, structural models of the Env
20 trimer have been proposed that place the base of the V1/V2 loop of one subunit in
21 proximity to the V3 loop of a neighboring subunit (32, 34). In the present study we
22 observed a reduction in the binding of anti-V3 MAbs to V2-mutated Env expressed on the
23 cell surface, whereas mutations in V2 did not have an effect on the binding of the MAbs to

1 monomeric gp120. These results suggest the association of V2 mutations with anti-V3
2 antibody accessibility in the context of the oligomeric conformation of the functional
3 envelope. It has been proposed that the gp120 of TCLA strains forms a relatively open
4 conformation and that the primary isolate trimeric complex has a more closed conformation
5 (2, 51). These findings suggest that antibody-induced V2 mutations may affect envelope
6 oligomers on the viral surface so that they form a more closed conformation; thus
7 neutralization epitopes become less accessible to antibodies. The essential amino acid
8 residues responsible for neutralization resistance located at the center of the V2 region may
9 have a role in the interaction with the V3-loop of neighboring gp120 molecules.

10 We previously described the in vitro selection and characterization of a
11 KD-247-escape mutant of JR-FL (67). The amino acid substitution that was critical for the
12 resistant phenotype was Gly to Glu at residue 314 (G314E) in the V3-tip region. The
13 genetically engineered mutant was completely resistant to neutralization by KD-247. Other
14 researchers have also reported the induction of V3-mutated viruses by strain-specific
15 anti-V3 MAb in in vitro culture systems (8, 37, 65). In earlier studies, combinations of
16 genetically cloned viruses and highly potent NAb were used for in vitro selection (8, 37,
17 65). The escape mutants were induced in the presence of high concentrations of MAbs to
18 acquire V3-tip mutation(s). In contrast to these observations in vitro, the Gly-Pro-Gly
19 amino acid sequence in the V3-tip varies to a negligible extent in clinical isolates from
20 HIV-1-infected patients (35). The important role of the V3-tip in forming the β -turn of the
21 V3 loop and in interacting with chemokine receptors may partly explain the discrepancy
22 between in vivo and in vitro studies (19). In addition to V3 loop, variation in or near the
23 V1/V2 region is known to contribute to coreceptor usage of HIV-1 (17, 21, 27, 28, 47, 56,

1 64, 66). However, it is possible that HIV-1 suffers critical damage with respect to
2 replication and infectivity through mutation in the V3 region, especially in the tip region,
3 because the V3 loop plays a major role in the interaction of gp120 with coreceptors. Thus,
4 mutations in the V2 region may be important, not only to avoid anti-V3 pressure but also to
5 maintain replication efficiency at a suitable level.

6 In the present study, which used a MOKW primary virus for selection, the virus
7 underwent acquisition of resistance via V2 mutations and then V2 plus V3 mutations in
8 response to increases in the concentration of MAb. By contrast, no V2 mutations were
9 selected in JR-FL by KD-247 pressure in our previous study (67). Because the primary
10 isolate, MOKW, was a few passaged viruses that contained quasi-species of related but
11 distinct viruses, relatively resistant variants with mutations in V2 were easily selected for
12 replication. Pinter et al. found that inherent neutralization resistance in JR-FL is mediated
13 by the V1/V2 domain (50). It is therefore possible that the V1/V2 sequence (or the
14 conformation of this sequence) in JR-FL already had a resistant phenotype against anti-V3
15 antibodies, because the escape variant underwent mutation directly in the V3-tip of the
16 KD-247-reacting epitope (67).

17 In the in vitro selection process, the 175th amino acid (Pro or Leu) in the V2
18 region of MOKW played a crucial role in dramatically changing the oligomeric state of the
19 envelopes. However, MOKW-RDP obtained by prolonged culture in vitro without KD-247
20 became neutralization-sensitive compared with MOKW. 175P was the amino acid
21 responsible for the change to the neutralization-sensitive phenotype, whereas viruses with
22 175L became highly resistant to the MAbs and rsCD4. The same phenomenon was
23 observed in the relatively resistant strain JR-FL. 175L is highly conserved among HIV-1

1 strains, and is located at the center of the V2 loop (35), and the V2 region also mediates
2 gp41-independent intersubunit contact (5). It is therefore possible that the V2 region,
3 including the 175th residue, by mediating changes in the conformation of the gp120
4 oligomer, contributes to resistance to neutralization by limiting the exposure of epitopes.

5 Although the MOKW-RDL virus had highly resistant phenotype against KD-247,
6 MOKW with R166K/D167N and P175L in the V2 region and with the C3 mutations, which
7 were less neutralization-resistant than MOKW-RDL, were expanded in vitro selection.
8 Substitutions at residues 165-167 during the adaptation of various HIV-1 strains to
9 replication in vitro have been reported; the adaptation is associated with an increase of the
10 positive charge of this amino acid motif (1, 13, 39, 52, 57, 63). In our present study, the
11 amino acid change at 166/167 in the V2 region in passaged MOKWs with KD-247 was RD
12 to KN, again increasing the positive charge. MOKW-RDL was partially sensitive to
13 anti-CD4 MAb (RPA-T4) compared with MOKWs with 175P, MOKW-RDP, and other
14 166/167-mutated MOKWs. Pugach et al. also showed that charged aa at 165-167 with 175L
15 in the V2 region emerged during in vitro replication and that those viruses also had their
16 sensitivity to rsCD4 and resistance to the anti-CD4 antibody slightly changed by the
17 165-167 aa charge (52). It is therefore possible that the 166th/167th amino acid mutations
18 are necessary to compensate variants with 175L for interactions with CD4 molecules on the
19 target cell membrane. As shown in Fig. 9, KN sequences at positions 166 and 167 with the
20 175L variant confer replication advantage in PM1/CCR5 cells. Therefore, 166/167th aa
21 may help compensate for the reduced fitness of the viruses with Leu as the 175th aa in
22 PM1/CCR5 cells. C3 mutations may also be involved in a minor compensation effect in
23 replication cycles under moderate selective pressure from KD-247 (45, 60).

1 The neutralization resistance of primary HIV-1 variants is considered instrumental
2 for HIV-1 persistence in the presence of NAb in vivo. Various immunological pressures
3 always induce escape variants by eliciting appropriate mutation(s) (14, 60, 62). In the
4 present study, we found that HIV-1 could escape from the broadly reactive anti-V3 MAb,
5 KD-247, by stepwise mutation in the V2 and V3 regions. These observations strongly
6 support the idea that the major problem facing the development of V3-based immunogens
7 is not sequence variation within V3 but, rather, that access of most V3-directed antibodies
8 to their epitopes in functional Env complexes is blocked, often by the V1/V2 domain (29,
9 50).

10 Our observations support the hypothesis that neutralization-escape in a primary
11 isolate is mainly mediated by amino acid substitutions in the V2 region in vivo, because
12 only a moderate selective pressure by neutralizing antibodies against autologous viruses has
13 been reported for infected individuals (9, 14, 26, 50, 62). The large sequence diversity
14 observed for V2 in quasi-species existing in patients may represent the accumulation of
15 escape mutants early in HIV-1 infection in response to NAb pressure. Our observations may
16 also explain why the V3 sequence in quasi-species existing in patients is relatively
17 conserved in the face of a vigorous antibody response, especially in early HIV-1 infection.
18 A recent study by Deeks et al. has important implications for understanding the NAb
19 response against autologous virus (9): although NAb responses against contemporaneous
20 autologous viruses are absent in early HIV infection, they can be detected at low levels in
21 some patients with chronic infection. These data suggest the existence of an NAb response
22 that overcomes the emergence of escape mutants. Further characterization of the response
23 in humans who have potent and broadly neutralizing activities not affected by V1/V2

1 blocking effects may allow the identification of additional neutralization sites in HIV-1 Env,
2 which might allow new targets to be identified for vaccine development.

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ACCEPTED

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9 Infectious Diseases.

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1 **Figure legends**

2 Fig. 1. Selection of neutralization-resistant virus variants against KD-247. (A) The selection
3 was carried out in PM1/CCR5 cells, as described in Materials and Methods. (B) Sensitivity
4 of MOKW5p(200) and MOKW9p(2000) to KD-247 as determined by MTT assay.
5 PM1/CCR5 cells (2×10^3) were exposed to 100 TCID₅₀ of MOKW9p(-), MOKW5p(200) or
6 MOKW9p(2000), and were cultured in the presence of various concentrations of KD-247.
7 The IC₅₀ values were determined by MTT assay on day 7 of culture. The assay was
8 conducted in duplicate. The values shown are representative of three separate experiments.

9
10 Fig. 2. Amino acid sequences of gp120 from the supernatants of MOKW-infected
11 PM1/CCR5 cells passaged in the presence or absence of KD-247. Viral RNA from the cell
12 culture supernatants at several concentrations of KD-247 was reverse-transcribed. After
13 subjecting the obtained cDNAs to PCR amplification and cloning, the *env* regions in the
14 viruses passaged in the presence or absence of KD-247 were sequenced. The V2, V3 and
15 C3 regions are denoted. The top amino acid sequence represents one of major sequences
16 from supernatants of MOKW-infected PBMCs. The locations and numbers of specific
17 amino acids, based on the HXB2 sequence, are shown above the consensus line. The
18 numbers show the number of clones with the listed sequence among the total number of
19 clones tested. For each set of clones, the deduced amino acid sequence of the gp120 was
20 aligned by the single amino acid code. Dots denote sequence identity.

21

22 Fig. 3. Schematic representation of recombinant MOKW *env* genes used for analysis of the
23 genetic basis for resistance to KD-247. MOKW-RDP, MOKW-KNL/C3m and

1 MOKW-KNL/V3m *env* genes were amplified from passaged MOKW-infected PM1/CCR5
2 cells in the absence or presence of KD-247. MOKW-KNL, MOKW-RDP/V3m and
3 MOKW-RDP/C3m *env* genes were constructed by replacing each region of MOKW-RDP
4 with the corresponding MOKW-KNL/C3m or MOKW-KNL/V3m sequence. MOKW-KNP,
5 MOKW-RDL, MOKW-KDL and MOKW-RNL were constructed by site-directed
6 mutagenesis. Construction of the clones and mutagenesis procedures are described in the
7 Materials and Methods. The locations and numbers of specific amino acids, based on the
8 HXB2 sequence, are shown above the MOKW-RDP sequence.

9
10 Fig. 4. Neutralization sensitivities of pseudoviruses with *env* genes from passaged MOKW
11 viruses to MAbs, rsCD4 and CCR5 inhibitors. Pseudoviruses that have envelope sequences
12 listed in Fig. 4 were prepared as described in the Materials and Methods. KD-247, 447-52D,
13 rsCD4 and 17b were preincubated with 100 TCID₅₀ of each MOKW pseudotype virus for
14 15 min, followed by addition of the mixtures to the target cells (GHOST-hi5). The target
15 cells were treated with TAK-779 and 2D7 for 15 min, followed by inoculation of the
16 pseudotype clones. The inhibitory effects were determined by measuring the luciferase
17 activities on day 2 of culture.

18
19 Fig. 5. Comparison of antibody binding to cell surface-expressed MOKW Envs. (A) 293T
20 cells transfected with MOKW Env-expression vectors were harvested at 24 h
21 post-transfection and stained with KD-247. Flow cytometry data for binding of the KD-247
22 (black lines) to cell surface MOKW Envs are shown among GFP-gated 293T cells along
23 with the control antibody (normal human IgG; dotted lines). The number at the top right of

1 each graph shows the mean fluorescence intensity (MFI). (B) Each bar indicates the relative
2 binding of KD-247, 447-52D and 17b to MOKW Env-expressing cell surfaces. Data were
3 normalized to each antibody's MFI for MOKW-RDP.

4

5 Fig. 6. Neutralization sensitivities of pseudoviruses with *env* genes from MOKW9C(-) with
6 selected V2 mutations to MAbs, rsCD4 and CCR5 inhibitors. Pseudoviruses that have
7 envelope sequences with the selected V2 mutations listed in Fig. 4 were prepared as
8 described in Materials and Methods. KD-247, 447-52D, rsCD4 and IgGb12 were
9 preincubated with 100 TCID₅₀ of each MOKW pseudotype virus for 15 min, followed by
10 addition of the mixtures to the target cells (GHOST-hi5). Target cells were treated with
11 TAK-779 and RPA-T4 for 15 min, followed by inoculation of the pseudotype clones.
12 Inhibitory effects were determined by measuring the luciferase activities on day 2 of
13 culture.

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15 Fig. 7. Binding affinity of anti-V3 MAbs to monomeric gp120. Viral lysates for each
16 MOKW pseudovirus were used. Gp120 was captured onto microtiter wells using a sheep
17 polyclonal antibody specific for the C terminus of gp120. Serial dilutions of KD-247 or
18 447-52D were tested for binding by ELISA. Because of differences in the amount of bound
19 gp120, optical density at 405 nm (OD₄₀₅) values were normalized to saturating levels of
20 antibody (5 µg/ml) for comparison.

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22 Fig. 8. Comparison of antibody binding to cell surface-expressed MOKW Envs with V2
23 mutations. (A) 293T cells transfected with MOKW Env-expression vectors were harvested

1 at 24 h post-transfection and stained with KD-247. Flow cytometry data for binding of the
2 KD-247 (black lines) to cell surface MOKW Envs are shown for GFP-gated 293T cells
3 along with data for the control antibody (normal human IgG; dotted lines). The number at
4 the top right of each graph shows the mean fluorescence intensity (MFI). (B) Each bar
5 indicates relative binding of KD-247, 447-52D and IgGb12 to MOKW Env-expressing cell
6 surfaces. Data were normalized to each antibody's MFI for MOKW-RDP.

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8 Fig. 9. Replication kinetics of infectious molecular clone NL-MOKW-RDL and
9 NL-MOKW-KNL. PM1/CCR5 cells were exposed to NL-MOKW-RDL (open square) or
10 the NL-MOKW-KNL (filled square) and cultured for 10 days. Virus replication was
11 monitored by measuring the amounts of p24 gag protein produced in the culture
12 supernatants. The data is representative of the results from two independent experiments.

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