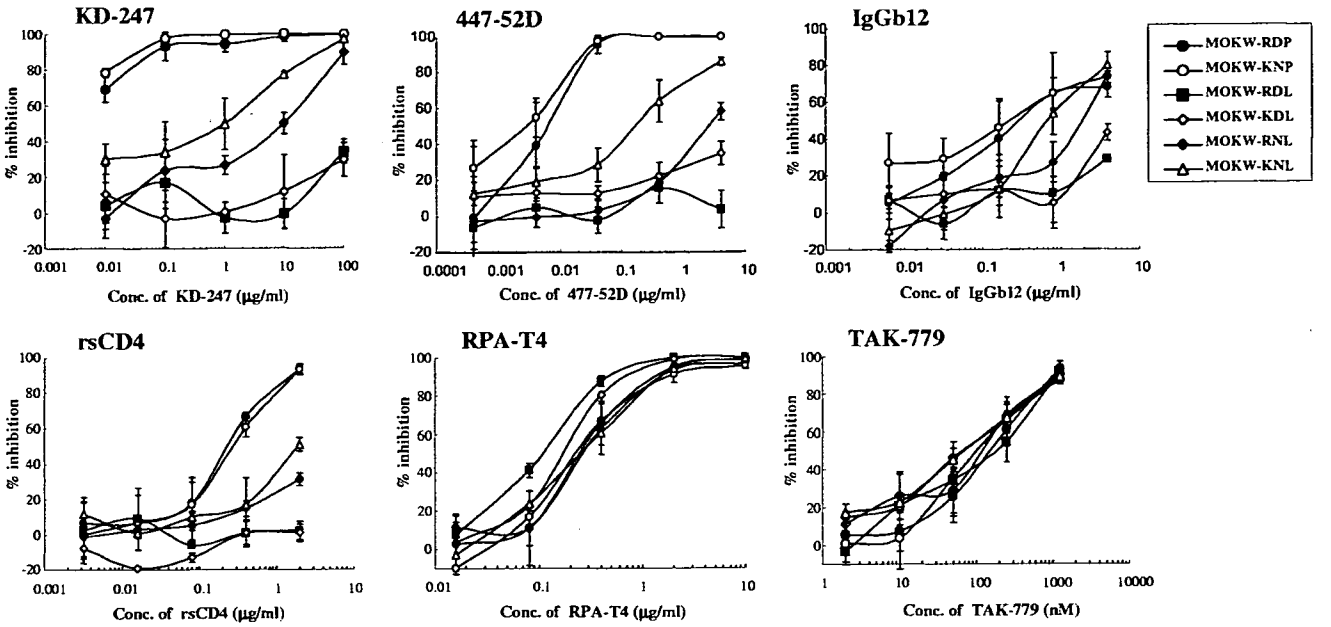

FIG. 6. Neutralization sensitivities of pseudoviruses with *env* genes from MOKW9C(−) virus with selected V2 mutations to MAbs, rsCD4, and CCR5 inhibitors. Pseudoviruses that have envelope sequences with the selected V2 mutations listed on Fig. 4 were prepared as described in Materials and Methods. KD-247, 447-52D, rsCD4, and IgGb12 were preincubated with 100 $TCID_{50}$ of each MOKW pseudotype virus for 15 min, followed by addition of the mixtures to the target cells (GHOST-hi5). Target cells were treated with TAK-779 and RPA-T4 for 15 min, followed by inoculation of the pseudotype clones. Inhibitory effects were determined by measuring the luciferase activities on day 2 of culture. Conc, concentration.

the relative binding of KD-247, 447-52D, and IgGb12 to Env expressed on the cell surface was no different than for MOKW-RDP and MOKW-KNP.

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

Comparison of replication kinetics between the NL-MOKW-RDL and NL-MOKW-KNL viruses. Although the MOKW-RDL variant was much more resistant against KD-247 than the MOKW-KNL variant (Fig. 6) and the RD sequence was

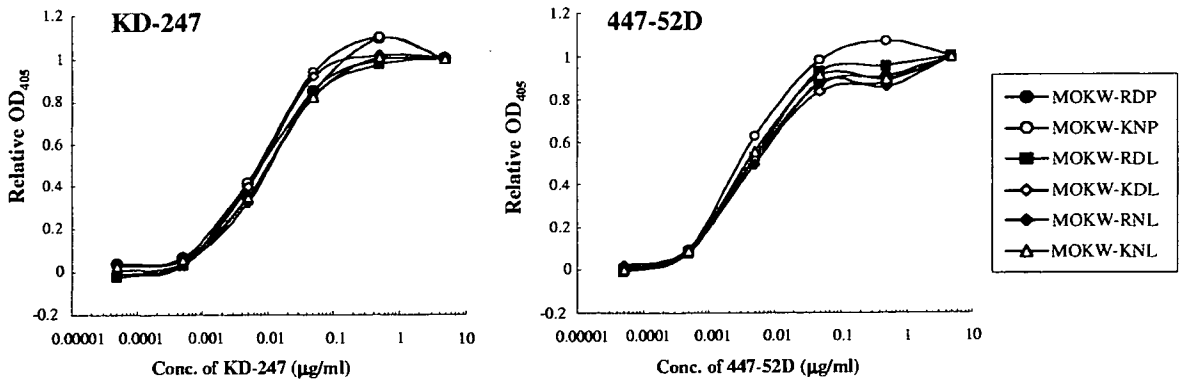

FIG. 7. Binding affinity of anti-V3 MAbs to monomeric gp120. Viral lysates for each MOKW pseudovirus were used. gp120 was captured onto microtiter wells using a sheep polyclonal antibody specific for the C terminus of gp120. Serial dilutions of KD-247 or 447-52D were tested for binding by ELISA. Because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

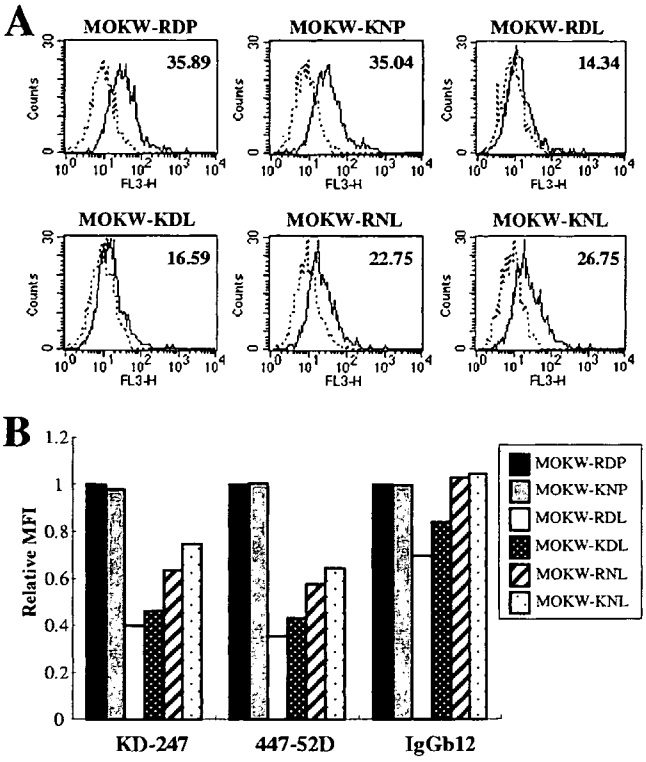

FIG. 8. Comparison of antibody binding to cell surface-expressed MOKW Env proteins with V2 mutations. (A) 293T cells transfected with MOKW Env-expression vectors were harvested at 24 h posttransfection and stained with KD-247. Flow cytometry data for binding of the KD-247 (black lines) to cell surface MOKW Env proteins are shown for GFP-gated 293T cells along with data for the control antibody (normal human IgG; dotted lines). The number at the top right of each graph is the MFI. (B) Each bar indicates relative binding of KD-247, 447-52D, and IgGb12 to MOKW Env-expressing cell surfaces. Data were normalized to each antibody's MFI for MOKW-RDP virus. FL3-H, relative fluorescence.

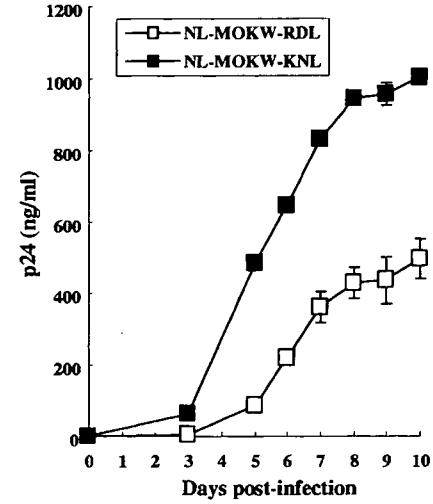

FIG. 9. Replication kinetics of infectious molecular clones NL-MOKW-RDL and NL-MOKW-KNL. PM1/CCR5 cells were exposed to NL-MOKW-RDL (open square) or NL-MOKW-KNL (filled square) and cultured for 10 days. Virus replication was monitored by measuring the amounts of p24 Gag protein produced in the culture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

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

DISCUSSION

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

The V1/V2 region of gp120 is highly diverse, not only in respect to virus subtypes but also in respect to intraspecies diversity in the same patient (16, 61, 66). The primary isolate, MOKW, also displayed diversity in the V2 region (Fig. 2), and the first-passaged virus already harbored mutations in the V2 region. Many researchers have reported that the V1/V2 domain strongly influences neutralization of the anti-V3 MAbs, MAbs to the other epitopes, and rsCD4 (12, 13, 25, 27, 30, 44, 49, 50). Moreover, structural models of the Env trimer have been proposed that place the base of the V1/V2 loop of one subunit in proximity to the V3 loop of a neighboring subunit (32, 34). In the present study we observed a reduction in the binding of anti-V3 MAbs to V2-mutated Env expressed on the cell surface, whereas mutations in V2 did not have an effect on the binding of the MAbs to monomeric gp120. These results suggest the association of V2 mutations with anti-V3 antibody

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

We previously described the in vitro selection and characterization of a KD-247-escape mutant of JR-FL (67). The amino acid substitution that was critical for the resistance phenotype was Gly to Glu at residue 314 (G314E) in the V3 tip region. The genetically engineered mutant was completely resistant to neutralization by KD-247. Other researchers have also reported the induction of V3-mutated viruses by strain-specific anti-V3 MAb in in vitro culture systems (8, 37, 65). In earlier studies, combinations of genetically cloned viruses and highly potent NAbs were used for in vitro selection (8, 37, 65). The escape mutants were induced in the presence of high concentrations of MAbs to acquire V3 tip mutation(s). In contrast to these observations in vitro, the Gly-Pro-Gly amino acid sequence in the V3 tip varies to a negligible extent in clinical isolates from HIV-1-infected patients (35). The important role of the V3 tip in forming the β-turn of the V3 loop and in interacting with chemokine receptors may partly explain the discrepancy between in vivo and in vitro studies (19). In addition to the V3 loop, variation in or near the V1/V2 region is known to contribute to coreceptor usage of HIV-1 (17, 21, 27, 28, 47, 56, 64, 66). However, it is possible that HIV-1 suffers critical damage with respect to replication and infectivity through mutation in the V3 region, especially in the tip region, because the V3 loop plays a major role in the interaction of gp120 with coreceptors. Thus, mutations in the V2 region may be important not only to avoid anti-V3 pressure but also to maintain replication efficiency at a suitable level.

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

In the in vitro selection process, amino acid residue 175 (Pro or Leu) in the V2 region of MOKW virus played a crucial role in dramatically changing the oligomeric state of the envelopes. However, MOKW-RDP obtained by prolonged culture in vitro without KD-247 became neutralization sensitive compared with MOKW virus. Residue 175P was the amino acid responsible for the change to the neutralization-sensitive phenotype, whereas viruses with 175L became highly resistant to the MAbs and rsCD4. The same phenomenon was observed in the relatively resistant strain JR-FL. Residue 175L is highly conserved among HIV-1 strains and is located at the center of the V2 loop (35), and the V2 region also mediates gp41-independent intersubunit contact (5). It is therefore possible that the V2 region, including residue 175, by mediating changes in the conformation of the gp120 oligomer, contributes to resistance to neutralization by limiting the exposure of epitopes.

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

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

Our observations support the hypothesis that neutralization escape in a primary isolate is mainly mediated by amino acid substitutions in the V2 region in vivo, because only a moderate selective pressure by neutralizing antibodies against autologous viruses has been reported for infected individuals (9, 14, 26, 50, 62). The large sequence diversity observed for V2 in quasi-species existing in patients may represent the accumulation of escape mutants early in HIV-1 infection in response to NAb pressure. Our observations may also explain why the V3

sequence in quasi-species existing in patients is relatively conserved in the face of a vigorous antibody response, especially in early HIV-1 infection. A recent study by Deeks et al. has important implications for understanding the NAb response against autologous virus (9): although NAb responses against contemporaneous autologous viruses are absent in early HIV infection, they can be detected at low levels in some patients with chronic infection. These data suggest the existence of an NAb response that overcomes the emergence of escape mutants. Further characterization of the response in humans who have potent and broadly neutralizing activities not affected by V1/V2 blocking effects may allow the identification of additional neutralization sites in HIV-1 Env, which might allow new targets to be identified for vaccine development.

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