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Characterization of Mutants of Human Immunodeficiency Virus Type 1 That Have Escaped Neutralization by a Monoclonal Antibody to the gp120 V2 Loop

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The biologically cloned human immunodeficiency virus type 1 (HIV-1) RF isolate is sensitive to neutralization by the murine monoclonal antibody (MAb) G3-4 to a conformationally sensitive epitope in the V2 loop of HIV-1 gp120. To assess how variation in the V2 amino acid sequence affects neutralization by this MAb, we cultured RF in the presence of G3-4 to select neutralization escape mutants. Three such mutants resistant to G3-4 neutralization were generated from three independent experiments. Solubilized gp120 from each of these escape mutants had a reduced affinity for G3-4 and also for two other V2 MAbs that were able to bind the wild-type RF gp120. PCR sequencing of the entire gp120 of the wild-type RF virus and the escape mutants showed that amino acid substitutions had occurred only at two positions, Y177H and L179P, both in V2. Experimental introduction of the Y177H substitution into the RF V2 loop in the context of the NL4-3 molecular clone re-created the G3-4-resistant phenotype. The L179P mutant was not viable. Thus, our findings confirm that the HIV-1 V2 loop contains the conformationally sensitive neutralization epitope recognized by G3-4 and that a single amino acid substitution within this region can result in escape variants that arise from immune selection pressure.

A significant fraction of the humoral immune response to human immunodeficiency type 1 (HIV-1) infection in humans is directed against the viral envelope glycoproteins gp120 and gp41 (1, 26), which contain epitopes for neutralizing antibodies (17, 22). Since chimpanzees have been protected against HIV-1 infection by immunization with gp120 (4, 8), the envelope glycoproteins are a focus for vaccine development. However, one of the major problems facing gp120-based vaccines is that of virus variability (15, 17, 20, 21, 28). Two of the better-characterized neutralization sites on gp120 lie in variable regions of the protein: the V2 and V3 loops, which are formed by disulfide bonds at their bases (13). The role of V3 as a determinant of antibody neutralization and cellular tropism has been known for some time (17, 22), but it is becoming clear that the V2 region also contains neutralization epitopes in both HIV-1 (7, 10, 19, 29) and simian immunodeficiency virus (3, 12). V2 sequences are also important for HIV-1 infectivity (29) and tropism (31) and are subject to considerable variation (9, 24). It is widely believed that the variation in these domains arises from the selection pressure mediated by the host immune system (6, 11). Thus, sequence changes both within and outside an antibody epitope can enable HIV-1 or simian immunodeficiency virus to escape neutralization while not necessarily harming its replicative capacity (2, 4a, 5, 14, 23, 25, 32).

To explore whether and how escape mutants can be generated against an antibody directed against the V2 domain, we cultured HIV-1 RF in the presence of the murine monoclonal antibody (MAb) G3-4 (10). This is a moderately strong neutralizing MAb raised against HIV-1 IIIB gp120 that has been mapped by antibody competition and mutagenesis experiments to a conformationally sensitive epitope at the crown of the V2 loop (7, 10, 19, 29). We report on the selection in vitro of three independent G3-4 escape mutants, each of which has a sequence change within the V2 loop.

MATERIALS AND METHODS

Generation of biologically cloned HIV-1 RF. The procedure was similar to that described previously (6a). One milliliter of Molt-4 cells (2×10^5/ml) was mixed with the same volume of HIV-1 RF at a multiplicity of infection of 0.5 and then incubated for 1 h at 37°C. The cells were then washed twice with RPMI 1640 medium before they were resuspended at a density of 10 cells per ml; 50 μl of suspension was added to each well of a U-bottom 96-well plate. After 6 days, 50 μl of fresh medium was added to each well. One month later, 15 cell clones were obtained from individual wells, and the culture supernatant was collected and examined for p24 antigen production. Two HIV-positive cell clones were obtained, and one of them (clone 7) was used as a producer of biologically clonal RF.

Generation of neutralization-resistant virus. Culture supernatant from the clonal RF stock was incubated with MAb G3-4 (4 μg/ml) for 30 min at 37°C. A 0.5-ml aliquot of the virus-antibody mixture was then added to 0.5 ml of MT-4 cells at 6×10^5 cells per ml. After 3 days of infection, supernatant fluids from infected MT-4 cultures were harvested and inoculated onto fresh MT-4 cells in the presence of 20 μg of G3-4 per ml. The MAb was continuously present in the cultures. The same procedure was repeated five times with 100 μg of G3-4 per ml to increase the selection pressure on the virus. Three escape mutants (AD1, AD2, and AD3) were obtained from three independent experiments.

HIV-1 neutralization assay. Neutralization assays were performed as described previously (33). One milliliter of virus (1.2×10^4 50% tissue culture infective doses per ml) was mixed with 1 ml of serially diluted G3-4 for 30 min at room

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temperature before addition of 50 μl of the mixture to 50 μl of MT-4 cells, adjusted to a density of 6×10^5 cells per ml. To provide a calibration curve, the same viral inoculum (300 50% tissue culture infective doses per well), as well as 1:3 and 1:9 dilutions of the inoculum, was added to 50 μl of MT-4 cells. On day 3 postinfection, 100 μl of fresh medium was added to the cultures; on day 4, 1 μCi of [^{3}H]thymidine (20 Ci/mmol; New England Nuclear) was added. After incubation for 16 h, the cells were harvested and the extent of [^{3}H]thymidine incorporation into DNA was determined in a scintillation counter. All datum points represent the means of triplicate determinations. Percentage neutralization was calculated by determining the reduction in supernatant p24 antigen production in the presence of the MAb compared to that in control cultures lacking the MAb.

Amplification of proviral DNA and nucleotide sequencing. Proviral HIV-1 DNA was extracted from at least 10^7 infected MT-4 cells by standard procedures. Nested PCR was performed to amplify the gp120 coding region. For the first round of PCR, REC-07, which hybridizes to the minus strand at positions 5969 to 5988 of the NL4-3 sequence, and REC-09, which hybridizes to the plus strand at positions 7826 to 7845, were used. For the second round of nested PCR, REC-27, hybridizing to the minus strand at positions 6203 to 5988, and REC-12, hybridizing to the plus strand at positions 7728 to 7747, were used. The products of nested PCR were digested with *Bam*HI and *Eco*RI and inserted into the M13mp18 bacteriophage vector. Recombinant phages were propagated in *Escherichia coli* JM101 cells, and single-stranded DNA was collected from the culture supernatant. The dideoxynucleotide chain termination method was used for DNA sequencing.

gp120 binding assay. The binding of MAbs to gp120 was assessed by enzyme-linked immunosorbent assay (ELISA) essentially as described previously (16, 18–20). Virus-containing culture supernatants were inactivated with 1% Nonidet P-40 nonionic detergent to provide a source of gp120. A sheep anti-gp120 antibody, D7324, which recognizes a 15-amino-acid peptide from the C terminus of gp120 was used to capture gp120 to Immulon-2 plates. Unbound gp120 was removed by washing the cells twice with Tris-buffered saline, and MAbs were added in Tris-buffered saline containing 2% nonfat milk powder, 20% sheep serum, and 0.5% Tween 20. After 1 h, unbound antibodies were removed by washing twice, and bound antibodies were detected by incubation for 1 h with the appropriate alkaline phosphatase-conjugated anti-species antibody and the AMPAK system (Dako Diagnostics). Binding curves of optical density at 492 nm against MAb concentration were plotted, and the MAb concentrations giving 50% binding were determined as a measure of MAb-gp120 affinity.

Site-directed mutagenesis. Since an infectious molecular clone of RF is not available, a recombinant HIV-1 RF/NL4-3 proviral molecular clone was constructed by digesting the pNL4-3 clone with *Eco*RI and *Bam*HI and then ligating the large fragment to the corresponding sites in the polylinker region of the pALTER-1 vector, generating plasmid pALTER-1-NL4-3_{EB}. The *Eco*RI (positions 5260 to 5265 of the RF sequence)-to-*Stu*I (6389 to 6394) fragment of RF proviral DNA, which includes the V2 loop and flanking sequences, was amplified by nested PCR. This fragment was then cloned into pALTER-1-NL4-3_{EB} to create plasmid pALTER-1-RF_{ES}-NL4-3_{SB}. For the first round of PCR amplification, primers PRM1 (5′-AGATGGAACAAGCCCCAGAAGACCA-3′) and PRM-5 (5′CTCTTCTTCTGCTAGACTGCCATT-3′), corresponding to bases 5074 to 5098 and 6588 to 6565, respectively, of the RF sequence, were used. For the second round, the primers were PRM2 (5′-GAAGCTGTCAGACATTTTCCTAGG-3′) and PRM4 (5′-TTCCATGTGTACATTGTACTGTGC-3′), corresponding to bases 5160 to 5183 and 6532 to 6509, respectively. Site-directed mutagenesis at amino acid residue 177 or 179 or at both residues was carried out on plasmid pALTER-1-RF_{ES}-NL4-3_{SB}, using the Altered Site *in vitro* Mutagenesis System (Promega Inc.) as instructed by the manufacturer. Finally, the *Eco*RI-to-*Bam*HI fragment from recombinant RF/NL4-3, or from mutagenized versions thereof, was reconstructed into pNL4-3. In every step of the mutagenesis protocol, the nucleotide sequence was verified. Recombinant pNL4-3-RF or mutagenized variants (20 μg of DNA) were transfected into COS-1 cells by using a standard calcium phosphate precipitation procedure. On day 2 posttransfection, the culture medium was changed; at 72 h posttransfection, the medium was collected and filtered to provide a source of infectious HIV-1.

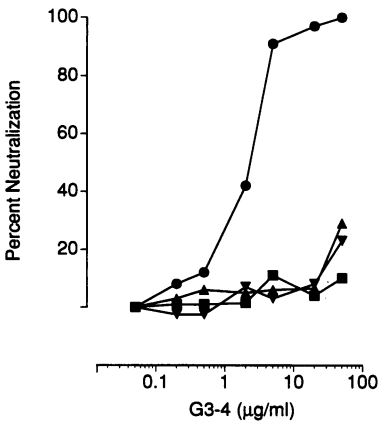

FIG. 1. G3-4 neutralization of HIV-1 RF and the escape mutants. Shown are data for RF (●), AD1 (■), AD2 (▲), and AD3 (▼).

RESULTS

HIV-1 RF escape from G3-4 neutralization. In the absence of a molecular clone for HIV-1 RF, we generated a biological clone of this virus by established procedures (6a) and used this cloned virus in experiments designed to assess the feasibility of neutralization escape from a V2 MAb, G3-4. Three separate escape mutants (AD1, AD2, and AD3) were generated by in vitro selection with this MAb. Neutralization of HIV-1 RF by G3-4 is depicted in Fig. 1 together with evidence that the variants are indeed resistant to the selecting antibody. Wild-type RF was progressively neutralized by G3-4 concentrations of between 1 and 10 μg/ml, whereas none of the three escape mutants was significantly inhibited by G3-4 in doses of up to 50 μg/ml.

Reactivity of MAbs with soluble gp120 from escape mutants. The observed resistance of AD1, AD2, and AD3 to neutralization by G3-4 could be due to sequence changes that result in the lost of binding by the MAb. To address this possibility, we lysed wild-type and mutant virions contained in culture supernatants with a nonionic detergent to provide a source of soluble gp120 and then tested their reactivities with G3-4 in an ELISA (Fig. 2). Equivalent amounts of gp120 were captured onto the solid phase, as judged by their binding of polyclonal antibodies in a pool of HIV-1$^+$ serum (Fig. 2A). Each of the variants AD-1, AD-2, and AD-3 had an average affinity for G3-4 that was approximately fivefold less than that of the parental virus (Fig. 2B). However, each of the variants and the wild-type gp120 bound equivalently to human MAb 48d (data

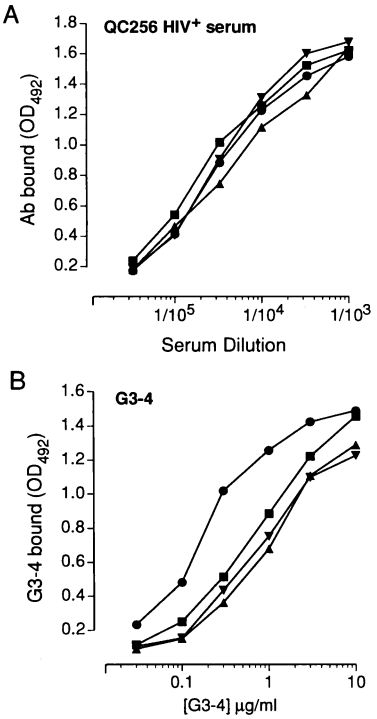

FIG. 2. G3-4 binding to gp120s from HIV-1 RF and the escape mutants. Nonidet P-40-solubilized gp120s from culture supernatants containing wild-type RF (●), AD1 (■), AD2 (▲), or AD3 (▼) were reacted with a pooled HIV-1$^+$ human serum or MAb G3-4 at the concentrations indicated, and the amounts of gp120-bound antibodies were determined. OD_{492}, optical density at 492.

not shown), an antibody whose epitope is sensitive to many mutations on the HxB2 background (27, 30).

We also tested the abilities of RF and the escape mutants to bind seven other MAbs shown also to be sensitive to mutations in the V2 loop of HIV-1 IIIB (19). Five of these (BAT-085, 110.B, 684-238, CRA-3, and CRA-4) were unreactive with the parental RF gp120 and also failed to bind to the variant gp120s (data not shown). However, two other MAbs (G3-136 and SC258) reacted well with RF gp120 but had significant reductions in their affinities for gp120 from each of the three escape mutants (Table 1).

gp120 sequences of escape mutants. To determine the genetic basis of the resistance of variants AD1, AD2, and AD3 to G3-4, we derived sequences corresponding to the entire gp120 molecule from the wild-type RF virus and each of the three variants. Four M13 clones derived from the parental, biologically cloned RF were sequenced, as were five, six, and six clones for AD1, AD2, and AD3, respectively. The predicted amino acid sequences for the V2 region are shown in Fig. 3. All four clones of the parental, biologically cloned RF stock had identical V2 sequences (Fig. 3). Despite the generation of three escape mutants in independent experiments, mutations were found only at two residues near the crown of the V2 loop. All three mutants contained a unique tyrosine-to-histidine change at position 177 (Y177H) in several of the M13 clones. Two of the AD3 clones contained a leucine-to-proline change at position 179 (L179P). One or two M13 clones from each mutant still contained the wild-type sequence. None of the four sequenced M13 clones of the parental RF biological clone contained either a histidine at residue 177 or a proline at residue 179.

TABLE 1. Percent binding of MAbs to escape mutants relative to the wild-type virus (RF)

MAb	Binding[a]		
	AD1	AD2	AD3
G3-4	22 ± 3	18 ± 6	17 ± 7
G3-136	25 ± 4	22 ± 5	18 ± 3
SC258	47 ± 26	45 ± 23	16 ± 4

[a] For each MAb, the concentration giving 50% binding to wild-type RF gp120 in ELISA was assigned a value of 100. The data presented for variants AD1, AD2, and AD3 reflect their relative abilities to bind to the same MAbs: a value of 20 corresponds to a fivefold increase in the Mab concentration giving half-maximal binding, which equates to a fivefold reduction in affinity. The data represent the means ± standard deviations of triplicate determinations.

Neutralization of mutagenized HIV-1 RF. To assess whether the observed amino acid substitutions were responsible for the resistance of the three escape mutants to neutralization, we first inserted the C1-to-V2 sequence (amino acids 1 to 210) of the parental RF virus in place of the corresponding gp120 region of the NL4-3 infectious molecular clone. We then mutagenized the chimeric molecular clone to create the Y177H and L179P substitutions as well as the Y177H/L179P double mutation. This two-stage procedure was necessary to overcome our lack of an infectious molecular clone of RF. The chimeric virus retained infectivity, as did the Y177H mutant (Fig. 4). However, the L179P mutant and the double mutant containing Y177H and L179P were noninfectious (data not shown) and were not subject to further analysis.

The parental clone with the wild-type RF V2 sequence was sensitive to neutralization by the V2 MAbs G3-4 and SC258 and also by the V3 MAb BAT-123 (Fig. 4). However, the chimeric virus containing the Y177H substitution was resistant to neutralization by G3-4 and SC258 but was still sensitive to the V3 MAb BAT-123 (Fig. 4). MAb-gp120 binding assays on lysates of these two viruses confirmed that the Y177H substitution completely eliminated binding of each of G3-4, G3-136, and SC258 (Fig. 5).

```
          160                 180                 200
           *         *         *         *         *        Clones
RF V2  CSFQVTTSKRDKTQKEYALF YKLDVVPIEKGNISPKNNTS YGNYKLIHC   4/4
AD1    ..................... H..................... .........  3/5
AD2    ..................... H..................... .........  5/6
AD3    ..................... H..................... .........  3/6
AD3    ..................... ..P..................... .........  2/6
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FIG. 3. Amino acid seq/uences of the V2 region of gp120 from HIV-1 RF and the escape mutants.

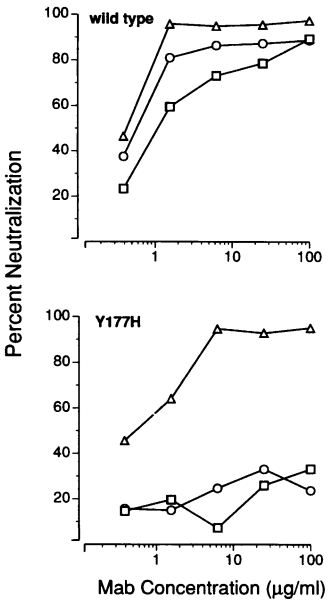

FIG. 4. G3-4 neutralization of wild-type and Y177H mutant viruses. The chimeric viruses containing the wild-type RF V2 sequences or Y177H mutation were incubated with V2 MAb G3-4 (○), V2 MAb SC258 (□), or V3 MAb BAT-123 (△) at the concentrations indicated, and the amount of residual infectivity was determined.

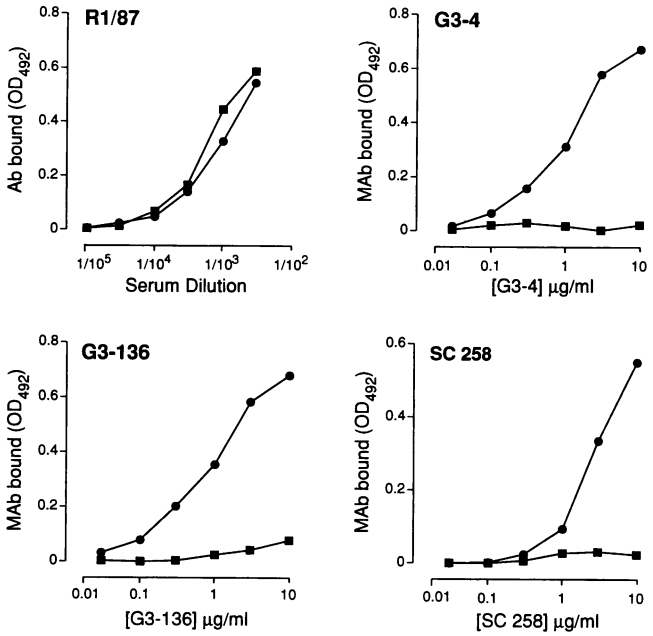

FIG. 5. Binding of V2 MAbs to wild-type and Y177H mutant viruses. Nonidet P-40-solubilized gp120s from the wild-type chimera (●) or the Y177H mutant (■) were reacted with R1/87 polyclonal anti-gp120 serum, MAb G3-4, MAb G3-136, or MAb SC258 at the concentrations indicated, and the amounts of gp120-bound antibodies were determined. The data are corrected for nonspecific antibody absorption in the absence of gp120. OD_{492}, optical density at 492 nm.

DISCUSSION

Starting with a biological clone of HIV-1 RF, we have demonstrated that a selection pressure exerted in vitro by the neutralizing MAb G3-4 against the V2 region of gp120 has caused the generation of escape mutants with a single amino acid substitution (Y177H) within the antibody epitope. Four M13 clones of the parental RF biological clone had identical V2 sequences (Fig. 3), with a tyrosine at residue 177. We believe that the histidine substitution arose de novo as a result of selection pressure exerted by MAb G3-4, but it is also possible that a minor variant containing a histidine at residue 177 was present in the biologically clonal parental virus stock and was expanded to dominate the virus population grown in the presence of MAb G3-4. The Y177H substitution, both naturally arising and experimentally introduced into an infectious molecular clone, reduced the average affinity of soluble gp120 molecules for the selecting MAb and also for two other MAbs directed to a similar V2 epitope. In the context of the molecular clone, the Y177H mutation completely abolished V2 MAb binding (Fig. 5). Thus, we suppose that the residual binding of G3-4 and the other V2 MAbs to the naturally arising escape mutant (Fig. 2) reflects the presence of an in homogeneous population of gp120 molecules, some retaining the wild-type sequence (Fig. 3).

Previous studies using solid-phase peptides from HxB2 have demonstrated that the core epitope for G3-4 probably lies within amino acids 170 to 180, although the peptide reactivity of G3-4 is weak and the epitope is very sensitive both to the overall gp120 conformation and to amino acid substitutions outside this linear sequence (19, 29). Within the corresponding RF V2 sequence, QKEYALFYKLD, there is only a single amino acid change from HxB2: L175F. This is clearly unimportant for the G3-4 epitope, as the antibody binds to both RF and HxB2 gp120. Analysis with HxB2 gp120 mutants shows that the binding of G3-4 to HxB2 gp120 is destroyed by the substitutions FY176/177AT, LD179/180DL, and PI183/184SG and significantly reduced by the substitution YSL192/194GSS (19, 29). The last two sets of changes may affect the G3-4 epitope indirectly; the antibody binding site is likely to be sensitive to the identities of flanking amino acids (19, 29). However, it is notable that the two mutations that arose spontaneously in RF gp120 through G3-4 selection pressure, Y177H and L179P, correspond in position to two experimentally introduced mutations in HxB2 gp120 that destroy the MAb epitope (19, 29). The results of our escape mutant study are therefore entirely consistent with prior knowledge of the V2 loop structure and its role as a neutralizing antibody epitope (19, 29). Thus, while the V2 domain is important to the functioning of the gp120 molecule (9, 29), a certain degree of sequence flexibility is tolerable. This is a prerequisite for the generation of neutralization escape mutants. Our study therefore demonstrates that neutralization via the V2 domain of gp120 is fraught with the same problems as neutralization via V3: virus variability and neutralization escape. As most MAbs to V2 described to date are of lower affinity and neutralization potency than V3 MAbs (7, 10, 19, 29), it seems that V2 may have all of the problems associated with V3 but few of its advantages.

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