JOURNAL OF VIROLOGY, Dec. 1993, p. 7493–7500
0022-538X/93/127493-08$02.00/0

Vol. 67, No. 12

Immune Escape by Human Immunodeficiency Virus Type 1 from Neutralizing Antibodies: Evidence for Multiple Pathways

BRYNMOR A. WATKINS, MARVIN S. REITZ, JR., CAROLYN A. WILSON,† KRISTINE ALDRICH, ALBERTA E. DAVIS, AND MARJORIE ROBERT-GUROFF*

Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Maryland 20892

Received 24 June 1993/Accepted 21 September 1993

Sera from many HIV-1-infected individuals contain broadly reactive, specific neutralizing antibodies. Despite their broad reactivity, variant viruses, resistant to neutralization, can be selected in vitro in the presence of such antisera. We have previously shown that neutralization resistance of an escape mutant with an amino acid substitution in the transmembrane protein (A582T) occurs because of alteration of a conformational epitope that is recognized by neutralizing antibodies directed against the CD4 binding site. In this report we demonstrate that immune escape via a single-amino-acid substitution (A281V) within a conserved region of the envelope glycoprotein gp120 confers neutralization resistance against a broadly reactive neutralizing antiserum from a seropositive individual. We show this alteration affects V3 and additional regions unrelated to V3 or the CD4 binding site. Together with previous studies on escape mutants selected in vitro, our findings suggest that immune-selective pressure can arise by multiple pathways.

Numerous studies have shown an association of neutralizing antibodies with lesser disease manifestation and improved clinical outcome for both human immunodeficiency virus type 1 (HIV-1)-infected children and adults (37; for a review, see reference 33). Moreover, the ability of neutralizing antibodies to protect against HIV-1 infection has been clearly established by passive immunization of a chimpanzee with a monoclonal antibody specific for the principal neutralization determinant (PND) (10, 27, 38) of the gp120 of isolate HIV-1_{IIIB} and its subsequent protection from a live HIV-1_{IIIB} challenge (5). The PND of gp120 is a linear peptide epitope which lies between two cysteine residues within the third variable (V3) region and exhibits considerable variation between different strains of HIV-1 (11, 12, 43, 51). While some antibodies to the conserved tip of the V3 loop are able to neutralize several HIV-1 isolates (9, 16), this variability in general limits neutralizing cross-reactivity between different isolates and restricts the utility of immune responses elicited by any single specific PND sequence for controlling HIV-1 infection (45). Neutralizing antibody responses with broader specificity have been described, including those directed against the CD4 binding site on gp120 (for a review, see reference 44) and other, undefined conformational envelope epitopes (45). Responses to these epitopes have yet to be efficiently elicited by vaccine preparations. The progression of HIV-1 infection to AIDS has been associated with the development of neutralization-resistant virus strains within an infected individual over time (1, 2) which generates outbreaks of "mini-viremia" (25). According to this model, these neutralization-resistant variants would contribute to erosion of the $CD4^+$ lymphocyte population, leading to the eventual collapse of $CD4^+$ count and the development of AIDS. From these observations, it has been suggested that with regard to humoral immunity, protection against HIV-1 will best be achieved by a vaccine, or immunotherapeutic agent, which elicits broadly reactive neutralizing antibodies against epitopes of HIV-1 that show minimal variability (7, 13, 21, 25, 30).

One way to identify such sites and elucidate immune escape mechanisms is to analyze in vitro variants immune selected with heterotypic antisera. This approach has previously been used to identify a single-amino-acid substitution (A582T) in the gp41 transmembrane protein of HXB2 (32), which confers neutralization resistance to approximately one-third of sera from seropositive individuals capable of neutralizing the parental virus (50). The mechanism of neutralization escape by A582T has recently been identified as the loss of binding by antibodies which block the binding of gp120 to CD4 and therefore inhibit viral infectivity (17). In this report, we have employed the approach used in the selection and characterization of variant A582T (32, 36, 50) to select and characterize another neutralization-resistant variant of HIV-1, HXB2/O925. Analysis of envelope clones from variant HXB2/O925 demonstrated that a single point mutation in a relatively conserved region of gp120, leading to a valine-for-alanine substitution at position 281, was sufficient for resistance to neutralization by the selecting antiserum. Further analysis indicated that position 281 does not make up part of a linear neutralization epitope. Moreover, the pathway for immune escape was not via diminished antibody recognition of either the PND of gp120 or the CD4 binding site. We surmise from these observations that the neutralization escape observed with the A281V substitution must be due to a conformational change in gp120 that results in yet another pathway for immune escape by HIV-1.

MATERIALS AND METHODS

Cell culture and virus strains. Cos-1 cells were cultured in Dulbecco modified Eagle medium supplemented with 25 μg of gentamicin per ml–2 mM L-glutamine–10% fetal bovine serum (FBS), passaged, and subcultured by trypsinization using standard techniques. H9 cells were grown in RPMI 1640 medium supplemented with 1 to 2 mM L-glutamine, 10% FBS, and either 25 μg of gentamicin per ml or 100 U of penicillin per ml and 100 μg streptomycin per ml. Viral strain HXB2 was expressed from a plasmid containing a full-length infectious

* Corresponding author.
† Present address: The Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD 20892.

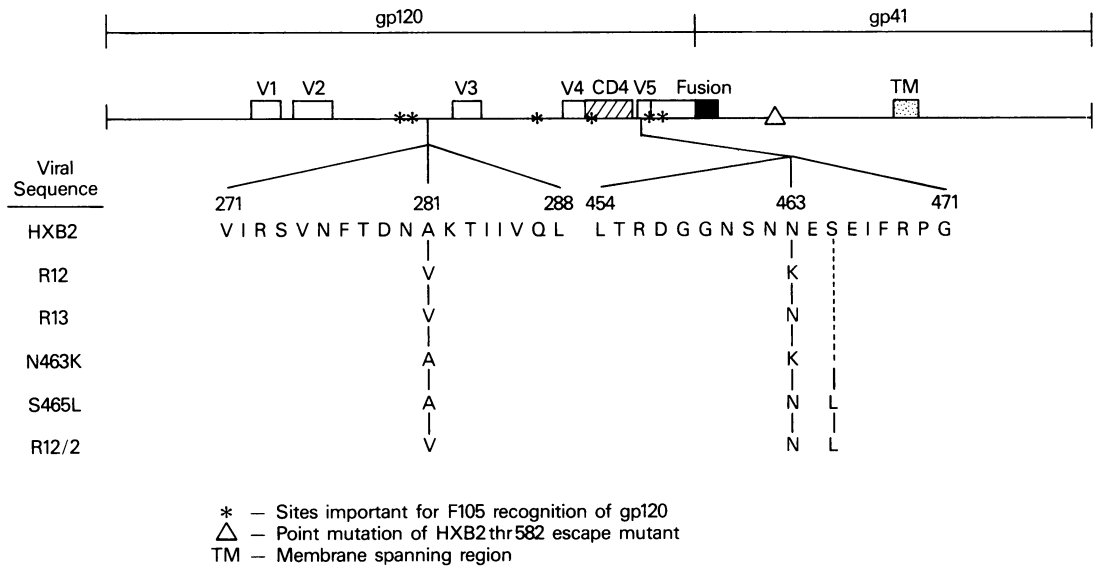

FIG. 1. Amino acid substitutions in immune-selected and molecularly constructed HXB2 variants. Several of the prominent features of the HIV-1 envelope are depicted, including the variable, CD4 binding, fusion, and transmembrane regions. The HXB2A582T mutation leading to immune escape via the CD4 binding region (17) is also shown. The F105 recognition sites were taken from reference 47. Amino acid substitutions resulting from point mutations in naturally derived and molecularly constructed infectious clones are shown. The sequences of peptides 271ala288 and 454asn471 are also represented.

molecular clone (pHXB2) (6) by electroporating 40 μg of DNA into 10^7 trypsinized Cos-1 cells by using the Bio-Rad gene pulser. These cells were plated into a 75-cm^2 tissue culture flask and grown for 24 h before the addition of 10^7 H9 cells. After 48 h of cocultivation, the H9 cells were removed and grown separately for an additional 5 to 7 days before the extent of the infection was assessed by immunofluorescence assay as previously described (34). The neutralization-resistant mutant, HXB2/O925, was generated in vitro as previously described (36). Briefly, 10^6 irradiated H9 cells infected with HXB2 were cocultured with 3×10^6 polybrene-treated uninfected H9 cells in the presence of 5% FBS and 5% WO925 serum obtained from an HIV-1-seropositive healthy male. The culture was monitored periodically for expression of HIV-1 p24 by indirect immunofluorescence assay. Molecularly cloned variant viruses derived from HXB2/O925 and variant constructs based on sequences of the cloned variants were expressed and cultured as described for HXB2.

Synthetic peptides and anti-peptide sera. Synthetic peptides were purchased from Multiple Peptide Systems (San Diego, Calif.) as 80% pure preparations. Anti-peptide sera were produced in goats or guinea pigs by immunization with keyhole limpet hemocyanin-conjugated peptides emulsified in complete Freund's adjuvant. Booster inoculations were given in incomplete Freund's adjuvant. The sequences of the V3 peptides were NNTRKRIRIQRGPGRAFVTIGKIGC (HXB2) and YNKRKRIHIGPGRAFYTTKNIIGC (MN). The carboxy-terminal cysteines were added to facilitate keyhole limpet hemocyanin conjugation. The amino acid sequences of peptides 271ala288 and 454asn477 are shown in Fig. 1. Enzyme-linked immunosorbent assay (ELISA)-binding titers to peptides, determined as described in reference 35 by using horseradish peroxidase conjugates of rabbit anti-goat immunoglobulin G (IgG), goat anti-guinea pig IgG, or goat anti-human IgG as appropriate, were defined as the reciprocal of the serum dilution at which the absorbance of the test serum was twice that of a control serum diluted 1:100. The titer of goat anti-271ala288 serum against peptide 271ala288 was 6,400, and the titer of guinea pig anti-271ala288 serum was 650. The titer of guinea pig anti-454asn471 serum was 430,000. Selecting antiserum WO925 showed no detectable binding to either peptide and had titers of 260 and 930, respectively, against HXB2 and MN V3 loop peptides.

Immunologic assays. Neutralization sensitivity or resistance was assessed by using dilutions of fresh supernatant virus from H9 cells infected with parental or variant viruses and a final concentration (1:10 dilution) of immune-selecting serum WO925 or normal human serum (NHS) as previously described (36). Following transmission to H9 cell targets, comparisons of neutralization sensitivity were made by using viral dilutions at which infectivity rates in the presence of NHS were similar for the four viruses. Neutralization titers of HIV-1-positive human sera obtained from U.S. and European healthy individuals; human monoclonal antibodies which recognize the conformationally dependent CD4 binding site on gp120 (F105 [29], I5e [15], 120-1B1 [Virus Testing Systems Corp., Houston, Tex.]); murine monoclonal antibodies specific for overlapping epitopes of the HXB2 V3 loop (M77 [26], IIIB-V3-13 [18], 0.5β [20]); soluble CD4 (American Biotechnologies, Inc., Cambridge, Mass.); and anti-peptide antisera were determined as previously described (34). Neutralizing titers were defined as the reciprocal of the serum dilution or the antibody or soluble (sCD4) concentration at which infectivity levels were 60% of control following normalization of the data to control values.

Construction of variant viruses. DNA was isolated from H9 cells infected with HXB2/O925 by standard protocols (39), digested with the restriction enzyme *Sac*I, selected for fragments of 3 to 5 kb, and cloned into lambda Zap (Stratagene, La Jolla, Calif.). The resulting library was screened with a DNA probe for the HXB2 envelope using standard techniques (39). Two positive clones (R12 and R13) were subcloned into pGEM 4 (Promega, Madison, Wis.) and sequenced by dideoxy techniques (14, 40). *Sac*I fragments R12env and R13env were

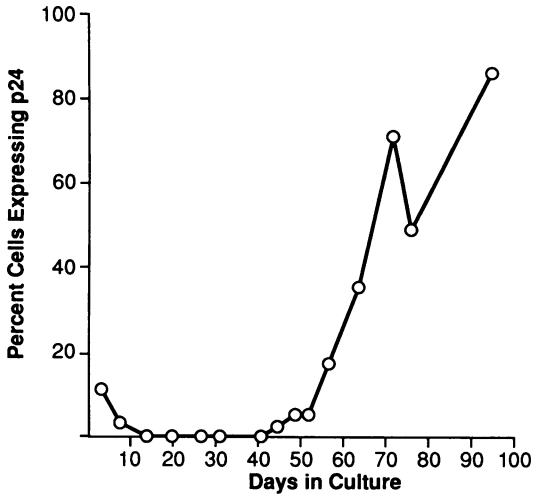

FIG. 2. Kinetics of appearance of neutralization escape mutant HXB2/O925 in the presence of serum WO925. On the days indicated, cells were removed from the culture, washed, spotted on slides, and fixed for 10 min with 50% acetone–50% methanol at room temperature prior to assessment of p24 expression by indirect immunofluorescent assay. Under similar conditions in the presence of normal human serum, 80 to 90% infected cells appear within 7 to 14 days.

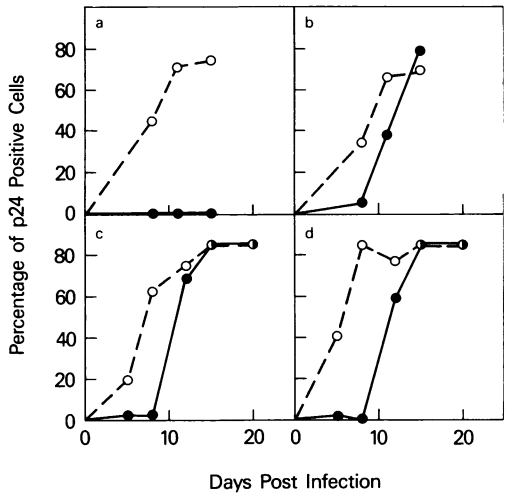

FIG. 3. Cell-free transmission of (a) parental HXB2, (b) HXB2/O925, (c) R12, and (d) R13 variant viruses in the presence of selecting neutralizing antiserum WO925 (closed circles), and NHS (open circles). Transmissions were repeated two to six times; representative data are depicted.

cloned into pGEM4 containing the *Sal*I-*Bam*HI fragment of HXB2, and R12env and R13env *Sal*I-*Bam*HI fragments were then subcloned into pHXB2 (6), producing plasmids containing infectious molecular clones R12 and R13.

The mutations N463K and S465L were generated by a polymerase chain reaction mutagenesis technique described elsewhere (49), and the resulting variant *Pvu*II-*Bam*HI fragments were subcloned into pGEM4 with the *Sal*I-*Pvu*II fragment of HXB2 to generate variant *Sal*I-*Bam*HI fragments, which were then subcloned into pHXB2 as described above. Variant R12/2 was made by cloning the *Sal*I-*Pvu*II fragment of R13 and the *Pvu*II-*Bam*HI fragment of S465L into pGEM4 and then subcloning this *Sal*I-*Bam*HI fragment into pHXB2.

RESULTS

Immune selection of a neutralization-resistant variant in vitro. To study neutralization escape mutations occurring within the envelope of HIV-1, we employed an experimental protocol previously used to identify an escape mutant with an alanine (A) to threonine (T) mutation at position 582 (A582T) in gp41 (32, 36, 50). HXB2, an infectious molecular clone of HIV-1_{IIIB}, was grown in H9 cells (28), which were then lethally irradiated. Transmission of HXB2 to nonirradiated H9 cells was conducted in the presence of antiserum WO925, a broadly reactive neutralizing antiserum from an HIV-1 seropositive individual. The kinetics of appearance of a virus resistant to neutralization by serum WO925 are illustrated in Fig. 2. Following transient p24 expression in the culture, attributable to residual irradiated HXB2-infected H9 cells, virus expression was not evident until the sixth week of culture. At this point, the resistant virus was steadily propagated, and by day 72 a productive culture was firmly established. Day 72 infected cells were subsequently expanded for nucleic acid extraction and the molecular analysis detailed below. Confirmation that the emergent virus, designated HXB2/O925, was indeed resistant to neutralization by the selecting serum was carried out by a secondary transmission experiment. Cell supernatant from the HXB2/O925 infected culture was tested for its ability to infect fresh H9 cells in the presence of antiserum WO925 and shown to be resistant to neutralization (Fig. 3b). In contrast, the parental virus was efficiently neutralized by the selecting antiserum (Fig. 3a).

Determination of the molecular basis of neutralization resistance. A bacteriophage lambda library was generated from HXB2/O925 DNA and screened for clones containing the HIV-1 envelope gene. Two positive clones, R12env and R13env, were subcloned and sequenced. Clone R12env contained two amino acid substitutions relative to HXB2: a valine (V) for alanine (A) substitution at envelope amino acid 281 (A281V) (numbering from reference 31) and a lysine (K) for asparagine (N) substitution at amino acid 463 (N463K). Clone R13env contained only the A281V substitution. Both envelope genes were subcloned into pHXB2 to give two infectious molecular clones of HIV-1, R12 and R13. R12 and R13 were both resistant to neutralization by WO925 serum (Fig. 3c and d) and exhibited the same initial lag in infection kinetics as the original variant, HXB2/O925 (Fig. 3b). These results suggested that the A281V substitution alone confers resistance to neutralization by selecting antiserum WO925.

To determine whether the N463K substitution, present in R12 but not R13, had any effect on neutralization of HIV-1 by antiserum WO925, three further variant viruses, N463K, S465L and R12/2, were designed (Fig. 1), constructed, and tested. Variant N463K contained only the N463K substitution found in variant R12. Variant S465L contained a leucine (L) for serine (S) substitution at position 465, thereby disrupting the N-linked glycosylation motif (N-X-S/T, where X is any amino acid except proline). Variant R12/2 combined both the A281V and S465L substitutions. The S465L and R12/2 variants were designed to discriminate between the possible involvement of either the amino acid at position 463 or the N-linked carbohydrate residues at this site in neutralization escape. When tested for resistance to neutralization by WO925 serum, variants N463K and S465L were both sensitive to neutralization, whereas variant R12/2 was resistant (Fig. 4). This finding demonstrated that neither the N463K amino acid substitution nor the removal of the N-linked carbohydrate at position 463

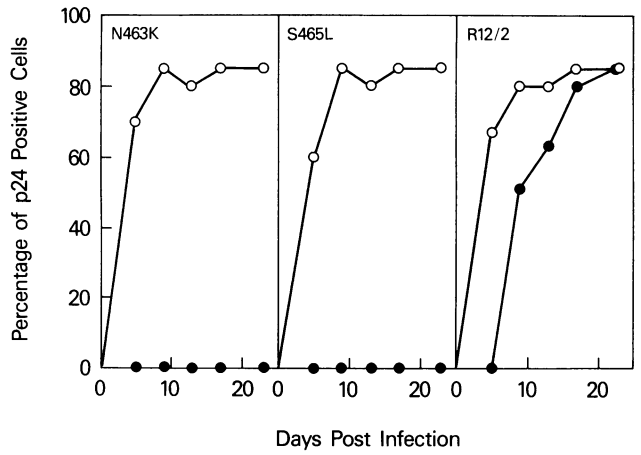

FIG. 4. Cell-free transmission of HXB2 variant viruses N463K, S465L, and R12/2 in the presence of selecting antiserum WO925 (closed circles) and NHS (open circles). The variant viruses were assessed twice with similar results. Control experiments with HXB2, run in parallel, produced results similar to those shown in Fig. 3.

TABLE 1. Neutralization of HXB2 and variant HXB2/O925 by antisera from HIV-1-seropositive individuals

Serum	Neutralizing titer on:	
	HXB2	HXB2/O925
W6235	115	100
WN510	140	265
WN512	325	300
WN518	275	90
WN524	25	65
RT	965	390
WN529	50	40
WO380	115	65
WO395	90	100
WO426	175	100
WO745	140	50
WO747	565	90
WO925	890	<25
Mean	298	127
SEM	87	32

influences the neutralization of HXB2 by antiserum WO925. Thus, the A281V substitution alone confers resistance to neutralization by WO925 serum.

Determination of the immunologic basis for immune escape. To further characterize the mechanism of the immune escape by variants of HXB2 containing the A281V mutation, a synthetic peptide from the 281 region of HXB2 (271ala288) (Fig. 1) was synthesized, and antisera were raised to this peptide. These reagents were used to determine whether the 281 region constitutes a contiguous binding site for neutralizing antibodies. The addition of an excess of peptide 271ala288 to incubation mixtures did not abrogate neutralization of HXB2 by the immune selecting serum, WO925, nor did it significantly affect syncytial inhibition of the parental virus by the same serum. A synthetic peptide from the N463K region (454asn477) gave similar results. The neutralizing titer of WO925 serum on HXB2 in the absence of peptide was 940, in the presence of 271ala288 was 950, and in the presence of 454asn471 was 1,090. Percent syncytial inhibition of HXB2 by WO925 serum in the absence of peptide or in the presence of peptides 271ala288 or 454asn471 was 75, 57, and 83, respectively. Antisera raised in a guinea pig and a goat against 271ala288 bound to the peptide yet failed to neutralize HXB2 cell-free virus infection or inhibit syncytium formation by HXB2-infected cells. Similarly, a guinea pig antiserum to peptide 454asn477 recognized the peptide 454asn471 but failed to neutralize HXB2.

Additional analysis of the neutralization resistance conferred by the A281V mutation was conducted by analyzing the resistance of the original variant, HXB2/O925, to neutralization by a panel of broadly neutralizing antisera from HIV-1-seropositive individuals. The results of these experiments showed that HXB2/O925 was still neutralized by all antisera tested, except for the immune-selecting serum (WO925). However, the overall mean neutralizing titer of these antisera was lower against variant HXB2/O925 than against HXB2 (Table 1). Of 12 nonselecting sera tested, 4 (W6235, WN512, WN529, and WO395) showed little difference in their ability to neutralize either HXB2 or HXB2/O925. Two of the sera (WN510 and WN524) showed increased neutralizing capabilities on HXB2/O925 compared with those on HXB2. The remaining six sera (WN518, RT, WO380, WO426, WO745, and WO747) showed a reduction in neutralizing titer against HXB2/O925. Although the neutralizing titers of the 12 nonselecting sera were not sufficiently different to distinguish variant from parental virus, the twofold decrease in mean neutralizing titer against HXB2/O925 suggests the sera did recognize subtle differences in epitope presentation between the two viral envelopes.

A previous neutralization escape variant, A582T, selected by using the same protocol resembles the A281V variant in possessing a mutation which leads to immune escape in a region shown not to be a linear neutralizing epitope (50). Recent work has shown that the mechanism of neutralization escape by variant A582T is through the alteration of conformational binding sites for antibodies which block the binding of gp120 to CD4 (17). This effect has been clearly demonstrated in the case of variant A582T with several monoclonal antibodies which recognize the CD4 binding site of gp120, most notably the human monoclonal antibody F105, which neutralizes HXB2 but not variant A582T. Serum RT used to select variant A582T exhibits a 2.5-fold greater neutralizing titer against HXB2 than against HXB2/O925 (Table 1), suggesting that the latter might possess an envelope conformation poorly recognized by conformation-dependent antibodies to the CD4-binding region of gp120 present in the selecting serum. Therefore, to examine the possibility that variant A281V utilized the same mechanism as A582T for neutralization escape, we tested the abilities of monoclonal antibodies F105, I5e, and 120-1B1 as well as sCD4 to neutralize variants HXB2/O925, R12, and R13. The results of these experiments (Table 2) showed no significant differences between HXB2 and the variants in sensitivity to neutralization by F105, I5e, 120-1B1, or sCD4, indicating that the mechanism of neutralization escape for A281V variant viruses is different than for the A582T variant, which required more than 81-fold more F105 for neutralization than HXB2 (17).

By logical extension, we reasoned that if a mutation in gp41 (A582T) can affect distant conformational antibody binding sites on a different protein (gp120), then the A281V mutation could affect antibody binding sites within the V3 loop of gp120, which lies between amino acids 296 and 331, relatively close to the site of the mutation. To test this possibility, we examined the ability of neutralizing monoclonal antibodies 0.5β, M77, and IIIB-V3-13 to neutralize variants HXB2/O925, R12, and

TABLE 2. Neutralization of HXB2 and variant viruses by anti-HIV-1 monoclonal antibodies and sCD4

Virus strain	Titer[a]						
	F105[b]	I5e[c]	120-1B1[c]	sCD4[c]	M77[d]	IIIB-V3-13[c]	0.5β[c]
HXB2	290	6.8	10.8	130	1125	6.2	3.8
HXB2/O925	340	10.0	18.6	93	500	9.6	8.8
R12	250	6.4	20.0	200	1625	10.0	12.2
R13	200	8.0	14.0	110	500	9.8	7.4

[a] For low-titer antibodies, average values were calculated by taking the highest antibody concentration used as the value for samples which were not neutralized.
[b] Titer defined as the reciprocal of the antibody dilution at which infectivity was reduced by 75%.
[c] Concentration in micrograms per milliliter (nanograms per milliliter for sCD4) at which infectivity was 60% of the control value is listed.
[d] Titer defined as the reciprocal of the antibody dilution at which infectivity was 60% of the control.

R13. These data showed that a slight increase in the concentration of monoclonal antibodies 0.5β and IIIB-V3-13 compared with HXB2 was required to neutralize all three variant viruses, with variant R12 showing the greatest resistance and monoclonal antibody 0.5β showing the largest increases. The increases observed, ranging from 2.3- to 3.2-fold for 0.5β, were not sufficient to explain the escape from serum WO925, since 6- to 38-fold more WO925 serum was necessary to neutralize the variant viruses compared with HXB2. The neutralization titers of WO925 against HXB2, HXB2/O925, R12, and R13 were 940, 25, 65, and 150, respectively. Corresponding percentages of syncytium inhibition were 75, 14, 39, and 35.

DISCUSSION

Using an established technique for the derivation of neutralization-resistant variants of HIV-1, we selected variant HXB2/O925 by growing an HIV-1 infectious molecular clone in the presence of a broadly neutralizing antiserum, WO925. Molecular cloning of the envelope of HXB2/O925 gave two clones, R12env and R13env, which were tested for resistance to neutralization by antiserum WO925 following subcloning into the parental virus, HXB2, and using the resulting variant viruses, R12 and R13, in neutralization assays. The results of these assays, and tests on additional variant viruses (N463K, L465S, and R12/2), demonstrated that a single-amino-acid substitution (a change from alanine to valine at amino acid 281 in gp120) found in both R12 and R13 gave rise to the neutralization-resistant phenotype.

Searches of the AIDS data base (23) to assess the degree of variation at amino acid 281 in gp120 among different HIV-1 isolates revealed that four amino acids have been observed at this site. Of 41 available sequences for review, 33 contain alanine at position 281, 5 contain valine, 2 contain threonine, and 1 contains isoleucine. To compare relative antibody reactivities to both the A281 parent and the V281 variant, we conducted comparative studies of HXB2 and HXB2/O925 with a panel of human sera from HIV-1-seropositive individuals. HXB2/O925 was sensitive to neutralization by 12 of 13 human sera which neutralize HXB2 and was resistant to WO925. This observation has two alternative explanations: either serum WO925 differs from the sera of most HIV-1-seropositive individuals in its inability to recognize A281V variant viruses, or most sera contain other anti-HIV-1 reactivities which neutralize regardless of 281 variation. Our data favor the latter possibility. The lower range of neutralizing titer seen with natural antisera against HXB2/O925 (Table 1) and the twofold decrease in the mean neutralizing titer exhibited against HXB2/O925 compared with HXB2 indicate that while the majority of seropositive individuals produce antibodies which neutralize both parent and variant viruses, recognition of the variant is weaker, suggesting that recognition of the epitope disrupted by the A281V mutation may have been lost. This would also agree with the much lower incidence of V281 in the data base, which, if it accurately reflects the relative incidence of V281 among infected individuals, would suggest that only 10 to 15% of sera from seropositive individuals would contain this reactivity. It is not yet known whether the substitution of threonine or isoleucine for alanine at position 281 in HXB2 would result in similarly reduced antibody reactivity and enhanced neutralization resistance. Analysis of either T281 or I281 variants may allow a distinction to be made between the two alternative mechanisms, since recognition of either variant should be very rare. It is clear, however, that there are additional pathways for neutralization that are unaffected by the amino acid present at position 281.

To establish whether the neutralization escape we observed was due to the disruption of a linear neutralization epitope by the substitution of valine for alanine at position 281, we used two approaches. First, we attempted to block the neutralization of HXB2 by WO925 serum with a synthetic peptide (271ala288) to this region and secondly, we used antisera raised against this peptide in neutralization studies to determine whether anti-peptide activity to peptide 271ala288 neutralized HXB2. These studies did not demonstrate any loss of neutralizing activity by WO925 serum in the presence of peptide 271ala288 or any neutralizing activity in antiserum to peptide 271ala288. From these results, we concluded that the mechanism of immune escape by variants containing the A281V mutation could not be attributed to the disruption of a linear neutralization epitope that included amino acid 281. These observations are consistent with previous reports that no detectable HIV-1 neutralizing activity has been associated with antibodies that bind to peptides from this region of gp120; however, it is impossible to eliminate the possibility that neutralizing antibodies to this region may not recognize synthetic peptides. As the mechanism of immune escape of the variant viruses described here could not be attributed to alteration of a linear neutralizing epitope in the 281 region, we carried out additional experiments to elucidate the immunologic basis for the neutralization resistance.

In previous studies of a neutralization-resistant variant (36) attributed to a point mutation in gp41 (32) (Fig. 1), we concluded that resistance was due to a conformational change at a distant location (50). Recently, Klasse et al. (17) have shown that escape for this variant (A582T) occurred within or near the CD4 binding site, as the resistant variant is less readily neutralized by conformationally dependent antibodies directed to this region, including monoclonal antibody F105 (29). No effect on neutralization of the A582T variant via the V3 loop was observed. In addition, binding of antibody F105 has been shown to be impaired by mutations at discontinuous sites within gp120 (47) (Fig. 1); however, position 281 was not

evaluated in these experiments. Experiments with F105 showed no significant differences in neutralization titers against HXB2, HXB2/O925, R12, or R13 (Table 2). In a related experiment, we found the amount of sCD4 necessary to inhibit infectivity was similar for HXB2, HXB2/O925, R12, and R13 (Table 2). Additionally, monoclonal antibodies I5e and 120-1B1, which neutralize HIV-1 via the CD4 binding site, were equally able to neutralize HXB2 and the A281V-containing variant viruses. Previous studies of neutralizing monoclonal antibodies that recognize gp120 after binding to CD4 have examined A281V variants but did not find a significant contribution of this mutation to resistance to neutralization by these antibodies (46). Thus, the A281V mutation does not appear to affect the conformation of the CD4 binding site, the binding of antibodies to this site, or the affinity of gp120 for the CD4 receptor. Therefore, we conclude that the observed neutralization resistance to serum WO925 must involve a different site.

In pursuit of this alternative, we compared neutralization of the parental and variant viruses by monoclonal antibodies M77 (26), IIIB-V3-13 (18), and 0.5β (20), which neutralize HIV-1_{IIIB}-related isolates (including HXB2) through binding to the V3 domain. Between 2.3- and 3.2-fold more 0.5β was required to neutralize HXB2/O925, R12, and R13 than HXB2, a difference which is insufficient to account for the 6- to 38-fold difference in the neutralizing antibody titer of WO925 against the variant viruses compared to HXB2 (Table 2). Antibodies M77 and IIIB-V3-13 showed smaller differences in neutralizing titer between HXB2 and the variant viruses than 0.5β. Our data suggest that the variant viruses only minimally resist neutralization because of a conformational alteration of their V3 loop resulting from a substitution of V for A at position 281. That this effect on the V3 loops of HXB2/O925, R12, and R13 is only partial is not surprising considering that the selecting serum, WO925, possesses negligible antibody capable of recognizing the HXB2 V3 loop peptide, and only a modest binding antibody titer to a V3 loop peptide of the HIV-1_{MN} strain, an early HIV-1 isolate (8, 41) representative of the consensus sequence of the V3 loop described for isolates in the United States (19). Most sera from HIV-positive individuals in the United States and Europe recognize the V3 loop of MN (3). We conclude the immunologic basis for the neutralization resistance of HXB2/O925 is not escape via a change in the CD4 binding site, or primarily escape via a change in V3, but may depend on local and/or more distant conformational changes induced by the A281V alteration. Compared to HXB2/O925, R12 and R13 are somewhat less resistant both to cell-free neutralization and syncytial inhibition by the WO925-selecting serum. This may indicate that further variants exist within the HXB2/O925 population with greater neutralization resistance than R12 or R13. Analysis of additional neutralization-resistant clones of HXB2/O925 and their immunologic characterization may elucidate the alternate mechanism of immune escape suggested by our results.

It has recently become clear that immune escape can occur via the CD4 binding site region on gp120 (17) as well as via V3. The latter can involve amino acid substitution within a V3 epitope leading to alteration of a linear (22) or conformational (4) V3 loop epitope. Amino acid changes outside the V3 loop can also lead to escape from V3-specific neutralizing antibodies (24). The change from alanine to valine at position 281 has been observed as one of three amino acid changes in the gp120 sequences of matched viruses in which one virus is more resistant to soluble CD4 neutralization (48). Our data suggest that the A281V mutation alone is not responsible for this increase in relative resistance to soluble CD4 neutralization with time in culture, since all three of our A281V-containing variants were as susceptible to sCD4 neutralization as the parental virus HXB2 (Table 2).

The region around position 281 has also been implicated in influencing the structure and function of the V3 loop. A change from aspartate (D) to asparagine (N) at a position equivalent to 279 in HXB2 gp120 has been shown to cooperate with a histidine (H) for tyrosine (Y) substitution at position 317 in the V3 loop of HIV-1_{SF13} with regard to viability (42) and to be associated with decreased syncytium-forming ability in HIV-1_{SF2} (42). From these observations, it would appear that the 281 region may interact with the V3 loop, affecting its structure and function. We, however, have detected only a modest change in the neutralizing titer of monoclonal antibodies that recognize the V3 loop of HXB2 against variants containing the A281V mutation (Table 2).

On the basis of our studies here, we conclude that immune escape by HIV-1 from neutralizing antisera occurs via multiple discrete mechanisms, which in vivo may operate in concert. This circumstance poses a severe challenge to vaccine development and suggests that a successful AIDS vaccine may require identification of highly conserved, immunogenic (and probably conformational) regions of critical functional importance, such that mutation within them would result in a noninfectious virus. Identification of further mutations that give rise to immune escape will allow this hypothesis to be tested, and, if correct, efficient presentation of the relevant epitopes may lead to the development of a vaccine against HIV-1.

ACKNOWLEDGMENTS

We thank William A. Blattner for the selecting serum, WO925; Shuzo Matsushita for monoclonal antibody 0.5β; Fulvia di Marzo Veronese for monoclonal antibody M77; David D. Ho for monoclonal antibody I5e; Maria Myagkikh for performing the binding ELISAs; Anna Mazzuca for expert editorial assistance; and Robert C. Gallo for support. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: F105 monoclonal antibody from Marshall Posner and IIIB-V3-13 monoclonal antibody from Jon D. Laman.

REFERENCES

1. **Albert, J., B. Abrahamsson, K. Nagy, E. Aurelius, H. Gaines, G. Nystrom, and E. M. Fenyo.** 1990. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. AIDS **4**:107–112.
2. **Arendrup, M., A. Soonerborg, B. Svennerholm, L. Akerblom, C. Nielsen, H. Clausen, S. Olofsson, J. O. Nielsen, and J.-E. S. Hansen.** 1993. Neutralizing antibody response during human immunodeficiency virus type 1 infection: type and group specificity and viral escape. J. Gen. Virol. **74**:855–863.
3. **Devash, Y. A., T. J. Matthews, J. E. Drummond, K. Javaharian, D. J. Waters, L. O. Arthur, W. A. Blattner, and J. R. Rusche.** 1990. C-terminal fragments of gp120 and synthetic peptides from five HIV variants: prevalence of antibodies in HIV-infected individuals and cross-reactivity of goat sera. AIDS Res. Hum. Retroviruses **6**:307–316.
4. **Di Marzo Veronese, F., M. S. Reitz, Jr., G. Gupta, M. Robert-Guroff, C. Boyer-Thompson, A. Louie, R. C. Gallo, and P. Lusso.** J. Biol. Chem., in press.
5. **Emini, E. A., W. A. Schleif, J. H. Nunberg, A. J. Conley, Y. Eda, S. Tokioshi, S. D. Putney, S. Matsushita, K. E. Cobb, C. M. Jett, J. W. Eichberg, and K. K. Murthy.** 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature (London) **355**:728–732.
6. **Fisher, A. G., E. Collati, L. Ratner, R. C. Gallo, and F. Wong-Staal.** 1985. A molecular clone of HTLV-III with biologic activity. Nature (London) **316**:262–265.
7. **Fultz, P. N., P. L. Nara, F. Barre-Sinoussi, A. Chaput, M. L.**

Greenberg, E. Muchmore, M.-P. Kieny, and M. Girard. 1992. Vaccine protection of chimpanzees against challenge with HIV-1-infected peripheral blood mononuclear cells. Science **256:**1687–1690.

8. **Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, G. White, P. Foster, and P. D. Markham.** 1984. Human T-lymphotropic retrovirus, HTLV-III, isolated from AIDS patients and donors at risk for AIDS. Science **224:**500–503.
9. **Gorny, M. K., A. J. Conley, S. Karwowska, A. Buchbinder, J. Xu, E. A. Emini, S. Koenig, and S. Zolla-Pazner.** 1992. Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. J. Virol. **66:**7538–7542.
10. **Goudsmit, J., C. Debouck, R. H. Meleon, L. Smit, M. Bakker, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., and D. C. Gajdusek.** 1988. Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. Proc. Natl. Acad. Sci. USA **85:**4478–4482.
11. **Hahn, B. H., M. A. Gonda, G. M. Shaw, M. Popovic, J. Hoxie, R. C. Gallo, and F. Wong-Staal.** 1985. Genomic diversity of the AIDS virus HTLV-III: different viruses exhibit greatest divergence in their envelope genes. Proc. Natl. Acad. Sci. USA **82:**4813–4817.
12. **Hahn, B. H., G. M. Shaw, M. E. Taylor, R. R. Redfield, P. D. Markham, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, E. S. Parks, and W. P. Parks.** 1986. Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS. Science **232:**1548–1553.
13. **Haigwood, N. L., C. B. Barker, K. W. Higgins, P. V. Skiles, G. K. Moore, K. A. Mann, D. R. Lee, J. W. Eichberg, and K. S. Steimer.** 1990. Evidence for neutralizing antibodies directed against conformational epitopes of HIV-1 gp120, p. 313–320. *In* R. M. Chanock, H. Ginsberg, and R. A. Lerner (ed.), Vaccines 1990: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
14. **Hattori, M., and Y. Sakaki.** 1986. Dideoxy sequencing method using denatured plasmid templates. Anal. Biochem. **152:**232–238.
15. **Ho, D. D., J. A. McKeating, X. L. Li, T. Moudgil, E. S. Daar, N.-C. Sun, and J. E. Robinson.** 1991. Conformational epitope on gp120 important in CD4 binding and human immunodeficiency virus type 1 neutralization identified by a human monoclonal antibody. J. Virol. **65:**489–493.
16. **Javaherian, K., A. J. Langlois, G. J. LaRosa, A. T. Profy, D. P. Bolognesi, W. C. Herlihy, S. D. Putney, and T. J. Matthews.** 1990. Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. Science **250:**1590–1593.
17. **Klasse, P. J., J. A. McKeating, M. Schutten, M. S. Reitz, Jr., and M. Robert-Guroff.** 1993. An immune-selected point mutation in the transmembrane protein of human immunodeficiency virus type 1 (HXB2-Env: Ala582(→Thr)) decreases viral neutralization by monoclonal antibodies to the CD4 binding site. Virology **196:**332–327.
18. **Laman, J. D., M. M. Schellekens, Y. H. Abacioglu, G. K. Lewis, M. Tersmette, R. A. M. Fouchier, J. P. M. Langedijk, E. Claassen, and W. J. A. Boersma.** 1992. Variant-specific monoclonal and group-specific polyclonal human immunodeficiency virus type 1 neutralizing antibodies raised with synthetic peptides from the gp120 third variable domain. J. Virol. **66:**1823–1831.
19. **LaRosa, G. J., J. P. Davide, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreesman, R. N. Boswell, P. Shadduck, L. H. Holley, M. Karplus, D. P. Bolognesi, T. J. Matthews, E. A. Emini, and S. D. Putney.** 1990. Conserved sequence and structural elements in HIV-1 principal neutralizing determinant. Science **249:**932–935.
20. **Matsushita, S., M. Robert-Guroff, J. Rusche, A. Kioto, T. Hattori, H. Hoshino, K. Javaharian, K. Takatsuki, and S. Putney.** 1988. Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. J. Virol. **62:**2107–2114.
21. **Matthews, T. J., A. J. Langlois, W. G. Robey, N. T. Chang, R. C. Gallo, P. J. Fischinger, and D. P. Bolognesi.** 1986. Restricted neutralization of divergent human T-lymphotropic virus type III isolates by antibodies to the major envelope glycoprotein. Proc. Natl. Acad. Sci. USA **83:**9709–9713.
22. **McKeating, J. A., J. Gow, J. Goudsmit, L. H. Pearl, C. Mulder, and R. A. Weiss.** 1989. Characterization of HIV-1 neutralization escape mutants. AIDS **3:**777–784.
23. **Meyers, G., J. A. Berzofsky, B. Korber, R. F. Smith, and G. N. Pavlakis.** 1992. Human retroviruses and AIDS 1992. Los Alamos National Laboratory, Los Alamos, New Mexico.
24. **Nara, P. L., and J. Goudsmit.** 1990. Neutralization resistant variants of HIV-1 escape via the hypervariable immunodominant region (303-331 aa): evidence for a conformational neutralization epitope, p. 297–306. *In* R. M. Chanock, H. Ginsberg, and R. A. Lerner (ed.), Vaccines 1990: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Nowak, M. A., R. M. Anderson, A. R. McLean, T. F. W. Wolfs, J. Goudsmit, and R. M. May.** 1991. Antigenic diversity thresholds and the development of AIDS. Science **254:**963–969.
26. **Pal, R., F. di Marzo Veronese, B. C. Nair, R. Rahman, G. Hoke, S. W. Mumbauer, and M. G. Sarngadharan.** 1992. Characterization of a neutralizing monoclonal antibody to the external glycoprotein of HIV-1. Intervirology **34:**86–93.
27. **Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randell, D. P. Bolognesi, and B. F. Haynes.** 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic antibodies. Proc. Natl. Acad. Sci. USA **85:**1932–1936.
28. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. A method for the detection, isolation, and continuous production of cytopathic human T-lymphotropic retroviruses of the HTLV family (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497–500.
29. **Posner, M. R., T. Hideshima, T. Cannon, M. Mukherjee, K. H. Mayer, and R. A. Byrn.** 1991. An IgG human monoclonal antibody that reacts with HIV-1/gp120, inhibits virus binding to cells, and neutralizes infection. J. Immunol. **146:**4325–4332.
30. **Profy, A. T., P. A. Salinas, L. I. Eckler, N. M. Dunlop, P. L. Nara, and S. D. Putney.** 1990. Epitopes recognized by the neutralizing antibodies of an HIV-1 infected individual. J. Immunol. **144:**4641–4647.
31. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. R. Starcich, S. F. Josephs, E. R. Dotan, J. A. Rafalski, E. A. Whitwhoen, K. Baumeister, L. Ivanoff, S. R. Petteway, M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus HTLV-III. Nature (London) **313:**277–284.
32. **Reitz, M. S., C. A. Wilson, C. Naugle, R. C. Gallo, and M. Robert-Guroff.** 1988. Generation of a neutralization resistant variant of HIV-1 is due to selection for a point mutation in the envelope gene. Cell **54:**57–63.
33. **Robert-Guroff, M.** 1990. HIV-neutralizing antibodies: epitope identification and significance for future vaccine. Int. Rev. Immunol. **7:**15–30.
34. **Robert-Guroff, M.** 1990. Neutralizing antibodies, p. 179–185. *In* A. Aldovini and B. Walker (ed.), Techniques in HIV research. Stockton Press, New York.
35. **Robert-Guroff, M., K. Aldrich, R. Muldoon, T. L. Stern, G. P. Bansel, T. J. Matthews, P. D. Markham, R. C. Gallo, and G. Franchini.** 1992. Cross-neutralization of human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus isolates. J. Virol. **66:**3602–3608.
36. **Robert-Guroff, M., M. S. Reitz, W. G. Robey, and R. C. Gallo.** 1986. In vitro generation of an HTLV-III variant by neutralizing antibody. J. Immunol. **137:**3306–3309.
37. **Robert-Guroff, M., E. Roilides, R. Muldoon, D. Venzon, R. Husson, D. Marshall, R. C. Gallo, and P. A. Pizzo.** 1993. Human immunodeficiency virus (HIV) type 1 strain MN neutralizing antibody in HIV-infected children: correlation with clinical status and prognostic value. J. Infect. Dis. **167:**538–546.
38. **Rusche, J. R., K. Javaharian, C. McDanal, J. Petro, D. L. Lynn, R. Grimaila, A. Langlois, R. C. Gallo, L. O. Arthur, P. J. Fischinger, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1988. Antibodies that inhibit fusion of human immunodeficiency virus-infected

cells bind a 24-amino acid sequence of the viral envelope, gp120. Proc. Natl. Acad. Sci. USA **85:**3198–3202.

39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
41. **Shaw, G. M., B. H. Hahn, S. K. Arya, J. E. Groopman, R. C. Gallo, and F. Wong-Staal.** 1984. Molecular characterization of human T-cell leukemia (lymphotrophic) virus type III in the acquired immune deficiency syndrome. Science **226:**1165–1171.
42. **Stamatatos, L., and C. Cheng-Meyer.** 1993. Evidence that the structural conformation of envelope gp120 affects human immunodeficiency virus type 1 infectivity, host range, and syncytium-forming ability. J. Virol. **67:**5635–5639.
43. **Starcich, B. R., B. H. Hahn, G. M. Shaw, P. D. McNeeley, S. Modrow, H. Wolf, E. S. Parks, W. P. Parks, S. F. Josephs, R. C. Gallo, and F. Wong-Staal.** 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell **45:**637–648.
44. **Steimer, K. S., P. J. Klasse, and J. A. McKeating.** 1991. HIV-1 neutralization directed to epitopes other than linear V3 determinants. AIDS **5**(suppl. 2)**:**s135–s143.
45. **Steimer, K. S., C. J. Scandella, P. V. Skiles, and N. L. Haigwood.** 1991. Neutralization of divergent HIV-1 isolates by conformation-dependent human antibodies to gp120. Science **254:**105–108.
46. **Thali, M., J. P. Moore, C. Furman, M. Charles, D. D. Ho, J. Robinson, and J. Sodroski.** 1993. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. J. Virol. **67:**3978–3988.
47. **Thali, M., U. Olshevsky, C. Furman, D. Gabuzda, M. Posner, and J. Sodroski.** 1991. Characterization of a discontinuous human immunodeficiency virus type 1 gp120 epitope recognized by a broadly reactive neutralizing human monoclonal antibody. J. Virol. **65:**6188–6193.
48. **Turner, S., R. Tizard, J. Demarinis, R. B. Pepinsky, J. Zullo, R. Schooley, and R. Fisher.** 1992. Resistance of primary isolates of human immunodeficiency virus type 1 to neutralization by soluble CD4 is not due to lower affinity with the envelope glycoprotein gp120. Proc. Natl. Acad. Sci. USA **89:**1335–1339.
49. **Watkins, B. A., A. E. Davis, F. Cocchi, and M. S. Reitz.** A rapid method for site-specific mutagenesis using larger plasmids as templates. BioTechniques, in press.
50. **Wilson, C. A., M. S. Reitz, Jr., K. Aldrich, P. J. Klasse, J. Blomberg, R. C. Gallo, and M. Robert-Guroff.** 1990. The site of an immune-selected point mutation in the transmembrane protein of human immunodeficiency virus type 1 does not constitute a neutralization epitope. J. Virol. **64:**3240–3248.
51. **Wong-Staal, F., G. M. Shaw, B. H. Hahn, S. Z. Salahuddin, M. Popovic, P. D. Markham, R. Redfield, and R. C. Gallo.** 1985. Genomic diversity of human T-lymphotropic virus type III. Science **229:**759–762.