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The Site of an Immune-Selected Point Mutation in the Transmembrane Protein of Human Immunodeficiency Virus Type 1 Does Not Constitute the Neutralization Epitope

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We previously reported the in vitro generation of a neutralization-resistant variant of the molecularly cloned isolate of human immunodeficiency virus type 1 (HIV-1), HXB2D. The molecular basis for the resistance was shown to be a point mutation in the *env* gene, causing the substitution of threonine for alanine at position 582 of gp41. Here, we show the variant to be resistant to syncytium inhibition as well as to neutralization by the immune-selecting serum. Moreover, 30% of HIV-positive human sera able to neutralize the parental virus have significantly decreased ability to neutralize the variant. As the A-to-T substitution thus has general relevance to the interaction of HIV-1 with the host immune system, we investigated further the biologic and immunologic bases for the altered properties. Synthetic peptides corresponding to the 582 region failed to compete in infectivity, neutralization, or syncytium inhibition assays and did not elicit neutralizing antibodies. Furthermore, human antibodies, affinity purified on synthetic peptide resins, bound to gp41 and peptides from the 582 region but did not possess neutralizing antibody activity. Some viral constructs in which the AVERY sequence in the 582 region was altered by site-directed mutagenesis were not infectious, indicating that the primary structure in this region is crucial for viral infectivity. Constructs predicted to possess a local secondary structure similar to that of the variant nevertheless behaved like the parental virus and remained neutralization sensitive. These results suggest that the requirements for neutralization resistance in this region are very precise. Our results with synthetic peptides show that the 582 region does not by itself constitute a neutralization epitope. Moreover, the degree of flexibility in amino acid substitution which allows maintenance of neutralization sensitivity suggests that position 582 does not form part of a noncontiguous neutralization epitope. The basis for neutralization resistance of the immune-selected variant is more likely a conformational change altering a neutralization epitope at a distant site.

The human immunodeficiency virus type 1 (HIV-1) exhibits extensive genetic heterogeneity, especially in the envelope gene (8, 37). The HIV-1 *env* gene products, gp120 (the external surface glycoprotein) and gp41 (the transmembrane protein), possess many functions related to virus-host interaction, including recognition of the surface receptor on target cells, cell fusion, initial events in viral entry into host cells, and elicitation of humoral and cellular immune responses. While natural selection processes driven by viral requirements for infectibility, replication, and regulatory controls limit the extent of viral variation in many functionally important regions, immune selection can allow for propagation of replication-competent mutants which have escaped immunologic control. The extensive variability of the viral envelope provides a basis for immune selection of viral variants which may arise during the course of infection and which are not neutralized by the host immune system. This generation of so-called escape mutants is well documented both in vivo and in vitro for a number of lentiviruses, including visna virus (22, 34) and equine infectious anemia virus (15, 20), and also occurs in the HIV-1 system (20a, 30). We previously reported on in vitro immune selection of a neutralization-resistant variant generated during propagation of a molecularly cloned HIV-1 isolate, HXB2D, in the presence of a high-titer neutralizing antiserum from an infected individual (30). Selection of this variant provided the first indication that individuals naturally infected with HIV-1 possess type-specific neutralizing antibodies. Type specificity, which enables immune selection, subsequently was confirmed in numerous studies in which the HIV-1 envelope, envelope subfragments, or recombinant envelope proteins were used to elicit type-specific monoclonal or polyclonal antibodies with high-titer neutralizing activity (18, 19, 23, 31). Recent studies of HIV-1-infected chimpanzees also demonstrate that neutralization-resistant variants arise naturally in vivo during the course of infection (20a). The possibility that HIV-1 may thus elude host immunologic control mechanisms must be carefully considered in designing putative vaccines.

Whether the phenomenon of immune selection and generation of neutralization-resistant variants influences the course of HIV-1 infection in vivo and contributes to disease progression has not been established. The capability of developing such variants in vitro, however, provides a system which can be exploited for identification of neutralization epitopes. A single, major, immunodominant neutralizing epitope has been identified within the third variable (V3) region of HIV-1 (7, 18, 23, 26, 31), which is strictly type specific. Antibodies from infected humans possess broadly group-specific neutralizing activity and are able to neutralize a wide variety of HIV-1 isolates (36). While apparent group-specific reactivity may arise from a collection of type specificities directed against the V3 domain, it is also possible that additional neutralizing epitopes exist elsewhere in the

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envelope. Several studies, in fact, have pointed to alternate epitopes (1, 10, 11, 13, 33). In general, these additional sites have elicited neutralizing antibodies of very low titer. Recent work has described a region in the transmembrane protein able to elicit antibodies with broader neutralizing activity when presented as a poliovirus chimera (4). This finding awaits independent confirmation.

Neutralization by antibody is an ill-defined term, signifying a variety of possible events. A block of viral infectivity can occur during either binding or postbinding processes. Depending on detection methods, apparent neutralization could also result from fusion inhibition or a block in virus assembly or release. Therefore, an in vitro immune-selection system can use natural antibody interactions to identify several viral envelope regions which may be involved in important functions.

We previously showed that the molecular basis for the neutralization resistance of our in vitro immune-selected variant was the substitution of threonine for alanine at position 582 in the transmembrane protein (27). We have subsequently examined the biologic, immunologic, and conformational properties of the variant more fully in order to understand how a change in this region would allow neutralization escape. Experiments were designed to determine whether amino acid 582 lies within a neutralization epitope or whether its substitution was responsible for neutralization resistance at a distant site.

MATERIALS AND METHODS

Cells and viruses. The infectious molecular clone of HIV-1, designated HXB2D, served as the parental virus and has been previously described (5). MXB2 is HXB2D with the majority of the *env* gene substituted with the *env* gene of the immune-selected variant, MX (27). Except as noted in the Results section, we used HXB2Dthr582, a viral construct prepared by oligonucleotide-directed mutagenesis, as the variant virus. This construct is identical to the cloned variant MX with respect to neutralization properties and proved that the original neutralization resistance to the selecting serum observed with the variant selected in vitro was the result of a point mutation of alanine to threonine at position 582 of the envelope protein (27). Additional viral constructs with alterations in the 582 region were similarly prepared, transfected into COS-1 cells, and subsequently transmitted to H9 cells (25) for in vitro propagation as previously described (27). Fresh supernatant media from producing cultures were used as the source of virus in all neutralization assays. CEM-SS cells (21) were used as targets in syncytium inhibition assays. All cell cultures were propagated in RPMI 1640 containing 1 mM glutamine, 10% fetal calf serum, and penicillin/streptomycin (R10).

Immunologic assays. Neutralizing antibody assays were carried out essentially as described previously (28). Briefly, serum samples were diluted 1:10 in R10 and 20 μl of threefold serial dilutions was incubated with 20 μl of cell-free virus preparations for 30 min at 4°C. Polybrene-treated H9 cells (10 μl of 4×10^6/ml) were added, and the plates were incubated at 37°C for 1 h. Portions (15 μl) of each reaction mixture were plated in duplicate into wells of a microdilution plate containing 200 μl of R10 and were cultured at 37°C in a 5% CO_2 atmosphere. Cells were harvested onto toxoplasmosis slides and fixed in 50% methanol–50% acetone for immune fluorescence assay at day 7 and every 2 or 3 days thereafter. Neutralizing antibody titers were determined within 1 to 2 weeks when 50 to 80% of target cells in the presence of normal human serum were infected. Viral infectivity was assessed by p24 expression with a monoclonal antibody to HIV-1 p24 (2) and fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin G in an immune fluorescence assay (28). Percentages of cells expressing p24 in the presence of test serum were normalized to values obtained in the presence of normal control serum. Neutralizing antibody titer of the test serum was then expressed as the reciprocal of the test serum dilution at which 60% of the H9 target cells were p24 positive.

Neutralizability of viral constructs was determined as previously described (27) with 1:10 dilutions of either normal control serum, the original immune-selecting serum from a healthy homosexual male (30), or a second neutralizing antibody-positive human serum capable of neutralizing both the parental and variant viruses. Neutralization resistance of viral constructs was defined as the ability to infect H9 cells in the presence of the immune-selecting serum with kinetics of infection and infectivity levels similar to those obtained in the presence of normal control serum as was previously reported for the HXB2Dthr582 construct (27). Constructs were determined to be neutralization sensitive if they behaved like the parental HXB2D virus and were completely neutralized by the immune-selecting serum.

Syncytium inhibition assays were carried out by incubating 25 μl of 1×10^5 to 4×10^5 virus-infected cells per ml with 25 μl of a 1:10 dilution of serum in 0.5-volume 96-well microdilution plates for 30 min at room temperature. CEM-SS cells (50 μl of 5×10^5/ml) were added, and the mixtures were incubated overnight at 37°C in a 5% CO_2 environment. Syncytia were counted by using an inverted microscope.

Synthetic peptides were solubilized and filter sterilized before use in immunologic assays. They were added to infectivity assays or syncytium formation assays prior to the addition of target cells. Their effect on serum neutralization or syncytium inhibition was assessed by first incubating the peptide with the test serum for 30 min at room temperature and then adding the additional components of the assay.

Sera. Human sera used to determine the frequency of neutralization of the parental and HXB2Dthr582 viruses were obtained from HIV-1 antibody-positive healthy male homosexuals or thalassemia patients (29). Sera used in affinity purification experiments were from eight seropositive individuals; seven were healthy, and one had peripheral generalized lymphadenopathy. Hyperimmune rabbit sera were prepared by an injection of 0.1 mg of keyhole limpet hemocyanin-coupled peptide. The sera were obtained following the eleventh immunization. All serum samples were heat inactivated at 56°C for 30 min prior to use in immunologic assays.

Synthetic peptides. Peptide 576b was synthesized by Peninsula Laboratories, Inc. (Belmont, Calif.). All other peptides were synthesized, purified, and characterized as previously described (14). The sequences are shown in Fig. 2. Two different control peptides, both free and resin bound, were used and had the following jumbled sequences of peptide 574: QLVRALQIKAEQRYKQDLL and AEKDL YIAQRQLKLVQIRL. Neither of these peptides reacted with HIV-1 antibody-positive sera in solid-phase enzyme-linked immunosorbent assays (ELISAs) or bound HIV-1 antibodies in affinity purifications. Therefore, results obtained with only one of them are reported (see Table 4).

For affinity purification of human antibodies, six human HIV-1 antibody-positive serum samples (200 μl) were inactivated with 0.5% Triton X-100 and purified on polystyrene

resins (20 mg) to which 10 mg of the various synthetic peptides were left coupled through phosphoamidite bonds after synthesis. Following overnight incubation at 4°C, the resins were washed twice with phosphate-buffered saline containing 4% bovine serum albumin and twice with phosphate-buffered saline only. Bound antibodies were eluted with phosphate buffer (pH 2.9) and were neutralized to pH 7.4. Two additional human serum samples were not detergent inactivated but were simply affinity purified as described above on peptide resins. Bound antibodies were eluted at either pH 2.7 or pH 2.5. Purified antibodies were tested for binding to specific peptides by ELISA as previously described (14) and to *env* gene products gp160, gp120, and gp41 by electroimmunoblotting. Although the original sera and wash fractions had a full electroimmunoblotting pattern, *gag* and *pol* bands were not detected in the eluates from resin with peptide 574. Eluates from resins with random peptides were not reactive. The recovery of anti-peptide and anti-gp41 activity determined by titration ranged from 1 to 15% (data not shown).

The ability of sera or affinity-purified antibodies to recognize native envelope proteins was tested in a dot blot assay. Purified recombinant gp160 from Vero cells infected with vaccinia carrying an HIV-1 *env* gene insert was a generous gift of Friedrich Dorner, Immuno, Vienna, Austria. This native gp160 in Tris hydrochloride buffer, (pH 8.3) containing 0.9% NaCl and 5% sucrose, was spotted onto nitrocellulose (1 μg per spot) and dried. Following blocking of the nitrocellulose sheet with BLOTTO (12a), the spots were reacted overnight at 4°C with 1:10 dilutions of the preimmune or hyperimmune rabbit sera or with affinity-purified antibodies. Following washing, the nitrocellulose was reacted for 30 min at room temperature in BLOTTO containing 5×10^5 cpm of iodinated protein G per ml (2.3×10^8 cpm/μg), washed, and autoradiographed.

Construction of mutants. The viral mutants were constructed by a variety of methods for oligonucleotide-directed mutagenesis and confirmed by dideoxy sequencing (32). Substitution of Thr-582 (to make the TVERY mutant) for Ala-582 (the AVERY parental form) has been previously described (27), and involved mutagenesis of a *Sal*I-*Bam*HI 2.7-kilobase (kb) envelope-containing fragment (Fig. 1) in M13 phage by the method of Zoller and Smith (38). For other mutagenesis experiments with M13, a 1.3-kb *Bam*HI-*Pvu*II fragment bearing the TVERY mutation was coligated into the *Bam*HI-*Eco*RI site of M13mp18 with a linker consisting of two partially complementary oligonucleotides, 5′-AAT TCGAGCTCGGTACCCGGGCAG-3′ and 5′-CTGCCCGGG TACCGAGCTCG-3′. This construct was termed M13-PB. The Gly-582 mutant (GVERY) was made with the M13-PB with an antisense mutagenic oligomer 5′-CTTTCCACAC CCAGGATTCTTGC-3′ as described previously (27). The mutant in which Val-583 and Arg-585 were replaced by Ile and Lys, respectively, (to make the AIEKY mutant) was generated by the gapped heteroduplex method of Inouye and Inouye (12) using as a starting material the 2.7-kb *Sal*I-*Bam*HI fragment of pHXB2D in the plasmid vector pGEM4 (Promega Biotec, Madison, Wis.). Briefly, as outlined in Fig. 1, a 3.0-kb *Stu*I-*Bam*HI fragment and a 4-kb *Xmn*I-*Bgl*I fragment were isolated and annealed to form a gapped heteroduplex circle. Mutagenesis was performed with the sense mutagenic oligomer 5′-CCAGGCAAGAAT CCTGGCTATCGAAAAATACCTAAAGGATCAACAG-3′. Mutants were selected by several rounds of differential screening with a shorter version of the mutagenic oligomer and retransformation. The 2.7-kb *Sal*I-*Bam*HI mutagenized

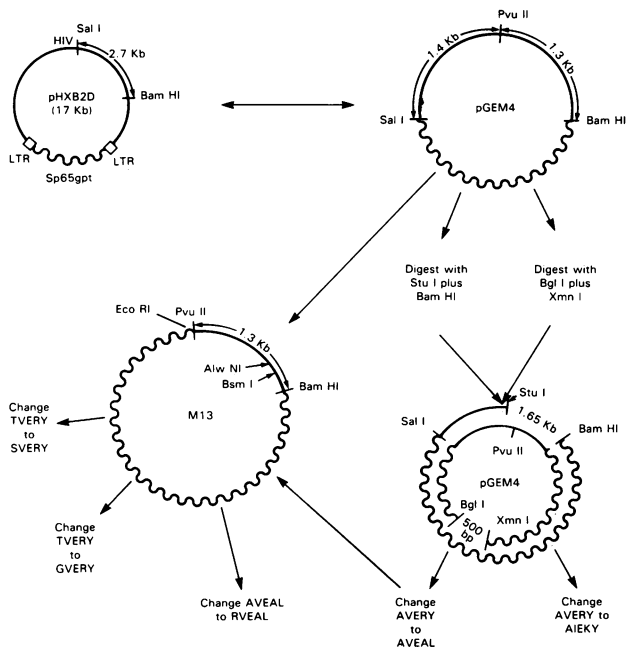

FIG. 1. Construction of viral mutants. Mutations were introduced into the biologically active molecular clone pHXB2D as described in Materials and Methods. As shown, the source for mutagenized fragments was the 2.7-kb *Sal*I-*Bam*HI *env*-containing fragment from either the wild-type pHXB2D, placed into the plasmid vector pGEM4, or the TVERY mutant previously described, placed into M13. LTR, Long terminal repeat. Plasmid sequences are represented as wavy lines. The AIEKY and AVEAL mutants were made by the gapped heteroduplex method, which involved digesting separately the pGEM4 *Sal*I-*Bam*HI construct with *Stu*I plus *Bam*HI and *Bgl*I plus *Xmn*I, annealing the large fragments to give the gapped heteroduplex shown, and performing the indicated mutagenesis. The 1.3-kb *Pvu*II-*Bam*HI AVEAL mutant fragment was placed into M13 as described, mutagenized to RVEAL, and put back into pHXB2D. The GVERY mutant was made from the TVERY 1.3-kb *Pvu*II-*Bam*HI fragment placed into M13 with a linker and then put back into pHXB2D. The SVERY mutant was made the same way, except that a 150-base-pair (bp) *Alw*NI-*Bsm*I fragment was first put back into the pGEM4 vector containing the 2.7-kb *Sal*I-*Bam*HI fragment, which in turn was substituted into pHXB2D. All final constructs were verified by direct DNA sequencing.

fragment was substituted into the pHXB2D plasmid to generate a biologically active mutant clone. The mutant in which Arg-585 and Tyr-586 were replaced with Ala and Leu, respectively (to make the AVEAL mutant), was generated in a similar fashion, except that the mutagenic sense oligomer was 5′-CCAGGCAAGAATCCTGCGTGTGGAAGCGCT GCTAAAGGATCAACAGCTC-3′. Differential screening was again used to select mutants, but DNA sequencing of the mutant revealed that only the GCGCTG mutation was present and that the other two mutations were lacking. To create the mutant in which, besides the Arg-Ala and Tyr-Leu substitutions, Ala-582 was replaced with Arg (to make the RVEAL mutant), the 1.3-kb *Pvu*II-*Bam*HI fragment with the AVEAL mutation was placed into M13mp18 and the additional mutation was introduced with an antisense mutagenic oligomer (5′-CGCTTCCACACGCAGGATTCTTGCCTGG-3′) exactly as described for the construction of the GVERY mutant. Both of these were then subcloned back into HXB2D. First, the replicative form of M13 was prepared and the 1.3-kb *Pvu*II-*Bam*HI fragment was isolated. This frag-

TABLE 1. Effect of alanine-to-threonine substitution at position 582 on neutralization and syncytium inhibition

Viral construct	Neutralization of cell-free virus infection[a]		Inhibition of syncytium formation[b]	
	Selecting serum	Second positive serum	Selecting serum	Second positive serum
HXB2D	+	+	+ (46)	+ (59)
MXB2	−	+	− (209)	− (146)
HXB2Dthr582	−	+	− (153)	− (113)

[a] From references 27 and 30.
[b] Average number of syncytia in control cultures was 86 (HXB2D), 62 (MXB2), and 48 (HXB2Dthr582). Values in parentheses represent syncytia as percentage of control in the presence of normal human serum.

ment was then coligated with a *Sal*I-*Pvu*II fragment from HXB2D into HXB2D in which the 2.7-kb *Sal*I-*Bam*HI fragment was deleted.

The Ser-582 mutant (SVERY) was prepared from M13-PB (the 1.3-kb *Pvu*II-*Bam*HI fragment of the TVERY mutant) as described above for the GVERY mutant, except that the antisense mutagenic primer was 5′-GGTATCTTTCCACT GACAGGATTCTTGC-3′. Mutagenized SVERY clones were identified by differential screening, and a 1.3-kb fragment was obtained from the single-stranded mutagenized M13 DNA by the polymerase chain reaction by using a thermal cycler and GeneAmp kit (Perkin Elmer Cetus) according to the instructions of the manufacturer. The primers used in the amplification were the M13 universal reverse primer (New England BioLabs, Inc., Beverly, Mass.) and 5′-CCTTGGGTTCTTGGGAG-3′. A 0.15-kb *Alw*NI-*Bsm*I fragment which encompassed the mutation was excised from the polymerase chain reaction product, isolated on a gel, and substituted into the same region of the 2.7-kb *Sal*I-*Bam*HI fragment of pHXB2D in pGEM4. The mutant *Sal*I-*Bam*HI fragment was then substituted into pHXB2D to form the biologically active mutant clone (Fig. 1).

RESULTS

HXB2Dthr582 resists syncytium inhibition by immune-selecting serum. Our earlier finding that a point mutation occurring at position 582 within the transmembrane protein of HIV-1 was responsible for neutralization resistance of the immune-selected variant was unexpected. A change in the external surface glycoprotein, gp120, the site of the major immunodominant neutralizing epitope, had been anticipated. Therefore, it was of interest to examine more fully the biologic consequences of the mutation at position 582. This alteration occurs near the putative fusion region (residues 512 through 527) of the transmembrane protein (16), so we first examined the ability of the parental and variant viruses to form syncytia. The viruses readily form syncytia (Table 1); however, the escape mutant was resistant to syncytium inhibition by the immune-selecting serum as well as to neutralization. Results were similar regardless of whether the variant construct, MXB2, in which the variant envelope was substituted for the envelope of HXB2D, or the variant generated by site-directed mutagenesis, HXB2Dthr582, was used. Syncytium formation was in fact enhanced over that observed in the presence of normal control serum for both variant viruses in the presence of two different neutralizing sera. While the variants were resistant to both neutralization and syncytium inhibition by the selecting serum, a second antibody-positive serum was able to neutralize the variants but did not inhibit syncytium formation. This observation illustrates that epitopes responsible for neutralization and syncytium inhibition are distinguishable.

Alanine-to-threonine point mutation is distinguished by 30% of neutralizing sera. Before investigating further the biological basis for the observed neutralization resistance of the HXB2Dthr582 variant, we investigated the frequency with which the variant might escape neutralization by antibodies generated during natural infection in humans. Serum samples from 37 HIV-1-positive individuals, including the immune-selecting serum, which were able to neutralize HXB2D were assayed for the ability to neutralize HXB2Dthr582. The results are presented in Table 2. Of the 37 serum samples, 26 possessed neutralization titers which were similar on both viruses. Eleven (30%) of the serum samples, however, were unable to neutralize the variant or did so with substantially reduced titers. The mean titer on HXB2D of these latter serum samples was higher than that of the former group, suggesting that antibodies unable to neutralize the variant virus have greater type specificity for the parental virus. The fact that one-third of the serum samples tested had decreased ability to neutralize the variant suggests that the epitopic change which occurred in the variant is generally relevant and not peculiar to the single neutralizing serum used in the original immune-selection process.

Competition studies with synthetic peptide. To investigate the biological basis for the observed neutralization resistance, we initially asked whether the 582 transmembrane region itself constituted a neutralization epitope. To answer this question, we synthesized a 19-amino-acid-long peptide, 576b, encompassing the 582 region (Fig. 2). Experiments were carried out to determine whether this peptide could inhibit directly cell-free or cell-associated virus infection and whether it could compete with infectious virus in neutralization and syncytium inhibition assays. The presence or absence of peptide 576b had no effect on the interaction of

TABLE 2. Decreased ability of one-third of sera which neutralize HXB2D to neutralize HXB2Dthr582[a]

Serum type	No. of sera/total tested (%)	Neutralizing antibody titer			
		Range		Mean ± SEM	
		HXB2D	HXB2Dthr582	HXB2D	HXB2Dthr582
With equivalent titers	26/37 (70.3)	10–440	0–500	94 ± 25	75 ± 25
To which HXB2Dthr582 is more resistant	11/37 (29.7)	20–1,250	0–95	240 ± 112	17 ± 9

[a] Sera to which HXB2Dthr582 was judged to be more resistant possessed neutralizing antibody titers on the variant virus which were at least fivefold lower than titers on HXB2D. Sera were also included in this group if they were neutralizing antibody negative on HXB2Dthr582 and possessed a titer of at least 20 on HXB2D.

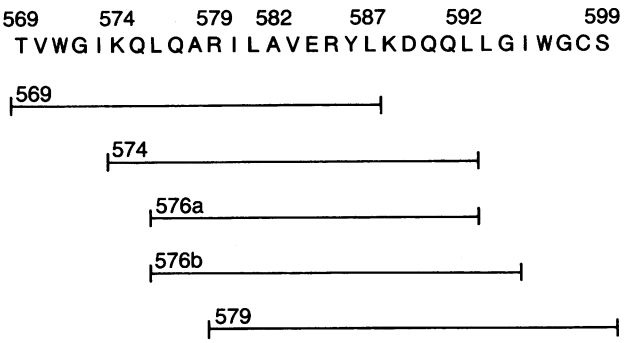

FIG. 2. Synthetic peptides used for analysis of the 582 region as a neutralizing epitope. Peptide 576b was used for direct competition experiments. Peptides 576a and 579 were used to prepare hyperimmune rabbit sera. Peptides 569, 574, and 579 and a peptide comprised of the amino acids of peptide 574 in random sequence were used for affinity purification of human sera.

the parental or variant viruses with target cells or antibodies (Table 3). Cell-free infectivity levels of both viruses were the same regardless of the presence of the peptide. Similarly, HXB2D was neutralized by the immune-selecting serum and HXB2Dthr582 was resistant whether the peptide was present or not. Peptide 576b also failed to influence cell-associated infectivity of either virus. Again, syncytium inhibition of HXB2D by the immune-selecting serum and the relative resistance to this phenomenon by the variant were not influenced by the peptide.

The 582 region peptides do not elicit neutralizing antibody. To further investigate the 582 region as a possible neutralizing epitope, hyperimmune sera were prepared in rabbits against peptides 576a and 579 (Fig. 2) coupled to keyhole limpet hemocyanin. While the antisera were able to bind to the immunizing peptide with a titer from 1:1,000 to 1:10,000 in ELISAs, they failed to neutralize either the parental or variant virus (data not shown).

Hyperimmune sera prepared against synthetic peptides may not recognize peptide epitopes in their native conformation, and in fact, we were unable to demonstrate binding of the rabbit sera to native gp160 in a dot blot assay. However, HIV-infected individuals develop antibodies to native viral proteins expressed in vivo and would be expected to recognize those peptides accessible in the native state. Therefore, we affinity purified human antibodies on resins complexed with peptides 569, 574, and 579 and a control peptide consisting of the amino acids of peptide 574 in a random sequence (Fig. 2). That human sera possess such antibodies to peptides in the 582 region is illustrated in Table 4, in which the affinity-purified antibodies are shown able to bind in an ELISA to peptides 574 and 579 but not to peptide 569 or control peptide 574 in random sequence. Moreover, when purified antibodies from sera E through H were tested in a dot blot assay, direct binding to native gp160 by three of the six positive affinity-purified antibodies tested was demonstrated. Those able to bind to the native protein were purified in the absence of detergent and included sample G purified on resin 574 and sample H purified on resins 574 and 579 (data not shown). Compared with the eight starting serum samples, all of the purified antibodies possessed a 10-fold-lower binding capacity to one or more of the 582 region peptides. While the starting serum samples neutralized the parental virus with titers ranging from 20 to 245, none of the affinity-purified antibodies were able to neutralize the infectivity of either HXB2D or HXB2Dthr582 (Table 4). Thus, although these experiments confirm that this region of gp41 is immunogenic (35), as all eight serum samples

TABLE 4. Failure of affinity-purified human antibodies to neutralize HXB2D and HXB2Dthr582[a]

Serum	Peptide resin	Binding to peptide	Neutralizing antibody titer on: HXB2D		HXB2Dthr582	
			Whole serum	Affinity purified	Whole serum	Affinity purified
A	569	NB[b]	120		90	
	574	NB				
	574R	NB		—[c]		—
	579	579		—		—
B	569	NB	20		—	
	574	574		ND		ND
	574R	NB		—		—
	579	574		—		—
C	569	NB	80		15	
	574	NB				
	574R	NB		—		—
	579	574, 579		—		—
D	569	NB	110		30	
	574	NB				
	574R	NB		—		—
	579	579		—		—
E	574R	NB	105	—	20	—
	579	574, 579		—		—
F	574R	NB	245	—	85	—
	579	574, 579		—		—
G	574	574, 579	160	—	115	—
	574R	NB		—		—
	579	574, 579		—		—
H	574	574, 579	ND	—	ND	—
	574R	NB		—		—
	579	574, 579		—		—

[a] Neutralization assays were carried out as described in Materials and Methods with threefold serially diluted affinity-purified antibodies beginning at a dilution of 1:4 or 1:10 or unfractionated serum beginning at a dilution of 1:10. 574R represents a peptide in which the amino acids of the 574 peptide are arranged in random sequence as detailed in Materials and Methods. Sera G and H were affinity purified without prior Triton X-100 inactivation. ND, Neutralization assay not done.

[b] NB, No binding.

[c] —, No detectable neutralizing activity.

TABLE 3. Effect of peptide 576b on cell-free and cell-associated infectivity, neutralization, and syncytium inhibition[a]

Peptide 576b	Infectivity (% + cells)		Neutralization by selecting serum (% + cells)		No. of syncytia formed		Syncytium inhibition by selecting serum (% control)	
	HXB2D	HXB2Dthr582	HXB2D	HXB2Dthr582	HXB2D	HXB2Dthr582	HXB2D	HXB2Dthr582
+	82	74	0	83	49	49	2	19
−	87	77	6	63	37	31	0	23

[a] Final concentration of 576b peptide used in the various assays was as follows: cell-free infectivity, 115 μg/ml; serum neutralization, 160 μg/ml; cell-associated transmission, 200 μg/ml; syncytium inhibition, 160 μg/ml.

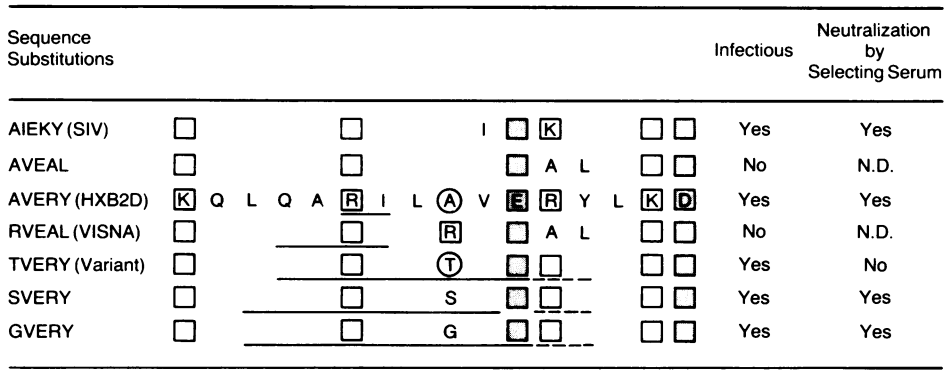

FIG. 3. Effect of alterations in 582 region on predicted secondary structure, infectivity, and neutralizability. The sequence of the parental HXB2D virus is listed from amino acid 574 to amino acid 589. Predicted secondary structure is indicated by solid underline for beta sheet, dashed line for beta turn, and no underline for alpha helix. Substituted amino acids are indicated. Positively charged amino acids are within open squares, and negatively charged amino acids are within shaded squares. The alanine-to-threonine substitution at position 582 is marked by open circles. N.D., Not determined.

tested possessed binding activity to one or more of the synthetic peptides, it does not elicit neutralizing antibody.

Effects of predicted secondary structural alterations on neutralizability. As the experiments with the synthetic peptides indicated that the 582 region did not itself constitute a neutralizing epitope, we further analyzed this region by using computer algorithms to predict the local secondary structure. We previously observed that the alanine-to-threonine substitution at position 582 might alter the predicted alpha-helical conformation of the parental virus to that of a beta strand with a beta turn in the variant (27). To further explore the consequences of secondary structural alterations on neutralizability, we prepared viral constructs in which the secondary structure was predicted to resemble more that of the variant virus. Figure 3 summarizes the viral constructs prepared by site-directed mutagenesis. The 582 region is highly conserved among HIV-1 isolates and is also conserved among lentiviruses in general. Therefore, in addition to preparing two constructs predicted to possess secondary structure very similar to that of the variant (SVERY and GVERY), we included analyses of viruses in which the AVERY sequence of HXB2D was substituted with amino acids found in the same region of the simian immunodeficiency virus (AIEKY) and visna virus (RVEAL). Figure 3 lists the constructs, beginning with those predicted to possess predominantly alpha-helical structure in this region and proceeding to those with increasing predicted propensity to form a beta sheet with beta turns. The parental virus HXB2D is predicted to form predominantly an alpha helix in the AVERY region, while the variant virus is predicted to possess a beta sheet configuration with a beta turn, as are the SVERY and GVERY constructs. The AIEKY simian immunodeficiency virus construct and the AVEAL construct, possessing only two of the amino acid substitutions needed to complete the visna sequence in this region, are predicted to be primarily alpha helical. Each of the constructs was transfected into COS-1 cells and was subsequently cocultured with H9 cells in order to transmit virus and establish a permanently producing cell line. In contrast to the five other constructs, both the visna RVEAL and the partial visna AVEAL constructs failed to yield productive viral infections following repeated coculture attempts. We were not able to detect any viral infectivity of the target H9 cells. The RVEAL substitution results in a shift in the position of a positively charged amino acid, and the AVEAL substitution results in the loss of a positively charged amino acid in this region (Fig. 3). Both the AVEAL and RVEAL constructs are predicted to have altered hydrophilicity profiles (17) in this region compared with that of the parental AVERY virus, the TVERY variant, and the other three constructs (data not shown). In addition, plots of hydrophobic moment, which measures the amphiphilicity of alpha-helical regions (3), are predicted to be markedly different for the AVEAL and RVEAL constructs, compared with that of the parental virus. The AIEKY construct, also predicted to be predominantly alpha helical with a hydrophobic moment plot much more similar to that of the parental virus, is infectious (Fig. 3). The failure of the RVEAL and AVEAL constructs to yield productive infections thus suggests that amphiphilicity and hydrophilicity profiles within the 582 region must be rigidly maintained in order to allow viral infectivity.

The five infectious viral constructs were tested subsequently for neutralizability by the original immune-selecting serum and additional control positive and negative sera. All of the constructs, except the variant with the TVERY sequence, were sensitive to neutralization by the selecting serum (Fig. 3). Representative data showing the neutralization resistance of the variant virus with the TVERY sequence compared with the neutralization sensitivity of the other infectious viruses, including the parental virus with the AVERY sequence and the AIEKY, GVERY, and SVERY constructs, are presented in Fig. 4.

DISCUSSION

We previously established that a point mutation in the *env* gene of the infectious molecularly cloned virus HXB2D, resulting in the alteration of an alanine residue to a threonine residue at position 582 within the transmembrane protein, was the molecular basis for immune selection of a neutralization-resistant variant (27). We have now explored the biologic and immunologic bases for the observed resistance. Elucidation of the mechanisms contributing to the phenomenon is important, not only for understanding the generation of a single escape mutant but also for our further understanding of the overall functioning of the HIV-1 envelope protein. As we show here, the point mutation affects syncytium inhibition as well as neutralization. Moreover, we found that one-third of serum samples able to neutralize the HXB2D virus are significantly less able to neutralize the variant.

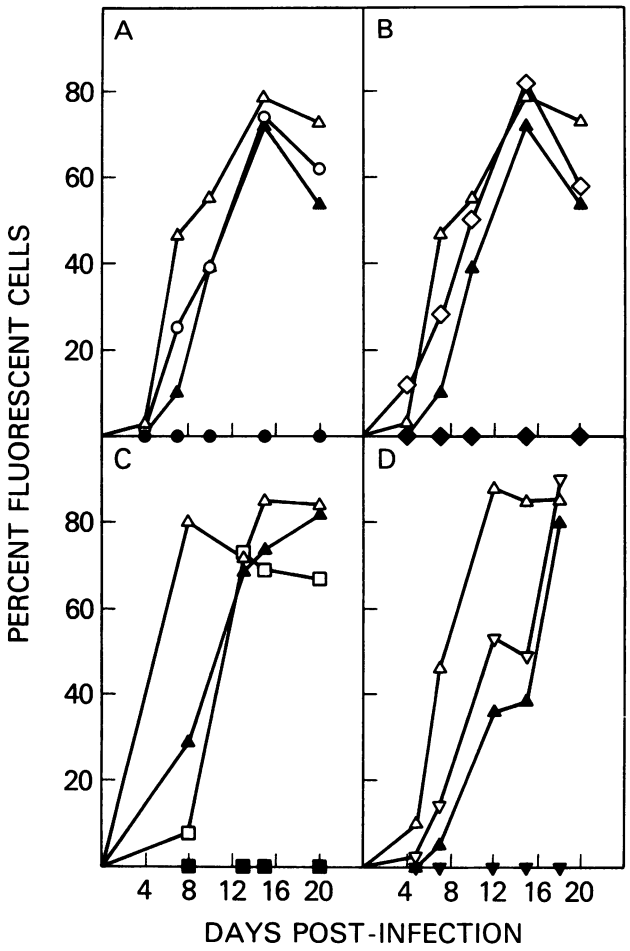

FIG. 4. Neutralizability of parental and variant viruses and mutant viral constructs. Viruses were tested for neutralization resistance or sensitivity in the presence of the original immune-selecting serum as described in Materials and Methods. Kinetics of infection over 3 weeks of culture are illustrated for viruses in the presence of normal human control serum (open symbols) or the immune-selecting serum (filled symbols). Panel A compares the parental HXB2D virus (○, ●) with the variant HXB2Dthr582 virus (△, ▲). In the remaining panels, we compare the variant virus with the AIEKY construct (◇, ◆) (B), the GVERY construct (□, ■) (C), and the SVERY construct (▽, ▼) (D). Neutralizability of all viral constructs was always analyzed in experiments including the parental and variant viruses as controls and a second neutralizing serum capable of neutralizing both. For simplicity, we have not included all of these control data in the figure.

Therefore, the change(s) occurring as a result of the alanine-to-threonine substitution is distinguishable by a significant proportion of natural antibodies. Finally, as indicated by the failure of two of our constructs to productively infect H9 cells, the overall conformation of the region is crucial for viral infectivity. This is not unexpected, as this region of the transmembrane protein is highly conserved, not only among HIV-1 isolates but also among lentiviruses in general, emphasizing its functional importance. In this regard, Gallaher et al. (6) have recently proposed a model for the HIV-1 envelope protein in which the 582 region comprises part of an extended amphipathic alpha-helical portion of the transmembrane protein which may play a role in maintaining appropriate interactions of the gp41 with gp120. In fact, Kowalski et al. (16) earlier pointed out that mutations near the 582 region (at amino acids 530, 537, and 640) abrogate association of gp41 with gp120.

In order to determine the structural or immunologic basis for the observed neutralization resistance of the variant virus, we first asked whether the 582 region by itself constituted a neutralization epitope. We addressed this question by using several synthetic peptides corresponding to overlapping segments within this region in three different approaches. The results indicate that the 582 region is not by itself a neutralizing epitope.

First, a synthetic peptide corresponding to the 582 region failed to compete in viral infectivity studies, either by cell-free or cell-associated virus, showing that the region represented by the peptide was not directly involved in infection. Nor did the peptide interfere with serum neutralization of cell-free virus infectivity or with serum inhibition of syncytium formation by cell-associated virus. Interpretation of these competition experiments is limited by the possibility that failure of the 576b peptide to compete may be the result of its failure in solution to assume the conformation existing in the native protein. Therefore, the additional approaches described below were carried out. We also considered that, even if the synthetic peptide possessed a configuration which allowed binding, the binding affinity might be low. The gp41 protein tends to form oligomers (24) which in the virus particle might bind antibody more avidly than do the monomeric fragments of competing peptide in solution. This possibility seems remote, as the competing peptide was added at a vast molar excess compared with viral gp41 and should have overcome any failure to compete because of low affinity. On the basis of protein determinations of concentrated and purified viral preparations, we generously estimate the concentration of viral protein in our cell-free culture supernatant to be approximately 1 μg/ml. Of this, 0.1% is attributed to gp120 and gp41 (9), for a concentration in preincubation mixtures of 2.1 pM envelope protein. The competing peptide was added at a concentration of 63 μM, or a $>10^7$-fold excess.

Second, antisera were raised against two different synthetic peptides corresponding to the 582 region. While these rabbit sera readily bound synthetic peptides in ELISAs, they were not able to neutralize virus infection.

Third, we affinity purified human sera and tested the purified antibodies eluted from synthetic peptide resins for their ability to neutralize the parental and variant viruses. The rationale was that antibodies elicited by proteins in their native conformation, as found in virus particles and infected cells in vivo, might possess specificities nonexistent in hyperimmune sera generated against synthetic peptide in solution. We showed that HIV-1-seropositive human sera possessed antibodies capable of reacting with peptides of the 582 region; however, none of these affinity-purified antibodies, including those demonstrably able to bind native gp160, were able to neutralize virus infectivity. Thus, this series of experiments clearly demonstrated that the 582 region does not by itself constitute a neutralizing epitope.

As a simple epitope alteration cannot explain the neutralization resistance of our immune-selected variant, we considered other possibilities. Synergistic antibodies, one binding the 582 region and the second binding an alternate site, might be required in order to achieve neutralization. This possibility can be ruled out in our system by the competition experiments with the 576b peptide. A vast molar excess of peptide would abolish binding to the 582 region, thus block-

ing any neutralization involving a second synergistic antibody as well. No such inhibition occurred (Table 3).

Blocking antibodies present in the immune-selecting serum and recognizing the TVERY region of the variant might also explain the neutralization resistance. We did not attempt competition experiments with a peptide encompassing the TVERY sequence, however. In separate studies, we have found that only a small percentage of sera which bind to peptides with the AVERY sequence bind to TVERY analogues (Klasse et al., unpublished data). Thus, the possibility of blocking antibodies to this region competing with neutralizing antibodies specific for the analogous region in the parental virus is unlikely.

Finally, conformational models can be suggested to explain the neutralization resistance of our immune-selected variant. The first proposes that the alanine-to-threonine substitution at position 582 causes a conformational change within the viral envelope, resulting in the alteration of a neutralization epitope at a distant location. The second requires that position 582 itself is a contact point in a noncontiguous conformational epitope.

We examined the first possibility by constructing viral variants which were predicted to possess local secondary structure similar to that of either the parental or variant virus. These experiments revealed that the structure of the 582 region is crucial for viral infectivity, as two constructs with significant changes in charge distribution were not infectious. The highly conserved nature of the 582 region underscores its functional importance, a conclusion emphasized here by the results of our infectivity studies. Unexpectedly, both viral constructs with secondary structure predicted to be highly similar to that of the variant virus were sensitive rather than resistant to neutralization by the immune-selecting serum. Thus, we could not show that a putative local beta sheet conformation is important for resistance to neutralization by the immune-selecting serum. We can conclude, however, that the structural requirements within this region which allow neutralization escape are very precise. Protein structure can be determined accurately only by X-ray crystallographic analysis or by nuclear magnetic resonance studies. Therefore, predictions concerning the secondary structure of the 582 region must be viewed with caution. The precise contribution of conformation in this region to neutralizability must remain open.

The second model invoking a noncontiguous neutralizing epitope is not directly testable with information currently available. In this model, peptides corresponding to the 582 region would not be expected to compete with infectious virus for neutralizing antibody sites. The native conformation of position 582 together with one or more additional viral sites would be expected to possess significantly greater binding affinity for neutralizing antibodies specific for a conformational epitope compared with a single amino acid or epitopic fragment. Similarly, anti-peptide antibodies in hyperimmune sera would not be elicited against the entire noncontiguous epitope. Moreover, affinity-purified human antibodies, selected on specific peptide resins, would also be expected to possess low affinity for native noncontiguous epitopes. Therefore, our experiments with synthetic peptides do not appropriately examine this model. The fact, however, that by using several viral constructs we observed relatively broad flexibility in the particular amino acids which can reside at position 582, including A, S, and G, and still allow recognition by neutralizing serum, suggests this particular residue is not of major importance as part of a noncontiguous epitope. Therefore, we conclude that the most likely mechanism conferring neutralization resistance in our variant is a conformational change, altering a neutralizing epitope at a distant location.

We can merely speculate as to the identity of the affected epitope, which we can characterize only as outside the immediate 582 region. In this regard, we have earlier reported that the 0.5β monoclonal antibody (18) which has been mapped to amino acids 301 to 324, an immunodominant region of gp120 which elicits type-specific neutralizing antibody, neutralizes both the parental and variant viruses. This suggests that another epitope critical for neutralization by at least one-third of HIV-1-seropositive individuals is involved.

We have not investigated here one final possibility that the neutralization resistance of the variant is due to changes in the quaternary structure of the envelope protein. Pinter et al. (24) have shown that gp41 tends to oligomerize, forming mainly trimers or tetramers. The substitution of threonine for alanine in our variant might result in slight changes in the interactions between the gp41 subunits, resulting in shielding of a normally exposed epitope. This question awaits future experimentation.

In designing effective vaccine preparations, it is essential to thoroughly understand the functionally and immunologically important regions of the virus envelope. Because HIV-1 exhibits such genetic heterogeneity allowing the possibility of immune selection, regions of the virus important in maintaining virus conformation and hence antigenic integrity must be elucidated. In this study, we have shown that a region of the virus which is not itself a neutralizing epitope can have profound effects on the ability of a variant virus to be propagated in the presence of an immune serum. Study of additional HIV-1 variants immune selected in vitro should be helpful in elucidating additional viral regions important in infectivity and neutralization.

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