

A Human Monoclonal Antibody to a Complex Epitope in the V3 Region of gp120 of Human Immunodeficiency Virus Type 1 Has Broad Reactivity within and outside Clade B

JOHN P. MOORE,^{1*} ALEXANDRA TRKOLA,¹ BETTE KORBER,² LYNN J. BOOTS,³
JOSEPH A. KESSLER II,³ FRANCINE E. McCUTCHAN,⁴ JOHN MASCOLA,⁴ DAVID D. HO,¹
JAMES ROBINSON,⁵ AND ANTHONY J. CONLEY³

Aaron Diamond AIDS Research Center, New York University School of Medicine, New York, New York 10016¹; Los Alamos National Laboratory, Los Alamos, New Mexico 87545²; Merck Research Laboratories, West Point, Pennsylvania 19486³; Henry M. Jackson Foundation for Military Medicine and Division of Retrovirology, Walter Reed Army Institute of Research, Rockville, Maryland 20850⁴; and Department of Pediatrics, University of Connecticut Health Center, Farmington, Connecticut 06030⁵

Received 8 September 1994/Accepted 17 October 1994

We have used virus neutralization and antibody-binding techniques to define the epitope for a human monoclonal antibody, designated 19b, within the V3 region of the gp120 surface glycoprotein of human immunodeficiency virus type 1. Unusually, the 19b epitope encompasses residues on both flanks of the V3 loop. However, 19b binding to gp120 is independent of sequences at the crown of the V3 loop, provided that they are compatible with the formation of a type II β turn that is presumably necessary to juxtapose the antigenic residues on the V3 flanks. By comparing the V3 sequences of virus gp120s able and unable to bind 19b, we were able to define the canonical 19b epitope as -I---G--FY-T, where residues at the positions indicated by the gaps do not contribute directly to the 19b-binding site. A few conservative substitutions at the more critical residues are also compatible with 19b binding. Inspection of V3 sequences in the human immunodeficiency virus database indicated that the canonical 19b epitope is well conserved among isolates from the North American-European clade B and also among clade E isolates from Thailand and clade F isolates from Brazil. A minority of gp120s from clades A and C also possess the 19b epitope. Consistent with the theoretical predictions of its cross-clade reactivity, 19b was found to bind to gp120s from clades A, B, C, E, and F in immunoassays. However, 19b was not able to reduce the infectivity of primary viruses from clades A, E, and F that were predicted to possess the 19b epitope and only modestly reduced the infectivity of a clade C virus at low input virus concentrations. Cross-clade neutralization via V3-directed antibodies may, therefore, be difficult, even if the antibodies show broad reactivities in binding assays and the viruses theoretically possess the relevant binding site.

Among neutralization sites on the envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1), the V3 region of the surface glycoprotein gp120 has attracted particular attention (20, 21, 24, 28–30, 42, 46, 50, 52). This is attributable not only to its being one of the few regions of gp120 identified to be a continuous neutralization epitope (20, 24, 50, 52) but also to the potency with which anti-V3 antibodies neutralize T-cell-line-adapted viruses such as HIV-1 LAI or HIV-1 MN. Thus anti-V3 murine monoclonal antibodies (MAbs) raised against recombinant gp120 or V3-based peptides can neutralize certain T-cell-line-adapted viruses at concentrations considerably below 1 $\mu\text{g/ml}$ (26, 28, 56, 57), as can some human MAbs (HuMAbs) (17–19, 54). Furthermore, under appropriate conditions, a passively administered V3 MAb could protect a chimpanzee from subsequent challenge by HIV-1 LAI (14).

It has been widely considered that any vaccine immunogen designed to elicit humoral immunity to HIV-1 should be able to stimulate the production of virus-neutralizing V3 antibodies (4, 33, 42, 46). Furthermore, the V3 loop is a possible target for passive immunotherapy of HIV-1-infected individuals by using HuM Abs (60, 61). There are, however, clear problems asso-

ciated with focussing on the V3 loop as a target for active or passive humoral immunity. Not least among these is sequence variability in the V3 region, which is apparent both within and between individuals (5, 23, 27, 35, 49). Notwithstanding the infidelity of retrovirus replication, V3 variation also reflects changes in the biological phenotype of the infecting virus (11, 15) and the evolution of neutralization escape mutants (13). Variation in V3 sequences is particularly apparent between HIV-1 clades (3, 12, 34, 40, 44, 45, 47, 48), a feature likely to bedevil the development of any single, globally effective vaccination or immunotherapeutic stratagem. Furthermore, the general resistance of primary HIV-1 isolates to neutralization by antibodies, including V3-directed antibodies, is likely to complicate all facets of intervention against HIV-1 that are based on humoral immunity (7, 9, 32, 33, 39). It would therefore be useful to know more about the antigenic properties of the V3 loop, especially those that relate to the production of broadly reactive V3 antibodies. It is also important to understand how such antibodies interact with variant determinants present in the gp120 of primary isolates among the different clades present worldwide.

Here we characterize an anti-V3 HuMAb, 19b, that recognizes an epitope spanning both flanks of the V3 loop that is strongly conserved in clade B viruses and is also conserved in a significant fraction of viruses from clades A, C, E, and F. HuMAb 19b was able to reduce the infectivity of four clade B

* Corresponding author. Mailing address: Aaron Diamond AIDS Research Center, New York University School of Medicine, 455 First Ave., New York, NY 10016. Phone: (212) 725-0018. Fax: (212) 725-1126.

TABLE 1. Virus isolate gp120s reactive with HuMAb 19b^a

Isolate	V3 sequence	19b concn (ng/ml) for half-maximal 19b binding
MN	<u>RIHIGP</u> GRAFYTT	40
JR-FL	<u>SIHIGP</u> GRAFYTT	45
JR-CSF	<u>SIHIGP</u> GRAFYTT	50
AD-13	<u>SIHIGP</u> GRAFYAT	40
5010	<u>SIHIGP</u> GRAFYTT	35
5016	<u>SIHIGP</u> GRAFYAT	20
205	<u>SIHIGP</u> GRAFYAT	25
N70-2a	<u>SIHIGP</u> GRAFYAT	40
N70-2b	<u>SIHMGP</u> GRAFYAT	
5001	<u>GIHIGP</u> GRAFYTT	40
5003	<u>GIHIGP</u> GRAFYTT	40
MR452	<u>GIHIGP</u> GRAFYAT	50
120	<u>SINIGP</u> GRAFYTT	15
NYC-1	<u>SIPIGP</u> GRAFYTT	15
AD-11	<u>SIPIGP</u> GRAFYTT	70
SF-162	<u>SITIGP</u> GRAFYAT	50
146	<u>SIHIAP</u> GRAFYAT	15
108	<u>SIHIAP</u> GRAFYTT	10
5006	<u>SIHIAP</u> GRTFYAT	10
AN608	<u>SIHMGP</u> GRAFYAT	20
CM28a	<u>SIHLGP</u> QSFYAT	20
CM28b	<u>SIHLGP</u> GRSIFYAT	
CM238	<u>SITIGP</u> QVIFYRT	60
AD-6	<u>SITMGP</u> GKVFYVT	15
439	<u>SIHMGP</u> GKAFYAT	15
5009	<u>SIPMGP</u> GKAFYTT	15
WM	<u>GIHIGP</u> GKAFYTT	130
ACP-1	<u>GIGIGP</u> GRTVYTT	60
US 1	<u>SIHIGP</u> GRAIFYAT	70
BK 130	<u>RIHIGP</u> GRALYTT	100
BK 132	<u>RTMGP</u> GRVYVYTT	20

^a The V3 sequences given are those of the most abundant clone identified for each virus stock. For isolates N70-2 and CM28, two clones (designated a and b) with sequences differing by one amino acid were present at approximately equal abundance; both sequences are presented. Conserved amino acids are underlined.

primary viruses that possessed its epitope and was weakly active against a clade C virus. However, 19b could not significantly reduce the infectivity of primary viruses from clades A, E, and F even though the canonical 19b epitope was predicted or detected on these viruses.

MATERIALS AND METHODS

MAbs. HuMAb 19b was isolated from an individual infected with a clade B virus and has been previously mapped to the V3 region of gp120 by using peptides (54). HuMAb 447-D (r1) to the V3 region of gp120 is a variant of HuMAb 447-52D and has been described elsewhere (9, 17-19, 37). Murine MAbs 50.1, 58.2, and 83.1 were raised against a V3 peptide from HIV-1 MN (57) and were donated by Repligen, Inc. Purified gp120 derived from HIV-1 MN-infected cells was a gift from Larry Arthur (National Cancer Institute, Frederick, Md.).

Viruses. The clade B viruses described in Tables 1 and 2 were isolated from individuals residing in the United States, Western Europe, or Brazil. Several of these isolates have been described elsewhere (9, 10, 38, 39, 59). The virus isolates from clades A to F used to derive the data presented in Table 4 and Fig. 4 were collected from various regions of the world by three organizations: the World Health Organization, the Henry M. Jackson Foundation for the Advancement of Military Medicine, and the National Institute of Allergy and Infectious Diseases. Viruses were expanded in mitogen-stimulated peripheral blood mononuclear cells (PBMC) (9, 10, 31, 32, 39), and culture supernatants containing infectious virus were stored in central repositories at -70°C. The designation of viruses into clades was made on the basis of sequence information based on the gag gene or on the V2-C5 region of gp120 or, in some cases, after heteroduplex mobility analysis (12). Further information on these isolates will be described elsewhere (40).

For use in infectivity reduction tests with HuMAb 19b, primary HIV-1 isolate stocks were prepared as described elsewhere (9, 10). Briefly, mitogen (phytohe-

TABLE 2. Virus isolate gp120s unreactive with HuMAb 19b^a

Isolate	V3 sequence
DU 6587-3 ^b	<u>RL</u> SIGPGRVSFYAT
P-17	<u>MS</u> LGGPGRVYVYTT
430	<u>RIHIGR</u> GRAFYAT
5002	<u>GIHMGP</u> GK-FYAT ^c
437	<u>GIHIGP</u> GKTLVYTT
441	<u>SIHMGP</u> GKALVYTT
RF	<u>SITKGP</u> GRVIVYAT
DU 7887-7 ^b	<u>GIRIGP</u> GRAILLAT
DU 6587-5 ^b	<u>RIHIGP</u> GRAFHVTT
AL-1	<u>RIHIGP</u> GRAFHVTT
SF-2	<u>SIYIGP</u> GRAFHVTT
IMB	<u>S/RIHIQR</u> GPGRAFVYTI

^a V3 sequences are those of the most abundant clones for each virus. Amino acid variations in the consensus 19b epitope, or other significant sequence variations, are underlined.

^b Isolates DU 6587-3, DU 7887-7, and DU6587-5 were not tested in the 19b-gp120-binding assay; the inability of 19b to react with gp120 from these viruses was inferred from neutralization data presented in reference 54.

^c -, amino acid deletion.

magglutinin [PHA]-P)-stimulated PBMC cultures were infected with either a low-passage primary isolate or an aliquot of virus obtained from the central repositories. Virus was accumulated during the last 72 h of a culture infected for 7 days, to provide a stock for analyses.

Virus neutralization assay. An infectivity reduction assay was performed essentially as described previously (9, 10). Briefly, serial dilutions of freshly prepared, low-passage primary virus stocks were mixed with MAb (100 µg/ml), human HIV-1-positive serum (1:50 dilution), or media and incubated for 1 h at 37°C. Titers of the virus on PBMC were determined, and virus replication was assessed after 7 days of culture by p24 antigen-capture enzyme-linked immunosorbent assay (ELISA; Coulter Diagnostics).

MAb-gp120-binding assay. Infectious culture supernatants containing virus and free gp120 were treated with 1% Nonidet P-40 nonionic detergent to provide a source of gp120 (38, 40, 44). An appropriate volume of inactivated supernatant was diluted with a buffer comprising Tris-buffered saline (TBS), 1% Nonidet P-40 nonionic detergent, and 10% fetal calf serum. A 100-µl aliquot was then added for 2 h at room temperature to microplate wells (Immulon II; Dynatech Ltd.) coated with sheep polyclonal antibody D7324. This antibody was raised to peptide APTKAKRRVVQREKR, derived from the C-terminal 15 amino acids of the clade B LAI isolate. Unbound gp120 was removed by washing with TBS, and test MAbs, diluted in TMTSS buffer (TBS, 2% nonfat milk, 20% sheep serum, 0.5% Tween 20), were titrated as described previously (41, 43). Bound MAb was then detected with an appropriate alkaline-phosphatase-conjugated anti-immunoglobulin G (IgG), followed by AMPAK (Dako Diagnostics). The absorbance (optical density at 492 nm [OD₄₉₂]) was determined.

In the experiment for which the results are presented in Table 4, each virus was tested against CD4-IgG (1 µg/ml) in triplicate and against HuMAb 19b (0.3 µg/ml) in duplicate. All OD₄₉₂ values were corrected for nonspecific antibody binding in the absence of added gp120 (buffer blank). The mean, blank-corrected OD₄₉₂ values for CD4-IgG and 19b were then calculated, and the OD₄₉₂ ratios of 19b to CD4-IgG were determined. This normalization procedure enables allowance to be made for the different amounts of gp120 captured onto the solid phase via antibody D7324 when comparing 19b reactivity with a panel of viruses. Binding ratios of 0.50 or greater were deemed to represent strong MAb reactivity; ratios from 0.25 to 0.50 were considered indicative of weak reactivity; values <0.25 were designated as representative of essentially negative MAb reactivity (40). Alternatively, 19b was titrated to determine the 19b concentration that resulted in 50% binding. A 50% binding value of <100 ng/ml was designated as strong 19b reactivity; values from 100 to 250 ng/ml were deemed to represent weak 19b reactivity; values of >250 ng/ml were considered negative for 19b reactivity.

Cleavage of MN gp120 with thrombin. Lyophilized bovine thrombin (Boehringer, Mannheim, Germany) was reconstituted in 1 ml of water. MN gp120 (20 ng/ml in TBS plus 10% fetal calf serum) was captured onto Immulon II plates by antibody D7324 and then incubated for 1 h at 37°C in TBS containing 0.5% fetal calf serum, with or without a 1:10 dilution of thrombin (6, 53). Excess enzyme was washed away, MAbs were added in TMS buffer (no Tween 20) for 1 h, and the bound MAb was detected.

Selection and identification of recombinant phage that bind HuMAb 19b. The recombinant phage 15-mer display library with a complexity of approximately 9 × 10⁷ was constructed by using the fUSE5 vector (55). The screening and sequencing methods used to identify sequences that bound 19b have been previously described (25). Briefly, HuMAb 19b was bound to polystyrene beads, and the beads were incubated with approximately 10¹¹ phages from the library.

Selected phages were eluted, expanded, and used in an additional round of antibody selection. After confirmation of clonal selection, the sequence of each selected phage was determined as described elsewhere (25).

RESULTS

Characterization of epitope for HuMAb 19b. HuMAb N701.9b, now designated 19b, was isolated from an HIV-1-infected, asymptomatic individual, N70 (54). The 19b epitope was mapped to the V3 region of gp120 by virtue of its reactivity with V3 peptides in solid-phase binding assays (54). By determining the effect of alanine substitutions in peptide KRIHIG PGRAFYT from the MN V3 sequence on 19b binding, the critical amino acids for 19b recognition were considered to be --I--PGRAFYT. The ability of 19b to neutralize a limited panel of T-cell-line-adapted viruses of known V3 sequence was consistent with this epitope designation (54). However, subsequent studies showed that 19b could also bind strongly to gp120 and a V3 peptide from HIV-1 isolate AD-6 (37, 38). The V3 sequence of this isolate was KSITMGPGKVFYVT, which was clearly different in several positions from the putative 19b epitope outlined above. Of particular note was the apparent insensitivity of 19b to the presence of a lysine residue instead of the more common arginine in the GPGR/K tetrad at the crown of the V3 loop. This feature clearly distinguished 19b from other V3 HuMAbs described to date, which generally only bind to or neutralize viruses possessing the GPGR motif (9, 17–19). It therefore seemed possible that 19b might display an unusual breadth of reactivity with viruses within clade B and even outside clade B.

We therefore explored the ability of 19b to react with gp120s from clade B viruses with known V3 sequences, in an attempt to delineate the requirements for 19b binding more precisely than has been done hitherto. To do this, we used an antigen-capture ELISA, in which nonionic detergent-treated virus culture supernatants were used as gp120 antigen sources for the measurement of 19b binding (40). This procedure was chosen in lieu of peptide-based assays, to eliminate artifacts associated with the absorption of peptides onto plastic (36): for example, 19b binds strongly to a 20-mer peptide from the AD-6 V3 sequence in a solution-phase competition assay and binds to AD-6 gp120, but does not bind the AD-6 V3 peptide, in a solid-phase peptide-binding assay (37, 38). In addition, MAb epitopes are more likely to be displayed in a relevant configuration on gp120 than on a peptide.

The ability of 19b to bind to the different gp120s was assessed by titrating the antibody in the presence of a fixed amount of captured gp120 and then measuring the amount of bound gp120. Titration curves derived by using 10 viruses with widely differing V3 sequences (Table 1) are depicted in Fig. 1. Each of these viruses is from clade B, except for the Thailand clade E virus CM 238. Eight of the 10 viral gp120s shown bound strongly to 19b, with half-maximal binding occurring at 19b concentrations from 15 to 50 ng/ml (0.1 to 0.3 nM), whereas 2 of the gp120s (from strain AL-1 and isolate 437) were unable to bind 19b. Comparison of the V3 sequences for the gp120s (Table 1) with the binding curves shows that 19b binding is not affected by variation in the tetrad at the N-terminal side of the V3 crown: 19b bound to JR-FL (SIHI), 5003 (GIHI), 120 (SINI), NYC-1 (SIPI), and AN608 (SIHM). Neither was 19b binding influenced by the identity of the residues at the crown of the loop, for it recognized JR-FL (GPGR), 146 (APGR), AN608 (GWGR), 5009 (GPGK), and CM238 (GPGQ) (Fig. 1). However, all eight of the reactive gp120s possessed the FY-T motif in the C-terminal side of the V3 loop, and the two unreactive gp120s showed variation in

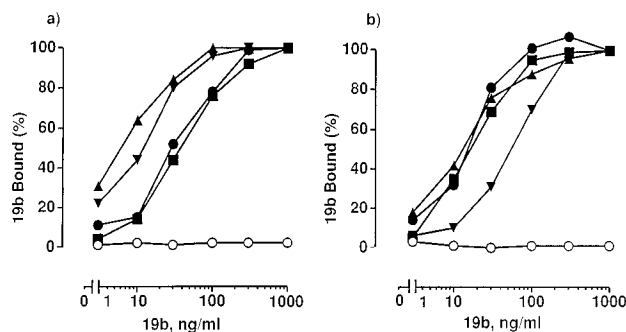


FIG. 1. Reactivity of HuMAb 19b with gp120s with different V3 sequences. HuMAb 19b at the concentrations indicated was reacted with D7324-captured gp120 from various HIV-1 isolates, and bound 19b was detected. Data are expressed relative to the maximum OD₄₉₂ value obtained for each isolate, except for the 19b-unreactive isolates AL-1 and 437, for which the 100% values were those obtained by using 1 μ g of CD4-IgG per ml. The 100% OD₄₉₂ values for the isolates used were (a) 0.92 for JR-FL (●), 1.03 for 5003 (■), 1.50 for 120 (▲), 1.11 for NYC-1 (▼), and 1.28 for AL-1 (○); and (b) 1.31 for 146 (●), 1.18 for AN 608 (■), 1.14 for 5009 (▲), 0.87 for CM 238 (▼), and 1.30 for 437 (○).

this motif: AL-1 (FHIT) and 437 (LYTT). The conclusion that the FY-T motif was an integral component of the 19b epitope was reinforced by inspection of the sequences of an extended panel of viruses that were able or unable to bind 19b (Tables 1 and 2). Of the 19b-reactive gp120s, 25 of 29 possessed the FY-T motif, the sole exceptions being from viruses ACP-1 (VYTT), US 1 (IYTT), BK 130 (LYTT), and BK 132 (YYTT). A valine, isoleucine, leucine, or tyrosine residue may therefore be able to replace the phenylalanine residue in the context of certain V3 sequences without complete loss of 19b reactivity. However, such substitutions are not invariably tolerated (Table 2) (40). For example, the Thai clade B isolate CM 237, like BK 132, has the YYTT motif but does not detectably bind 19b (40). Presumably, the overall conformation of the V3 loop influences the 19b epitope, and the context in which the above substitutions occur will determine whether a binding site for 19b can be formed. The tyrosine residue in the FY-T motif does, however, seem to be an invariant requirement for 19b binding; in particular, a histidine residue is unacceptable (Table 2).

The only other conserved residues in the 19b-reactive V3 sequences were the first isoleucine on the N-terminal flank (e.g., RIHI), the second glycine at the crown (e.g., GPGR), and the final threonine (e.g., FYTT). Taken together, these observations suggest that the canonical sequence for 19b recognition is -I---G--FY-T, which spans both sides of the V3 loop. Most substitutions in the indicated amino acids are apparently incompatible with 19b binding. However, no virus isolates lacking the second glycine residue at the crown of the V3 loop and the final threonine were available for testing, so any direct contribution of these invariant residues to the 19b binding site is a matter of conjecture. In any case, the glycine residue could contribute only peptide backbone to any antibody epitope. Preliminary results with additional virus isolates suggest that the first isoleucine in the above sequence may be replaced by a valine residue in some circumstances. Thus, viral gp120s from clade A isolates that have a valine at this position (SVRI or SVHI motif) tend to have at least a 5- to 10-fold-lower affinity for 19b than most of those possessing an isoleucine, but at least a subset of these valine-containing gp120s are able to bind 19b (data not shown) (40). Consistent with this finding, among the four phage sequences selected for their abilities to bind 19b (see below), two had a valine, one had a

TABLE 3. Consensus epitope for HuMAb 19b with tolerated substitutions^a

Epitope	Sequence												
Minimal.....	-	I	-	-	-	-	G	-	-	F	Y	-	T
Tolerated ..	R	I	H	I	G	P	G	R	A	F	Y	A	T
	S	(V)	N	L	A	W		K	T	(I)		T	
	G		P	M		L		Q	S	(L)		V	
			G						V	(V)		R	
			T							(Y)			

^a The epitope for 19b is inferred from data presented in Tables 1 and 2. Residues in parentheses may not be tolerated for 19b binding in the context of all V3 loops and are often associated with a reduction in 19b affinity.

leucine, and one had an isoleucine residue at the relevant position (see Table 4). Thus an aliphatic hydrocarbon chain on the amino-terminal side of the V3 loop appears to be a necessary component of the 19b epitope, with an isoleucine being, perhaps, the preferred amino acid.

The only gp120s possessing the canonical -I---G--FY-T 19b motif that were unable to bind 19b were from viruses 430 and 5002 (Table 2). The former gp120 has a highly unusual GRGR sequence at the V3 crown that may be incompatible with the formation of a β turn (58), and the latter has a single amino acid deletion immediately before the FY-T motif; these sequence variations are presumably incompatible with 19b binding. A Brazilian clade B virus gp120 (BZ167) which lacks a residue between the tyrosine and threonine in the above motif (i.e., FYT) was also unable to bind 19b (data not shown). Those V3 amino acids which are and are not compatible with 19b binding are summarized in Table 3.

Reactivity of 19b with peptidic epitopes on recombinant bacteriophage. Further characterization of the 19b epitope was performed by selecting, from a library of recombinant, display bacteriophage expressing random peptide sequences, those phage that were able to bind strongly to 19b (Table 4). Four phages sequenced from the group had inserts that were broadly compatible with the 19b epitope as defined above by gp120-binding assays. Thus, an isoleucine, leucine, or valine residue was present in the amino-terminal side of each insert, and there was an invariant phenylalanine residue eight amino acids downstream. The next residue was always a bulky hydrophobic, amino acid: tyrosine, phenylalanine, or tryptophan. In the positions corresponding to those at the crown of the V3 loop, four motifs were found: DGNR, GSGR, APDR, and DPGR. Each of these sequences is compatible with the formation of a β turn (58), which we suggest is necessary to juxtapose the isoleucine, leucine, or valine residue on the amino-terminal flank appropriately with the bulky, hydropho-

TABLE 4. Amino acid sequences selected by phage library screening with HuMAb 19b^a

MAb	Insert sequence selected																
19b	. . .	<u>I</u>	<u>H</u>	<u>I</u>	<u>G</u>	<u>P</u>	<u>G</u>	<u>R</u>	<u>A</u>	<u>F</u>	<u>Y</u>	<u>T</u>	. . . ^b				
Clone																	
19b.1009-6			<u>E</u>	<u>V</u>	<u>D</u>	<u>M</u>	<u>D</u>	<u>G</u>	<u>N</u>	<u>R</u>	<u>T</u>	<u>F</u>	<u>F</u>	<u>Y</u>	<u>V</u>	<u>G</u>	<u>R</u>
19b.1009-8	<u>S</u>	<u>T</u>	<u>E</u>	<u>I</u>	<u>L</u>	<u>W</u>	<u>M</u>	<u>G</u>	<u>S</u>	<u>G</u>	<u>R</u>	<u>Q</u>	<u>F</u>	<u>Y</u>	<u>M</u>		
19b.1009-7		<u>S</u>	<u>V</u>	<u>I</u>	<u>M</u>	<u>L</u>	<u>A</u>	<u>P</u>	<u>D</u>	<u>R</u>	<u>G</u>	<u>F</u>	<u>W</u>	<u>R</u>	<u>G</u>	<u>E</u>	
19b.1009-10		<u>V</u>	<u>M</u>	<u>V</u>	<u>V</u>	<u>G</u>	<u>M</u>	<u>D</u>	<u>P</u>	<u>G</u>	<u>R</u>	<u>S</u>	<u>F</u>	<u>F</u>	<u>G</u>	<u>H</u>	

^a Sequences of the insert in recombinant bacteriophage reactive with HuMAb 19b are presented, along with the clade B consensus V3 sequence. The amino acids at positions important for 19b binding are underlined.

^b Clade B consensus sequence.

bic residues on the carboxy-terminal side and form the 19b epitope. Note that in the context of the short peptidic phage insert, diversity in the identity of the second hydrophobic residue in the FY-T motif can be tolerated for 19b binding (Table 4). Whether a phenylalanine or tryptophan residue can substitute for tyrosine in the context of the gp120 protein and still allow 19b binding is unresolved, as the FF-T or FW-T motifs occur very infrequently in published HIV-1 V3 sequences (45) and we have not yet tested one of these isolates for 19b reactivity.

Sensitivity of 19b epitope to proteolysis. We studied the binding of several V3 MAbs to gp120s derived from virus culture supernatants. It was noted that it was sometimes necessary to use three- to fivefold-more supernatant medium to be able to detect 19b binding than was required for the detection of the binding of other V3 MAbs such as 447-D (r1) (data not shown). This phenomenon was not observed when we used purified MN gp120. The data were consistent with the specific loss of the 19b epitope from a fraction of the gp120 molecules in the virus culture supernatants. We reasoned that one explanation could be that proteolytic cleavage of a fraction of the gp120 molecules had occurred during the 3- to 10-day incubation of mature virus and gp120 during cell culture. Proteolysis of gp120 has been described under similar conditions (1, 22), and we noted that a cleavage site for thrombin-like proteinases was within the 19b epitope (6, 53). Scission of gp120 at this site would divide the putative 19b-binding site.

To explore whether this explanation was plausible, we treated purified MN gp120 with thrombin and measured the effect of this on the binding of a panel of V3 MAbs, including 19b (Fig. 2). On the basis of prior studies with BH10 gp120, thrombin is predicted to cleave MN gp120 on the C-terminal side of the V3 loop, as indicated by the arrow: RIHIGPGR ↓ AFYTT (6). Treatment of MN gp120 for 1 h with thrombin had no effect on its ability to bind either HuMAb 447-D (r1), whose epitope is centered on the GPGR motif (17–19), or murine MAb 50.1, which recognizes the RIHIG sequence (57) (Fig. 2a and b). Binding of MAb 83.1 to the I-IGPGR motif (57) was also unaffected (data not shown). However, thrombin treatment caused a major reduction in 19b binding (Fig. 2c) and a minor reduction in the binding of MAb 58.2 (Fig. 2d); this MAb is dependent upon the HIGPGRAF sequence (57), which contains the thrombin cleavage site. Residual binding of 19b probably reflects incomplete scission of the entire population of gp120 molecules. Thus, the thrombin cleavage experiment establishes that the 19b epitope does not merely encompass the FY-T sequence. Instead, the results are consistent with the notion that the epitope spans both sides of the V3 loop. Furthermore, gp120 scission in culture could well account for the reduced 19b binding seen with certain virus-derived gp120s (40).

Reactivity of HuMAb 19b with gp120s from clades A to F. The independence of the 19b epitope on sequences at the crown of the V3 loop suggested that this antibody, although raised against a virus from clade B, might be able to react with gp120s from other clades. We therefore scanned a database of V3 sequences from viruses of known clades to see what proportion of these viruses possessed the 19b epitope (Table 5). The canonical 19b epitope is well conserved in clade B, C, E, and F viruses, is present in a minor proportion of viruses from clade A, but is absent among clade D viruses. Relaxing the criteria for the 19b epitope to include the F/I, F/V, F/Y and F/L substitutions that are sometimes compatible with 19b binding in certain sequence contexts did not markedly increase the percentage of viruses from clades B, C, and E predicted to

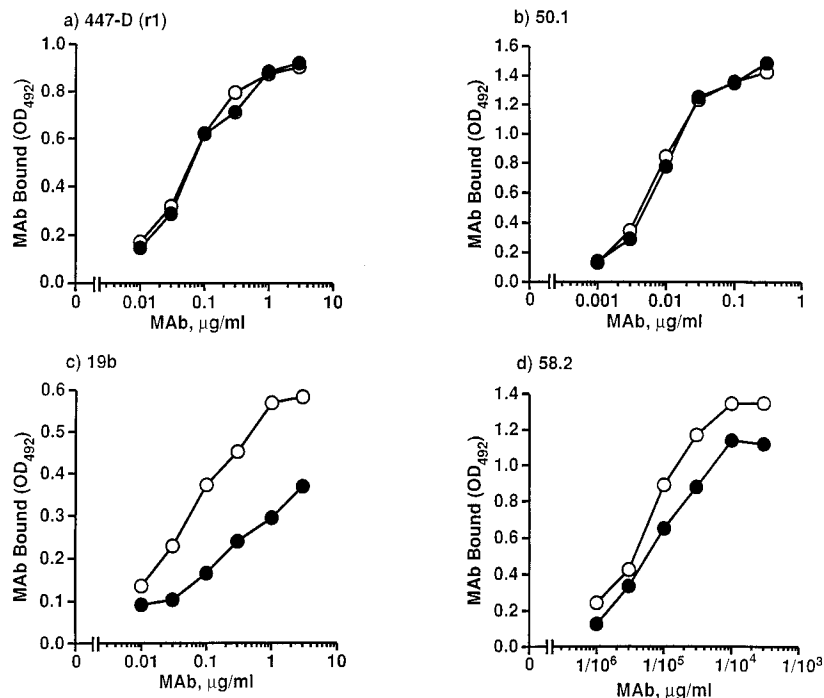


FIG. 2. (a to d) Effect of thrombin cleavage on binding of V3 MAbs to MN gp120. MN gp120, captured by antibody D7324, was incubated for 1 h with (●) or without (○) thrombin before removal of the enzyme by washing. MAbs (as indicated in each panel) were then added at the concentrations indicated, and the amount of bound MAb was detected.

be reactive with 19b, for these substitutions occur relatively infrequently in the database. However, allowing the I/V substitution in the amino-terminal flank caused a large increase in the proportion of clade A viruses that would be predicted to be reactive with 19b, as the clade A consensus sequence has a valine at the relevant position (Table 5). Furthermore, a significant number of clade D viruses (35%) contain the LY-T motif, which is the consensus for clade D and so would be predicted to bind 19b by the relaxed criteria (Table 5). In

TABLE 5. Prevalence of 19b epitope among viruses of clades A through F^a

Clade	V3 consensus sequence	No. positive/total no. (%)		
		Predicted frequency of:		Measured frequency of 19b reactivity
		-I---G--FY-T epitope	19b epitope allowing substitutions	
A	<u>S</u> VHIGP <u>Q</u> AFYAT	9/52 (17)	37/52 (71)	3/8 (38)
B	<u>S</u> IHIGP <u>G</u> RAFYT <u>T</u>	265/341 (78)	285/341 (84)	19/29 (66)
C	<u>S</u> IRIGP <u>Q</u> TFYAT	15/24 (63)	21/24 (88)	1/8 (13)
D	RTHIGPQALYT T	0/57 (0)	8/57 (14)	0/8 (0)
E	<u>S</u> ITIGP <u>Q</u> VFYRT	30/42 (71)	36/42 (86)	10/12 (83)
F	<u>S</u> IHLGP <u>Q</u> AFYAT	10/10 (100)	10/10 (100)	2/2 (100)
Total	<u>S</u> IHIGP <u>Q</u> AFYAT	329/526 (63)	397/526 (75)	35/67 (52)

^a The consensus sequences for each HIV-1 clade are presented as described by Myers et al. (45); residues contributing to 19b binding are underlined. The proportion of the total V3 sequences in the Los Alamos database that possess the 19b canonical epitope is listed for each HIV-1 clade. These values were adjusted to allow for conservative substitutions (I/V, F/I, F/L, F/V, and F/Y) that may be compatible with 19b binding. Data for 19b binding to gp120s from a panel of viruses of clades A to F were derived from the data in reference 40.

practice, this relaxation appears not to be acceptable, for no clade D gp120s actually bound 19b and 3 of 8 isolates tested contained the LY-T motif (40). Note, however, that the clade D consensus sequence contains a threonine residue on the amino-terminal side of the 19b epitope, which may prevent recognition. Thus, the overall context in which variations on the 19b epitope are compatible with binding may be critical, and a substitution that may be tolerated in a V3 loop from a clade B virus might not be in a virus from a clade other than B.

To explore whether the predictions of 19b binding to gp120s from clades other than clade B were accurate, we accumulated a panel of viruses from clades A to F without initial knowledge of their V3 sequences and then measured 19b binding to monomeric gp120s released from these viruses (Table 5). The experimental results were broadly consistent with the predictions made from sequence analyses; thus a majority of B, E, and F clade gp120s were able to bind 19b, but only a minority of A and C clade gp120s could do so, and 19b was unreactive with all the clade D gp120s (Table 5). However, a lower-than-expected percentage of clade C gp120s was able to react with 19b (Table 5). A post hoc analysis of the sequences of the test panel viruses, when these were available, revealed a few minor inconsistencies between the binding data and the predicted presence of the 19b epitope. For example, three unreactive clade C isolates (BR 92/025, ZAM 18, and UG 268) and one unreactive clade E isolate (TH 92/022) failed to bind 19b, despite their predicted possession of the canonical 19b epitope. This may be attributable to proteolytic destruction of the epitope, as the OD₄₉₂ signals were generally low in experiments using these isolates, and one of the cultures of a clade C virus (ZAM 18) did contain some 19b-reactive gp120. Alternatively, variation in other regions of gp120 from these isolates may influence indirectly the presence of the 19b epitope. In addition, only a few of the clade A gp120s with the

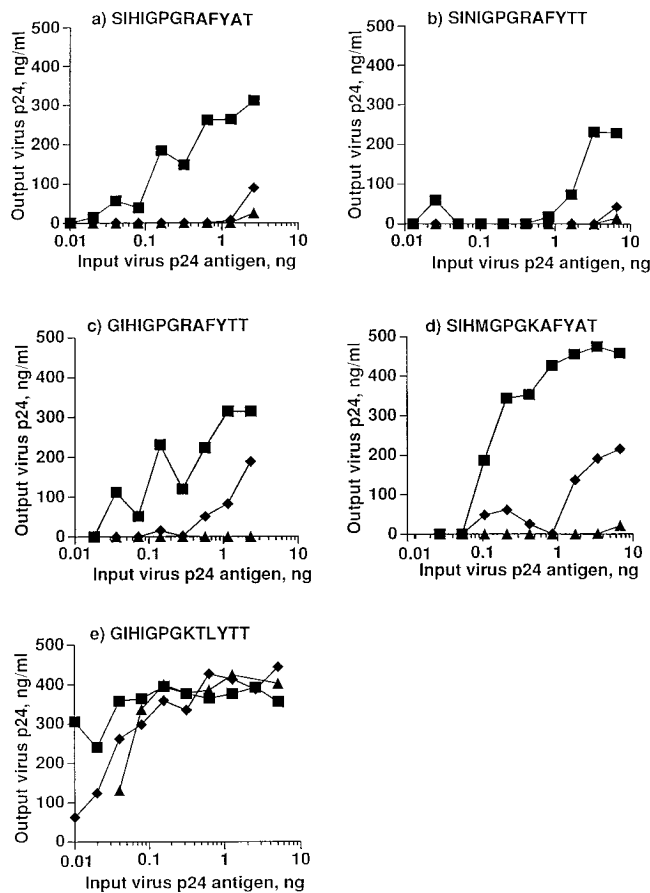


FIG. 3. Effect of 19b on infectivity of clade B primary viruses. Various amounts of the indicated viruses were incubated with 100 µg of 19b (▲) per ml, a 1:50 dilution of HIV-1-positive human serum (◆), or with no added reagent (■) before addition to phytohemagglutinin-stimulated PBMC. The extent of virus replication was monitored by measurement of extracellular p24 antigen production. The viruses tested were 5016 (a), 120 (b), 5001 (c), 439 (d), and 437 (e). The central V3 sequence of each virus is shown in the appropriate panel.

consensus SVRI motif on the amino-terminal side of the V3 loop were able to bind 19b, and they did so with reduced affinity (data not shown). This suggests that a valine residue substitutes only poorly for the conserved isoleucine in the canonical 19b epitope. A more detailed analysis of the reactivity of a large panel of MAbs with this panel of A to F viruses is presented elsewhere (40).

Neutralizing activity of 19b against primary viruses. The breadth of reactivity of 19b in binding assays suggested that it might be a useful agent for passive immunotherapy and as a standard for assessment of the presence of potentially protective virus-neutralizing antibodies in sera raised against experimental vaccines. However, the ability of a MAb to reduce the infectivity of primary viruses is the most relevant parameter for judging its worth for immunotherapy (33). For example, we have observed that the ability of a MAb to bind to monomeric gp120 from a primary virus was a poor predictor of the virus-neutralizing activity of that MAb (39). The inclusion of 100 µg of 19b per ml in cultures of four clade B primary viruses that possessed the canonical 19b epitope reduced the infectivity of these viruses in the range of 16- to 64-fold (geometric mean titer reduction for the four viruses, 48-fold) compared with control cultures, whereas a 1:50 dilution of HIV-1-positive human serum, used as an internal reference standard, was less

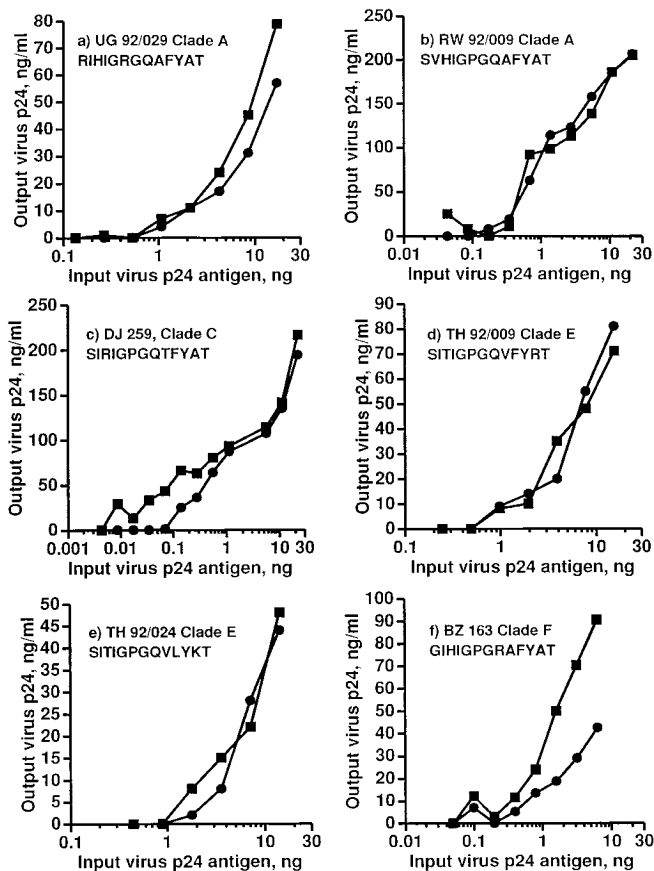


FIG. 4. Effect of 19b on infectivity of primary viruses from clades A, C, E, and F. Various amounts of the indicated viruses were incubated with (●) or without (■) 100 µg of 19b per ml before addition to phytohemagglutinin-stimulated PBMC. The extent of virus replication was monitored by measurement of extracellular p24 antigen production. The viruses tested, their clades, and their central V3 sequences are listed in the appropriate panels.

effective (Fig. 3a to d). In contrast, the infectivity of clade B isolate 437, which lacks the predicted 19b epitope and is unable to bind 19b (Table 2), was not reduced by 19b (Fig. 3e). Thus 19b has the ability to at least partially reduce the infectivity of clade B HIV-1 strains, provided they possess the 19b epitope. Additional information on the potency of HuMAb 19b at neutralizing clade B primary viruses, in comparison with other MAbs directed at V3 and non-V3 gp120 epitopes is described elsewhere (39).

To explore whether the virus-neutralizing activity of 19b extended beyond clade B, we selected a panel of A, C, E, and F clade primary viruses on the grounds that the presence of the 19b epitope was predicted by the V3 primary sequences, along with A and E clade viruses that had sequence variations within the canonical epitope (RW 92/009 and TH 92/024, respectively). The V3 sequences of the viruses in the panel are listed in Fig. 4. No clade D viruses were tested, as none available had the predicted 19b epitope (40). At 100 µg/ml, 19b was able to reduce the infectivity of the clade C virus DJ 259, albeit only eightfold and at low input virus concentrations (Fig. 4c). However, 19b had no significant effect (\pm threefold) on the infectivity of the clade A viruses UG 92/029 and RW 92/009, and the clade E viruses TH 92/009 and TH 92/024 (Fig. 4). The clade F virus BZ 163 was weakly sensitive to 19b, but only at high input virus concentrations, and we do not deem this

significant (Fig. 4f). A second clade F virus, BZ 162, was also insensitive to 19b (data not shown). The presence of the 19b epitope on gp120 solubilized from several of these viruses was confirmed by ELISA. Half-maximal 19b binding occurred at 65 ng/ml for DJ 259, at 100 ng/ml for BZ 162, and at 150 ng/ml for BZ 163. Insufficient gp120 was present in the cultures of UG 92/029, RW 92/009, TH 92/009, and TH 92/024 for accurate values to be derivable from titration curves. We were, however, able to confirm that TH 92/009 gp120 bound 19b strongly, that UG 92/029 gp120 did so weakly, and that RW 92/009 gp120 was unable to bind 19b (40).

There was no obvious correlation between the infectivity of the viral isolates used (i.e., the extent to which an isolate can be diluted while retaining infectivity) and their sensitivity to 19b; thus the most-infectious isolates, 437 (Fig. 3e) and DJ 259 (Fig. 4), were both resistant to MAb 19b. However, there was a modest tendency for those isolates that produced the highest amount of virus output to be the most sensitive to 19b (Fig. 3 and 4), for reasons that are presently obscure.

DISCUSSION

We have characterized a complex epitope for an HuMAb reactive with the V3 region of gp120. Taken together, our data suggest that the binding site for 19b spans both sides of the V3 loop but is not significantly influenced by residues at the V3 loop crown, provided that they are compatible with the formation of a type II β turn. These features distinguishes 19b from previously described V3 loop HuMAbs (17–19) and from murine MAbs against this region (26, 28, 56, 57). Our best assessment of the requirements for 19b binding is that an isoleucine residue is essential on the amino-terminal side of the V3 loop, although a valine residue might be tolerable in certain circumstances: valine and isoleucine differ by only a single methylene group. A type II β turn formed by a tetrad such as GPGR or GLGO at the crown of the loop is required to juxtapose the isoleucine or valine residue on the amino-terminal flank of the V3 loop with residues on the carboxy-terminal flank to form a complex epitope. The thrombin cleavage experiments suggest that scission of the V3 loop divides the 19b epitope. On the carboxy-terminal side, the FY-T motif seems critical, although an isoleucine, valine, leucine, or tyrosine residue may sometimes be acceptable in place of the phenylalanine. It is possible that the branched-chain methylene groups present in isoleucine, leucine, or valine may substitute for the hydrocarbon moieties normally provided by phenylalanine's (or tyrosine's) aromatic ring structure. The experiments with recombinant bacteriophage suggest that the tyrosine residue in the above motif might also be replaceable by phenylalanine or tryptophan. However, confirmation of this will require the use of gp120 molecules containing such substitutions, in that conformational constraints on the 19b epitope may be significantly greater in the context of the gp120 protein than in a peptide fragment (37). A histidine substitution for the tyrosine does, however, seem to be incompatible with 19b binding (Table 2). More precise delineation of the structural features of the 19b epitope may, perhaps, be obtained by crystallography of Fab from this antibody with a V3 loop peptide; this would yield information more relevant to the true conformation of the V3 loop than has been obtained hithertofore from crystallographic studies of murine anti-peptide MAbs and V3 peptides (16, 51).

The consensus epitope for 19b is strongly conserved among clade B, E, and F viruses and is present on a minor fraction of viruses from clades A and C. The predictions of the prevalence of the 19b epitope among these different clades are supported

by experimental observations, which increases our confidence in the validity of the methods that we employed. The antigen-capture ELISA approach to epitope definition relies on the use of viral culture supernatants combined with V3 sequence analysis. In simplicity, this approach is superior to one relying on synthesis of a panel of V3 peptides; more importantly, studies with the complete gp120 molecule are not subject to artifacts associated with the adsorption of peptides to plastic or to uncertainties as to whether the conformation of synthetic and substituted peptides reflect the structure of those locations in gp120. These factors can yield misleading information on the nature of antibody-binding sites such as that of 19b (36, 37). However, proteolysis of a fraction of the gp120 molecules during the prolonged culture must be borne in mind, for it can lead to false-negative results when defining the epitopes for V3 MAbs like 19b that span a cleavage site. Conversely, the presence of gp120 molecules derived from clones with minor abundance in the viral stock can also lead to false-positive results. With care, these potential errors can be discerned and discounted.

Whereas the absence from monomeric gp120 of an epitope for a MAb like 19b means, in all probability, that the virus will not be neutralized by the MAb, it is important to realize that the converse is not necessarily true; the presence of a MAb epitope on a primary virus gp120 does not predict, a priori, that the virus will be neutralized by the MAb with any potency (39). It now seems clear that primary viruses are relatively resistant to neutralization by MAbs and HIV-1-positive sera compared with T-cell-line-adapted viruses (4a, 9, 32, 33, 39). Nonetheless, the binding data suggested that 19b would be able to reduce the infectivity of at least some primary viruses from clades A, B, C, E, and F, and this was tested experimentally. Continuous exposure of four clade B primary viruses to 100 μ g of 19b per ml caused up to a 50-fold reduction in their infectivity. These viruses were predicted, and shown experimentally, to possess the 19b epitope, whereas the clade B isolate 437 with gp120 unreactive with 19b was not neutralized by this antibody. A clade C virus, DJ 259, was also weakly sensitive to 19b, albeit only at low concentrations of input virus. However, none of the isolates from clades A, E, and F tested in the infectivity reduction assay was sensitive to 19b, notwithstanding the predicted and experimentally confirmed presence of the 19b epitope on several of the gp120s solubilized from these viruses. Thus, the context in which the 19b epitope is presented may be critical for it to serve as a neutralization target, and this context may vary among gp120s from different clades.

While the extent of infectivity reduction of sensitive clade B viruses caused by 19b is comparable to that achieved by other HuMAbs, such as the anti-gp41 MAb 2F5 and an independent anti-V3 MAb 447-52D, that possess some neutralizing activity against primary viruses displaying the appropriate determinants (9, 10), it must be of concern that greater potency was not observed. The proportion of an input primary virus that is neutralized is a function of the MAb concentration used, as demonstrated elsewhere with 2F5 and 447-52D (9, 10). Titration of 19b against a different panel of clade B primary viruses indicated that $\geq 90\%$ virus neutralization was not achieved at 19b concentrations of ≤ 25 μ g/ml (39). This stands in stark contrast to the activity of 19b against T-cell-line-adapted viruses (39, 54) and supports the notion that primary viruses are inherently hard to neutralize, probably because of an inaccessibility of important neutralization epitopes such as the V3 loop and the CD4-binding site on the oligomeric form of the virus envelope (4a, 39). For passive immunotherapy with HuMAbs to be effective, a greater degree of potency than is possessed by 19b and most other antibodies tested to date

might be necessary (9, 10), although it may be noted that circulating concentrations of passively administered MAbs of at least 100 µg/ml have been achieved in chimpanzees (8) and may be achievable in humans.

The breadth of 19b's reactivity in binding assays with gp120s from several clades is impressive, for we are not aware of any other V3 MAb with this property. The data imply that some structural and antigenic features of the V3 loop can withstand the primary sequence variation common to this region of gp120. Yet the minimal action of 19b in infectivity reduction experiments with viruses from clades other than B demonstrates just how difficult it will be to induce V3-directed neutralizing antibodies that are effective across all clades. However, given the perception that the induction of broadly active neutralizing antibodies would be a desirable feature of an effective AIDS vaccine, it might be fruitful to investigate how antibodies such as 19b, which have some cross-clade reactivity, might best be induced. For example, should our deduction that the 19b epitope spans both sides of the V3 loop be correct, then constrained peptides or recombinant proteins might be more effective than free peptides at mimicking the natural gp120 antigen for 19b. Improvement of the affinity of 19b by genetic manipulation of the relevant immunoglobulin gene might improve its performance as a broadly active passive immunotherapeutic agent (2). Further studies on the nature of the 19b epitope therefore seem warranted, not only for the development of effective immunotherapeutic reagents but also to assist in the generation of broadly effective HIV-1 vaccines based on the induction of humoral immunity. However, in view of recent indications that the V3 loop is relatively inaccessible on the oligomeric form of primary virus envelopes (4a, 39), it may be that more attention should now be focussed on other gp120 or gp41 epitopes, perhaps oligomer-dependent ones, that may be better targets for neutralizing antibodies.

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