

## Neutralization of Diverse Human Immunodeficiency Virus Type 1 Variants by an Anti-V3 Human Monoclonal Antibody

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**The third variable region (V3) of the HIV-1 gp120 envelope glycoprotein is thought to induce potent neutralizing antibodies which are generally defined as type specific and reactive with individual viral isolates. In contrast, the CD4-binding domain is thought to induce neutralizing antibodies that are group specific and capable of neutralizing all isolates of HIV-1. However, in this study, we used a panel of human monoclonal antibodies to these regions of gp120 which displays specificities and neutralizing activities that challenge these tenets. In particular, we used a human monoclonal antibody to the V3 domain with exceptionally potent and broad neutralizing activity against many diverse HIV-1 isolates. The anti-CD4-binding domain antibodies, on the other hand, showed a more restricted pattern of activity.**

Antibodies (Abs) are responsible for the neutralization of infectivity of many viruses. Usually, effective neutralization is mediated only by Abs specific for discrete epitopes on the surface of the viral particle (4, 29, 38, 40, 45). With human immunodeficiency virus type 1 (HIV-1), potent neutralization of virus infectivity has been associated primarily with Abs to two regions of the gp120 envelope glycoprotein: the CD4-binding domain (CD4bd) and the third hypervariable (V3) region (16, 18, 19, 28, 32, 37, 41).

The CD4bd is composed of discontinuous regions of gp120 including portions of the second, third, and fourth conserved regions of gp120 (21, 31). Polyclonal Abs and monoclonal Abs (MAbs) to this region block the interaction of CD4 and gp120 and are capable of neutralizing many, but not all, strains of HIV-1 (15, 19, 20, 23, 25, 34, 36, 41-43). For this reason, Abs to the CD4bd have been classified as group-specific Abs. V3 of gp120 has been described as the principal neutralizing domain of HIV-1 (28, 32, 37) and consists of a loop of 32 to 34 amino acids bridged at the base by a disulfide bond. Most of the residues in this loop are highly variable, but the amino acids at the base of the loop are relatively well conserved, as is the structure of the loop and the GPCR motif at the tip of the loop (22, 27). Originally, polyclonal Abs and MAbs to the V3 region were described as being specific for individual or closely related isolates owing to the extreme variability of this region of gp120 (10, 12, 32, 37, 39). However, recently anti-V3 Abs elicited in rodents and baboons which neutralize several divergent HIV-1 isolates have been described (1, 14, 17, 30).

To characterize the protective antibody response and ascertain whether cross-reactive neutralizing antibodies exist in infected humans, a series of heterohybridomas which produce immunoglobulin G human MAbs (HuMAbs) to the V3 loop and CD4bd was generated as previously described (9, 10, 20). HuMAbs to the V3 loop were defined by their reactivity with a synthetic 23-mer peptide from the V3<sub>MN</sub> loop: YNKRKRHIHGPGRAFYTTKNIIG (10). HuMAbs

specific for the CD4bd were identified by the ability to block the interaction between gp120 and CD4 (20).

Three HuMAbs to the V3 loop (447-52-D, 694/98-D, and 537-D) and three anti-CD4bd HuMAbs (448-D, 559-D, and 558-D) were studied. Epitope mapping of HuMAb 447-52-D was performed by enzyme-linked immunosorbent assay (ELISA) as previously described (10), on overlapping hexapeptides synthesized on polyethylene pins by the method of Geysen et al. (8); analysis of amino acids of the V3 loop critical for antibody binding was performed similarly, by using a set of hexapeptides in which each amino acid of the peptide HIGPGR was replaced with the 19 other amino acids. The dissociation constants,  $K_d$ , were determined by the ELISA method of Friguet et al. (6) as described previously (10), by using 20- to 23-mers of various V3 peptides and recombinant gp120 proteins from SF-2 (provided by N. Haigwood and K. Steimer) and HXB2.

For neutralization tests of lymphotropic HIV-1 isolates tested on MT-4 cells, twofold serial dilutions of HuMAbs were made and a 100- $\mu$ l volume was used in each test well. All virus stocks were made from available chronically infected H9 cells (IIIB, MN, and RF) or from newly established chronically infected FDA/H9 cells (isolates SF-2, AL-1, WMJ-2, DU 6587-5, and DU 7887-7). Virus stocks were prepared from clarified culture medium 72 h after suspension of the respective chronically infected cell line in fresh medium at a density of  $2 \times 10^5$  cells per ml. For each virus stock of each isolate, an MT-4 cell infectivity endpoint was defined as the last dilution of the virus stock that killed essentially all MT-4 cells in a 7-day assay. For consistency in neutralization tests, a dilution representing approximately 10-fold more than the minimum amount of the virus stock necessary to achieve complete killing was chosen. The HIV-1 isolates used included IIIB, RF (33), MN (7), AL-1 (2), SF-2 (24), WMJ-2 (13), DU 6587-5, and DU 7887-7 (39). These viruses were used in neutralization studies to characterize a variety of MAbs and polyclonal antibodies. The performance of each virus isolate in these studies was consistent. In addition, sequence analysis was used to reconfirm the unique gp120 V3 loop of each isolate and

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demonstrate the lack of discernible contamination among the isolate stocks. Each virus stock (100  $\mu$ l) was added to each test well, and the virus-antibody mixtures were incubated at 37°C for 1 h, after which 10<sup>4</sup> MT-4 cells in 50  $\mu$ l of culture medium were added to each well. The cultures were incubated for 7 days, after which the antibody neutralization endpoint was determined (35). The neutralization endpoints were determined as the last dilution of the antibody preparation that prevented MT-4 cell killing. Uninfected MT-4 cells were cultured with each test, and a virus stock reiteration was performed with each analysis.

Neutralization of monocytotropic isolate SF-162 was also tested. This virus stock was treated with or without HuMAb 447-52-D as described above and added to microtiter wells containing 2.0  $\times$  10<sup>5</sup> primary macrophage-monocytes derived from peripheral blood cells separated by Ficoll-Paque centrifugation, adherence to gelatin-coated plates, and 7 days of culture in monocyte-macrophage growth medium (complete Dulbecco modified Eagle medium with 10% fetal calf serum and 10% horse serum and supplemented with 125 U of macrophage colony-stimulating factor [Genzyme] and 1,250 U of granulocyte macrophage colony-stimulating factor [Amgen] per ml) in Teflon culture jars. All macrophage-monocyte cultures were fed with fresh medium at days 5, 8, 12, 14, and 21 postinfection. Medium was collected at days 18, 21, and 24 for quantitation of p24 by ELISA to determine virus production. Also, the cells were lysed at day 24 to determine the intracellular virus by p24 ELISA. The neutralization endpoint was determined as the last dilution of the HuMAb that prevented macrophage-monocyte infection in vitro as evidenced by absence of p24 production.

The anti-V3 immunoglobulin G HuMAbs were examined and found to bind to most synthetic peptides corresponding to the tip of the V3 loops of 10 HIV-1 isolates (Table 1) but not to irrelevant peptides derived from p24 (data not shown). None of the HuMAbs tested bound to the corresponding V3 peptides which contain GPGK or GLGQ in place of GPGR. These HuMAbs bound to several V3 peptides coated on ELISA plates at <0.1  $\mu$ g/ml (Table 1), suggesting that they possess high relative binding affinities.

These anti-V3 HuMAbs and three more immunoglobulin G HuMAbs specific for CD4bd (20) were tested for neutralization against eight HIV-1 isolates which contain the GPGR motif in the V3 loop but represent divergent branches of the HIV-1 family (Table 2 and reference 27). Anti-V3 HuMAb 447-52-D was the most broadly neutralizing of the anti-V3 HuMAbs tested. Neutralization endpoints with 447-52-D were attained with geometric mean concentrations ranging from 0.04 to 1.35  $\mu$ g/ml (Table 2). By the same assay, the potency of 447-52-D was comparable to that of type-specific murine and human-murine chimeric anti-V3 MAbs 0.5 $\beta$  and C $\beta$ 1, respectively (unpublished data), which prevent HIV-1<sub>IIIB</sub> infection in chimpanzees when administered at a dose of 36 mg/kg (3, 5, 26). Moreover, 447-52-D appears to be more broadly reactive than the cross-reactive anti-V3 murine MAbs and rodent polyclonal antibodies previously described (1, 14, 17, 30). Moreover, while 447-52-D neutralized eight of eight isolates, none of the three anti-CD4bd HuMAbs were capable of neutralizing all of the tested isolates, and significantly higher concentrations of anti-CD4bd antibodies were generally required for neutralization compared with 447-52-D. HuMAbs to the CD4bd described by others were similarly inconsistent in the ability to neutralize various laboratory HIV strains (15, 44).

Given the unique features of HuMAb 447-52-D, additional studies were performed to characterize its biological and

TABLE 1. Immunochemical characterization of anti-V3 HuMAbs

HuMAb	ELISA reactivity <sup>a</sup> to:													K <sub>d</sub> (10 <sup>-6</sup> M) vs:			
	V3-MN (GPGRAF)	V3-SF-2 (GPGRAF)	V3-HXB2 (GPGRAF)	V3-RF (GPGRV1)	V3-WM52 (GPGRAF)	V3-NY5 (GPGRTL)	V3-SC (GPGRAF)	V3-CD4 (GPGRVW)	V3-SF33 (GPGKVL)	V3-EL1 (GLGOSL)	V3-MN	V3-SF-2	V3-HXB2	r <sub>gp</sub> 120 <sub>gp</sub>	r <sub>gp</sub> 120 <sub>gp</sub>		
447-52-D	0.01	0.01	0.2	0.4	0.2	0.1	0.01	0.1	—	—	0.56	0.9	24.0	0.01	3.9		
694/98-D	0.01	0.01	0.1	1.0	0.6	1.0	0.02	1.0	—	—	ND	1.8	12.0	0.17	0.0085		
537-D	0.6	1.0	—	—	2.0	0.8	1.0	1.0	—	—	1.3	8.5	>100	ND	ND		

<sup>a</sup> ELISA reactivities of HuMAbs used at 10  $\mu$ g/ml with minimum antigen concentrations giving positive results are shown in micrograms of the V3 peptide used to coat ELISA plates per milliliter. —, lack of reactivity at antigen concentrations of 0.01 to 2.0  $\mu$ g/ml. ND, not done.

TABLE 2. Neutralization of HIV-1 infectivity in vitro by HuMAbs

HIV-1 isolate	V3 loop sequence	Neutralization endpoint ( $\mu\text{g/ml}$ ) of in vitro MT-4 cell-killing assay <sup>a</sup>					
		Anti-V3			Anti-CD4bd		
		447-52-D	537-D	694/98-D	448-D	559-D	588-D
IIIB	TRKSIRIQRGPGRAFVTIGKIG	1.29	>50	0.78	6.25	6.25	6.25
MN	YNKRKRRIHIGPGRAFYTTKNII	0.37	6.25	3.13	>25	1.56	6.25
AL-1	IYRKGRIHIGPGRAFHTTRQII	0.15	3.13	0.04	0.09	0.39	0.78
SF-2	NNTRKSIYIGPGRAFHTTGRII	0.04	>50	0.04	$\leq 0.04$	$\leq 0.09$	0.39
DU 6587-5	SNVNRRIHIGPGRAFHTTKRIT	0.62	ND	ND	ND	ND	ND
WMJ-2	NNVRRSLSIGPGRAFRTREIIG	1.35	>50	6.25	>12.5	50	>50
DU 7887-7	NNTSRGIRIGPGRAILATERII	0.78	ND	>25	>12.5	>50	>50
RF	NNTRKSITKGPGRVIYATGQII	0.62	>50	>25	>12.5	>50	6.25

<sup>a</sup> Data for 447-52-D represent the geometric mean titers from two to eight experiments; data for other HuMAbs are derived from single experiments. ND, not done.

immunochemical properties further. In primary monocyte-macrophage cultures, 447-52-D was shown to neutralize monocytotropic isolate SF-162, with an endpoint neutralization titer of 1.98  $\mu\text{g/ml}$ . To define the epitope recognized by 447-52-D more precisely, it was tested for binding with a set of overlapping hexapeptides spanning the aforementioned region of V3<sub>MN</sub>; each peptide was offset from its neighbor by one amino acid. Figure 1 shows that HuMAb 447-52-D reacted with three hexapeptides, HIGPGR, IGPGRA, and GPGRAF, suggesting that it binds to hiGPGRaf, where the capital letters of the amino acid code represent the core of the epitope and the lowercase letters represent flanking amino acids which may also contribute to binding of the epitope. The binding determinant was defined further by reacting the HuMAb with a set of peptides in which each residue of the HIGPGR hexapeptide was substituted with each of 19 amino acids. It was found that the first, second, and fifth residues of HIGPGR could be varied considerably while still allowing detectable reactivity (Fig. 2). The substitutions not permitted, e.g., C, D, and E for I at the second position, have never or only rarely been seen in virus isolates sequenced (27). However, the third, fourth, and sixth residues of this hexapeptide could not be changed without abrogating the ability of the peptide to bind to the

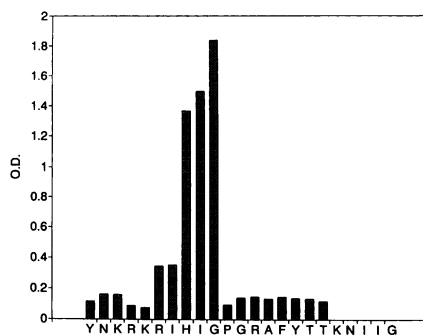


FIG. 1. ELISA reactivity of HuMAb 447-52-D with overlapping hexapeptides from the region of amino acids 306 to 328 of the gp120<sub>MN</sub> envelope. The reactivity of each hexapeptide with supernatants from the heterohybridoma is shown on the ordinate. Each hexapeptide is designated by the single-letter code of its N-terminal residue and the subsequent five amino acids. Thus, the sequence appearing on the abscissa is that of a peptide of amino acids 306 to 328. O.D., optical density at 410 nm.

HuMAb. These data suggest that the core epitope of HuMAb 447-52-D is most correctly represented as GPXR.

Although the V3 peptides with which HuMAb 447-52-D reacted contain GPGR, there were 43-fold differences in the  $K_d$  values of antibody binding to these various peptides (Table 1). The relevance of these data is underscored by the finding that the neutralizing capacity of both mouse and human anti-V3 MAbs is inversely correlated with antibody affinity (11). Further studies indicated that the reactivity of HuMAb 447-52-D for recombinant gp120 was as much as 90-fold stronger than it was for the homologous V3 peptide (Table 1), suggesting that sequences outside of the core epitope, as well as protein conformation, contribute to the strength of antibody binding.

To detect the level of antibodies in patients' sera capable of competing with 447-52-D, sera from 29 HIV-infected subjects were used; 12 had CD4 cell counts of <250, four had 251 to 500 CD4 cells, eight had 501 to 750 CD4 cells, and five had >750 CD4 cells. The sera of these subjects were tested for the ability to inhibit by 50% the binding of biotin-labeled 447-52-D to the V3<sub>MN</sub> synthetic peptide. Titers ranged from <1:2 to 1:256 (data not shown) and were 1 to 3 orders of magnitude lower than titers obtained in similar competition experiments with HuMAbs specific for gp41 determinants (46). Since several anti-V3 HuMAbs block the binding of 447-52-D to V3<sub>MN</sub> (unpublished data), these serum titers may reflect the levels of antibodies to the several epitopes at the crown of the V3 loop, including antibodies specific for GPGR. No correlation exists between progression of disease, as reflected by the number of patients' circulating CD4<sup>+</sup> cells, and the titer of antibodies capable of blocking 447-52-D.

The studies reported here demonstrate that an anti-V3 domain HuMAb exhibits greater virus-neutralizing potency and is more broadly reactive than comparable antibodies to the CD4bd. However, such anti-V3 domain antibodies appear to be present only at low levels in the sera of HIV-1-infected individuals. The broad neutralizing activity of 447-52-D is due to its ability to bind to the GPXR determinant. This sequence is present in approximately 80% of V3 domain sequences that have been derived from HIV-1 isolates in North America and Europe (22). This sequence is not as prevalent among African HIV-1 isolates (27). However, within the limits of this geographical restriction, the potential exists for use of this antibody as an immunoprophylactic and immunotherapeutic agent. These results have implications for the design of active vaccine immunogens in that they

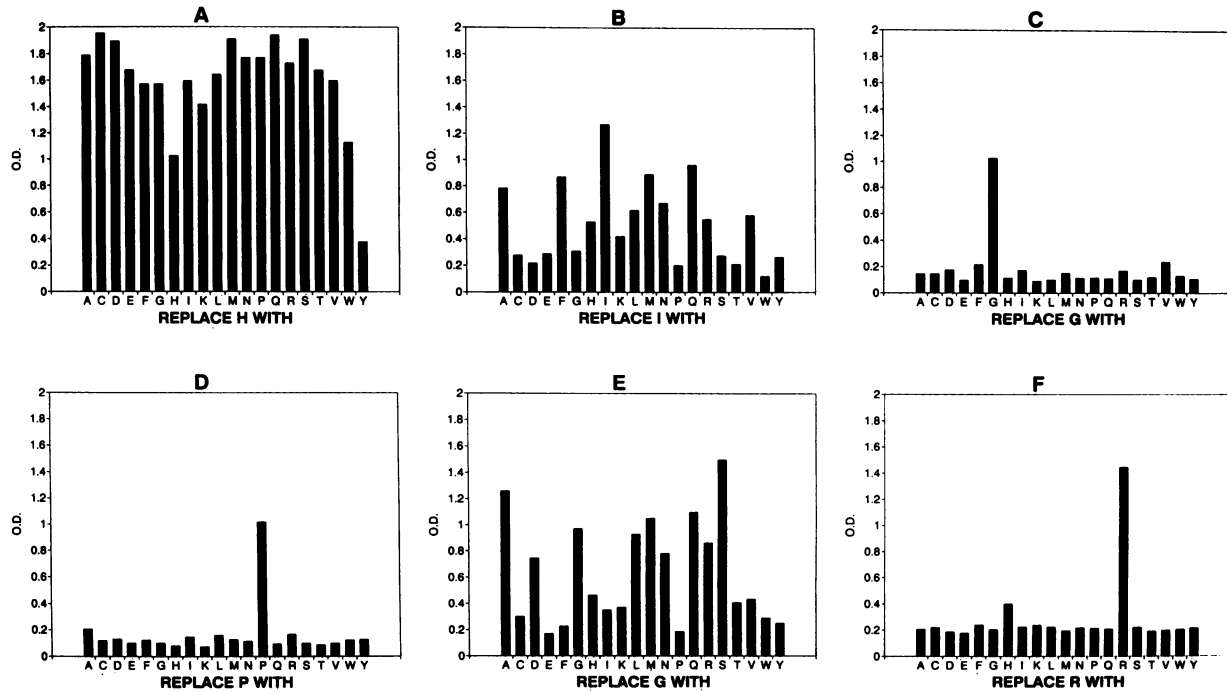


FIG. 2. Replacement set analysis. The replacement set of peptides is based on the parent peptide HIGPGR (amino acids 313 to 318) located at the tip of the V3<sub>MN</sub> loop. Each block of 20 ELISA values, determined as described in the legend to Fig. 1, represents the results obtained with peptides containing the single amino acid substitution identified by the single-letter code beneath each bar. Panels A through F represent substitutions at positions H, I, G, P, G, and R, respectively. O.D., optical density at 410 nm.

directly demonstrate that the human immune system, given an appropriate antigenic stimulus, is capable of mounting a potent and broadly reactive anti-V3 antibody response.

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