Human Anti-V2 Monoclonal Antibody That Neutralizes Primary but Not Laboratory Isolates of Human Immunodeficiency Virus Type 1

MIROSLAW K. GORNY,¹ JOHN P. MOORE,² ANTHONY J. CONLEY,³ SYLWIA KARWOWSKA,^{1†}
JOSEPH SODROSKI,⁴ CONNIE WILLIAMS,⁵ SHERRI BURDA,⁵ LYNN J. BOOTS,³
AND SUSAN ZOLLA-PAZNER^{1,5*}

*New York University Medical Center,¹ Aaron Diamond AIDS Research Center,² and Veterans Affairs Medical Center,⁵
New York, New York; Merck Research Laboratories, West Point, Pennsylvania³; and Division of Human
Retrovirology, Dana-Farber Cancer Institute, Department of Pathology,
Harvard Medical School, Boston, Massachusetts⁴*

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A human immunoglobulin G1 lambda monoclonal antibody (MAb), 697-D, was developed that recognizes the V2 region of human immunodeficiency virus type 1 (HIV-1) gp120. Substitutions at amino acid positions 176/177, 179/180, 183/184, and 192 to 194 in the V2 loop of gp120 each completely abolished the binding capacity of 697-D in an enzyme-linked immunosorbent assay format. Competition analysis with three different neutralizing murine anti-V2 MAbs confirmed the specificity of 697-D. The 697-D epitope is primarily conformation dependent, although there was weak reactivity of the MAb with a V2 peptide spanning residues 161 to 180. Treatment of recombinant gp120 HIV_{IIIB} with sodium metaperiodate, which oxidizes carbohydrates, abolished the binding of the MAb, showing the dependence of the epitope on intact carbohydrates. The broad reactivity of 697-D was displayed by its binding to the gp120 molecules from four of four laboratory isolates and five of five primary isolates. The MAb 697-D neutralized three out of four primary isolates but failed to neutralize any of four laboratory strains of HIV-1. 697-D and a human anti-V3 MAb, 447-52-D, displayed similar potency in neutralizing primary isolates, indicating that the V2 region of gp120, like the V3 region and the CD4-binding domain, can induce potent neutralizing antibodies against HIV-1 in humans.

Characterization of the immunodominant and neutralizing regions in the envelope proteins of human immunodeficiency virus type 1 (HIV-1) is essential for both the design of a vaccine and the preparation of polyclonal and/or monoclonal antibodies (MAbs) for immunoprophylaxis. The generation of human MAbs has allowed the characterization of several neutralizing sites on gp160; these include the V3 loop and the CD4-binding domain (CD4bd) of gp120 and an epitope in the gp41 ectodomain (11, 13, 16, 29, 35, 37, 42). Another region in the V1 loop of gp120 that induces potent neutralizing antibodies was identified by affinity purification of antibodies from the serum of an HIV_{IIIB}-infected laboratory worker (33). Two additional neutralizing regions have been localized by using various MAbs from rodents and nonhuman primates to the V2 and the C4 regions of gp120 (6, 23, 27, 31, 43), and the binding of CD4 to gp120 exposes yet another set of neutralizing epitopes (15, 41).

The V2 region is contained in a loop stabilized by a disulfide bond and is closely linked to the structure of the first variable (V1) region (22). A conserved region of no more than 12 amino acids (aa) is located between the V1 and V2 regions (24). Recent studies have shown that the V2 region contributes to various functions of the virus. By using mutants with amino acid substitutions in the V2 region, fusion-deficient phenotypes were demonstrated. This may indicate that the V2 region plays

a role in post-receptor binding events in the membrane fusion process (38). On this basis, it was postulated that anti-V2 antibodies would interfere with virus-cell fusion and subsequently neutralize the virus. Furthermore, the V1 and V2 regions interact with the V3 loop, contributing to the cell tropism of macrophage-tropic viruses (20). Also, the V2 region contributes to properties that render HIV-1 isolates sensitive to neutralization by soluble CD4 (sCD4) (20).

The V2 loop contains both linear and conformation-dependent epitopes (6, 23, 27). The conformational epitopes of the V1/V2 regions were recognized by about half of the serum samples tested from HIV-infected individuals (19), whereas only one-fifth of the patients' serum samples were reactive with V2 peptides that contain only linear epitopes (23, 27).

In this paper, we describe the first human MAb to the V2 loop. This MAb neutralizes primary isolates of HIV-1 but not laboratory strains of HIV-1. While binding weakly to a V2 peptide, it is also conformation dependent and binds to the monomeric gp120 glycoproteins from various laboratory and primary isolates of HIV-1.

MATERIALS AND METHODS

MAbs, recombinant proteins, and viral lysates. The MAbs used in these studies are listed in Table 1. The human MAb 860, which neutralizes cytomegalovirus (CMV), was produced from the peripheral blood mononuclear cells (PBMCs) of an HIV-seropositive subject.

Our method for the production of human MAbs has been previously described (11). Briefly, PBMCs were isolated from heparinized blood from HIV-1-seropositive individuals and transformed with Epstein-Barr virus. Cultures that were posi-

* Corresponding author. Mailing address: Veterans Affairs Medical Center, 423 E. 23rd St., Room 18124N, New York, NY 10010. Phone: (212) 951-3211. Fax: (212) 951-6321. Electronic mail address: ZOLLA@MCCLB0.MED.NYU.EDU.

† Present address: Miles, Inc., Diagnostic Division, Tarrytown, NY 10591.

TABLE 1. MAbs used in this study

MAb	Epitope	Neutralizing activity	Source or reference(s)
Murine			
G3-4	V2	+	6, 12, 27, 38
BAT-085	V2	—	6, 12, 27, 38
SC258	V2	+/-	27
684-238	V2	+	27
G3-536	C4	—	28, 39
G45-60	C4	—	28, 39
Human			
697-D	V2 (see text)	+	This paper
447-52-D	V3 (GPGR)	+	10
694/98-D	V3 (GRAF)	+	10
654-D	CD4bd (conformational)	—	18
450-D	C terminus (PTKAKRR)	—	16
860	CMV (gB specific)	—	This paper

tive for the desired antibodies were expanded and then fused with the SHM-D33 human $\times$ mouse heteromyeloma (40). The resultant heterohybridomas were rescreened for the production of the desired antibody, and the positive cultures were sequentially cloned at limiting cell concentrations until monoclonality was achieved.

HIV_{IIIB} recombinant (r) gp120 (BH10 clone) expressed in Chinese hamster ovary (CHO) cells by Celltech, Ltd. (Slough, United Kingdom) was obtained from the United Kingdom Medical Research Council AIDS Directed Program (26). rgp160 from HIV_{IIIB} (LAI strain), commercially denatured by urea, mercaptoethanol, and sodium dodecyl sulfate and prepared in baculovirus-infected insect cells, and rgp120 were purchased from Repligen (Cambridge, Mass.), and rgp120 from HIV_{SF-2} produced by CHO cells was kindly supplied by K. Steimer (Chiron, Emeryville, Calif.). Strain MN gp120, purified from culture supernatants of MN-infected cells, was a gift from L. Arthur (National Cancer Institute, Frederick, Md.). Virus lysates of the laboratory strain RF and of primary isolates JR-CSF, VS, CB24, MG47, CJ48, MP49, and JC50 were used for immunochemical studies. sCD4, an engineered protein made in CHO that lacks the transmembrane and intracellular domains of CD4, was purchased from Du Pont (Boston, Mass.).

Viruses. Virus stocks of laboratory isolates HIV_{IIIB}, HIV_{RF} (34), and HIV_{MN} (7) were prepared from the chronically infected H9 human T-lymphoid cell line. Isolate HIV_{AL-1} (2) was prepared from the chronically infected FDA/H9 cell line. Virus stocks were prepared from culture media 3 days after the suspension of the respective chronically infected cell line in fresh medium at a density of 2×10^5 cells per ml. For each virus stock of each isolate, the 50% tissue culture infective dose (TCID₅₀) was determined on CEM-SS cells as described by Johnson and Byington (14).

Primary isolates were generated by the coculture of infected and uninfected PBMCs according to the method described by Gartner and Popovic (8). The culture supernatants were harvested every second day after the seventh day of culture and used as stock virus. For the neutralization assays, stock virus of primary isolates MG47, CJ48, MP49, and JC50 was titrated on normal PBMCs and each TCID₅₀ per ml was defined. Lysates were made by treating stock virus preparations with 1% Nonidet P-40 or 1% Empigen detergent (Calbiochem, La Jolla, Calif.).

Extraction of viral RNA and sequence analysis. The gp120

V1-V2 region sequences of the primary isolates used in this study were determined by methods described previously (3, 4). Extracted viral RNA was used to amplify the V1-V2 region by a combinational use of the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.) and the CloneAmp System (Life Technologies, Inc., Gaithersburg, Md.). The oligonucleotide primer used in the reverse transcriptase reaction was 5'-(CAU)₄GTACATTGTACTGTGCTGACATT-3'. The 13th nucleotide represents nucleotide pair number 727 of the *env* gene (subtype B) consensus (30). The second primer used in the PCR mix addition was 5'-(CUA)₄AAGCCATGTGTA AAATTAACCC-3', where the 13th nucleotide represents nucleotide pair number 351 of the *env* gene consensus (30). PCR products were cloned by reacting with uracil DNA glycosylase while annealing to the pAMP vector plasmid. Sequence analysis of verified insert clones was performed as previously described with a modification of the Sequence version 2.0 (UAB) protocol (3, 4).

Binding of MAbs to monomeric gp120. Virus-derived gp120 and rgp120 were captured onto enzyme-linked immunosorbent assay (ELISA) plates via adsorbed sheep antibody D7324 specific for the C terminus of gp120 (25, 28). Viral gp120 derived from the RF strain of HIV-1 was used at a concentration of approximately 100 ng/ml, whereas SF-2 gp120 was used at 1,000 ng/ml and BH10 gp120 was used at 25 ng/ml. The gp120 levels in stocks of primary isolates ranged between 2.4 and 4.8 ng/ml. Bound human MAbs were detected with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Accurate Chemicals or Zymed), followed by color development with the AMPAK or ELAST amplification system (Dako Diagnostics or GIBCO, respectively).

For competition experiments, murine MAbs were added to anti-C-terminus-captured gp120 for 30 min before the addition of human MAb.

To detect the ability of MAbs to block gp120-CD4 binding, human MAbs at various concentrations were mixed with sCD4 and with rgp120 HIV_{IIIB}. The mixture was incubated for 2 h at 37°C and then added to a microplate coated with murine anti-V3 HIV_{IIIB} antibodies. After incubation, the presence of sCD4 was detected with horseradish peroxidase-labeled mouse anti-CD4 MAb (American BioTechnologies, Cambridge, Mass.). This reaction was amplified with the ELAST amplification system. The blocking anti-CD4bd MAb 654-D was used as a positive control, and the MAb 450-D was used as a nonblocking control. The percentage of blocking of binding of sCD4 to rgp120 was calculated by using a formula described previously (16).

Treatment of gp120 with sodium metaperiodate. The oxidation of the carbohydrate moieties of gp120 was performed with sodium metaperiodate according to the method of Woodward et al. (44). Briefly, rgp120_{SF-2} or gp120 from primary isolate CB24 was captured by immobilized anti-C-terminus antibody and incubated with NaIO₄ at concentrations ranging from 0 to 12.5 mM at pH 4.65 for 2 h in the dark at 4°C. After blocking the NaIO₄ with 2% glycine at pH 9.6, the human MAbs were added and their binding was detected with alkaline phosphatase-coupled goat anti-human immunoglobulin G.

Epitope mapping with gp120 mutants. The binding site for the 697-D MAb was assessed with mutant gp120 molecules. The mutants were produced by transfecting COS-1 cells with 10 μ g of pSVIIIenv plasmid expressing either wild-type or mutant HIV-1 (HxBc2) envelope glycoproteins. The culture supernatants from transfected cells were used in an ELISA format in which the gp120 molecules were captured onto a solid phase via antibody D7324. Calculation of binding ratios

and the criteria for assessing the significance of the data have been described elsewhere (28).

Determination of MAb affinity. The dissociation constant (K_d) for the human MAb was determined by ELISA according to the method of Friguet et al. (5). Briefly, the culture supernatants containing 0.5 to 2.5 μ g of MAb 697-D per ml were incubated overnight at room temperature with rgp120 of SF-2 or IIIB at various concentrations (10^{-5} to 10^{-8} M). The mixture was tested by ELISA for the presence of excess antibody not complexed with antigen by adding the mixture to microplate wells precoated with homologous antigen at 1 μ g/ml. The optical density (OD) and antigen concentration values were plotted according to the Friguet modification of the Klotz equation to determine K_d .

Determination of neutralizing activity. The ability of the MAbs to neutralize HIV_{IIIB}, HIV_{RF}, HIV_{AL-1}, and HIV_{MN} was assessed by a modification of the Nara method (32), wherein the microscopic readout is replaced by a p24 ELISA (21). Briefly, serial twofold dilutions of MAbs were incubated for 1 h at room temperature with 100 TCID₅₀ of the respective isolate. The virus-antibody mixture was incubated for 1 h at 37°C with CEM-SS cells that had been made to adhere to a poly-L-lysine-coated 96-well plate, after which the virus-antibody mixture was removed. The cells were then cultivated for 5 days, and the amount of p24 in culture supernatants was assayed with an antigen-capture ELISA (21).

The ability of the MAbs to neutralize primary HIV-1 isolates was determined by two methods. For the neutralization assay, 1.5 to 100 μ g of human MAbs per ml was incubated with 100 TCID₅₀ of input virus for 1 h at 37°C. The MAb-virus mixture was then plated on 100,000 PBMCs per well. The PBMCs had been stimulated 3 days earlier with 5 μ g of phytohemagglutinin per ml. Cultures of virus-infected cells were incubated for 7 to 14 days, and the supernatant was harvested for p24 quantitation. The second test, an infectivity reduction assay, was performed according to the method described by Conley et al. (4). Serially diluted primary virus stock containing 20 to 0.01 ng of p24 per well was incubated with a constant concentration of MAb (100 μ g/ml) for 1 h at 37°C. Next, 50,000 cells (PBMCs stimulated for 3 days with 5 μ g of phytohemagglutinin per ml and stimulated for a subsequent 2 days with interleukin 2 [Boehringer, Mannheim, Germany] at 20 U/ml) were added to each MAb-virus mixture. Virus and MAbs were maintained in culture for 7 days, and the p24 levels in the supernatants were then measured. The reduction of infectivity was determined by comparison of p24 levels in the presence and absence of the test MAb.

Nucleotide sequence accession number. All sequences reported in these studies have been submitted to GenBank and assigned accession numbers U14537 to U14547.

RESULTS

Mapping the specificity of MAb 697-D. The human MAb 697-D binds strongly in ELISA to rgp120_{IIIB} but binds very weakly to denatured rgp160_{IIIB}, suggesting that the epitope is primarily conformation dependent (Table 2). Furthermore, MAb 697-D was found not to react in ELISA with a gp120 mutant lacking the V1 and V2 loops (deletion of aa 119 to 205) or with a mutant lacking the V1, V2, and V3 regions (deletion of aa 121 to 204 and 298 to 329) (Table 3). The ability of MAb 697-D to inhibit sCD4-gp120 binding also was tested. The results demonstrated that 697-D and the negative control MAb 450-D do not significantly inhibit the interaction of rgp120 with sCD4 at any concentration up to the maximum used (3.3 μ g/ml). In contrast, MAb 654-D, which recognizes the CD4bd,

TABLE 2. Binding properties of V2 MAbs

Antigen	Binding of MAb ^a :	
	697-D	BAT-085
rgp120 _{IIIB}	++++	++++
rgp160 _{IIIB} (denatured)	+	++++
V2 peptide ^b		
aa 151-170	—	—
aa 161-180	+	++++
aa 171-190	—	++
NaIO ₄ -treated rgp120 _{IIIB}	—	NT ^c

^a Binding is expressed as follows: +++++, OD of >1.5; +++, OD of 1.0 to 1.5; ++, OD of 0.5 to 1.0; +, OD of 0.22 to 0.5; —, OD below cutoff (OD = 0.21).

^b V2 peptides had the following sequences: aa 151 to 170, KGEIKNCSFNIST-SIRGKVQ; aa 161 to 180, ISTSIRGKVQKEYAFFYKLD; aa 171 to 190, KEYAFFYKLDIIPIDNDTTS.

^c NT, not tested.

required 0.11 μ g/ml for 50% blocking of rgp120-sCD4 binding (data not shown). These data show that 697-D is not specific for the CD4bd and that gp120 recognition by 697-D depends upon the presence of the V1 region and/or V2 region.

In view of the weak reactivity of MAb 697-D with denatured rgp160_{IIIB}, we screened MAb 697-D against three overlapping peptides spanning the V2 loop (Table 2). The MAb did not react with peptides of aa 151 to 170 or 171 to 190 (OD₄₉₂ of 0.107 and 0.084, respectively) but showed weak reactivity with the aa 161 to 180 peptide (OD₄₉₂ of 0.239). In contrast, murine MAb BAT-085 reacted strongly with the peptides of aa 161 to 180 and 171 to 190 (OD₄₉₂ of 1.730 and 1.321, respectively). The weak reactivity of 697-D with the peptide of aa 161 to 180 was similar to that observed with murine MAb G3-4 (27); these MAbs cross-compete (described below).

Next, we used as antigens a previously characterized set of mutant HxBc2 gp120 molecules. The mutants were secreted as

TABLE 3. Binding of 697-D to mutant HxB2 gp120 molecules^a

Virus or mutant	Domain	MAb/HIV-1 serum binding ratio ^b
Panel average		1.00
Wild type		1.14
Deletion of aa 119-205	V1/V2	0.00
Deletion of aa 121-204 and 298-329	V1/V2/V3	0.00
168 K/L	V2	0.48
176/177 FY/AT	V2	0.00
179/180 LD/DL	V2	0.00
183/184 PI/SG	V2	0.10
192-194 YSL/GSS	V2	0.00
256 S/Y	C2	2.06
262 N/T	C2	2.00
384 Y/E	C3	1.39
420 I/R	C4	1.77

^a Binding to the other mutants tested was not significantly different from that to the wild-type gp120 glycoprotein. A listing of the entire panel of mutants tested was published previously (27).

^b The MAb concentration used was 3 μ g/ml. Binding ratios are expressed relative to the average value across the panel of mutants (0.31 ± 0.08), which was defined as 1.00 for purposes of normalization. Values of <0.5 or >1.5 are considered indicative of inhibitory or enhancing substitutions, respectively. The binding ratio for the 384 Y/E mutant does not meet these criteria but is included because of its probable proximity to residue 420 at the base of the V4 loop.

FIG. 1. ELISA experiments to map the specificity of MAb 697-D. (A) Inhibition of binding of human MAb 697-D by murine MABs BAT-085 (▲), SC-258 (*), and G3-4 (□). The murine MABs were incubated at a fixed concentration of 10 μ g/ml with BH10 gp120 before the addition of various concentrations of 697-D and the subsequent detection of bound human MAB. The binding of 697-D in the absence of murine MABs is also shown (■). (B) Enhancement of binding of 697-D to BH10 gp120 in the presence of murine anti-C4 MABs G3-536 (▲) and G45-60 (□). Binding of 697-D alone with (■) or without (+) gp120 captured on the plate is also shown. Points on the curves with error bars are the means of triplicate determinations $\pm$ 1 standard deviation (A and B). (C) ELISA reactivity of 697-D with gp120 from different HIV-1 strains. rgp120 and native gp120 from virus preparations lysed with 1% Nonidet P-40, RF (×), BH10 (⊗), VS (*), MN

soluble molecules from transfected COS-1 cells (28). 697-D was unable to bind to mutants containing amino acid changes near the tip of the V2 loop (mutants 176/177 FY/AT and 179/180 LD/DL) or on the carboxy-terminal side of the V2 loop (mutants 183/184 PI/SG and 192–194 YSL/GSS) (Table 3). A few other changes outside the V2 loop had a minor effect on the binding of the V2 MAB. Several amino acid substitutions in the C2 domain (256 S/Y and 262 N/T), the C3 domain (384 Y/E), and the C4 domain (420 I/R) enhanced the binding of 697-D. These substitutions presumably indirectly increase the exposure of the V2 epitope for 697-D.

Blocking experiments with three murine anti-V2 MABs were performed to compare their epitopes with the binding specificity of 697-D. Murine MABs BAT-085, SC 258, and G3-4 were each incubated at 10 μ g/ml with captured BH10 gp120. The subsequent binding of 697-D to gp120 was inhibited by SC 258 and G3-4 (Fig. 1A). The blocking effect of BAT-085 was less pronounced, perhaps because of its substantially lower affinity compared with that of the other two murine MABs (27). These data confirm that 697-D recognizes an epitope in the V2 loop and that this epitope is related to that recognized by several murine anti-V2 MABs.

Because ligands to the CD4-binding domain have been reported to induce conformational changes in the structure of gp120/41 oligomers leading to enhanced or decreased binding of some V2 MABs to gp120 (23, 27), the effect of anti-C4 MABs on binding of 697-D to monomeric gp120 was tested (Fig. 1B). BH10 gp120 was captured on an ELISA microplate via anti-C-terminus antibodies and then was incubated with 10 μ g of the murine anti-C4 MAB G3-536 or G45-60 per ml, followed by incubation with 697-D at concentrations ranging from 0.05 to 10 μ g/ml. The anti-C4 MABs, particularly G3-536, enhanced the binding of 697-D to gp120. This suggests that interaction of anti-C4 MABs with gp120 leads to the enhanced exposure of the V2 epitope recognized by 697-D. Similar results have been obtained with murine anti-V2 MABs in place of MAB 697-D (data not shown).

Next, the cross-reactivity of 697-D was determined by measuring its ability to bind rgp120 (BH10, SF-2), native gp120 from laboratory isolates (MN and RF), and native gp120 from primary isolates (JR-CSF, VS, MG47, CJ48, MP49, and JC50). The MAB reacted with all of the gp120 preparations. The strongest reactivity with gp120 from laboratory strains was observed with gp120 from RF, and the weakest was observed with rgp120 of SF-2 (Fig. 1C). Among four primary isolates tested, gp120 from all isolates was well recognized (Fig. 1D).

Because gp120 of HIV-1 is heavily glycosylated, we studied the effect of altering the carbohydrate residues on the binding of 697-D (Fig. 2). rgp120_{SF-2} and gp120 from the primary isolate CB24, both captured by polyclonal anti-C-terminus antibodies, were treated with concentrations of NaIO₄ ranging from 0 to 12.5 mM. This treatment results in mild oxidation of carbohydrate moieties and abrogates the binding of sCD4 to rgp120 (data not shown). The binding of the anti-V3 MAB 447-52-D to treated and untreated gp120s of SF-2 (Fig. 2A)

(▲), JR-CSF (□), and SF-2 (⊗) were captured on the solid phase and then allowed to react with 697-D at concentrations between 0.01 and 10 μ g/ml. (D) ELISA reactivity of 697-D with native gp120 from primary isolates. gp120 from MG47 (■), CJ48 (□), MP49 (▲), and JC50 (*) and rgp120 of IIIB (⊗) at concentrations ranging from 2.4 to 4.8 ng/ml were captured on the solid phase in the presence of 1% Empigen. rgp120_{IIIB} (LAI) was tested at a concentration of 50 ng/ml. 697-D reacted with gp120 at the concentrations indicated.

FIG. 2. Reactivity of MAbs with gp120 treated with sodium meta-periodate. Anti-V2, 697-D (■), anti-V3, 447-52-D (▲), and anti-CD4bd 654-D (□) human MAbs were allowed to bind to rgp120 SF-2 (A) or native gp120 from primary isolate CB24 (B) treated with sodium metaperiodate at various concentrations.

and CB24 (Fig. 2B) was unchanged. In contrast, treatment of both gp120s with NaIO_4 significantly affected the binding of 654-D (an anti-CD4bd MAb) and 697-D (Fig. 2). Because low levels of metaperiodate do not denature proteins (44), these results suggest that the binding of 697-D (like that of 654-D) depends on intact carbohydrates and/or the protein structures that they stabilize.

The K_d s of 697-D for rgp120 of HIV_{IIIB} and HIV_{SF-2} were found to be 1.5 and 3.3 nM, respectively; these values are in the range of the K_d s of anti-V3 and anti-CD4bd MAbs, which are able to neutralize HIV_{IIIB} and HIV_{SF-2} (10, 16), suggesting that the absence of neutralizing activity by 697-D against laboratory strains (see below) is not due to a high K_d .

Neutralizing activity of MAb 697-D. The MAb 697-D and three control MAbs (694/98-D [human anti-V3], G3-4, and 684-238 [murine anti-V2]) were tested for their ability to neutralize cell-free HIV_{IIIB} (Fig. 3A). The anti-V3 MAb 694/98-D was able to neutralize 50% of IIIB infectivity (IC_{50}) at a concentration of 0.15 µg/ml. (Anti-V3 MAb 447-52-D had previously been shown to yield 50% neutralization of IIIB at 1.8 µg/ml [10].) The mouse MAbs 684-238 and G3-4 were weakly neutralizing, with IC_{50} s for each of 84 and 53 µg/ml, respectively. The anti-V2 human MAb 697-D did not neutralize IIIB at the highest concentration used, 100 µg/ml. Three more laboratory isolates were tested for their ability to be neutralized. HIV_{RF}, HIV_{AL-1}, and HIV_{MN} were all neutralized by the human anti-V3 MAb 447-52-D with an IC_{50} of <1 µg/ml, while the MAb 697-D was unable to achieve 50% neutralization at concentrations up to and including 50 µg/ml (data not shown).

Furthermore, MAb 697-D was tested for its ability to neutralize four primary isolates that were generated by coculture from the cells of HIV-1-seropositive individuals. Because

FIG. 3. Neutralization of laboratory strains (A) and primary isolates (B) of HIV-1. (A) HIV_{IIIB} neutralization by MAbs with the syncytium-forming assay with a p24 ELISA readout. Serially diluted human MAbs anti-V2 697-D (■) and anti-V3 694/98-D (▲) and murine anti-V2 MAbs G3-4 (*) and 684-238 (□) were incubated with cell-free virus and then added to target cells. One-hundred percent virus is equal to 39 ng of p24 per ml, which was produced in cultures of cells exposed to virus alone. (B) Neutralization of primary isolate CJ48 by 697-D (■), 447-52-D (anti-V3) (▲), and 860 (anti-CMV) (□). In the neutralization assay, a constant virus input, 100 TCID_{50} , was incubated with serially diluted MAbs, after which the mixture was added to 3-day phytohemagglutinin-stimulated PBMCs. The p24 level was measured in culture supernatant on day 7.

the anti-V3 MAb 447-52-D had been shown to efficiently neutralize the majority of primary isolates tested (3), we compared its activity to that of 697-D and to the activity of an irrelevant MAb, 860, which is specific for CMV. The neutralization curves of these three MAbs tested against primary isolate CJ48 are shown in Fig. 3B. The IC_{50} s of 447-52-D and 697-D are identical, 2.0 µg/ml, while the control anti-CMV MAb did not achieve 50% neutralization. This experiment shows that the anti-V2 MAb 697-D is comparable to an anti-V3 MAb in its ability to neutralize this primary isolate. 697-D was then tested against three other primary isolates. Two of the three (MG47 and MP49) were neutralized with IC_{50} s of 10.0 and 11.0 µg/ml and IC_{90} s of 12.5 and 50.0 µg/ml, respectively. The third isolate, JC50, was not neutralized by 447-52-D, 697-D, or 860 at concentrations up to 50 µg/ml (data not shown).

One of the primary isolates that was relatively resistant to neutralization, MP49, with an IC_{90} with 697-D of 50 µg/ml, was retested in an infectivity reduction assay with 100 µg of 697-D per ml. There was a fourfold difference between the neutralization end point (90% or greater) observed with MAb 697-D and the end point of the virus stock titration in the absence of

FIG. 4. Infectivity reduction assay with primary isolate MP49. Serially diluted primary virus stock in the range 1.0 to 0.01 ng of p24 was incubated in the absence of MAb (□) and with a constant concentration (100 μ g/ml) of 697-D (■) and then was added to 5-day-stimulated PBMCs. The p24 level was determined on day 7. Each point on the curves represents the mean of duplicate determinations, with the individual values varying by a maximum of 15%.

MAb (Fig. 4). In previous experiments (3), we showed that, on this basis, this virus would be classified as belonging to the group of least-sensitive primary isolates.

In order to discern why some viruses were sensitive and some were resistant to neutralization by MAb 697-D, the V1 and V2 regions of the primary isolates that were used were sequenced. Virus MG47 RNA gave five identical clones in the V2 region (Table 4). CJ48 gave five different sequences from the eight clones that were sequenced. Clones CJ48-b and CJ48-e had identical V2 regions yet were different in V1 (V1 not shown). Virus MP49 had the largest V2 region, 54 residues, and had two different V2 sequences. Virus JC50, which displayed resistance to neutralization by MAb 697-D had the

smallest V2 region, 40 residues, and had three unique sequences that differed from the sequences of the primary isolates that displayed susceptibility to MAb 697-D. These unique sequences were SD at positions corresponding to LD in HxB2 (residues 179/180), residues LI (or SI) at positions corresponding to PI in HxB2 (residues 183/184), and an S at residue 164 in place of an N residue in all other virus strain primary isolates studied herein (Table 4). The only correlation that can be made between the sequence and neutralization data is that MAb 697-D fails to neutralize the viruses that have an S residue at position 164. Susceptibility of primary isolates to neutralization by MAb 697-D did not appear to be related to the glycosylation sites that varied greatly among the V1-V2 regions of these primary isolate sequences (data not shown).

DISCUSSION

We have described a human MAb against the V2 region of gp120 of HIV-1 that shows broad immunochemical reactivity with several laboratory and primary isolates and the ability to neutralize three of four primary isolates but none of four laboratory strains. The mapping of this MAb with gp120 mutants and competing murine anti-V2 MAbs indicates that the 697-D epitope spans the region from the tip to the C-terminal side of the V2 loop. The V2 region contains both linear and conformation-dependent epitopes (6, 23, 27). The epitope at the tip of the V2 loop that is recognized by murine MAb BAT-085 is continuous in nature because the murine MAb binds exceptionally well to V2 peptides representing this part of the loop (aa 170 to 180) and to denatured gp120 (27). 697-D bound very weakly to denatured gp160 and to the aa 161 to 180 peptide of the V2 region and competed only weakly with BAT-085 for binding to gp120; therefore, it appears that the 697-D epitope is primarily conformation dependent with weak recognition of a linear epitope. Similar features have been displayed by the murine anti-V2 MAbs G3-4 and G3-136,

TABLE 4. Amino acid sequence of the V2 region of HIV-1 gp120 of primary and laboratory isolates used in neutralization assays

Virus	Sequence	Total found/no. sequenced
MG47	CSFNITTNIRDKVQENYALFYKLDVVPIDGSDNTSYRLISC	5/5
CJ48		
-a	CSFNITTNIRDKIKKEYALFDKLDIVQIDNEIDNDSDSYRLINC	4/8
-b	-----R-----S-----	1/8
-c	-----F-----P-N-- --T-----	1/8
-d	-----V-----F-----NV-P-N-- --T-----	1/8
-e	-----R-----S-----	1/8
MP49		
-a	CSFNITTNMRDKMQQYATFYKLDVEQIDDNKNTNHNNTTDNASKDYRLISC	3/4
-b	-----N-----	1/4
JC50		
-a	CSFNITT ^S IRDKIEKEYALFYK ^S DVILIDENTTSYRLTSC	2/4
-b	-----VS-E-----I--	1/4
-c	-----MQ-----VS-E-----I--	1/4
LAI	CSFNIST ^S IRGKVQKEYAFFYKLDIIPIDNDTTSYTLTSC	
AL-1	CSFNITT ^S IRDKMQKESALFHKLDDVVPIDNNNTSYILISC	
MN	CSFNITT ^S IRDKMQKEYALLYKLDIVSIDNDSTSYRLISC	
RF	CSFQVTT ^S RRDKTQKKYALFYKLDVVP ^I EKGNI ^S PKNNTSNNTSYGNYTLIHC	

which react weakly with the same V2 peptide of aa 161 to 180 and which are conformation dependent (27).

Unusual features of 697-D are its broad reactivity and its ability to neutralize primary isolates. While several murine and rat anti-V2 MAbs display restricted binding with gp120 from several laboratory isolates (23, 27), 697-D binds to all nine gp120 molecules tested, which were derived from laboratory and primary isolates of the B clade. The murine and rat anti-V2 MAbs were obtained by immunization and selection with virus-derived gp120_{IIIB} or rgp120_{BH10}, while the human MAb was induced by natural infection and selected on rgp120_{IIIB}. This may account for the relatively narrow reactivity of the rodent MAbs as opposed to the broad cross-reactivity of the human MAb. Similar cross-reactivity was observed among human MAbs against the V3 loop, another variable region that induces neutralizing antibodies (9, 11, 17). The broad cross-reactivity of human anti-V2 and anti-V3 MAbs suggests the existence of an immunologic resemblance between diverse isolates even in variable regions of the envelope.

The anti-V2 MAbs of animal origin are relatively restricted in the number of laboratory strains they neutralize but vary greatly in potency (6, 23, 27, 43). In contrast, the human MAb 697-D neutralizes three out of four primary isolates tested with a potency similar to that of the human anti-V3 MAb 447-52-D. However, 697-D lacks the ability to neutralize laboratory strains HIV_{IIIB}, HIV_{RF}, HIV_{AL-1}, and HIV_{MN}, whereas the broadly reactive anti-V3 MAb 447-52-D neutralizes both laboratory and primary strains of HIV-1 (3, 9, 10). Both MAbs 697-D and 447-52-D react in ELISA with rgp120 HIV_{IIIB}, but the affinity of 697-D is 1 order of magnitude greater than that of 447-52-D (10). Thus, the failure of 697-D to neutralize HIV_{IIIB} must depend on factors other than its affinity for the monomeric gp120 glycoprotein. Rat and chimpanzee MAbs to V2 neutralized both the BH10 clone and HIV_{IIIB} with potencies comparable to those of anti-V3 MAbs (23, 43). In contrast, three out of eight murine anti-V2 MAbs displayed some neutralizing capability for HIV_{IIIB}, although much less than murine anti-V3 MAbs (27). Such differences in neutralization could be attributable to the ability of humans, chimps, and rats to recognize neutralizing epitopes in the V2 region that are poorly recognized by mice. Alternatively, differences in the antigen and immunization schemes could account for the differences in neutralization.

The fact that MAb 697-D is the first human anti-V2 MAb to be described may reflect the relatively poor immunogenicity of the V2 region. Indeed, the frequency of peptide-reactive serum anti-V2 antibodies is low. Twenty-one and 12% of HIV-positive serum samples from two separate studies contained antibodies against peptides representing the consensus and HXB2 V2 sequence, respectively (23, 27). These results, however, do not reflect all humoral activity against the V2 loop, because peptides present only linear epitopes. Recently, Kayman et al. (19) demonstrated that 50 and 44% of hemophilic and homosexual male patients' serum samples, respectively, recognized a fusion glycoprotein containing the native HXB2 V1/V2 domains. Because patients are unlikely to have been exposed to HIV_{IIIB}, and the aforementioned studies examined the patients' reactivity to V2 sequences of IIIB clones, only antibodies to conserved epitopes in the V2 region would have been measured. Type-specific immune responses against the V2 region of a patient's own isolates could be more frequent.

The conserved V2 epitope for which MAb 697-D is specific can be discerned in general terms from several of the studies described above. Thus, MAb 697-D binds to the peptide of aa 161 to 180 (Table 2); mouse MAbs SC258 and G3-4 block the

activity of 697-D (Fig. 1A); the binding of 697-D to gp120 is abrogated when residues 176/177, 179/180, 183/184, and 192 to 194 of gp120 are substituted (Table 3); and 697-D fails to neutralize viruses tested with an S residue at position 164 of HxB2 (Table 4) (which corresponds to position 149 of the clade B consensus), although this MAb binds to the gp120 derived from these resistant viruses. These data suggest that the binding site of MAb 697-D spans a region from residue 164 to residue 194. Because, however, the size of an antibody combining site normally encompasses up to only about 7 aa (36), the epitope of 697-D must be recognizing a conformational grouping of only a few of the residues within this span between residues 164 and 194.

Another factor that may influence neutralization is the glycosylation of HIV-1 gp120. Recently, it was demonstrated that *N*-glycans localized within the V3 region may inhibit neutralization of HIV-1 by anti-V3 and anti-CD4bd MAbs (1). The probable mechanism of the *N*-glycan interference with antibody neutralization was related to the induction of glycan-induced conformational changes in gp120, which limited the accessibility of the V3 neutralizing epitopes. In contrast, we show that the same glycosylation sites appear in the V2 region regardless of whether the isolates are resistant or sensitive to neutralization by MAb 697-D. Theoretically, distant carbohydrate side chains such as those found in V1 could also affect the neutralization just as *N*-glycans of the V3 loop influence neutralization by anti-CD4bd MAb (1). However, no pattern was apparent in the V1 glycosylation sites that could distinguish sensitive from resistant strains.

Finally, the potent neutralization of primary isolates and the broad cross-reactivity of the human anti-V2 MAb reveal a new feature of the natural humoral immune response in humans against HIV-1. Because the V2 region seems capable of inducing neutralizing antibodies as potent as those that are specific for V3 and the CD4bd of gp120, V2 may participate in the protective immune response against HIV-1.

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