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Probing the Structure of the V2 Domain of Human Immunodeficiency Virus Type 1 Surface Glycoprotein gp120 with a Panel of Eight Monoclonal Antibodies: Human Immune Response to the V1 and V2 Domains

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We have analyzed a panel of eight murine monoclonal antibodies (MAbs) that depend on the V2 domain for binding to human immunodeficiency virus type 1 (HIV-1) gp120. Each MAb is sensitive to amino acid changes within V2, and some are affected by substitutions elsewhere. With one exception, the MAbs were not reactive with peptides from the V2 region, or only poorly so. Hence their ability to bind recombinant strain IIIB gp120 depended on the preservation of native structure. Three MAbs cross-reacted with strain RF gp120, but only one cross-reacted with MN gp120, and none bound SF-2 gp120. Four MAbs neutralized HIV-1 IIIB with various potencies, and the one able to bind MN gp120 neutralized that virus. Peptide serology indicated that antibodies cross-reactive with the HxB2 V1 and V2 regions are rarely present in HIV-1-positive sera, but the relatively conserved segment between the V1 and V2 loops was recognized by antibodies in a significant fraction of sera. Antibodies able to block the binding of V2 MAbs to IIIB or MN gp120 rarely exist in sera from HIV-1-infected humans; more common in these sera are antibodies that enhance the binding of V2 MAbs to gp120. This enhancement effect of HIV-1-positive sera can be mimicked by several human MAbs to different discontinuous gp120 epitopes. Soluble CD4 enhanced binding of one V2 MAb to oligomeric gp120 but not to monomeric gp120, perhaps by inducing conformational changes in the oligomer.

The human immunodeficiency virus type 1 (HIV-1) surface glycoprotein gp120 allows viral attachment to the cell surface glycoprotein CD4 in the initial stage of the virus-cell fusion reaction that is mediated by the viral transmembrane glycoprotein gp41 (29). gp120 is an extensively glycosylated molecule with a complex domain structure delineated by nine disulfide bonds (24, 29). These domains can be distinguished by their variability among different HIV-1 isolates; five relatively conserved segments of the protein are interleaved with five sections that are variable to differing extents (25, 34, 46). The conserved domains can be presumed to contain structural elements necessary for correct functioning of gp120; thus, amino acid residues whose substitution interferes with CD4 binding or gp41 association have been shown to lie in the conserved domains and to be invariant (or largely so) among widely divergent HIV-1 isolates (12, 13, 21, 35). Conversely, their very variability implies that the variable domains are less sensitive to disabling mutations, although both the V2 and V3 regions contain amino acids whose identity influences HIV-1 fusion and infectivity (7, 12, 16, 31, 48). Consistent with this, conserved features can be observed by inspection of primary amino acid sequences in both the V2 and V3 domains (11, 22, 23, 34, 39).

Variation in retroviral proteins is usually driven in part by immune selection pressures (4, 18), and both the V2 and V3 domains are binding sites for antibodies capable of neutralizing HIV-1 infectivity. The role of the V3 region as the principal neutralizing determinant of HIV-1 gp120 is well documented (31), but it has become clear recently that the V2 domain is also a target for neutralizing antibodies (8, 11a, 14, 48). Indeed, the corresponding section of the simian immunodeficiency virus (SIV) surface glycoprotein gp130 contains epitopes for neutralizing antibodies (1, 5, 19), unlike the SIV gp130 domain corresponding to the HIV-1 V3 loop (17). Furthermore, sequence variation in the V1 and V2 regions of gp120 is associated with alterations in viral tropism for both HIV-1 (2, 11, 55) and SIV (43), and there is evidence that immune selection drives the variation in the V1 amino acid sequences of these viruses (22, 36, 37).

We have used a panel of eight monoclonal antibodies (MAbs) to probe the structure of the V2 loop of HIV-1 gp120. A panel of gp120 mutants from the HxB2 clone of HIV-1 IIIB (35, 48, 50–52) was used to assess the effect of mutations within and outside V2 on the binding of these MAbs and to investigate their neutralizing properties. Furthermore, we have monitored the effects of soluble CD4 (sCD4), other gp120 MAbs, and HIV-1-positive sera on the binding of V2 MAbs to gp120, and we have explored the prevalence in HIV-1-positive sera of antibodies to the V1 and V2 domains.

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MATERIALS AND METHODS

MAbs, recombinant gp120s, and peptides. MAbs G3-4, G3-136, and BAT-085 have been described previously and were available as purified antibodies (8, 14, 48). MAbs CRA-3 (ADP-324) and CRA-4 (ADP-325) were raised against recombinant BH10 gp120 by Mark Page and colleagues at the National Institute for Biological Standards and Control, Potters Bar, United Kingdom. These MAbs were obtained from the United Kingdom Medical Research Council (MRC) AIDS Directed Programme (ADP) reagent repository in the form of ascites fluid (CRA-3) or hybridoma culture supernatant (CRA-4). MAbs 52-581-SC258 (SC258) and 52-684-238 (684-238) were raised against gp120 derived from strain IIIB-infected cells at Abbott Laboratories and were provided as purified antibody. MAb 110-B was raised against recombinant LAI gp120 (Transgène) at Hybridolabs (Institut Pasteur, Paris, France) and was provided as purified antibody. MAbs RSD-33 and 6C4/5 were raised against recombinant BH10 gp120 and were gifts from Rod Daniels (National Institute for Medical Research, London, United Kingdom) and Shaheen Ranjbar (National Institute for Biological Standards and Control), respectively. MAbs G3-508 and G3-519 to the C4 domain of gp120 have been described elsewhere (33, 49), as has MAb 110.5 to the V3 loop (20). All the above MAbs are murine. Human MAbs (HuMAbs) 15e, 21h, F-91, A-32, 211-C, 48d, and 17b to discontinuous gp120 epitopes have been described previously (15, 42, 51). HuMAb 447-D, broadly reactive with the V3 regions of several isolates, was a gift from Susan Zolla-Pazner (9). sCD4 was obtained from Biogen Inc., Cambridge, Mass. (6).

Sera from HIV-1-infected individuals from London and New York were not selected on clinical grounds and have been described elsewhere (27, 28). A serum sample (FF3346; blood sample of 5/7/92) from a laboratory worker infected accidentally with HIV-1 IIIB about 7 years previously (54) was a gift from William Blattner (National Cancer Institute, Frederick, Md.). Its use in similar assays has been described previously (27, 28).

IIIB gp120 (BH10 clone) expressed in CHO cells by Celltech Ltd. (Slough, United Kingdom) was obtained from the MRC ADP (30). SF-2 gp120 expressed in CHO cells (47) was donated by Kathy Steimer and Nancy Haigwood, Chiron Inc., Emeryville, Calif. MN gp120 purified from culture supernatants of MN-infected cells was a gift from Larry Arthur, National Cancer Institute, Frederick, Md. Culture supernatants containing mutants of IIIB gp120 (HxBc2 clone) were obtained by transient transfection of COS cells as described previously (12, 13, 35, 48, 50–52).

A set of 20-mer gp120 peptides (BH10 sequence) overlapping by 10 amino acids was obtained from the ADP reagent programme (ADP 740). A separate set of 20-mer peptides from the HxB2 sequence, also overlapping by 10 amino acids but slightly out of phase with the first set, was purchased from Cambridge Research Biochemicals (CRB), Hartston, United Kingdom.

Neutralization assays. Neutralization of HIV-1 was assessed by a [^{3}H]thymidine incorporation assay (59). A fixed amount of virus (80 μl) was incubated with a range of concentrations of MAb (80 μl) for 30 min at room temperature, and then 50 μl of the mixture was added to 50 μl of MT-4 cells (6×10^5 cells per ml) in microplate wells. For studies with strain IIIB, the virus input was 900 tissue culture infectious doses ($TCID_{50}$) per culture. MN, a virus less cytopathic than IIIB for MT-4 cells, was used at 3,000 $TCID_{50}$ per culture. Three- and ninefold dilutions of the virus inocula were routinely prepared to provide a standard curve. Three days after infection, 100 μl of fresh medium was added to the cultures and on day 4, they were pulsed for 18 h with 1 μCi of [^{3}H]thymidine (20 Ci/mmol; New England Nuclear, Boston, Mass.). The cells were harvested onto glass-fiber filters, and the amount of DNA-incorporated radiolabel was determined by scintillation counting. All datum points were derived from triplicate cultures, and standard deviations of the mean values for [^{3}H]thymidine incorporation rarely exceeded 10% of the mean value. In some experiments, neutralization was assessed by inhibition of the production of supernatant p24 antigen after 4 days of culture, using a twin-site enzyme-linked immunosorbent assay (ELISA) described elsewhere (38).

Binding of MAbs to monomeric gp120 or solid-phase peptides. Soluble gp120 molecules were captured onto a solid phase via adsorbed antibody D7324 to the carboxy-terminal 15 amino acids as described previously (26, 28, 33). For experiments on native gp120, the buffer was Tris-buffered saline (TBS) supplemented with 10% fetal calf serum (FCS). In some experiments, gp120 was denatured by boiling with 1% sodium dodecyl sulfate (SDS)–50 mM dithiothreitol (28). BH10 gp120 was routinely used at 25 ng/ml. However, for comparative experiments, the concentrations of BH10, MN, and SF-2 gp120 used had been determined to give comparable levels of captured gp120 in the absence of detergent, as judged by sCD4 binding assays (26), and were 40 ng/ml, 60 ng/ml, and 1 μg/ml, respectively.

MAbs were bound onto gp120 in TMSS buffer (2% non-fat milk powder, 20% sheep serum in TBS), which was supplemented with 0.5% Tween 20 for experiments on denatured gp120. The use of detergents was completely avoided in experiments on native gp120, except when specified. Bound murine MAb was detected with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Dako Diagnostics), and HuMAb was detected with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Accurate Chemicals), followed by the AMPAK amplification system (Dako Diagnostics).

For competition experiments with HIV-1-positive sera or HuMAbs, sera or HuMAbs diluted in TMSS at twice the final concentration were added to D7324-captured gp120 in a volume of 50 μl for 30 min before the addition of an equal volume of TMSS containing a murine MAb.

Peptide binding assays were performed by adsorbing peptides at 10 μg/ml in TBS, pH 7.5, onto Immulon II ELISA plates (Dynatech Ltd.). After washing away unbound peptides and blocking the plates for 30 min with a solution of 2% non-fat milk powder in TBS, MAbs or HIV-1-positive sera were added in TMSS buffer plus 0.5% Tween 20 (27). Bound MAbs were detected as outlined above.

Binding of MAbs to oligomeric gp120: fluorescence-activated cell sorter (FACS) analysis. H9 cells were maintained in RPMI 1640 medium with 10% FCS. When semiconfluent, 10^7 cells were transfected with 10 μg of the HIV-1 molecular clone pHxB2 by a standard procedure, using DEAE-dextran. The cells were cultured for 7 days, after which there was no detectable CD4 present on the cell surfaces and no further cytopathic effect. Envelope glycoprotein expression is maximal 1 to 4 weeks after transfection (32, 44, 45), so all experiments were carried out during this period.

Cells were washed twice in growth medium and resuspended at 6×10^6 cells per ml in ice-cold wash buffer (phosphate-buffered saline [PBS], 1% FCS, 0.02% sodium azide). HuMAbs or sCD4 was titrated in round-bottomed

TABLE 1. Properties of V2 MAbs

MAb	Binding to V2 peptide	Binding to denatured BH10 gp120	Binding to native gp120 from:				Neutralization of BH10 virus
			BH10	MN	RF	SF-2	
G3-4	+	−	+	−	+	−	+
G3-136	+	−	+	−	+	−	+
BAT-085	+++	+++	+	−	−	−	−
SC258	−	−	+	+	+	−	+/−
684-238	−	−	+	−	−	−	+
CRA-3	−	−	+	−	−	−	−
CRA-4	−	−	+	−	−	−	−
110-B	−	−	+	−	−	−	−

microtiter wells in 25 μl of wash buffer, and then 25 μl of cell suspension was added. After incubation for 2 h on ice, murine MAb was added to each well and the incubation was continued for a further 1 h on ice or at 37°C. The cells were washed three times in cold wash buffer, and phycoerythrin-labelled anti-mouse immunoglobulin (Immunotech, Luminy, France) was added to each well in a total volume of 50 μl. After incubation for 30 min on ice, the cells were washed as before and then resuspended in 200 μl of PBS–1% FCS–1% formaldehyde per well and stored overnight at 4°C. The fixed cells were analyzed by flow cytometry on a Becton Dickinson FACScan (BDIF, San Jose, Calif.). Results are presented as the mean fluorescence intensity of the test samples minus the background staining with the fluorochrome conjugate alone.

Characterizing MAb epitopes with gp120 mutants. The binding of MAbs to mutant gp120 molecules was assessed by using culture supernatants (100 μl) from COS-1 cells transfected 48 h previously with 10 μg of pSVIIIenv plasmid expressing either wild-type or mutant HIV-1 (HxBc2) envelope glycoproteins. These were used in an ELISA format, in which the gp120 molecules were captured onto a solid phase via antibody D7324, essentially as described previously (33, 51). No detergent was used at any stage of the assays, except with MAb 110-B when the low level of binding necessitated the use of nonionic detergent when testing the MAb against the non-V2 mutant panel (see Table 3). Binding ratios were calculated as described elsewhere, as are the criteria for assessing the significance of the data (33).

We also mapped MAbs separately on a small panel of V2 mutants, assessing the significance of the data by a method similar to that used with radioimmunoprecipitation (RIPA)-based assays (12, 13, 35, 48, 50–52). The binding of each MAb and of a pool of HIV-1-positive sera to each mutant and to wild-type gp120 was determined in triplicate. The ratios of the mean and net optical density at 492 nm (OD_{492}) values for MAb to HIV-1-positive serum were determined for each mutant and then normalized on the ratio derived using wild-type gp120, which was defined as 1.00. The average value for this normalized ratio was determined from two to four separate experiments and is presented. Our cutoff for significance are ratios <0.5 and >1.5 which translate into approximately threefold changes in MAb affinity, but values close to the cutoff may not be significant in practice.

RESULTS

Reactivities of V2 MAbs with native and denatured gp120s. Three MAbs reactive with the V2 region of gp120 have been described previously. MAbs BAT-085 and G3-136, raised against virus-derived IIIB gp120, were shown to map to a peptide epitope VQKEYAFFYKLDIIP (residues 169 to 183) in the V2 domain (8). MAb G3-4, raised in the same way (14), has been mapped to the V2 domain, using gp120 mutants with small numbers of amino acid changes (48). To identify additional V2-reactive MAbs we screened MAbs against a panel of HxBc2 gp120 mutants using an ELISA (33). Five MAbs in addition to G3-4, G3-136, and BAT-085 were found not to react with a gp120 mutant lacking the V1 and V2 loops (deletion of amino acids 119 to 205). These were CRA-3, CRA-4, SC258, 684-238, and 110-B, all raised against LAI-related gp120. The above eight MAbs constitute our core V2 MAb panel. Each of these MAbs has been shown subsequently to react with a construct comprising the glycosylated V1 and V2 loops of HIV-1 gp120 engrafted onto gp70 from murine leukemia virus (40), confirming our identification of them as V2 (or possibly V1) MAbs. Their properties, detailed below, are summarized in Table 1.

We tested the eight MAbs for their reactivities with two sets of 20-mer peptides with 10-amino-acid overlaps that spanned the deleted V1-V2 region. At a MAb concentration of 5 μg/ml (or 1:100 and 1:10 dilutions for CRA-3 and CRA-4), only BAT-085 reacted strongly with any of the peptides, giving OD_{492} values of 1.529 against peptide 162-181, 1.573 against peptide 161-180, and 0.214 against peptide 171-190 in a typical experiment. BAT-085 did not react with peptide 172-191 (OD_{492}, 0.085). Taken together with previous data (8), these results suggest that BAT-085 binds within the sequence from 170 to 180, QKEYAFFYKLD. G3-4 and G3-136 were very similar to each other in their peptide reactivities, both binding weakly only to a single peptide, peptide 162-181, with mean OD_{492} values of 0.243 and 0.208, respectively. Although weak, the binding of these MAbs to peptide 162-181 was consistent between experiments. Titration of the MAbs against a fixed amount of adsorbed peptide indicated that the affinities of G3-4 and G3-136 for peptide 162-181 were approximately 100-fold less than that of BAT-085. None of CRA-3, CRA-4, SC258, 684-238, and 110-B bound consistently to any of the 20-mer peptides.

We also tested two other MAbs found by their donors to be reactive with V2 peptides. MAb RSD-33 (1:300 dilution of ascites fluid) bound to peptides 152-171 (OD_{492}, 0.483) and 162-181 (OD_{492}, 1.404) and similarly to the equivalent peptides in our second set of 20-mers, indicating that its epitope is likely to be within approximately amino acids 162 to 171. MAb 6C4/5 (1/2 dilution of culture supernatant) bound only to peptide 151-170 (OD_{492}, 0.224) in one set of peptides, but only to peptide 162-181 (OD_{492}, 0.208) in the second set. Thus, its epitope is probably much the same as that of MAb RSD-33 and is located in the amino-terminal flank of the V2 loop.

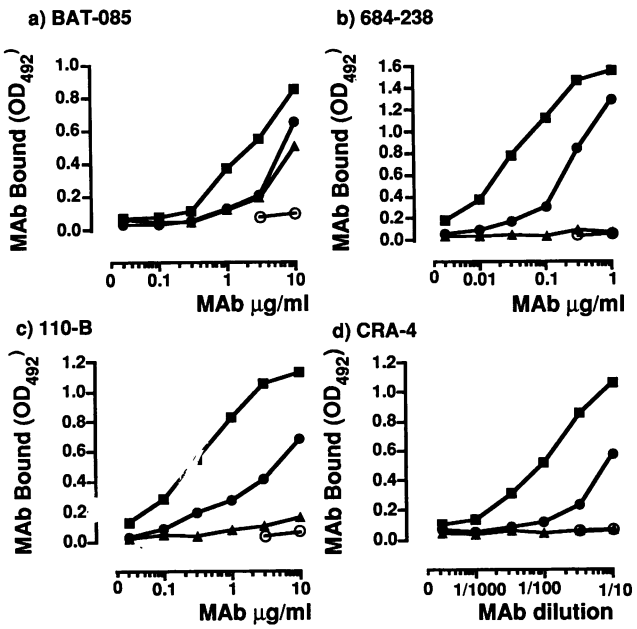

FIG. 1. Reactivity of V2 MAbs with native and denatured gp120. BH10 gp120 captured in the solid phase without detergent (●), in the presence of 1% NP-40 (■), or after boiling with SDS-dithiothreitol and dilution into 1% NP-40 (▲) was reacted with MAbs at the concentrations indicated, and the amount of gp120-bound MAb was determined. ○, no gp120 in the presence of 1% NP-40. Datum points represent single ELISA wells, but each titration curve is representative of several similar experiments.

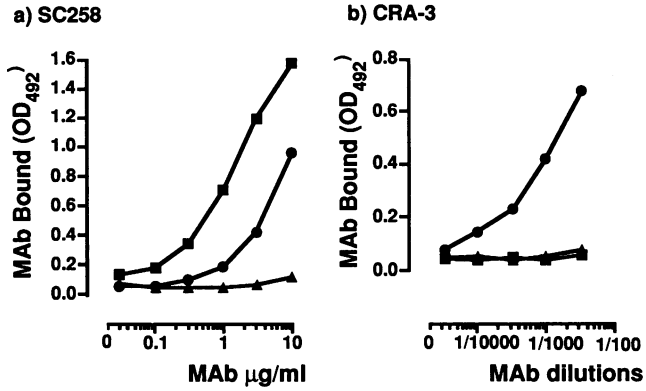

FIG. 2. Reactivity of V2 MAbs with BH10, MN, and SF-2 gp120s. BH10 gp120 (●), MN gp120 (■), or SF-2 gp120 (▲) captured in the solid phase in the absence of detergent was reacted with SC258 or CRA-3 at the concentrations indicated, and the amount of gp120-bound MAb was determined. Datum points represent single ELISA wells, but each titration curve is representative of several similar experiments.

We next assessed whether the eight V2-sensitive MAbs in our core panel reacted preferentially with BH10 gp120 either in native form or after denaturation by boiling with SDS-dithiothreitol (Fig. 1). Except for BAT-085, which reacted almost as well with denatured gp120 as with the native molecule (Fig. 1a), none of the MAbs reacted significantly with denatured gp120, exemplified by 684-238 (Fig. 1b), 110-B (Fig. 1c), and CRA-4 (Fig. 1d). Thus, BAT-085 is clearly distinguishable from the other seven MAbs (Table 1). All eight MAbs reacted with native BH10 gp120, with MAb 684-238 having an affinity 10- to 50-fold greater than that of the other purified MAbs (Fig. 1 and data not shown). Treatment of gp120 with nonionic detergent increased the binding of all eight V2 MAbs (Fig. 1), a phenomenon we observe also with MAbs to the V3 loop and to discontinuous epitopes overlapping the CD4 binding site (data not shown) (33, 57). The two MAbs RSD-33 and 6C4/5 that were reactive with peptides in the amino-terminal flank of the V2 loop (see above) bound very weakly to native gp120, but their binding was increased by fivefold when gp120 was denatured (data not shown). The binding of MAb 6C4/5, but not of RSD-33, to gp120 was also increased significantly by nonionic detergent (Nonidet P-40 [NP-40]). Thus, RSD-33 and 6C4/5 are distinguishable from the other eight V2 MAbs. Their epitopes, approximately amino acids 160 to 175, are relatively poorly exposed on the surface of native, recombinant gp120 compared with the epitopes at the crown of the V2 loop. This is consistent with observations with MAbs to the corresponding regions of SIV_{mac} gp120 (5). Because of their weak reactivity with gp120 in the absence of detergent, we did not study MAbs 6C4/5 and RSD-33 further.

The type specificity of the eight V2 MAbs was determined by measuring their reactivities with recombinant SF-2 gp120 and with virus-derived MN gp120, in comparison with that of BH10 gp120. None of the eight MAbs reacted with SF-2 gp120, and only SC258 reacted with MN gp120 (Fig. 2, Table 1). The affinity of SC258 for MN gp120 was about three- to fivefold greater than that for BH10 gp120, although the latter is more closely related genetically to the IIIB gp120 used as the immunogen for this MAb. Thus, SC258 is a heteroclitic MAb. We also tested the MAbs for reactivity in ELISA with RF gp120 solubilized from virions with NP-40 detergent. G3-4, G3-136, and SC258 bound to RF gp120 with affinities comparable to those for BH10, whereas none of the other MAbs bound detectably (Table 1). SC258 also bound in ELISA to several primary virus gp120s, including JR-CSF, and studies using RIPA techniques showed that SC258 reacted with gp120 from the IIIB, MN, RF, and ALA isolates, but not with SF-2, Z-34, Z-84, and AC gp120 (data not shown). MAb SC258 is therefore a relatively broadly reactive V2 MAb. In contrast, MAb 684-238 reacted only with IIIB gp120 in these RIPA experiments, and the other six MAbs were not tested.

The binding of MAb G3-4 to IIIB gp120 is abolished by deglycosylation of gp120 with endoglycosidase H (14). A similar analysis using SC258 and 684-238 showed that both endoglycosidase H and F treatments of gp120 substantially impaired its ability to bind these MAbs, whereas neuraminidase treatment was without effect (data not shown). Thus, the epitopes for SC258 and 684-238 require the presence of carbohydrates. These moieties could either directly contribute to the antibody-gp120 binding energy or they could be necessary for the presentation of a wholly peptidic antibody-binding site in an appropriate configuration.

Characterizing the epitopes for V2 MAbs with gp120 mutants. To determine the effect of amino acid substitutions in the V2 domains on the binding of our panel of MAbs to gp120, we used a set of mutant HxB2 gp120s secreted as soluble molecules from transfected COS-1 cells (48). These were used in an ELISA with which we compared the binding of MAbs to the gp120 panel with the binding of a pool of HIV-1-positive sera (Tables 2 and 3). The binding of each of the eight V2 MAbs was destroyed, or virtually so, by either of two sets of amino acid changes at the crown of the V2 loop

TABLE 2. Effects of amino acid changes in the V1 and V2 loops on MAb binding to HxB2 gp120

Virus or mutant	Binding ratio (MAb to HIV-1-positive serum)[a]									
	CRA-3	CRA-4	G3-4	G3-136	BAT-085	SC258	684-238	110-B	15e	48d
Wild type	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Δ119-205	**0.02**	**0.01**	**0.00**	**0.03**	**0.00**	**0.00**	**0.00**	**0.04**	1.09	0.03
152/153GE/SM	0.67	**0.39**	0.68	1.01	1.00	0.84	0.92	0.51	0.84	0.77
168K/L	0.87	0.87	0.92	1.15	1.48	0.89	0.82	**0.17**	0.67	0.83
176/177FY/AT	**0.00**	**0.07**	**0.02**	**0.05**	**0.00**	**0.00**	**0.00**	**0.00**	0.73	1.22
179/180LD/DL	**0.00**	**0.09**	**0.00**	**0.02**	**0.00**	**0.00**	**0.00**	**0.00**	0.73	0.87
183/184PI/SG	**0.00**	**0.08**	**0.00**	**0.28**	3.33	**0.16**	**0.14**	**0.00**	0.63	0.80
192-194YSL/GSS	**0.00**	**0.00**	**0.30**	0.65	4.10	**0.00**	**0.16**	**0.12**	0.88	1.01

[a] The MAbs indicated were tested against the panel of V1 and V2 mutants listed. The MAb concentrations used were: CRA-3, 1:250; CRA-4, 1:10; G3-4, 1 μg/ml; G3-136, 5 μg/ml; BAT-085, 10 μg/ml; SC258, 10 μg/ml; 684-238, 1 μg/ml; 110-B, 10 μg/ml; 15e, 1 μg/ml; and 48d, 1 μg/ml. Significantly inhibitory amino acid substitutions are in bold type. The binding ratios of MAb to HIV-1-positive serum are normalized on the value for wild-type gp120 (-1.00).

(mutants 176/177FY/AT and 179/180LD/DL). Both of these changes are within the peptide epitope for BAT-085. Consistent with its ability to bind strongly to a peptide and to denatured gp120, BAT-085 binding to native HxB2 gp120 was not inhibited by other changes in V2 outside its peptide epitope. Indeed, the 183/184PI/SG and 192-194YSL/GSS substitutions in the carboxy-terminal side of the V2 loop increased the reactivity of BAT-085 with HxB2 gp120 (Table 2).

In contrast to BAT-085, G3-4 and G3-136 were strongly inhibited by the 183/184PI/SG substitutions and G3-4 was weakly inhibited by the 192-194YSL/GSS change. The sensitivity of G3-4 and G3-136 to substitutions outside the peptide epitope probably accounts for the 100-fold reduction in the reactivity of these MAbs with peptide 162-181 compared with that of BAT-085 and for their inability to bind to denatured gp120. The other five MAbs, unable to bind peptides and requiring a native gp120 conformation for binding, were also all sensitive to amino acid changes away from the crown of the V2 loop. Thus, the changes 183/184PI/SG and 192-194YSL/GSS caused the abolition or significant impairment of the binding of CRA-3, CRA-4, SC258, 684-238, and 110-B (Table 2). CRA-4 and 110-B were also weakly sensitive to the 152/153GE/SM substitution at the carboxy-terminal base of V1, and 110-B was unique in its inability to bind well to the 168K/L mutant. Whether the above amino acid changes directly or indirectly affect the epitopes for the V2 MAbs has not yet been determined.

As controls, we tested two other MAbs to different conformationally sensitive gp120 epitopes: 15e, which binds to a complex epitope overlapping the CD4 binding site on gp120 (15, 50), and 48d, which binds to an epitope on gp120 whose exposure is increased after CD4 binding (42, 51). The binding of 15e or 48d was unaffected by any of the V2 amino acid substitutions in our panel, although 48d binding was abolished by the complete deletion of the V1 and V2 loops (Table 2), consistent with a prior analysis of 48d using RIPA (51).

Substitutions outside the V2 loops generally had a minor effect on binding of the V2 MAbs (Table 3) (see Addendum in Proof). It was, however, notable that the 117K/W and 120/121VK/LE substitutions in the C1 domain at the amino-terminal base of the V2 loops abolished the binding of CRA-3 and the 207K/W substitution in the C2 region at the carboxy-terminal base of these loops significantly impaired CRA-3 binding (Table 3). CRA-4 and 684-238 were also sensitive to some of the amino acid changes at the bases of the V2 loops, but not to the same extent as CRA-3. Conversely, the 125L/G substitution in C1 moderately enhanced the binding of BAT-085 and may have the same functional effect as the enhancing substitutions 183/184PI/SG and 192-194YSL/GSS (Table 2) at residues which are topologically adjacent to Leu-125 in the stem of the V2 loop. The 125L/G and 120/121VK/LE changes were also weakly enhancing for the binding of MAb 110-B but did not increase the binding of the other V2 MAbs (Table 3). Presumably these amino acid changes in the base and stem of the V2 loops alter the geometry of the loops. However, their effect on CRA-3 binding might be more direct and affect a structure outside V2 that is a component of the MAb epitope. It may be relevant that the 117K/W, 120/121VK/LE, and 207K/W substitutions also abolish or significantly impair the binding of HuMAb 48d, whereas the 125L/G change moderately enhances the binding of this MAb (51). The binding of HuMAb 48d to the gp120 glycoprotein is cross-blocked by CRA-3 (51).

A few other changes had mild effects on the binding of V2 MAbs (Table 3). Several amino acid substitutions in the C2 domain (257T/R, 262N/T, and 269E/L) enhanced the binding of some V2 MAbs (note that the apparently strong enhancement of MAb 684-238 binding to the 262N/T mutant is based on a relatively small OD_{492} change because this mutant was secreted only at very low levels in the panel used to map the 684-238 epitope). A substitution (298R/G) at the base of the V3 loop moderately increased the binding of CRA-4 and 110-B. This change also increases the binding of MAbs to the V3 and C4 domains and to more complex, discontinuous epitopes; it may alter the geometry of the V3 loop with respect to the rest of gp120 (33, 57). Several substitutions between amino acids 450 and 470 in C4 were weakly inhibitory for various of the V2 MAbs, the most frequently occurring inhibitory changes being 450T/N and 470P/L. In contrast, amino acid changes in C3 (380G/F, 381E/P, 382F/L, and 384Y/E) and C4 (420I/R, 421K/L, and 432K/A) located at the bases of the V4 loop increased the binding of most of the V2 MAbs (Table 3). The binding ratios for these mutants were almost always close to the cutoff value of 1.50 we set for significance, but are only listed in Table 3 when they were actually above that value. Substitutions in C3 and C4 have been shown previously to increase the binding of some MAbs to the V3 loop (33, 57) but to destroy the binding of HuMAb 48d (51).

Neutralization properties of V2 MAbs. The neutralization characteristics of MAbs G3-4, G3-136, and BAT-085 have been reported previously (8, 14). The first two MAbs neutralize HIV-1 IIIB and also a range of other isolates (8, 14). This was confirmed by our results (Table 4); G3-4 and G3-136 neutralized molecular clones of IIIB and also RF, but

TABLE 3. Effects of amino acid substitutions outside the V1 and V2 loops on V2 MAb binding

gp120 mutation[b]	Binding ratio (MAb to HIV-1-positive serum)[a]							
	CRA-3	CRA-4	G3-4	G3-136	BAT-085	SC258	684-238	110-B
Mean ratio ± SD	0.43 ± 0.20	0.58 ± 0.20	0.60 ± 0.18	0.76 ± 0.21	0.47 ± 0.16	0.62 ± 0.18	1.63 ± 0.48	0.54 ± 0.28
Inhibitory								
45W/S								0.33
117K/W	0.00						0.42	
120/121VK/LE	0.00	0.36					0.38	
207K/W	0.37							
281A/V	0.42							
356N/I								0.31
450T/N	0.40			0.30				0.19
456R/K								0.28
457D/A								0.28
463N/D								0.33
470P/L	0.30	0.40	0.37					
Enhancing								
120/121VK/LE								1.72
125L/G					2.00	1.84		1.98
257T/R	1.86		1.52					
262N/T							3.07	
269E/L					1.57			
298R/G		1.57						2.03
308-310		1.55						
380G/F								1.94
381E/P	2.09	1.81	1.57					
382F/L								1.78
384Y/E		1.60						
395W/S		1.62						
420I/R			1.57			1.68		2.39
421K/L	2.47	1.86	1.62	1.66	1.51	1.68		2.11
432K/A				1.53	1.68			
438P/R	1.77							
463N/D					1.57			
475M/S							1.81	
485K/V							1.52	

[a] Data for each of the MAbs were derived from quadruplicate determinations except those for G3-4, BAT-085, SC258, and 684-238, which were analyzed in triplicate. The mean ratio for MAb to HIV-1-positive serum binding to the entire panel is given at the top of each column. This value is defined as 1.00 for normalization of the ratios for each mutant. Mutants with normalized binding ratios outside the range 0.50 to 1.50 are listed.

[b] The following mutants, plus wild-type HxB2 gp120, comprised the test panel: 36V/L, 40Y/D, 45W/S, 69W/L, 80N/R, 88N/P, 102E/L, 103Q/F, 106E/A, 113D/A, 113D/R, 117K/W, 120/121VK/LE, 125L/G, Δ119-205, 207K/W, 252R/W, 257T/R, 257T/G, 262N/T, 266A/E, 267E/L, 269E/L, 281A/V, 298R/G, 308-310RIQ/RPELIPVQ, 314G/W, 356N/I, 368D/R, 368D/T, 370E/R, 370E/Q, 380G/F, 381E/P, 382F/L, 384Y/E, 386N/G, 392N/E + 397N/E, 395W/S, 406N/G, 420I/R, 421K/L, 427W/V, 427W/S, 429K/L, 430V/S, 432K/A, 433A/L, 435Y/H, 438P/R, 450T/N, 456R/K, 457D/A, 457D/R, 463N/D, 470P/L, 475M/S, 485K/V, 491I/F, and 493P/K. 308-310 is an abbreviation for the V3 loop insertion mutant 308-310RIQ/RPELIPVQ. MAbs 684-238 and 110-B were tested against a separate set of gp120 transfection supernatants than was used for the other five MAbs. This panel included also the V1 and V2 mutants listed in Table 2. Data derived from these point mutants are not listed in the present table but were qualitatively similar to those presented in Table 2. MAb concentrations used were as described in footnote *a* of Table 2 except that for 110-B, which was used at 5 μg/ml. The binding of MAb 110-B, but not that of the other MAbs, to the gp120 panel was tested in the presence of nonionic detergent.

did not neutralize MN. BAT-085 is a much weaker neutralizing antibody and is relatively type specific (8, 14) (Table 4). This is consistent with the lower affinity of BAT-085 for BH10 gp120 compared with G3-4 and G3-136 (Fig. 1 and data not shown). We were unable to demonstrate specific neutralization of any virus tested by CRA-3, CRA-4, or 110-B (Table 4), probably because of the low affinity of these MAbs for gp120 (Fig. 1). SC258 neutralized MN, RF, and IIIB clones, whereas 684-238 was specific for IIIB clones (Table 4). These results are consistent with the gp120 binding data in Fig. 1. However, none of the V2 MAbs matches the neutralization potency of typical V3 MAbs; the V2 loop is probably not a particularly strong neutralization site on HIV-1 gp120.

Reactivity of HIV-1-positive sera with V1 and V2 peptides. We wished to examine whether sera from HIV-1-infected humans contained antibodies to the gp120 V1 and V2 domains. One commonly used method is peptide serology. However, given that V2 MAbs other than BAT-085 are essentially unreactive with peptides, such an approach would be likely to identify only a minor subset of V2-reactive antibodies in HIV-1-positive sera. Most anti-gp120 antibodies in such sera recognize conformationally sensitive epitopes and are poorly reactive with denatured gp120 and, by inference, with short peptides (28). Furthermore, in a preliminary screen, we noted that one of our HIV-1-negative control sera was strongly reactive with peptide 171-190 (OD_{492}, 0.617) and weakly reactive with peptide 151-170 (OD_{492}, 0.166) from the CRB set. This presumably reflects the presence in this serum sample of antibodies that do not bind to gp120 but are able to cross-react adventitiously with a gp120-derived peptide. The ADP peptide set tended to give higher levels of nonspecific binding with control sera than the CRB peptides set, so we focussed on the latter. Together

TABLE 4. Neutralization properties of V2 MAbs

Clone or strain	Ratio of MAb to HIV-1-positive serum[a]							
	CRA-3	CRA-4	G3-4	G3-136	BAT-085	SC258	684-238	110-B
HxB2	>1/100	>1/10	10.0	10.0	100	100	10.0	>50
BH10	>1/100	>1/10	5.0	2.0	100	50	4.0	>100
IIIB	ND[b]	ND	ND	ND	>100	>100	>10	>50
MN	>1/100	>1/10	>50	>50	>50	10.0	>50	>50
RF	>1/100	>1/10	5.0	2.0	>100	20.0	>100	ND
NL4-3	>1/100	>1/10	5.0	3.0	100	20.0	20.0	ND

[a] Values are concentrations of antibodies (microgram per milliliter) or reciprocal dilutions of ascites fluid (CRA-3) or culture supernatant (CRA-4) which caused 50% inhibition of p24 production (for strain RF) or [^{3}H]thymidine incorporation compared with controls.
[b] ND, not done.

with the other factors outlined above, the problem of cross-reactivity highlights the limitations of peptide serology for analyzing the human immune response to HIV-1 infection (27).

Notwithstanding the above factors, we tested whether HIV-1-positive sera contained antibodies able to react with six 20-mer peptides (CRB set) spanning residues 121 to 190 in V1 and V2. These peptides are from the HxB2 sequence, but peptide 151-170 corresponds to the segment of gp120 between V1 and V2 that is relatively conserved among HIV-1 isolates (34). Our serum panel contained 14 samples from randomly selected individuals who were infected with unknown HIV-1 strains (27, 28), 1 sample from a laboratory worker infected with HIV-1 IIIB (54), and 2 samples from uninfected individuals. Eight of the 14 HIV-1-positive sera were obtained from New York hospitals, the other 6 were from London.

At 1:1,000 dilutions, all eight HIV-1-positive New York sera (no. 47, 48, 50, 51, 52, 54, 55, and 56) reacted significantly with peptide 151-170 from the relatively conserved segment between V1 and V2, with OD_{492} values ranging from 0.36 to 0.85. Curiously, none of the six HIV-1-positive sera from London was significantly reactive with the same peptide (OD_{492}, <0.060). This pattern was reproducible when the corresponding peptide (peptide 152-171) from the ADP peptide set was used (OD_{492} range for the eight positive sera, 0.24 to 0.59). It is not obvious why there should be such a distinction between the New York and London sera, but one possibility is variation in the types of virus circulating in the two cities at the times when these sera were obtained. That the reactive sera bound peptide 151-170 but not the flanking peptides suggests that the immunodominant region lies within the central section of the peptide, at approximately amino acids 155 to 165. Two of the New York sera (no. 51 and 54) recognized peptide 131-150 from V1 (OD_{492}, 0.291 and 0.361), one (no. 54) bound weakly to V2 peptide 161-180 (OD_{492}, 0.166), and two (no. 51 and 55) reacted very weakly with V2 peptide 171-190 (OD_{492}, 0.121 and 0.125). None of the London sera (1:1,000 dilutions) was significantly reactive with any of the six V1-V2 peptides. At serum concentrations greater than 1:1,000, several sera reacted with several V2 peptides. However, nonspecific antibody binding to irrelevant peptides becomes significant at these higher serum concentrations, so reactions seen only at high serum concentrations (1:500 to 1:100) are of uncertain significance.

The IIIB-infected laboratory worker's serum (1:1,000 dilution) did not bind significantly to any of the V2 peptides nor to peptide 121-140. However, this serum bound strongly to the V1 peptides 131-150 (OD_{492}, 0.694) and 141-160 (OD_{492}, 1.154), a pattern of reactivity that was confirmed with the corresponding peptides from the ADP set. Titration showed that half-maximal binding to peptides 131-150 and 141-160 occurred at serum dilutions of 1:1,000 and 1:4,000, respectively, and that significant binding was detectable at a 1:50,000 dilution. Thus, there is a strong linear epitope spanning approximately amino acids 140 to 150 that is recognized by antibodies in the IIIB-infected laboratory worker's serum. Peptide competition experiments (as described in reference 28) indicate that antibodies to this V1 epitope account for almost all of the antibodies able to bind to linear epitopes on denatured BH10 gp120 that are not inhibited by V3 peptides (data not shown). Antibodies reactive with denatured gp120 constitute a minor fraction of the total anti-gp120 response of the laboratory worker (28). The failure of randomly selected sera to react significantly with HxB2-derived V1 peptides is probably accounted for by the extensive sequence variation in V1 (34); more consensus-like V1 peptides may increase the proportion of V1-reactive sera.

Some HIV-1-positive sera enhance the binding of V2 MAbs to monomeric and oligomeric gp120. As an alternative to peptide serology, we determined whether HIV-1-positive sera could compete for the binding of V2 MAbs to monomeric gp120. All 17 sera were screened at a 1:100 dilution for the presence of antibodies able to inhibit G3-4 (3 μg/ml), SC258 (5 μg/ml), or BAT-085 (10 μg/ml) from binding to BH10 gp120 or those able to inhibit SC258 (1 μg/ml) from binding to MN gp120. Neither HIV-1-negative serum significantly affected the binding of any of the MAbs, but variable effects were observed with the HIV-1-positive sera at 1:100 dilutions (Fig. 3). Three sera, no. 55, QC1, and QC5, were consistently able to inhibit the V2 MAbs from binding to BH10 or MN gp120 (Fig. 3A and D). An additional serum sample (no. 51) weakly inhibited MAb SC258 from binding to BH10 gp120 (Fig. 3C). Note the lack of correlation between the ability of sera QC1 and QC5 to compete for V2 MAb binding to gp120 and their inability to bind V2 peptides. The IIIB-infected laboratory worker's serum was weakly inhibitory only for binding of G3-4 to BH10 gp120 (Fig. 3A). Indeed, the laboratory worker's serum actually increased the binding of BAT-085 to BH10 gp120 (Fig. 3B). Enhancement of the binding of several V2 MAbs to BH10 gp120 was also consistently seen with another serum, no. 50, and five sera (no. 47, 50, 51, 52, and 54) at 1:100 dilutions slightly increased the binding of SC258 to MN gp120 (Fig. 3D).

Because the laboratory worker's serum sample contains high-titer antibodies to BH10 gp120 (25, 26), we had expected it to be among the most potent at inhibiting V2 MAb binding to BH10 gp120. To understand why it was not, we

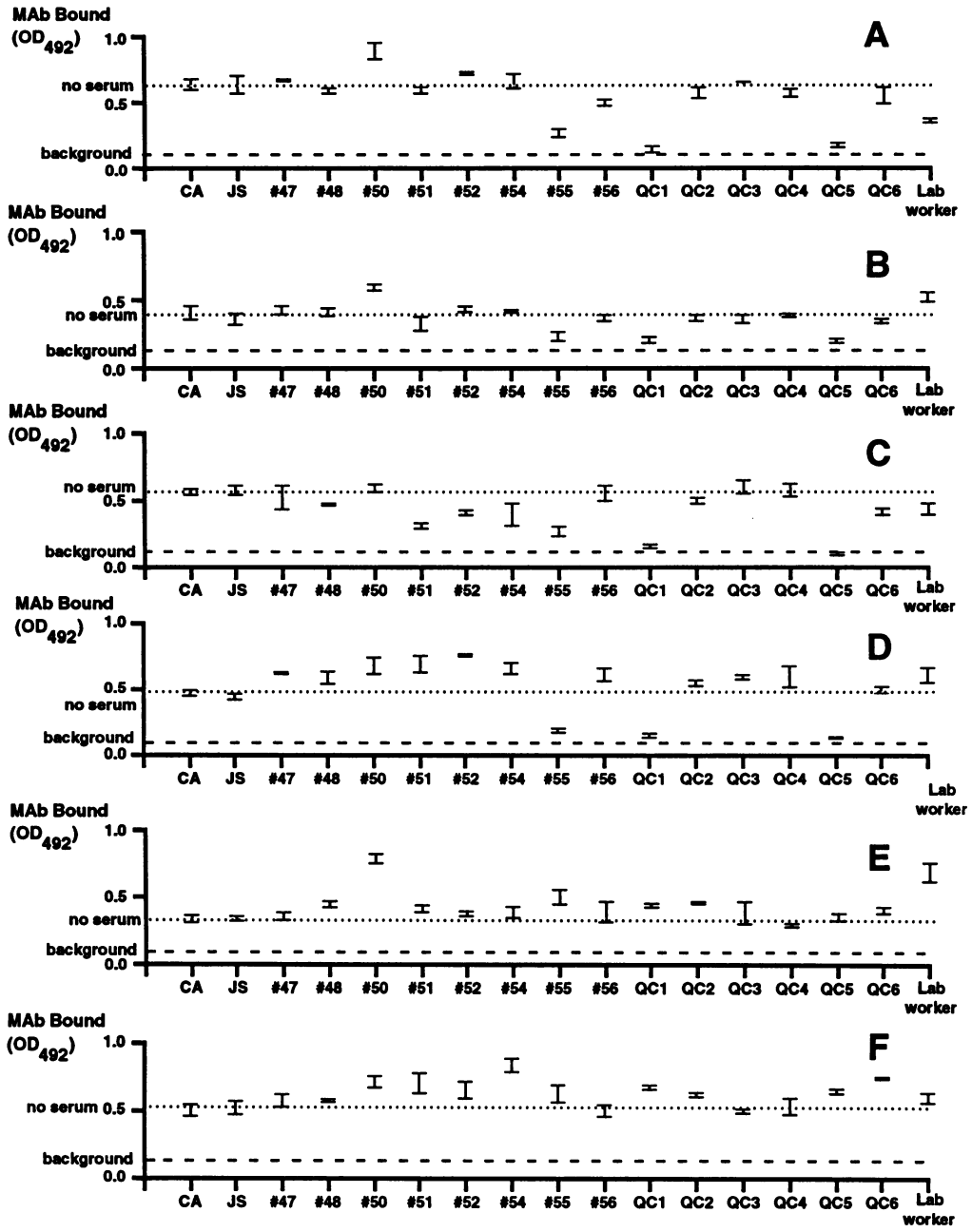

FIG. 3. Prevalence in HIV-1-positive sera of antibodies inhibiting and enhancing binding of V2 MAbs to gp120. HIV-1-positive or HIV-1-negative sera were incubated with BH10 or MN gp120 before the addition of a fixed concentration of MAb and determination of the amount of gp120-bound MAb. Data are presented as OD_{492} values and are the means ± standard deviation of triplicate wells for the HIV-1-negative sera (CA and JS) and for the no-serum controls. For the HIV-1-positive serum samples, data are the means of duplicates ± the range of the individual values. The upper and lower dotted and dashed lines indicate the OD_{492} values obtained in the absence of added serum and in the absence of gp120 (assay background), respectively. (A) BH10 gp120 + sera at 1:100 + G3-4; (B) BH10 gp120 + sera at 1:100 + BAT-085; (C) BH10 gp120 + sera at 1:100 + SC258; (D) MN gp120 + sera at 1:100 + SC258; (E) BH10 gp120 + sera at 1:5,000 + BAT-085; (F) MN gp120 + sera at 1:5,000 + SC258. For MAb concentrations, see text.

titrated it and another HIV-1-positive serum (QC5) and assessed their effects on the binding of several of the V2 MAbs to gp120 (Fig. 4). The laboratory worker's serum had a biphasic effect on the binding of BAT-085 to BH10 gp120. At low serum concentrations (1:30,000 to 1:1,000), prior binding of this serum to gp120 caused a strong enhancement of the binding of BAT-085 (Fig. 4a). At higher serum concentrations, the enhancement of BAT-085 binding disappeared, the shape of the curve suggesting that an inhibitory activity was counteracting the enhancing one (Fig. 4a). Enhancement of binding was observed using each of the V2 MAbs in place of BAT-085, although the magnitude of the

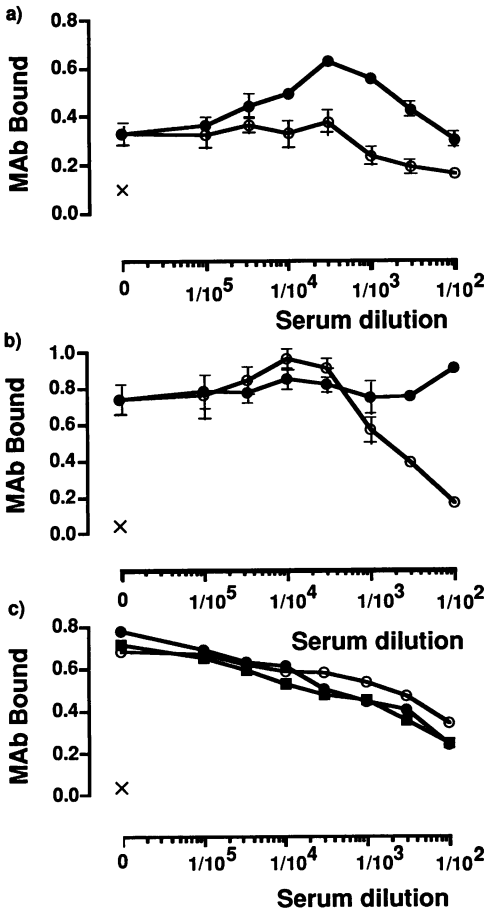

FIG. 4. Effect of HIV-1-positive sera on binding of V2 MAbs to monomeric gp120. Sera from a IIIB-infected laboratory worker (●) or from two individuals infected with unknown HIV-1 isolates (QC5, ○ and no. 55, ■) were titrated against gp120 before the addition of a fixed concentration of MAb and the detection of gp120-bound MAb. Assay background in the absence of gp120 is indicated (×) and was unaffected by the presence of HIV-1-positive serum at concentrations up to 1:100. Datum points are the means ± standard deviation of triplicate determinations (a and b) or the means of duplicate determinations (c). (a) BH10 gp120 + BAT-085; (b) MN gp120 + SC258; (c) BH10 gp120 + G3-508.

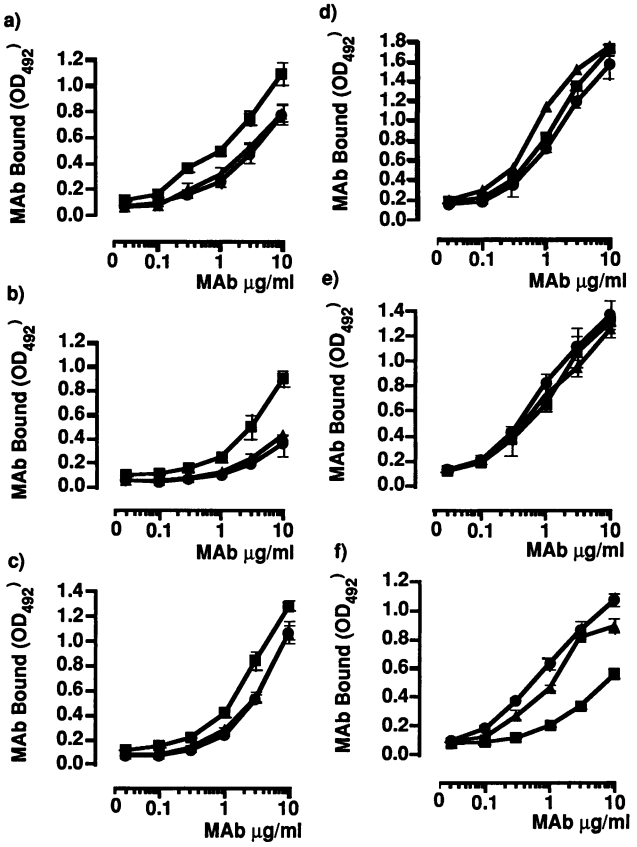

FIG. 5. Effect of HIV-1-positive sera on binding of V2 MAbs to monomeric gp120. Serum from a IIIB-infected laboratory worker (■) or from an individual infected with an unknown HIV-1 isolate (QC5, ▲) was incubated at a fixed concentration with gp120 before titration of MAb and detection of gp120-bound MAb. Binding of MAb in the absence of serum is also shown (●). The gp120 was from BH10 except for the results in panel d for which MN gp120 was used. Sera were added at 1:3,000 dilutions except those in panels b and d which were 1:1,000 and 1:5,000, respectively. The MAbs used were G3-4 (a), BAT-085 (b), SC258 (c), SC258 (d), G3-519 (C4) (e), and 110.5 (V3) (f). Datum points are the means ± standard deviation of triplicate determinations.

effect was usually lower than that seen with BAT-085 (data not shown, but see Fig. 5). In contrast to the laboratory worker's serum, serum QC5 did not enhance BAT-085 binding to BH10 gp120 but inhibited its binding when added in the concentration range of 1:1,000 to 1:100 (Fig. 4a), which is consistent with the data shown in Fig. 3B for this serum. Similar data were obtained when each of the V2 MAbs was substituted for BAT-085 and when serum no. 55 was tested against each of the V2 MAbs (data not shown). The enhancement effect observed with the V2 MAbs was not nonspecific; thus, the laboratory worker's serum and sera QC5 and no. 55 did not enhance the binding of MAb G3-508 to the C4 domain of BH10 gp120 but actually inhibited it (Fig. 4c).

To explore whether this enhancement was specific for the laboratory worker's serum against IIIB gp120 or perhaps more general, we titrated this serum and serum QC5 against MN gp120 and then added MAb SC258, the only one in our panel reactive with MN gp120. We observed the converse of that seen with the V2 MAbs and BH10 gp120; the laboratory worker's serum did not enhance MAb SC258 binding to MN gp120, but a weak yet consistent enhancement was observed at low concentrations (1:30,000 to 1:3,000) of serum QC5. Higher QC5 concentrations were strongly inhibitory (Fig. 4b), as predicted from Fig. 3D.

The enhancement effect was investigated further by titrating MAbs against gp120 with or without a fixed concentration (usually 1:3,000 dilution) of laboratory worker serum or of serum QC5 (Fig. 5). Data consistent with those described above were obtained, with the binding of V2 MAbs to BH10 gp120 being consistently enhanced by the laboratory worker's serum but not by QC5 (Fig. 5a, b, and c). Conversely, QC5 enhanced the binding of SC258 to MN gp120 (Fig. 5d). Neither serum (1:3,000 dilution) had any effect on the binding of MAb G3-519 to the C4 domain of BH10 gp120 (Fig. 5e), although the laboratory worker's serum, but not QC5, strongly inhibited binding of MAb 110.5 to the V3 loop of BH10 gp120 (Fig. 5f). The latter result is consistent with prior analyses of the V3-reactive antibodies in these two sera

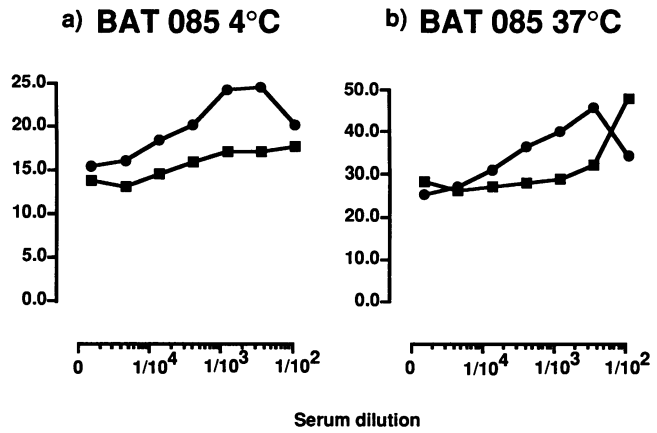

FIG. 6. Effect of HIV-1-positive sera on binding of BAT-085 to cell surface oligomeric gp120. Sera from a IIIB-infected laboratory worker (●) or from an individual infected with an unknown HIV-1 isolate (QC6, ■) were incubated at a range of concentrations with HxB2-infected cells for 2 h at 4°C before the addition of BAT-085 (10 μg/ml) for a further 1 h at 4°C (a) or 37°C (b) and detection of bound MAb by FACS analysis. Data are the means of duplicate determinations, with the individual fluorescence intensity values varying by approximately 15%.

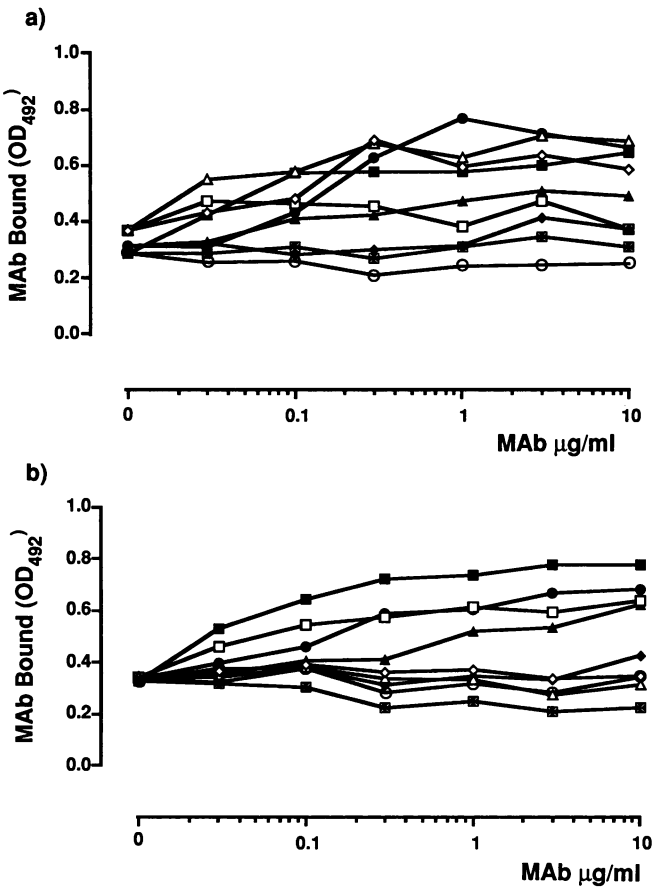

FIG. 7. Effect of MAbs and sCD4 on binding of V2 MAbs to monomeric gp120. HuMAbs at twice the concentrations indicated were reacted with gp120 for 1 h before the addition of an equal volume of buffer containing a V2 MAb and continuation of the incubation for a further 1 h. The amount of gp120-bound MAb was determined. (a) IIIB gp120 + 5 μg of BAT-085 per ml; (b) MN gp120 + 1 μg of SC258 per ml. The HuMAbs used were: A-32 (■); 211-C (●); 48d (△); 17b (◇); 15e (▲); 21h (○); F-91 (□); 447-D (◆). sCD4 (◪) was also tested. Each datum point is the mean of triplicate determinations. Error bars are omitted for clarity but were similar in magnitude to those shown in other figures describing experiments of similar design. Assay background (no gp120) was approximately OD_{492} = 0.070.

(27, 28). Of the other V2 MAbs, 110-B binding was enhanced by the laboratory worker's serum about as strongly as that of BAT-085, whereas the magnitude of the enhancement of G3-136 and 684-238 binding was similar to that seen with G3-4 (data not shown). CRA-3 and CRA-4 binding were only weakly enhanced by the laboratory worker's serum (1:3,000), and serum QC5 (1:3,000) was significantly inhibitory for these MAbs (data not shown). CRA-3 and CRA-4 may be more sensitive than the other V2 MAbs to inhibitory antibodies in the polyclonal sera, which would counter any antibodies enhancing their binding.

To estimate the prevalence of antibodies able to enhance the binding of V2 MAbs to gp120, we tested our panel of HIV-1-positive sera (1:5,000 dilutions) for their effects on the reactions between BAT-085 and BH10 monomeric gp120 and those between SC258 and MN gp120 (Fig. 3E and F). Four sera (no. 48, 50, 55, and the laboratory worker's serum) significantly increased the binding of BAT-085 to BH10 gp120, and eight sera (no. 50, 51, 52, 54, 55, QC1, QC5, and QC6) increased the binding of SC258 to MN gp120, although the magnitude of the increase was usually very weak for MN gp120 (Fig. 3F). Thus, antibodies able to enhance to some extent the binding of V2 MAbs to gp120 are at least as common as those that inhibit the binding of these MAbs. The enhancing antibodies tend to be observed at low serum dilutions, indicating that they are either of higher affinity or are present at higher concentrations than those able to block the binding of V2 MAbs.

We eliminated the possibility that the enhancement of V2 MAb binding to gp120 observed in the previous experiments was an artifact of the use of monomeric, recombinant gp120 by performing a similar experiment using gp120/gp41 oligomers on the surfaces of HxB2-infected cells (Fig. 6). At both 4 and 37°C, prebinding of the laboratory worker's serum to gp120 over a wide concentration range caused a substantial increase in subsequent BAT-085 binding. The bell-shaped dose-response curve was very similar to that seen with monomeric gp120 in ELISA (cf. Fig. 4a). A much weaker effect was observed with serum QC6, except at the highest serum concentration tested at 37°C, when the enhancement of BAT-085 binding was quite pronounced (Fig. 6b).

In view of the enhancement of V2 MAb binding to gp120 by the laboratory worker's serum, we tested whether this and other HIV-1-positive sera could synergize with BAT-085 or G3-4 to neutralize HIV-1 IIIB. The addition of serum concentrations of 1:400 to 1:4,000 to BAT-085 or G3-4 increased virus neutralization, but there was no clear synergistic effect (data not shown). Furthermore, because HIV-1-positive serum contains antibodies to several neutralization epitopes, dissecting the mechanism of additive or synergistic neutralization is difficult; therefore, we did not pursue this further.

Effect of HuMAbs and sCD4 on binding of V2 MAbs to monomeric gp120. The above data, taken together, suggest that the effects of HIV-1-positive sera on V2 MAb binding to gp120 can be separated into two components; a high-affinity or prevalence antibody population that enhances V2 MAb

binding and a low-affinity or prevalence antibody population that inhibits their binding, perhaps by direct competition. We tested whether the enhancement of V2 MAb binding to gp120 could be mimicked by HuMAbs to partially defined epitopes (Fig. 7). The most consistently enhancing HuMAbs were A-32 and 211-C, a pair of nonneutralizing antibodies with very similar properties (42). These increased the binding both of BAT-085 to BH10 gp120 (Fig. 7a) and of SC258 to MN gp120 (Fig. 7b). Enhancement of BAT-085 binding to IIIB gp120 was also observed with HuMAbs 48d and 17b (Fig. 7a), two closely related neutralizing MAbs with properties distinct from those of A-32 and 211-C (42, 51). Neither 48d nor 17b enhanced SC258 binding to MN gp120 (Fig. 7b), although both HuMAbs bound strongly to MN gp120 (data not shown). HuMAbs that block CD4 binding to gp120 by binding to epitopes overlapping the CD4-binding site had variable effects on V2 MAb binding to gp120; 15e weakly enhanced V2 MAb binding to both BH10 and MN gp120s, whereas 21h was ineffective (Fig. 7); F-91 was significantly enhancing for MN gp120, but only marginally so for BH10 gp120. The V3 HuMAb 447-D (9) did not enhance V2 MAb binding to either BH10 or MN gp120 (Fig. 7), although note that while 447-D binds avidly to MN gp120, its binding to BH10 gp120 is fairly weak (data not shown) (9).

The enhancing effect of HuMAbs and HIV-1-positive sera on the binding of V2 MAbs to gp120 was not mimicked by sCD4. This had no effect on BAT-085 binding to BH10 gp120 (Fig. 7a) and weakly inhibited SC258 binding to MN gp120 (Fig. 7b). These effects of sCD4 are consistent with previous reports that the binding of G3-4 and G3-136 but not that of BAT-085 to IIIB gp120 was inhibited by sCD4 (8, 14). The conformationally insensitive BAT-085 linear epitope at the crown of the V2 loop is presumably unaffected by sCD4 binding, whereas the conformationally sensitive epitopes for SC258, G3-4, and G3-136 must be modulated in some way.

The epitopes for HuMAbs A-32 and 211-C, which increase the binding of the V2 MAbs to monomeric gp120 (Fig. 7), are destroyed by substitutions 69W/L and 262N/T in C1 and C2 and significantly impaired by changes in C4, most notably 427W/S (data not shown). The V2 change 168K/L weakly enhances the binding of A-32 and 211-C. Descriptions of the amino acid substitutions affecting the binding of HuMAbs 15e and 21h (50), 48d and 17b (51), and sCD4 (35) have previously been reported. The binding of HuMAb F-91 to HxB2 gp120 in ELISA is abolished or strongly impaired by amino acid substitutions at 113D/R, 368D/T, and 370E/R and moderately reduced by changes 113D/A, 368D/R, 384Y/E, and 420I/R. In contrast, amino acid substitutions 168K/L, 262N/T, 485K/V, 491I/F, and 493P/K increase F-91 binding to HxB2 gp120. We cannot be certain from the epitope mapping data why some of the above HuMAbs increase the binding of V2 MAbs to monomeric gp120 and why others and sCD4 do not. One possibility, however, is that interaction of a MAb with the C4 region is important for increasing the exposure of the V2 loop; this would account for the failure of 21h, insensitive to C4 changes (50), to enhance V2 MAb binding to BH10 gp120, whereas MAbs 48d, 17b, A-32, 211-C, 15e, and F-91 are all sensitive to C4 substitutions to a greater or lesser extent. Consistent with this notion, we have observed that binding of a HuMAb to the V2 loop of BH10 gp120 is strongly increased by the prior binding of murine MAbs to the C4 region spanning amino acids 430 to 438 (10).

Effect of HuMAbs and sCD4 on binding of V2 MAbs to oligomeric gp120. The data presented in Fig. 7a were all obtained with monomeric gp120 in an ELISA format. We were concerned that this approach was oversimplistic. For example, the ability of MAbs to block sCD4 binding to monomeric gp120 in vitro is poorly predictive of their ability to neutralize the same HIV-1 strain (45). We therefore used FACS techniques to assess whether the HuMAbs were able to enhance the binding of V2 MAbs to gp120/gp41 oligomers on the surfaces of HIV-1-infected cells (Fig. 8). MAb 211-C did not enhance the binding of either BAT-085 or G3-136 to HxB2 gp120 under these conditions (Fig. 8). However, simultaneous determination of the extent of 211-C binding revealed that this MAb was virtually unable to react with oligomeric gp120 (data not shown). This probably explains why 211-C and the related HuMAb A-32 do not neutralize IIIB virus (42). However, HuMAb 48d caused strong enhancement of BAT-085 and G3-136 binding to oligomeric gp120 at both 4 and 37°C (Fig. 8), and HuMAb 15e had a weaker effect (data not shown).

Low sCD4 concentrations caused increased binding of both BAT-085 and G3-136 to oligomeric HxB2 gp120 (Fig. 8). Higher sCD4 concentrations counteracted this effect, but only partially so for BAT-085 (Fig. 8A and B). The overall enhancement by sCD4 of BAT-085 binding to oligomeric gp120 (Fig. 8A and B) contrasts with its lack of effect on the binding of the same MAb to monomeric gp120 (Fig. 7a) (8). In contrast to BAT-085, G3-136 binding was strongly inhibited by sCD4 doses greater than 1 μg/ml at both 4 and 37°C (Fig. 8C and D). That the inhibition was similar in extent at both temperatures indicates that it is not due to gp120 shedding, which occurs to a much lesser extent at 4°C than at 37°C (44). We suggest that sCD4-induced conformational changes in the structure of gp120/gp41 oligomers initially increase the accessibility of the V2 loop to MAbs (45), just as we have reported previously for V3 MAbs (44). However, binding of sCD4 to gp120 also partially occludes, or indirectly alters the structure of, the binding site for conformationally sensitive V2 MAbs like G3-136, leading to net inhibition. With monomeric gp120, only inhibition of binding is seen. Further studies on these phenomena will be described elsewhere (45).

DISCUSSION

The V1 and V2 regions have an important role in the functioning of HIV-1 gp120 in that the amino acid sequence of these domains can influence the replication competence of the virus (48) and also its tropism (2, 11, 55). However, it is unclear what this role is. The deletion or mutation of the V1 and V2 loops does not significantly impair the ability of recombinant gp120 to bind CD4 strongly, implying that the function of the loops is restricted to a stage subsequent to CD4 binding (35, 41, 48). Consistent with this, MAbs that bind to these loops only weakly reduce the ability of gp120 to bind CD4 (8, 14, 45). Single amino acid changes in the V2 domain decrease the association between gp120 and gp41 (48), but whether there is a direct interaction between V2 amino acids and gp41 is uncertain; the amino acid changes in V2 could indirectly affect the structure of a separate gp41-associative region of gp120. This possibility is supported by the observation that the gp120 glycoprotein can stably associate with the gp41 glycoprotein even when the V1 and V2 loops are deleted (56).

One of the V2 MAbs, BAT-085, recognizes a linear epitope (position 170 to 180) at the crown of the V2 loop that can be mimicked by peptides. Substitutions outside this linear sequence abolish or significantly reduce binding of all the other V2 MAbs. Thus, either amino acids elsewhere in V2 (or perhaps in some cases outside the loop) contribute to

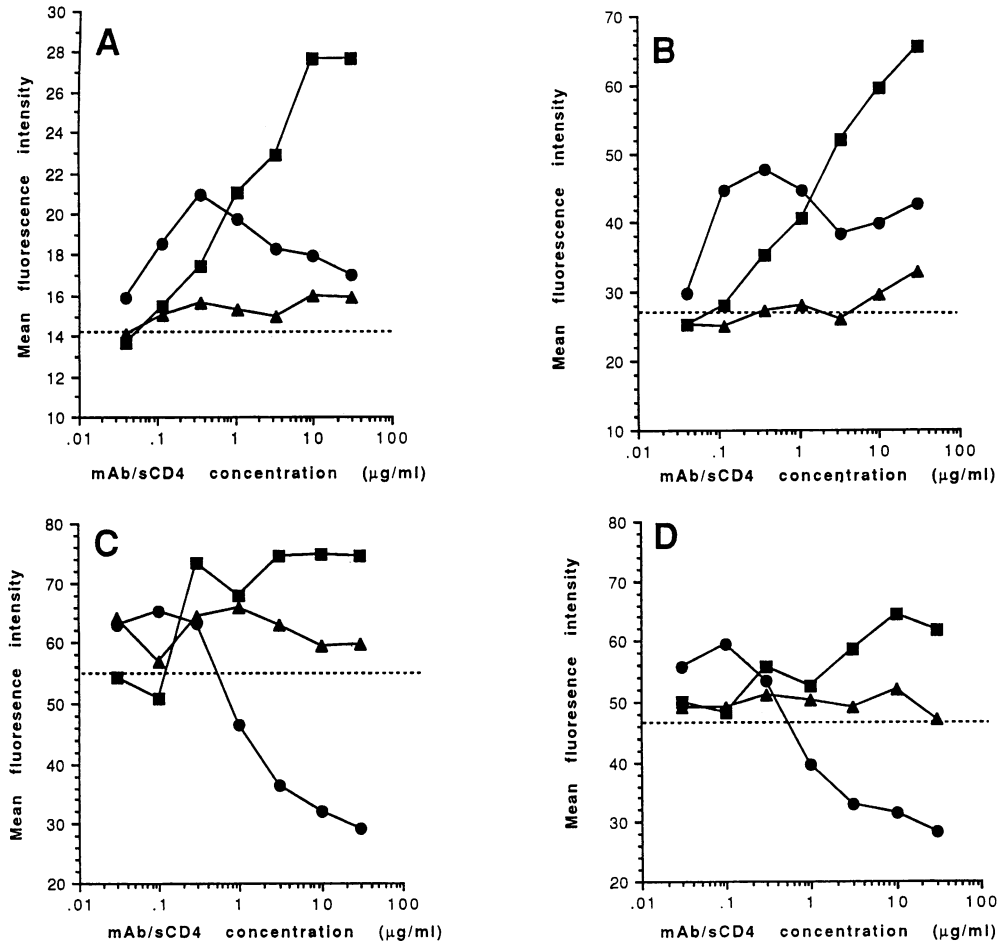

FIG. 8. Effect of HuMAbs and sCD4 on BAT-085 binding to cell surface oligomeric gp120. sCD4 (●), 48d (■), or 211-C (▲) at the concentrations indicated was incubated with HxB2-infected cells for 2 h at 4°C before the addition of BAT-085 (10 μg/ml) (A and B) or G3-136 (10 μg/ml) (C and D) for a further 1 h at 4°C (A and C) or 37°C (B and D) and detection of bound murine MAb by FACS analysis. The level of MAb binding in the absence of serum is represented by the dotted line. Data are the means of duplicate determinations, with the individual fluorescence intensity values varying by approximately 15%.

their epitopes or linear epitopes are very sensitive to the way in which they are presented on the surface of the glycoprotein. Either is possible: we have noted that several V3 MAbs which are clearly reactive with a linear peptide in solid-phase assays are poorly reactive with denatured gp120 (33). However, two other neutralizing MAbs, G3-4 and G3-136, bind weakly to the same peptide as BAT-085. Thus, it is probable that BAT-085, G3-136, and G3-4 bind to similar epitopes composing amino acids in a stretch from position 170 to 180 at the crown of the V2 loop but that the way in which this sequence is presented strongly influences antibody affinity. Within the HxB2 sequence 170-QKEYAFFYKLD-180, the amino acids conserved between HxB2 and RF are underlined. Because G3-4 and G3-136 bind to both HxB2 and RF gp120, the underlined amino acids might be crucial to their epitopes. Consistent with this, an RF mutant that escapes neutralization by G3-4 has an amino acid change at residue 177 (58). The failure of BAT-085 to bind to RF gp120 indicates that either or both of the nonconserved amino acids Gln-170 and Phe-175 are components of its epitope. It is also notable that MN gp120, to which G3-4 and G3-136 fail to bind, has an additional change within the 170 to 180 sequence (176F/L) (34) that might affect the epitopes for these MAbs. Presumably, neither of these changes significantly alters the epitope for SC258, which binds MN gp120. SF-2 gp120, not bound by any of the V2 MAbs, has three additional changes from HxB2 in the above sequence, 173Y/N, 177Y/R, and 178K/N (34).

Certain amino acid substitutions in the flanks and base of the V2 loop increase the binding of BAT-085. We assume that these mutations improve the exposure of the linear epitope for BAT-085 at the crown of the V2 loop. The same substitutions did not enhance, but actually inhibited, binding of the peptide-reactive but conformationally sensitive MAbs G3-4 and G3-136. The other five MAbs in our core panel are not detectably peptide reactive and are generally sensitive to amino acid substitutions in the carboxy-terminal flank of the V2 loop and at its base. The epitopes for these MAbs may be genuinely discontinuous, but we cannot be dogmatic about this, given the available data; they are certainly very sensitive to the overall structure of the V2 loop. MAb SC258 is broadly reactive with gp120 from a range of viruses, including primary isolates. This suggests that its epitope is well conserved. The available data from the gp120 mutants do

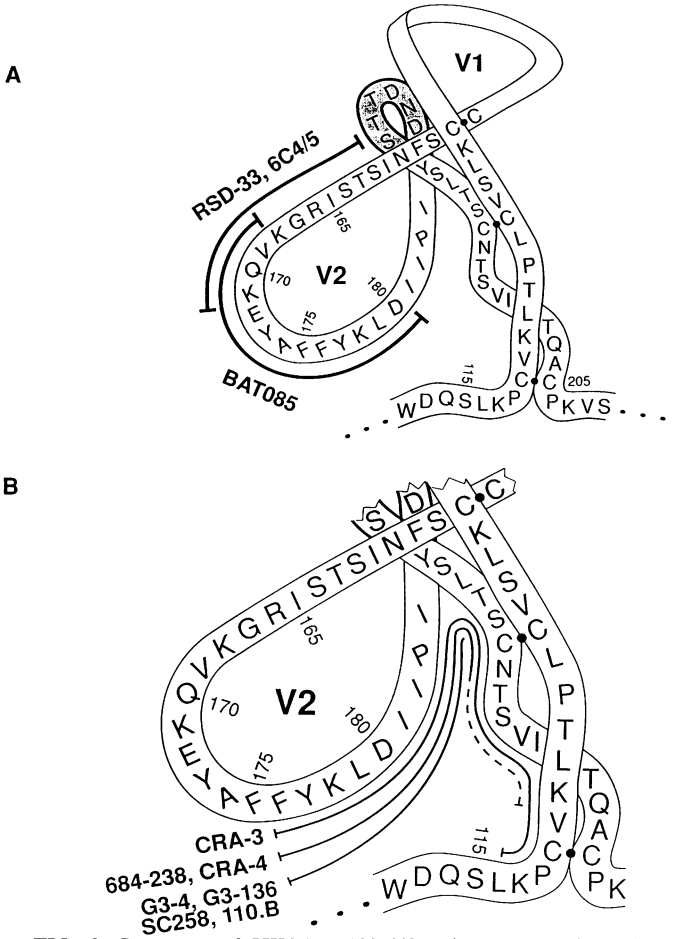

FIG. 9. Summary of HIV-1 gp120 V2 epitopes. A schematic diagram of the HIV-1 gp120 V1-V2 stem-loop structure based on the disulfide bonds (24) and epitope mapping data is shown. The shaded loop represents a structure that is highly variable in length among different HIV-1 strains. The sequence of the HxB2 envelope glycoprotein, with the corresponding numbers, was used in this diagram. Linear epitopes with minimal dependence on gp120 conformation are shown at the top, whereas conformation-dependent epitopes are depicted at the bottom. The extent of the linear epitopes was determined primarily by peptide mapping; the extent of the conformation-dependent epitopes was deduced from the effects of amino acid substitutions on antibody recognition. These epitopes are, however, only estimates.

not, however, allow us to say why SC258 is broadly reactive but, for example, 684-238 is not. Both are similarly sensitive to the amino acid substitutions in our current panel of V2 mutants. Several of these substitutions may be fairly disruptive to the overall structure of the V2 loop. It is notable that SF-2 gp120, to which none of the V2 MAbs binds, has a seven-amino-acid insert in the loop on the carboxy-terminal side of the V2 domain (34). Amino acid substitutions near this region of the HxB2 sequence (e.g., 183/184PI/SG and 192-194YSL/GSS) impair the binding of most of the V2 MAbs. However, RF gp120 has a more extensive insertion in the same region of V2, but still binds G3-4, G3-136, and SC258. We are now building on the current set of data by designing new gp120 mutants that might enable more precise definition of the epitopes for several of the V2 MAbs.

A schematic representation of the way in which the V1 and V2 loops might be folded with respect to the C1 and C2 structures at their base is presented in Fig. 9, with estimates of the epitopes for the various V2 MAbs depicted. Note that on this model the shaded loop on the carboxy-terminal flank of V2 depicts a region that is highly variable in length among HIV-1 isolates (34); the length, amino acid content, and extent of glycosylation of this hypervariable segment have been correlated with the phenotypic properties of syncytium-inducing or non-syncytium-inducing viruses (11). In HIV-2 and SIV, the corresponding region of V2 is predicted to be a disulfide-linked loop protruding from the main V2 loop structure.

We cannot be certain how amino acid changes outside the V2 structure affect the presentation of this region to antibodies. The influences of distant amino acid changes are weak, but there is a tendency for substitutions around the base of the V4 loop to enhance binding of MAbs to the V2 loop. These same changes also increase binding of certain MAbs to the V3 loop and to discontinuous epitopes overlapping the CD4 binding site (33, 57). We have previously reported a close structural interaction between the V3 and C4 domains of gp120 (33, 57); now we suggest that the V4-C4 structure might be folded into proximity with the V2 loops in the native gp120 molecule. This would be consistent with the observation that interactions of MAbs with the C4 region increase the binding of MAbs to V2 (Fig. 7) (10). Furthermore, HuMAb 48d is strongly cross-blocked not only by MAbs mapping to the C4 domain, but also by CRA-3, whereas other V2 MAbs are relatively ineffective (51). We show here that CRA-3 binds to an epitope destroyed by amino acid substitutions in V2 and at the base of the V1-V2 stem-loop structure (Fig. 9). Like CRA-3, 48d is extremely sensitive to amino acid substitutions in C1 and C2 at the base of the V1 and V2 loops (51), and it seems possible that structures in C1 and C2 may contribute to the epitopes of both CRA-3 and 48d. However, unlike CRA-3, 48d binding is also abolished by amino acid substitutions in C3 and C4 (51). The cross-blocking effect of these MAbs could be explained by a proximity of the base of the V1-V2 stem-loop and the C3-C4 structures at the base of the V4 loop in the folded gp120 glycoprotein.

It is clear that the binding of antibodies to the V2 region can neutralize HIV-1 infectivity (8, 11a, 14, 48) and that the equivalent regions of SIV gp120 also constitute neutralization epitopes (1, 5, 19). Some of our V2 MAbs are fairly potent neutralizing antibodies for IIIB, notably 684-238. Presumably, binding of MAbs to the V2 domain hinders the virus-cell fusion reactions at a stage subsequent to the binding of virions to CD4, although this has not been proven unequivocally. Irrespective of the mechanism, we suggest that neutralization of both HIV-1 and SIV by V2 MAbs occurs by a comparable mechanism, given that the epitopes for these MAbs are so similar in their positions in the respective glycoproteins. Both SIVmac- (1, 5, 19) and HIV-1 (8)-neutralizing V2 MAbs recognize a gp130/gp120 sequence of limited, but obvious, homology.

It is not, however, certain how important the V2 loop is as a neutralization site in the natural human immune response to HIV-1 infection. To assess the prevalence of V1 and V2 antibodies in HIV-1-positive sera, we initially used IIIB sequence-based 20-mer peptides. In general, V1 and V2 peptide-reactive antibodies were scarce in HIV-1-positive sera, although there was a modest but inconsistent reaction of serum antibodies with the conserved section between V1 and V2 and the IIIB-infected laboratory worker's serum contained a strong V1, but not V2, response. However, this method can only identify the subset of antibodies reactive

with linear epitopes and is thus of limited value (28). The generation of artifactual results using short peptides is also a factor to consider (27). Too much weight should not be put on peptide serology to analyze in detail the human immune response to gp120, particularly the variable loops. But consensus peptides from primary isolates might be of use for initial screens for antibodies to V1 and V2. It is also quite clear that the peptide reactivity of V2 MAbs does not predict their neutralization potency. Of BAT-085, G3-4, and 684-238, for example, BAT-085 was by far the most reactive with V2 peptides but had the lowest affinity for BH10 gp120 and was the weakest neutralizing MAb. MAb 684-238, by contrast, did not react with V2 peptides but bound strongly to gp120 and was among the most potent of the V2 MAbs at neutralizing IIIB.

As an alternative approach, we performed competition analyses. A subset of serum samples in our test panel was able to block strongly the binding of V2 MAbs to recombinant gp120, suggesting that antibodies to the V2 loops are made in infected humans. Consistent with this finding, a fairly broadly reactive human monoclonal antibody to the V2 loop has recently been identified that has properties similar to those of SC258 (10). However, two factors complicate cross-competition analyses; one prosaic, the other of interest. First, cross-competitive effects can be indirect, especially if different variable loops are folded into proximity in the native gp120. Second, there are antibodies in HIV-1-positive sera that enhance the binding of V2 MAbs to gp120 and thereby counteract any inhibitory antibodies that might be present. Whether net inhibition or enhancement will be observed under any given set of conditions depends on the relative abundances and affinities of the different types of antibodies present and the particular gp120 molecule used. Notwithstanding these factors, it seems reasonable to assert that the V2 loop is a target for antibodies during the human immune response to HIV-1 infection, although in the one truly homologous situation—the IIIB-infected laboratory worker—we could find no evidence for a strong anti-V2 response either by peptide or competition analyses.

The enhancement of V2 MAb binding to both monomeric and oligomeric gp120 by serum antibodies from the IIIB-infected laboratory worker and by some HuMAbs is noteworthy. We presume that the effects of the laboratory worker's serum and the HuMAbs are related mechanistically, but this is not certain. For example, the enhancing effect of the laboratory worker's serum on V2 MAb binding could be due to the anti-V1 antibodies that are present in this serum. We are currently exploring this possibility. Overall, there may be multiple effects of serum antibodies of different specificities. Some of these may only be relevant in the context of monomeric gp120; HuMAbs A-32 and 211-C, for example, enhance V2 MAb binding to monomeric gp120 but not to oligomeric gp120 to which they fail to bind. Conversely, sCD4 enhances BAT-085 binding to oligomeric gp120 but not to monomeric gp120, probably through the induction of conformational changes within a gp120 oligomer.

Antibody-induced conformational changes in proteins are unusual, but have been described for HIV-1 gp120 (53). Furthermore, sCD4 binding increases the exposure of the V3 domain to both oligomeric and monomeric gp120 (3, 44, 45). Combinations of MAbs more effective than individual antibodies at neutralizing gp120 have been described previously (reviewed in reference 60); the efficacy of combinations including V2 MAbs and others that enhance V2 MAb binding might be worthy of further study if V2 MAbs of sufficient potency and broadness of neutralization can be identified.

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ADDENDUM IN PROOF

McKeating et al. (J. A. McKeating, C. Shotton, J. Cordell, S. Graham, S. Graham, P. Balfe, N. Sullivan, M. Charles, M. Page, A. Bolmstedt, S. Oloffson, S. C. Kayman, Z. Wu, A. Pinter, C. Dean, J. Sodroski, and R. A. Weiss, J. Virol. **67**:4932–4944, 1993) report that CRA-4 and another V2 MAb are sensitive to substitutions at residue 435 in C4. We did not observe this (Table 3). We presume that the differences arise from variations in methodology; we used gp120 mutants secreted as soluble molecules, whereas McKeating et al. used cell lysates containing partially folded gp160 in addition to correctly folded envelope glycoproteins.

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