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Identification and Characterization of Monoclonal Antibodies Specific for Polymorphic Antigenic Determinants within the V2 Region of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein

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We have identified six monoclonal antibodies (MAbs) mapping to both linear and conformation-dependent epitopes within the V2 region of the human immunodeficiency virus type 1 clone HXB10. Three of the MAbs (12b, 66c, and 66a) were able to neutralize the molecular clones HXB10 and HXB2, with titers in the range of 9.5 to 20.0 μg/ml. MAbs mapping to the crown of the V2 loop (12b, 60b, and 74) bound poorly to cell surface-expressed oligomeric gp120, suggesting an explanation for the poor or negligible neutralizing activity of MAbs to this region. In contrast, MAbs 12b and 60b demonstrated good reactivity with recombinant gp120 in an enzyme-linked immunosorbent assay format, suggesting differential epitope exposure between the recombinant and native forms of gp120. Cross-competition analysis of these MAbs and additional V1V2 MAbs for gp120 binding enabled us to assign the MAbs to six groups (A to F). Selection of neutralization escape mutants with MAbs 10/76b and 11/68b, belonging to nonoverlapping competition groups, identified amino acid changes at residues 165 (I to T) and 185 (D to N), respectively. Interestingly, these escape variants remained sensitive to neutralization by the nonselecting V2 MAbs. All MAbs demonstrated good recognition of IIIB viral gp120 yet failed to neutralize nonclonal stocks of IIIB. In addition, MAbs 12b and 62c bound MN and RF viral gp120, respectively, yet failed to neutralize the respective isolates. Cloning and expression of a library of gp120 and V1V2 fragments from IIIB-, MN-, and RF-infected H9 cultures identified a number of polymorphic sites, resulting in antigenic variation and subsequent loss of V2 MAb recognition. In contrast, the V3 region from the clones of the same isolates showed no amino acid changes, suggesting that the V2 region is polymorphic in long-term-passaged laboratory isolates and may account for the reduced antibody recognition observed.

Two major regions of gp120 have been identified as neutralization targets, the third variable domain (V3) (reviewed in reference 25) and the CD4-binding site (4, 10, 21, 23, 30; reviewed in reference 37). Antibodies against the V3 region inhibit human immunodeficiency virus (HIV)-induced syncytium formation and are thought to block infectivity post-CD4 binding. Mutations within this region affect both the fusogenicity (7, 29) and tropism (3, 11, 33, 34, 36) of the virus, suggesting that the V3 region plays a critical role in the infectious pathway of HIV type 1 (HIV-1). A number of human and rat monoclonal antibodies (MAbs) have been reported to bind to regions overlapping the CD4-binding site (4, 10, 23, 30, 37) and are thought to block infectivity by inhibiting gp120-CD4 interaction. Recently, a number of neutralizing MAbs mapping to the V2 region of HIV-1 gp120 and to the corresponding region of simian immunodeficiency virus gp130 have been reported (2, 6, 9, 13, 14, 22, 38), implying that this variable loop may also have a functional role in the entry of viruses into target cells. The V2 region has been reported to interact with the V3 region in determining macrophage tropism and sensitivity to neutralization by soluble CD4 (sCD4) (1, 5, 15, 39). Single-amino-acid changes in the V2 region have been reported to affect both the fusogenicity and the gp120-gp41 association (35). Furthermore, the overall length of the carboxy side of the V2 loop has been correlated with a switch from the non-syncytium-inducing to the syncytium-inducing phenotype of primary HIV-1 isolates (8).

Characterization of neutralizing MAbs is important both for vaccine design and for the identification of gp120/gp41 regions with functional roles in the infectious pathway. We have identified six new MAbs, mapping to both linear and discontinuous epitopes within the V2 region, three of which neutralize HXB10 infectivity. Epitope mapping of the MAbs identifies three MAbs mapping to the crown of the V2 loop, all of which demonstrated poor reactivity with cell surface-expressed gp120, suggesting an explanation for their poor or negligible neutralizing activity. In contrast, the same MAbs bound well to recombinant gp120 (rgp120), suggesting differential epitope exposure between recombinant monomeric and oligomeric gp120. Analysis of competition between the MAbs for gp120 binding enabled us to group MAbs into six groups, thereby identifying a number of distinct epitopes within V2. Variants were selected which were resistant to neutralization by two MAbs, 10/76b and 11/68b, recognizing distinct nonoverlapping epitopes. However, these variants remained sensitive to neutralization by MAbs in different competition groups. All of the V2 MAbs we have raised to date fail to neutralize nonclonal preparations of virus. To investigate this further, we cloned and expressed both gp120 and V1V2 from IIIB-, MN-, and RF-infected cultures. The data presented here identify a number of polymorphic sites within V2 which affect MAb recognition, implying that in vitro the V2 domain is more variable than the V3 domain. The potential variability of this region in vivo leads us to question whether antibodies to the V2 domain will be effective against nonclonal primary virus populations.

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MATERIALS AND METHODS

Sources of reagents. The following gp120 MAbs were used for neutralization and/or gp120-binding studies: 11/65a and 33/79, specific for amino acids (aa) 102 to 121 within C1; 11/4c, 11/41e, and 10/76b, specific for aa 162 to 171 (22); 11/68b, CRA-3, CRA-4, and CRA-5, specific for conserved conformation-dependent epitopes within V2 (22); 39.13g, specific for a conserved conformation-dependent epitope involved in CD4 binding (4, 23); 38.1a, specific for aa 430 to 447 (4, 21); 41.1, specific for a conformation-dependent epitope within V3 (19); and MAb 11/85b, mapping to aa 311 to 321 in V3 (19).

Recombinant CHO-expressed gp120 and peptides 740.13 (GEIKNCSFNIST SIRGKVQK), 740.14 (STSIRGKVQKEYAFFYKLDI), and 740.15 (EYAFFY KLDIIPIDNDTTSY) are members of the full set of gp120 overlapping peptides, which were obtained from the Medical Research Council AIDS Directed Programme. The control Raf-c peptide (IVQQFGYQRRASDDGKLTD) was a gift from C. Marshall, Institute of Cancer Research, London, United Kingdom. IIIB, MN, and RF viral stocks were obtained from the Medical Research Council AIDS Directed Programme. Yeast-expressed IIIB gp120 was a gift from K. Steimer, Chiron Corp., Emeryville, Calif. (34). The expression plasmid pEE14 was a gift from P. Stephens, CellTech, Slough, United Kingdom.

MAb production. CBH/Cbi rats were immunized (via their Peyer's patches) with rgp120 (HIV-1 LAI:BH10 clone expressed in CHO cells) emulsified in complete Freund's adjuvant. Hybridomas were screened for anti-gp120 activity by a solid-phase radio-binding assay as previously described (4).

Neutralization assay. HIV (10^3 50% tissue culture infective doses) in a volume of 40 μl was incubated at 37°C for 1 h with 10 μl of a dilution of the MAb being tested. The virus-Ab mixture was then incubated with 100 μl of C8166 cells at a concentration of 2×10^5 cells per ml per well in a microtiter plate in triplicate. At 5 days postinfection the wells were scored for the presence of syncytia, and the extracellular supernatant was collected from the wells by centrifugation, inactivated with 1% Empigen, and assayed for soluble p24 antigen as described previously (18, 20). The lowest concentration of Ab resulting either in a complete blocking of syncytial formation or in a >90% reduction in p24 antigen produced was defined as the reciprocal neutralization titer.

Cloning and expression of gp120 and V1V2 proteins. gp120 or V1V2 coding regions were amplified from DNA purified from IIIB-, MN-, and RF-infected H9 cultures either with primers 788 (cct cct acc tgc gga gga tcc AGT GCT GCA GAA AAA TTG TGG GTC ACA G, sense; vector-specific sequence shown in lowercase type) and 786 (gga gga acc tgc gga gaa ttc tta TCT TTT TTC TCT CTG CAC CAC TG antisense) or with primers PR13 (ga gga tcc TTT AAC ATG TGG AAA, sense) and 40002R (ga gaa ttc tta AAT TGG CTC AAA GG antisense), respectively. Amplification was performed for 30 cycles (94°C for 40 s, 50°C for 35 s, and 72°C for 210 s) with 16 ng of each primer, 200 μM each deoxynucleoside triphosphate, 50 μM TMAC, 1× pfu polymerase buffer 3, and 0.5 U of pfu polymerase (Stratagene). A 10-μl volume of the PCR product was visualized on an agarose gel, and the remainder was purified by a Gene-Clean procedure. After digestion with *Eco*RI-*Bam*HI (Promega Biotec), the PCR product was ligated into *Eco*RI-*Bgl*II-digested pEE14 and cloned into *Escherichia coli* "SURE" (Stratagene). Colonies were screened for inserts by PCR, with the same primers as for the generation of the template DNA, and plasmids were prepared from positively identified colonies. Constructs were expressed transiently in CHO cells by the calcium phosphate DNA coprecipitation method as supplied by CellTech. Plasmids demonstrated to express proteins were sequenced in the V1V2 region with an Applied Biosystems automated sequencer. The V1V2 construct expresses aa 93 to 212, including aa 93 to 126 of C1, aa 127 to 195 of V1V2, and 196 to 212 of C2 (based on the numbering of the HXB10 clone).

Ab binding to rgp120 and V1V2 proteins. (i) Peptide reactivity. Peptides were applied onto a solid support (Immulon II 96-well plates; Dynatech) at 2.5 μg/ml in Tris-buffered saline (TBS) overnight. Plates were blocked with blocking buffer (4% Marvel–TBS) and incubated for 1 h at room temperature with MAbs at 10 μg/ml diluted in 4% Marvel–20% sheep serum–TBS (TMSS). Unbound Abs were removed by washing with TBS, and bound Abs were detected with anti-rat immunoglobulin G (IgG) conjugated to horseradish peroxidase (Seralabs, Crawley, United Kingdom) and visualized with the substrate TMB (Murex Diagnostics, Beckenham, United Kingdom).

(ii) Binding of MAbs to V1V2 proteins. Soluble V1V2 proteins were obtained from the extracellular medium 72 h after transfection of CHO cells and were used to directly coat Immulon II 96-well plates. Plates were blocked as above, and the ability of the MAbs to bind to the V1V2 was assessed in the same way as for the peptide reactivity assay.

(iii) gp120 reactivity. rgp120 and Nonidet P-40-inactivated viral gp120/160 were bound to the solid phase via sheep Ab D7324 to the C terminus of gp120 (Aalto Bioreagents, Dublin, Ireland) in the presence of 2% Marvel (Cadbury) in TBS at an input concentration of 100 ng/ml. The ability of MAbs to bind to the captured gp120 was assessed in the same way as for the peptide reactivity assay.

(iv) Binding of MAbs to gp120 mutants. Mutations were introduced into the HXBc2 *env* gene, and mutant glycoproteins were transiently expressed in COS-1 cells as described previously (35). Cells were lysed in the presence of 50 mM Tris, 150 mM NaCl, 1% Triton, and 0.1% sodium dodecyl sulfate and the concentration of gp120 in the lysates was determined by twin-site enzyme-linked immunosorbent assay (ELISA) as previously described, with rgp120 as a calibrant (24). Mutant and wild-type envelope glycoproteins were allowed to bind to the solid-phase D7324 (see above) at an input concentration of 15 ng/ml. All MAbs were tested for their binding to the various mutant envelope glycoproteins at a predetermined saturating concentration of 10 μg/ml. Bound MAbs were detected with horseradish peroxidase-conjugated anti-rat IgG (Seralabs). The results are expressed as a ratio of the optical density (OD) of the mutant protein to wild-type protein and are termed the relative binding index.

(v) MAb binding to sCD4-rgp120 complexes. D7324-bound rgp120 (as above) was incubated with increasing concentrations of sCD4 (kind gift of R. Ward, Genentech Inc., South San Francisco, Calif.) diluted in TMSS for 1 h at room temperature. Unbound sCD4 was removed by three washes with TBS, and the ability of MAbs to bind to rgp120 in the presence or absence of sCD4 was assessed as described above. Bound sCD4 was detected with a polyclonal rabbit anti-sCD4 serum (rabbit 178) and horseradish peroxidase-conjugated anti-rabbit IgG (Seralabs).

(vi) MAb cross-competition analysis for rgp120 binding. V2 MAbs were tested for their ability to compete with iodinated preparations of a panel of V2 MAbs for binding to D7324-bound rgp120. The competing or unlabeled MAbs, at a concentration sufficient to saturate the gp120 (10 μg/ml), were mixed with an equal volume of iodinated MAb at a concentration resulting in half-maximal binding, and the mixture was incubated with gp120 for 1 h. Amounts of labeled MAb that bound to gp120 in the presence or absence of the competing MAb were determined.

Cell surface MAb binding. H9 cells infected 8 to 14 days previously with the HXB10 clone of HIV-1 were washed twice in prechilled phosphate-buffered saline (PBS), resuspended at a concentration of 10^7 cells per ml, and chilled on ice. A 50-μl volume of the cell suspension was added to each well of a U-bottom microtiter plate and incubated for 2 h with 50 μl of MAb at the concentrations indicated. For sCD4 inhibition experiments, the same number of cells was preincubated for 1 h with 10 μg of sCD4 per ml before being washed; MAb was then added. Bound sCD4 was detected with MAb L120 (Medical Research Council AIDS Directed Programme Repository), specific for domain 4. The cells were incubated for 30 min with the appropriate dilution of anti-rat or anti-mouse IgG conjugated to phycoerythrin (Immunotech, Luminy, Marseilles, France) before being subjected to overnight fixation in PBS–2% formaldehyde. The fixed cells were analyzed on a Becton Dickinson FACScan with Consort 30 software. All incubations and washes were carried out on ice.

TABLE 1. Neutralization titers for various MAbs and sCD4

Ligand	Isotype	Neutralization titer (μg/ml) for virus:			
		HXB10	IIIB	MN	RF
66a	IgG1	12.0	NDN[a]	NDN	NDN
66c	IgG1	9.5	NDN	NDN	NDN
62c	IgG1	NDN	NDN	NDN	NDN
12b	IgG2a	20.0	NDN	NDN	NDN
60b	IgG2a	NDN	NDN	NDN	NDN
74	IgG1	NDN	NDN	NDN	NDN
10/76b	IgG2a	3.0	NDN	NDN	NDN
11/68b	IgG1	2.4	NDN	NDN	NDN
sCD4		0.4	0.8	0.6	0.7
41.1	IgG2a	0.8	0.5	NDN	NDN

[a] NDN, no detectable neutralizing activity.

RESULTS

We screened a panel of hybridoma culture supernatants for antibodies that were capable of recognizing rgp120 but failed to react with a mutant gp120 protein in which both the V1 and V2 domains had been deleted (deletion of aa 119 to 205) (35). Six gp120-specific MAbs (66a, 66c, 62c, 12b, 60b, and 74) which failed to react with the V1V2-deleted protein were identified. The new MAbs were compared with two previously reported neutralizing V2 MAbs, 10/76b and 11/68b (22), and sCD4 for their ability to neutralize HXB10. Three of the six new MAbs tested, 66a, 66c, and 12b, were found to neutralize HXB10 with titers ranging from 9.5 to 20 μg/ml, in contrast to 10/76b and 11/68b, which neutralized HXB10 at 1.2 and 2.3 μg/ml, respectively (Table 1). MAbs 62c, 60b, and 74 failed to neutralize HXB10 at the concentrations tested (up to 100 μg/ml). In addition, all of the MAbs tested failed to neutralize IIIB, MN, and RF virus, whereas sCD4 neutralized all three isolates within the 0.4- to 0.8-μg/ml range. The inability of the V2 MAbs to neutralize IIIB may suggest V2 amino acid variation within the nonclonal preparation of virus being used, which

TABLE 2. Reactivity of MAbs with rgp120 and peptides

MAb	Concn of MAb resulting in half-maximal binding to protein:			
	rgp120	740.13	740.14	740.15
66a	0.25	—[a]	—	—
66c	0.15	—	—	—
62c	0.40	—	—	—
12b	0.60	—	0.08	—
60b	0.80	—	1.50	6.0
74	5.40[b]	—	8.50[b]	10.6[b]
10/76b	0.12	0.04	0.40	—

[a] —, no detectable binding.
[b] MAb 74 failed to saturate gp120 or peptides; hence, the value is shown is the concentration of MAb resulting in 50% of the maximal values obtained.

may be sufficient to reduce MAb recognition. Additional IIIB viral preparations, which were consistently neutralized by MAbs to the V3 domain, were not neutralized by any of the V2 MAbs tested (data not shown).

This inability of some V2 MAbs to neutralize viral infectivity may be explained either by a low affinity of the MAb for gp120 or by epitopes of the V1V2 domain not being neutralization targets. We therefore monitored the ability of the MAbs, in the absence of detergent, to bind to rgp120 in an ELISA format. With the exception of MAb 74, all the MAbs bound to rgp120, with half-maximal binding at concentrations of 0.15 to 0.80 μg/ml (Table 2). MAb 74 failed to saturate rgp120 at concentrations of up to 50 μg/ml (data not shown), suggesting that the inability of this MAb to neutralize HIV infectivity is due primarily to its low affinity. However, the nonneutralizing MAbs, 62c and 60b, bound gp120 with relative affinities close to that of 12b, which did neutralize HXB10 at 20 μg/ml. It is therefore likely that epitope specificity also plays a role in determining whether a MAb is neutralizing. We therefore tested the ability of these MAbs to bind to a series of overlapping peptides encompassing the HXB10 V1V2 sequence and a series of V2 mutant gp120 proteins. Three of the MAbs (12b, 60b, and 74) were able to bind to a denatured and unglycosylated yeast (*Saccharomyces cerevisiae*) expressed gp120, suggesting that they were recognizing linear epitopes (34). All of the MAbs failed to react with a control peptide (Raf-c) and both V1 peptides (740.11 and 740.12) (data not shown), MAbs 60b and 74 bound to peptides 740.14 and 740.15, whereas MAb 12b reacted only with peptide 740.15 (Table 2). MAbs 66a, 66c, and 62c, which failed to react with the yeast gp120 protein, also failed to bind all the peptides tested (Table 2), suggesting that they recognize conformation-dependent epitopes. Since MAbs 60b and 74 bind to the overlapping peptides STSIRGKVQKEYAFFYKLDI (ADP 740.14 aa 162 to 181) and EYAFFYKLDIIPIDNDTTSY (ADP 740.15 aa 172 to 191), the residues common to both peptides (EYAFFYKLDI) may form their epitope.

To identify amino acids important for recognition of gp120 by MAbs 66a, 66c, and 62c and to confirm the peptide-binding data, all of the MAbs were tested for their reactivity with a set of gp120 mutants altered in conserved residues within the V2 loop. These mutant glycoproteins have been previously characterized with respect to gp160 precursor processing, gp120-gp41 association, and CD4-binding ability (35). The wild-type and mutant gp120s were compared for their ability to bind a saturating concentration (with the exception of MAb 74, which was tested at 20 μg/ml) of the six new V1V2 MAbs and MAb 11/85b mapping to the V3 domain. The results are expressed as the ratio of MAb bound to the mutant gp120 in comparison with the wild-type protein (binding index) (Table 3). The V3 MAb, 11/85b, bound equivalently to all the mutants with binding indices ranging from 0.86 to 1.12 (data not shown), serving as an internal control for gp120 levels. Changes at residues 176 and 177, 179 and 180, 183 and 184, and 191 to 193 abrogated the binding of MAbs 66a, 66c, and 62c, suggesting that changes within the crown and C-terminal side of the V2 loop affect the reactivity of this set of MAbs (Table 3). The binding of MAbs 12b and 60b was abrogated only by changes at 179 and 180 (LD/DL) and at both 179-180 and 191–193 (YSL/GSS), respectively. Residues 179 and 180 reside within peptides 740.14 and 740.15; however, residues 191 to 193 are not all contained within peptide 740.15, implying that changes outside the defined minimal peptide epitope may have effects on MAb gp120 reactivity. Interestingly, the binding of MAb 74, which exhibited a weak binding affinity both for gp120 and for peptides 740.14 and 740.15, is sensitive to changes at residues 168, 176 and 177, 179 and 180, 183 and 184, and 191 to 193 (Table 3).

To define the number of distinct epitopes recognized by our complete panel of V2 MAbs, we compared the ability of the V1V2 MAbs to cross-compete with each other for gp120 recognition (Table 4). On the basis of these results, the MAbs were grouped into six competition groups (A to F). Not surprisingly, linear MAbs 12b and 60b completely cross-block each other for gp120 reactivity and compete weakly with the conformation-dependent MAbs 11/68b, 66c, 66a, 62c, CRA-3, CRA-4, and CRA-6 for gp120 reactivity, suggesting that the group A MAbs bind to epitopes near the crown of the V2 loop. This is further supported by the inability of the group A and F MAbs (aa 162 to 171) to cross-compete with each other for gp120 reactivity. MAb 74 (group D), which bound weakly to the same peptides as 60b (Table 2), failed to compete with both 60b and 12b; furthermore, its binding to gp120 was enhanced by linear MAbs within group F and by two of the conformation-dependent MAbs (11/68b and 62c). On the basis of this enhancement, MAbs 11/68b and 62c (group B) were classified

TABLE 3. Reactivity of MAbs for gp120 V2 mutants

MAb	Binding index[a] for gp120:								
	Wild type	ΔV1V2	152–153 (GE to SM)	166 (R to L)	168 (K to L)	176–177 (FY to AT)	179–180 (LD to DL)	183–184 (PI to SG)	191–193 YSL to GSS
12b	+++	−	+++	+++	+++	+++	−	+++	+++
60b	+++	−	+++	+++	+++	+++	−	+	−
74	+++	−	+++	+++	−	−	−	−	−
62c	+++	−	+++	+++	+++	−	−	−	−
66c	+++	−	+++	+++	+++	−	−	−	−
66a	+++	−	+++	+++	+++	−	−	−	−

[a] +++, binding index 0.8 to 1.20; ++, binding index 0.5 to 0.79; +, binding index 0.2 to 0.49; −, binding index 0 to 0.19.

TABLE 4. Cross-competition of V1V2 MAb for gp120 reactivity

Group	Competing MAb	% Binding of labeled MAb[a]:										
		12b	60b	11/68b	62c	66c	66a	CRA4	74	CRA3	CRA6	11/4c
A	12b	**1**	**1**	38	40	39	54	**13**	**15**	**22**	34	101
	60b	**15**	**1**	66	85	59	55	**33**	50	48	61	94
B	11/68b	69	69	**3**	**12**	**15**	**11**	**1**	256	**1**	**2**	127
	62c	64	48	**1**	**1**	**3**	**1**	**9**	323	**9**	**4**	117
C	66c	66	53	**1**	**4**	**12**	**1**	**3**	35	**2**	**1**	105
	66a	66	55	**1**	**2**	**8**	**1**	**15**	**30**	**1**	**5**	108
	CRA4	72	63	**24**	**25**	**25**	**31**	**6**	66	**10**	35	119
D	74	95	88	120	174	123	—[b]	81	**1**	50	—	94
E	CRA3	85	80	76	55	69	59	76	**26**	34	48	103
	CRA6	83	77	47	44	36	**32**	**24**	**1**	**15**	**6**	91
F	11/4c	87	96	199	169	148	147	169	631	**19**	**2**	**2**
	10/76b	106	96	151	186	147	114	124	641	**2**	**1**	**1**
	11/4b	96	79	196	241	150	140	164	673	**18**	**1**	**1**

[a] Boldface type corresponds to a percent binding of ≤33%.
[b] —, not tested.

differently from MAbs 66a and 66c (group C) (Table 3). As a further control, we demonstrated that a panel of MAbs mapping to V3, the CD4-binding site, and to linear epitopes within C1 and C5 had no effect on the ability of MAb 74 to recognize gp120 (data not shown).

Selection of neutralization-resistant escape variants. To further define amino acids critical for the binding and subsequent neutralization of HIV-1 by V2 MAbs, variants of the molecular clone HXB2 were selected that could replicate in the presence of neutralizing concentrations of MAbs 10/76b and 11/68b. Variants 11 and 16 which were resistant to neutralization by 11/68b and 10/76b, respectively, were obtained. Both variants remained sensitive to neutralization by V2 MAbs within different competition groups (Table 5). Variant 16 was resistant to neutralization by all MAbs within group F but remained sensitive to neutralization by MAbs within noncompeting groups. MAbs in groups B and C, which competed with 11/68b for gp120 recognition, were still able to neutralize variant 11 with comparable sensitivities to that of the parental virus (Table 5). Sequencing the gp120 gene from the two variants and from the parental virus identified single-amino-acid changes in each envelope gene: variant 11 had a single base change, GAT to AAT (nucleotide 6776), predicting an amino acid change of D to N at residue 185, while variant 16 had a base change of ATA to ACA (nucleotide 6717), predicting an amino acid change of I to T at residue 165. We monitored the ability of a range of antibodies to bind to detergent (Nonidet P-40)-inactivated wild-type and variant viral gp120 in an ELISA format (Fig. 1). Variant 11 showed a reduced binding index of 0.55 for the selecting MAb 11/68b, resulting in complete resistance to 11/68b neutralization at MAb concentrations up to 50 μg/ml. Similarly, variant 16 showed a reduced binding index of 0.25 for the selecting MAb 10/76b, also resulting in resistance to neutralization; this suggested that relatively modest reductions in the affinity of the MAb for gp120 result in considerable effects on the ability of the MAb to neutralize (Fig. 2). All of the MAbs within group F demonstrated reduced binding for variant 16-gp120, whereas MAbs 62c, 66c, and 66a within groups B and C bound to variant 11-gp120 with binding indices of 0.85, 0.71 and 0.74, respectively (Fig. 1).

Ability of V2 MAbs to bind to native cell surface-expressed gp120. We compared the ability of the nonneutralizing MAbs (60b, 62c, and 74) and the neutralizing MAbs (66c, 12b, and 10/76b) to bind to rgp120 and to oligomeric gp120 expressed at the surface of HXB10-infected cells (Fig. 3). MAbs 60b, 12b,

TABLE 5. Neutralization titers for escape variants

Ligand	Competition group	Neutralization titer (μg/ml) for virus:		
		Wild type	Variant 11	Variant 16
12b	A	22.0	21.5	22.4
11/68b	B	2.4	NDN[a]	3.3
66c	C	10.2	12.4	11.4
66a	C	12.6	10.8	11.8
CRA-4	C	3.2	4.5	4.2
11/4c	F	5.0	5.6	NDN
10/76b	F	3.2	2.8	NDN
11/4b	F	3.6	3.8	NDN
39.13g		4.3	3.8	4.7

[a] NDN, no detectable neutralization.

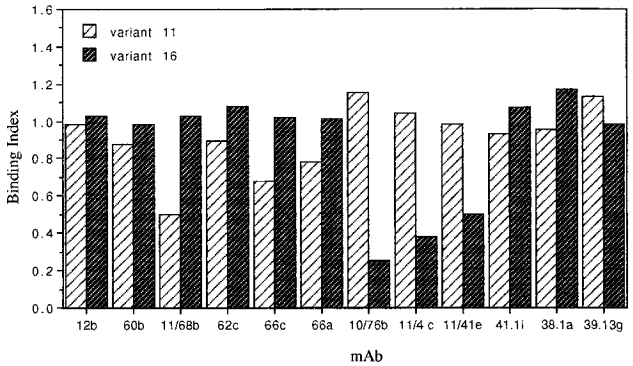

FIG. 1. Binding of MAbs to variants 11 and 16 gp120/160. MAbs (at a saturating concentration of 10.0 μg/ml) that map to V2 epitopes within competition groups A (12b, 60b), B (11/68b, 62c), C (66c, 66a), and F (10/76b, 11/4c, and 11/41e), to a V3 epitope (41.1i), to a linear C4 epitope (38.1a), and to an epitope overlapping the CD4-binding site (39.13g) were compared for their ability to bind to Nonidet P-40-solubilized gp120/160 from variant 11 and 16 viral stocks. The results are expressed as the ratio of MAb (OD_{450}) bound to the variant relative to the wild type and are termed the relative binding index.

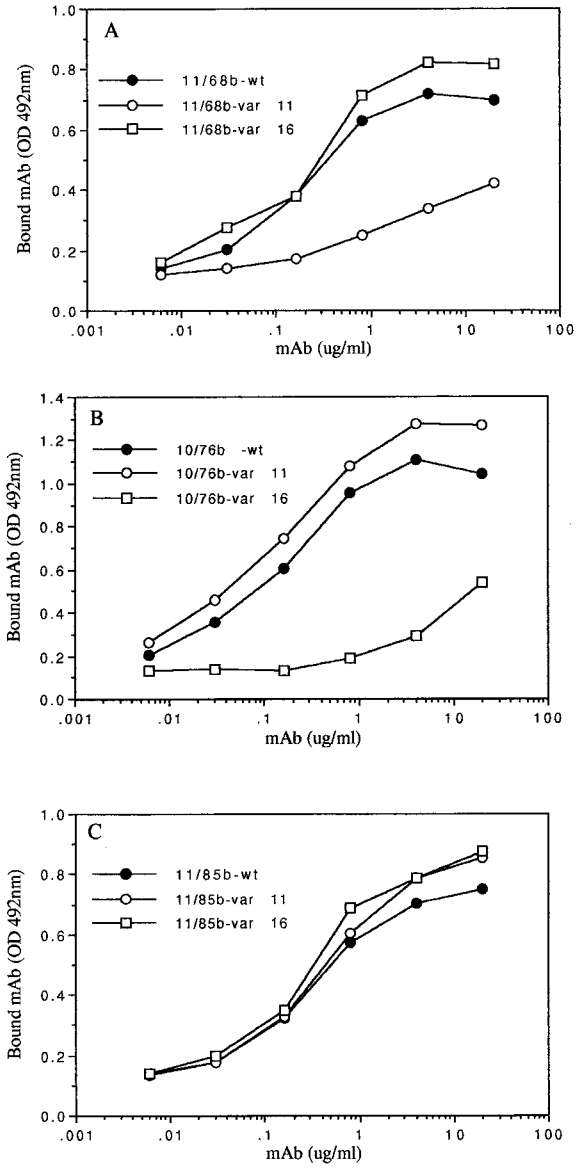

FIG. 2. Binding of MAbs to wild-type and variant 11 and 16 gp120/160. Increasing concentrations of MAbs 11/68b, mapping to a conformation-dependent epitope within V2 (A), 10/76b, mapping to a linear epitope within V2 (B), and 11/85b, mapping to a linear epitope within V3 (C), were analyzed for their ability to bind to Nonidet P-40-solubilized viral gp120 from wild-type (wt) and variant 11 and 16 viral stocks.

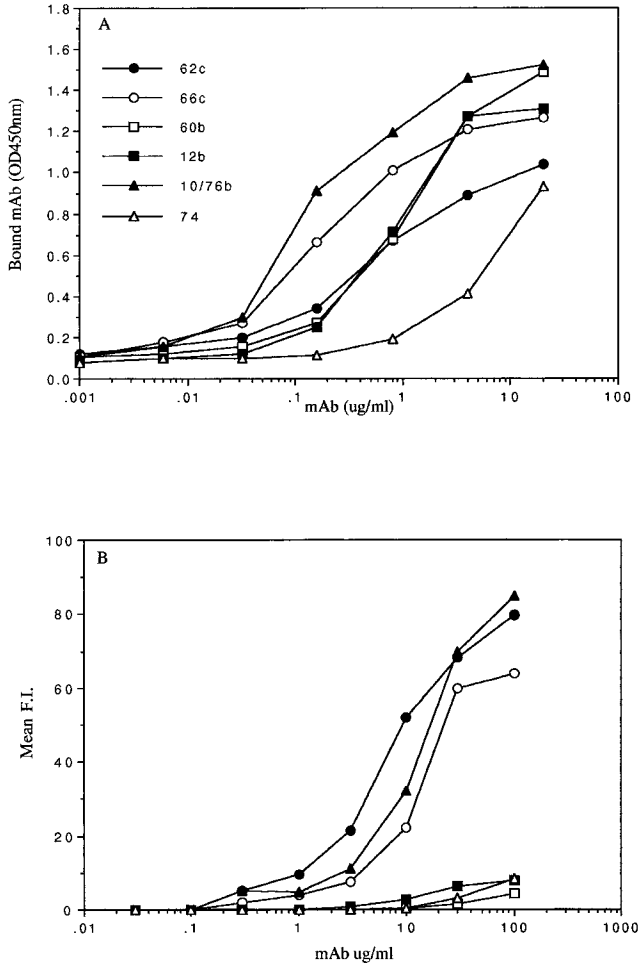

FIG. 3. Binding of MAbs to recombinant and native cell surface-expressed gp120. Increasing amounts of MAbs, 62c, 66c, 60b, 12b, 10/76b, and 74 were monitored for their ability to bind recombinant gp120 (clone BH10) in an ELISA (A) and to bind to the surface of HXB10-infected H9 cells (B). Results are expressed as OD_{450} (A) and mean fluorescence intensity (F.I.) (B).

and 74 bound very weakly to oligomeric gp120, even though the first two MAbs showed good reactivity with rgp120, suggesting that their epitopes are not well exposed on oligomeric gp120 and thereby offering an explanation both for the inability of MAb 60b to neutralize and for the low neutralizing activity of MAb 12b. These data suggest that there are differences in V2 epitope exposure on monomeric gp120 and gp120 in the context of the oligomeric envelope glycoprotein.

We have previously reported that the gp120 recognition of MAbs 11/68b and CRA-4, members of competition groups B and C, respectively (Table 4), was inhibited by prior incubation of gp120 with sCD4 (22). Since we now have a larger panel of V2 MAbs mapping to nonoverlapping epitopes spanning the V2 loop, we were able to investigate whether sCD4 induced change in V2 exposure was epitope specific. We therefore measured the ability of the panel of V2 MAbs to bind to monomeric and oligomeric gp120 in the presence or absence of a saturating concentration of sCD4 (10 μg/ml). All the MAbs that recognized conformation-dependent epitopes (66c, 66a, 62c, and 11/68b) exhibited reduced envelope recognition in the presence of sCD4, with both recombinant and oligomeric gp120 (Fig. 4). However, MAbs that recognized linear epitopes within groups A and F did not show a significant reduction in gp120 binding in the presence of sCD4; this was also observed for MAb recognition of oligomeric gp120 (Fig. 4).

Antigenic variation within the V2 domain. Ho and colleagues reported that some V2 MAbs were capable of cross-neutralizing divergent HIV-1 isolates (6, 9). However, we found that all of our MAbs were capable of neutralizing (as determined by >90% inhibition of p24 antigen production) only HXB10 and HXB2 molecular clones and failed to neutralize IIIB, MN, and RF. We therefore investigated the ability of the MAbs to bind to gp120 from detergent-inactivated viral stocks of IIIB, MN, and RF. All of the MAbs were able to recognize IIIB gp120; however, MAbs 12b and 62c also reacted with MN and RF gp120, respectively, giving OD values in a capture ELISA of 0.54 and 0.42. The binding of MAbs 12b and 62c to MN and RF gp120, respectively, was lower than that observed with IIIB gp120 (OD values of 1.05 and 0.84). MAb

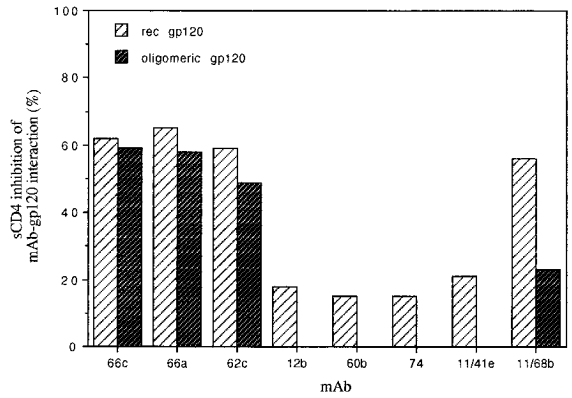

FIG. 4. Effect of sCD4 on V2 MAb recognition of native cell surface-expressed gp120 and rgp120. MAbs mapping to conformation-dependent V2 epitopes (66c, 66a, 62c, and 11/68b) and linear V2 epitopes (12b, 60b, 74, and 11/41e) were tested for their ability to bind to recombinant gp120 and to HXB10-infected H9 cells, with or without prior incubation with sCD4 (10.0 μg/ml). Results are expressed as the sCD4-induced percent inhibition of MAb-gp120 binding.

38.1a (binding to an epitope conserved among the viral strains tested) bound with equivalent OD values to all three gp120 preparations, suggesting that there were equivalent gp120 concentrations in the three viral lysates used in this assay (data not shown). These results suggest that the reduced binding exhibited by MAbs 12b and 62c may be due either to a reduced affinity of the MAb for the gp120 in question or to antigenic variation present in the polymorphic viral stock leading to loss of MAb recognition. To address this question, we cloned and expressed both gp120 and a V1V2 subfragment from the IIIB, MN, and RF viral stocks used for neutralization studies and monitored the ability of the cloned proteins to bind a panel of V2 MAbs. Most of the cloned IIIB gp120 proteins (12 of 16) failed to bind MAb 10/76b and other MAbs within group F (data not shown), whereas 9 of 16 clones bound the conformation-dependent MAbs 11/68b and 66c (Fig. 5). In contrast, all of the clones bound MAbs 12b and 60b. Sequencing the V2 domain from these clones identified five polymorphic sites at residues 163, 164, 165, 177, and 193, suggesting that isoleucine at residue 165 is critical for determining the binding of all group F MAbs. In contrast, it was difficult to predict the amino acid residues important for recognition by the conformation-dependent MAbs 11/68b and 66c, suggesting that amino acid changes in regions outside the V2 domain may have an effect on the tertiary structure of the V2 loop and hence its recognition by conformation-dependent antibodies. Only 4 of 12 of the MN gp120 and 5 of 11 of the MN V1V2 proteins bound MAb 12b (Fig. 5). Sequencing of the V1V2 regions from the MN clones identified three polymorphic sites, at residues 188, 193, and 195, common to both gp120 and V1V2 constructs (Fig. 5). A proline at residue 188 is critical for recognition by MAb 12b. The importance of the proline at residue 188 (P-188) was confirmed by expressing the V1V2 domains alone and demonstrating, in the absence of any interaction with the rest of the gp120 molecule, that P-188 determined MAb 12b recognition. In addition, 17 of 24 MN clones had lost a predicted glycosylation site because of amino acid changes at residues 191 and 193. MAb 12b bound less well to V1V2 protein containing P-188, N-191, S-193, and S-195 than to a protein with P, S, S, and S or P, N, N, and R at the same residues, suggesting that the loss of the predicted glycosylation site at residue 191 led to increased binding of MAb 12b (Fig. 6). We were unable to measure this effect for 12b-gp120 reactivity, since all of the cloned gp120 proteins able to bind MAb 12b coded for a predicted glycosylation site at residue 191. Similarly, 3 of 11 cloned RF gp120 proteins bound to MAb 62c, and sequencing of these clones identified a single-amino-acid change of E to K at residue 193 (Fig. 5). The importance of this residue for MAb 62c reactivity was further confirmed by expressing V1V2 domains which differed only at this position; proteins with a glutamine at residue 193 bound MAb 62c, whereas those with a lysine did not. In contrast, the sequences obtained from the V3 regions of IIIB, MN, and RF gp120 clones showed no amino acid polymorphisms (data not shown).

DISCUSSION

We have cloned six new V1V2 MAbs which recognize both linear and conformation-dependent epitopes within the V2 loop. Three MAbs, 66c, 66a, and 12b, were found to neutralize HXB10 in the range 9.5 to 20.0 μg/ml, which is comparable to the neutralization efficiency of previously reported V2 MAbs (27). The remaining three MAbs, however, failed to neutralize at concentrations up to 100 μg/ml. In contrast, two previously described V2 MAbs, 10/76b and 11/68b, neutralized HXB10 at 3.0 and 2.4 μg/ml, respectively (22). Epitope-mapping data demonstrated that MAbs 12b, 60b, and 74 bind to epitopes covering the crown of the V2 loop (Tables 2 and 3). However, only MAb 12b was able to neutralize HXB10 at relatively high concentrations of MAb (Table 1). Interestingly, MAbs 12b, 60b, and 74 demonstrated poor binding to cell surface-expressed gp120 (at 50 μg/ml), giving maximal mean fluorescence intensities of 15.7, 9.3, and 7.4, respectively, compared with 197.9 for MAb 10/76b, which recognizes an epitope (aa 162 to 170) in the N-terminal region of V2. Recently, Warrier et al. reported that a chimpanzee MAb, C108G, which recognizes a glycan-dependent epitope within aa 162 to 169 and is capable of cross-competing with all of our group F MAbs for gp120 binding (data not shown), was able to neutralize HXB2 at 0.1 μg/ml (38). These data further support the conclusion that the N-terminal region of V2 on oligomeric gp120 is well exposed and capable of binding neutralizing antibodies. The low affinity of MAbs 12b, 60b, and 74 for oligomeric cell surface gp120 may explain their weak or undetectable neutralizing activity. It is of interest that both MAbs 12b and 60b bound well to rgp120 in an ELISA, with affinities comparable to those of the other V2 MAbs, suggesting differential epitope exposure on recombinant monomeric gp120 and gp120 in the context of the oligomeric envelope glycoprotein complex. The reduced exposure of the crown of the V2 loop may be due to oligomerization of gp120 and possible interaction(s) of V2 with other regions of gp120 and/or gp41 (1, 5, 15). A number of residues in the crown of the loop, overlapping with the epitope(s) of MAbs 12b and 60b, are hydrophobic and therefore may not be exposed at the surface of the molecule. Furthermore, Sattentau et al. (32) found that epitopes in the V2 domain of HIV-2 and simian immunodeficiency virus, corresponding to the crown of the respective loops, were not well exposed on the native molecule. In contrast, Moore et al. (26, 27) reported that the crown of the V2 loop was better exposed than the amino-terminal side, since a MAb mapping to the N-terminal side of the V2 loop (6C4/5) failed to recognize oligomeric gp120 but a mAb mapping to the crown of the loop, BAT-085, bound to HXB10-infected H9 cells with a mean fluorescence intensity of 9.38. We agree with these data and found that MAb 6C4/5 exhibited weak affinity for both recombinant and cell surface-expressed gp120, suggesting that the inability of the MAb to bind native gp120 is purely a sensitivity problem associated with its low affinity. Furthermore, these data are based only on the IIIB isolate; many laboratory-adapted isolates have V2 loops of different lengths,

Virus	Protein	aa at residue[a]: 163	164	165	177	193	No of Clones	Reactivity with MAb[b]: 11/65a	12b	11/68b	66c	10/76b
IIIB	gp120	T	S	K	Y	L	6	+++	+++	++	++	-
		T	S	K	Y	L	2	+++	+++	+	+	-
		T	S	K	Y	L	2	+++	+++	-	-	-
		T	S	I	Y	L	1	+++	+++	+++	+++	+++
		T	S	I	Y	L	1	+++	+++	-	-	-
		T	G	K	Y	L	1	+++	+++	-	-	-
		T	S	K	Y	M	1	+++	+++	-	-	-
		T	S	I	H	L	1	+++	+++	+++	+++	+++
		A	S	I	Y	L	1	+++	++	++	++	++

		169	187	188	191	193	195		11/65a	12b
MN	V1V2	S	E	S	N	N	R	5	+++	-
		S	E	P	S	S	S	2	+++	+++
		G	V	P	N	N	R	2	+++	+++
		S	E	P	N	S	S	2	+++	+
		S	E	E	N	N	R	1	+++	-
	gp120	S	E	S	N	N	R	6	+++	-
		S	E	P	N	S	S	4	+++	+++
		S	E	S	N	S	R	1	+++	-
		S	E	S	N	N	K	1	+++	-
	Los Alamos	S	V	S	N	S	S			

		193		11/65a	62c
RF	gp120	E	8	+++	-
		K	3	+++	+++

FIG. 5. Polymorphic residues identified within the V2 domain of IIIB, MN, and RF [a], numbered as in reference 28. [b], ELISA readings at 450 nm: −, <0.1; +, 0.10 to 0.40, ++, 0.41 to 0.80; +++, >0.80.

varying from 38 residues (HIV-1 GUN) to 53 residues (HIV-1 RF), which may dramatically affect epitope exposure in the native molecule (16, 28).

All of the conformation-dependent MAbs, 62c, 66a and 66c, appeared to be affected equally by mutations in the crown and C-terminal side of the V2 loop (Table 3), suggesting that they were recognizing common epitopes. This idea is further supported by the ability of the three MAbs to compete with each other for gp120 recognition (Table 4). However, MAb 62c failed to neutralize HXB10, despite binding to the cell surface with intensities equivalent to or greater than those of MAbs 66c and 66a. MAb 62c could be distinguished from both 66c and 66a in two assays; first, MAb 62c was able to enhance the binding of MAb 74 to gp120 (Table 4), and second, it was unique in its ability to recognize the RF gp120. These data suggest that 62c recognizes an epitope distinct from that recognized by MAbs 66c and 66a and therefore cannot be directly compared with them.

Cross-competition analysis has enabled us to group the MAbs alongside a number of previously reported V2 MAbs (22) with respect to their ability to compete with each other for rgp120. Competition groups A and F, comprising MAbs that bind to linear epitopes in the N terminus and the crown of the V2, respectively, fail to compete with each other, and they therefore define two nonoverlapping groups. MAb 12b clearly recognizes a closely related but distinct epitope from that of 60b on the basis of differential peptide reactivity, gp120 mutant reactivity, and recognition of MN gp120. It is interesting that MAbs in group F compete with MAbs in group E (CRA-3 and CRA-6), whereas the reciprocal competition does not occur (Table 4 and data not shown). MAbs 11/68b, 62c, 66c, 66a, and CRA-4 cross-compete with each other and compete with MAbs in group E for gp120 reactivity but differ in their ability to enhance the binding of MAb 74. MAb 74 appears unique in that it fails to compete with any other MAb, even 12b and 60b, and its binding to gp120 is enhanced by MAbs in groups B and E. These data suggest that antibodies binding to both linear and conformation-dependent epitopes can affect V2 loop exposure. MAbs in group E compete only with each other for gp120 recognition, whereas MAbs in both groups B and C effectively compete with all MAbs in group E for gp120 binding. This analysis has identified a number of putative epitope groups in V2; clearly, MAbs which appear to recognize similar epitopes as measured by their ability to recognize gp120 V2 mutants (Table 3) can behave differently in competition assays.

We previously reported that prior incubation of both recombinant and oligomeric gp120 with sCD4 inhibited the subse-

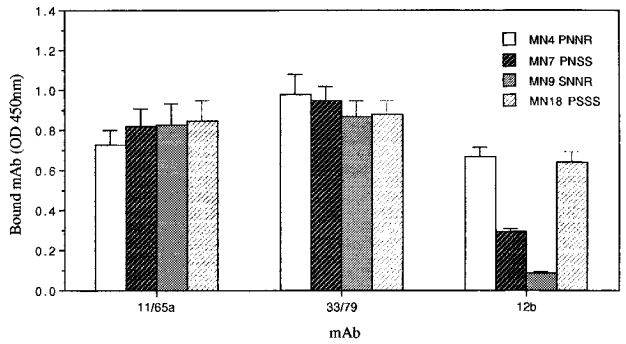

FIG. 6. Binding of MAbs to MN variant V1V2 proteins. MAbs 11/65a and 33/79, mapping to amino acids 102 to 121 in C1, and MAb 12b, mapping to amino acids 162 to 181 in V2, were monitored for their ability to bind to a panel of MN V1V2 proteins. The MN clones were sequenced and shown to be polymorphic at residues 188, 191, 193, and 195, respectively.

quent recognition of gp120 by two conformation-dependent V1V2 MAbs, 11/68b and CRA-4 (22). Since we now have a larger panel of V2 MAbs that recognize a number of nonoverlapping epitopes distributed within the V2 domain, we have been able to investigate the effect of sCD4 on the modulation of the various V2 epitopes. Generally, all of the conformation-dependent MAbs (66c, 66a, 62c, and 11/68b) demonstrated reduced recognition of gp120 complexed with sCD4 (Fig. 4). In contrast, MAbs that recognized linear epitopes within the N terminus and the crown of the V2 loop bound to gp120 with equivalent values irrespective of the presence of sCD4 (Fig. 4 and data not shown). These data suggest that sCD4 binding to gp120 affects V2 configuration generally and that such changes are more readily detected with MAbs that recognize conformation-dependent epitopes. Sattentau et al. (32) reported that the V2 MAb G3-4 demonstrated enhanced binding to cell surface-expressed gp120 in the presence of sCD4. This observation would appear to rule out a general disruption of V2, since MAb G3-4 recognizes a conformation-dependent V2 epitope, but would support the model that sCD4-induced movement of the V2 loop results in selective masking of some conformation-dependent V2 structures. The effect of sCD4 on V2 MAb-gp120 recognition was similar both with recombinant and native cell surface-expressed envelope (Fig. 4). None of the V2 MAbs affected the ability of sCD4 to bind to rgp120, whereas some inhibition (MAbs 11/68b, 66a, and 62c) and enhancement (MAbs 10/76b and 11/4c) (19 to 28%) of sCD4 binding to cell surface-expressed gp120 was observed (data not shown).

To further delineate the critical residues for V2 MAb recognition, we selected variants resistant to neutralization by MAbs (40) from two nonoverlapping epitope groups. Two variants were selected which were resistant to 11/68b and 10/76b (Table 5) and which had single-amino-acid changes at residues 185 (D to N) and 165 (I to T), respectively. The amino acids at these two residues appear relatively well conserved, with 27 of 32 viral sequences having a D at position 185 and 28 of 32 sequences having an I at position 165 (28). Since we have already demonstrated that a single-amino-acid change at residue 166 (R to L) (22) affects all the linear MAbs mapping to group F, it is not surprising that a change at neighboring residue 165 reduces MAb recognition and hence neutralization for all MAbs within this group. In contrast, we have reported that changes in residues 179 and 80 (LD to DL), 183 and 184 (PI to SG), and 191 to 193 (YSL to GSS) affect the binding of all of the conformation-dependent MAbs (11/68b, 62c, 66c, and 66a); however, the single-amino-acid change at residue 185 (D to N) in variant 11 affected the binding and subsequent neutralization of only MAb 11/68b (Fig. 1; Table 5). These data suggest that the conformation-dependent MAbs in groups B and C may recognize closely related but distinct epitopes. Furthermore, if antibodies to the V2 region play a neutralizing role in vivo, it is encouraging to observe that both variants 11 and 16 remained sensitive to neutralization by all the other V2 MAbs (Table 5).

A number of polymorphic residues in the V2 domain were observed in the gp120 and V1V2 proteins cloned from IIIB-, MN-, and RF-infected cultures, resulting in the loss of V2 MAb recognition (Fig. 5). The antigenic polymorphism present within laboratory isolates explains the inability of the V2 MAbs to neutralize nonclonal preparations of IIIB, MN, and RF (Table 1). Only 4 of 16 IIIB gp120 clones, all of which contained an isoleucine at residue 165, were able to bind MAb 10/76b. Similarly, 6 of 16 IIIB gp120 clones were able to bind the conformation-dependent MAbs 11/68b and 66c; however, it was impossible to predict from the V2 sequence which clones would be able to bind the conformation-dependent MAbs (Fig. 5), suggesting that amino acid changes outside the V2 domain may affect its conformation and ability to bind antibodies that recognize discontinuous epitopes. Sequencing of the V1 domain from the 16 IIIB gp120 clones failed to identify any amino acid changes between the clones. In contrast, all 16 gp120 clones bound MAbs 12b and 60b, which recognize linear epitopes covering the crown of the loop, suggesting that this region of V2 is more highly conserved and that this may be due to the inaccessibility of these epitopes on native oligomeric gp120. Furthermore, only a minority (9 of 23) of MN gp120 and V1V2 cloned proteins, all of which contained a proline at residue 188 in place of a serine, were able to bind MAb 12b. It is interesting that the sequence for MN at residue 188 in the Los Alamos database is a serine but that all other HIV-1 sequences have a proline at the equivalent residue. In addition, changes at residues 191 and 193 in the MN V1V2 proteins resulted in the loss of a predicted glycosylation site (17), which led to an increase in the binding of MAb 12b (Fig. 5 and 6). These data suggest that the presence of glycans may affect the ability of V2 MAbs to bind and subsequently neutralize HIV infectivity. Only a single coding change was observed in the 11 RF gp120 clones sequenced, resulting in a change at residue 193 (E to K), which modulated the ability of MAb 62c to bind (Fig. 5).

Sequencing of the V3 region from the MN and RF gp120 clones identified no polymorphic sites within the clones and no differences from the published Los Alamos sequence. Furthermore, we noted a number of other amino acid differences of our consensus sequences for the V2 loop of MN (five coding changes) and RF (six coding changes) from those published in the Los Alamos database (Fig. 5), supporting the idea that the V2 loop is highly variable within an isolate. However, we and others (12) have reported the presence of V1V2-specific antibodies in sera obtained from infected individuals and capable of reacting with a HXB10 V1V2 subfragment of gp120, suggesting the presence of cross-reactive epitopes. Clearly, laboratory-adapted isolates of HIV are a mixture of antigenic variants, suggesting that multiple envelope clones must be sequenced before the diversity remaining even after long-term propagation can be appreciated. Future experiments need to monitor the level of V1V2 variation present in viruses circulating in the infected population to determine the level of antigenic variation present and to assess whether the variation will affect the ability of antibodies to this region to recognize and subsequently neutralize viral infectivity.

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