

Human Monoclonal Antibodies Specific for Conformation-Sensitive Epitopes of V3 Neutralize Human Immunodeficiency Virus Type 1 Primary Isolates from Various Clades

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The epitopes of the V3 domain of the human immunodeficiency virus type 1 (HIV-1) gp120 glycoprotein have complex structures consisting of linear and conformational antigenic determinants. Anti-V3 antibodies (Abs) recognize both types of elements, but Abs which preferentially react to the conformational aspect of the epitopes may have more potent neutralizing activity against HIV-1, as recently suggested. To test this hypothesis, human anti-V3 monoclonal Abs (MAbs) were selected using a V3 fusion protein (V3-FP) which retains the conformation of the third variable region. The V3-FP consists of the V3_{JR-CSF} sequence inserted into a truncated form of murine leukemia virus gp70. Six human MAbs which recognize epitopes at the crown of the V3 loop were selected with the V3-FP. They were found to react more strongly with molecules displaying conformationally intact V3 than with linear V3 peptides. In a virus capture assay, these MAbs showed cross-clade binding to native, intact virions of clades A, B, C, D, and F. No binding was found to isolates from subtype E. The neutralizing activity of MAbs against primary isolates was determined in three assays: the GHOST cell assay, a phytohemagglutinin-stimulated peripheral blood mononuclear cell assay, and a luciferase assay. While these new MAbs displayed various degrees of activity, the pattern of cross-clade neutralization of clades A, B, and F was most pronounced. The neutralization of clades C and D viruses was weak and sporadic, and neutralization of clade E by these MAbs was not detected. Analysis by linear regression showed a highly significant correlation ($P < 0.0001$) between the strength of binding of these anti-V3 MAbs to intact virions and the percent neutralization. These studies demonstrate that human MAbs to conformation-sensitive epitopes of V3 display cross-clade reactivity in both binding to native, intact virions and neutralization of primary isolates.

The third variable region (V3) of the gp120 envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) is critical for the infectivity of the virus, and certain single mutations in this region render the virus inactive (26, 35). The sequence of the V3 loop plays a primary role in determining the cell tropism of the virus (8, 25), which is a function of its ability to differentially bind to the CCR5 and/or CXCR4 coreceptors on the target cell (22, 55). As a consequence of the key functions attributable to the V3 loop, anti-V3 antibodies (Abs) can be expected to block HIV-1 infectivity. Indeed, serum neutralization of T-cell line-adapted (TCLA) strains was found to be mediated by anti-V3 Abs (50, 65) and could be abolished by absorption with V3 peptides (2, 51) or by depletion of anti-V3 Abs (57). Moreover, monoclonal Abs (MAbs) against the V3 loop were shown to have potent neutralizing activity against TCLA strains, and this neutralization sensitivity to anti-V3 Abs led to the designation of the V3 loop as the principal neutralizing domain of the virus (27, 47).

Several studies, however, have weakened the enthusiasm for anti-V3 Abs over the past decade. For example, due to the

extreme sequence variation in the V3 loop (31), it was suggested that anti-V3 Abs could only be type specific and lacked broad reactivity. While this view is still widely held, it is based on studies of V3 Abs from animals that had been immunized with V3 peptides for short periods (27, 42). In contrast, anti-V3 Abs from HIV-infected humans in whom the humoral immune response has matured over a period of many months to years show broad reactivity (1). Indeed, type-specific anti-V3 Abs from humans are the exception rather than the rule, with some anti-V3 MAbs showing not only broad cross-clade binding to V3 peptides and to intact, native virions but also neutralizing activity against multiple strains of primary isolates (9, 17, 36, 40, 41, 56, 64). The generally negative consensus about the relevance of anti-V3 Abs was also promoted by the hypothesis that the V3 loop was “cryptic,” i.e., poorly displayed on the surfaces of virions of primary isolates (4). It is possible that the accessibility of the V3 loop may differ among primary isolates (7), but the majority of primary isolates reveal good exposure of the V3 region on the surfaces of intact virions (40, 41).

The V3 region consists of ~35 amino acids embedded in a three-dimensional structure which contributes to the formation of conformational epitopes that were described by studying the escape mutants from anti-V3 neutralizing Abs (35, 38). The binding of anti-V3 Abs is most effective when the V3 domain retains its conformation. This was shown in compara-

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tive studies in which serum Abs displayed much higher reactivity with a conformationally correct V3 fusion protein (V3-FP) than with linear V3 peptides (28, 29, 49). Most human anti-V3 MAbs, which were selected with V3 peptides, target primarily linear epitopes and have poor neutralizing activity against HIV primary isolates (17). Abs which react with conformation-sensitive epitopes in the V3 region could be more potent in neutralization of primary isolates, as recently suggested (43). To test this hypothesis, human MAbs were selected with a V3-FP, instead of V3 peptides, to produce MAbs which would preferentially bind to conformation-sensitive V3 epitopes. This study presents the characteristics of six such anti-V3 human MAbs which were selected with a V3-FP and which show cross-clade reactivity both in binding to native, intact virions and in neutralizing primary isolates.

MATERIALS AND METHODS

Human MAbs. The human MAbs were produced according to the method previously described (15, 19). Briefly, peripheral blood mononuclear cells (PBMC) derived from asymptomatic HIV-1-infected individuals were transformed with Epstein-Barr virus. The cells were cultured for 3 weeks, and the supernatant was screened by enzyme-linked immunosorbent assay (ELISA) for reactivity to V3-FP (see below). Cells from positive cultures were fused with the human \times mouse heteromyeloma SHM-D33 (54), and the resulting hybridomas were cloned at limiting dilutions until monoclonality was achieved. Five previously characterized human MAbs were used as controls: anti-V3 MAb 447-52D (20), anti-CD4bd MAb 654 (30), anti-C5 MAb 1331A (18), anti-gp41 MAb 246 (63), and anti-parvovirus B19 MAb 1418 (14).

HIV-1 isolates. Sixteen primary HIV-1 isolates from group M, clades A to F (see Fig. 3), were used in this study. Four isolates, CA1, VI191, CA5, and 2036, were obtained from the Institute of Tropical Medicine, Antwerp, Belgium, and have been previously described (40). The isolates JR-FL, Bx08, 93IN904, 98TZ017, 92UG021, 92TH009, 92TH011, 93BR019, and 93BR029 were supplied by the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program; isolate MNp, which has never been grown in cell lines, was obtained from J. Sullivan, University of Massachusetts Medical School, Worcester, Mass. The primary isolates NYU1457 and NYU1545 were isolated from PBMC obtained from infected individuals living in Cameroon and were provided by Philippe Nyambi (63a). All primary isolates use coreceptor R5, with the exception of two viruses, MNp and 92UG021, which use X4. The viruses were amplified in 3-day phytohemagglutinin (PHA)-stimulated PBMC from healthy donors. After 2 to 3 weeks of culture, the supernatants from infected PBMC were filtered, aliquoted, and frozen in liquid nitrogen. The level of p24 in each virus stock was determined using a noncommercial ELISA, as previously described (39).

In addition to the neutralization assay, all viruses were used in the binding assay, and for this purpose they were first inactivated by treatment with aldrithiol-2 (AT-2) (Sigma, St. Louis, Mo.) according to the method described by Rossio et al. (46). Briefly, AT-2 suspended in dimethyl sulfoxide was added to the virus preparation at a final concentration of 1,000 μ M for 1 h at 37°C. To remove the AT-2, a centrifugal filtration device with a 100-kDa cutoff was used (Centricon YM-100; Amicon, Bedford, Mass.). The stock virus, diluted in phosphate-buffered saline (PBS), was quantitated for p24 as described previously (39) and was kept frozen until it was used.

Proteins and peptide. The V3-FP is composed of a 263-amino-acid fragment of the murine leukemia virus gp70 joined to a 45-amino-acid domain of gp120 containing the V3_{JR-CSF} sequence; it was expressed in CHO cells as described previously (28). The V3 fragment in this fusion protein contains the disulfide-bonded loop and three sites for N-linked glycosylation, one within the V3 loop and one on each flank (28). Monomeric gp120₄₅₁ was affinity purified from cell cultures infected with HIV-1₄₅₁ and was purchased from Advanced BioScience Laboratories (Kensington, Md.). The 35-mer V3 peptide of JR-CSF with two cysteine residues was synthesized by Sigma-Genosys (Woodland, Tex.) to 70% purity and analyzed by high-performance liquid chromatography.

Binding assays. Binding of MAbs to V3-FP, V3 peptide, and gp120₄₅₁ was determined by standard ELISA (17). Briefly, ELISA plates were coated with the antigens overnight at 4°C, blocked for 1 h at 37°C with 2% bovine serum albumin in PBS, and then washed with PBS containing 0.05% Tween-20, pH 7.4, before incubation for 1.5 h at 37°C with human MAbs. The plates were washed, and for

detection of bound MAbs, alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG; γ specific) (Zymed, San Francisco, Calif.) was added for 1.5 h at 37°C. After being washed, the substrate was added for 30 min to develop color, and the plates were read at 410 nm.

The epitopes in the V3 loop recognized by the new MAbs were determined by a competition ELISA in which unlabeled MAbs were tested for their ability to inhibit the binding of biotinylated anti-V3 MAb 447-52D to the V3-FP. Microplate wells were coated with the V3-FP at 0.4 μ g/ml, and a mixture containing unlabeled MAb at concentrations ranging from 0.06 to 20.0 μ g/ml and biotinylated 447-52D at a concentration of 0.1 μ g/ml was added for 1.5 h of incubation at 37°C. The binding of biotinylated 447-52D to V3-FP was detected with alkaline phosphatase-conjugated streptavidin (Life Technologies, Gaithersburg, Md.) followed by incubation with substrate. MAbs 447-52D and 1418 were used as positive and negative controls, respectively. Biotinylation of 447-52D was performed using the EZ-link Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockford, Ill.) according to the manufacturer's protocol.

The relative affinity of each MAb was assessed by ELISA by measuring the amount of MAb required for 50% maximal binding to antigens. To compensate for the different molecular weights (MW) of the V3-FP (MW, 33,880) and the V3 peptide (MW, 3,850), the ELISA plate was coated with both antigens at an equimolar concentration, 59 nM. The antigen-coated plates were incubated with MAbs at concentrations ranging from 0.003 to 10.0 μ g/ml, followed by the steps described above for the standard ELISA. The concentration of MAb that gave 50% maximal binding was calculated when the binding reached the saturation point. The half-maximal binding was obtained mathematically by linear interpolation using Quatro Pro software (Corel, Ottawa, Canada).

The effect of disulfide bond reduction on the binding of MAbs to gp120₄₅₁ was tested by ELISA. The plate was coated with monomeric gp120₄₅₁ at a concentration of 1.0 μ g/ml and then treated for 1 h at 37°C with dithiothreitol (DTT) at concentrations ranging from 0.1 to 10.0 mM. The DTT was washed out, and 5 nM iodoacetic acid was added to each well for 1 h at 37°C; then, after the plates were washed, a standard ELISA was performed as described above.

To study the role of carbohydrate residues in the binding of MAbs to gp120₄₅₁, the carbohydrate determinant was destroyed by mild periodate oxidation according to the method described by Woodward et al. (61). Briefly, plates were coated with 1.0 μ g of gp120₄₅₁/ml and rinsed with 50 mM sodium acetate buffer (pH 4.5) before sodium metaperiodate was added at concentrations between 0.1 and 10.0 mM for 1 h at room temperature. The aldehyde groups generated by periodate oxidation were blocked with 1% glycine. The binding of MAbs was determined by ELISA as described above.

The binding of MAbs to intact virions was determined in a capture assay with some modifications to the protocol already described (39). Briefly, a microtiter plate was coated overnight with goat anti-human IgG Fc (ICN Biomedicals, Aurora, Ohio) at 2.0 μ g/ml, and after the plates were washed, each MAb (at 10 μ g/ml) was added to the wells for 1.5 h of incubation at 37°C. The plates were washed and blocked with 0.5% bovine serum albumin in PBS containing 10% goat serum and 10.0 μ g of human IgG/ml. After being washed, aldrithiol-treated virions at 100 ng/ml of p24 were incubated overnight at room temperature, and the captured virus was lysed with 1% Triton-X in PBS. The p24 in the virus lysate was quantitated by a noncommercial ELISA as described previously (39). MAbs 447-52D (anti-V3) and 246 (anti-gp41) and an irrelevant MAb, 1418 (anti-parvovirus B19), were used as controls.

Neutralization assays. The GHOST cell neutralization assay was used as described previously (5). Briefly, GHOST-R5 (CD4⁺ CCR5⁺) and GHOST-X4 (CD4⁺ CXCR4⁺) cells (kindly provided by Dan Littman, New York University) were used as target cells for primary isolates. One day prior to the assay, 6×10^4 GHOST cells/well/0.5 ml were seeded into 24-well tissue culture plates. Virus stocks were diluted to a predetermined concentration which would result in \sim 1,000 fluorescence-positive cells per 15,000 total cells when analyzed by flow cytometry at the end of the assay. A mixture of diluted virus stock and MAb at a final concentration of 25 μ g/ml was incubated for 1 h at 37°C and then added to the GHOST cells in the presence of DEAE-dextran at 8.0 μ g/ml. After overnight incubation, the MAb-virus-containing medium was aspirated, and the cells were washed once and incubated for an additional 3 days. At the end of the culture period, the cells were retrieved from the wells with 5 mM EDTA, fixed in a final concentration of 2% formaldehyde, and analyzed using a FACScan flow cytometer (Becton-Dickinson). The percent neutralization was calculated using the formula (1 - number of infected cells in the presence of MAb/number of infected cells in the absence of MAb) \times 100. The control human MAbs 447-52D (anti-V3) and 1418 (anti-B19) were used in each experiment. The neutralizing activities of the MAbs were determined using a cutoff based on the 95% confidence level calculated from the fitted normal (Gaussian) distribution of data from 39 experiments using the irrelevant MAb 1418.

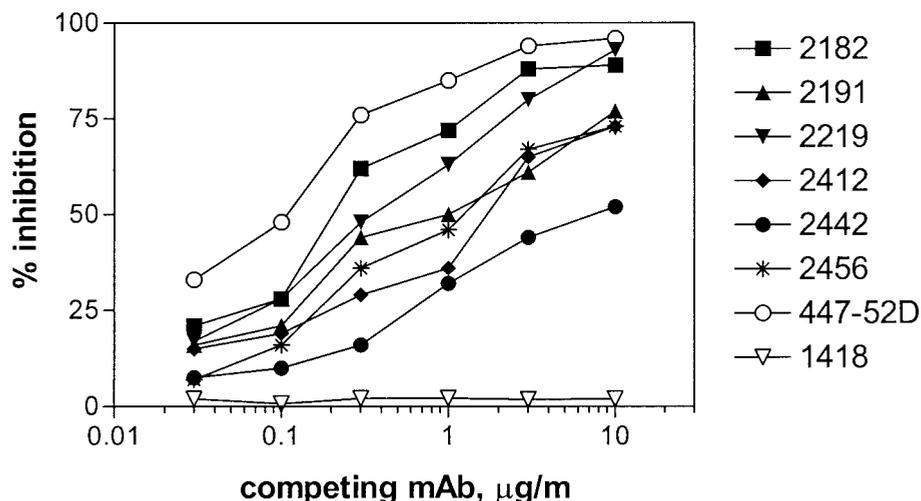


FIG. 1. Competition of various MABs with biotinylated anti-V3 MAB 447-52D for binding to V3-FP. Each of the unlabeled MABs, diluted from 20.0 to 0.06 $\mu\text{g/ml}$, was mixed 1:1 with biotinylated 447-52D and then incubated on V3-FP-coated ELISA wells. Binding of biotinylated 447-52D was detected by streptavidin-alkaline phosphatase in ELISA format. The results are shown as percent inhibition of bound biotinylated 447-52D by unlabeled MABs. The positive control was unlabeled 447-52D; the negative control was anti-parvovirus B19 MAB 1418.

The PBMC neutralization assay was performed as described by Mascola (33). The assay was performed in quadruplicate wells (with eight virus-only control wells) using a dilution of virus stock that yielded >10 ng of supernatant p24 protein/ml in control (virus-only) wells by days 3 to 6, depending upon the viral growth kinetics. The pretitered virus stock and MAB at 50 $\mu\text{g/ml}$ were preincubated for 30 min at 37°C in 96-well plates, and an aliquot containing 150,000 PHA-stimulated HIV-negative donor PBMC was added to each well. After overnight incubation, the cells were washed with RPMI containing 2% fetal bovine serum and 1% penicillin/streptomycin and then resuspended in 15% fetal bovine serum-RPMI medium containing interleukin-2 (20 IU/ml; Boehringer-Mannheim) and transferred to the corresponding wells of a 96-well U-bottom plate. On day 3, 100 μl of culture supernatant was collected and replaced with 100 μl of medium containing interleukin-2. At the end of the culture period, a 100- μl pool of 25 μl from each of the quadruplicate wells was prepared from all samples, and p24 was quantitated for each pooled sample. The percent neutralization was calculated as the percent reduction of p24 in experimental wells compared to wells with virus alone. The neutralizing activities of MABs were determined using a cutoff based on the 95% confidence level calculated from the fitted normal (Gaussian) distribution of data from 13 negative control experiments using human anti-parvovirus MAB 1418.

The neutralization of two pseudoviruses expressing envelope glycoproteins derived from SF162 and JR-FL was measured by using a luciferase assay (6, 29). Pseudotyped virions were produced by cotransfecting HEK293 cells with the *env*-expressing plasmid pLRB826 and with the complementing viral-genome reporter gene vector, pNL4-3.Luc.E⁻R⁻ (NIH AIDS Research and Reference Reagent Program). Culture supernatant containing pseudoviruses at a final concentration of 0.5 ng/ml of p24 was preincubated with MAB for 1.5 h at 37°C and added to 10,000 U87-T4-CCR5 cells which had been seeded 1 day before in a 96-well plate. After 24 h at 37°C, the cells were refed with fresh medium containing 10.0 μg of Polybrene/ml, and incubation was continued for an additional 2 days. The cells were washed twice with PBS and lysed with lysis buffer (Promega). The lysate was transferred to a new plate, and luciferase substrate (Promega) was added to each well. The relative light units were measured on a Dynex MLX microtiter plate luminometer.

RESULTS

Generation of heterohybridoma cell lines. Six heterohybridomas were generated from cells of asymptomatic HIV-1-infected individuals by using the V3-FP for selection of Ab-producing cells from Epstein-Barr virus-transformed PBMC. Five MABs were derived from individuals infected with HIV-1 in the United States, presumably with clade B, and one MAB,

2182, was derived from an individual who was infected abroad with clade A and is presently living in New York city. The clade A *env* region was determined using a heteroduplex mobility assay (63a). Two MABs, 2412 and 2456, were produced from cells obtained in a span of 2.5 months from the same individual, while the other MABs were each generated from different subjects. All hybridoma cell lines produce subclass IgG1 with lambda light chains.

Immunochemical characterization of MABs. All six MABs bind to epitopes located in the crown of the V3 loop as determined by a competition ELISA. Each of the unlabeled MABs inhibited the binding of biotinylated anti-V3 MAB 447-52D to V3-FP in a dose-dependent fashion (Fig. 1). Inhibition by the six MABs varied but was lower than inhibition mediated by the unlabeled autologous 447-52D. The most highly competing MAB was 2182, which, at concentrations ranging from 0.03 to 10.0 $\mu\text{g/ml}$, inhibited 20 to 95% of the binding of biotinylated 447-52D to the V3-FP. MAB 2442 was the least competitive; at 10.0 $\mu\text{g/ml}$, it inhibited only 50% of the binding of biotinylated 447-52D. The selection of the six new MABs with the V3-FP and the abilities of these MABs to compete with MAB 447-52D for binding to the V3-FP demonstrate their specificity for the V3 loop and show that the tip of the V3 loop (for which 447-52D is specific) (16) is recognized as part of the epitope of these newly selected MABs.

The relative affinities of MABs to V3_{JR-CSF}-FP and corresponding linear V3_{JR-CSF} peptide were tested in parallel ELISAs to determine whether they were directed at V3 conformation-sensitive epitopes. The ELISA plates were coated with both antigens at equimolar concentrations, 59 nM, to compensate for the different MW of the two antigens. The half-maximal binding to the two antigens was calculated at the time point when the MABs reached saturation, showing that the relative affinities of five of the six MABs for binding to the V3-FP were significantly higher ($P < 0.05$; Student *t* test) than the affinities for the V3 peptide (Table 1). Measurement at

TABLE 1. Reactivities of human MAb with V3_{JR-CSF}-FP and V3_{JR-CSF} linear peptides

MAb	Half-maximal binding to ^a :		P value
	V3-FP	V3 peptide	
2182	0.02 ± 0.01	0.14 ± 0.03	0.071
2191	0.04 ± 0.02	0.83 ± 0.09	0.015
2219	0.02 ± 0.01	0.84 ± 0.16	0.036
2412	0.02 ± 0.01	2.21 ± 0.01	0.0001
2442	0.02 ± 0.01	1.40 ± 0.2	0.02
2456	0.03 ± 0.02	2.10 ± 0.4	0.035
447-52D ^b	0.01 ± 0.01	0.16 ± 0.07	0.17

^a ELISA plates coated with V3-FP and V3 peptide at 59 nM concentration; mean of two experiments (micrograms per milliliter ± standard deviation).

^b Control human anti-V3 MAb selected with V3_{MN} peptide.

several time points (up to 30 min) revealed a consistent difference in MAb binding to the V3-FP versus the V3 peptide (data not shown), suggesting that the difference in half-maximal binding was not due to differential binding of the two antigens to the plate. Two MAbs, 2182 and the positive control, 447-52D (which was selected previously with the linear V3_{MN} peptide), also reacted better with the V3-FP than with the V3 peptide, but the differences were not significant, showing a diminished dependence of these two MAbs on the V3 conformation.

The roles of disulfide bonds and carbohydrate residues in creating the V3 conformation recognized by the anti-V3 MAbs were studied by treating gp120₄₅₁ with DTT and sodium metaperiodate. Gp120₄₅₁ is an HIV-1 envelope glycoprotein purified from the culture supernatant of chronically infected cells, and its reactivity with anti-V3 MAbs is similar to that of the V3-FP (data not shown). DTT reduces the disulfide bonds which contribute to the tertiary structure of proteins, and periodate oxidation destroys the carbohydrate determinants which may influence the epitopes recognized by MAbs. gp120₄₅₁ was treated with increasing concentrations of DTT or sodium metaperiodate from 0.1 to 10 mM and then used in ELISA for testing the binding activity of MAbs (Fig. 2). The binding of MAb 1331A to gp120₄₅₁, which recognizes a linear epitope in the C5 region of gp120 and is not dependent on conformation, is not affected by DTT or periodate used at various concentrations, and MAb 1331A was included as a negative control (Fig. 2A). In contrast, MAb 654-D (anti-CD4bd) is strictly conformation dependent, and its binding is almost completely abolished by treatment with either DTT or periodate at concentrations between 1 and 10 mM. This MAb was used as a positive control (Fig. 2B). Treatment of gp120₄₅₁ with DTT or periodate affected the binding of each anti-V3 MAb to gp120₄₅₁ to various degrees. All six new anti-V3 MAbs displayed reduced binding activity to gp120₄₅₁ treated with DTT compared to binding to untreated protein. This reduction was dependent on the DTT concentration, and at the highest concentration tested, 10 mM, ranged from 39 to 84% for various MAbs. Binding to three of four recombinant gp120s was less affected by DTT compared to the effect that DTT had on gp120₄₅₁ (data not shown).

Treatment of gp120₄₅₁ with 10 mM sodium metaperiodate significantly reduced the binding activities of four anti-V3 MAbs, 2191, 2219, 2412, and 2456. The binding of MAbs 2182,

2442, and 447-52D was not significantly affected by NaIO₄. Similar decrease in the binding of mouse anti-V3 MAbs to gp120 treated with sialidase and increasing concentrations of NaIO₄ (over 60% reduction at 3.2 mM of NaIO₄) was previously described by Bolmstedt et al. (3). The involvement of N-glycans was shown when the gp120 was treated with peptide N-glycosidase, and the binding of the anti-V3 MAb was decreased (3).

Binding of MAbs to native, intact virions. The cross-reactivities of anti-V3 MAbs to primary isolates were determined by using virus capture ELISA. Sixteen primary isolates representing clades A, B, C, D, E, and F were tested against nine MAbs, and the specific binding to intact virions was determined by the cutoff (mean + 3 standard deviations), which was calculated separately for each virus on the basis of p24 values obtained with the irrelevant human MAb 1418 (anti-parvovirus B19). MAb 246, specific for gp41, was used as a positive control, since as previously shown, it binds to all viruses (40). In this study, MAb 246 was also highly cross-reactive, indicating that MAb 246 recognizes a conserved and well-exposed epitope of the envelope glycoprotein (Fig. 3). Among the new anti-V3 MAbs, 2219, 2456, and 2442 were the most cross-reactive and captured 13, 12, and 12 isolates, respectively, out of the 16 virions tested. These three MAbs and MAb 447-52D had similar patterns of cross-reactivity with isolates from clades A, B, C, D, and F. None of the newly described MAbs reacted with either of two clade E viruses, and in general, binding to viruses from clades C and D was less frequent than binding to clade A, B, and F viruses. MAb 2191 differed from the first group of MAbs by missing recognition of viruses from clade D. The least cross-reactive MAbs were 2182 and 2412, which bound to eight and seven isolates, respectively, from clades A, B, and F. Notably, MAb 2182 is derived from a clade A virus-infected individual, and its specificity could be related to a different pattern of binding.

Neutralization of primary isolates. The neutralizing activity of the MAbs against primary isolates was determined in three different assays performed at three different sites. In each assay, the six new anti-V3 MAbs were tested with two controls, anti-V3 MAb 447-52D and anti-parvovirus MAb 1418. Eleven primary isolates were employed in the GHOST cell assay, and five were also used in the PHA-stimulated PBMC assay. The viruses were from the same source but were amplified separately in each laboratory. In the third assay, two cloned pseudotyped clade B viruses, SF162 and JR-FL, were used. The specific neutralizing activities of MAbs in each assay were determined by cutoff values based on the 95% confidence level of values obtained with the irrelevant MAb 1418.

In the GHOST cell assay, all seven anti-V3 MAbs showed cross-clade neutralization (Table 2). Based on 39 experiments with the negative control anti-parvovirus B19 MAb 1418, neutralization values of ≥20% were statistically significant at the 95% confidence level. Using this criterion, 39 of 77 (51%) of the virus-MAb combinations showed significant neutralization. The most cross-neutralizing MAb was 2442. MAb 2442, from an individual infected in the United States, presumably with clade B, neutralized 7 of 11 primary isolates, and MAbs 2182, 2456, and 447-52D each neutralized 6 of 11 primary viruses. The remaining three MAbs neutralized 4 or 5 of the 11 primary isolates. In general, these new MAbs displayed reproducible cross-neutralization among clades A, B, and F, with little or no

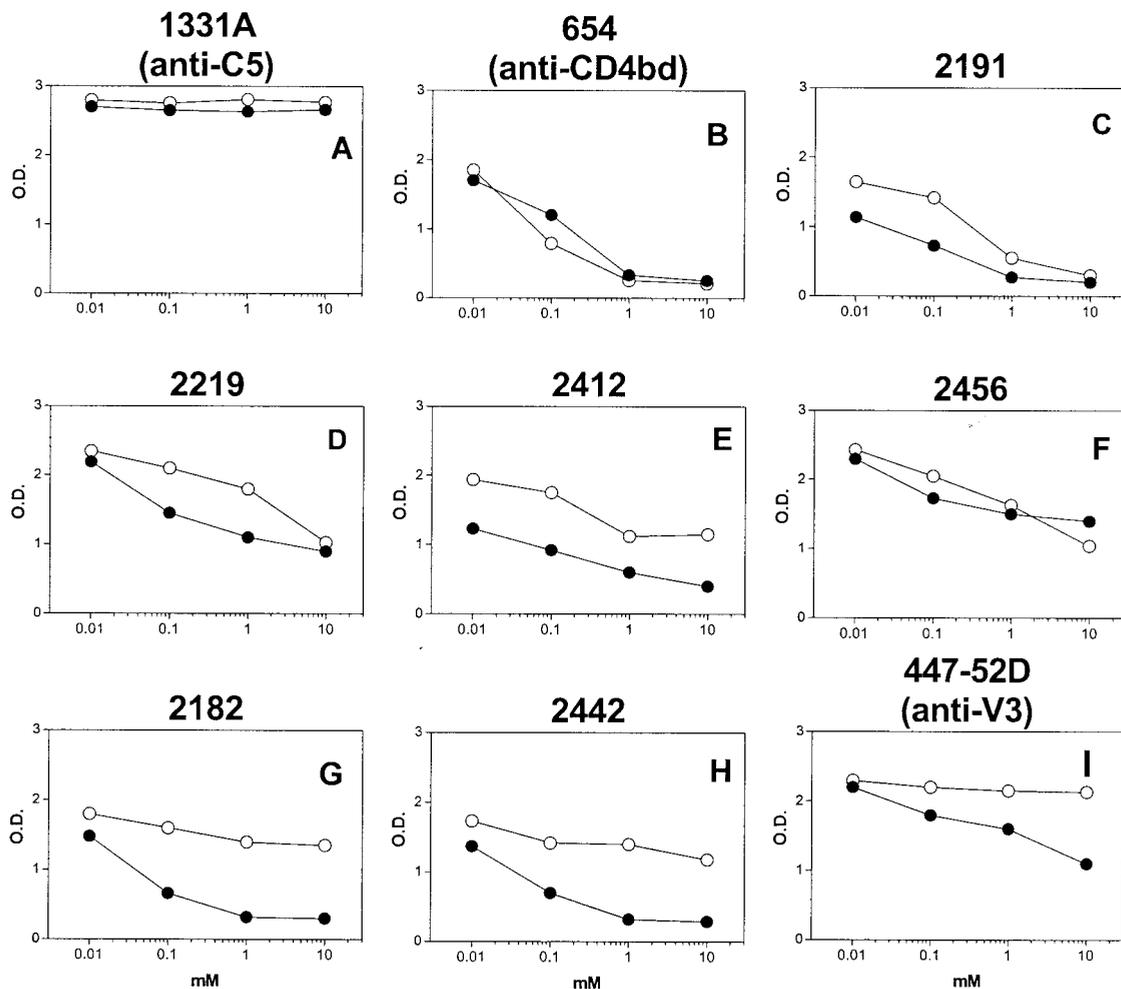


FIG. 2. Effect of disulfide bond reduction and carbohydrate oxidation on binding of MABs to gp120₄₅₁. gp120₄₅₁ was immobilized on an ELISA plate, treated with DTT (●) or with sodium metaperiodate (○) at concentrations from 10 to 0 mM, and then exposed to MABs at 10 μg/ml. Two MABs were used as controls: 1331A (anti-C5) (A) recognizes a linear epitope, and its binding is not affected by DTT or NaIO₄, while 654 (anti-CD4bd) (B) is conformation dependent, and its binding is abolished upon treatment with either reagent. Anti-V3 MABs (C to I) displayed various degrees of reduced reactivity with gp120₄₅₁ treated with DTT or NaIO₄. The results from one of three similar experiments are represented. O.D., optical density at 410 nm.

significant activity against the isolates of clades C, D, and E that were tested.

Similarly, the PHA blast assay showed cross-neutralizing activity for each of the MABs (Table 3). In this series of experiments, the cutoff value for significant neutralization was $\geq 25\%$, and based on this criterion, 24 of 35 (68%) virus-MAB combinations showed significant neutralization, with the most notable activity against primary isolates from clades A, B, and F.

Analysis of neutralization data by linear regression revealed a highly significant correlation ($P < 0.0001$) between the GHOST cell and PHA blast assays (Fig. 4). The two assays employed different target cells and detection systems and were performed in two different laboratories. Comparisons of results were based on 28 virus-MAB combinations in which four primary isolates (VI191, MNp, Bx08, and 2036) were tested with seven anti-V3 MABs.

In the third assay, the neutralization of pseudotyped viruses, SF162 and JR-FL, was determined. The cutoff values for significant neutralization, based on the 95% confidence level,

were 19 and 8% for SF162 and JR-FL, respectively. All of the MABs neutralized SF162 at between 0.4 and 2.0 μg/ml, while JR-FL was more resistant, being neutralized by six of the seven MABs at concentrations between 0.1 and 50 μg/ml (Fig. 5). The relative resistance of JR-FL to neutralization described previously (5, 58) is confirmed here by data showing that maximal neutralization of the JR-FL pseudovirus was not achieved even at 50 μg/ml. Similarly, JR-FL was resistant to neutralization in the GHOST assay (Table 2).

Relationships between MAB neutralization and binding to intact virions. Analysis of results from the binding of MABs to intact virions and from the neutralization of the same viruses (Tables 2 and 3 and Fig. 3 and 5) indicate that only bound viruses are neutralized by anti-V3 MABs. For example, MABs which reacted well with intact virions also potently neutralized MNp, BX08, and, to some extent, CA5. On the other hand, the intact virions, like 92UG021, 92TH009, 2036, and 93IN904, which were not reactive or bound very weakly in the capture assay, were not neutralized or were neutralized very poorly. In

Virus	clade	2219	2456	2442	2191	2182	2412	447-52D	246	1418 (cut-off)
CA1	A	386	236	159	117	451	127	677	664	11 (40)
VI191	A	517	352	145	116	446	125	754	580	13 (34)
MNp	B	558	477	153	456	505	219	790	310	32 (78)
JR-FL	B	144	141	125	114	182	85	407	323	10 (33)
CA5	B	787	763	755	783	737	730	785	795	70(187)
BX08	B	561	487	358	417	512	302	836	869	53(112)
2036	C	411	339	400	360	48	78	474	975	64(180)
93IN904	C	65	27	23	59	14	5	28	892	13 (36)
98TZ017	C	249	287	305	97	23	39	88	759	46(155)
92UG021	D	20	15	33	4	60	18	58	551	16 (55)
NYU1457	D	518	401	228	141	116	95	626	707	52(178)
NYU1545	D	504	207	376	28	22	17	462	700	27(123)
92TH011	E	141	162	163	103	107	125	124	1041	91(172)
92TH009	E	68	73	69	21	18	39	49	942	42 (76)
93BR019	F	506	362	337	446	62	80	485	843	98(137)
93BR029	F	570	398	552	550	436	321	580	934	104(189)

FIG. 3. Pattern of binding of MABs to native, intact virions representing HIV-1 clades A to F. The binding study was performed using the virus binding assay, in which virions are bound to MABs that are immobilized on ELISA plates. Bound virus is determined by measuring the level of p24 released when bound virus is lysed with detergent. Each value represents the mean p24 value (in picograms per milliliter) of three experiments. MAB 246 against gp41 was used as a positive control. The irrelevant MAB 1418 against parvovirus B19 served as a negative control, and the binding values of 1418 versus each virus served to establish the cutoff value (shown in parentheses), which was defined as the mean binding with MAB 1418 + 3 standard deviations. The boxes are coded as follows: open, less than the cutoff value; light shading, binding up to 50% of the level of binding achieved with MAB 246; dark shading, >50% of MAB 246 values.

the luciferase assay, the two MABs 2182 and 447-52D, which neutralized pseudotyped JR-FL at 9.2 and 6.8 $\mu\text{g/ml}$ for 50% neutralizing doses, respectively, showed the highest percent of binding in the capture assay (Fig. 3 and 5). The two variables, percent neutralization and p24 concentration in picograms per milliliter, reflecting the amount of virus bound to MABs, were analyzed for all the viruses and MABs tested and showed a highly significant correlation whether the neutralization data came from the GHOST cell assay ($P < 0.0001$) or the PHA

blast assay ($P = 0.001$) (Fig. 6). These correlations indicate that the neutralization of primary isolates by anti-V3 MABs is strictly dependent on the abilities of these MABs to bind to intact virions.

DISCUSSION

Six new human MABs with specificities for conformation-sensitive epitopes mapping to the V3 region of the gp120

TABLE 2. Cross-clade neutralization of primary isolates in the GHOST cell assay^a

Virus	Clade	% Neutralization							
		2219 V3-FP	2456 V3-FP	2442 V3-FP	2191 V3-FP	2182 V3-FP	2412 V3-FP	447-52 V3	1418 parvovirus
CA1	A	42	NN	69	NN	94	NN	91	NN
VI191	A	NN	28	39	NN	56	56	44	NN
MNp	B	100	91	97	99	99	86	96	NN
JR-FL	B	NN	NN	NN	NN	38	NN	NN	NN
CA5	B	57	50	41	52	60	43	49	NN
BX08	B	79	65	71	81	92	57	90	NN
2036	C	NN	NN	NN	NN	NN	NN	NN	NN
93IN904	C	NN	39	NN	NN	NN	NN	NN	NN
92UG021	D	NN	NN	NN	NN	NN	NN	NN	NN
92TH009	E	NN	NN	25	NN	NN	NN	NN	NN
93BR019	F	59	46	40	44	NN	35	32	NN

^a All MABs tested at 25 $\mu\text{g/ml}$; means of three to five experiments. The cutoff is 20% based on the 95% confidence level of 39 experiments with the irrelevant MAB 1418 (see Materials and Methods). Thus, only values of $\geq 20\%$ represent statistically significant neutralization. Any value of $< 20\%$ is shown as nonneutralizing (NN).

TABLE 3. Cross-clade neutralization of primary isolates tested in the PHA blast assay^a

Virus	Clade	% Neutralization							
		2219 V3-FP	2456 V3-FP	2442 V3-FP	2191 V3-FP	2182 V3-FP	2412 V3-FP	447-52 V3	1418 parvovirus
VI191	A	44	NN	NN	29	68	NN	63	NN
MNp	B	85	72	88	91	93	63	88	NN
BX08	B	70	25	63	66	80	NN	79	NN
2036	C	30	NN	NN	37	NN	NN	NN	NN
93BR029	F	52	NN	35	48	43	NN	35	NN

^a All MABs tested at final concentration of 25 µg/ml; means of two or three experiments. Neutralization was determined by the cutoff, which was 25% based on the 95% confidence level of 13 experiments with the irrelevant MAb 1418. A value of ≥25% represents statistically significant neutralization. Any value of <25% is shown as nonneutralizing (NN).

envelope glycoprotein of HIV-1 were selected. In most cases, the relative affinities of these MABs were 1 to 2 orders of magnitude greater for the V3 region retaining its conformation than for the same V3 region represented by a linear peptide. The conformational structures recognized in the V3 region of gp120 were destroyed by reduction of the disulfide clasp at the base of the V3 loop and/or by oxidation of carbohydrate moieties. These MABs could recognize the V3 loop exposed on the surfaces of intact, native virions of primary isolates from several clades and displayed broad cross-clade neutralizing activity against primary isolates. Indeed, the data from these experiments demonstrate for the first time that the strength of binding of anti-V3 MABs to HIV virions correlates with neutralizing activity.

The new MABs described here were selected using the V3_{JR-CSF}-FP, which mimics the V3 conformation on the virus. Previous human anti-V3 MABs were selected on linear V3 peptides (16, 17, 19, 20), with the exception of MAB 694/98D; this MAB was selected by its reactivity for gp120_{LAI} and is relatively specific for HIV_{LAI} because of the QR insert at the tip of its V3 loop (20). Since the selection process determines MAB specificity, it follows that the new anti-V3 MABs, selected with a conformationally correct molecule, are better able to react with intact virions than are MABs selected with linear

peptides. Like many conformation-dependent Abs, the new anti-V3 MABs can bind to the linear peptide but with lower affinity than they bind to the correctly folded V3 loop (Table 1). Interestingly, the specificities of MABs 2182 and 2442, selected on the V3-FP, and 447-52D, selected on the V3_{MN} linear peptide, were similar in that they were minimally affected by the oxidation of carbohydrate moieties, but these MABs, like the other anti-V3 MABs described here, were sensitive to destruction of the disulfide clasp at the base of the V3 loop.

The shape of the V3 loop is constrained by its relatively constant length (~35 amino acids) and the disulfide bond at its base. The secondary structure of the V3 domain, predicted from the sequence, consists of two β strands on both sides of the tip of the loop and a type II β-turn around residues GPGR

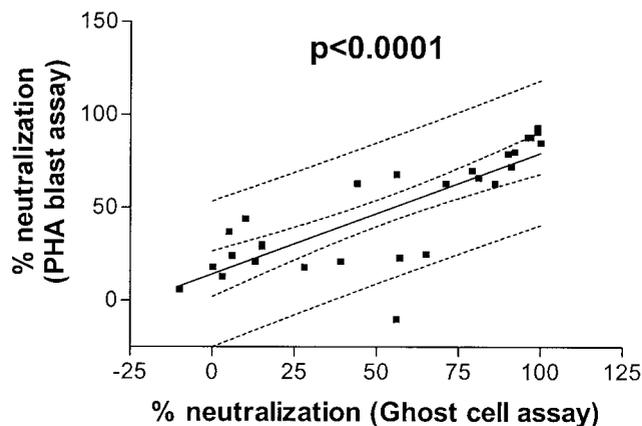


FIG. 4. Analysis by linear regression of neutralization results obtained by two assays, the GHOST cell assay and the PHA blast assay. The 95% confidence interval boundary (dashed lines near the best-fit regression line) and the 95% prediction interval (outer dashed lines) are shown. The latter area is expected to include 95% of all data points. The highly significant correlation indicates similar neutralizing activities of MABs measured by two different assays.

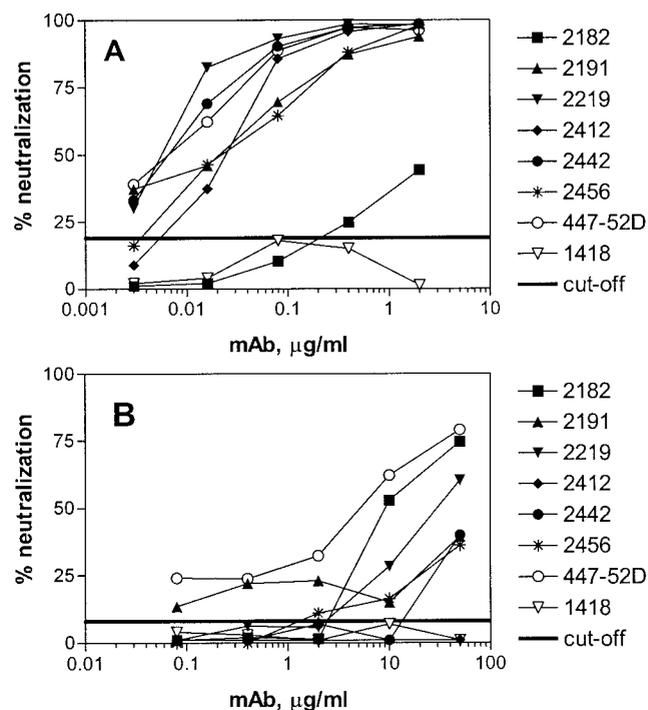


FIG. 5. Neutralization of pseudotyped viruses SF162(NL4-3luc) (A) and JR-FL(NL4-3luc) (B) by anti-V3 MABs. The cutoff values, based on the 95% confidence level calculated from data with the irrelevant MAb 1418, were 19 and 8% for SF162 and JR-FL, respectively.

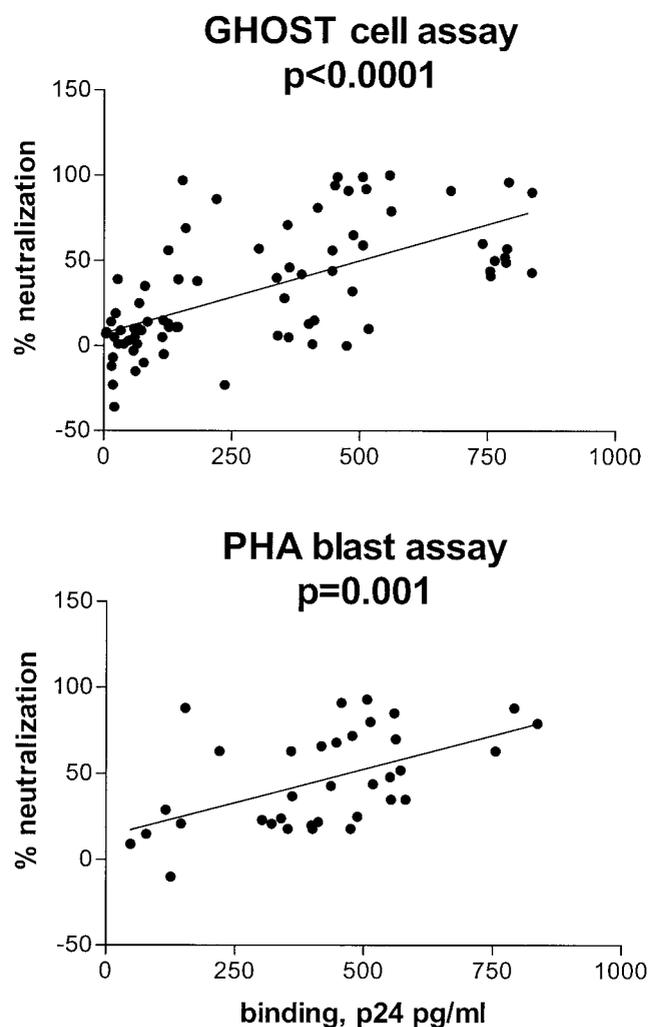


FIG. 6. Correlation between the abilities of MAbs to bind to and neutralize primary isolates of various HIV-1 clades. (Top) Data for 77 MAb-virus combinations tested in the GHOST cell neutralizing assay and the virus binding assay with seven anti-V3 MAbs and 11 viruses (CA1 and VI191, clade A; MNp, JR-FL, CA5, and Bx08, clade B; 2036 and 93IN904, clade C; 92UG021, clade D; 92TH009, clade E; and 93BR019, clade F). (Bottom) Data for 35 MAb-virus combinations tested in the PHA blast neutralization assay with seven anti-V3 MAbs and five viruses (VI191, clade A; MNp and Bx08, clade B; 2036, clade C; and 93BR029, clade F). The best-fit regression lines and *P* values from linear regression analyses are shown.

(31). The type II β -turn is quite stable among virus strains and could be responsible for the cross-reactivity of some MAbs, such as MAb 447-52D, which are specific for the tip. The new MAbs competed with biotinylated 447-52D for binding to the V3-FP but with lower activity than the unlabeled autologous 447-52D, indicating that their epitopes may overlap with the type II β -turn. Their cross-reactivity can also be partly explained by data from molecular modeling, which confirm that the type II β -turn is a constant feature, which may translate into shared antigenic structures (10). Additional studies of the tertiary conformation of the V3 domain added a new explanation for the phenomenon of cross-reactivity. X-ray crystallography analysis revealed that the V3 loop can adopt at least two

different conformations while bound to neutralizing Fabs, and two conformations for the tip of the loop were also seen using the nuclear magnetic resonance method (12, 13, 44, 52, 56a). As the exact nature of the V3 domain remains to be elucidated, these results suggest that the V3 region is probably conformationally variable and can adapt more easily to anti-V3 MAbs which are conformationally sensitive, resulting in cross-reactivity.

The cross-reactivity of the anti-V3 MAbs described here and elsewhere (17, 40, 41, 64) reflects the adapted conformation, rather than the variable sequence, of the V3 loop. All six new MAbs, as well as most of 19 other anti-V3 loop MAbs previously studied (41), display cross-clade binding to virions from multiple clades. This broad cross-reactivity of anti-V3 MAbs derived from infected humans stands in sharp contrast to the type specificity associated with mouse anti-V3 MAbs derived from immunized animals (21, 42). Both the nature of the immunogen and the length of exposure of the host to the immunogen account for this difference. Thus, in this study, as in previous studies of human anti-V3 MAbs (36, 40, 41, 64), cross-reactivity rather than type specificity is the rule, with individual MAbs, such as 2219, 2456, 2442, 2191, and 447-52D, binding to virions of clades A, B, C, D, and F. Interestingly, we have observed no cross-reaction with two clade E primary isolates, a finding which is consistent with previously published data suggesting that the V3 loop of clade E is immunologically distinct (34).

The new anti-V3 MAbs selected with the V3-FP display neutralizing activity against primary isolates from several clades. Thus, neutralization of clade A, B, and F primary isolates was a consistent feature of most of these new MAbs, while neutralization of C and D viruses was observed sporadically. Neutralization of clade E viruses was not observed. These data are consistent with previous findings showing that Abs purified from sera of infected subjects using an immunoaffinity column coupled with the V3-FP were able to efficiently neutralize primary isolates (29). Indeed, both the new V3-FP-selected human anti-V3 MAbs and the V3-FP-specific serum Abs display preferential reactivity for the V3-FP protein compared to the corresponding linear V3 peptide (Table 1) (28, 29) and can neutralize primary isolates (Tables 2 and 3) (29). These results stand in contrast to several earlier studies which suggested that most MAbs selected with linear V3 peptides, as well as polyclonal anti-V3 Abs from HIV⁺ serum, can neutralize TCLA strains but not primary isolates (51, 57, 60). The latter findings were based on absorption of sera with linear V3 peptides, which abolished the neutralizing activity against TCLA viruses but had no effect on activity against primary viruses (2, 51, 57). Clearly, Abs directed against linear V3 peptides have limited reactivities, and absorption of polyclonal anti-HIV sera with linear V3 peptides removes only a subset of anti-V3 Abs (2, 51), leaving V3 Abs against conformation-sensitive epitopes which mediate broader neutralization.

The abilities of conformation-dependent V3 MAbs to bind to intact virions and to mediate neutralization of primary isolates indicates that the V3 region must be well exposed on the virus surface. These results are consistent with our previous work showing binding of a large panel of anti-V3 MAbs to 26 primary isolates from diverse clades (40, 41). Of the 16 primary isolates tested in the present study, all but two clade E primary

isolates bound to two or more of the anti-V3 MABs tested (Fig. 3). The data do not distinguish whether the absence of binding of the clade E isolates is due to epitope heterogeneity or to the lack of exposure of the V3 loop. For all other primary isolates, V3 exposure on intact virions seems to be a general characteristic. Exceptions occur and have been documented. Thus, V3 appears to be occluded on the surface of some viruses, such as JR-CSF (4), and only partially exposed on the closely related JR-FL (Fig. 3). The resultant neutralization of these viruses with anti-V3 Abs was similarly poor (Table 2) (4). Given that binding to detergent-solubilized envelope proteins from JR-FL was strong (data not shown), it is clear that the V3 loop of this virus is recognized by anti-V3 Abs but it does not act as a neutralizing epitope on these particular viruses. A similar case can probably be made for primary isolate DH012, which does not induce anti-V3 Abs in infected chimpanzees; however, its escape mutant is very sensitive to neutralization by guinea pig anti-V3 Abs (7). Thus, the accessibility of the V3 loop for Ab binding may vary among primary isolates, but except for the examples cited, V3 exposure is the rule rather than the exception.

Previous studies have suggested that the degree of V3 exposure on the virus surface may be linked to anti-V3-mediated neutralization (24, 48). Our studies establish a clear relationship between the binding of anti-V3 MABs to intact virions and neutralization. Experiments with several primary isolates revealed that only those viruses which were strongly reactive with human MABs directed to the V3 loop were neutralized. Thus, when 77 MAB-virus combinations were tested for strength of binding and degree of neutralization in the GHOST cell assay, a highly significant correlation was found ($P < 0.0001$). A similarly strong correlation was found when binding data and neutralization data from the PHA blast assay were analyzed ($P = 0.001$). Although experiments of this sort cannot be done with polyclonal HIV⁺ sera (because the presence of anti-p24 Abs interferes with the virus binding assay readout), pooling the results obtained with MABs approximates the results that would be obtained with polyclonal sera and a random selection of infectious viruses. Thus, the results can reasonably be extrapolated to apply to the human polyclonal Ab response to diverse HIV strains. However, these conclusions do not imply that binding of any single anti-V3 MAB to any particular virus will result in virus neutralization, and the conclusions are only applicable to anti-V3 MABs. Abs specific for other HIV epitopes clearly follow other rules. Thus, anti-C5 and anti-gp41 Abs, which bind avidly to the envelope glycoproteins of native primary isolates, have no neutralizing activity (11, 23, 40).

Abs targeting the V3 loop may mediate neutralization by interfering with one or several steps in the process of infection. These Abs may inhibit the interaction of V3 with the coreceptor. It has been shown that anti-V3 MABs block the ability of gp120-CD4 complexes to occupy CCR5 (22, 55, 62). The V3 loop may also be indirectly involved in coreceptor binding, because the V3-associated CCR5-binding region was found in the cavity close to the bridging sheet at the bases of the V3 and V1/V2 loops (45). Abs to the tip of the V3 loop (the most immunogenic portion of V3) could induce conformational changes affecting the CCR5-binding region. Postadsorption neutralizing effects have also been proposed, although these

effects have been documented to date only using anti-V3 MABs with TCLA strains (37).

While the MABs described here were shown to mediate statistically significant *in vitro* neutralization of primary isolates from several HIV-1 clades at levels that can be readily achieved *in vivo* (25 $\mu\text{g/ml}$), it remains for future studies to determine the threshold of neutralizing activity which is relevant *in vivo*. In recent experiments with rhesus monkeys, it was shown that even a low titer of anti-V3 Abs has biological relevance: the V3 peptide-elicited neutralizing Abs to SHIV-89.6 reached a titer of only 1:2 when tested in a PBMC-based neutralization assay but were able to provide partial protection against viremia following virus challenge. The authors speculated that eliciting a higher titer of anti-V3 Abs might provide more efficient protection and possibly a complete barrier to infection (32). Indeed, extremely low neutralizing titers confer protection against poliovirus and hepatitis B virus (53, 59). Thus, low levels of broadly reactive Abs to selected epitopes may yet prove to be protective against a diverse range of HIV isolates. Given the broad activity of human Abs against conformation-sensitive epitopes of V3 demonstrated here, the V3 region of HIV-1 gp120 should once again be considered one of the critical epitopes responsible for inducing protective anti-HIV Abs.

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