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Identification and Characterization of a Neutralization Site within the Second Variable Region of Human Immunodeficiency Virus Type 1 gp120

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Two monoclonal antibodies designated BAT085 and G3-136 were raised by immunizing BALB/c mice with gp120 purified from human immunodeficiency virus type 1 (HIV-1) IIIB-infected H9 cell extracts. Among three HIV-1 laboratory isolates (IIIB, MN, and RF), BAT085 neutralized only IIIB infection of CEM-SS cells, whereas G3-136 neutralized both IIIB and RF. These antibodies also neutralized a few primary HIV-1 isolates in the infection of activated human peripheral blood mononuclear cells. In indirect immunofluorescence assays, BAT085 bound to H9 cells infected with IIIB or MN, while G3-136 bound to H9 cells infected with IIIB or RF, but not MN. Using sequence-overlapping synthetic peptides of HIV-1 IIIB gp120, the binding site of BAT085 and G3-136 was mapped to a peptidic segment in the V2 region (amino acid residues 169 to 183). The binding of these antibodies to immobilized gp120 was not inhibited by the antibodies directed to the principal neutralization determinant in the V3 region or to the CD4-binding domain of gp120. In a competition enzyme-linked immunosorbent assay, soluble CD4 inhibited G3-136 but not BAT085 from binding to gp120. Deglycosylation of gp120 by endo-β-*N*-acetylglucosaminidase H or reduction of gp120 by dithiothreitol diminished its reactivity with G3-136 but not with BAT085. These results indicate that the V2 region of gp120 contains multiple neutralization determinants recognized by antibodies in both a conformation-dependent and -independent manner.

Intensive efforts in AIDS research have been focused on the development of an effective vaccine for protection of individuals at risk for human immunodeficiency virus type 1 (HIV-1) infection (4, 45). It has become evident that humoral responses play crucial roles in protective immunity against infection by HIV-1 and its progression to disease states. In this respect, Wendler et al. (49) showed that higher neutralizing antibody titers correlated with better prognosis and low or decreasing neutralizing antibody titers correlated with disease progression. Jackson et al. (19) and Karpas et al. (22, 23) demonstrated that AIDS-related complex and AIDS patients treated with hyperimmune plasma from asymptomatic HIV-1-seropositive individuals showed clinical improvement, which correlated with reduction in HIV-1 viremia. More recently, preliminary studies by Emini et al. (7) showed that chimpanzees injected with a mouse-human chimeric HIV-1-neutralizing monoclonal antibody (MAb) were protected from infection by the virus.

The envelope proteins of HIV-1, gp120 and gp41, are known to be the major targets for HIV-1-neutralizing activity in the sera of HIV-1-infected individuals (13, 18, 33, 48) and of animals immunized with the viral proteins (3, 28, 40). Several neutralization sites have been identified in gp120, including an immunodominant epitope in the V3 region (amino acid residues 308 to 322), commonly known as the principal neutralization determinant (PND) (9, 21, 24, 27, 32, 43, 44). Antibodies raised against this continuous epitope usually exhibit type-specific neutralizing activity against the homologous viral isolates (11, 12, 33, 40, 43). However, recent studies by Javaherian et al. (20) and White-Scharf et al. (50) showed that antibodies raised in animals against the relatively conserved portion of the PND displayed broadened reactivity with multiple HIV-1 isolates. Anti-PND antibodies do not block virus binding to CD4 but prevent virus internalization subsequent to CD4 binding (31, 44). Another continuous neutralization site on gp120 is located in the C4 region (amino acid residues 413 to 447), which is found to be involved in CD4-gp120 interaction (29, 47). Antibodies to this region exhibit broad neutralizing activity against divergent HIV-1 isolates, but this neutralization site appears to be rather immunosilent in HIV-1-infected individuals (47).

Sera from most HIV-1-infected individuals usually have low levels of broad neutralizing activity against HIV-1 (2, 13, 48), which were found to be associated with antibodies directed to conformational or discontinuous epitopes on gp120 (13). Consistent with this finding, we have reported a human MAb (I5e) which recognizes a discontinuous epitope on gp120 involved in CD4 binding and in antibody neutralization of multiple HIV-1 isolates (16). We also generated a HIV-1-neutralizing murine MAb (G3-4), which identified another distinct conformational epitope on gp120 involved in the interaction with CD4 (15).

In this report, we describe the generation and characterization of two murine MAbs designated BAT085 and G3-136 which define a unique neutralization domain in the V2 region of gp120.

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MATERIALS AND METHODS

MAb reagents and polyclonal antibodies. Murine HIV-neutralizing MAb BAT123 immunoglobulin G1(κ) [IgG1(κ)] binds to the PND in the V3 region of IIIB gp120 (amino acid residues 308 to 322) (32). Murine MAb G3-519 [IgG1(κ)] recognizes a component of the CD4-binding domain in the C4 region of gp120 (amino acid residues 423 to 437) (47). Murine MAb G3-4 [IgG2a(κ)] recognizes a conformational neutralizing epitope on HIV-1 gp120 (15). I5e [IgG1(κ)] is a HIV-1-neutralizing human MAb (a gift from J. Robinson, Louisiana State University School of Medicine, New Orleans) which also recognizes a distinct conformational epitope on gp120 (16). The murine MAbs were produced from ascitic fluid of BALB/c mice (Harlan Sprague-Dawley, Inc., Houston, Tex.) injected with the hybridomas and affinity purified by using recombinant protein A-Sepharose (Repligen, Boston, Mass.). I5e was produced from culture supernatant of the hybridoma and purified by protein A affinity chromatography. Polyclonal anti-HIV-1 gp120 antibodies (D6205) (International Enzymes, Inc., Fallbrook, Calif.) were generated by immunizing sheep with a synthetic peptide encompassing a highly conserved peptidic segment (APTKAKRRVVQREKR) in the carboxy terminus of HIV-1 (BH-10 strain) gp120 (amino acid residues 497 to 511) (35–37). The antibodies were isolated from the sheep hyperimmune sera by affinity chromatography using the specified synthetic peptide coupled to Sepharose.

Cells and HIV-1 isolates. CEM-SS cells, a syncytium-sensitive clone of CEM cells (a gift from P. Nara, National Cancer Institute), were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. H9 cells chronically infected with HIV-1 IIIB, MN, or RF, were maintained in RPMI 1640 medium with 15% heat-inactivated fetal bovine serum. These infected cells were derived from single-cell clones which were tested to be almost 100% expressing gp120 on the cell surface by immunofluorocytometric methods. The culture supernatants from these HIV-1-infected cells containing high reverse transcriptase activities were kept at −160°C and used for HIV neutralization assays. Peripheral blood mononuclear cells (PBMCs) were isolated from HIV-1-seronegative donors by Ficoll-Paque density gradient centrifugation, and were stimulated for 3 days with 2 μg of phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, Mo.) per ml in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 10% human interleukin-2 (Cellular Products, Inc., Buffalo, N.Y.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. After stimulation, PBMCs were washed and maintained in the complete growth medium for infectivity assays. Primary HIV-1 isolates (LS, RP, CC, LL, TB, and AC) were obtained from plasma specimens from HIV-1-seropositive individuals, as previously described (5, 16, 17). The JR-CSF isolate was kindly provided by I. Chen (University of California at Los Angeles, School of Medicine, Los Angeles).

Generation and screening of MAbs against HIV-1 gp120. Affinity-purified HIV-1 IIIB gp120 was used to immunize male BALB/c mice. Our standard procedures for purification of gp120 from infected H9 cell lysates, immunization of mice, and generation of hybridomas were previously described in detail (10, 47). Briefly, 3 days after the final immunization, the mice were sacrificed, and spleen cells were isolated and fused with Sp2/0 myeloma cells. After selection using hypoxanthine-aminopterin-thymidine (HAT) medium, culture supernatants from the wells of microdilution plates with hybridomas were tested for reactivity with gp120 as coating antigen by enzyme-linked immunosorbent assays (ELISAs). Culture supernatants showing strong gp120 reactivity were tested for staining live HIV-1 IIIB-infected cells by an indirect immunofluorescence method (47). Hybrids from selected fusion wells were expanded and cloned. MAbs from hybridomas selected for further characterization were produced in mouse ascitic fluid and purified by protein A affinity chromatography (47).

gp120 ELISA for screening of hybridomas. The procedures were similar to those described earlier (10, 47). Wells of Immulon 2 microdilution plates (Dynatech, Chantilly, Va.) were coated overnight at room temperature with 100 μl of purified gp120 (0.1 μg/ml) in phosphate-buffered saline (PBS). The wells were then treated with 5% BLOTTO in PBS for 1 h at room temperature and washed with PBS containing 0.05% Tween 20 (PBST). Next, 100 μl of test culture supernatant was added to each well for 1 h at room temperature. The wells were then washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted in PBST containing 1% bovine serum albumin (PBSTB). After another washing step, bound antibodies were detected by reaction with 0.1% tetramethyl benzidine and 0.0003% hydrogen peroxide as substrates. The optical density at 450 nm (OD_{450}) of the reaction solution was read with an ELISA reader.

Neutralization assays of HIV-1 laboratory isolates using CEM-SS cells. The syncytium-forming microassays using CEM-SS cells were performed as previously described (10, 39). Briefly, 50 μl of diluted MAb was mixed with 50 μl of viral culture supernatant containing 200 syncytium-forming units (SFU) of HIV-1 IIIB, MN, or RF and incubated for 1 h at room temperature. The mixtures were added into microculture wells containing 5×10^4 DEAE-dextran-treated CEM-SS cells, and the cell cultures were maintained in 5% CO_2 at 37°C for 3 to 4 days. The syncytia were then enumerated under an inverted microscope. The neutralizing activity was expressed as ID_{50}, defined as the concentration required to achieve 50% inhibition of the infection (i.e., Vn/Vo = 50%), where Vn is the number of SFU in the test wells and Vo is the number of SFU in the control without test antibodies. In the study of the epitope specificity using peptide V15P, the neutralizing activity of BAT085 at 12.5 μg/ml (ID_{50}) was tested for inhibition by incubating the antibody with various concentrations of the peptide for 20 min at room temperature, prior to the addition of a viral inoculum of 100 SFUs of HIV-1 IIIB. The irrelevant peptide α-endorphin (Peninsula Laboratories, Belmont, Calif.) was used for a control.

Neutralization assays of primary HIV-1 isolates using PHA-activated PBMCs. The titration of primary HIV-1 isolates and their neutralization assays were performed by our standard procedures (5, 17). An inoculum of 50 50% tissue culture infective doses was incubated with various concentrations of MAbs (0.01 to 25 μg) in 1 ml of final culture for 30 min at 37°C. The control contained no MAb. On day 4 to 7, depending on the replication kinetics of the primary HIV-1 isolates, the HIV-1 p24 antigen levels of the antibody-treated and untreated cell cultures were measured with Abbott HIV-1 Antigen Kits (Abbott Laboratories, North Chicago, Ill.). The neutralizing activity of the antibody is expressed as the percent reduction of p24 antigen levels in the antibody-treated wells compared with that of the control.

Flow cytometry. The binding of BAT085 and G3-136 to H9 cells infected with HIV-1 IIIB, MN, or RF was analyzed by flow cytometric methods using a Coulter EPIC cell analyzer (Coulter Electronics, Hialeah, Fla.) as previously described (25). The bound MAbs were detected by fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.).

Generation of HIV-1 gp120 peptides. All peptides used in epitope mapping were synthesized by using the RaMPs peptide synthesis system (Du Pont Co., Wilmington, Del.) by the 9-fluorenylmethoxycarbonyl synthesis protocol described in the manual (6a). The purity of the RaMPS-synthesized peptides was characterized by high-performance liquid chromatography, and the predicted structure was assessed by fast-atom bombardment mass spectrometry analysis. Amino acid sequences of the synthetic peptides for IIIB (HXB2 clone), MN, and RF isolates of HIV-1 were obtained from Myers et al. (38).

Epitope mapping using synthetic HIV-1 gp120 peptides. Preliminary epitope mapping was performed by using nitrocellulose strips impregnated with sequence-overlapping synthetic oligopeptides (9 to 15 amino acid residues) encompassing the entire length of HIV-1 IIIB gp120 polypeptide chain (24) (from S. Petteway formerly of Du Pont Co.). The amino acid sequences were derived from HIV-1 clone HXB2 (38). The reactivities of anti-HIV-1 gp120 MAbs with these peptides were determined by the methods previously described (32). Briefly, the nitrocellulose strips were incubated overnight at room temperature with test MAbs at 10 μg/ml in 5% BLOTTO. The strips were then washed with PBST. The bound antibody was then detected by incubating the strips with diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. The strips were washed, and the color reaction was developed with a substrate solution containing 0.3% 4-chloro-1-naphthol and 0.005% hydrogen peroxide.

Further fine epitope mapping was performed by peptide ELISA. Wells of Immulon 2 microtest plates were coated overnight at room temperature with 100 μl of the synthetic peptides (1 μg/ml) in PBS. The wells were then treated with 5% BLOTTO for 1 h at room temperature. After the wells were washed with PBST, 100 μl of BAT085 or G3-136 (1 μg/ml) was added to the wells for reaction for 1 h at room temperature. The wells were then washed, and the bound MAb was then detected by horseradish peroxidase-conjugated goat anti-mouse IgG. The color reaction was developed by incubation with the peroxidase substrate solution as described above. Reactivity was regarded as positive if the OD_{450} was twofold higher than that of the wells coated with the irrelevant peptide α-endorphin.

Competition ELISA. The effects of anti-HIV-1 gp120 MAbs, peptide V15P and recombinant soluble CD4 (sCD4) on the binding of BAT085 and G3-136 to HIV-1 IIIB gp120 were determined by the procedures previously described (35–37). sCD4 was obtained from Biogen, Inc., Cambridge, Mass. Wells of Immulon 2 microtest plates were coated overnight at room temperature with 50 μl of a solution containing 5 μg of affinity-purified sheep anti-HIV-1 gp120 (D6205, International Enzymes, Inc.) per ml. The wells were then treated with 5% BLOTTO for 1 h at room temperature. Fifty microliters of purified gp120 (0.5 μg/ml) was then added to each well for incubation for 2 h at room temperature. The plates were then washed. One hundred microliters of diluted biotinylated MAb was added to the wells with or without various amounts of competing MAbs (including BAT085, G3-136, G3-4, BAT123, G3-519, and I5e), peptide V15P or sCD4. The biotinylated MAbs were used at dilutions which gave half-maximal binding to the captured gp120. The incubation time was 1 h at room temperature. The plates were washed and reacted with 100 μl of horseradish peroxidase-conjugated streptavidin (diluted 1:2,000 in PBSTB) (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. The plates were washed again and allowed to react with the peroxidase substrate solution as described above. The degree of inhibition was expressed as the percent decrease in OD_{450} in the test wells compared with the OD of control wells without the inhibitors.

RIPA. The radioimmunoprecipitation (RIPA) procedure was carried out as described earlier (34, 47). Briefly, HIV-1 IIIB-infected H9 cells were metabolically labeled for 4 h with [^{35}S]cysteine and [^{35}S]methionine (100 μCi/ml; ICN Pharmaceuticals, Inc., Irvine, Calif.) and suspended in RIPA lysing buffer (50 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride). To examine the effect of antibody binding to gp160 and gp120 after reduction of disulfide linkages, the metabolically labeled cell lysates was treated with 0.1 M dithiothreitol in PBS at 37°C for 1 h. Dithiothreitol was then removed by dialysis. The effect of deglycosylation of gp160 and gp120 on antibody binding was also examined by treating the cell lysates with 100 mU of endo-β-*N*-acetylglucosaminidase H for 2.5 h at 37°C. The treated or untreated lysates were precleared with protein A-Sepharose bound to rabbit antiserum to mouse kappa light chain (κ-PAS) for 3 h at room temperature. RIPA was performed by adding 3 μg of purified MAb (BAT085, G3-136, or G3-4) and 0.2 ml of a 10% suspension of κ-PAS to 200 μl of labeled and clarified lysate. The samples were incubated for 18 h at 4°C, and the beads were washed with RIPA lysing buffer. The pellets were suspended in electrophoresis sample buffer and boiled for 3 min. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by autoradiography.

RESULTS

Generation and characterization of MAbs BAT085 and G3-136. Affinity-purified gp120 prepared from lysates of HIV-1 IIIB-infected H9 cells was used to immunize mice. The culture supernatants of hybrids were tested for reactivity with gp120 by ELISA. Antibodies exhibiting strong gp120 reactivity were tested for staining live HIV-1 IIIB-infected H9 cells by an indirect immunofluorescence method in order to determine whether the antibodies reacted with gp120 on infected cells. The MAbs from cloned hybrids were tested for neutralizing activity against HIV-1 IIIB using a syncytium-forming assay. Two neutralizing MAbs designated BAT085 and G3-136 [both IgG1(κ)] were identified for further characterization.

In the flow cytometric assay shown in Fig. 1A, BAT085 at 10 μg/ml stained 87 and 35% of H9 cells infected with HIV-1 IIIB and MN, respectively. It did not stain H9 cells infected with HIV-1 RF. G3-136 stained 73 and 91% of H9 cells infected with HIV-1 IIIB and RF, respectively (Fig. 1B) but did not stain H9 cells infected with HIV-1 MN.

In the in vitro neutralization assays against HIV-1 laboratory isolates using CEM-SS cells, BAT085 neutralized IIIB with ID_{50} of 12.5 μg/ml (Fig. 2A). It did not neutralize MN and RF. G3-136 neutralized IIIB and RF with ID_{50} of 16 and 2 μg/ml, respectively (Fig. 3A). It did not neutralize MN.

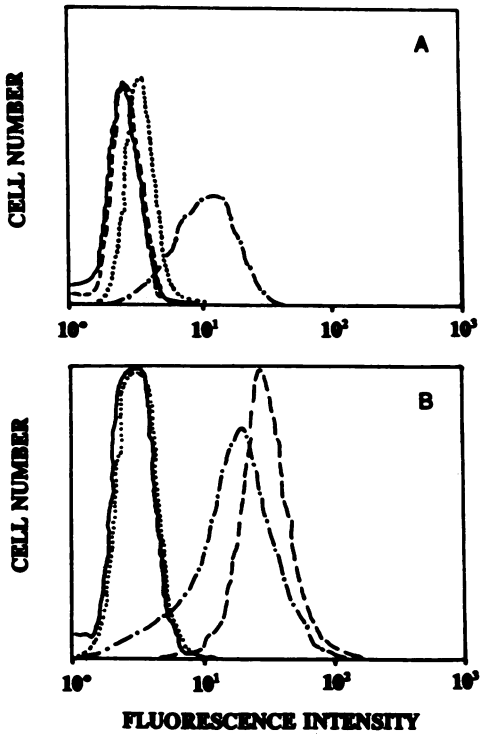

FIG. 1. Binding activities of BAT085 (A) and of G3-136 (B) to H9 cells infected with HIV-1 IIIB (—•—•—), MN (••••), or RF (— — —). Uninfected H9 cells (————) were used for a control. The H9 cells (10^7 cells per ml) were incubated with or without the antibodies for 30 min on ice. After the cells were washed, they were incubated with goat anti-mouse IgG coupled with fluorescein isothiocyanate for 30 min, washed, and fixed with paraformaldehyde. Fluorescence intensity was analyzed by flow cytometry.

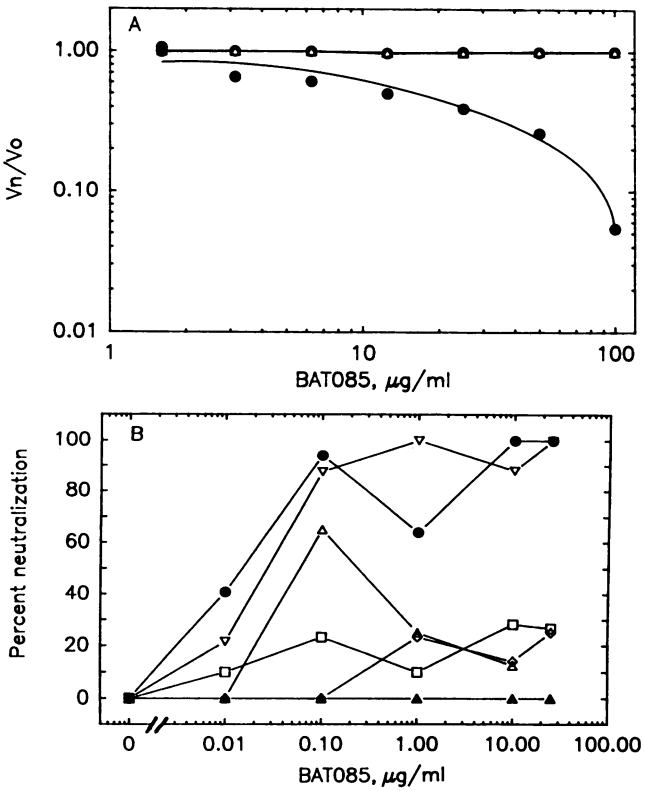

FIG. 2. Neutralization of HIV-1 by BAT085. (A) Inhibition of infection of CEM-SS cells by laboratory HIV-1 isolate IIIB (●), MN (○), and RF (△). A viral inoculum of 100 SFU was used to infect the cells in the presence or absence of various concentrations of BAT085. Vn is the mean number of syncytia in duplicate test wells, and Vo is the mean number of syncytia in the duplicate control wells. (B) Inhibition of infection of PHA-activated PBMCs from HIV-1-seronegative donors by primary HIV-1 isolates LS (●), RP (▽), CC(□), JR-CSF (◇), LL(△), and TB (▲). A viral inoculum of 50 50% tissue culture infective doses was used to infect the cells in the presence or absence of various concentrations of BAT085. The neutralizing activity of the antibody is expressed as the percent reduction of p24 antigen levels in the antibody-treated wells compared with that of the control.

Neutralizing activities against the infectivity of several primary HIV-1 isolates were also tested by using PHA-activated PBMCs. BAT085 neutralized two (LS and RP) of six isolates tested with an ID_{50} of less than 1 μg/ml (Fig. 2B); G3-136 neutralized three (AC, LS, and TB) of seven isolates with ID_{50} ranging from 0.03 to 0.4 μg/ml (Fig. 3B).

Epitope mapping of BAT085 and G3-136. To delineate the epitopes on gp120 that these two MAbs recognize, we first tested the effect of other previously characterized neutralizing anti-HIV-1 gp120 MAbs on the binding of biotinylated BAT085 and G3-136 to gp120. These other MAbs include BAT123, G3-519, G3-4, and I5e; the last two MAbs recognize distinct conformational neutralizing epitopes on gp120. In the competition ELISA, purified HIV-1 IIIB gp120 was captured by affinity-purified sheep anti-HIV-1 gp120 antibodies which are specific for a highly conserved peptidic segment in the carboxy terminus of gp120. The immobilization of gp120 did not hinder the binding of BAT123, G3-519, G3-4, I5e, BAT085, and G3-136. Figure 4A shows that the binding of biotinylated BAT085 to gp120 was partially inhibited by G3-4 (80%) and G3-136 (65%) at 40 μg/ml. In contrast, BAT123, G3-519, and I5e had no effect. Unconjugated BAT085 at 40 μg/ml completely abolished binding. When the binding of biotinylated G3-136 to gp120 was tested, G3-4 inhibited the binding as effectively as G3-136 did (Fig. 4B). However, BAT085 inhibited the binding by only 33% even at 40 μg/ml, and BAT123, G3-519, and I5e had no effect. These results were confirmed by examining the effects of BAT085 and G3-136 on the binding of biotinylated G3-4, BAT123, G3-519, and I5e to gp120. As expected, BAT085 and G3-136 competed with G3-4 but not with BAT123, G3-519, and I5e (16; also unpublished results).

To define the binding epitope(s) of BAT085 and G3-136 on gp120, the MAbs were examined by using nitrocellulose strips impregnated with sequence-overlapping synthetic peptides encompassing the entire length of HIV-1 IIIB gp120 polypeptide chain. It was found that BAT085 and G3-136 reacted specifically with a peptidic segment, VQKEYAFFY KLDIIP (referred to as V15P) in the V2 region of gp120, amino acid residues 169 to 183. To confirm this observation, overlapping oligopeptides encompassing the peptidic segment (amino acid residues 159 to 198) in HIV-1 IIIB gp120 (Table 1) were synthesized for ELISAs and liquid-phase competition assays. When these peptides were used as the solid-phase antigens at 1 μg/ml, BAT085 reacted strongly with V15P (OD_{450} = 1.4), and weakly with L20T (OD_{450} = 0.13). It did not react with F15Y, V10K, and A10P, giving OD_{450}s less than twofold greater than the negative control α-endorphin (OD_{450} = 0.01). G3-136 also reacted with V15P

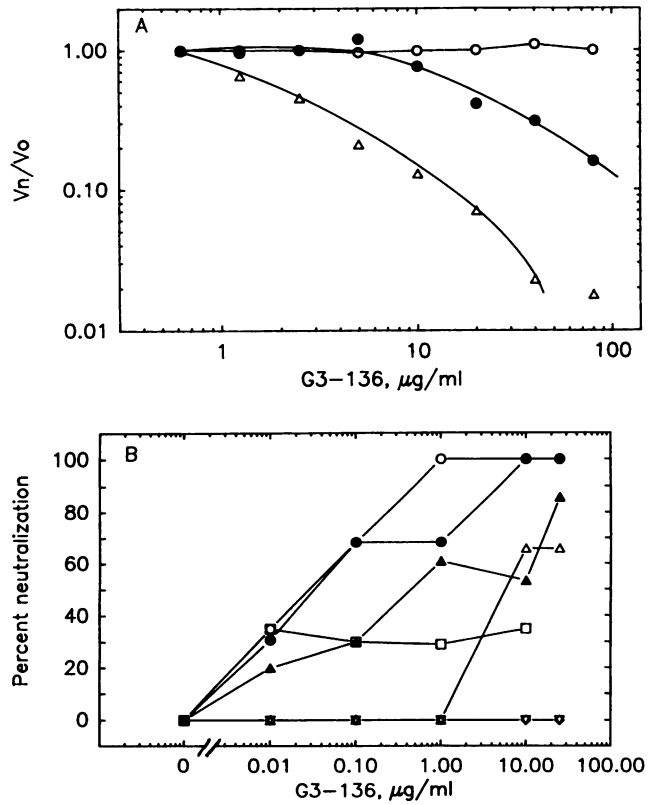

FIG. 3. Neutralization of HIV-1 by G3-136. (A) Inhibition of infection of CEM-SS cells by laboratory HIV-1 isolates IIIB (●), MN (○), and RF (△). (B) Inhibition of infection of PHA-activated PBMCs from HIV-1-seronegative donors by primary HIV-1 isolates AC (○), LS (●), LL (△), TB (▲), CC (□), JR-CSF (◇), and RF (▽). The neutralizing activities of the antibody were determined as described in the legend to Fig. 2.

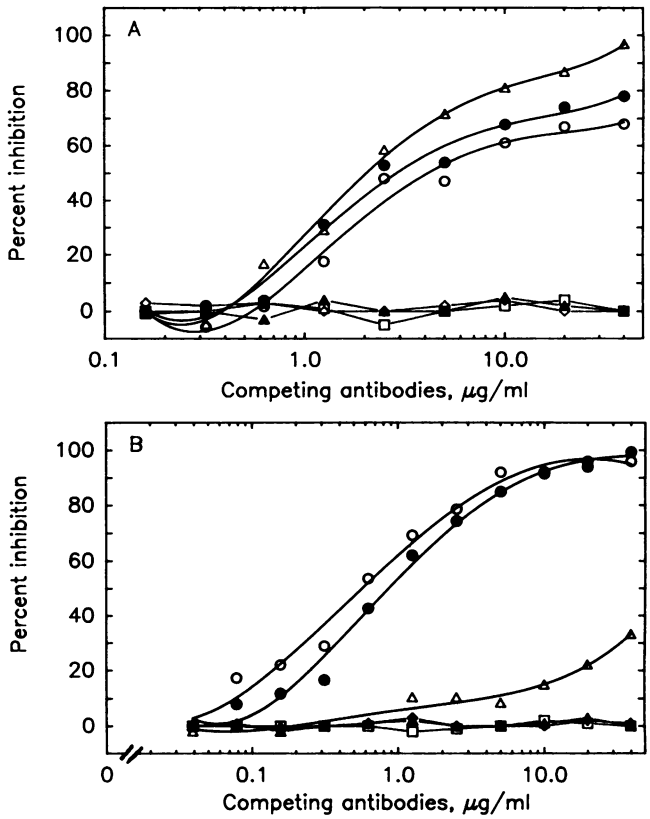

FIG. 4. Inhibition of the binding of biotinylated BAT085 (A) and biotinylated G3-136 (B) to captured HIV-1 IIIB gp120 in ELISA by BAT085 (△), G3-136 (○), G3-4 (●), BAT123 (▲), G3-519 (□), and I5e (◇). The competition ELISA procedure is described in Materials and Methods.

(OD_{450} = 0.44) but did not react with F15Y, L20T, V10K, and A10P. In the determination of reactivity with different HIV-1 isolates, BAT085 did not react with peptides M15S and T15P, which correspond to the same region of peptide V15P in the gp120 of HIV-1 MN and RF, respectively. G3-136 reacted with T15P (OD_{450} = 0.23), but not with M15S.

In the liquid-phase competition binding assay examining the effects of peptides on the binding of biotinylated BAT085 to captured gp120, V15P inhibited the binding of BAT085 to

TABLE 1. Epitope mapping of BAT085 and G3-136 to the V2 region of HIV-1 gp120 by peptide ELISA

Viral peptide and antigen	Sequence (amino acids 159–198[a])	Reactivity (OD_{450})	
		BAT085	G3-136
HIV HXB2	160 ▼ 170 ▼ 180 ▼ 190 ▼		
F15Y	FNISTSIRGKVQKEY	0.01	0.03
V15P	VQKEYAFFYKLDIIP	1.40	0.44
L20T	LDIIPIDNDTTSYSLTSCNT	0.13	0.02
V10K	VQKEYAFFYK	0.02	0.01
A10P	AFFYKLDIIP	0.03	0.01
HIV MN			
M15S	M- - - -LL- - - -VS	0.01	0.02
HIV RF			
T15P	T--K--L- - - -VV-	0.01	0.23

[a] Deduced amino acid sequences are obtained from Myers et al. (38). Homologous amino acids are represented by dashes. The OD_{450} of the blank was between 0.01 and 0.03.

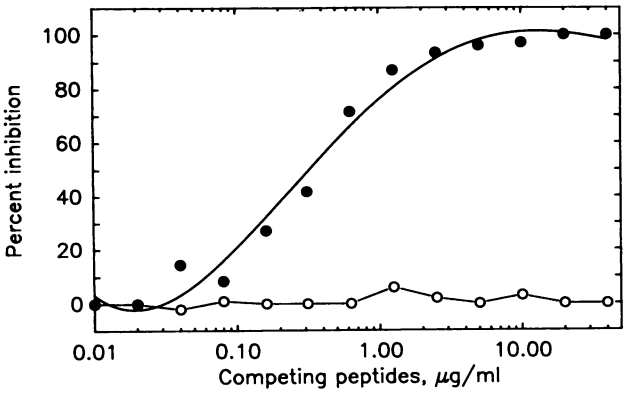

FIG. 5. Competition of the binding between biotinylated BAT085 and captured HIV-1 IIIB gp120 by the epitope peptide V15P (●) and the irrelevant peptide α-endorphin (○). The amino acid sequence of V15P is shown in Table 1. The ELISA procedure is described in Materials and Methods.

gp120 in a dose-dependent manner (Fig. 5). The α-endorphin peptide had no effect, even at a concentration of 40 μg/ml. The binding specificity of V15P to BAT085 was also examined in the neutralization assay of HIV-1 IIIB using CEM-SS cells. Figure 6 shows that V15P exhibited a dose-dependent inhibition of the neutralizing activity of BAT085, while α-endorphin had no effect. Either peptide alone did not affect the infectivity of HIV-1 IIIB.

Effects of sCD4 on the binding of BAT085 and G3-136 to gp120. To examine whether the neutralizing epitope mapped to the V2 region was involved in the interaction between CD4 and gp120, the effect of sCD4 on the binding of the biotinylated MAbs to captured gp120 was tested. sCD4 inhibited the binding of G3-136, G3-4, and G3-519 to gp120 (Fig. 7), although not completely even at a concentration as high as 40 μg/ml. The binding of BAT085 and BAT123 to gp120 is not affected by sCD4. These results suggest that the

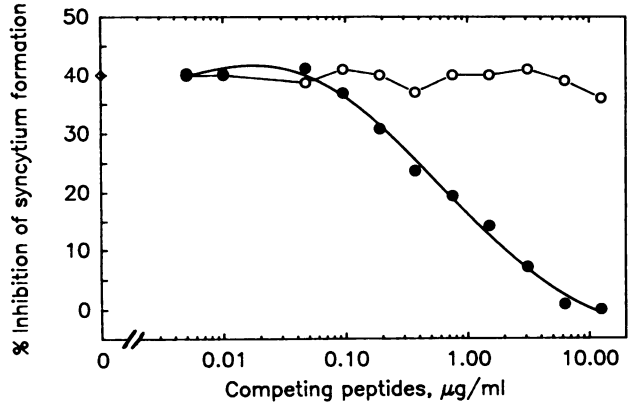

FIG. 6. Effects of oligopeptides on the neutralizing activity of BAT085 against HIV-1 IIIB. As described in Materials and Methods, CEM-SS cells (5×10^4) were infected with 100 SFU of HIV-1 IIIB in the presence of BAT085 at 12.5 μg/ml equivalent to ID_{50} (◇). Various concentrations of the epitope peptide V15P (●) or the irrelevant peptide α-endorphin (○) were mixed with the antibody as described in Materials and Methods. The percent inhibition of syncytium formation is defined as the percent decrease in the number of syncytia in the test wells compared with that of the control wells without the antibody.

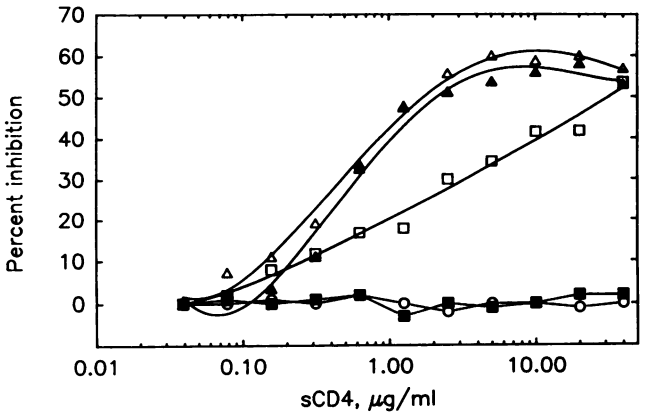

FIG. 7. Inhibitory activity of sCD4 on the binding of biotinylated MAbs to captured HIV-1 IIIB gp120 in ELISA. These biotinylated antibodies, including BAT085 (■), G3-136 (△), G3-4 (▲), G3-519 (□), and BAT123 (○) were used at dilutions which gave half-maximal binding to the captured gp120. Various concentrations of sCD4 were mixed with the labeled antibodies for binding to the gp120 for 1 h at room temperature as described in Materials and Methods.

epitopes recognized by BAT085 and G3-136 may be different, and that of G3-136 is near a CD4-binding domain of gp120.

Effect of reduction and deglycosylation of gp120 on its binding by BAT085 and G3-136. To study whether the binding of BAT085 and G3-136 to gp120 was affected by conformational changes of gp120, the effects of reduction of disulfide linkages and deglycosylation of gp120 were examined. As summarized in Table 2, the reactivity of G3-136 with gp120 in a RIPA was reduced when the viral lysate was first denatured by dithiothreitol. When a radiolabeled HIV-1 lysate was subjected to endo-β-*N*-acetylglucosaminidase H digestion, the partially deglycosylated product of 90 to 100 kDa was weakly recognized by G3-136 (Table 2). In parallel experiments, the reactivity of BAT085 with reduced or deglycosylated gp120 was unaltered, whereas the reactivity of G3-4 with gp120 was completely abolished under these conditions.

DISCUSSION

We report here that a unique neutralization domain in the V2 region of HIV-1 gp120 has been identified by two murine MAbs, BAT085 and G3-136, raised against HIV-1 IIIB gp120. These two MAbs can neutralize in vitro the infectivity of certain laboratory and primary HIV-1 isolates. The immunologic and biologic properties of these MAbs are summarized in Table 2.

By using sequence-overlapping synthetic peptides encompassing the entire length of the HIV-1 gp120 polypeptide chain, the binding site of BAT085 and G3-136 was mapped to the peptidic segment V15P (amino acid residues 169 to 183) in HIV-1 IIIB gp120 (Table 1). Confirming our findings, Stephens et al. (46) recently reported that antisera to the synthetic peptide defining the corresponding segment in the V2 region of HIV-1 SF2 gp120 (amino acid residues 171 to 185) neutralized in vitro the infectivity of the virus. The specificity of peptide V15P for BAT085 has been confirmed by the liquid-phase competition assays as illustrated in Fig. 5 and 6. The absence of reactivity of these MAbs with the shorter peptides (V10K and A10P) of this region indicates

TABLE 2. Properties of neutralizing MAbs to the V2 region of HIV-1 gp120[a]

MAb	Neutralization of HIV			Staining cells infected with HIV			sCD4 inhibition of gp120 binding	Effect of deglycosylation on gp120 binding	Effect of disulfide bond reduction on gp120 binding	Binding to epitope peptide (amino acids 169-183)
	IIIB	MN	RF	IIIB	MN	RF				
BAT085 [IgG1(κ)]	+	−	−	++	+	−	−	None	None	++
G3-136 [IgG1(κ)]	+	−	++	+	−	++	+	Reduced	Reduced	+
G3-4 [IgG2a(κ)]	+	−	++	+	−	++	+	Abolished	Abolished	−

[a] Symbols: +, reactive; −, unreactive; ++, strongly reactive.

that the actual binding site may be composed of several amino acid residues interspersed within peptide V15P. The identities of the crucial amino acid residues remain to be elucidated. Despite the fact that both BAT085 and G3-136 can bind to peptide V15P, only G3-136 reacts with peptide T15P of HIV-1 RF and strongly stains H9 cells infected with this viral isolate. It is still unclear which amino acid residues in these peptides actually determine the binding specificities of different HIV-1 isolates.

Inasmuch as the binding of G3-136 to gp120 is partially sensitive to deglycosylation and reduction (Table 2), its binding epitope is likely to be conformation dependent. In contrast, the binding of BAT085 to gp120 is not sensitive to conformational changes of gp120. This subtle difference in the binding characteristics between G3-136 and BAT085 is consistent with the inability of BAT085 to compete effectively with the binding of G3-136 to gp120 (Fig. 4B). We recently reported that a murine HIV-1-neutralizing MAb, G3-4, recognized a conformational epitope in HIV-1 IIIB gp120 (Table 2) (15). Although G3-4 did not react with peptide V15P, it effectively inhibited the binding of G3-136 but not BAT085 to gp120, suggesting that the binding epitopes of G3-4 and G3-136 are overlapping (Table 2). The binding of G3-4 to gp120 is strictly conformation dependent, because its binding is completely abolished when gp120 is deglycosylated or reduced (15). On the basis of these findings, we conclude that the V2 region of gp120 may contain multiple neutralization determinants constituted by either continuous or discontinuous structures.

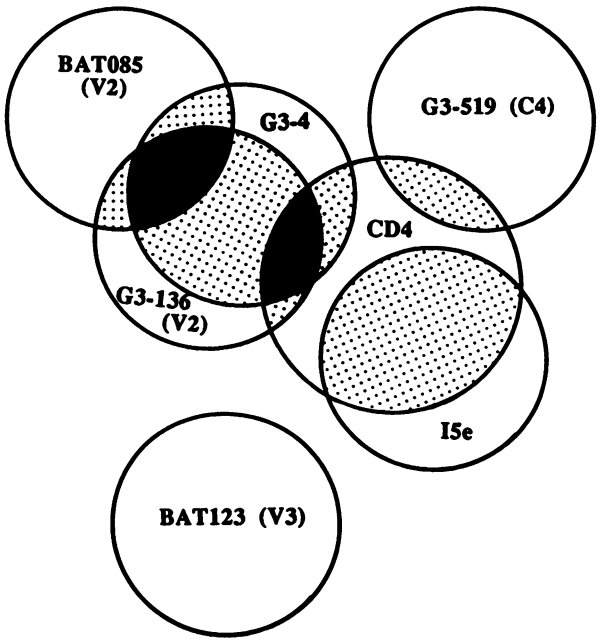

FIG. 8. Schematic representation of the epitopes for BAT085, G3-136, G3-4, G3-519, BAT123, and I5e on gp120 and their spatial relationship with one another and with the CD4-binding site as determined by competition ELISA.

Results from this study also indicate that the neutralization determinants in the V2 region recognized by BAT085 and G3-136 are likely to be topographically removed from the PND in the V3 region and the CD4-binding domain in the C4 region, since the binding of BAT085 or G3-136 to gp120 cannot be inhibited by BAT123 and G3-519 in ELISA. In addition, BAT085 and G3-136 do not inhibit the gp120 binding of I5e, which recognizes a distinct conformational epitope yet to be defined (16). This observation provides credence to the possibility of combining HIV-1 MAbs and various neutralization epitopes in passive immunotherapy to confer improved efficacy and broadened viral reactivity. In vitro experiments are in progress in our laboratories to address the functional interactions between these neutralization sites.

It is interesting to note that G3-136 neutralizes a heterologous HIV-1 isolate (RF) more efficiently than the homologous isolate (IIIB) (Fig. 3A), although its immunoreacitivity is stronger with peptide V15P of IIIB than with the corresponding peptide T15P of RF. A possible explanation for this observation is that the binding site on native HIV-1 IIIB gp120 is not as accessible as that of HIV-1 RF. The accessibility of the neutralization sites on gp120 can be affected by the intricate folding of gp120, as well as the variable extent of glycosylation of gp120 (8, 14, 30). The juxtaposition of discontinuous regions of gp120 has been implicated in the association between gp120 and gp41 (26) and in the escape of HIV-1 variants from neutralization by antibodies (41, 51). In line with this, recent studies by Davis et al. (6) indicated that glycosylation of gp120 could affect the binding of anti-peptide antibodies to gp120. Another possible explanation for the higher neutralizing activity of G3-136 against HIV-1 RF is that since the binding of G3-136 to gp120 is partially conformation dependent (Table 2), G3-136 may fit the three-dimensional configuration of the binding site on the gp120 of the RF isolate better.

In order to understand the possible mechanisms of neutralization mediated by BAT085 and G3-136, we examined whether their binding to gp120 can affect CD4-gp120 interaction. Indeed, like G3-519, the binding of G3-136 and G3-4 to gp120 can be inhibited by sCD4. In contrast, the binding of BAT085 to gp120 is not affected in the presence of sCD4. These data suggests that the neutralizing activities of G3-136 and G3-4 may be mediated by interfering with the interaction between CD4 and gp120, whereas BAT085 may have a distinct and as yet undefined mechanism. The inability of sCD4 to completely abolish the binding of G3-136, G3-4, or G3-519 to gp120 can be explained by the fact that the

structural determinants recognized by these antibodies are either only sterically proximal to the actual CD4 binding sites or are forming parts of the multiple discontinuous regions responsible for CD4 binding (1, 42). The spatial relationship of the newly identified V2 neutralization epitopes relative to the other previously defined neutralization sites (V3, C4, and the I5e conformational determinant) and to the CD4-binding site is schematically shown in Fig. 8.

On the basis of the findings in this study, we conclude that the V2 region of gp120 bears multiple and overlapping neutralization determinants. To better design active and passive immunization strategies, it is important to study whether neutralizing antibodies to this region play an active role in the protective immunity against HIV-1 infection in humans.

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