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Characterization of a Human Immunodeficiency Virus Neutralizing Monoclonal Antibody and Mapping of the Neutralizing Epitope

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A monoclonal antibody was produced to the exterior envelope glycoprotein (gp120) of the human T-cell lymphotropic virus (HTLV)-III$_B$ isolate of the human immunodeficiency virus (HIV). This antibody binds to gp120 of HTLV-III$_B$ and lymphadenopathy-associated virus type 1 (LAV-1) and to the surface of HTLV-III$_B$- and LAV-1-infected cells, neutralizes infection by cell-free virus, and prevents fusion of virus-infected cells. In contrast, it does not bind, or weakly binds, the envelope of four heterologous HIV isolates and does not neutralize heterologous isolates HTLV-III$_{RF}$ and HTLV-III$_{MN}$. The antibody-binding site was mapped to a 24-amino-acid segment, using recombinant and synthetic segments of HTLV-III$_B$ gp120. This site is within a segment of amino acid variability known to contain the major neutralizing epitopes (S. D. Putney, T. J. Matthews, W. G. Robey, D. L. Lynn, M. Robert-Guroff, W. T. Mueller, A. J. Langlois, J. Ghrayeb, S. R. Petteway, K. J. Weinhold, P. J. Fischinger, F. Wong-Staal, R. C. Gallo, and D. P. Bolognesi, Science 234:1392–1395, 1986). These results localize an epitope of HIV type-specific neutralization and suggest that neutralizing antibodies may be effective in controlling cell-associated, as well as cell-free, virus infection.

Human immunodeficiency virus (HIV) is the cause of acquired immunodeficiency syndrome (2, 16), and the HIV external envelope glycoprotein, gp120, is associated with viral infectivity and cytopathology including cell fusion (12, 24). gp120 binds the cellular receptor of the virus, CD4 (5, 15), and cells expressing the envelope fuse with CD4-positive cells in culture (12, 24). Fusion of infected and noninfected cells and infection by cell-free virions are important routes of HIV infection. It will be important for subunit vaccines to prevent infection by both of these routes.

gp120 purified from virus-infected cells elicits antibodies that neutralize the infectivity of HIV (22). Recombinant gp120 or gp160 expressed in mammalian cells (10), insect cells (23), or yeast cells (26) also elicits neutralizing antibodies. The amino acid sequence of the envelope varies between different HIV isolates (6, 25), and the neutralizing antibodies elicited by the envelope of one HIV isolate are effective against only a subset of heterologous isolates (14, 18, 27). This suggests that these neutralizing antibodies are directed to a region of envelope sequence variability. This concept is supported by neutralizing antibodies elicited by an *Escherichia coli*-produced recombinant fragment from the carboxyl-terminal region of gp120, PB1, that contains 37% of gp120 and is from a more variable region of the envelope (17). This fragment contains the dominant neutralizing epitopes, and a more complete understanding of the number, the location, and the potential role of antibodies to these epitopes in preventing viral infection may facilitate development of a subunit vaccine able to induce immunity to diverse HIV isolates.

In this report, we describe a monoclonal antibody produced by using purified gp120 which is capable of neutralizing cell-free and cell-associated virus infection in an isolate-specific fashion. This epitope was mapped to 24 amino acids which are within a highly variable segment of PB1.

MATERIALS AND METHODS

Monoclonal antibody production. The glycoprotein fraction used as an immunogen was prepared from H9/HTLV-III$_B$ cell extracts. H9/HTLV-III$_B$ cells were washed with phosphate-buffered saline (pH 7.2) and lysed in 0.15 M NaCl–0.05 M Tris hydrochloride (pH 7.2)–1% Triton X-100–0.1% sodium dodecyl sulfate (SDS). The extracts were heat inactivated, cleared by Sepharose conjugated with fetal calf serum, and reacted with concanavalin A-Sepharose. The concanavalin A-bound fraction was then eluted and incubated with Sepharose coupled with an immunoglobulin fraction of pooled sera from healthy carriers of HIV. The viral glycoprotein fraction was eluted and used as an immunogen. BALB/c mice were immunized on day zero with 100 μg of protein in Freund complete adjuvant, and three subsequent immunizations of 50 μg in Freund incomplete adjuvant were given at 14-day intervals. Three days after the final immunization, splenic cells were fused with cells of the x63 mouse myeloma line. The cell fusion, hypoxanthine-aminopterin-thymidine selection, and cloning of hybridoma lines were essentially as described by Robert-Guroff et al. (21).

Immunologic assays. For fluorescent staining of cell surfaces, 5×10^5 cells were incubated for 30 min on ice with 10 μg of 0.5β antibody or control immunoglobulin G1 (IgG1) antibody (MOPC 21) per ml. Following extensive washing, cells were incubated on ice with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG for 30 min, washed, and analyzed by laser flow cytometry.

Western blot (immunoblot) analysis of 0.5β reactivity used lysates of human T-cell lymphotropic virus (HTLV)-III$_B$ virions purified by sucrose density gradient centrifugation.

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Viral antigens were fractionated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose. Individual nitrocellulose strips were reacted with dilutions of ascites fluids containing mouse monoclonal antibodies or of human sera. Bound antibodies were visualized by enzyme immunoassay, using biotinylated anti-mouse or anti-human antibody and horseradish peroxidase-conjugated avidin. In some cross-precipitation experiments, HTLV-III_B lysates were first absorbed by 0.5β or MOPC 21 bound to Sepharose. The cleared supernatants were electrophoresed, transferred to nitrocellulose, and used in Western blot analysis.

For Western blot analysis of purified envelope protein or envelope subfragments, proteins were first electrophoresed on 15% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were reacted with a 1:1,000 dilution of 0.5β ascites for 2 h at room temperature. After washing, the blots were incubated with 5×10^5 cpm of ^{125}I-goat anti-mouse IgG per ml, washed again, and autoradiographed.

Radioimmunoprecipitation analyses were carried out essentially as previously described (13). H9 cells infected with HTLV-III_B were labeled with [^{35}S]cysteine (100 μCi/ml), washed, disrupted with 0.05 M Tris hydrochloride buffer (pH 7.2) containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 0.15 M NaCl. The lysate was cleared once with Sepharose-conjugated normal human IgG and reacted with 20 μl of Sepharose-conjugated antibodies. Precipitates were electrophoresed on 12% SDS-polyacrylamide gels and autoradiographed. For some cross-precipitational analyses, radiolabeled lysate was first absorbed once or twice with either 0.5β-Sepharose or MOPC 21-Sepharose. Precipitates resulting from reaction of the cleared supernatants with anti-HIV IgG-Sepharose prepared from serum from a healthy HIV carrier were then analyzed as described above.

Enzymatic treatment. Disrupted HTLV-III_B virions were deglycosylated by incubation for 3 h at 37°C in the presence of 0.25 μg of endoglycosidase H (New England Nuclear Corp., Boston, Mass.) and were subsequently used as an antigen in the Western blot assay. Reactions were visualized by enzyme immunoassay as described above.

Syncytial inhibition assay. H9 cells infected with HTLV-III_B (2.5×10^4) were preincubated in 98-well microtiter plates with various concentrations of purified 0.5β or MOPC 21 antibody in 100 μl of RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. After 30 min of incubation at 37°C, 5×10^4 target CEM cells were added. CEM cells alone and H9/HTLV-III_B cells alone were also included as controls. The plates were cultured at 37°C in 5% CO_2 containing humidified air. The cultures were examined after 18 h and 3 days of culture, using an inverted microscope.

Neutralization of cell-free HIV. Neutralization of cell-free HIV infection of H9 cells by 0.5β antibody was assayed as previously described (21), using titered stocks of various HIV isolates. After 5 to 6 days of culture, neutralization of infection was determined by measuring virus expression by using an indirect immunofluorescence assay and monoclonal antibody to HIV p24.

Generation of gp120 recombinant fragments and peptides. gp160-III_B (or r160-III_B) was produced in insect cells by using a recombinant baculovirus expressing the gp160 gene of the BH10 clone (19). Fragments containing nucleotides 405 to 2190 and 838 to 1421 from the HAT3 clone of the RF isolate (25) were substituted for the corresponding fragments to generate baculovirus vectors expressing r160-RF and r160-hybrid, respectively. Extracts of infected insect cells were used in the Western blot experiments. Subfragments of PB1-III_B were expressed by excising fragments of the BH10 genome and expressing them in *E. coli* as fusion proteins containing additional non-HIV expression vector-derived amino acids on each end. The cloned fragments correspond to envelope nucleotide positions 838 to 1046 (Sub 1), 838 to 1199 (Sub 2), 1046 to 1421 (Sub 6), 1046 to 1367 (Sub 7), and 1046 to 1199 (Sub 8). Sub 2 was treated with cyanogen bromide to generate CN1 and CN2. Each of these proteins was purified or partially purified by ion exchange and gel filtration chromatography.

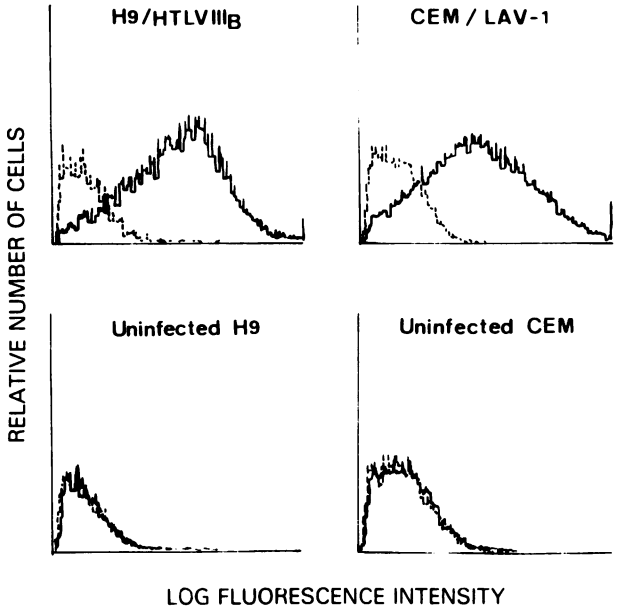

FIG. 1. Representative cell sorter profiles of cells labeled with the 0.5β monoclonal antibody. Profiles representing cell populations labeled with 0.5β are shown by solid lines, while control populations labeled with control mouse IgG1 are shown by broken lines.

Peptides were synthesized by using an Applied Biosystems synthesizer and purified by using high-performance liquid chromatography. Residue positions correspond to the BH10 clone from HTLV-III_B (19), and each peptide corresponds to the sequence of the envelope from BH10. RP134 has five additional non-HIV amino acids on the amino terminus.

RESULTS

Isolation and characterization of gp120 specific monoclonal antibody. Purified viral glycoproteins used as immunogens were prepared from H9/HTLV-III_B cell extracts, and immunizations and fusions were done as described in Materials and Methods. Culture fluids from growing hybridomas were screened for antibodies to HTLV-III_B proteins by enzyme-linked immunosorbent assay, and supernatants that scored positive were further assayed by immunofluorescence surface staining of virus-producing cells. Of the several hybridomas tested, one, designated 54′C, secreted antibody [denoted 0.5β; IgG1(κ)] that reacted with the surface of H9/HTLV-III_B cells and lymphadenopathy-associated virus type 1 (LAV-1)-infected CEM cells (Fig. 1). The antibody did not react with uninfected H9 or CEM cells or with HTLV-I-infected HUT 102 cells, MJ tumor cells, HTLV-II-infected C3/44 cells, or normal peripheral blood mononuclear cells (not shown). These data indicate that the 0.5β antibody specifically recognizes HTLV-III_B/LAV-1-infected cells.

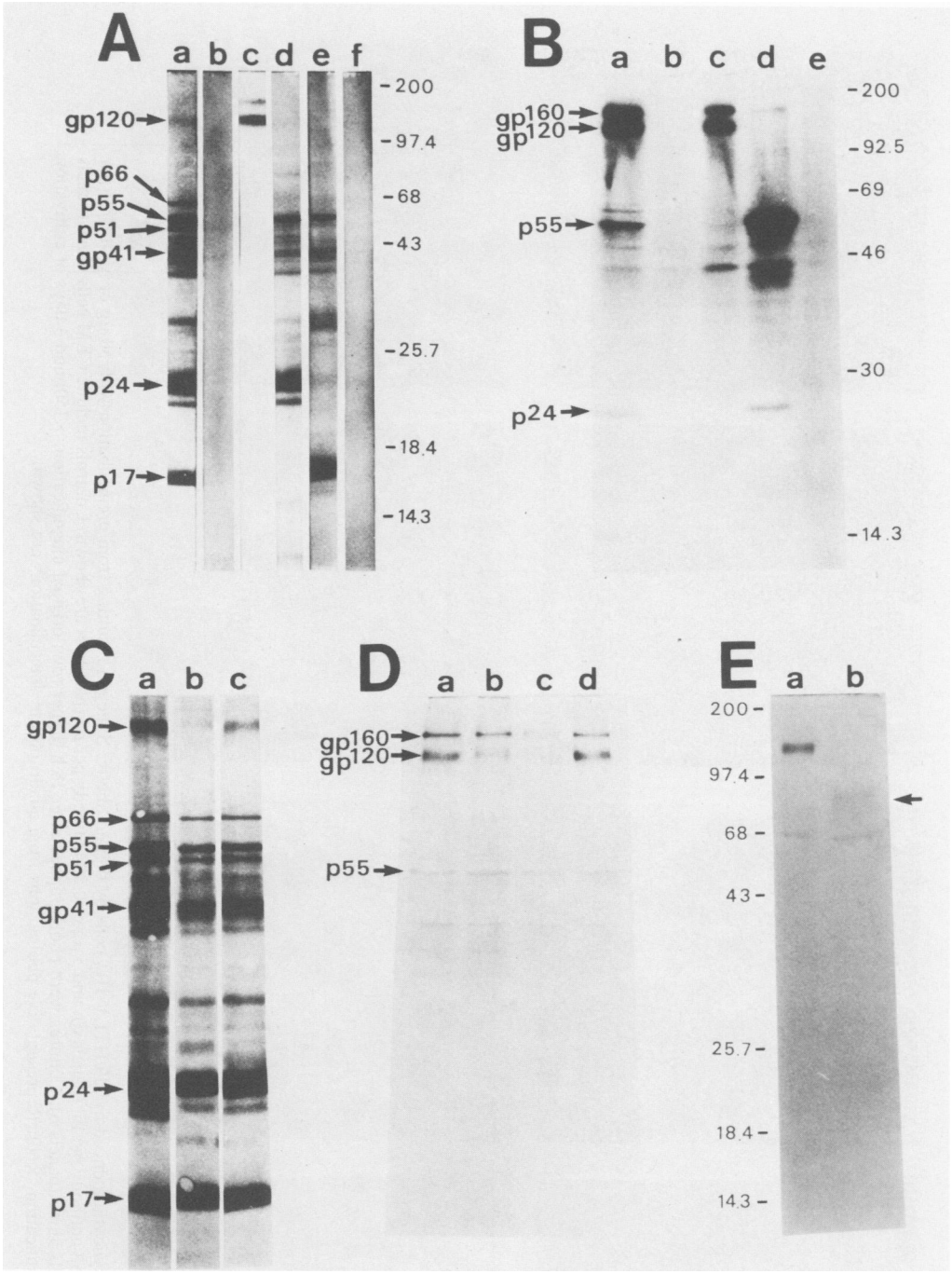

FIG. 2. Detection of gp120 of HTLV-III$_B$ by 0.5β antibody. (A) Western blot analysis for reactivity with HTLV-III$_B$ antigens was carried out as described in Materials and Methods. Individual strips were reacted with serum from an asymptomatic HIV carrier (lane a), normal human serum (lane b), 0.5β antibody (lane c), monoclonal anti-p24 antibody (VAK5) (7) (lane d), monoclonal anti-p17 antibody (52E5) (lane e), or MOPC-21 ascites as a control (lane f). Monoclonal antibodies were used at a 1:500 dilution of ascites, and human sera were diluted 1:100. (B) Immunoprecipitation of gp160 and gp120 from metabolically labeled H9/HTLV-III$_B$ cell lysate by 0.5β antibody. Radioimmunoprecipitates were obtained by using purified IgG from an asymptomatic HIV carrier (lane a), normal human IgG (lane b), 0.5β antibody (lane c), anti-p24 (VAK5) monoclonal antibody (lane d), or MOPC 21 ascites (lane e). (C) Cross-precipitation analysis using Sepharose-conjugated antibodies. HTLV-III$_B$ lysate (lane a) or the lysate absorbed with 0.5β-Sepharose (lane b) or MOPC 21-Sepharose (lane c) was subjected to Western blot analysis with 1:100-diluted serum from an HIV carrier. (D) Sequential cross-precipitational analyses of metabolically labeled H9/HTLV-III$_B$ lysates. [^{35}S]cysteine-labeled H9/HTLV-III$_B$ cell lysate was absorbed once (lane b) or twice (lane c) with 0.5β-Sepharose antibody or twice with MOPC 21-Sepharose (lane d). Resulting supernatants and unabsorbed lysate (lane a) were reacted with anti-HIV IgG-Sepharose. (E) Effect of glycosylation on binding of 0.5β. A Western blot is shown following treatment of disrupted HTLV-III$_B$ with (lane b) or without (lane a) endoglycosidase H (New England Nuclear Corp., Boston, Mass.). The blot was reacted with a 1:200 dilution of 0.5β ascites. The arrow indicates the reactivity of 0.5β with deglycosylated gp120. Molecular mass standards show protein sizes in kilodaltons.

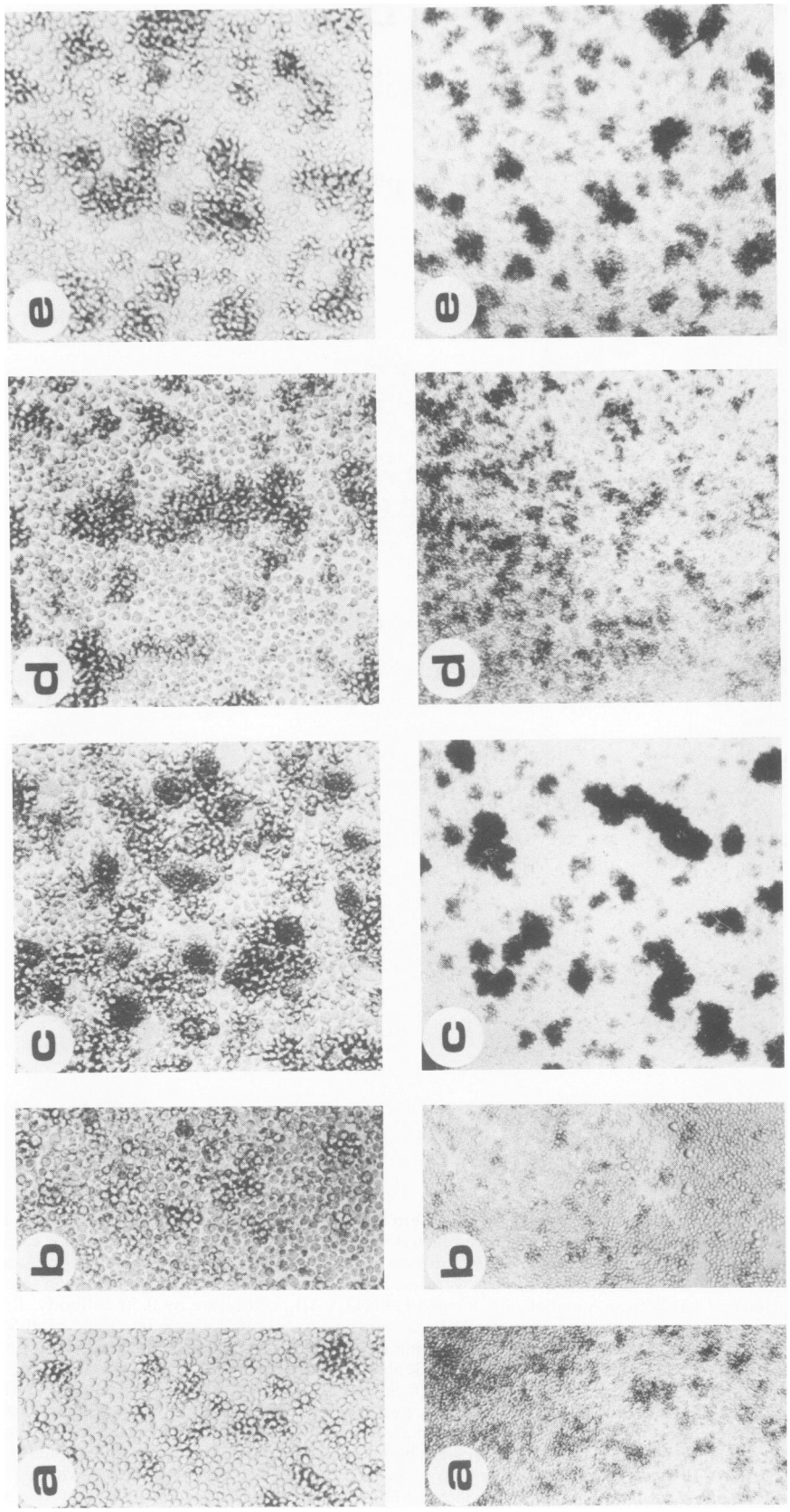

FIG. 3. Inhibition of syncytia induced by H9/HTLV-III$_B$ cells by 0.5β antibody. Syncytia resulting from preincubation with 50 μg of MOPC 21 per ml (panels c), 50 μg of 0.5β antibody per ml (panels d), and 5 μg of 0.5β antibody per ml (panels e) are shown. Controls include CEM cells alone (panels a) and H9/HTLV-III$_B$ cells alone (panels b). Cultures were photographed after 18 h (upper row; original magnification, ×150) and 3 days of cultivation (lower row; original magnification, ×60). Representative photographs from quadruplicate determinations are shown.

TABLE 1. Type-specific neutralization of HIV by 0.5β antibody

Isolate	Neutralizing antibody titer[a]		
	Purified 0.5β	0.5β ascites	Human seropositive sera
HTLV-III$_B$	100 ng/ml	>6250	280
RF	–	–	750
MN	–	ND	750

[a] Neutralizing antibody titer is expressed as nanograms of purified monoclonal IgG per milliliter or the reciprocal of the serum of ascites dilution at which infectivity was neutralized more than 75%. –, No neutralization with a 1:10 dilution of ascites or 60 μg of purified 0.5β antibody per ml; ND, not done.

Recognition of gp120 by the antibody was shown by Western blotting of disrupted HTLV-III$_B$ virions (Fig. 2A, lane C). Metabolically radiolabeled cell lysates were also examined by radioimmune precipitations, and Sepharose-conjugated 0.5β specifically precipitates both gp120 and the primary *env* gene product, gp160 (Fig. 2B, lane c). As expected, serum antibodies from an HIV-seropositive individual recognize gp120 and gp160 (Fig. 2A and B, lanes a), whereas monoclonal antibodies to *gag* gene products p24 or p17 do not (Fig. 2A, lanes d and e, and Fig. 2B, lane d). In addition, 0.5β does not recognize HTLV-I or -II proteins on Western blots (results not shown).

To confirm that the molecules recognized by 0.5β are the same as those recognized by patient antibodies, purified HTLV-III$_B$ proteins were cleared by immunoprecipitation with 0.5β-Sepharose or MOPC 21 (control IgG1)-conjugated Sepharose and subjected to Western blotting analyses. The blots were reacted with antibodies from an HIV-seropositive individual positive for *gag*, *pol*, and *env* gene products, and the reactivity of the anti-HIV serum against gp120 was markedly decreased when the virus was cleared by 0.5β-Sepharose (Fig. 2C, lane b). In contrast, the reactivity with gp120 was similar to that of the untreated control (lane a) when the antigens were cleared by MOPC 21-Sepharose (lane c). These results were confirmed by using sequential radioimmunoprecipitation of H9/HTLV-III$_B$ lysates (Fig. 2D). When lysates are cleared once (lane b) or twice (lane c) with 0.5β-Sepharose, the gp160 and gp120 precipitated by anti-HIV IgG-Sepharose is decreased, whereas the amounts of precipitated gp160 and gp120 in the MOPC 21 control (lane d) are similar to those in the untreated cell lysate control (lane a). These results confirm that the molecule recognized by 0.5β is gp120.

The effect of the extensive glycosylation of gp120 on the binding of 0.5β was assayed by endoglycosidase H treatment of disrupted virions. Reactivity of the antibody with deglycosylated gp120 is detected (Fig. 2E, lane b), and recognition of a range of molecular masses from 70 to 84 kilodaltons is observed. The molecular weight of deglycosylated gp120 is 70 to 80 kilodaltons (1).

HIV isolate-specific neutralization by 0.5β. Because gp120 is the major target of HIV neutralizing antibodies (3, 10, 14, 17, 23, 26) the ability of 0.5β to neutralize in vitro virus infection was assessed. One of the two assays used measures the ability of antibody to block fusion of HIV-infected and uninfected cells. Following the mixing of target CEM cells with HTLV-III$_B$-infected H9 cells in the presence of control mouse IgG1 antibody, numerous syncytia are observed after 18 h (Fig. 3, upper row, panel c). In contrast, in the presence of 0.5β, syncytia formation is completely inhibited at an antibody concentration of 50 μg/ml and partially inhibited (with small syncytia) at 5 μg/ml (Fig. 3, upper row, panels d and e). After 72 h, large clumps of cells form in the control culture with few viable cells while cultures protected by 0.5β do not form such clumps at the higher concentration of antibody (Fig. 3, lower row, panels c and d). At the lower concentration of 0.5β, many healthy cells exist between smaller cell clumps (Fig. 3, lower row, panel e).

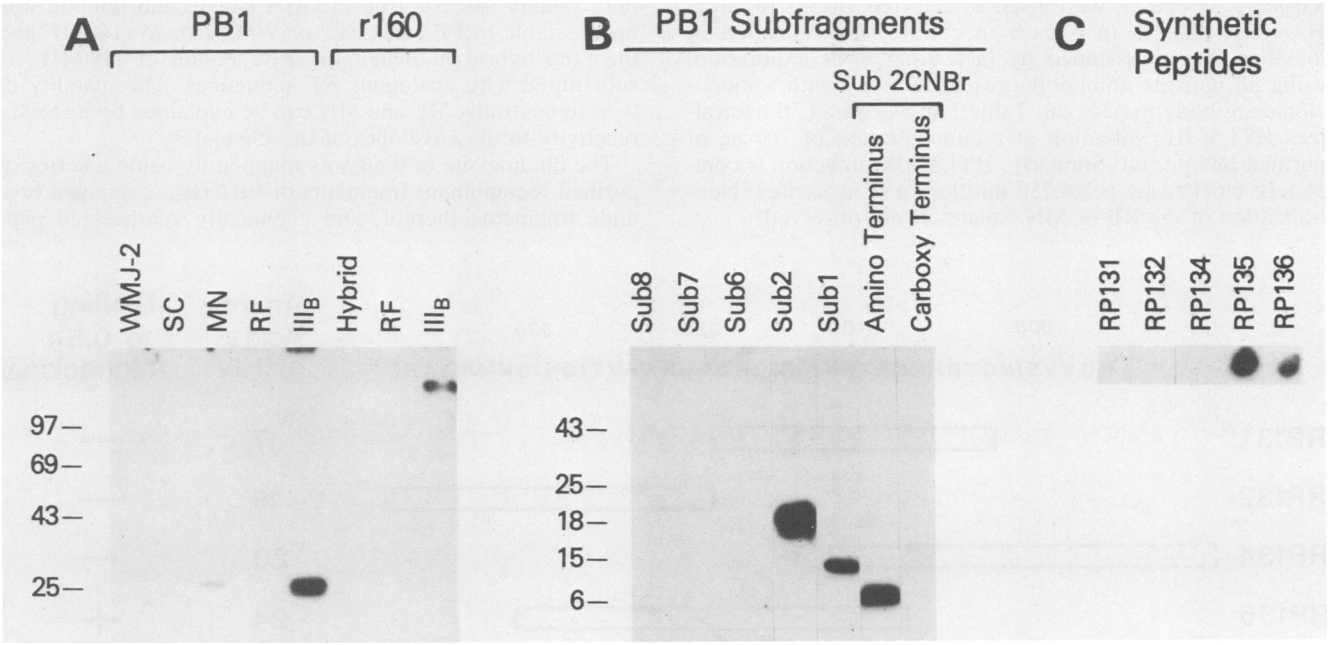

FIG. 4. Reactivity of 0.5β with gp160 and PB1 from several HIV isolates and with subfragments of PB1-III$_B$. In panels A and B, Western blots were carried out on proteins separated on 15% SDS-polyacrylamide gels. In panel C, 1 μg of synthetic peptides was spotted on a nitrocellulose strip which was then reacted as for panels A and B. III$_B$, HTLV-III$_B$ isolate.

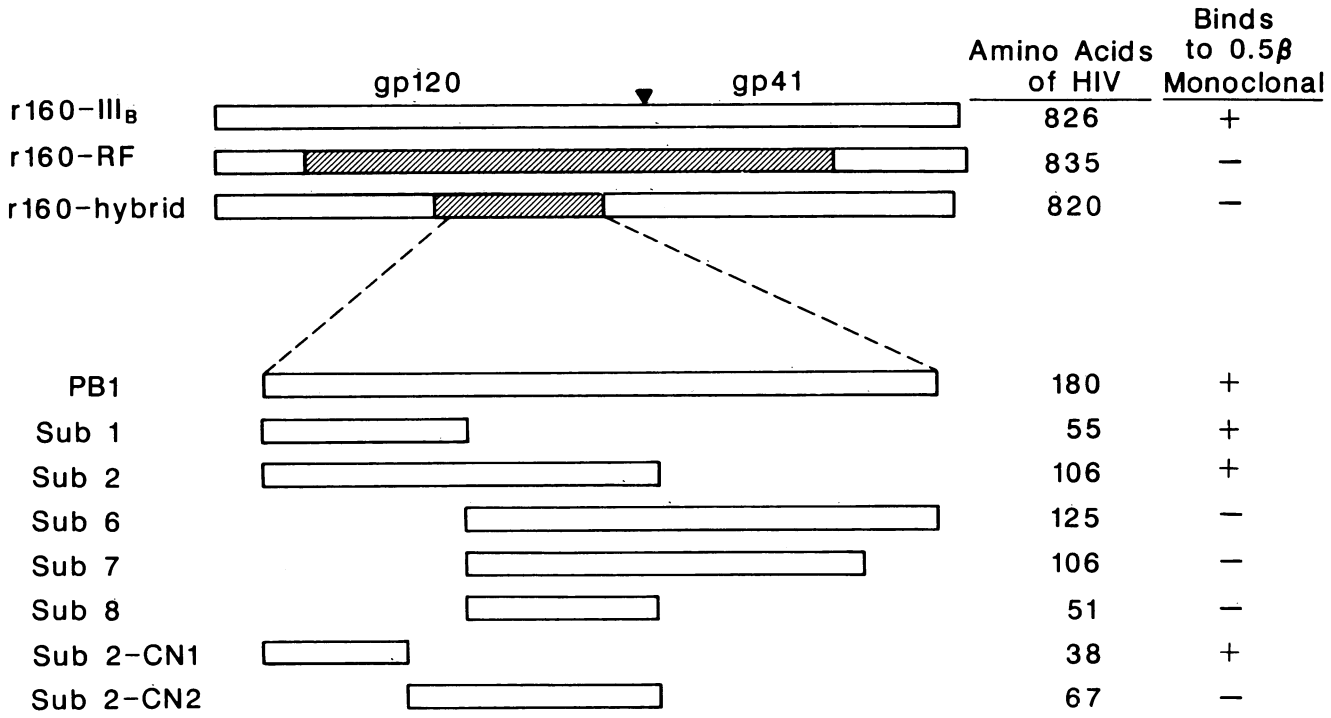

FIG. 5. Location of PB1 and recombinant subfragments within gp120.

The second assay measures neutralization of free virus. Because neutralizing antibodies elicited by purified gp120 or by recombinant gp120 fragments show HIV type-specific neutralization (14, 18, 27), the cross-isolate neutralizing activity of 0.5β was examined. In addition to HTLV-III_B, the HTLV-III_{MN} (MN) and HTLV-III_{RF} (RF) isolates, which differ by 18 and 21% from HTLV-III_B, respectively, in the amino acid sequence of gp120 (25; C. Gurgo et al., Virology, in press), were used as cell-free virions to infect H9 cells. After 5 to 6 days in culture, neutralization of infection was determined by measuring virus expression, using an indirect immunofluorescence assay with a monoclonal antibody to p24 (20). Table 1 shows that 0.5β neutralizes HTLV-III_B infection at a concentration of 100 ng of purified IgG per ml. Similarly, HTLV-III_B infection is completely blocked by a 1:6,250 dilution of 0.5β ascites. Neutralization of the RF or MN isolates is not observed.

Binding of 0.5β to a 24-amino-acid-long peptide. To explore the basis of the type-specific neutralization, the ability of 0.5β to bind purified recombinant gp160 (r160) and a gp120 fragment from several HIV isolates (HTLV-III_B, RF, MN, SC, and WMJ-2) (6, 25; Gurgo et al., in press) was assayed. On Western blots, 0.5β binds strongly to PB1-III_B and to r160-III_B produced by a recombinant baculovirus in insect cells (23) (Fig. 4A and 5). In contrast, the antibody binds only weakly to PB1 from the MN isolate and binding was undetectable to PB1-RF, SC, or WMJ-2 or to r160-RF and the r160 hybrid in which the PB1 region of r160-III_B is substituted with analogous RF sequences. The inability of 0.5β to neutralize RF and MN can be explained by its lesser reactivity to the envelopes of these isolates.

The binding site of 0.5β was mapped by using a series of purified recombinant fragments of PB1-III_B, cyanogen bromide fragments thereof, and chemically synthesized pep-

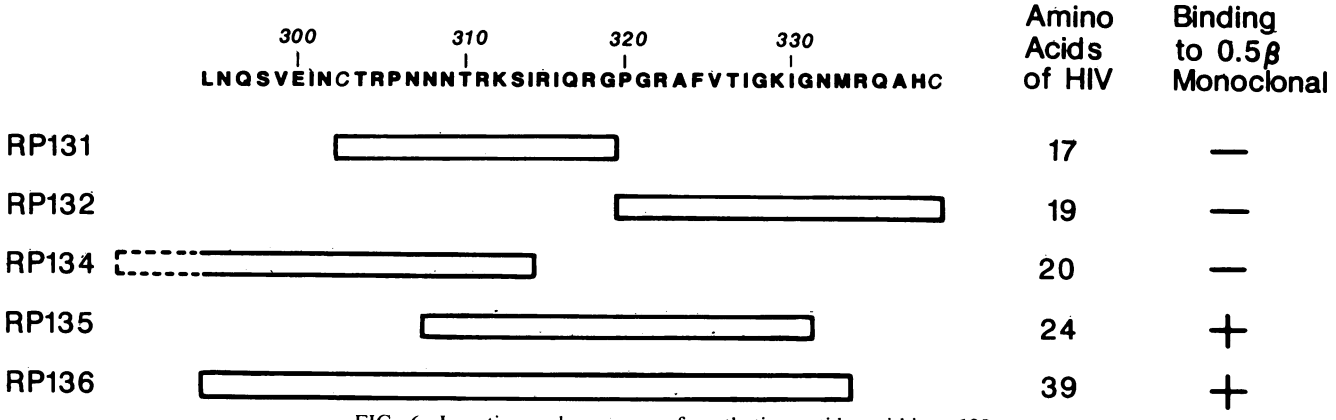

FIG. 6. Location and sequence of synthetic peptides within gp120.

TABLE 2. Sequence variability of RP135

Isolate	Sequence[a]
HTLV-III$_B$ (BH10)	C*TRPNNNTRKSIRIQRGPGRAFVTIGKI GNMRQAHC*
RF (HAT3)	C---------- *TKGPG--VI--AT--Q---I*---D-----K-----C
MN	C--------*Y-K*----- -----*HIGPG*---------*TKN---I*----T----------C

[a] The sequence RP135 is italicized. The cysteines, conserved among all HIV-1 isolates, and the Gly-Pro-Gly, conserved among most isolates, are underlined.

tides (Fig. 5 and 6). On Western blots, the antibody binds PB1-Sub 1, Sub 2, and the amino-terminal half of Sub 2 which is generated by cyanogen bromide (Fig. 4B). 0.5β also binds synthetic peptides RP 135 and PR 136 but not peptides RP 131, 132, and 134 (Fig. 4C). RP 135 contains 24 amino acids and is from a highly variable region of gp120 (25).

DISCUSSION

These results show that infections by both free and cell-associated viruses are prevented by a monoclonal antibody directed to gp120. The binding site of this antibody, and hence at least one neutralization and syncytium formation-inhibiting epitope, has been mapped to 24 amino acids. Nucleotide sequencing of gp120 from several HIV isolates reveals amino acid sequence variability, and the binding site of 0.5β, RP135, is from a highly variable region. This region has also been shown to block the majority of HIV-1 type-specific fusion-inhibiting antibody elicited by PB1, gp120, gp160, or infectious virus (J. R. Rusche et al., Proc. Natl. Acad. Sci. USA, in press).

Although the mechanism of neutralization or syncytium formation inhibition by 0.5β has not been elucidated, possible mechanisms are the prevention of the binding of gp120 to CD4 or prevention of steps subsequent to binding required for virus entry. Because of the high variability of the RP135 region and because the regions of gp120 that have been reported to interact with CD4 do not lie within RP135 (9, 11), it is unlikely that this portion of gp120 directly binds to CD4. It may be that binding of 0.5β, even though it does not bind directly to the CD4 binding domain, sterically interferes with a critical envelope-CD4 interaction. These questions regarding the mechanism of HIV neutralization and cell fusion can now be probed by using this antibody.

HIV neutralizing and fusion-inhibiting antibodies are elicited by RP135 and other synthetic peptides from this region (8; Rusche et al., in press). In other studies, it has been shown that sera from HIV-1-infected humans contains antibodies that recognize RP135 (Y. Devash, J. Drummond, D. Waters, and S. Putney, unpublished results). The fact that these peptides are deglycosylated and that 0.5β binds to enzymatically deglycosylated gp120 confirms earlier work that glycosylation is not required to elicit or bind neutralizing antibody. This strengthens the prospects for using deglycosolated proteins, such as those made in *E. coli*, as HIV vaccines.

The variability of the RP135 region has important implications for vaccine development. Analysis of the amino acid sequence of this region from numerous HIV sequences reveals that, in general, 30 to 50% of the amino acids in the RP135 region vary from one molecularly cloned isolate to another (6, 25). For example, there are 11 amino acid differences, including deletions, among HTLV-III$_B$ and RF and MN (Table 2). However, there are several conserved amino acids in this region that include flanking cysteines and an internal Gly-Pro-Gly. The cysteines are conserved among all HIV-1 envelope sequences elucidated to date, and the Gly-Pro-Gly residues are conserved among the large majority. It is possible that the flanking cysteines are linked via a disulfide bond and that the RP135 region protrudes and is accessible to bind neutralizing antibody.

The data show that 0.5β binds HIV-infected cells with high affinity and specificity. This property may be exploited in HIV therapy development. Even though binding of 0.5β to the envelope sequence is type specific, this will allow initial studies to begin to test the feasibility of targeting conjugated toxins or radiolabels to infected cells.

It is not yet known what role neutralizing antibodies may play in prevention of HIV infection, and these results imply that an effective HIV envelope subunit vaccine, capable of eliciting broadly reactive neutralizing and fusion-inhibiting antibodies, may need to be composed of antigens from a mixture of viral isolates. Although the RP135 region appears to contain the principal neutralizing epitope, other epitopes, with more conserved amino acid sequences, involved in virus neutralization or other potentially protective immune responses, such as antibody-dependent cellular cytotoxicity, may be present on the envelope. For example, neutralizing epitopes on gp41 have been reported (4, 8), and this is a more conserved portion of the envelope.

These results also show that antibody binding at a single site on the envelope will prevent infection by both cell-free virus and cell fusion of HIV-infected cells. It is not known to what extent HIV infection involves cell-free or cell-associated virus, and a vaccine may have to protect against both infectious routes. Further delineation of this and other epitopes may define the components of a polyvalent envelope-based subunit vaccine capable of eliciting protective immunity to multiple HIV types.

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