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Human Immunodeficiency Virus Type 1-Neutralizing Monoclonal Antibodies Which React with p17 Core Protein: Characterization and Epitope Mapping

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Monoclonal antibodies (MAbs) to human immunodeficiency virus type 1 were produced. Two antibodies reacted with the 17-kilodalton core protein (p17) of the virus and with its polyprotein precursor. To various degrees, each MAb neutralized infection by the cell-free virus. With a series of sequential overlapping hexapeptides which represent the p17 gene product, the epitopes identified by the MAbs were defined. The epitopes localize to overlapping regions near the amino terminus of the protein. Soluble synthetic peptides which span the antibody-binding sites of interest were demonstrated to competitively inhibit the reactivity of p17 MAbs, thus confirming the location of virus-neutralizing sites within the core protein.

Human immunodeficiency virus type 1 (HIV-1) is tropic for cells that express the CD4 molecule and is the etiologic agent associated with acquired immunodeficiency syndrome. In vitro infection by HIV-1 can be blocked by antibodies obtained from sera of infected individuals (7, 17, 26), although a precise relationship between antibody titers and disease course is not apparent.

Several reports have also demonstrated that HIV-1-neutralizing antibodies to various immunogens, including glycoprotein extracts (10), recombinant proteins (8, 9), and synthetic peptides (6, 7, 13), can be developed. These antibodies react with HIV-1 *env* gene products, indicating the role of viral surface glycoproteins and cell receptor interactions. In addition, antibodies to the CD4 molecule are capable of inhibiting virus-binding activity, as are anti-idiotypic reagents to these antibodies (2).

Although HIV-1 envelope glycoproteins are of strong interest with respect to virus inhibition, recent information indicates that *gag*-encoded proteins participate as a target for the neutralizing immune response. For example, it has been demonstrated that core protein p17 is modified at the posttranslational level to express an amino-terminal myristic acid group (23), thus increasing the hydrophobicity of the molecule. Also, antibodies to the thymic hormone thymosin α1 appear to inhibit HIV-1 infection in vitro (22). Since amino acid sequence homology occurs between that hormone and the carboxy-terminal region of p17 (12, 22), the role of p17 in immune recognition and virus neutralization warrants further examination.

The present study was initiated to define the characteristics of monoclonal antibodies (MAbs) to HIV-1 which demonstrated reactivity to core protein p17, to define their virus-neutralizing capacity, and to map the immunoreactive epitopes of interest.

MATERIALS AND METHODS

MAb production. HIV-1 was purified from the supernatant of a producer cell line as described previously (16). Virions were disrupted in detergent-high salt (0.5% Triton X-100, 0.6 M KCl) under sonication and then ether extracted to remove the detergent. Female BALB/c mice were primed with 100 μg of viral protein emulsified in complete Freund adjuvant and boosted at monthly intervals with 50 μg of protein in incomplete adjuvant. At 3 days after boost 4, the spleens were obtained for cell fusion. The fusion protocol, using Sp2/0-Ag14 myeloma cells in the presence of 50% polyethylene glycol, was as described previously (14).

Immunologic assays. Western blotting (WB; immunoblotting) was performed essentially as described before (16), with antigen strips provided by DuPont, Biotechnology Systems Division, Wilmington, Del. Briefly, WB strips were incubated for 18 h at room temperature with MAbs at 10 μg/ml. Strips were washed in PBS-T (50 mM sodium phosphate [pH 7.2], 150 mM sodium chloride, 0.05% Tween 20) and then allowed to react with biotin-labeled goat antibody to murine immunoglobulin G (IgG; Jackson Laboratory, West Grove, Pa.) for 1 h at 37°C. After the strips were washed, streptavidin-peroxidase conjugate (Jackson Laboratory) was applied for 0.5 h at 37°C. Washed strips were developed in a solution containing phosphate-buffered saline–0.01% 4-chloro-1-naphthol–0.03% hydrogen peroxide.

Solid-phase synthetic peptides were examined for immunoreactivity by enzyme immunoassay (EIA). Polyethylene pins with peptides on their surfaces (see below) were countercoated in EIA buffer (phosphate-buffered saline, 1% ovalbumin, 1% bovine serum albumin, 0.1% Tween 20) for 18 h at 4°C. After being washed with PBS-T (four times for 10 min each time), the pins were incubated in microplates containing a MAb or a control antibody, each at 10 μg/ml, for 18 h at 4°C. After the pins were washed as described above, incubation was allowed to proceed for 1 h in enzyme-conjugated anti-immunoglobulin (anti-murine IgG-peroxidase; Jackson Laboratory). The washed pins were next immersed in wells containing ABTS substrate solution (azino-bis-3-ethylbenzthiazoline-6-sulfonic acid [0.5 mg/ml] in pH 4.0 citrate buffer–0.03% hydrogen peroxide). Reactions were stopped after 30 min by removal of the pins, and A_{450} measurements were taken with a microplate reader.

Competitive inhibition experiments were performed with soluble synthetic peptides dissolved in PBS-T. The solid-phase target antigen was 96-well microplates coated with HIV-1 (5 μg/ml) for 18 h at 4°C. For competition analysis,

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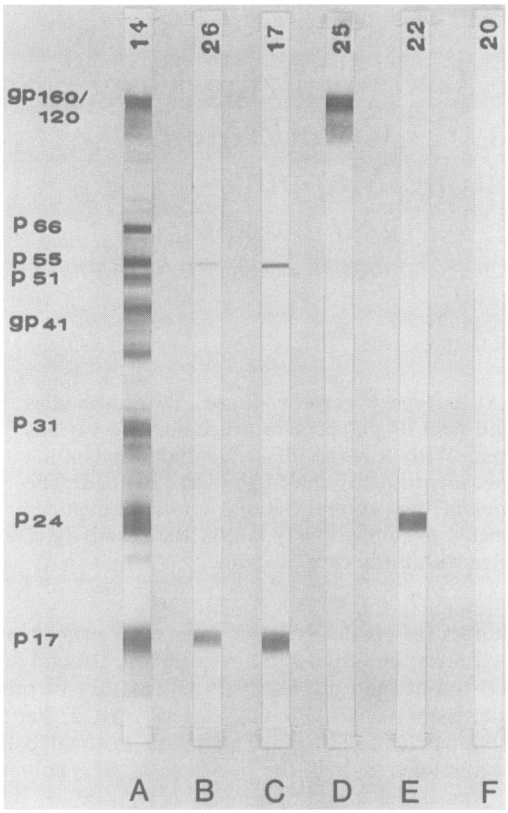

FIG. 1. Reactivity of HIV-1 with MAbs as determined by WB analysis. Lanes: A, HIV-1-seropositive human serum H-731 diluted 1/1,000; B, anti-p17 MAb, clone 32/5.8.42; C, anti-p17 MAb, clone 32/1.24.89; D, anti-gp160 MAb, clone 10E9; E, anti-p24 MAb, clone 32/5.17.76; F, control IgG subclass MAb, clone F5 (anti-prostate antibody, reference 15).

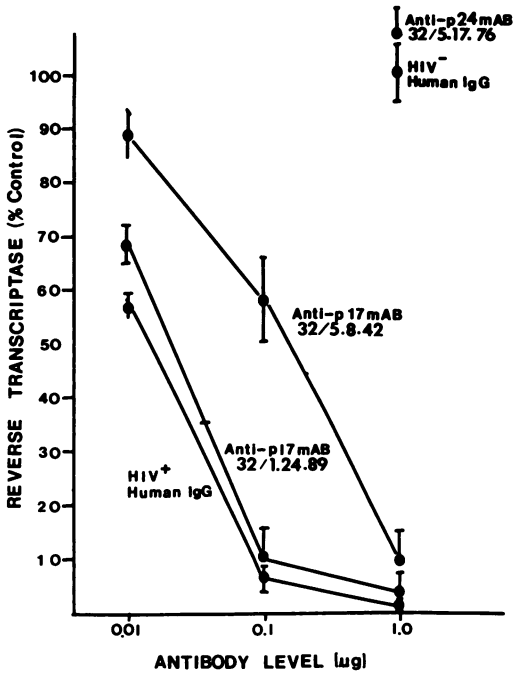

FIG. 2. Inhibition of cell-free HIV-1 infectivity of HUT-102 cells by anti-p17 MAbs. HUT-102 cells were challenged with 10^3 50% tissue culture infective doses of HIV-1 in the presence of antibodies as described in Materials and Methods. Reverse transcriptase measurements were taken at 10 days postinfection. Abscissa, Concentrations of control or experimental antibodies; ordinate, reverse transcriptase activity (mean of triplicates) as a percentage of positive control cultures (virus only, no inhibitor). HIV^+, IgG fraction of seropositive human serum; HIV^-, IgG fraction of seronegative human serum. The results shown are the mean and the standard error of the mean of two separate experiments. The control reverse transcriptase levels were 2.6×10^6 (experiment 1) and 1.6×10^6 (experiment 2) cpm.

various concentrations of synthetic peptides were allowed to react within HIV-1-coated microwells in the presence of biotin-labeled MAbs for 90 min at 37°C. Biotin derivitization was performed with *N*-hydroxysuccinimide-*d*-biotin (Calbiochem-Behring, La Jolla, Calif.) (5). The concentration of biotin-MAb chosen corresponded to about 40% of the maximal binding activity. After aspiration of the probe-inhibitor mixture, the wells were washed five times with PBS-T, and streptavidin-peroxidase (Jackson Laboratory) was added for a further 30 min. Thereafter, washed wells received ABTS substrate solution, and absorbance was monitored as described above. Specific inhibition was calculated by the following formula: % specific inhibition = $[(A_{max} - A_x)/(A_{max} - A_{min})] \times 100$, where A_{max} is maximal absorbance in the presence of buffer, A_{min} is minimal (background) absorbance in the presence of a specific inhibitor (10 μg of a homologous, unlabeled MAb per ml), and A_x is absorbance in the presence of the test peptide.

Epitope scanning. The strategy used for epitope scanning consisted of the construction of sequential, overlapping hexapeptides which completely spanned the entire HIV-1 p17 amino acid sequence (4). Since the anti-p17 MAbs under study strongly reacted with the prototype human T-cell lymphotropic virus type IIIB strain of HIV-1, its published sequences were used to construct peptide homologs (18). Peptides were synthesized in situ on plastic pins which conform in configuration to a standard 96-well microplate by using reagents and a kit (Epitope Mapping Kit) provided by Cambridge Research Biochemicals, Inc., Valley Stream, N.Y. After stepwise solid-phase syntheses, the peptides were deprotected (20% piperidine in dimethyl formamide), washed, and air dried. Included in the experiment were concurrently synthesized peptide controls with known reactivity to available antisera. These peptides represented the sequences Pro-Leu-Ala-Gln and Gly-Leu-Ala-Gln. One of these peptides (Pro-Leu-Ala-Gln) is known to react with an antibody to sperm whale myoglobin, while the other is nonreactive but similar in structure. These EIA-testable peptides were included in each assay run.

Soluble synthetic peptides. Peptides were synthesized by the strategy of Merrifield (11) at Peninsula Laboratories, Inc., Belmont, Calif. The acid-labile, *tert*-butyloxycarbonyl group was used for temporary amino-terminal protection. Peptides were cleaved from the resin with HF-anisole (9:1) containing 2% ethanedithiol and purified by gel filtration (Sephadex G-25 in 0.1 M acetic acid), followed by reversed-phase high-performance liquid chromatography. The sequence of each peptide was confirmed by amino acid analysis.

HIV-1-neutralization assay. Serial dilutions of various MAbs or control antibodies were mixed with 10^3 50% tissue culture infective doses (25) of infectious HIV-1 and incubated in complete medium for 1 h at 4°C. One milliliter of the mixture was used to infect 10^6 permissive HUT-102 cells in the presence of Polybrene (2 μg/ml in RPMI 1640). After 1 h

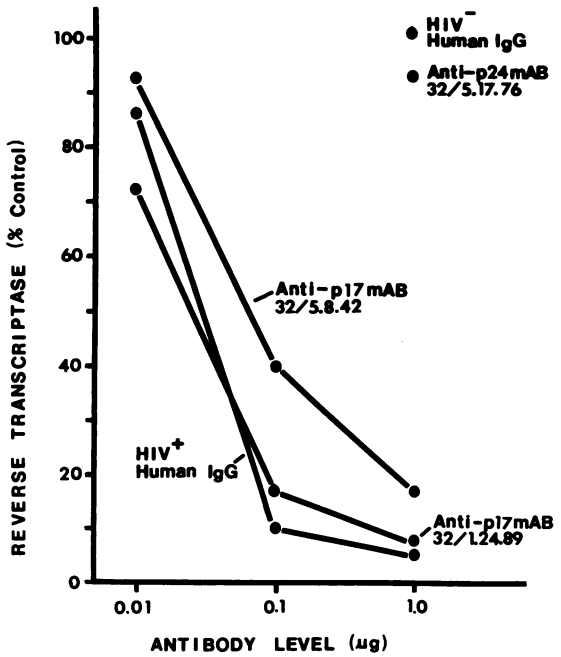

FIG. 3. Neutralization of cell-free HIV-1 infectivity of T lymphocytes by MAbs. Normal donor peripheral blood mononuclear cells were prestimulated with phytohemagglutinin–interleukin-2 as described in Materials and Methods, washed, and challenged with 10^3 50% tissue culture infective doses of HIV-1 in the presence of antibodies or control immunoglobulins. After 10 days, virus replication was determined by reverse transcriptase activity measurements. The same antibodies as for Fig. 2 were used. The control reverse transcriptase activity was 3.5×10^5 cpm.

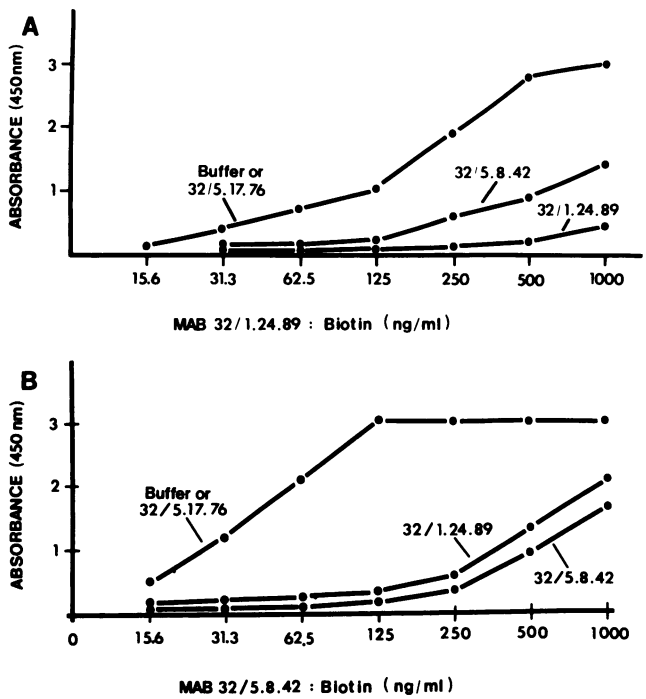

FIG. 4. Reciprocal competitive binding inhibition analysis of anti-p17 MAbs. Increasing concentrations of biotin-antibodies (abscissa) were allowed to react with solid-phase HIV-1 in the absence or presence of competing antibodies, each at 5 µg/ml. Thereafter, the reactions were developed with streptavidin-peroxidase and the substrate and the A_{450} was measured (ordinate). Results are expressed as optical density versus input level of a biotin-labeled MAb in the presence or absence of inhibitors. Panels: A, reactivity of biotin-labeled anti-p17 MAb, clone 32/1.24.89; B, reactivity of biotin-labeled anti-p17 antibody, clone 32/5.8.42. Buffer, No inhibitor; 32/5.17.76, anti-p24 MAb.

of incubation at 37°C, the cells were washed and placed in culture in complete medium containing RPMI 1640–10% fetal bovine serum–antibiotics. Virus replication was monitored at 10 days by measuring reverse transcriptase activity (19). Counts per minute were corrected for background levels of reverse transcriptase as constitutively released by the HUT-102 cells.

For some experiments, peripheral blood mononuclear cells prestimulated with phytohemagglutinin were used as the permissive cell substrate, as previously described (3). In brief, washed lymphocytes at 10^6 cells per ml were incubated for 3 days in the presence of 1 µg of PHA-P (Difco Laboratories, Detroit, Mich.). Thereafter, the washed cells were suspended in complete medium containing 10% interleukin-2 (Cellular Products, Inc., Buffalo, N.Y.) and 2 µg of Polybrene per ml. The lymphocyte cultures containing activated T cells were then used for virus transmission experiments.

RESULTS

Spleen cells from an animal immunized with HIV-1 lysate were subjected to cell fusion, and the resultant crude hybridoma cultures were screened for antibody activity by solid-phase EIA and WB. Cultures of interest were cloned, reassayed, and subcloned. Three cloned lines were studied in more detail; two hybridomas secreted anti-p17 antibodies (clones 32/5.8/42 and 32/1.24.89), and one produced an antibody reactive with p24 (clone 32/5.17.76), each of the IgG class. On WB examination (Fig. 1), the p17 MAbs bound to polyprotein precursor in addition to mature virus core protein. No cross-reactivity was observed with human T-cell lymphotropic virus type I-infected T cells.

To study the biological activity of the MAbs, HIV-1 neutralization assays were performed. Assays were performed with cell-free virus which was allowed to propagate in HUT-102 permissive cells. In addition to the anti-core MAbs, the neutralizing capacities of IgGs purified from seropositive (HIV^+ IgG) and seronegative (HIV^- IgG) donors were evaluated. MAb 32/5.17.76 (anti-p24) and HIV^- IgG failed to perturb the infectivity of the cell-free virus (Fig. 2). In contrast, submicrogram concentrations of HIV^+ IgG and MAb 32/1.24.89 were potent inhibitors of virus replication. The other anti-p17 antibody (MAb 32/5.8.42) also demonstrated a significant level of virus inhibition, although at higher input levels of immunoglobulin. Indistinguishable results were obtained when phytohemagglutinin-stimulated peripheral blood lymphocytes were used as the permissive cells (Fig. 3), indicating that antibody-mediated virus inhibition was a cell substrate-independent event.

To determine whether the two MAb reagents to p17 identify the same antigenic site on the core protein, antibody competition assays were run. The reactivity of biotin-conjugated MAb 32/1.24.89 versus solid-phase HIV-1 was undisturbed in the presence of 1,000-fold excess levels of the anti-p24 MAb (Fig. 4). In contrast, complete binding inhibition was observed with homologous, unlabeled antibody 32/1.24.89. It is interesting that anti-p17 MAb 32/5.8.42 also produced significant inhibition (about 80%), indicating that these antibodies reacted with sterically related epitopes of

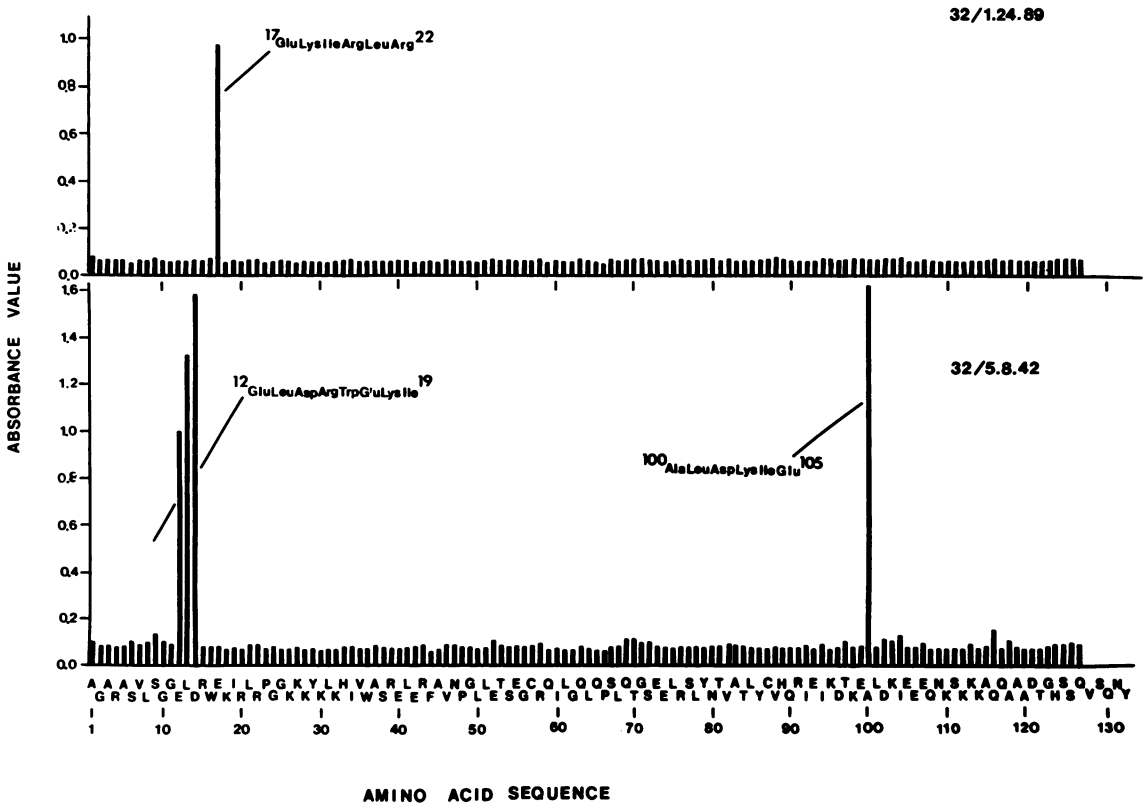

FIG. 5. Epitope mapping of MAbs to p17 by epitope scanning (Geysen technique). A series of sequential overlapping hexapeptides was synthesized in situ on solid-phase pins, as described in Materials and Methods. The peptide series corresponds to the entire HIV-1 p17 reading frame, beginning at the ATG (Met) start codon. The peptides were probed for immunoreactivity to anti-p17 MAbs (clones 32/5.8.42 and 32/1.24.89) at 10 μg/ml. The reactions were developed with biotin-labeled goat antibodies to murine IgG, followed by streptavidin-peroxidase and then the substrate. The results are expressed as optical density (ordinate) versus peptide number (abscissa). Control MAbs F5 (15) and 32/5.17.76 (anti-p24) demonstrated no reactivity to any peptide (data not shown).

the p17 molecule. Reciprocal-inhibition experiments (Fig. 4B) yielded similar data.

To define precisely the p17 epitopes of interest, epitope scanning was performed with a series of overlapping hexapeptides which completely spanned the HIV-1 p17 gene product. The solid-phase peptides were individually screened for reactivity to each p17 MAb by EIA (Fig. 5). The results clearly indicated that MAb 32/5.8.42 strongly bound to three adjacent peptides occupying the amino-terminal region of p17 and to a single hexapeptide much further downstream. MAb 32/1.24.89 produced a distinct pattern of binding (Fig. 5); that MAb strongly bound to a single peptide which partially overlapped the antigenic region recognized by MAb 32/5.8.42. No downstream binding was observed with MAb 32/1.24.89.

To confirm the data obtained from epitope scanning experiments, soluble peptides were synthesized which corresponded to the amino-terminal MAb 32/5.8.42-binding site (epitope A, residues 12 to 19), the MAb 32/1.24.89-binding site (epitope B, residues 17 to 22), and a region containing both binding sites (epitope A/B, residues 12 to 22); these synthetic peptides were termed SP-17-A, SP-17-B, and SP-17-A/B, respectively. Each soluble peptide was allowed to compete with solid-phase HIV-1 for binding of both MAbs. SP-17-A effectively inhibited the binding activity of MAb 32/5.8.42, exhibiting a 50% inhibitory dose of about 1 μg/ml (Fig. 6). This peptide was immunologically specific, inasmuch as no effect was noted on the reactivity of MAb 32/1.24.89. SP-17-B, corresponding to the binding site of MAb 32/1.24.89, was capable of inhibiting homologous antibody but only at very high concentrations (50% inhibitory dose, about 3.2×10^3 μg/ml), indicating a low-affinity interaction. However, the inhibition was immunologically specific. Further studied was a synthetic peptide which contained both MAb-binding sites. This peptide, SP-17-A/B, was a strong inhibitor of each anti-p17 antibody (Fig. 6). The 50% inhibitory dose versus MAb 32/5.8.42 was similar to that observed with SP-17-A (0.4 versus 1.06 μg/ml). In distinction, SP-7-A/B was almost 500 times more effective than SP-17-B in its capacity to compete with MAb 32/1.24.89 (50% inhibitory dose, 6.03 μg/ml versus 3.2×10^3 μg/ml). Of the three synthetic peptides studied, none demonstrated detectable inhibition of an irrelevant MAb (anti-HIV-1 p24, clone 32/5.17.76) at dose ranges of up to 10^3 μg/ml.

DISCUSSION

The *gag* gene of HIV-1 is expressed as a 55-kilodalton precursor protein which gives rise to at least three mature virus core proteins (18). Core protein p17 is the N-terminal product of the polyprotein precursor and itself is modified posttranslationally at its amino terminus to express a myristic acid moiety (23). This specific modification of the N-terminal *gag* protein has been shown for all human retroviruses (23) and likely imparts a significant function, such as integration within membrane components.

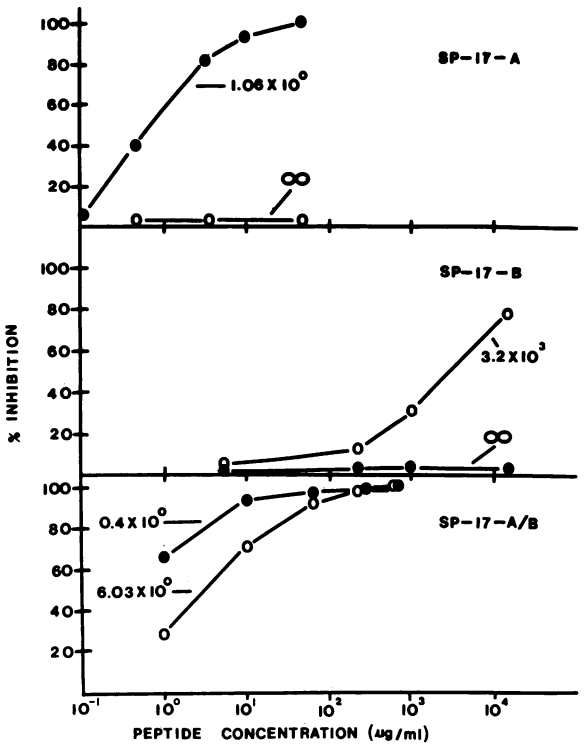

FIG. 6. Competitive inhibition of anti-p17 MAbs by soluble synthetic peptides. Peptides were synthesized which corresponded to the antibody-reactive epitopes identified by epitope scanning (Fig. 5). Increasing concentrations of soluble peptides (abscissa) were allowed to compete for the reaction of MAbs with the HIV-1 target antigen. The reactions were developed with biotin-avidin enzyme reagents and then the substrate. Panels: SP-17-A, synthetic peptide corresponding to the reactive site of anti-p17 MAb clone 32/5.8.42 (Glu-Leu-Asp-Arg-Trp-Glu-Lys-Ile); SP-17-B, synthetic peptide corresponding to the reactive site of anti-p17 MAb clone 32/1.24.89 (Glu-Lys-Ile-Arg-Leu-Arg); SP-17-A/B, synthetic peptide containing both of the antibody-reactive sites described above (Glu-Leu-Asp-Arg-Trp-Glu-Lys-Ile-Arg-Leu-Arg). Symbols: ●, MAb 32/5.8.42; ○, MAb 32/1.24.89. The concentrations of peptides which produced 50% inhibition of antibody-binding activity are indicated.

Of therapeutic interest, antisera to thymosin have been shown to neutralize the in vitro infectivity of HIV-1 (22), putatively because of cross-reactivity with p17. The cross-reactivity observed may be a result of the partial sequence homology between the two molecules (12), although a recent investigation failed to demonstrate immunological relatedness between p17 and thymosin α1 (20).

The present data clearly indicate that p17 represents a potential target for immunotherapy. Two distinct MAbs reactive with the core protein effectively neutralized the infectivity of cell-free HIV-1 in vitro. These effects were independent of the permissive cell type chosen and were quantitatively similar to the neutralization produced by the IgG fraction of high-titered seropositive serum. Although one antibody (MAb 32/5.8.42) demonstrated binding to a scanning peptide located within the thymosin α1-related domain, the predominant epitopes were mapped to regions far upstream of that domain. Also, MAb 32/1.24.89 failed to react with any regions of p17 associated with thymosin α1 homology. Thus, it appears that at least two disparate areas exist within the p17 sequence which are involved in antibody-mediated virus inhibition.

Two lines of evidence indicate that the MAb reagents under study recognize separate antigenic determinants. Antibody competition experiments demonstrated significant, but not quantitative, reciprocal cross-inhibition. This suggests the occurrence of sterically related antibody-binding sites. At the epitope-scanning level of resolution, similar data were obtained, indicating detection of sterically related, directly adjacent binding sites. This novel domain may represent a highly immunogenic portion of the p17 core protein and has been shown at the nucleic acid level to be highly conserved among several virus isolates which have been sequenced (1, 18, 21, 24).

The underlying mechanism of antibody-mediated virus inhibition remains to be determined. Previous investigations using antivirus antibodies have shown that certain domains of the gp120 virus surface glycoprotein are prominent targets for neutralization (8–10, 13). These antibodies, in addition to those reactive with binding sites of the CD4 receptor, effectively inhibit virus adsorption and subsequent penetration. Since p17 has not been shown to play a role in receptor binding, antibodies to that core protein are unlikely to prevent virus adsorption to CD4 and a distinct mechanism must exist. However, recent data indicate that antibodies to a conserved region of gp120 not involved in receptor binding also can exert HIV-1 neutralization without affecting virus binding to the CD4 receptor (6). It is conceivable that novel HIV-1 epitopes contribute to virus infection by interacting with undefined components of the T-cell membrane or the virus-processing machinery of the T cell (or both).

Yet to be defined is the role that neutralizing antibodies may play in prevention of HIV-1 infection and, relatedly, what antigens and epitopes must be present in a vaccine which will elicit effective immunity which is broadly reactive. Although the epitopes described here appear to be conserved in sequence, questions remain regarding their capacity to elicit fusion-inhibiting antibodies and antibody-dependent cellular cytotoxicity. Also of considerable interest, the present antibodies may be of value in generating anti-idiotypic reagents which may represent immunogens more effective than short peptides.

ACKNOWLEDGMENTS

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