Neutralizing Human Monoclonal Antibodies to Conformational Epitopes of Human T-Cell Lymphotropic Virus Type 1 and 2 gp46

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Ten human monoclonal antibodies derived from peripheral B cells of a patient with human T-cell lymphotropic virus (HTLV)-associated myelopathy are described. One monoclonal antibody recognized a linear epitope within the carboxy-terminal 43 amino acids of HTLV gp21, and two monoclonal antibodies recognized linear epitopes within HTLV type 1 (HTLV-1) gp46. The remaining seven monoclonal antibodies recognized denaturation-sensitive epitopes within HTLV-1 gp46 that were expressed on the surfaces of infected cells. Two of these antibodies also bound to viable HTLV-2 infected cells and immunoprecipitated HTLV-2 gp46. Virus neutralization was determined by syncytium inhibition assays. Eight monoclonal antibodies, including all seven that recognized denaturation-sensitive epitopes within HTLV-1 gp46, possessed significant virus neutralization activity. By competitive inhibition analysis it was determined that these antibodies recognized at least four distinct conformational epitopes within HTLV-1 gp46. These findings indicate the importance of conformational epitopes within HTLV-1 gp46 in mediating a neutralizing antibody response to HTLV infection.

Human T-cell lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) are members of the primate T-cell lymphoma/leukemia viruses. HTLV-1 causes adult T-cell leukemia (reviewed in reference 6) and a neurological disorder, HTLV-associated myelopathy or tropical spastic paraparesis (HAM/TSP) (21). HTLV-2 was originally isolated from a patient with atypical hairy-cell leukemia, and infected individuals with chronic myelopathy have been identified (7, 28, 29). At the molecular level, HTLV-1 and HTLV-2 have similar genomic organizations and are approximately 60% homologous at the nucleotide level. Both viruses are distributed worldwide but exhibit relatively little interisolate variation (22). Variants of HTLV-1 are commonly found in many old-world primates (57, 62), and HTLV-1 infection has been successfully transmitted in the laboratory to rabbits and rats (40, 63).

The involvement of HTLV-1 envelope glycoproteins in eliciting a protective immune response is well established (8, 33, 60). Multiple linear epitopes within both the surface (gp46) and transmembrane (gp21) glycoproteins of HTLV-1 and HTLV-2 have been identified (reviewed in reference 23), and several of these epitopes induce type-specific neutralizing antibodies in immunized animals (2, 48, 65, 66, 68). However, there is little information about the role of conformational epitopes within HTLV-1 or HTLV-2 gp46 in virus neutralization. Nor have any monoclonal antibodies (MAbs) recognizing conformational epitopes in HTLV-1 gp46 been reported. The importance of conformational epitopes in eliciting a protective immune response is suggested by the following. First, antibodies to conformational epitopes are a dominant component of the neutralizing antibody response for other retroviruses, most

notably human immunodeficiency virus (HIV) (41, 42). Similar observations have been reported for bovine leukemia virus (BLV), which is genetically much more closely related to HTLV-1. Studies of murine monoclonal antibodies (mMAbs) recognizing BLV gp58 have indicated that three antibodies capable of virus neutralization all recognized conformational epitopes (4, 56). Second, it is known that HTLV-1 is transmitted by cell-to-cell-mediated mechanisms involving the Env proteins (6), which possess a complex three-dimensional structure. Either the introduction of random mutations or the deletion of one or more of the five glycosylation sites is sufficient to prevent correct processing and cell surface expression of HTLV-1 gp46 and gp21 (53, 54). These observations strongly suggest that a significant fraction of the antibody responses of infected individuals should be to epitopes present only on correctly folded Env proteins expressed on the cell surface. Third, the existence of a significant antibody response to conformational epitopes is suggested by experiments demonstrating that incubation of HTLV antisera with synthetic peptides encoding the major linear neutralization epitopes of HTLV-1 gp46 fails to reverse antibody-mediated syncytium inhibition (14, 16, 34). Thus, a significant fraction of the neutralizing antibody response to HTLV-1 infection is directed at epitopes that have not yet been characterized.

To better define the role of antibodies to conformational epitopes in HTLV-1 infection, we produced and characterized a panel of human monoclonal antibodies (HMAbs) from peripheral B cells from a patient with HAM/TSP. Ten HMAbs were produced, and their reactivities with HTLV proteins were characterized by Western blotting, immunoprecipitation, and immunofluorescence. Three HMAbs recognized HTLV-1 gp46 or HTLV-1 gp21 according to Western blot analysis, and seven HMAbs recognized denaturation-sensitive epitopes within HTLV-1 gp46. Eight HMAbs exhibited significant virus neutralization activity in a syncytium inhibition assay. By compet-

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FIG. 1. Western blot analysis of representative HTLV-1 HMAbs. The indicated HMAbs at a concentration of 1 (6C2), 5 (PRH-8), or 10 μ g/ml (all others) were incubated overnight with Western blots containing extracts prepared from HTLV-1-infected (lanes MT-2 and HUT), HTLV-2-infected (lanes MoT), and control cell lines (lanes UNINF). R04 is an HMAb that recognizes a 64-kDa protein of cytomegalovirus. Western blots were prepared and bound antibody was detected as described in Materials and Methods.

itive inhibition analysis four conformational epitopes were defined. One epitope was present in both HTLV-1 and HTLV-2 gp46, and three epitopes were specifically present in HTLV-1 gp46. The implications of these findings for the development of an HTLV-1 vaccine and for the structural analysis of HTLV-1 Env proteins are discussed.

MATERIALS AND METHODS

Cell lines. The HTLV-1-infected cell lines HUT-102 (55) and MT-2 (38), the HTLV-2-infected cell line MoT (7), the human T-cell line SUP T1, and the human osteosarcoma (HOS) (43) cells were grown in RPMI media (Life Technologies, Bethesda, Md.) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (growth medium). The human lymphoid T-cell line RPMI-8402 (31) was grown in Iscove's modified Dulbecco's medium supplemented with 10% FCS, 2 mM glutamine, 0.4 mM sodium pyruvate, 0.24 IU of bovine insulin per ml and 1 mM *cis*-oxaloacetic acid. Heteromyloma cell lines K₆H₆B5 and H73C11, used in the production of HMAbs, were cultured as described previously (51, 52).

Generation and identification of HMAbs. Human B-cell hybridomas secreting specific antibodies were derived from peripheral B cells isolated from a patient with HAM/TSP. Human hybridomas were produced via the electrofusion of B cells, either immediately upon isolation (HMAb PRH-3) or after *in vitro* activation with Epstein-Barr virus (EBV; all other HMAbs), to heteromyloma cells by methods described previously (18, 51, 52). Immunoglobulin G (IgG) levels in culture supernatants were determined by an enzyme immunoassay (EIA) (17) and ranged between 12 and over 100 μ g of IgG/ml. IgG subtypes of the HMAbs were determined with a commercially available kit (The Binding Site Inc., San Diego, Calif.).

HTLV-1-specific antibodies were detected with an immunofluorescence assay (IFA). HTLV-1-infected cells (MT-2) or HTLV-2-infected cells (MoT) were combined with uninfected cells (RPMI-8402) at a ratio of one to three and were fixed onto HTC Super Cured 24-spot slides (Cel-Line Associates, Newfield, N.J.) with 100% acetone for 10 min at room temperature (RT). Fixed cells were incubated with undiluted culture media from EBV-activated B cells or hybridomas for 30 min at 37°C and washed for 5 min with phosphate-buffered saline (PBS), pH 7.4. Slides were then incubated for 30 min at 37°C with a 0.001% solution of Evan's blue counterstain and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Zymed, South San Francisco, Calif.). Bound antibody was revealed by fluorescence microscopy.

Live-cell immunofluorescence assay. Various dilutions of test antibody in 100 μ l of staining solution (PBS plus 1% FCS and 0.1% sodium azide) were combined with 10^6 viable HTLV-1-infected or control cells, suspended in 100 μ l of staining solution, and incubated at 4°C for 45 min. After an additional 800 μ l to 3 ml of staining solution was added, the cells were pelleted by centrifugation for 10 min at $500 \times g$ at RT and resuspended in 100 μ l of FITC-conjugated goat anti-human IgG diluted in staining solution. After 45 min at 4°C, 900 μ l to 3 ml of staining solution was added and the cells were pelleted as described above. The cells were then suspended in 1 ml of fixative solution (3.8% formaldehyde in PBS), and the amount of HMAb bound to the surfaces of cells was analyzed on either a FACS Vantage flow cytometer (Becton-Dickinson, San Jose, Calif.) or an EPICS Profile II flow cytometer (Coulter Corporation, Miami, Fla.).

Purification and biotinylation of antibodies. Tissue culture supernatants from secreting hybridomas were pooled and purified with protein G-Sepharose (HMAb PRH-21) or with protein A-Sepharose CL-4B (all other HMAbs) as described previously (27). Purified antibodies were biotinylated with a commercially available kit (ECL; Amersham, Arlington Heights, Ill.) or as follows. Approximately 1 to 2 mg of purified antibody was dialyzed into 4 liters of borate buffer (100 mM sodium borate, pH 8.8), followed by incubation with 40 μ l of biotin-C₁₁-N-hydroxysuccinimide ester (Amersham) per mg of antibody for 4 h at RT. Unreacted biotin was then quenched by the addition of 20 μ l of 1 M

NH₄Cl and subsequently removed by dialysis for 24 to 48 h against 4 liters of PBS, pH 7.4, with one change of buffer. Biotinylated antibodies were aliquoted and stored at -20°C until use. The IgG concentration of purified biotinylated antibody was determined by an IgG assay, as described above. The appropriate dilution of biotinylated antibody to be used in competition assays was determined in titration experiments and was between 0.8 and 2 μ g/ml for all biotinylated antibodies.

Western blotting. HMAbs were tested against whole-cell lysates of HTLV-1 (HUT-102 and MT-2), HTLV-2 (MoT), and uninfected (RPMI-8402) cell lines. Extracts were prepared by washing cultured cells with PBS and resuspending the cells in lysis buffer (150 mM NaCl, 20 mM Tris [pH 7.5], 0.5% deoxycholate, 1.0% Nonidet P-40, 1 mM EDTA) to which the protease inhibitors Pefabloc (Boehringer Mannheim, Indianapolis, Ind.), aprotinin, leupeptin, and pepstatin were added to final concentrations of 0.5 mg/ml, 2 μ g/ml, 2 μ g/ml, and 1 μ g/ml, respectively. Fifty microliters of lysis buffer was added for every 10^6 cells harvested. Nuclei were then pelleted by centrifugation at $18,000 \times g$ at 4°C for 10 min, and the supernatant was either used directly or stored at 4°C for not more than 4 days prior to use.

For Western blot analysis, 10 μ l of lysis buffer extract was combined with 10 μ l of 2 $\times$ sodium dodecyl sulfate (SDS) sample buffer (20% glycerol, 10% β -mercaptoethanol, 4.8% SDS, 0.125 M Tris [pH 6.8] [58]), heated to 95°C for 5 min, and fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (35) employing 12% polyacrylamide gels. The fractionated proteins were then electrotransferred to nitrocellulose and incubated overnight with purified antibody or HMAb-containing culture media diluted to an IgG concentration of 1 to 10 μ g/ml in BLOTTO (2.5% nonfat dry milk, 2.5% normal goat serum, 0.1% Tween-20 [Sigma, St. Louis, Mo.], 0.02% sodium azide in Tris-buffered saline [TBS; 150 mM NaCl, 20 mM Tris, pH 7.5]). The blots were washed and then incubated with the appropriate alkaline phosphatase-conjugated secondary antibody, washed again, and incubated with substrate as described previously (24, 25).

Immunoprecipitation. For immunoprecipitation experiments, either 1 or 5 μ l of serum or 1 μ g of HMAb was adsorbed to 100 μ l of a 10% solution of protein A-Sepharose CL-4B (Pharmacia, Piscataway, N.J.) in lysis buffer for 30 min at RT. The protein A beads were washed one time with 1 ml of lysis buffer, resuspended in 50 μ l of lysis buffer, and combined with 50 μ l of extract from HTLV-1-infected and uninfected cells, prepared as described above. The antibodies were incubated with extract for 2 h at 4°C with gentle agitation, at which point the protein A beads were pelleted by centrifugation for 10 min at $18,000 \times g$. After being washed two times with 1 ml of lysis buffer, the beads were resuspended in 50 μ l of 1 $\times$ SDS sample buffer, heated to 95°C for 5 min, and pelleted in a microcentrifuge. Then, 20 μ l of supernatant was fractionated via SDS-PAGE employing 12% polyacrylamide gels, followed by electrotransfer to nitrocellulose membranes. The resulting blots were incubated overnight with either HTLV-1 gp46 mAb clone 65/6C2.2.34 (abbreviated 6C2; obtained from Cellular Products, Buffalo, N.Y.) diluted to 1 μ g/ml in BLOTTO or biotinylated PRH-1 HMAb diluted 1/50 in BLOTTO. Blots incubated with 6C2 were subsequently washed three times with TBS and incubated for 1 h with goat anti-mouse alkaline phosphatase conjugate (Promega, Madison, Wis.), followed by incubation with alkaline phosphatase substrate, as described previously (24, 25). Blots incubated with biotinylated PRH-1 were incubated for 1 h at RT with streptavidin-conjugated horseradish peroxidase (Amersham) diluted 1/1,000 in PBS plus 0.1% Tween-20, followed by incubation with a 1-mg/ml solution of diaminobenzidine plus 0.1% hydrogen peroxide.

Epitope mapping. A λ gt11 library expressing 100- to 500-nucleotide fragments of MT-2 HTLV-1 was constructed as described previously (36) and screened with the PRH-21 HMAb by standard methods (36, 72). Immunoreactive clones were isolated, and their inserts were DNA sequenced with an automated sequencer (Applied Biosystems, Foster City, Calif.). The shortest of the clones, designated CC2-5-1, was expressed in a modified version of the pGEX vector (61, 70), as described previously (24). Clones expressing portions of the carboxy-terminal region of HTLV-1 gp46 (see Fig. 4) were constructed by designing PCR primers with either *Nco*I or *Bam*HI restriction sites at their 5' ends to amplify the indicated sequences of the MT-2 strain of HTLV-1. After PCR amplification under standard conditions (25 cycles of 94°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min to amplify 1 ng of plasmid pMT-2) the DNA fragments were cloned into the *Nco*I and *Bam*HI sites of a modified pGEX-2 vector, essentially as previously described (24, 25). For all HTLV-1 inserts expressed in the pGEX vector, Western blot analysis of recombinant protein expression and immunoreactivity was performed as described previously (24).

Neutralization assays. Syncytium inhibition assays were performed essentially as described previously (43). Briefly, various dilutions of antibodies were combined with 1.25×10^4 HUT-102 or MT-2 cells in duplicate wells of 96-well tissue culture plates. Control wells with nonrelevant antibodies or no added antibody were also prepared. Plates were incubated for 60 min at 37°C, followed by the addition of 1.25×10^4 HOS cells in growth medium, after which cells were incubated overnight at 37°C in 6% CO₂. Cells were gently washed with growth medium, followed by fixation of cells with methanol and staining with Giemsa stain (Life Technologies). Syncytia (cells with four or more nuclei) were counted with an inverted microscope at $\times 250$ magnification, counting four fields per well. The counts of the duplicate wells were averaged, and the percent inhibition for any HMAb was calculated as follows: (average number of syncytia in the absence

of antibody – average number of syncytia in the presence of antibody/the average number of syncytia in the absence of antibody) $\times$ 100.

HTLV syncytium inhibition assays were performed essentially as described previously (46). The SUP T1 cell line was infected with HIV primary isolate DV for 1 h at 37°C, after which cells were washed, and 2×10^4 cells were plated in individual wells of 96-well plates already containing media, HTLV HMAs, or a control antisense oligonucleotide. After 4 days in culture at 37°C syncytia were counted and the results from duplicate wells were averaged. An antisense oligonucleotide previously demonstrated to have potent syncytium-inhibiting activity was employed as a positive control (47). HTLV-1 and control HMAs were tested for activity at 20 μ g/ml and at serial twofold dilutions down to a concentration of 0.04 μ g/ml.

Competitive EIA. Antibody competition assays were performed in a manner similar to that described by Moore and Sodroski (42). Briefly, 100 ng of purified MAb diluted in 100 μ l of PBS was allowed to adsorb to microtiter plates (Maxisorp; Nalge Nunc International, Rochester, N.Y.) for 1 h at 37°C. After being washed one time with TBS, the plates were blocked by incubation for 1 h at RT with 150 μ l of BLOTTO/well. Plates were washed two times with TBS, followed by the addition of 100 μ l of extract from the HUT-102 or RPMI-8402 cell lines diluted 1:1 with BLOTTO. After incubation for 1 h at RT, plates were washed three times with TBS, followed by the addition of unlabeled antibodies at various concentrations in 50 μ l of BLOTTO to duplicate wells. Plates were incubated for 30 min, at which point biotinylated HTLV HMA diluted in BLOTTO was added. After 60 min at RT, the plates were washed three times with TBS, and 100 μ l of streptavidin-conjugated horseradish peroxidase (Amersham) diluted 1/1,000 in PBS plus 0.1% Tween-20 was added. After incubation for 1 h at RT, the plates were washed four times with TBS, followed by incubation with a 0.5-mg/ml solution of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) with 0.1% hydrogen peroxide. Substrate development was allowed to proceed for 15 to 30 min, and the absorbance of the wells at 405 nm was determined with a multiwell plate reader (Du Pont Co., Wilmington, Del.). Results obtained from duplicate wells were averaged.

RESULTS

Production and initial characterization of HMAs. Peripheral B cells were isolated from a patient with HAM/TSP and were either fused directly or EBV activated in microtiter plates. Wells containing HTLV-1 antibodies were identified by IFA with fixed MT-2 cells as targets. In initial testing, 175 of 360 cultures of EBV-activated cells were found to have anti-HTLV-1 reactivity, and cells derived from 44 of these cultures were fused the day after HTLV-reactive antibodies were detected. Both selected HTLV-reactive EBV-activated B cells and hybrids obtained from the fusions were maintained in culture for an extended period and retested for HTLV reactivity by IFA and Western blotting. Individual hybrids were selected for cloning based on both the reactivity observed in the assays and the amount of antibody secreted. Eleven hybridomas derived from six fusions were stabilized to secrete significant amounts of HTLV-reactive antibody. All of the

FIG. 2. Epitope mapping of HMA PRH-21. (A) Diagram indicating fragments of HTLV-1 gp21 expressed by the various recombinant proteins employed. The location of the transmembrane helix of gp21 is indicated (TM). The shaded portions at the 5' ends of clones p21E and 1A2B indicate sequences derived from HTLV-1 gp46 present in these clones. The numbering at the bottom is from the first amino acid of gp21. (B) Immunoreactivity of the PRH-21 HMA, serum from an HTLV-1-infected individual (H-1), and serum from an uninfected control (UNINF) with whole-cell lysates of *Escherichia coli* expressing the indicated recombinant HTLV-1 proteins. pGX refers to whole-cell lysate from nonrecombinant modified pGEX-2. HMA PRH-21 was tested at a concentration of 1 μ g/ml, and all sera were diluted 1/100 in BLOTTO. The migration of molecular mass standards is indicated at the left (in kilodaltons). Western blots were prepared and bound antibody was detected as described in Materials and Methods.

isolated HMAs strongly stained fixed HTLV-1-infected MT-2 cells but not control cell lines (data not shown).

Western blot analysis with HTLV-1 and HTLV-1 proteins showed that one HMA, WA05-D5, recognized HTLV-1 p24, and details of its characterization will be presented elsewhere (data not shown). Of the remaining 10 HMAs, PRH-1 recognized HTLV-1 gp46 and PRH-8 recognized HTLV-1 gp46 derived from MT-2 but not from HUT-102 cells (Fig. 1). The forms of HTLV-1 gp46 reactive with HMAs PRH-1 and PRH-8 are similar to those detected by previously described mMAb 6C2, which reacts with HTLV-1 and HTLV-2 gp46 (50). HMA PRH-21 was strongly reactive with both unprocessed HTLV-1 gp61 and HTLV-1 gp21 but not with HTLV-1 gp46. Western blotting indicated that none of these three HMAs detected HTLV-2 Env proteins. The remaining seven HMAs were all found to be negative for reactivity with HTLV-1 and HTLV-2 proteins by Western blotting. The results obtained with PRH-4 and PRH-7A are representative of the results obtained with Western blot-negative HMAs (Fig. 1).

Epitope mapping of Western blot-reactive HMAs. The epitopes recognized by HMAs reactive with Western-blotted HTLV-1 Env proteins were determined. Four independent clones were isolated by immunoscreening a λ gt11 epitope library with the HMA PRH-21. DNA sequencing of the clones indicated that all clones expressed the carboxy-terminal region of HTLV-1 gp21. The shortest of these clones, designated CC2-5-1, contained the carboxy-terminal 43 amino acids of HTLV-1 gp21 and was expressed in the pGEX vector. Western blot analysis (Fig. 2B) confirmed that HMA PRH-21 was highly reactive with recombinant protein CC2-5-1, with reactivity to both the full-length protein (at 30 kDa) and the multiple proteolytic breakdown products observed. Similar reactivity to both the full-length and some of the more prominent proteolytic breakdown products was observed with serum from an HTLV-1-infected individual. Attempts to reduce the amount of proteolytic digestion of CC2-5-1 were unsuccessful. No reactivity of PRH-21 with any of the other immunoreactive

FIG. 3. Epitope mapping of HMAbs PRH-1 and PRH-8. (A) Diagram indicating the fragments of HTLV-1 gp46 expressed by the various recombinant proteins employed. The locations of the amino-terminal (48) and central (3) neutralizing epitopes of HTLV-1 are indicated. The numbering at the bottom is from the initiating methionine of the Env precursor protein. (B) Immunoreactivities of the indicated MAbs and sera with whole-cell lysates of *Escherichia coli* expressing the indicated recombinant HTLV-1 proteins. H-I, serum from an HTLV-1-infected individual; UNINF, serum from an HTLV-negative individual; RM-01, mMAb to glutathione S-transferase; 6C2, mMAb to HTLV-1 gp46 (50). HTLV-1 recombinant proteins were constructed as described in Materials and Methods. pGX refers to whole-cell lysate from nonrecombinant pGEX-2 (61). The 6C2 and PRH-1 antibodies were tested at a concentration of 1 μ g/ml, and the PRH-8 antibody was tested at a concentration of 5 μ g/ml. Sera were tested at a dilution of 1/100, and mMAb RM-01 was tested at a dilution of 1/1,000. All antibodies were diluted in BLOTTO. The migration of molecular mass standards is indicated at the left (in kilodaltons). Western blots were prepared and bound antibody was detected as described in Materials and Methods.

regions of HTLV-1 gp21, including GD21 and BA21 (24), was observed.

Initial testing of both the PRH-1 and PRH-8 antibodies indicated that they were negative for reactivity with MTA-1, a previously described immunodominant epitope of HTLV-1 gp46 (amino acids 162 to 209) (37). Therefore, the regions between the carboxy terminal of MTA-1 and the gp46/gp21 cleavage site of HTLV-1 strain MT-2 were expressed as a series of five recombinant proteins. The regions expressed were based upon previous data obtained from the analysis of the reactivity of HTLV-1 synthetic peptides (reviewed in reference 23). Testing of the recombinants with an mMAb (RM-01) specific for the glutathione S-transferase portion of the HTLV-1 recombinant proteins confirmed that roughly equivalent amounts of each protein were produced (Fig. 3B). Western blot analysis of the recombinant proteins with serum from an HTLV-1-infected individual confirmed that four of the five constructs contained at least one immunoreactive epitope (Fig. 3B, panel H-I). Testing of HMAb PRH-1 with these recombinant proteins indicated that the binding site of PRH-1 was located between amino acids 260 and 294 of HTLV-1 gp46. The epitope recognized by PRH-8 was located between amino acids 210 and 312 of HTLV-1 gp46 but could not be further defined. This suggests that the PRH-8 epitope is located at or near amino acids 260 to 262, which were at the amino or carboxy terminals of several small constructs but which were not expressed in the center of any construct other than that of the full-length construct expressing amino acids 210 to 312. Finally, mMAb 6C2 recognized constructs expressing amino acids 210 to 312 and 210 to 236 but not 210 to 262. Although we cannot satisfactorily explain the failure of mMAb 6C2 to bind a construct expressing amino acids 210 to 262, the strong reactivity of 6C2 with a construct expressing amino acids 210 to 236 further defines the location of the epitope recognized by

this mMAb as being between amino acids 210 and 236 (50), which are sequences just 3' of the central immunodominant epitope.

Immunoprecipitation analysis and cell surface binding of HTLV-I HMAbs. The seven HMAbs found to be nonreactive by Western blotting were analyzed by immunoprecipitation with HTLV proteins. mMAb 6C2 was used to detect immunoprecipitated HTLV-1 and HTLV-2 gp46 (Fig. 4A). As seen in Fig. 1, mMAb 6C2 efficiently detected both HTLV-1 and HTLV-2 Env proteins present in cytoplasmic extracts of HUT-102, MT-2, and MoT cells (Fig. 4A, panel EXTR). mMAb 6C2 did not cross-react with proteins present in extracts from uninfected T-cell lines, and the antimurine secondary antibody had minimal cross-reactivity with human antibody heavy chains (Fig. 4A, lanes marked 0). Nor was any Env protein detected when extracts were immunoprecipitated with a control HMAb (Fig. 4A, panel R04). In agreement with the data obtained by Western blot analysis, HMAb PRH-8 immunoprecipitated Env proteins from extracts of MT-2 cells but not from HUT-102 cells or from HTLV-2-infected MoT cells. The Western blot-positive HMAb PRH-1 efficiently immunoprecipitated Env proteins from both MT-2 and HUT-102 cells but not from MoT cells (data not shown). Five of the Western blot-negative HMAbs, PRH-4, PRH-3, and PRH-11A, -11B, and -11C immunoprecipitated HTLV-1 gp46 but not HTLV-2 gp46. Immunoprecipitation of both the immature and processed forms of gp61 and gp46 was observed to varying degrees with all HMAbs. The pattern of HTLV-1 Env proteins immunoprecipitated was essentially the same when biotinylated HMAb PRH-1 was used to evaluate the immunoprecipitation of HTLV-1 Env proteins (data not shown).

The remaining two Western blot-negative HMAbs, PRH-7A and PRH-7B, also immunoprecipitated HTLV-1 Env proteins from both HUT-102 cells (Fig. 4B) and MT-2 cells (data not

FIG. 4. Immunoprecipitation of HTLV-1 HMABs. (A) Fifty microliters of lysis buffer extract from HTLV-1-infected cell lines HUT-102 (lanes HUT) and MT-2 (lanes MT2), from HTLV-2-infected cell line MoT (lanes MoT), or from uninfected T-cell line RPMI-8402 (lanes UNINF) was immunoprecipitated with the indicated HMABs. The immunoprecipitated proteins were subjected to SDS-PAGE and were Western blotted as described in Materials and Methods. Lanes marked 0 are immunoprecipitations done with 50 μ l of lysis buffer in place of 50 μ l of extract. The blot marked R04 shows results obtained when the indicated extracts were immunoprecipitated with an HMAb to cytomegalovirus. The blot marked EXTR contains 10 μ l of the indicated extracts electrophoresed and Western blotted without any prior immunoprecipitation. Immunoprecipitated and Western-blotted proteins were detected by overnight incubation with mAb 6C2 at a concentration of 1 μ g/ml in BLOTTO. The migration of molecular mass standards (in kilodaltons) is indicated at the left. (B) Immunoprecipitation of HTLV-2 gp46 by HMABs PRH-7A and PRH-7B. Fifty microliters of lysis buffer extract from cell lines HUT-102, MoT, or RPMI-8402 was immunoprecipitated with the indicated HMABs. The blots marked H-I, H-II, and UNINF show the results obtained when the indicated extracts were immunoprecipitated with serum from an HTLV-1-infected, an HTLV-2-infected, or an HTLV-negative individual, respectively. Immunoprecipitated proteins were detected by overnight incubation with mAb 6C2 at a concentration of 1 μ g/ml in BLOTTO. The blots and lanes are marked as described for Fig. 4A.

shown). PRH-7A and PRH-7B also immunoprecipitated HTLV-2 gp46. The amounts of HTLV-2 protein detected were comparable to the amount of HTLV-2 gp46 immunoprecipitated by 1 μ l of HTLV-1 antiserum (panel H-I). Cross-reactivity of an HTLV-2 antiserum with HTLV-1 Env protein was also observed (panel H-II). Thus, all nine HMABs that recognized gp46 could immunoprecipitate protein in a native state, including the seven HMABs that were Western blot negative. Two of the Western blot-negative HMABs recognized an epitope common to both HTLV-1 and HTLV-2 gp46.

The ability of the HTLV-1 HMABs to bind to the surfaces of HTLV-1- and HTLV-2-infected cells was determined. The results obtained with a majority of the HMABs are presented in Fig. 5. The Western blot-negative HMABs, PRH-3, PRH-4, PRH-7A, PRH-7B, PRH-11A, PRH-11B, and PRH-11C (data not shown) all displayed significant binding to the surfaces of both HTLV-1-infected HUT-102 cells (shaded profiles in the HUT-102 column) and MT-2 cells (data not shown), with no binding to cells from an uninfected T-cell line (open profiles). Similar results were obtained with the Western blot-positive HMAb PRH-1 (data not shown). These observations are characteristic of antibodies specific for epitopes present in native Env protein expressed on the surfaces of infected cells. HMAb PRH-21, in contrast, bound HTLV-infected and control cell lines at a low level that was indistinguishable from that of a control HMAb, suggesting that its epitopes are not accessible on the surfaces of infected cells. The HMAb PRH-8 exhibited significant binding to MT-2 cells and detectable but reduced binding to HUT-102 cells (data not shown), suggesting that it may have the ability to recognize HUT-102 Env protein, albeit at a vastly reduced level.

When binding of the HMABs to HTLV-2-infected MoT cells was examined, HMABs PRH-1, PRH-3, PRH-4, PRH-8, and PRH-21 were found to be nonreactive (Fig. 5 and data not shown). Confirming the results of the immunoprecipitation experiments, HMABs PRH-7A and PRH-7B bound strongly to HTLV-2-infected MoT cells. HMABs PRH-11A, PRH-11B (Fig. 5), and PRH-11C (data not shown) all had levels of immunofluorescence with live MoT cells that were slightly elevated relative to the fluorescence obtained with an uninfected T-cell line or with a control HMAb. This might suggest a low-level affinity of HMABs PRH-11A, -11B, and -11C for HTLV-2 gp46. However, these HMABs neither immunopre-

cipitated HTLV-2 gp46 (Fig. 4) nor showed appreciable immunofluorescence staining of fixed MoT cells (data not shown). It is more probable that the slight binding of background seen in the live-cell IFA reflects a weak and nonspecific association.

Neutralization activity of HTLV-1 gp46 HMABs. A titration of the syncytium inhibition activities of the seven Western blot-negative HMABs is shown in Fig. 6, and the results obtained with all HMABs are summarized in Table 1. HMABs PRH-7A and PRH-7B strongly inhibited syncytium formation, with 50% inhibition occurring at an antibody concentration of 0.5 μ g/ml and greater than 80% inhibition obtained at 2 μ g/ml. PRH-4 had weaker neutralizing activity, with a concentration of 5 μ g/ml being required for 80% inhibition. HMABs PRH-3, PRH-11A, PRH-11B, and PRH-11C exhibited similar weak syncytium inhibition activity, with 50% inhibition occurring at an antibody concentration of 10 μ g/ml and 80% inhibition of syncytium formation occurring at approximately 30 to 40 μ g/ml. Although this is a relatively high antibody concentration, no syncytium inhibition was observed with control antibodies at similar concentrations. Additionally, all of the HTLV-1 HMABs recognizing conformational epitopes inhibited MT-2 cell-mediated syncytium formation at similar concentrations (data not shown).

Multiple controls were performed to ensure that the syncytium inhibition activity of the HMABs was specific. Similar results were obtained with the HMABs in experiments that utilized HMAb-containing tissue culture supernatants and protein A-purified antibodies, indicating that the results were antibody dependent and not due to some contaminating protein present in hybridoma tissue culture supernatants. Additionally, purified preparations of HMABs PRH-7A, PRH-4, and PRH-11A, as well as of the Western blot-positive HMAb PRH-1 and a control HMAb, were tested for activity in an HIV syncytium

FIG. 5. Live-cell immunofluorescence with HTLV-1 HMABs. HMABs were combined with 10^6 HUT-102, MoT, or RPMI-8402 cells, and the amount of antibody binding to the cell surfaces was determined by flow cytometry as described in Materials and Methods. The HMABs tested are as follows: A, PRH-7A; B, PRH-7B; C, PRH-4; D, PRH-21; E, PRH-11A; F, PRH-11B; G, PRH-3; H, the control HMAB R04. All HMABs were tested at a concentration of $10 \mu\text{g/ml}$. The x axis is a logarithmic scale of increasing fluorescence intensity. The y axis indicates the number of cells. The fluorescence profiles obtained with the HMABs on HUT-102 or MoT cells are shaded, and the fluorescence profiles obtained with the same HMABs on uninfected RPMI-8402 cells are open.

assay. None of these antibodies had any inhibitory activity at concentrations of up to $20 \mu\text{g/ml}$ (data not shown). In contrast, an antisense oligonucleotide previously demonstrated to have potent inhibitory activity (47) completely inhibited HIV-mediated syncytium formation at concentrations of $1 \mu\text{M}$ and higher. Thus, all seven of the HMABs recognizing conformational epitopes possessed varying degrees of HTLV-1-specific neutralization activity.

Of the three Western blot-positive HTLV-I HMABs only PRH-8 showed significant neutralization activity, inhibiting 88% of MT-2 cell-dependent syncytium formation at a concentration of $22 \mu\text{g/ml}$. In agreement with data obtained in the assays described above, HMAB PRH-8 did not show significant inhibition of syncytium formation by HUT-102 cells (Table 1). HMAB PRH-1 did not exhibit significant neutralization activity on either HUT-102 or MT-2 cells (Table 1). Nor did HMAB PRH-21 exhibit any effect on HTLV-1-mediated syncytium formation, as would be expected for a HMAB that recognizes an epitope not present on the surfaces of infected cells.

Antibody competition analyses. Analysis of the above data suggests that the seven HMABs to conformational epitopes can be assigned to three different groups by their reactivities in the various assays. HMABs PRH-3, PRH-11A, PRH-11B, and

PRH-11C all have similar properties, including strong binding to viable HTLV-1-infected cells, immunoprecipitation of HTLV-1 but not HTLV-2 gp46, and a 50% inhibition titer for syncytium formation of $>10 \mu\text{g/ml}$. HMAB PRH-4 is HTLV-1 specific and has stronger neutralization activity. HMABs PRH-7A and PRH-7B recognize an epitope common to both HTLV-1 and HTLV-2, with strong neutralizing activity against HTLV-1-infected cells. However, it is possible that antibodies within the broad groups might recognize different epitopes (i.e., PRH-11A need not recognize the same amino acids as PRH-11B). Conversely, the differences in neutralization titers observed between the HMABs could be the result of two unique antibodies with different affinities for HTLV-1 gp46 recognizing the same or very similar epitopes.

To confirm the number of independent neutralizing epitopes isolated, competition assays evaluating the abilities of the various HMABs to bind HTLV-1 gp46 in the presence of other HMABs were performed. The assay involves the capture of HTLV-1 gp46 expressed by HUT-102 or MT-2 cells with the HMAB PRH-1. This is followed by the addition of a biotinylated test antibody in the presence and absence of competing antibody. The binding of the biotinylated test antibody is detected with streptavidin-conjugated horseradish peroxidase. To

FIG. 6. Syncytium inhibition assay of HTLV-1 HMABs. Various concentrations of the indicated HMABs were combined with HUT-102 and HOS cells, and the numbers of syncytia formed were determined. The percent inhibition was calculated as described in Materials and Methods. The points represent the means of at least two separate assays except for PRH-11A and PRH-7A for which the points represent the means of three separate assays and R04 for which the points represent the means of five separate assays. The error bars indicate 1 standard deviation from the mean. —□—, PRH-7A; ■, PRH-7B; ---△---, PRH-4; ○, PRH-11A; ---□---, PRH-11B; ●, PRH-11C; ▲, PRH-3; —△—, R04, control HMAB.

confirm that PRH-1 was a suitable capture antibody, the ability of biotinylated PRH-1 to bind HTLV-1 gp46 captured by purified preparations of the HTLV-1 gp46 HMABs was assessed (Fig. 7A). None of the HMABs captured any protein reactive with biotinylated PRH-1 from extracts of uninfected RPMI-

8402 cells. Nor did HTLV-1 p24 HMAB WA05-D5 capture any reactive protein from either HUT-102 or RPMI-8402 extracts, confirming the specificity of the assay for HTLV-1 gp46. The failure of HMAB PRH-1 to capture any protein reactive with itself from HUT-102 extracts also suggested that the majority of the HTLV-1 gp46 present in the HUT-102 extract was monomeric.

The HMABs PRH-7A, PRH-7B, PRH-11A, PRH-11B, and PRH-4 bound significant quantities of HTLV-1 gp46 that was reactive with biotinylated PRH-1. In agreement with the locations of the epitopes determined above (Fig. 3) mAb 6C2 was also able to bind significant quantities of Env protein that could be recognized by biotinylated HMAB PRH-1. In contrast, HMAB PRH-3 did not capture any protein recognized by biotinylated PRH-1. This suggests that the epitope recognized by HMAB PRH-3 partially overlaps the binding site of PRH-1. However, it is also possible that HMAB PRH-3 does not have a sufficiently strong affinity for HTLV-1 gp46 to bind significant quantities in this assay. To address this, the ability of HMAB PRH-3 to bind gp46 was assessed with biotinylated HMAB PRH-7A in addition to PRH-1 (Fig. 7B). As expected, HMAB PRH-1 was very efficient at binding and displaying HTLV-1 Env protein to HMAB PRH-7A. Additionally HMAB PRH-7A recognized reduced but equivalent amounts of Env protein when either mAb 6C2 or PRH-3 was employed as the capture antibody. In contrast, HMAB PRH-1 did not recognize Env protein bound by PRH-3, even though both HMABs PRH-1 and PRH-7A bound similar amounts of Env protein captured by mAb 6C2. Therefore, the failure of PRH-1 to recognize Env protein captured by HMAB PRH-3 indicates that the epitopes of these HMABs partially overlap, a characteristic not seen with HMAB PRH-11A or -11B. Thus, PRH-3

TABLE 1. Summary of the properties of HTLV-1 Env HMABs

HMAb ^a	Subtype	Major proteins detected by ^b :				Live-cell IFA results ^c		Syncytium inhibition ^d (μg/ml)
		Western blotting		Immunoprecipitation		HTLV-1	HTLV-2	
		HTLV-1	HTLV-2	HTLV-1	HTLV-2			
PRH-1	IgG1	gp61, gp46	—	gp61, gp46	—	++	—	—
PRH-8 M/T-2	IgG1	gp46	—	gp46	—	++	—	22
PRH-8 HUT		—	—	—	—	+/—	—	—
PRH-4	IgG1	—	—	gp61, gp46	—	++	—	5
PRH-7B	IgG1	—	—	gp61, gp46	gp46	++	++	2
PRH-7A	IgG1	—	—	gp61, gp46	gp46	++	++	2
PRH-11A	IgG1	—	—	gp61, gp46	—	++	+/—	30
PRH-11B	IgG1	—	—	gp61, gp46	—	++	+/—	30
PRH-11C	IgG1	—	—	gp61, gp46	—	++	+/—	30
PRH-3	IgG1	—	—	gp61, gp46	—	++	—	40
PRH-21	IgG3	gp61, gp21	—	gp61, gp21	gp21	—	—	—

^a The nomenclature for these HMABs has been simplified for clarity. In abstracts presented at various conferences and in a recent report (1) these HMABs were identified by the original notation. The conversion is as follows: PRH-1 = WA07-2B10; PRH-3 = WA03-1B4; PRH-4 = WA04-2B10; PRH-7A = WA07-1G7; PRH-7B = WA07-2F7; PRH-8 = WA08-2E9; PRH-11A = WA11-1F5; PRH-11B = WA11-2F3; PRH-11C = WA11-2E2; PRH-21 = WA07-1E4.

^b —, no detectable HTLV protein. Note that the results for HUT-102 and MT-2 cells are listed separately for HMAB PRH-8 only. For all other HMABs equivalent results were obtained with extracts from HUT-102 and MT-2 cells.

^c ++, strongly positive; +, positive; +/-, peak of fluorescence with HMAB was shifted relative to peak obtained with control antibody but was not clearly separated; —, peak obtained with HTLV-1 HMAB overlapped peak obtained with control antibodies and uninfected cells. Similar results were obtained with all HMABs with MT-2 cells except for HMAB PRH-8 as indicated above.

^d Concentration of antibody routinely required to achieve 80% or greater inhibition of syncytium formation. The highest antibody concentration tested was 50 μg/ml. —, syncytium inhibition never exceeded 80% at any concentration.

FIG. 7. EIA of HTLV-1 gp46. (A) Fifty microliters of extract from HUT-102 cells (striped bars) or RPMI-8402 cells (solid bars) was captured onto microtiter plate wells precoated with 100 ng of the HMAbs indicated on the x axis. 1, PRH-1; 7A, PRH-7A; 7B, PRH-7B; 4, PRH-4; 11A, PRH-11A; 11B, PRH-11B; 3, PRH-3; D5, WA05-D5; 6C2, mAb 6C2. Bound proteins were detected with biotinylated HTLV-1 HMAb PRH-1 diluted 1/50 (final concentration, 0.82 $\mu\text{g/ml}$) in BLOTTO. Bound biotinylated antibody was detected by incubation with streptavidin-conjugated horseradish peroxidase followed by incubation with colorimetric substrate as described in Materials and Methods. The bars represent the means of two separate assays (except for HMAb PRH-11B, which was only tested once) and the error bars indicate the higher of the values obtained. AVG OD 405 nm, mean optical density at 405 nm. (B) Fifty microliters of extract from MT-2 cells or RPMI-8402 cells was captured onto microtiter plate wells precoated with 100 ng of the MAbs indicated on the x axis. Bound proteins from wells incubated with HTLV-1-infected MT-2 cells were detected with biotinylated HMAb PRH-7A (open bars) or biotinylated HMAb PRH-1 (striped bars). Bound protein from wells incubated with uninfected RPMI-8402 cells and detected with biotinylated HMAb PRH-7A or HMAb PRH-1 (black bars) are also indicated. Both biotinylated HMAbs were added at a concentration of 1 $\mu\text{g/ml}$. The bars represent the means of two separate determinations in one assay, and the error bars indicate the higher of the two values obtained.

and the three PRH-11 HMAbs have similar virus neutralization activities but the epitopes they recognize are distinct.

We next examined the ability of a representative member of each of the remaining three groups of HMAbs to bind HTLV-1 gp46 in the presence of competing antibodies. As expected, biotinylated preparations of HMAbs PRH-4, PRH-7A, and PRH-11A were all able to efficiently bind HTLV-1 gp46 captured by HMAb PRH-1 (data not shown). In competition assays, the binding of biotinylated PRH-7A to HTLV-1 gp46 was strongly inhibited by incubation with increasing amounts of unlabeled PRH-7A and PRH-7B (Fig. 8A). Minor amounts of inhibition of PRH-7A binding were also obtained with high concentrations of HMAb PRH-4. No inhibition was observed with either the p24 HMAb WA05-D5 or the gp46 HMAbs PRH-11A, PRH-11B, and PRH-11C (data not shown). When PRH-11A was the biotinylated antibody, nei-

FIG. 8. Antibody competition analysis of HTLV-1 HMAbs. (A) Fifty microliters of extract from HUT-102 cells was captured onto microtiter plate wells precoated with 100 ng of HMAb PRH-1. Bound protein was detected with biotinylated HMAb PRH-7A diluted 1/500 (final concentration, 0.83 $\mu\text{g/ml}$) in BLOTTO in the presence of increasing amounts of the indicated HMAbs. Each point represents the mean of two determinations, and the error bars indicate the individual values. Error bars were not plotted for HMAbs PRH-11A, PRH-11B, and WA05-D5 for clarity but did not exhibit a greater range than those observed with the other HMAbs. For the other HMAbs plotted, the lack of error bars indicates that both values were within the symbol for the mean. $\square$, PRH-7A; $\blacksquare$, PRH-7B; $\triangle$, PRH-4; $\circ$, PRH-11A; $\bullet$, PRH-11B; $\diamond$, WA05-D5. AVG OD 405 nm, mean optical density at 405 nm. (B) Fifty microliters of extract from HUT-102 cells was captured onto microtiter plate wells precoated with 100 ng of HMAb PRH-1. Bound protein was detected with biotinylated HMAb PRH-11A diluted 1/500 (final concentration, 1.3 $\mu\text{g/ml}$) in BLOTTO in the presence of increasing amounts of the indicated HMAbs (the symbols are the same as for panel A with one addition: $\blacklozenge$, PRH-11C). Error bars were plotted for all HMAbs except PRH-11C for which the values plotted are from one determination.

ther HMAb PRH-4 nor control HMAb WA05-D5 exhibited significant inhibition. In contrast, HMAbs PRH-11A, PRH-11B, and PRH-11C all strongly inhibited binding of HMAb PRH-11A (Fig. 8B), as did HMAbs PRH-7A and PRH-7B.

One explanation for the unidirectional inhibition of PRH-11A by PRH-7A and PRH-7B is that the PRH-11 HMAbs and the PRH-7 HMAbs recognize the same epitope but that the PRH-7 HMAbs have a much higher affinity for the epitope and are able to displace HMAb PRH-11A. To investigate this possibility, the ability of biotinylated HMAbs PRH-1, PRH-7A, and PRH-11A to bind HTLV-1 Env protein captured by HMAb PRH-1 was assessed (Fig. 9A). HMAbs PRH-7A and PRH-11A had similar apparent affinities for HTLV-1 Env protein, binding 50% of total Env protein at a concentration of approximately 0.4 $\mu\text{g/ml}$ and binding all available Env protein

FIG. 9. Binding of HMABs to Env protein captured by HMABs PRH-1 and PRH-11A. (A) Fifty microliters of extract from HUT-102 cells (solid lines) or RPMI-8402 cells (dashed lines) was captured onto microtiter plate wells pre-coated with 100 ng of HMAB PRH-1. Bound protein was detected with biotinylated HMABs at the indicated concentrations. □, biotinylated PRH-7A; ○, biotinylated PRH-11A. Each point represents the mean of two determinations, and the error bars indicate the individual values obtained. The lack of error bars indicates that both values were within the symbol for the mean. AVG OD 405 nm, mean optical density at 405 nm. (B) Fifty microliters of extract from HUT-102 cells (solid lines) or RPMI-8402 cells (dashed lines) were captured onto microtiter plate wells pre-coated with 100 ng of HMAB PRH-11A. Bound protein was detected with biotinylated HMABs at the indicated concentrations. □, biotinylated PRH-7A; ○, biotinylated PRH-11A; ◇, biotinylated PRH-1. Each point represents the mean of two determinations, and the error bars indicate the individual values obtained. The lack of error bars indicates that both values were within the symbol for the mean.

at an antibody concentration of 4 μ g/ml. No binding to extracts derived from an uninfected T-cell line was observed. We then examined the ability of biotinylated HMABs PRH-1, PRH-7A, and PRH-11A to bind HTLV-1 Env protein captured by HMAB PRH-11A (Fig. 9B). As seen in Fig. 7A, HMAB PRH-1 was quite efficient at binding Env protein captured by HMAB PRH-11A. In agreement with results obtained with HMAB PRH-1, only minor amounts of binding were observed when HMAB PRH-11A was employed to detect Env protein captured by itself. HMAB PRH-7A exhibited an intermediate level of binding to Env protein displayed by HMAB PRH-11A, recognizing 42% of the Env protein seen by HMAB PRH-1. The fact that HMAB PRH-7A exhibited any Env binding above that observed with HMAB PRH-11A is inconsistent with the hypothesis that the two HMABs recognize the same epitope. Since saturation of binding was not achieved, it is not possible

to estimate the concentration required for 50% binding with HMAB PRH-7A, but it is clear that it is substantially increased when HMAB PRH-11A, rather than PRH-1, is the capture antibody. This result is consistent with the two separate epitopes being adjacent but not overlapping. Thus, the PRH-7 and PRH-11 HMABs recognize distinct epitopes, increasing to four the total number of epitopes detected by the seven conformational HMABs.

DISCUSSION

Although effective vaccines have been developed against many viral pathogens, the development of a vaccine capable of preventing infection by a retrovirus has been an elusive goal. Both the high level of amino acid conservation among different isolates and the availability of small-animal models for infection have made HTLV-1 an attractive target for the development of a retroviral vaccine. Early evidence suggesting that antibodies capable of neutralizing HTLV-1 are present in infected individuals was obtained from studies studying passive immunization of rabbits with sera from HTLV-1-infected individuals (32, 39, 64). In one study, dilution of an HTLV-1 Ig preparation suggested that a neutralization titer (as measured by a vesicular stomatitis virus pseudotype assay [8]) of at least 1/500 in the passively immunized rabbit was required to protect rabbits from subsequent HTLV-1 challenge (32). The results of vaccination experiments employing recombinant HTLV-1 Env protein or synthetic peptides as immunogens have been less consistent (19, 26, 44, 60, 64). Although questions have been raised about the successful vaccination protocols described above (see reference 26), the failure of a given vaccination protocol to protect immunized animals has generally been linked to a failure to induce a strong neutralizing antibody response (26, 32, 64).

It is therefore important to define the individual epitopes that make up the neutralizing antibody response to HTLV-1 and HTLV-2. A number of studies showed that synthetic peptides encoding the immunodominant central region of HTLV-1 gp46 (amino acids 181 to 210) and peptides encompassing an amino-terminal region of HTLV-1 gp46 (amino acids 88 to 98) can induce a neutralizing antibody response against HTLV-1 or HTLV-2 in vaccinated animals (2, 14, 48, 66, 68). Studies of the immune responses of HTLV-1-infected individuals have confirmed that the overwhelming majority of HTLV-1-infected individuals develop a potent antibody response to a variety of short peptide sequences located between amino acids 170 and 210 of HTLV-1 gp46 (3, 30, 34, 37). In contrast, studies of the reactivities of peptides encoding the amino-terminal-neutralizing domain of HTLV-1 have not demonstrated a strong antibody response to this region in HTLV-1-infected individuals (30, 49, 71). The presence of neutralizing antibodies that recognize synthetic peptides from other regions of HTLV-1 gp46 has been reported (3, 14), but their importance relative to the two regions mentioned above is not well established. No information is currently available on the number of conformational epitopes present in HTLV-1 gp46 or the importance of these antibodies in virus neutralization.

To further our understanding of the protective immune response of HTLV-1-infected individuals, we generated HMABs to directly analyze the immune response of infected individuals to HTLV-1 proteins. The initial selection of specific HMABs was by IFA with HTLV-1-infected cells to maximize the identification of antibodies to conformational epitopes. Using this approach, we produced 10 HMABs, of which 9 bind HTLV-1 Env proteins expressed on the surfaces of infected cells and

three bind Western-blotted HTLV-1 envelope proteins. HMAb PRH-21 recognized Western-blotted HTLV-1 but not HTLV-2 gp21. However, PRH-21 immunoprecipitated small amounts of HTLV-2 gp21 (Table 1) and produced a weak immunofluorescence staining of fixed MoT cells. To finally resolve the type specificity of this epitope, immunization studies with synthetic peptides or recombinant proteins expressing the PRH-21 epitope would be required.

PRH-21 is the second HMAb recognizing HTLV-1 gp21 to be identified. The previously described gp21 HMAb 5G4 was shown to recognize an epitope located between amino acids 361 and 404 of the HTLV-1 Env protein (24). The epitope recognized by HMAb PRH-21 was localized to the carboxy-terminal 43 amino acids of HTLV-1 gp21. Since the first 19 amino acids of this sequence form the transmembrane-spanning alpha helix of gp21, they are unlikely to be involved in the epitope recognized by PRH-21. This restricts the epitope recognized by PRH-21 to the cytoplasmic domain of HTLV gp21, or amino acids 465 to 488. Evidence for the presence of antibodies to the cytoplasmic portion of gp21 has been observed before, especially in sera from patients with HAM/TSP (30, 45). In agreement with this proposal, little or no cell surface staining of HTLV-1-infected cells was observed with the PRH-21 antibody. Nor did this antibody display any neutralizing activity. As shown in Fig. 1, PRH-21 is useful for the specific detection of HTLV-1 gp21 and precursor proteins. We have found this HMAb to be useful in studying the expression and processing of HTLV-1 Env (data not shown).

Two other HMAbs recognizing linear epitopes within HTLV-1 gp46 were isolated. The epitope recognized by HMAb PRH-1 is located between amino acids 260 and 294 of HTLV-1 gp46. Evidence for an antibody response to this region has been described previously (3, 30). Although the epitope recognized by PRH-1 is accessible on the surfaces of HTLV-1-infected cells, this antibody had little or no ability to inhibit HTLV-1 syncytium formation. HMAb PRH-1 is, however, useful as a capture and detection antibody for monitoring HTLV-1 gp46 expression (Fig. 1 and 7–9). The other HMAb that recognizes a linear epitope, PRH-8, is unique in that it strongly recognizes gp46 from MT-2 cells but has little or no reactivity with gp46 expressed by HUT-102 cells. This antibody also displayed similar subtype specificity in neutralization assays, with an antibody concentration of 22 µg/ml required to achieve 88% inhibition of syncytium formation with MT-2 cells but not with HUT-102 cells (Table 1). By using recombinant proteins derived from the MT-2 strain of HTLV-1, the PRH-8 epitope was localized to amino acids 210 to 312 of HTLV-1 gp46. The nucleotide sequence of this region of HUT-102 has not been reported. However, it is known that both MT-2 and HUT-102 are within the cosmopolitan genotype of HTLV-1, with MT-2 being assigned to the Japanese subtype and HUT-102 being from the United States subtype (10, 59). Although the subtype specificity of the PRH-8 HMAb needs further confirmation with additional isolates of the subtypes, it is likely that the epitope recognized by PRH-8 is subtype specific. We are currently completing the sequencing of HUT-102 gp46. When this is completed, sequence comparisons between the HUT-102 and MT-2 strains of HTLV-1 should allow for a very precise identification of the amino acids that make up the epitope recognized by PRH-8.

During the course of our investigations, the epitope recognized by the mAb 6C2 was further defined from the previously reported range of amino acids 210 to 306 (50) down to a 27-amino-acid sequence (210 to 236). In the middle of this sequence, a block of nine amino acids (QLTLGSTNY) is highly conserved between HTLV-1, HTLV-2, and the new

simian retrovirus primate T-cell lymphotropic virus type L (69). The epitope recognized by the 6C2 antibody is likely to encompass this sequence, and thus the possibility exists that this antibody will be useful for the characterization of more distantly related retroviruses.

We also produced seven HMAbs that did not bind any HTLV proteins by Western blotting but that strongly immunoprecipitated HTLV Env proteins and bound to the surfaces of cells in HTLV-1-infected cell lines. To our knowledge, these are the first MAbs (human or murine) to conformational epitopes within HTLV-1 gp46 to be reported. All seven HMAbs that recognized conformational epitopes have some level of virus neutralization activity, although a 20-fold difference between the strongest and weakest neutralizing HMAb was observed. The strongest virus neutralization activity was exhibited by HMAbs PRH-7A and PRH-7B, which also immunoprecipitated HTLV-2 gp46 and bound to the surfaces of MoT cells. While other MAbs recognizing both HTLV-1 and HTLV-2 gp46 have been described (50, 67, 68), they do not have significant virus neutralization activity. PRH-7A and PRH-7B are the first MAbs to an epitope common to HTLV-1 and HTLV-2 with significant virus-neutralizing activity. Experiments to determine the ability of these HMAbs to neutralize HTLV-2 infection and simian/primate T-cell lymphotropic virus infection are in progress.

The number of conformational epitopes mediating virus neutralization was determined by antibody competition assays. The data obtained indicated that the seven HMAbs recognized four epitopes within HTLV-1 gp46. The first strongly neutralizing epitope is recognized with high affinity by HMAbs PRH-7A and PRH-7B and is made up of amino acids that are substantially conserved between HTLV-1 and HTLV-2. The second epitope is recognized by HMAb PRH-4 and is clearly distinct from that recognized by PRH-7A and PRH-7B, as indicated both by competition analysis and by the fact that it is an HTLV-1-specific epitope. The epitope with the weakest neutralizing activity is recognized by HMAb PRH-3. This antibody is HTLV-1 specific as determined by immunoprecipitation and IFA and was the only one of the seven conformational HMAbs to be unable to display Env protein to the linear HMAb PRH-1 (Fig. 7). This suggests that the epitope of PRH-3, at least in part, overlaps the linear epitope recognized by HMAb PRH-1. HMAbs PRH-11A, PRH-11B, and PRH-11C recognize an HTLV-1-specific epitope that induces weak virus neutralization activity. Binding of the PRH-11 HMAbs to gp46 can be inhibited by HMAbs PRH-7A and PRH-7B. The possibility that the epitope recognized by the PRH-11 and PRH-7 HMAbs is the same is refuted by the observation that HMAb PRH-7A and HMAb PRH-11A can both bind to HTLV-1 Env protein at the same time (Fig. 9). The observed inhibition could be a result of the binding of PRH-7A and PRH-7B to HTLV-1 gp46, causing a conformational change in the protein that alters the binding site of PRH-11A, -11B, and -11C. Alternatively, the epitopes recognized by the PRH-7A, PRH-7B, and PRH-11 HMAbs could be adjacent to each other. These explanations are not mutually exclusive. Final resolution of this question will require identification of individual amino acids involved in the epitopes recognized by the PRH-7 and PRH-11 HMAbs.

Two previous reports have described panels of HMAbs to HTLV-1 Env proteins (3, 34). Both studies resulted in the successful production of large numbers of antibodies recognizing synthetic peptides from the central region of HTLV-1 gp46. In both of the previous studies of HTLV-1 HMAbs only a minority of the isolated HMAbs had virus-neutralizing activity (3, 34). In one of these studies (3), nine neutralizing

HMAbs not reactive with synthetic peptides were described. However, these HMAbs all bound Western-blotted HTLV-1 gp46, and the epitopes recognized by them are unlikely to be conformation dependent. Our effort yielded a high percentage of HMAbs to conformational epitopes with significant virus neutralization activity (7 of 10). A likely explanation for the difference in antibody selection is the different screening assays, IFA of fixed HTLV-1-infected cells (this study) versus EIA with synthetic peptides (34) or immunoaffinity-purified HTLV-1 gp46 (3). Preservation of native antigens is probably greater in minimally disrupted virus-infected cells. Similar increases in the fraction of isolated MAbs with neutralization activity and conformation dependence have been observed when oligomeric instead of monomeric HIV gp120 was used as an immunogen and screening reagent (15). The use of intact virus-infected cells as a screening tool will be applicable to other viral systems in which the conformational integrity of the target antigen is thought to be important.

The availability of HMAbs recognizing conformational epitopes within HTLV-1 gp46 has important implications for study of the structure and function of the HTLV-1 envelope proteins. Previous studies of HTLV-1 mutants or chimeric proteins have generally resulted in the identification of large numbers of mutations whose major phenotype is the inhibition of processing of the gp61 precursor protein into gp46 and gp21 (13, 53, 54). The failure of these mutants to undergo correct processing has been thought to be the result of the mutants assuming an incorrect three-dimensional structure. As noted by others (12), a facile means for discriminating between mutations that destroy the tertiary structure of the Env protein and mutations that prevent processing directly has not been available. Additionally, the fact that antibodies recognizing four conformational epitopes were obtained from a survey of 10 HMAbs suggests that antibodies to conformational epitopes are a significant part of the antibody response of infected individuals to HTLV-1 gp46. This raises the possibility that the use of immunogens that can induce antibodies to linear and conformational epitopes may be required to induce a robust protective antibody response in vaccinees. The described conformational HMAbs allow for a very direct evaluation of the conformational integrity of an HTLV-1 mutant or potential vaccine construct (1). HMAbs PRH-4, PRH-7A, and PRH-7B are also prime candidates for exploring the isolation of peptide mimotopes, as has been done successfully with other viral systems (9). Such mimotopes could be used alone or in combination with synthetic peptides encoding linear neutralization epitopes.

Finally, several groups have proposed structural models for the oncoretroviral Env proteins (5, 20). These models identify similar features found in retroviral Env proteins (or other proteins) of very distant relationship. They suggest that the retroviral surface glycoprotein can be described as having one or more large globular domains that are supported by "stems" composed of the amino-terminal and carboxy-terminal amino acid sequences. The regions of the Env protein that are involved in receptor recognition and syncytium formation are thought to be clustered at the amino-terminal end and in the center of the large globular domain. For HTLV-1 and HTLV-2 the amino-terminal domain corresponds to the neutralizing epitope between amino acids 88 and 98 and the center of the large globular domain corresponds to the immunodominant neutralizing epitope between amino acids 181 and 210 (5). Mutational studies of the HTLV gp46 have implicated similar regions as mediating syncytium formation (11). The two stem regions include the regions outside of the large globular domain, approximately amino acids 1 to 70 and 255 to 312 of

HTLV-1 gp46. What is not known is how the various domains fold together in three-dimensional space and the proximity of the different chains to each other. Use of the conformational HMAbs in both epitope-mapping studies and antibody cross-competition studies can provide critical information on which regions of HTLV-1 gp46 are in close proximity and allow for the construction of a competition map, as has been done for HIV gp120 (42). Such information could have implications both for the development of HTLV vaccines and the design of therapeutic approaches to the treatment of the diseases associated with HTLV-1 infection.

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