Mapping of Homologous, Amino-Terminal Neutralizing Regions of Human T-Cell Lymphotropic Virus Type I and II gp46 Envelope Glycoproteins

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Twelve synthetic peptides containing hydrophilic amino acid sequences of human T-cell lymphotropic virus type I (HTLV-I) envelope glycoprotein were coupled to tetanus toxoid and used to raise epitope-specific antisera in goats and rabbits. Low neutralizing antibody titers (1:10 to 1:20) raised to peptides SP-2 (envelope amino acids [aa] 86 to 107), SP-3 (aa 176 to 189), and SP-4A (aa 190 to 209) as well as to combined peptide SP-3/4A (aa 176 to 209) were detected in the vesicular stomatitis virus-HTLV-I pseudotype assay. Higher-titered neutralizing antibody responses to HTLV-I (1:10 to 1:640) were detected with pseudotype and syncytium inhibition assays in four goats immunized with a combined inoculum containing peptides SP-2, SP-3, and SP-4A linked to tetanus toxoid. These neutralizing anti-HTLV-I antibodies were type specific in that they did not inhibit HTLV-II syncytium formation. Neutralizing antibodies in sera from three goats could be adsorbed with peptide SP-2 (aa 86 to 107) as well as truncated peptides containing envelope aa 90 to 98, but not with equimolar amounts of peptides lacking envelope aa 90 to 98. To map critical amino acids that contributed to HTLV-I neutralization within aa 88 to 98, peptides in which each amino acid was sequentially replaced by alanine were synthesized. The resulting 11 synthetic peptides with single alanine substitutions were then used to adsorb three neutralizing goat antipeptide antisera. Both asparagines at positions 93 and 95 were required for adsorption of neutralizing anti-HTLV-I antibodies from all three sera. Peptide DP-90, containing the homologous region of HTLV-II envelope glycoprotein (aa 82 to 97), elicited antipeptide neutralizing antibodies to HTLV-II in goats that were type specific. In further adsorption experiments, it was determined that amino acid differences between homologous HTLV-I and HTLV-II envelope sequences at HTLV-I aa 95 (N to Q) and 97 (G to L) determined the type specificity of these neutralizing sites. Thus, the amino-terminal regions of HTLV-I and -II gp46 contain homologous, linear, neutralizing determinants that are type specific.

Human T-cell lymphotropic virus type I (HTLV-I) was the first human retrovirus to be linked etiologically to human disease (15, 44-46, 62, 64). HTLV-I is regarded as the causative agent of adult T-cell leukemia/lymphoma (15, 44-46, 62, 64) and a neurological degenerative syndrome called HTLV-I-associated myelopathy/tropical spastic paraparesis (7, 10, 39, 50, 51, 63). HTLV-I infection is also associated with a wide array of secondary disease manifestations, including immunosuppression (35, 60), arthritis (15, 18, 25, 38), infectious dermatitis (29), and others (20, 32, 34, 52). A related human retrovirus, HTLV-II (21, 53), has been isolated from patients with a T-cell variant of hairy cell leukemia, although no consistent relationship between HTLV-II infection and human disease has yet been observed.

Sites on retroviral envelope glycoproteins are targets for neutralizing-antibody responses (1, 3, 11, 12, 22, 24, 26, 40, 54, 61) that, in some cases, are associated with protection from infection (2, 26) or disease (26, 57). Immunization with recombinant HTLV-I envelope glycoproteins has provided protection from HTLV-I infection in animal models (37, 58). A neutralizing region of the gp46 external envelope glyco-

protein of HTLV-I has been mapped to a central region between amino acids (aa) 186 and 196 (33, 48, 61). Antibodies directed to this region can inhibit the mitogenic effect of UV-inactivated HTLV-I virions on resting human T cells (9, 48) and can also inhibit HTLV-I-induced syncytium formation (61).

We report here that an amino-terminal portion of HTLV-I gp46 contains a second neutralizing site which is functionally conserved on HTLV-II gp46.

MATERIALS AND METHODS

Peptide synthesis. The deduced amino acid sequences of HTLV-I and HTLV-II envelope glycoproteins are from Seiki et al. (56) and Sodroski et al. (59), respectively. The hydrophilic amino acid sequences of HTLV-I envelope glycoproteins were identified by the method of Kyte and Doolittle (28) and used for the production of synthetic peptides SP-1-11 and SP-73. These peptides were synthesized on a Du Pont 2100 peptide synthesizer at Duke University with the chemicals and program cycles recommended by the manufacturer. The peptides were deprotected and cleaved from the supporting resin with HF. Amino acid analysis (Immunodynamics, Inc., La Jolla, Calif.) was performed on all of the peptides to monitor

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content; all of these peptides had the expected amino acid content.

Peptides SP-2R, SP-2L-90, and SP-2L-91 were purchased from Research Genetics, Huntsville, Ala. The remaining peptides for adsorption experiments were synthesized on Applied Biosystems 431A peptide synthesizers at the Howard Hughes Medical Institute, Durham, N.C. (peptides SP-2L-1, SP-2L-1.1 through SP-2L-1.11, SP-2.2, SP-2.8, SP-2.10, SP-2.2/8/10, SP-2L-1.6/8D, SP-2L-1.6/8S, SP-2L-1.3E, and SP-2L-1.3R), and at the Center for AIDS Research, Duke University Medical Center (peptides DP-90, DP-91, DP-104, and SP-2L). All of these peptides were greater than 90% pure as determined by reverse-phase high-pressure liquid chromatography (HPLC) analysis.

Immunization of animals. Synthetic peptides (4 to 6 mg) were covalently linked to tetanus toxoid (14 mg) with the heterobifunctional cross-linking reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as previously described (40). Conjugates (5 mg) emulsified in complete (day 0) or incomplete (days 14, 21, 28, and 35) Freund's adjuvant were injected subcutaneously at two to four sites into female New Zealand White rabbits for production of anti-peptide antisera. Sera collected before immunization and normal goat serum were used as negative controls. Goats obtained through the Duke University Vivarium were immunized intramuscularly and subcutaneously at two to four sites with synthetic peptide-tetanus toxoid conjugates (5 to 10 mg) in complete (day 0) or incomplete Freund's adjuvant. Preimmune and immune sera were collected and stored at -70°C until used.

Immunoassays. The radioimmunoassay was performed as described previously (43) by diluting synthetic peptides in coating buffer (15 mM NaHCO_3 , 35 mM Na_2CO_3 [pH 9.6]) and incubating 50- μl samples (1 to 10 μg) in Immulon 2 (Dynatech) microtiter wells overnight at 4°C . The wells were emptied, incubated with 5% nonfat dry milk in coating buffer for 2 h at 4°C , and washed once in phosphate-buffered saline (PBS) containing 0.5% Tween 20, 3% nonfat dry milk, and 0.1% azide (wash buffer). Antibodies diluted 1:50 to 1:100 in wash buffer were incubated in microtiter wells for 1 h at 23°C , and the wells were washed three times with wash buffer. After addition of 50 μl of anti-rabbit or anti-goat immunoglobulin G (IgG; 1:200 final dilution; BCA/Cappel Products, West Chester, Pa.), the wells were incubated for 45 min and then washed as before. The wells were further incubated with ^{125}I -labeled protein A (10^5 cpm per well) for 30 min, washed, and evaluated for bound radioactivity in a gamma counter. An enzyme-linked immunosorbent assay (ELISA) was also used to detect anti-peptide antibodies as described above, except that alkaline phosphatase-labeled anti-rabbit or anti-human IgG antibodies (Promega Corp., Madison, Wis.) or anti-goat IgG antibodies (Cappel-Organon Teknika Corp., West Chester, Pa.) were used.

Western immunoblot assays and radioimmunoprecipitation assays (RIPAs) were performed with affinity-purified HTLV-I envelope glycoproteins (41) as described previously (43). A recombinant HTLV-I gp21 protein fragment (55) was a gift from T. Papas, National Cancer Institute, Frederick, Md. Recombinant HTLV-I envelope proteins RE1 and RE3 were purchased from Repligen, Inc., Cambridge, Mass.

Neutralization assays. Pseudotype neutralization of vesicular stomatitis virus (VSV)-HTLV-I particles was performed as described previously (5). The HTLV-I and HTLV-II syncytium inhibition assays were performed as described before (36) with HTLV-II-producing MO-T cells, obtained from the American Type Tissue Collection, Rockville, Md., and with the HTLV-I-positive C91/PL cell line

and the non-HTLV-I-expressing C8166 T-cell line, both gifts from Robert Gallo, National Cancer Institute, Bethesda, Md. Cells were grown in RPMI 1640 medium with 20% heat-inactivated fetal calf serum, 5 mM L-glutamine, and 10 μg of gentamicin sulfate per ml in a humidified chamber at 37°C in 5% CO_2 and harvested in the log phase of growth.

For the HTLV-I syncytium inhibition assay, 45 μl each of C91/PL and C8166 cells at 10^6 cells per ml in cell culture medium were added to duplicate wells of a Costar 96-well microtiter plate containing 10- μl aliquots of heat-inactivated test or control serum and incubated for 24 h in a tissue culture incubator. Cell clumps were dispersed by pipetting, and the syncytia in five fields were then counted with the aid of an ocular grid under an Olympus OM-2 inverted microscope at $200\times$ magnification. Control wells containing C91/PL or C8166 cells only were also evaluated for syncytia. Preimmune serum samples were used as negative serum controls. Antisera that inhibited syncytium formation by greater than 90% were considered positive for neutralizing antibodies. For the HTLV-II syncytium inhibition assay, HTLV-II-producing MO-T cells grown in Iscove's medium with 20% fetal calf serum were substituted for the C91/PL cells, and the assay was performed as described above.

Adsorption experiments with heat-inactivated anti-peptide antisera and synthetic peptides were performed with serially diluted 4- $\mu\text{mol/ml}$ solutions of unpurified or HPLC-purified synthetic peptides in PBS (pH 7.2). Equal volumes of twofold serially diluted peptide solutions and either control or immune serum were incubated for 1 h at 23°C and then used in the syncytium assay as described above. At these dilutions, the peptides neither inhibited syncytium formation with cocultivated C8166 and C91/PL cells nor induced syncytium formation when incubated with either of these cell lines. The percent adsorption of neutralizing anti-peptide antisera or normal serum with peptides was calculated by counting syncytia as described above and calculating the ratio of mean numbers of syncytia (duplicate wells) obtained with immune and normal or preimmune sera. Three to four separate adsorption experiments were performed per peptide, and the mean percent adsorption and standard deviations were then calculated. Stock recombinant HTLV-I envelope fragments RE1 and RE3 were dialyzed twice for 48 h each against 6 liters of PBS and diluted 10 times with immune or control sera. The sera were then tested in syncytium assays as described above.

Sera. Sera from HTLV-I-seropositive individuals were obtained from William A. Blattner, Angela Manns, and Stefan Wiktor, National Cancer Institute, Bethesda, Md. A panel of 16 sera from HTLV-II-seropositive individuals whose HTLV-II infections were verified by polymerase chain reaction was purchased from Serologicals, Inc., Clarkston, Ga.

RESULTS

Immunochemical analysis of anti-peptide antibody reactivity. Synthetic peptides (Table 1) containing amino acid sequences of HTLV-I (SP-1 through SP-11) and HTLV-II (DP-90) envelope glycoproteins were coupled to tetanus toxoid and used to immunize rabbits or goats. Peptides SP-3/4A and DP-91 were also used without coupling to tetanus toxoid for immunization. The locations of HTLV-I envelope amino acid sequences selected for synthesis of peptides are shown in Fig. 1. Additional peptides shown were used in adsorption experiments (see below).

All of the rabbit antisera raised to peptides SP-1 through

TABLE 1. Amino acid sequences of peptides used for immunization and adsorption experiments^a

Synthetic peptide	Amino acids	Sequence ^b
HTLV-I		
SP-1	33–47	VSSYHSKPCNPAQPV
SP-2	86–107	(C)PHWTKKPNRNGGGYYSASYS DP
SP-3	176–189	(C)LNTEPSQLPPTAPP(Y)
SP-4	129–149	SSPYWKFQHDVNFTQEVSR LN(C)
SP-4A	190–209	(C)LLPHSNLDHILEPSIPWKS K(Y)
SP-3/4A	176–209	LNTEPSQLPPTAPP LPHSNLDHILEPSIPWKS K
SP-5	269–280	(Y)LPFNWTECFDPQ(C)
SP-6	296–312	(C)PPFSLSPVPTLGSR SR
SP-7	374–393	YAAQNRRGLDLLFWEQGGLC
SP-8	400–415	CRFPNITNSHVPILQE
SP-9	411–422	(C)PILQERPPLENR
SP-10	462–480	CILRQLRHLP SRVRYPHYS
SP-11	475–488	(C)RYPHYSLIKPES SL
DP-91 (T-cell/B-cell chimera)	183–209/88–98	LPPTAPP LPHSNLDHILEPSIPWKS KWTKKPNRNGGG
Mutations		
SP-2L-1.1	88–98	<u>A</u> TKKPNRNGGG
SP-2L-1.2	88–98	W <u>A</u> KKPNRNGGG
SP-2L-1.3	88–98	WT <u>A</u> KKPNRNGGG
SP-2L-1.4	88–98	WTK <u>A</u> PNRNGGG
SP-2L-1.5	88–98	WTKK <u>A</u> NRNGGG
SP-2L-1.6	88–98	WTKKP <u>A</u> RNGGG
SP-2L-1.7	88–98	WTKKPN <u>A</u> NGGG
SP-2L-1.8	88–98	WTKKPNR <u>A</u> GGG
SP-2L-1.9	88–98	WTKKPNRN <u>A</u> GG
SP-2L-1.10	88–98	WTKKPNRNG <u>A</u> G
SP-2L-1.11	88–98	WTKKPNRNGG <u>A</u>
HTLV-II		
DP-90	82–97	PHWIKKPNRQGLGYYS(C)
II-3	177–199	(C)TQPPPTSPPLVHDS DLEHVLTPS

^a Sequences are from Seiki et al. (56) for HTLV-I and Sodroski et al. (59) for HTLV-II. The sequences of SP-1 to SP-11 are listed sequentially from the N to the C terminus of the HTLV-I gp63 envelope precursor molecule with the exception of SP-4. The sequences in SP-1 to SP-6 are from the gp46 external envelope glycoprotein, whereas the sequences in SP-7 to SP-11 are derived from the gp21 transmembrane glycoprotein. Amino acid numbering begins with the initiation methionine (aa 1).

^b Each amino acid is represented in the single-letter code. Amino acids shown in parentheses were added to the peptides to facilitate coupling to the carrier protein (C) or iodination of the peptide (Y). Amino acids that differ from the native sequence of the HTLV-I envelope protein are underlined.

SP-6 reacted in a peptide-specific manner to the immunizing peptide in ELISA (not shown). When tested in the Western blot assay, antisera to peptides SP-1, SP-3, SP-4, SP-4A, and SP-6 reacted with affinity-purified gp46 and the gp63 envelope precursor, whereas preimmune sera did not (Fig. 2A). Anti-SP-6 envelope peptide reactivity to gp46 could be

completely inhibited by preincubating the sera with peptide SP-6 but not SP-7, suggesting that anti-peptide antibody reactivity was epitope specific (Fig. 2B). Peptide adsorption of other anti-peptide antisera in the Western blot assay was not tested because of the limited availability of affinity-purified gp46. Also in the Western blot assay, antisera raised

FIG. 1. Location within HTLV-I envelope glycoprotein of hydrophilic amino acid sequences used for peptide synthesis. The gp63 envelope precursor is proteolytically cleaved (Δ) to give rise to the gp46 external envelope glycoprotein and gp21 transmembrane glycoprotein. The positions of the synthetic peptide sequences are indicated below.

FIG. 2. Reactivity of anti-peptide antisera to affinity-purified HTLV-I gp63 and gp46 (A and B) and to a 15-kDa recombinant transmembrane fragment (55) of gp21 (C) under reducing conditions. (A) Anti-peptide antisera raised to peptides SP-1 (lane 2), SP-3 (lane 4), SP-4 (lane 6), SP-4A (lane 8), and SP-6 (lane 10) reacted with gp63 and gp46 envelope glycoproteins and with smaller products (28 kDa, lane 6; 24 kDa, lane 8). The reactivities of corresponding preimmune sera are shown in lanes 1, 3, 5, 7, and 9. Shown in lane 11 is the reactivity of monoclonal antibody 1C11 (43) raised to HTLV-I gp46 and gp63. Lane 12, P3X63 ascites fluid (negative control). (B) Adsorption of anti-SP-6 peptide antiserum with synthetic peptides and subsequent testing in the Western blot assay. Lane 1, adsorption with peptide SP-6; lane 2, adsorption with SP-7; lane 3, adsorption of preimmune rabbit serum with SP-6. Peptide SP-6 inhibited anti-SP-6 antibody reactivity to gp46. (C) Antisera to synthetic peptides SP-7, SP-8, and SP-9 containing gp21 sequences reacted in a Western blot assay with a recombinant fragment of gp21 produced in *Escherichia coli*. Expression of the recombinant transmembrane protein of HTLV-I in *E. coli* containing the pKS400 plasmid (55) is induced (i) at 42°C and uninduced (u) at 32°C. Lysates of transformed *E. coli* grown at 32 or 42°C were subjected to electrophoresis and Western blotting with preimmune serum (lanes 1, 3, and 5) or antisera to peptides SP-7, SP-8, and SP-9 (lanes 2, 4, and 6, respectively). Antisera to peptides SP-7, SP-8, and SP-9 reacted with the 15-kDa recombinant portion of gp21 in induced lysates of transformed *E. coli*, while preimmune sera did not react. Low levels of anti-peptide antibody reactivity in uninduced lanes could reflect a small amount of recombinant protein expression in *E. coli* at 32°C. These studies further characterized the reactivities of anti-SP-7, SP-8, and SP-9 antisera.

to peptides SP-7, SP-8, and SP-9, containing sequences from the HTLV-I gp21 transmembrane glycoprotein, reacted with a recombinant gp21 transmembrane protein (55) containing aa 374 to 422 (Fig. 2C). Antisera to SP-11 also reacted with gp63 (not shown), which contains the gp21 transmembrane protein.

Affinity-purified HTLV-I gp46 was radiolabeled and used in the RIPA to test antibody reactivity to soluble, nonde-

TABLE 2. Reactivity of antibodies raised to synthetic peptides

Antiserum	Specific reactivity (technique used)		
	Immunizing peptide (radioimmunoassay)	HTLV-I envelope (RIPA)	HTLV-I envelope (immunoblot)
SP-1	+	+	+
SP-2	+	+	—
SP-3	+	+	+
SP-4	+	+	+
SP-4A	+	+	+
SP-5	+	—	—
SP-6	+	—	+
SP-7	+	ND ^a	+ ^b
SP-8	+	ND	+ ^b
SP-9	+	ND	+ ^b
SP-10	+	ND	—
SP-11	+	ND	+ ^c

^a ND, not determined.

^b Tested with recombinant HTLV-I gp21 (aa 374 to 422).

^c Tested with affinity-purified gp63.

tured gp46 (not shown). Rabbit antisera to peptides SP-1, SP-2, SP-3, SP-4, and SP-6 precipitated ¹²⁵I-labeled gp46 in various amounts, whereas preimmune sera did not.

A summary of anti-peptide antibody reactivities to HTLV-I peptides and envelope glycoproteins is shown in Table 2. Overall, 10 of 12 anti-peptide antisera reacted with either nondenatured (RIPA) or denatured (Western blot) forms of affinity-purified HTLV-I envelope glycoproteins.

Neutralization of VSV-HTLV-I pseudotype particles and HTLV-I-induced syncytium formation with anti-peptide antisera. Antisera to peptides SP-1 through SP-11 were next tested for the ability to neutralize the infectivity of VSV-HTLV-I pseudotype particles. Low neutralizing titers (1:10) were seen only with antisera raised to peptides SP-2, SP-3, and SP-4A and to peptide SP-3/4A (aa 176 to 209) either coupled to tetanus toxoid or in an uncoupled, free form (SP-3/4A). Preimmune sera did not neutralize infectivity in this assay. However, these low-titered neutralizing antisera did not inhibit HTLV-I-induced syncytium formation. In an effort to raise antisera with higher neutralizing titers, four goats (no. 20, 21, 128, and 117) were inoculated with peptides SP-2 and SP-3/4A coupled to tetanus toxoid. Higher-titered neutralizing antisera, which inhibited HTLV-I syncytium formation at dilutions of 1:10 to 1:640, were obtained from all four goats. Similar neutralizing titers of 1:10 to 1:320 were obtained in the VSV-HTLV-I pseudotype assay with sera from goats 20 and 21. To evaluate which peptide, SP-2 or SP-3/4A, induced neutralizing antibodies in goats 20 and 21, antisera from both goats were adsorbed with immunizing or control peptides and then tested in the syncytium inhibition assay. The majority of neutralizing antibodies in both sera were adsorbed by SP-2 but not by equimolar amounts of negative control peptide SP-7 (Fig. 3). Some adsorption of neutralizing antibodies was seen with peptide SP-3/4A, although most of the neutralizing antibodies were adsorbed by SP-2. Thus, these studies mapped a novel neutralizing determinant to an amino-terminal portion of HTLV-I gp46 between aa 86 and 107.

Additional truncated peptides containing amino-terminal or carboxy-terminal portions of SP-2 were used in adsorption experiments (Fig. 4 and 5). Peptides SP-2L (aa 83 to 98) and SP-2L-90 (aa 90 to 98) could adsorb 90% or more of the neutralizing activity from sera from goats 20 and 21, while the C-terminal peptide SP-2R (aa 96 to 109) and peptide

FIG. 3. Adsorption of neutralizing anti-peptide antibodies with synthetic peptide SP-2. Neutralizing antisera were raised to both HTLV-I peptides SP-2 and SP-3/4A in two goats (no. 20 and 21). To determine which peptide, SP-2 or SP-3/4A, induced neutralizing antibodies, both antisera were adsorbed with equimolar amounts of peptides and then tested for the ability to inhibit HTLV-I-induced syncytium formation. Peptide SP-2 (■), but not SP-3/4A (○) or SP-7 (▲), adsorbed most of the neutralizing antibodies in both sera antibodies in a dose-dependent manner, as shown by the increasing numbers of HTLV-I-induced syncytia. The results indicated that antibodies to SP-2 can inhibit HTLV-I-induced syncytium formation.

DP-104 (aa 88 to 94) could not. Peptide SP-2L-91 (aa 91 to 98) adsorbed nearly 100% of the neutralizing antibodies from the serum from goat 20 but only 33% of those from the serum from goat 21, suggesting either that antibodies in these sera bound to slightly different epitopes or that the antibody affinities for the peptides differed between these two sera. These data allowed further mapping of a minimal neutralizing site of the HTLV-I envelope to aa 91 to 98.

Mutational analysis of the amino-terminal HTLV-I neutralizing domain. To determine which amino acids between aa 88 and 98 were required for peptide adsorption of neutralizing antibodies, the amino acids within this sequence were each sequentially replaced by alanine, and the resulting 11 peptides with single amino acid substitutions were used to adsorb antisera from three goats (20, 21, and 128) with anti-HTLV-I neutralizing antibodies to SP-2 (Fig. 6 and 7).

It was found that the asparagines at positions 93 and 95 were each required for adsorption of all three neutralizing antisera (Fig. 6A and 7). Also, the lysine in position 90 was required for peptide adsorption of neutralizing antibodies in the antisera from goats 21 and 128, whereas the proline in position 92 was needed to adsorb the antiserum from goat 20.

To evaluate further the effects of amino acid changes at HTLV-I envelope protein residues 90, 93, and 95, additional peptides were synthesized in which the lysine at position 90 (K-90) was replaced by either arginine (R-90) or glutamic acid (E-90) and the asparagines at positions 93 and 95 (N-93 and N-95) were both replaced either by aspartic acids or by serines. Equimolar amounts of peptides were used to adsorb anti-HTLV-I neutralizing antibodies in the anti-peptide antisera from goats 20 and 21, which were then tested in the HTLV-I syncytium assay (Fig. 6B). Replacement of K-90 with E-90 reduced the adsorptive capacity of peptide SP-2L-

FIG. 4. Adsorption of neutralizing anti-peptide antibodies with peptides and recombinant proteins. Peptides containing HTLV-I envelope protein amino acid sequences between aa 83 and 109 were used to adsorb neutralizing anti-peptide antibodies in serum from goat 20 (solid bars), serum from goat 21 (hatched bars), and normal serum (not shown). Adsorbed sera were then tested for the ability to inhibit HTLV-I-induced syncytium formation. Percent adsorption of neutralizing anti-peptide antisera or normal serum with saturating amounts of peptides was calculated by counting syncytia as described in Materials and Methods. Three to four separate adsorption experiments were performed per peptide. Error bars show standard deviations. Recombinant HTLV-I envelope fragments RE1 and RE3 were also used to adsorb goat 20 immune serum and normal serum, and the percent adsorption was calculated as described above. See Table 1, footnote b, for explanation of amino acid shown in parentheses.

1.3E by about 20 to 30%, whereas replacement of K-90 by a similarly charged R-90 had little or no measurable effect on peptide adsorption. In contrast, replacement of both N-93 and N-95 either by negatively charged aspartic acids or by serines reduced peptide adsorption by 60% or more. These results further supported the importance of these residues in peptide adsorption.

Mapping of HTLV-I and HTLV-II type-specific neutralizing sites. Three amino acids are different within homologous regions of the HTLV-I and HTLV-II envelope that are defined by peptide SP-2L-1 (aa 88 to 98). To evaluate whether amino acids unique for HTLV-II would restrict the ability of HTLV-I peptides to adsorb neutralizing anti-peptide antisera to HTLV-I, peptides that contained the HTLV-I envelope sequence aa 88 to 98 with one of three different HTLV-II amino acid substitutions (peptides SP-2.2, SP-2.8, and SP-2.10) as well as a peptide containing all three amino acids unique to HTLV-II (HTLV-II peptide SP-2.2/10) were synthesized (Table 3). Peptide SP-2.2, containing the HTLV-I to HTLV-II threonine to isoleucine change at position 89, could still adsorb neutralizing antibodies from anti-HTLV-I sera from goats 20 and 21. In contrast, when HTLV-I amino acid changes were made at positions 95 (asparagine to glutamine [N→Q]) and 97 (glycine to leucine [G→L]), peptides SP-2.8 and SP-2.10 both lost the ability to adsorb anti-HTLV-I-neutralizing antibodies. These data suggest that the amino acids at positions 95 and 97 are important for conferring HTLV-I and HTLV-II type specificity to these regions.

Neutralization of HTLV-II with anti-peptide antisera. We next tested whether synthetic peptide DP-90 (Table 1) could induce neutralizing antibodies to HTLV-II. Anti-HTLV-II antisera raised in two goats (120 and 135) to DP-90-tetanus toxoid conjugates neutralized HTLV-II but not HTLV-I in syncytium inhibition assays with titers of 1:40 and 1:20,

FIG. 5. Adsorption of neutralizing anti-peptide antiserum from goat 20 with peptides containing HTLV-I envelope aa 91 to 98. Neutralizing antiserum 20 was incubated with equimolar, saturating amounts of peptide and then tested for the ability to inhibit HTLV-I-induced syncytium formation (see Fig. 4 legend). Peptides containing HTLV-I envelope aa 91 to 98 (SP-2, SP-73, SP-2L, SP-2L-1, SP-2L-90, and SP-2L-91) adsorbed neutralizing antibodies, as shown by the emergence of HTLV-I-induced syncytia in culture. Peptides lacking this sequence (SP-2R and DP-104) did not adsorb neutralizing antibodies.

respectively (not shown). Neutralizing antibodies to HTLV-II in both antisera could be adsorbed by the immunizing peptide (DP-90) but not by the homologous HTLV-I peptide (SP-2L-1). To evaluate which amino acids were important for determining HTLV-II-specific neutralization, anti-HTLV-II antiserum from goat 120 was adsorbed with equimolar amounts of three peptides bearing single amino acid substitutions that reflect unique residues in the HTLV-II envelope protein. The antisera were then tested for the ability to neutralize HTLV-II syncytium formation (Fig. 8). Only peptides with the native HTLV-II sequence (DP-90 and SP-2.2/8/10) or with either an N→Q (position 95) or G→L (position 97) change in the HTLV-I sequence could adsorb neutralizing anti-HTLV-II antibodies. This study confirmed that these amino acid changes were important in conferring HTLV-II type specificity to this region.

DISCUSSION

In this study, antisera raised to synthetic peptides were used to study the homologous amino-terminal neutralizing regions of HTLV-I and HTLV-II envelope glycoproteins. Anti-peptide antisera raised to 10 of 12 peptides reacted with the HTLV-I envelope in either the Western blot or RIPA. Low titers of neutralizing antibodies raised to peptides SP-2 (aa 86 to 107), SP-3 (aa 176 to 189), and SP-4A (aa 190 to 209) were detected in the VSV-HTLV-I pseudotype assay but not in the syncytium inhibition assay. Prior studies with human monoclonal antibody 0.5α (33, 48) and murine monoclonal antibodies to gp46 (61) have mapped neutralizing domains to aa 186 to 195 and 191 to 196, respectively. Our data obtained in the pseudotype assay tended to support the

functional importance of regions contained within aa 176 to 209, although our rabbit anti-peptide antisera were not able to inhibit HTLV-I syncytium formation, possibly because of low antibody titers to neutralizing sites within aa 176 to 209.

Higher-titered neutralizing antisera were raised in four goats immunized with peptides SP-2 and combined peptide SP-3/4A (aa 176 to 209). The majority of neutralizing antibodies could be adsorbed by SP-2 or truncated peptides bearing an N-terminal portion of SP-2 (aa 90 to 98), whereas most neutralizing antibodies were not adsorbed by equimolar amounts of peptides lacking this region. Substitution with alanine of the naturally occurring asparagine at either position 93 or 95 interfered with the ability of the peptides to adsorb neutralizing antisera raised to SP-2. In addition to the asparagines at positions 93 and 95, the lysine at position 90 was required for adsorption of two anti-HTLV-I neutralizing antisera (from goats 21 and 128), while the proline at position 94 was needed for adsorption of neutralizing antibodies from the third anti-peptide antiserum (from goat 20). The results indicate that the majority of neutralizing antibodies in the serum from goat 20 recognize, at least in part, a region within aa 88 to 98 (PNRN) that is contained within a site (KKP NRN) that bound antibodies in the sera from goats 21 and 128.

The homologous amino acid sequences of HTLV-I and HTLV-II envelope glycoproteins differ by three amino acids within the envelope region defined by HTLV-I aa 88 to 98. Three peptides that contained the HTLV-I amino acid sequences with one amino acid unique to the homologous HTLV-II sequence were synthesized, while a fourth peptide contained the homologous HTLV-II sequence with all three amino acid changes. These peptides were then used to

FIG. 6. HTLV-I peptide mutagenesis. (A) To evaluate which HTLV-I envelope amino acids between aa 88 and 98 were important for adsorption of neutralizing antibodies, peptides SP-2L-1.1 through SP-2L-1.11 were synthesized, each containing an alanine substitution for each of the amino acids in the native sequence (see Table 1). The peptides were then used to adsorb neutralizing anti-peptide antisera from goats 20, 21, and 128. Adsorbed antisera were then tested in HTLV-I syncytium inhibition assays. The asparagines (N) at positions 93 and 95 were found to be important for adsorption of all three anti-peptide antisera. In addition, the lysine (K) at position 90 was important for adsorption of sera from goats 21 and 128, whereas the proline (P) at position 92 appeared to contribute to the adsorption of serum from goat 20. Since sera 21 and 128 recognized the K at position 90 while serum 20 did not, sera 21 and 128 reacted with a different epitope than did serum 20. The results are representative of independent experiments performed twice with peptides SP-2L-1.1 through SP-2L-1.11 and once with peptides SP-2L-1 (Table 1), SP-2L-1.1 (W-88→A), SP-2L-1.3 (K-90→A), SP-2L-1.5 (P-92→A), SP-2L-1.6 (N-93→A), and SP-2L-1.8 (N-95→A). Peptide SP-2L-1 contains the native HTLV-I sequence (bar at left of each graph). (B) Adsorption of neutralizing anti-peptide antibodies to HTLV-I with peptides substituted at positions 90, 93, and 95. Changing either the charge (K-90→E) or the amino acid side chain (K-90→R) at position 90 did not substantially affect peptide adsorption of neutralizing anti-HTLV-I antibodies. However, changing charge (N-93, N-95→D-93, D-95) or amino acid side chains (N-93, N-95→S-93, S-95) at positions 93 and 95 did alter the ability of peptides SP-2L-1.6/8D and SP-2L-1.6/8S to adsorb neutralizing antibodies in sera 20 (solid bars) and 21 (hatched bars).

adsorb neutralizing antisera raised to the HTLV-I sequence. Critical amino acid changes reflecting the presence of type-specific neutralizing regions of HTLV-I and HTLV-II were identified at HTLV-I envelope aa 95 (N→Q) and 97 (G→L). These changes governed the ability of the peptides to adsorb anti-peptide neutralizing antibodies to HTLV-I or HTLV-II. This was surprising, because both sets of amino acid changes are conservative. The presence of a type-specific neutralizing determinant in this region was confirmed with antisera raised to the homologous HTLV-II peptide DP-90. Antisera to this peptide neutralized HTLV-II but not HTLV-I in the syncytium inhibition assay; moreover, antibodies in these HTLV-II-specific antisera could be adsorbed only by peptides containing an amino acid sequence (HTLV-II aa 84 to 94) present in the HTLV-II envelope. Remarkably, replacement of N-95 by Q in peptide SP-2.8 resulted in no net change in charge and an increase of only 14 Da (one methylene group) in the molecular mass of the amino acid side chain, yet peptide SP-2.8 could specifically adsorb neutralizing anti-peptide antibodies to HTLV-II but not to HTLV-I. These studies thus defined the sequences that contributed to type-specific neutralizing epitopes within envelope aa 88 to 98 of HTLV-I gp46 and aa 84 to 94 of HTLV-II gp46.

HTLVs can be grouped phylogenetically with the simian T-cell leukemia viruses and bovine leukemia virus (BLV) (8, 19). While the envelope glycoproteins of HTLV-I and BLV are only 30% identical, Rice et al. (49) have reported that the envelope hydrophilicity profiles of these retroviruses are very similar, suggesting similar folding patterns. Moreover, seven cysteines have been conserved between BLV gp60 and HTLV-I gp63, suggesting similar patterns of disulfide cross-linking. Using anti-peptide antisera, Portet et al. (47) have mapped the neutralizing domains of BLV gp51 to central (aa 144 to 157) and amino-terminal (aa 78 to 92) regions. The locations of the BLV neutralizing domains correspond to the neutralizing sites of HTLV-I defined previously (48, 61) and in the present study. For example, a neutralizing site of HTLV-I which binds the human monoclonal antibody 0.5α is located in a region with homology to the central neutralizing site of BLV gp51 (48, 49). While the amino-terminal neutralizing sites of HTLV-I defined in this study lack homology to the amino-terminal neutralizing site of BLV, both neutralizing sites of the BLV envelope (aa 78 to 92) and HTLV-I (aa 88 to 98) are located in similar positions relative to the predicted N terminus of the respective molecules. Thus, the distributions of amino-terminal and central neutralizing epitopes within HTLV-I gp46 and BLV gp51 appear to be similar. Taken together with the previous observations of Rice et al. (49), these data further suggest fundamental similarities in the structure and function of HTLV-I gp46 and BLV gp51.

In a preliminary study, sera from HTLV-I- and HTLV-II-infected individuals were tested in an ELISA for reactivity to the amino-terminal neutralizing sites of HTLV-I and HTLV-II gp46 within peptides SP-2L-1 (HTLV-I envelope aa 88 to 98) and SP-2.2/8/10 (HTLV-II envelope aa 82 to 97). Only 2 of 40 (5%) HTLV-I-seropositive individuals and 1 of 16 (6%) HTLV-II-seropositive individuals made antibodies to these regions, whereas, with the same sera, 78% of HTLV-I-seropositive serum donors had antibodies to HTLV-I peptide SP-4A (aa 190 to 209) and 94% of HTLV-II-seropositive donors had antibodies to HTLV-II peptide II-3 (aa 177 to 199). The results indicate that these homologous neutralizing regions of HTLV-I and HTLV-II gp46 are only weakly immunogenic in infected individuals. Lal et al.

FIG. 7. No adsorption of neutralizing anti-HTLV-I antibodies with peptides mutated at N-93 or N-95. Aliquots of anti-peptide antiserum 20 were adsorbed with equimolar amounts of synthetic peptide (B) wild-type (WT) SP-2L-1, (C) SP-2L-1.6 (N-93→A-93), or (D) SP-2L-1.8 (N-95→A-95), and antisera were then tested for the ability to block HTLV-I-induced syncytium formation. Peptides in which the N-93 or N-95 asparagine was changed to an alanine were unable to adsorb neutralizing antibodies, as shown by a lack of syncytia in panels C and D. In contrast, peptide SP-2L-1 adsorbed neutralizing antibodies that block HTLV-I-induced syncytium formation (B). (A) HTLV-I-producing C91/PL T cells and C8166 T cells coincubated in the absence of immune serum and peptide.

(30) have previously reported that a peptide containing HTLV-II envelope aa 85 to 102 was recognized by antibodies from 73% of HTLV-II-seropositive individuals. Our data indicate that an HTLV-II type-specific neutralizing domain between aa 84 and 94 is recognized by only 6% of HTLV-II-seropositive individuals. Taken together, our data and those of Lal et al. (30) suggest that a highly immunogenic

region of the HTLV-II envelope resides at or near aa 94 to 102. Our finding that 94% of HTLV-II-seropositive sera react to peptide II-3 (HTLV-II envelope aa 177 to 199) is in approximate agreement with the 97% seroreactivity reported by Lal et al. (30) for HTLV-II peptide ENV 202 (aa 173 to 209).

In previous studies, we have shown that it is possible to

TABLE 3. Amino acids that contribute to HTLV-I type-specific neutralization

Peptide	Envelope sequence ^a (amino acids)	Substitution ^b	Sequence ^c	Adsorption of HTLV-I- neutralizing antibody ^d	
				Serum 20	Serum 21
SP-2L-1	HTLV-I (88-98)	None (wild-type HTLV-I)	WTKKPNRNGGG	+	+
SP-2.2/8/10	HTLV-II (84-94)	None (wild-type HTLV-II)	WIKKPNRQGLG	-	-
SP-2.2	HTLV-I (88-98), HTLV-II (85)	T-89 _I →I-85 _{II}	WIKKPNRNGG	+	+
SP-2.8	HTLV-I (88-98), HTLV-II (91)	N-95 _I →Q-91 _{II}	WTKKPNRQGGG	-	-
SP-2.10	HTLV-I (88-98), HTLV-II (93)	G-97 _I →L-93 _{II}	WTKKPNRNLG	-	-

^a Sequences are from reference 56 for HTLV-I and reference 59 for HTLV-II.

^b Substitutions are indicated as follows: T-89_I→I-85_{II}, the T at position 89 in the HTLV-I gp46 sequence has been changed to an I, which is the native amino acid at position 85 in HTLV-II gp46.

^c Amino acids in HTLV-II gp46 that differ from those in HTLV-I gp46 are underlined.

^d +, greater than 90% increase in mean numbers of syncytia (duplicate wells) relative to mean numbers obtained with immune serum plus negative control peptide SP-7 (0% adsorption) and immune serum plus positive control peptide SP-2L-1 (100% adsorption); -, less than 10% increase in mean numbers of syncytia relative to controls.

FIG. 8. Antiserum to HTLV-II peptide DP-90 (Table 1) was adsorbed with peptides containing native, amino-terminal neutralizing sites for HTLV-I (SP-2L-1 [●]) or HTLV-II (DP-90 [■] and SP-2.2/8/10 [△]) and with HTLV-I peptides containing one of three amino acid substitutions unique to HTLV-II: □, SP-2.2 (T→I); ○, SP-2.8 (N→Q); and ▲, SP-2.10 (G→L). Neutralizing antibodies to HTLV-II were adsorbed by both HTLV-II peptides DP-90 and SP-2.2/8/10 and by HTLV-I peptides containing HTLV-II amino acids Q (position 95) and L (position 97), as shown by increased numbers of HTLV-II-induced syncytia. These two amino acids are thus important components of the HTLV-II-specific neutralization site.

elicit neutralizing antibody responses to human immunodeficiency virus type 1 (HIV-1) with a chimeric synthetic peptide containing a predefined T-helper site linked N-terminally to a neutralizing site recognized by B cells (13, 42). In this way, it was possible to eliminate the need to link peptides to a heterologous carrier molecule in order to elicit neutralizing antibody responses to HIV-1. In a preliminary study, we have also constructed a similar T-cell/B-cell chimera by linking a known T-helper site (27) of HTLV-I gp46 (aa 190 to 209) to the N terminus of the neutralizing site defined here. After three immunizations of two goats with this peptide (DP-91 [Table 1]), neutralizing antibodies to SP-2 were detected in both animals (data not shown), suggesting that peptides containing a T-helper site and the neutralizing B-cell site of HTLV-I can be used to elicit neutralizing antibodies without linkage to a carrier molecule. If higher-titered neutralizing antisera can be obtained, such constructs could be useful for testing as prototype vaccines in animal models of HTLV-I infection.

Sequence similarity was observed and β -turn secondary-structure predictions were made between the neutralizing domains of HIV-1_{RF} gp120 between envelope aa 321 and 326 (TKGPGR) and the amino-terminal neutralizing domains of HTLV-I and HTLV-II gp46 between envelope aa 89 and 94 (HTLV-I, TKKPNR) and 86 and 90 (HTLV-II, IKKPNR). The sequence of HIV-1_{RF} gp120, like the HTLV-I and HTLV-II SP-2 regions, resides in the middle of the V3 loop region, which contains dominant type- and group-specific neutralizing determinants (11, 24, 31, 40, 54). β -Turns are predicted for this portion of V3 (31) and for similar sequences of HTLV-I and HTLV-II gp46 (4). The arginines at position 325 in HIV-1_{RF} gp120, position 94 in HTLV-I gp46, and position 90 in HTLV-II gp46 are also conserved. In the case of gp120, the arginine at position 325 is thought to provide a site for proteolytic cleavage of gp120 by a T-cell surface trypsinlike protease after gp120 has bound to the

CD4 receptor (6, 14, 23). Whether these similarities signify similar functions of the SP-2 region and the V3 loop of gp120 is not yet known. The V3 loop is critical for determination of HIV cellular tropism (16) and for sensitivity to neutralization by the soluble HIV receptor CD4 (17). Thus, it will be important to determine whether the gp46 SP-2 regions on HTLV-I and HTLV-II have functions analogous to that of V3 in gp120 when the HTLV-I and HTLV-II cellular receptors are identified.

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