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Induction of Antibody Responses That Neutralize Human T-Cell Leukemia Virus Type I Infection In Vitro and In Vivo by Peptide Immunization

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In order to define neutralization regions on the envelope antigen of human T-cell leukemia virus type I (HTLV-I), we have generated a number of new anti-envelope gp46 monoclonal antibodies from rats and mice. Epitopes recognized by new monoclonal antibodies which could neutralize HTLV-I in syncytium and transformation inhibition assays were localized to sequences in gp46 from amino acids 186 to 193, 190 to 195, 191 to 195, 191 to 196, and 194 to 199. Ovalbumin-conjugated synthetic gp46 peptides containing these neutralization epitopes, pep190-199 (a synthetic gp46 peptide containing amino acids 190 to 199) and pep180-204, but not pep185-194 or pep194-203, could give rise to HTLV-I-neutralizing antibody responses in rabbits. These immune or nonimmune rabbits were then challenged with HTLV-I by intravenous inoculation with 5×10^7 live HTLV-I-producing ILT-8M2 cells. By a PCR assay, it was revealed that HTLV-I provirus was detected in peripheral blood lymphocytes from nonimmune and pep288-312-immunized rabbits, whereas the provirus was not detected in peripheral blood lymphocytes from pep190-199- and pep180-204-immunized rabbits over an extended period. These results suggest that the induction of anti-gp46 neutralizing antibody responses by immunization with synthetic peptides has the potential to protect animals against HTLV-I infection in vivo.

Human T-cell leukemia virus type I (HTLV-I) is a type C retrovirus etiologically associated with a human malignant T-cell disorder, adult T-cell leukemia (4, 10, 33, 45), and some neurologic disorders (12, 28). The main route of HTLV-I infection is from infected mothers to their children by breast-feeding (9, 17, 20, 26). Transfusion of HTLV-I-contaminated blood and sexual intercourse with HTLV-I-infected persons can result in horizontal HTLV-I transmission (27, 38). HTLV-I has two glycoproteins encoded by its *env* gene, one of which is a major external glycoprotein with a molecular mass of 46 kDa (gp46) noncovalently linked to the other transmembrane glycoprotein, gp21 (8, 34). Both glycoproteins are also expressed on the surfaces of HTLV-I-bearing cells and have been implicated to be involved in the viral infection (18, 25, 32, 35, 43–45).

There are social requirements for developing a protective vaccine against HTLV-I infection, including the facts that epidemic infection with HTLV-I is now recognized to be worldwide, the cumulative lifetime risk of developing adult T-cell leukemia or HTLV-I-associated myelopathy or tropical spastic paraparasis is about 5%, there are 10 to 20 million infected people (7, 15, 23, 24, 37), and coinfection with HTLV-I and HIV accelerates progression to AIDS (3, 29). On the basis of results that animals are protected from HTLV-I infection by immunization with either recombinant HTLV-I envelope antigen (25) or HTLV-I *env* gene-expressing recombinant vaccinia virus (env-vac) (35), it is apparent that induction of anti-HTLV-I envelope-specific immune responses was critical for protection. These observations, together with the finding that passive immunization of animals with human anti-HTLV-I antibodies with HTLV-I-neutralizing activity is protective against HTLV-I infection in vivo (16), indicated that anti-HTLV-I envelope antibodies with neutralizing capabilities are protective effectors against HTLV-I infection in vivo. Therefore, a protective vaccine should be able to induce neutralizing antibody responses.

A new approach for constructing a vaccine is the utilization of synthetic peptides which constitute the relevant neutralization epitopes of infectious agents (1, 21). In order to design such a protective peptide vaccine against HTLV-I, it is first necessary to identify the immunodominant neutralization epitope(s). Lines of evidence suggested that there are multiple neutralization epitopes on the envelope antigens. We have identified for the first time one of the neutralization epitopes of HTLV-I envelope gp46 antigen which mapped to amino acid residues 191 to 196 and demonstrated that immunization of rabbits with a synthetic peptide containing this sequence could give rise to a gp46-specific neutralizing antibody response (43). Recently, new neutralization epitopes have been mapped to gp46 sequences corresponding to amino acids 187 to 193 and 193 to 199 by using human monoclonal antibodies (MAbs) (2, 19) and corresponding to amino acids 90 to 98 by using rabbit antipeptide sera (31).

In order to find additional neutralization epitopes on the gp46 molecule, we have attempted to generate new MAbs from several strains of rats and mice by immunization with various antigen preparations. In the present study, we have generated a number of new neutralizing MAbs for which epitopes were mapped to the central region of gp46 between amino acids 186 to 199. We have also evaluated the efficacies

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TABLE 1. Synthetic gp46 peptides used in this study

Peptide	Amino acid sequence	M_w
pep180-204	PSQLPPTAPPLLPHSNLDHILEPSI	2,684
pep180-189	PSQLPPTAPP	1,004
pep182-191	QLPPTAPPLL	1,046
pep183-192	LPPTAPPLLP	1,015
pep184-193	PPTAPPLLPH	1,039
pep185-194	PTAPPLLPHS	1,029
pep186-195	TAPPLLPHSN	1,046
pep187-196	APPLLPHSNL	1,057
pep188-197	PPLLPHSNLD	1,102
pep189-198	PLLPHSNLDH	1,142
pep190-199	LLPHSNLDHI	1,158
pep191-200	LPHSNLDHIL	1,158
pep192-201	PHSNLDHILE	1,174
pep193-202	HSNLDHILEP	1,174
pep194-203	SNLDHILEPS	1,124
pep195-204	NLDHILEPSI	1,150

of several gp46 synthetic peptides containing the neutralization epitopes in protecting animals from HTLV-I infection in vivo.

MATERIALS AND METHODS

Animals. LEW rats were purchased from Charles River, Kanagawa, Japan, and WKA rats, C57BL/6 (B6) mice, BALB/c mice, and New Zealand White rabbits were purchased from SLC, Shizuoka, Japan. F_1 hybrid rats derived from LEW and WKA parents were bred in the Institute of Laboratory Animal Science, School of Hygienic Sciences, Kitasato University. HTLV-I-challenged animals were maintained in P3-level facilities of Kitasato University and Institute for Virus Research, Kyoto University.

Cells. HTLV-I-bearing T-cell lines used were human T-cell lines MT-2 (22) and ILT-8M2 (14). HTLV-I-negative (HTLV-I^-) cell lines used were human T-cell line Molt-4 (36), mouse myeloma cell line Sp2/0-Ag14, and simian virus 40-transformed rat kidney cell lines W7KSV and L8KSV (41). All cell lines, except for ILT-8M2, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (referred to as RPMI-1 medium). ILT-8M2 cell lines were cultured in RPMI-1 medium supplemented further with 40 U of human interleukin 2 (Shionogi, Osaka, Japan) per ml. An HTLV-II-bearing cell line, Mo-T (13), was obtained from the American Type Culture Collection (Rockville, Md.).

HTLV-I env-vac. Recombinant vaccinia virus containing the entire HTLV-I *env* gene, WR-*proenv1*, has been reported previously (35). For the expression of the envelope antigen, rat kidney cells were infected with env-vac for 1 h at a multiplicity of infection of 10 and incubated for 16 to 18 h at 37°C.

Peptides. Synthetic 10-mer peptides used in this study, listed in Table 1, were purchased from the Research Laboratory, Research and Development Division, Fujiya Co., Ltd. (Kanagawa, Japan). Some peptides were synthesized by the stepwise solid-phase procedure of *N*α9-fluorenylmetoxycarbonyl chemistry on an automated peptide synthesizer (PSSM-8; Shimazu, Kyoto, Japan). All peptides used were greater than 90% pure as judged by high-pressure liquid chromatographic analysis (not shown).

Generation of MAbs. Animals were primed with two different antigen preparations. (i) Adult female F_1 rats and B6 mice were injected subcutaneously with sonicated L8KSV cells (10^7 cells per animal) which had been infected previously with WR-*proenv1* at a multiplicity of infection of 10 for 16 h. Two months later, the animals were injected intravenously (i.v.) with partially purified gp46 (50 μg per animal) obtained from MT-2 cell culture supernatants by affinity column chromatography as described previously (42). (ii) Female WKA rats were immunized with synthetic peptides conjugated to ovalbumin (OVA) as described below. Two months later, each animal was injected with 50 μg of carrier-free peptides. Immune spleen cells were isolated 3 days after the final booster immunization. The spleen cells and Sp2/0-Ag14 cells were fused by means of polyethylene glycol 1500, and hybridomas were grown selectively in hypoxanthine-aminopterin-thymidine (HAT) medium. Hybridomas producing HTLV-I-neutralizing antibodies, as determined by syncytium inhibition assays, were cloned by limiting dilutions, using BALB/c mouse thymocytes as feeder cells. MAbs were purified from ascitic fluid by using Sephadex G-200 or protein G columns.

Peptide immunization. Each gp46 peptide (10 mg) was conjugated to OVA (10 mg) in 1.5 ml of water containing 30 mg of 1-ethyl-3-dimethylaminopropyl-carbodiimide hydrochloride at room temperature for 2 h and used after extensive dialysis against water and phosphate-buffered saline (PBS). Conjugates (0.5 mg per animal) emulsified in complete (day 0) or incomplete (day 14, 28, and 42) Freund's adjuvant were injected intramuscularly into female New Zealand White rabbits or WKA rats. Immune sera from rabbits were collected on day 56.

Immunofluorescence and Western immunoblot analysis. Live cells and cells that were smeared onto glass slides and fixed with methanol for 5 min at −20°C were stained with MAbs by an indirect immunofluorescence method, using fluorescein isothiocyanate-labeled goat immunoglobulin G (IgG) anti-rat or anti-mouse IgG (Zymed Laboratories, South San Francisco, Calif.) as the secondary reagent, as described previously (42). Fluorescent cells were examined by flow cytometry with an Epics Profile II (Coulter) or by fluorescence microscopy. Western blot analysis was performed as described previously (42). Briefly, cell lysates or glycoprotein fractions from cell lysates of cell-free culture supernatants which were collected with concanavalin A-conjugated Sepharose 4B (Pharmacia) in the sample buffer were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to blotting sheets. Binding of MAbs was visualized as described previously (42).

Enzyme-linked immunosorbent assay (ELISA). Synthetic peptides at 5 μg/ml, gp46 antigen at 10 μg/ml, or OVA at 5 μg/ml, diluted in PBS, was dispensed into the wells of round-bottom 96-well plates (catalog number 25802; Costar) (50 μl per well) and incubated at 4°C overnight. The wells, saturated with 25% Block Ace (Dainippon Pharmaceutical, Tokyo, Japan) in PBS (200 μl per well) at 37°C for 2 h, were incubated with antibody samples for 30 min at room temperature. After the wells were washed with PBS containing 0.05% Tween 20, they were incubated with horseradish peroxidase-labeled goat IgG anti-rat IgG, anti-mouse IgG, or anti-rabbit IgG (Tago, Burlingame, Calif.) for 30 min at room temperature. Enzyme activities bound to wells were measured in 0.1% H_2O_2 and 0.2 mg of 2,2′-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ATBS) per ml in 0.1 M sodium citrate buffer, pH 5.5, by a plate reader at 405 nm.

Syncytium inhibition assay. HTLV-I syncytium inhibition assay was described previously (42, 43). Briefly, ILT-8M2 cells were suspended in RPMI-1 medium at 10^6 cells per ml, aliquots (50 μl per well) were incubated with 100-μl portions of heat-inactivated MAb culture supernatants, purified MAbs, human and rabbit sera, or medium alone in wells of U-bottom 96-well plates (catalog number 25850 Corning) at 37°C for 15

TABLE 2. Neutralizing anti-gp46 MAbs generated from rats and mice

MAb	Origin	Immunogen[a]	Minimum neutralizing concentration (μg/ml)[b]	Reactivity[c] with gp46 in:	
				ELISA	Western blot
LAT-27	WKA rat	env-vac	5	+	+
F1-194	(WKA × LEW) F_1 rat	env-vac	50	+	+
B1E191N43	B6 mouse	env-vac	10	+	+
W180/2	WKA rat	pep180-204	4	+	+
W180/7	WKA rat	pep180-204	200	+	−
W185/4	WKA rat	pep185-194	60	+	−

[a] Animals were immunized with HTLV-I *env*-expressing recombinant vaccinia virus (env-vac) or synthetic gp46 peptides conjugated to OVA (pep185-194 or pep180-204).
[b] Minimum antibody concentration at which HTLV-I-mediated syncytium formation is completely inhibited.
[c] Reactivity to natural gp46 purified from MT-2 cell culture supernatants in ELISA and Western blot assays. +, reactive; −, not reactive.

min, and then 50 μl of Molt-4 cell suspension (10^6 cells per ml) was added to each well. After incubation at 37°C for 16 h in a 5% CO_2 incubator, each well was examined for syncytia (giant multinuclear cells) with an inverted microscope. Neutralization titers of antibody samples were expressed as the reciprocal of the sample dilution at which the syncytium formation was completely (100%) inhibited in the microcultures. Because HTLV-I-producing ILT-8M2 cells apparently form syncytia with Molt-4 cells, it was easy to examine for syncytium formation (2, 19, 43). We believe that the criteria for neutralization (100% inhibition) is more significant for evaluation of vaccines than 50% inhibition. Vaccines that fail to induce enough neutralizing antibodies and allow even one HTLV-I to enter into a host T cell will be useless.

Transformation inhibition assay. The abilities of MAbs and antipeptide antibodies to interfere with HTLV-I transformation were determined as described previously (43). Briefly, normal human peripheral blood lymphocytes (PBL) (1×10^6 cells per well) were cocultured with mitomycin-treated MT-2 cells (5×10^5 cells per well) in 2 ml of RPMI-1 medium in each well of a 24-well culture plate (catalog number 3424; Costar) in the presence or absence of purified antibodies. Duplicate wells were established for each examination. Half the volume of medium in each well was changed twice weekly with fresh RPMI-1 medium with or without antibodies. During a 1-month culture period, wells were observed for cell transformation, as defined by continuous proliferation of cells expressing HTLV-I Gag protein p19 or $p40^{tax}$ antigen. To confirm that each antibody does not inhibit the growth of HTLV-I-transformed cells or phytohemagglutinin-stimulated PBL, MT-2 cells (2×10^5 cells per ml) in RPMI-1 medium and PBL (1×10^6 cells per ml) in 0.1% phytohemagglutinin M-containing RPMI-1 medium were cultured in the presence or absence of antibodies for 4 days, and total viable cell numbers were counted.

HTLV-I challenge and detection of provirus by PCR. Live ILT-8M2 cells were washed with PBS and injected i.v. into rabbits (5×10^4 to 5×10^7 cells per animal). These rabbits were maintained in a P3-level facility. Every 3 months postinfection, PBL were isolated from heparinized blood samples by the density separation medium for rabbit lymphocytes, Lympholyte-Rabbit (Cedarlane Laboratories, Hornby, Ontario, Canada).

DNA samples were prepared by sodium dodecyl sulfate-proteinase K treatment, followed by phenol and chloroform extraction from PBL, and 0.5-μg DNA samples were subjected to PCR analysis. The primers used for the first step of PCR were 5'TTCCCAGGGTTTGGACAGA3' (HTLV-I positions 7307 to 7325) and 5'GGGTAAGGACCTTGAGGGTC3' (HTLV-I positions 7571 to 7552), and those used for the second step of PCR were 5'CGGATACCCAGTCTACGT3' (HTLV-I positions 7336 to 7353) and 5'GAGCCGATAACG CGTCCATCG3' (HTLV-I positions 7494 to 7474). The final PCR mixture (25 μl) consisted of 0.25 mM each of dATP, dCTP, dTTP, and dGTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin, 0.75 U of *Taq* polymerase (Cetus, Emeryville, Calif.), and 100 ng of each oligonucleotide. The reaction mixture was overlaid with 25 μl of mineral oil and subjected to 30 cycles of PCR: denaturation for 1 min at 94°C, annealing for 1 min at 58°C (62°C in the first cycle), and polymerization for 1 min at 72°C. Samples (2 μl) of the product from the first step of PCR were subjected to the second PCR step under the same conditions. The PCR products (159 bp) were analyzed by electrophoresis on 8% polyacrylamide gels and stained with ethidium bromide.

RESULTS

Generation and characterization of HTLV-I-neutralizing MAbs. We have observed that BALB/c mice and LEW rats did not produce detectable HTLV-I-neutralizing antibodies upon immunization with HTLV-I env-vac or purified gp46 antigen and that all the neutralizing MAbs generated so far from WKA rats immunized with env-vac were directed against a single determinant within gp46, amino acids 191 to 196 (40a). In order to obtain new HTLV-I-neutralizing MAbs, we attempted to generate MAbs from F_1 hybrid rats from mating WKA and LEW strains and B6 mice immunized with env-vac. Because WKA rats have a strong response as measured by neutralizing levels, we also attempted to generate new MAbs from WKA rats immunized with gp46 peptides pep185-194 and pep180-204. From these experiments, a number of HTLV-I-neutralizing IgG-type MAbs were generated.

Table 2 summarizes data of these new MAbs concerning their origins, minimum IgG concentrations required for HTLV-I neutralization in syncytium inhibition assay, and reactivities to gp46 antigen in ELISA and Western blot assays. Two MAbs, B1E191N43 and W180/2, inhibited HTLV-I-mediated syncytium formation at an antibody concentration of less than 10 μg/ml. In contrast, higher concentrations of IgG were required for F1-194, W185/4, and W180/7. All the new MAbs reacted to gp46 antigen as determined by an ELISA. However, antipeptide MAbs W185/4 and W180/7 did not bind to denatured gp46 antigen in Western blot assay.

Epitope mapping for these new neutralizing MAbs is shown in Fig. 1. Among 12 neutralizing MAbs generated from the F_1 rats, 10 MAbs showed similar reactivities to a control LAT-27 MAb, specific for gp46 amino acids 191 to 196 (43). The other two MAbs (represented by F1-194) showed a different pattern

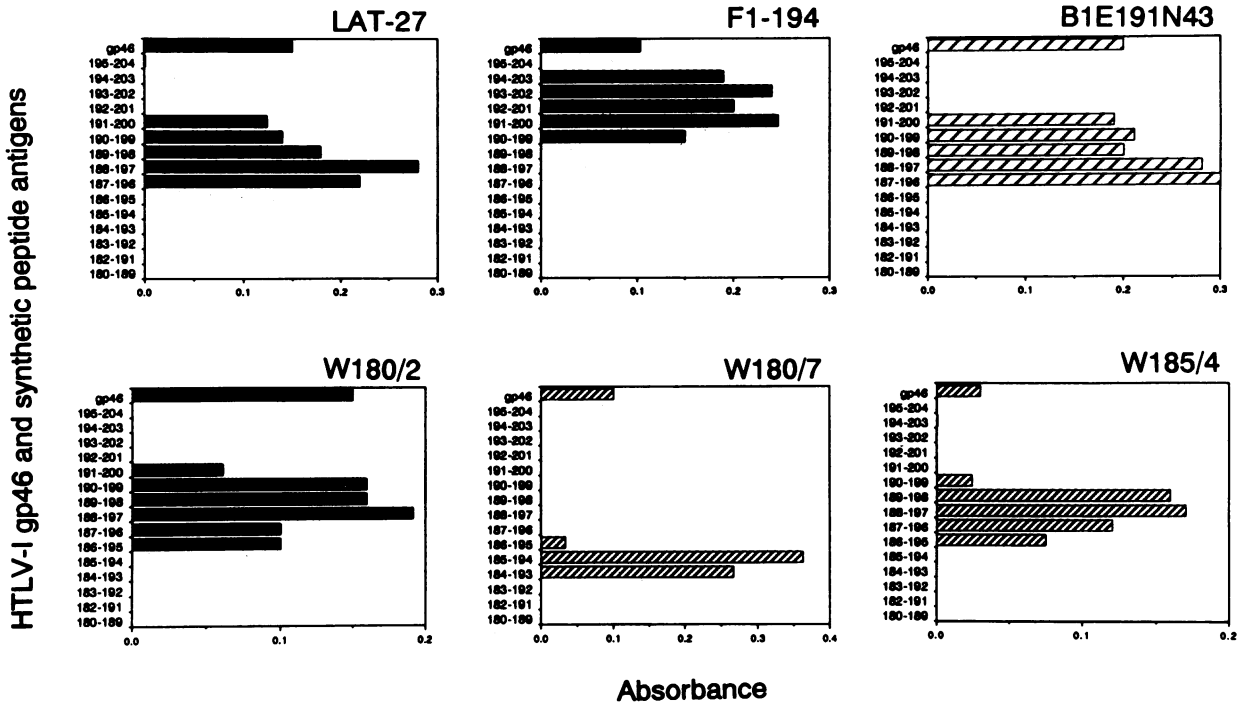

FIG. 1. Epitope mapping of HTLV-I-neutralizing MAbs. Plastic wells coated with various gp46 synthetic peptides (pep prefixes have been omitted in the figure) and native gp46 were incubated with each MAb. Binding of MAb was examined by using horseradish peroxidase-labeled goat IgG anti-mouse or rat IgG. LAT-27, which recognizes gp46 amino acids 191 to 196, was used as a positive control.

of reactivity. On the basis of its reactivity, the epitope recognized by F1-194 was localized to a new region, gp46 amino acids 194 to 199. All 10 neutralizing MAbs generated from the B6 mice reacted with the peptide panel similar to the pattern seen for LAT-27 MAb, indicating that these MAbs (represented by B1E191N43) recognize gp46 amino acids 191 to 196. These MAbs are the first neutralizing MAbs of mouse origin. Epitopes recognized by W185/4, W180/2, and W180/7 which were generated from WKA rats immunized with gp46 synthetic peptides were mapped to gp46 amino acids 186 to 193, 190 to 195, and 191 to 195, respectively. The present results, together with our previous findings (2, 19, 43), show that the central region of gp46 between amino acids 186 to 199 is a special region within which multiple neutralization epitopes appear to cluster.

The cell specificities of these new MAbs were further characterized by an immunofluorescence assay followed by flow cytometry (Table 3). All the neutralizing MAbs stained HTLV-I-positive (HTLV-I$^+$) cells but not HTLV-II$^+$ cells or HTLV-negative (HTLV$^-$) cells. The MAbs generated by env-vac immunization stained almost all the cells of MT-2 and ILT-8M2, but the other MAbs (W185/4, W180/2, and W180/7) generated by peptide immunization, stained only one- to two-thirds of cells of the HTLV-I$^+$ cell lines.

Induction of neutralizing antibody responses. Several synthetic peptides encompassing all or part the major neutralization region were tested for immunogenicity by induction of HTLV-I-neutralizing antibodies. New Zealand White rabbits were immunized with pep180-204, pep185-194, pep190-199, or pep194-204. All the peptides were conjugated to carrier protein OVA. OVA-conjugated pep288-313 and OVA alone were used as negative-control immunogens. Sera collected from these peptide-immunized rabbits reacted to respective peptides used as immunogens and OVA at high titers, as determined by ELISA (data not shown). As shown in Table 4, these antipeptide sera, except for anti-pep185-194 sera, contained high titers of anti-gp46 antibody. Among these sera, those from pep180-204- and pep190-199-immunized rabbits neutralized HTLV-I at dilutions of 1:8 to 1:16.

The HTLV-I-neutralizing activity was also determined in a transformation inhibition assay. IgG purified from sera of N-1 and N-2 rabbits immunized with pep180-204, and the MAbs LAT-27 (anti-gp46 amino acids 191 to 196), F1-194 (anti-gp46 amino acids 194 to 199), and W185/4 (anti-gp46 amino acids 186 to 193), inhibited the outgrowth of HTLV-I-transformed cells from normal PBL cocultured with HTLV-I$^+$ MT-2 cells in

TABLE 3. Immunofluorescence staining of the surface of HTLV-I$^+$ cells by anti-gp46 MAbs[a]

MAb	% Positive cells (mean fluorescence intensity[b])			
	HTLV-I$^+$		HTLV-II$^+$ Mo-T	HTLV-I$^-$ HTLV-II$^-$ Molt-4
	MT-2	ILT-8M2		
LAT-27	99.9 (9.8)	92.7 (6.8)	<0.5	<0.5
F1-194	99.8 (7.1)	92.8 (5.6)	<0.5	<0.5
W185/4	53.1 (2.9)	53.4 (3.8)	<0.5	<0.5
W180/2	68.9 (2.8)	43.6 (2.8)	<0.5	<0.5
W180/7	33.4 (2.5)	33.5 (2.8)	<0.5	<0.5
B1E1191N43	100.0 (15.4)	99.8 (10.7)	<0.5	<0.5
B1E288-12[c]	100.0 (11.5)	99.3 (8.5)	99.9 (12.6)	<0.5

[a] Negative control was the washing buffer.
[b] A linear scale was used in measuring mean fluorescence intensity.
[c] B1E288-12 is a B6 mouse MAb that recognizes HTLV-I gp46 amino acids 288 to 312 and cross-reacts to HTLV-II gp46 (40a).

TABLE 4. Induction of HTLV-I-neutralizing antibody by immunization with synthetic gp46 peptides in rabbits

Rabbit	Immunogen	Anti-gp46 titer[a]	Neutralization titer[b]
N-1	pep180-204	11,000	32
N-2	pep180-204	31,000	16
N-3	pep180-204	12,000	8
N-4	pep180-204	25,000	8
N-7	pep288-313	40,000	<2
N-8	pep288-313	70,000	<2
U-1	pep185-194	<100	<2
U-2	pep185-194	<100	<2
U-3	pep185-194	<100	<2
R-2	pep190-199	20,000	16
R-4	pep190-199	22,000	16
F-1	pep194-204	30,000	<2
F-2	pep194-204	30,000	<2
F-3	pep194-204	4,000	<2
YT-14	OVA alone	<100	<2
R-5	OVA alone	<100	<2
R-6	OVA alone	<100	<2

[a] Antibody titer was expressed as the reciprocal of serum dilution at which optical density at 405 nm of samples was greater than five times the background optical density obtained with diluent in the same test.
[b] Reciprocal of serum dilutions at which HTLV-I-mediated syncytium formation was completely inhibited.

a dose-dependent manner (Table 5). These antibodies did not inhibit growth of MT-2 cells or phytohemagglutinin-activated normal human T cells (data not shown). These results indicate that the inhibitory activity of syncytium formation and that of transformation activity of antibody correlate.

In order to examine epitopes recognized by antipeptide sera, their reactivities to a panel of gp46 peptides were examined by ELISA. The results in Fig. 2A suggest that rabbits immunized with pep180-204 produced heterogeneous antibodies reactive to various epitopes on pep180-204. In contrast, rabbits immunized with short peptides, pep190-199 and pep194-203, produced antibodies to restricted regions, amino acids 191 to 196 and 195 to 199, respectively (Fig. 2B).

Protection of rabbits from HTLV-I infection. The neutralizing antibody-positive rabbits immunized with pep190-199 and pep180-204, and negative-control rabbits immunized with pep288-313, OVA alone, or PBS alone, were challenged with HTLV-I by i.v. injection of $\sim 5 \times 10^7$ HTLV-I$^+$ ILT-8M2 cells. Every three months, PBL from the rabbits were examined for HTLV-I infection by a PCR assay. As shown in Fig. 3 and summarized in Table 6, the HTLV-I provirus was detected in the DNA samples from nonimmune and OVA- and pep288-313-immunized rabbits but has not been detected in PBL from pep190-199- and pep180-204-immunized rabbits for 3 and 12 months, respectively. The minimum dose of ILT-8M2 cells required for establishment of HTLV-I infection in the present rabbit system was less than 5×10^6 cells per animal.

DISCUSSION

The present in vivo HTLV-I challenge experiment showed that rabbits immunized with either HTLV-I gp46 pep190-199 or pep180-204 were protected from i.v. HTLV-I infection. Particularly, in the pep180-204-immunized animals, the provirus has not been detected for 12 months postinfection. Thus, the present study provides the first evidence that peptide-induced neutralization antibody responses are protective against primary i.v. cell-associated HTLV-I infection. This protective effect may be comparable to those induced by immunization with either recombinant HTLV-I envelope antigen (25) or HTLV-I env-vac (35).

Our strategy to identify neutralization epitopes has been to generate and characterize various MAbs with neutralization activity. We succeeded in identifying a number of neutralization epitopes on the envelope gp46 protein with rat and human MAbs. These epitopes include gp46 amino acids 187 to 193, 191 to 196, and 193 to 199 (2, 19, 43). In the present study, additional new neutralization epitopes were identified, which include gp46 amino acids 186 to 193, 190 to 195, 191 to 195, and 194 to 199. Key protocols for success in generating new MAbs may be both the choice of animal species and strains and the use of HTLV-I env-vac and synthetic peptides as immunogens. On the basis of these and our previous observations, it appears that the central region of gp46 between amino acids 186 to 199 contains multiple overlapping HTLV-I neutralization epitopes. Therefore, we conclude that a synthetic peptide vaccine against HTLV-I should contain this amino acid sequence.

It is of interest that the neutralization avidity of MAbs varied from one another. For complete inhibition of HTLV-I-mediated syncytium formation in the present conditions, 10 μg of antibody per ml was required for LAT-27, B1E191N43, and W180/2, whereas higher concentrations of antibody were required for F1-194, W185/4, and W180/7. These differences in antibody concentrations for neutralization cannot be explained by differences in epitope locations. Binding affinities of MAbs to each neutralization epitope of gp46 may influence the neutralization avidity.

We have generated a number of MAbs reactive with HTLV-I gp46 pep288-313 from mice and rats. These MAbs stained the surface of HTLV-I$^+$ cells and reacted with gp46 antigen in a Western blot assay. However, none of these MAbs neutralized HTLV-I in vitro in a syncytium inhibition assay. In

TABLE 5. Inhibition of HTLV-I transformation of normal human T cells by antipeptide and MAb antibodies[a]

Purified IgG antibody	Transformation[b] of HTLV-I$^+$ cells at the following antibody concentration (μg/ml):							
	1,000	500	250	125	62	31	16	8
N-1 rabbit anti-pep180-204	−	−	+	+	+	+	+	+
N-2 rabbit anti-pep180-204	−	−	+	+	+	+	+	+
YT-14 rabbit anti-OVA alone	+	+	+	+	+	+	+	+
LAT-27 (anti-env gp46 amino acids 191–196)	−	−	−	−	−	−	−	−
F1-194 (anti-env gp46 amino acids 194–199)	−	−	−	−	+	+	+	+
W185/4 (anti-env gp46 amino acids 186–193)	−	−	−	−	+	+	+	+
LAT-25 (anti-env gp46 amino acids 288–312)	+	+	+	+	+	+	+	+

[a] Normal human PBL and mitomycin-treated MT-2 cells were cocultured in the presence of each antibody for 1 month. HTLV-I transformation was determined by immunofluorescence assay of growing cells with anti-HTLV-I Tax and Gag MAbs.
[b] −, not transformed; +, transformed.

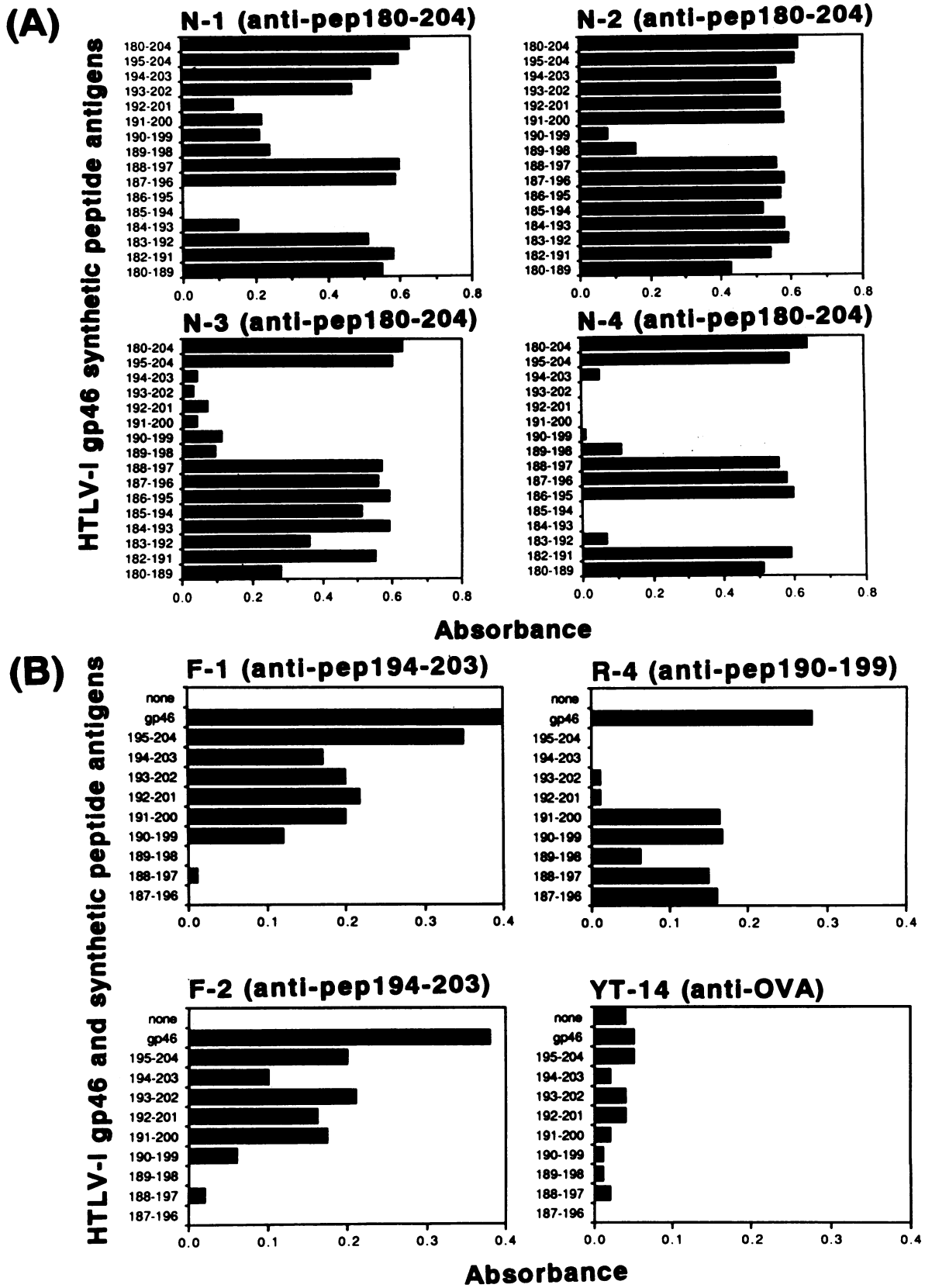

FIG. 2. Reactivities of rabbit antipeptide sera to gp46 and gp46 synthetic peptides. Plastic wells coated with various peptides (pep prefixes have been omitted in the figure) and gp46 were incubated with rabbit sera at 1:100 dilution. Binding of rabbit antibodies was examined by using horseradish peroxidase-labeled goat IgG anti-rabbit IgG. (A) Sera from rabbits immunized with pep180-204. (B) Sera from rabbits immunized with pep194-203, pep190-199, or OVA.

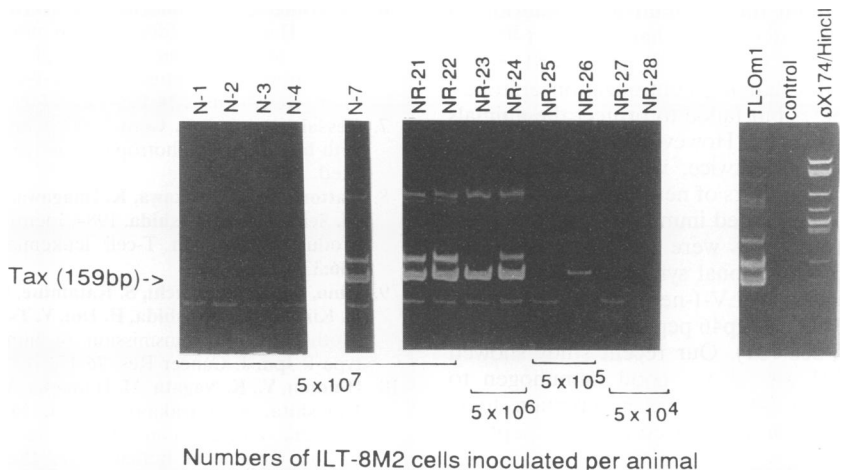

FIG. 3. Detection of HTLV-I provirus in PBL samples from HTLV-I-infected rabbits by PCR assay. An HTLV-I *tax* region (159 bp) was amplified from 0.5-μg DNA samples by a nested PCR. Amplified products were analyzed by electrophoresis on 8% polyacrylamide gels and visualized by staining with ethydium bromide. Rabbits immunized with pep180-204 (N-1, N-2, N-3, and N-4) or pep288-312 (N-7) and nonimmunized rabbits were infected with HTLV-I by i.v. inoculation with various numbers of ILT-8M2 cells, and PBL samples were examined for HTLV-I provirus 12 months postinfection. DNA from HTLV-I$^+$ human TL-Om1 cells was used as a positive control.

addition, rabbits immunized with OVA-conjugated pep288-313 produced anti-gp46 antibodies that were not able to neutralize HTLV-I as shown in Table 4. In contrast, the recent study done by Desgranges et al. (6) indicated that this region might be a putative neutralization domain, because preincubation of diluted HTLV-I$^+$ human serum with gp46 pep287-311 partially reduced the neutralizing activity of human serum in a syncytium inhibition assay. It is possible that the C-terminal region of gp46 may contain a neutralization epitope, which is recognized by human B cells and less immunogenic to rodents.

TABLE 6. Protection of rabbits from in vivo HTLV-I infection by synthetic peptide immunization[a]

Expt and rabbit	Immunogen	No. of ILT-8M2 cells inoculated per animal	Provirus[b] in PBL at the following time (mo) postinfection:			
			3	6	9	12
Expt 1						
R-2	pep190-199	5×10^7	−	ND	ND	ND
R-4	pep190-199	5×10^7	−	ND	ND	ND
R-5	OVA	5×10^7	+	ND	ND	ND
R-6	OVA	5×10^7	+	ND	ND	ND
Expt 2						
N-1	pep180-204	5×10^7	−	−	−	−
N-2	pep180-204	5×10^7	−	−	−	−
N-3	pep180-204	5×10^7	−	−	−	−
N-4	pep180-204	5×10^7	−	−	−	−
N-7	pep288-313	5×10^7	+	+	+	+
N-8	pep288-313	5×10^7	+	ND	ND	ND
NR-21	None	5×10^7	+	+	+	+
NR-22	None	5×10^7	+	+	+	+
NR-23	None	5×10^6	+	+	+	+
NR-24	None	5×10^6	+	+	−	+
NR-25	None	5×10^5	−	−	−	−
NR-26	None	5×10^5	−	−	+	+
NR-27	None	5×10^4	−	−	−	−
NR-28	None	5×10^4	−	−	−	−

[a] Each rabbit was inoculated i.v. with viable HTLV-I-producing ILT-8M2 cells. PBL were collected periodically, and then DNA (0.5 μg) from the PBL was examined for HTLV-I provirus by PCR using a *tax* gene primer pair as shown in Fig. 3.

[b] −, no provirus detected; +, provirus detected; ND, not done.

The mechanisms of HTLV-I neutralization by anti-gp46 antibodies remain to be revealed. One possible mechanism may be direct blockade of gp46 interactions with an as yet undefined HTLV-I receptor. The facts that multiple neutralization epitopes are clustered to a particular stretch of amino acids (gp46 amino acids 186 to 199) may support this idea. Alternatively, but not mutually exclusively, a conformational change in gp46 structure rather than direct blockade of a gp46 functional site(s) by specific binding of neutralizing antibodies may be a mechanism of HTLV-I neutralization. This hypothesis is supported by the fact that the introduction of single-amino-acid substitutions at multiple positions of HTLV-I envelope proteins gp46 and gp21 influenced the syncytium formation activity of HTLV-I (32). Because another neutralization epitope has been mapped to gp46 amino acids 90 to 98 by using antipeptide sera (31), it is possible that this site and the central neutralization region are located very close to each other on the native gp46 molecule.

In the present study, the OVA-conjugated peptides pep190-199 and pep180-204 were shown to give rise to anti-gp46 antibodies with neutralizing activity in the rabbit system. The other two peptides tested, pep194-203 and pep185-194, were not able to induce detectable neutralizing antibodies, although pep194-203 was effective in eliciting high titers of HTLV-I gp46-specific antibodies. These results indicate that even though a peptide may contain a neutralization epitope(s) and be recognized by a neutralizing MAb(s), it is not always true that such a peptide can induce neutralizing antibody responses. Several explanations are possible to explain the failure of pep185-194 and pep194-203. First, antibody titers against relevant epitopes of these antipeptide sera may not be sufficient for HTLV-I neutralization, because neutralization by either W185/4 or F1-194 MAb required more than 60 μg of IgG per ml (final concentration). Second, the anti-pep185-194 and anti-pep194-203 antibodies may not bind to the exact neutralization epitopes of the native gp46. It is also possible that nonneutralizing epitopes may become immunodominant

in oligopeptides even though they contain a predetermined neutralization epitope(s). Indeed, Takehara et al. (40) reported that immunization of rabbits with gp46 pep175-196, which contained several overlapping neutralization epitopes, induced anti-gp46 antibodies but failed to protect the animals from HTLV-I challenge in vivo. However, they only immunized rabbits with the peptide twice, which might not be sufficient to induce detectable titers of neutralizing antibodies. In the present experiment, repeated immunizations with peptides at least three or four times were necessary to induce neutralizing antibody. The additional synthetic peptides that have been reported to elicite HTLV-I-neutralizing antibodies in rabbits or goats were HTLV-I gp46 pep86-107, pep176-189, pep190-209, and pep176-206 (31). Our recent study showed that the gp46 pep185-204 was also a good immunogen to induce neutralizing antibodies (40a). However, it remains to be tested whether antibody responses induced by these peptides are protective in vivo.

Unlike HIV, it has been shown that sequence variation of HTLV-I *env* gene among various strains is very limited (5, 30). Recently, it has been shown that even Melanesian strains of HTLV-I, which exhibited relatively high divergence of the *env* gene sequence by ~7% from Japanese HTLV-I strains, were neutralized by MAb LAT-27, anti-gp46 amino acids 191 to 196 (11). These facts support the feasibility of peptide vaccines. Furthermore, induction of multiple neutralization epitope-specific neutralizing antibodies by a peptide vaccine will offer many advantages for the neutralization not only of prototype viruses but also for variants that may arise.

A protective role for maternal anti-HTLV-I antibodies in children born to HTLV-I-carrying mothers has been indicated (39). Therefore, vaccination of pregnant HTLV-I carriers with safe peptide vaccines capable of inducing or enhancing the production of HTLV-I-specific neutralizing antibodies, followed by passive immunization of their children with maternal neutralizing IgG, may reduce mother-to-child transmission of HTLV-I. This hypothesis will be determined in a future study. Further studies are in progress to design antigenic structures of peptide that elicit long-lasting and effective titers of neutralizing antibodies without using harmful adjuvant and carrier proteins.

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