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Linear Antigenic Regions of the Structural Proteins of Human T-Cell Lymphotropic Virus Type I Detected by Enzyme-Linked Immunosorbent Assays Using Synthetic Peptides as Antigens

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We synthesized 46 sequential peptides 21 to 39 amino acids long over the structural protein of human T-cell leukemia virus type I (HTLV-I; the p19 and p24 *gag* protein and the gp46 and p20E *env* proteins) and tested their reactivities against antibodies in sera from HTLV-I healthy carriers and patients diagnosed as having human T-cell leukemia-lymphoma (ATLL) and myelopathy (HAM) by using an enzyme-linked immunosorbent assay. Of the 46 synthetic peptides, 18 peptides (2 corresponding to the p19 *gag* protein, 2 corresponding to the p24 *gag* protein, 8 corresponding to the gp46 *env* protein, and 6 corresponding to the p20E *env* protein) reacted with antibodies in the sera from HTLV-I healthy carriers. In particular, the peptides comprising amino acids 100 to 119 and 119 to 130 of the *gag* and 175 to 199, 213 to 236, 253 to 282, and 288 to 317 of the *env* proteins reacted with antibodies in sera from more than 30% of HTLV-I healthy carriers. These peptides also showed high reactivities to the antibodies in the sera from patients with ATLL and HAM. The results indicate that the predominant antigenic regions of the structural protein of HTLV-I were located at the C-terminal end of the p19 *gag* protein and the C-terminal half of the gp46 *env* protein, and the corresponding peptides proved to be useful antigens in detecting antibodies in the sera from individuals infected with HTLV-I.

Human T-cell leukemia virus type I (HTLV-I) is closely associated with adult T-cell leukemia-lymphoma (ATLL) (3, 5, 10, 14) and human adult myelopathy (HAM)-tropical spastic paraparesis (4, 9). This virus is known to be transmitted from mother to infant by breast feeding (2), from husband to wife by sexual intercourse (13), and by blood transmission (8). In these transmissions, the HTLV-I infection is confirmed by the detection of antibodies to several gene (*gag*, *pol*, *env*, and *X*) products of HTLV-I. Sato et al. (11) reported that in recipients that were seroconverted by blood transfusion, immunoglobulin M (IgM) antibody against gp46 was first detected about 2 weeks after receiving blood, IgM and IgG antibodies to p19 and p24 were found in weeks 4 to 6, and then IgG antibody to gp46 was found about 9 weeks after transfusion. These results suggest that individuals infected with HTLV-I may show various antibody profiles to HTLV-I associated with the transmission routes and the amount of the virus acquired at the time of exposure. Accordingly, it is important to analyze the antibody profile of individuals infected with HTLV-I so that the most useful antigenic region of HTLV-I for diagnosis of HTLV-I infection can be identified.

We have previously shown that the synthetic peptides derived from the putative amino acid sequence of HTLV-I is a useful antigen in the detection of antibodies against HTLV-I (6). Therefore, using the sequential synthetic peptides which cover the structural proteins of HTLV-I, we were able to analyze the antibody profiles of HTLV-I healthy carriers and patients diagnosed as having ATLL and HAM.

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MATERIALS AND METHODS

Sera. Serum specimens from 96 blood donors in Fukuoka Red Cross Blood Center, 48 patients with ATLL, and 19 patients with HAM were used in this study. Patients with ATLL or HAM were physician diagnosed. All serum samples gave positive results in anti-HTLV-I antibodies tests by using geratin-particle aggulutination assay (Serodia HTLV-I; Fujirebio, Tokyo, Japan) and indirect immunofluorescence assay with acetone-fixed MT-2 cells on a slide (3).

Peptide synthesis. Peptides were synthesized according to predicted amino acid sequences on the basis of nucleotide sequences of the *gag* and *env* gene of HTLV-I (12). Peptide synthesis was performed by solid phase methods (7) with an automated peptide synthesizer (431A Peptide Synthesizer; Applied Biosystem, Foster City, Calif.). Protected amino acids were obtained from the Protein Institute (Osaka, Japan). The peptides were cleaved from resin with trifluoromethanesulfonic acid according to the manufacturer's instructions and lyophilized. After being dissolved in 5% acetic acid, the peptide solution was applied to a Sephadex G-25 column equilibrated with 5% acetic acid and eluted with the same solution. The purity of individual peptides was examined by reverse-phase high-performance liquid chromatography with an octadecyl (C_{18})-silicated column. The majority of the peptides were isolated as a single peak and were used for enzyme-linked immunosorbent assay (ELISA) without further purification.

ELISA. The wells of a 96-well microtiter plate (Costar, Cambridge, Mass.) were coated with 500 ng of each synthetic peptide dissolved in 0.01 M $NaHCO_3$ buffer (pH 9.55) at 4°C overnight. Untreated sites on the solid phase were blocked with 10% goat serum in 0.01 M phosphate-buffered saline (PBS), pH 7.2, at room temperature for 3 h. Next, serum specimens diluted 1:8 with PBS containing 10% goat

serum and 0.05% Tween 20 were added to the coated microtiter wells and incubated at 37°C for 1 h after six washes with PBS containing 0.05% Tween 20. The plate were further incubated with goat antibodies to human IgG conjugated with horseradish peroxidase (MBL Ltd., Nagoya, Japan) at 37°C for 30 min. The plates were washed six times and developed with 150 μl of 0.1% H_2O_2 and 0.04% *ortho*-phenylenediamine in 0.1 M sodium citrate buffer (pH 5.5). The reaction was stopped by the addition of 50 μl of 2.5 M H_2SO_4, and the color development was measured in a plate reader (SLT Labinstrument, Salzburg, Austria) at 492 nm. The cutoff level used to determine seropositivity was calculated by statistical analysis as the mean value of 31 serum samples which were PA negative and IF negative plus 3 standard deviations. In a competitive ELISA for confirmation, serum specimens preincubated with 5 μg of synthetic peptides or PBS at 37°C for 30 min were added to microtiter wells coated with the peptide. A serum sample showing more than a 30% decrease of absorbance in the competitive ELISA was judged to be positive.

RESULTS

To determine the antigenic regions of the structural proteins of HTLV-I, we synthesized (with an automated peptide synthesizer) 46 sequential peptides 11 to 39 amino acids long, overlapping by 1 to 5 amino acids, over the p19 and p24 *gag* proteins and the gp46 and p20E *env* proteins. By using the ELISAs with 96 wells of microtiter plates coated with the peptides as antigens, we were able to test the peptides reactivities against antibodies in the sera from 96 HTLV-I healthy carriers. Figure 1 shows the percentage of incidence of the positive serum samples for the antibody response of the individual peptide. Of the 46 synthetic peptides tested, 18 (2 derived from the p19 *gag* protein, 2 derived from the p24 *gag* protein, 8 derived from the gp46 *env* protein, and 6 derived from the p20E *env* protein) reacted with antibodies in the sera from HTLV-I healthy carriers. In the group of the p19 *gag* protein, the peptides from amino acids 100 to 119 and 119 to 130, which corresponded to the C-terminal of the p19 *gag* protein, reacted with antibodies in 84 (85%) and 38 (39%), respectively, of the 96 serum samples. As described in a previous paper, the peptide comprising amino acids 100 to 130 corresponding to the C-terminal end 30 amino acids of the p19 *gag* protein reacted with antibodies in all serum samples from HTLV-I healthy carriers (6). These results indicate that the C-terminal end 30 amino acids has at least two dominant antigenic epitopes. In the p24 *gag* protein, the two peptides (amino acids 131 to 160 and 288 to 317) reacted with antibodies in the sera, but their reactivities were low (4 and 18%, respectively). Of 23 peptides derived from the *env* protein, 14 peptides (amino acids 20 to 49, 89 to 115, 175 to 199, 213 to 236, 253 to 282, 277 to 292, and 288 to 317 of the gp46 protein and 332 to 352, 350 to 386, 382 to 403, 400 to 429, 426 to 448, and 458 to 488 of the p20E protein) reacted with antibodies in the sera. In particular, the peptides comprising amino acids 175 to 199, 213 to 236, 253 to 282, and 288 to 317 of the *env* protein reacted with antibodies in 87 (91%), 30 (31%), 46 (48%), and 82 (86%), respectively, of the 96 serum samples from healthy carriers. These results indicate that dominant antigenic regions of the envelope protein of HTLV-I are located at the C-terminal half of the gp46 *env* protein containing putative *N*-glycosylation sites (14).

Subsequently we tested the peptide reactivity to antibodies in the sera from the patients diagnosed as having HTLV-I-associated ATLL and HAM. Table 1 summarizes the results by ELISA for the peptide reactivity against antibodies in sera. The peptides (amino acids 100 to 119 and 119 to 130 of the *gag* protein and 175 to 199 and 288 to 317 of the gp46 *env* protein) which showed a high reactivity to the antibodies in the sera from HTLV-I healthy carriers also showed high reactivities to antibodies in the sera from patients. On the whole, the levels of peptide reactivities for antibody binding in patients with HAM were higher than those in healthy carriers and patients with ATLL.

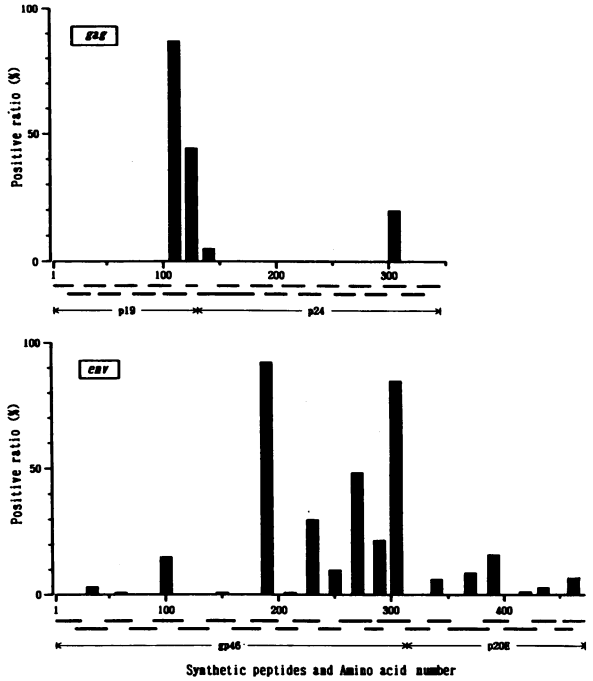

FIG. 1. Antigenic peptides in the *gag* and *env* proteins reacted with antibodies in sera from HTLV-I healthy carriers. Serum samples from 96 HTLV-I carriers were tested in ELISA by using individual peptides. The bars below the reactivity plots indicate the synthetic peptides. The residue numbers of the peptides are as follows: for the *gag* protein, 1 to 20, 15 to 34, 30 to 49, 43 to 62, 57 to 76, 73 to 92, 87 to 106, 100 to 119, 119 to 130, 131 to 160, 148 to 167, 161 to 180, 177 to 196, 190 to 209, 205 to 224, 220 to 239, 236 to 255, 251 to 270, 264 to 283, 279 to 298, 295 to 314, 312 to 331, and 325 to 344; for the env protein, 1 to 25, 20 to 49, 46 to 70, 68 to 92, 85 to 115, 111 to 138, 136 to 161, 159 to 183, 175 to 199, 197 to 216, 213 to 236, 235 to 254, 253 to 282, 277 to 292, 288 to 317, 313 to 333, 332 to 352, 350 to 386, 382 to 403, 400 to 429, 426 to 448, 445 to 462, and 456 to 488.

DISCUSSION

In the present study, we found that the peptides comprising amino acids 100 to 130 of the *gag* protein and 175 to 199 and 288 to 317 of the *env* protein were very sensitive antigenic peptides which reacted with the antibodies in the sera from HTLV-I healthy carriers and the patients diagnosed as having HTLV-I-associated ATLL and HAM.

These results indicate that the three peptides are useful antigenic peptides in ELISA for the detection of HTLV-I infections. However, one potential limiting factor in the use of synthetic peptides as an antigen in an immunoassay for mass screening is that a synthetic peptide may represent a single epitope. If an individual is infected with the virus but

TABLE 1. Peptide reactivities to antibodies in the sera from HTLV-I healthy carrier donors and patients with ATLL and HAM[a]

Peptide code	Amino acid sequences[b]	% Positive samples		
		Donor[c]	ATLL	HAM
p19 *gag* 100–119	PPPPSSPTHDPPDSDPQIPP	85	81	100
p19 *gag* 119–130	PPYVEPTAPQVL	39	62	94
p24 *gag* 131–160	PVMHPHGAPPNHRPWQMKDLQAIKQEVSQA	4	2	0
p24 *gag* 288–317	GLPEGTPKDPILRSLAYSNANKECQKLLQA	18	2	0
gp46 *env* 20–49	GDYSPSCCTLTIGVSSYHSKPCNPAQPVCS	3	2	0
gp46 *env* 89–115	TKKPNRNGGGYYSASYSDPGSLKCPVY	13	4	21
gp46 *env* 175–199	FLNTEPSQLPPTAPPLLPHSNLDHI	91	72	100
gp46 *env* 213–236	LVQLTLQSTNYTCIVCIDRASLST	31	64	94
gp46 *env* 235–254	STWHVLYSPNVSVPSSSSTP	8	25	52
gp46 *env* 253–282	TPLLYPSLALPAPHLTLPFNWTHCFDPQIQ	48	18	68
gp46 *env* 277–292	FDPQIQAIVSSPCHNS	22	43	52
gp46 *env* 288–317	PCHNSLILPPFSLSPVPTLGSRSRRAVPVA	86	66	94
p20E *env* 332–352	GGITGSMSLASGKSLLHEVDK	4	8	47
p20E *env* 350–386	EVDKDISQLTQAIVKNHKNLLKIAQYAAQNRRGLDLLF	7	4	26
p20E *env* 382–403	LDLLFWEQGGLCKALQEQCRFP	13	33	68
p20E *env* 400–429	CRFPNITNSHVPILQERPPLENRVLTGWGL	2	16	15
p20E env 426–448	GWGLNWDLGLSQWAREALQTGIT	4	10	5
p20E *env* 458–488	LAGPCILRQLRHLPSRVRYPHYSLIKPESSL	7	12	21

[a] The numbers of serum samples from healthy carriers and those with ATLL and HAM were 96, 48, and 19, respectively.
[b] Amino acids are represented by the single-letter code.
[c] The number of positive samples is the same as in Fig. 1.

fails to mount an antibody response to the particular epitope represented by the peptide, the immunoassay could give a false-negative result. To prevent this false-negative result in the immunoassay, two possible approaches may be considered: (i) the peptide selected for use as antigen in ELISA should correspond to an immunodominant epitope that elicits an antibody response in all infected individuals, and (ii) several different antigenic peptides should be combined in a single assay, or a single peptide that contains multiple epitope is used in a single assay. The peptide comprising amino acids 100 to 130, which corresponded to the C-terminal end 30 amino acids of the p19 *gag* protein, reacted with antibodies in all sera from individuals infected with HTLV-I as described in a previous paper, and this peptide also has two immunodominant epitopes. In this sense, the peptide comprising amino acids 100 to 130 of the *gag* protein may be a very useful peptide in detecting the HTLV-I infection. A second approach may be employed by using the peptide comprising amino acids 175 to 199 or 288 to 317 of the *env* protein as a second antigen. In our preliminary donor screening of the antibodies to HTLV-I by ELISA using the peptides comprising amino acid 100 to 130 of the *gag* protein and 175 to 199 of the *env* protein as antigens, 379 of 21,894 (1.7%) of the volunteer donors at the Fukuoka Red Cross Blood Center were judged to be positive. This result was also the same as results obtained by immunofluorescence assay with the MT-I cell and MT-2 cell lines (unpublished data).

The high antigenic regions of the structural protein of HTLV-I clustered at the C-terminal end 30 amino acids of the p19 *gag* protein and the C-terminal half of the gp46 protein. Recently, Chen et al. (1) reported that the recombinant p19 and p24 *gag* protein and the C-terminal region (amino acids 229 to 308) of the gp46 *env* protein were able to detect the antibodies in all eight serum samples from individuals found to be positive by particle agglutination assay, immunofluorescence assay and Western immunoblot analysis. The sensitive detection of the antibodies by using recombinant p19 *gag* and the C-terminal region of the gp46 *env* protein reported by them are in close agreement with data presented here. However, in the present study we failed to define the highly reactive peptides derived from the p24 *gag* protein for antibody binding. Although the discrepancy in reactivities for antibody binding by the synthetic peptides and the recombinant p24 *gag* protein could not be clarified, there are some possible explanations: (i) the synthetic peptide selected for use in this experiment did not cover an immunodominant epitope, (ii) the majority of antigenic determinants on the p24 *gag* protein may be conformational rather than sequential, and (iii) the antigen produced from infected tissue culture cells or by genetic engineering techniques with expression vectors inevitably contained some nonviral proteins that could have caused a false-positive assay in the results.

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