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Development and Evaluation of a Novel ELISA for Detection of Antibodies against HTLV-I Using Chimeric Peptides

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ABSTRACT

We aimed to develope a peptide-based indirect ELISA to detect antibodies against Human T-lymphotropic virus type I (HTLV-I). Two chimeric peptides (CP-1 and CP-2) were designed using linear immunodominant epitopes of gp-46-I, and gp21-I proteins, according to the sequence from Uniprot database.

These peptides were studied initially in the ELISA using infected sera. The most promising peptideCP-1, was used to develop a peptide ELISA for detection of HTLV-I infected sera. The optimal conditions for CP-1ELISA were: the optimum coating buffer was 100mM $NaHCO_3$, pH 9.6; coating peptide concentration was 10 μg/mL; the optimal blocking buffer was5% fetal bovine serum (FBS); the secondary antibody concentration was 1:2000; and serum dilution was 1:20. 20serum samples from HTLV-I infected patients were evaluated by ELISA developed. CP-1 showed high antigenicity while lacking any cross-reactivity with normal human sera.

The results of evaluations indicated that in comparison with commercial ELISA, CP-1 ELISA showed good sensitivity and specificity.

With further validation, CP-1as described in the present study could be introduced as novel reliable and cost-effective candidates for the high-specific screening of HTLV-I/-II infections in endemic regions.

Keywords: Antigenicity; gp-46-I; gp21-I; HTLV-I; p19; Synthetic peptides

INTRODUCTION

Human T-lymphotropic virus type I (HTLV-I) infection is associated with adult T-cell leukemia/lymphoma (ATLL) and HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP)diseases.[1,2] Most of HTLV-I infected individuals are asymptomatic and not aware of their infection.[3]

Such carriers transmit infection to healthy individuals through blood transfusion, breastfeeding, sharing of infected needles and sexual contact.[4]

The early detection of infection is the main strategy to limit the spread of HTLV-I. Viral-specific antibody

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detection is routinely used for HTLV-I screening. The recommended assay is ELISA because it is highly sensitive, very specific and suitable to analyze a large number of samples.[5]

Commonly used ELISA approaches use immunodominant peptides derived from virus glycoproteins as coating antigens to detect antibodies.[6] These peptides are prepared as recombinant Glutathione S-transferase (GST) fused protein in *E. coli* expression systems or synthesized by chemical methods.[7] The size of peptides in comparison to GST tag is very small (6 vs. 26 kDa). Due to the limited capacity of the solid phase to bind to antigens, in fusion proteins an inherent limitation is always imposed on epitope availability, thereby limiting sensitivity.[8] Also, antibodies against GST tag are present in serum samples of some healthy individuals. So, GST-fused antibodies are not suitable for use in indirect ELISA which has most sensitivity.[9] By using synthetic peptides as a coating antigen, higher density of epitopes on a solid surface may be achieved and sensitivity of ELISA may be enhanced. Also, the use of peptides could improve the specificity of diagnosis because synthetic peptides are free of contaminant proteins of expression host which remain in diagnostic reagents and may interfere with specificity of the assay.[10] However, there is no one peptide that could detect all HTLV-I-infected sera. Yamada et al showed that at least two peptides are most used together for detection of all HTLV-I positive sera.[11] Although two or more separate peptides could be coated on an ELISA microplate, the sensitivity of the test could be affected by the distribution of peptides bonded on the plate and equivalent binding of peptides on a solid surface.[10] For these disadvantages there is a general tendency to use a chimeric peptide instead of several distinct peptides.

In this study, for purposes of development of peptide ELISA, two chimeric peptides were designed using linear epitopes of gp46-I and gp21-I. Also, we synthesized the separate peptides identical to linear epitopes of HTLV-I proteins to compare with results of peptides containing single epitope of HTLV-I envelope glycoproteins.

MATERIALS AND METHODS

Serum Samples

The study was approved by the Ethics Committee of Sabzevar University of Medical Sciences. HTLV-I positive and negative sera were collected from blood donors referred to the Blood Transfusion Center of Mashhad city, Iran. All sera were evaluated by ELISA (PASTEO HTLV-I/II ELISA kits). The repetitive immune reactive specimens were analyzed by western blotting (MP biomedical) as confirmatory tests. If serum gives a positive result in western blotting it was considered as positive serum, otherwise serum was excluded from the study. Totally, 40 sera (20 HLTV-I infected and 20 negative) were selected and used in this study.

Chimeric Peptides Design and Synthesis

The selected sequences were linear epitopes of gp46-I (named H1: amino acids 173-207) and gp21-I (named H2: amino acid 374-400) were obtained from Uniprot database (www.Uniprot.org). The peptides were fused together by a glycine-rich flexible linker (sequence: GSGGSG), and two chimeric peptides were constructed which incorporating two epitopes in different order. The peptides were synthesized by PEPMIC (China) by purity of 95%.

Antigenecity Evaluation

To evaluate antigenecity of peptides, 50µL of diluted peptides (10 µg/mL) were coated on 96-well plates at 4°C overnight, followed by washing twice with phosphate buffered saline with Tween 20 (PBS-T) buffer. Then, 200 µL of blocking buffer (5% fetal bovine serum) was added to each well and incubated for 2h at 37°C and washed twice with PBS-T. Afterwards, 50 µL of the pooled HTLV-I infected serum with dilution of 1:20 was added to each well and incubated at 37°C for 1h, washed three times with PBS-T. 50 µL of secondary antibody (1:2000) was added and incubated at 37°C for 1h, and washed three times with PBS-T. Finally 50 µL of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate was added and incubated at room temperature for 15 min. The reaction was stopped by addition of 100 µL stop solution (H_2SO_4 2N) and absorption at OD450nm was measured by ELISA reader.

Optimizing of the Coating Buffer

Selected peptide (10 µg/mL) was dissolved in 100 mM $NaHCO_3$, pH 9.6 or 2 mM Tris-HCl, pH 8 buffers. To select optimal coating buffer, 50 µL of diluted peptide was coated on 96-well plates at 4°C overnight

and analyzed by ELISA, as described in the previous section. Optimal coating buffer was determined based on the results of OD450 nm and the value of P/N. The value of P/N was ratio of positive with negative, if the value is more than 2.1 then it is optimized.

Optimization of Blocking Buffer

200µL of different blocking buffer (2.4% skimmed milk, 1% BSA, 1% gelatin and 5% FBS) were added to each well and incubated at 37°C for 2h. Optimal blocking buffer was determined based on the results of OD450nm and the value of P/N.

Determination of Optimal Concentration of Peptides

Peptides were diluted to5, 10, 20, 30, 40, and 50 µg/mL. After overnight coating at 4°C, plates were washed three times with PBS-T and blocked by optimal blocking buffer at 37°C for 2h. The OD450 nm was measured to determine optimal concentration.

Optimization of HRP-Conjugated Secondary Antibody

HRP-conjugated rabbit anti-human antibody was used at dilutions 1:2000, 1:4000, 1:5000, 1:6000, 1:12000, 1:15000 in PBS-T buffer. Then 100 µL of these dilutions were added to each well and then incubated at 37°C for 1h, followed by adding 100µLTMB to each well and incubating the plates for 15min at room temperature. The absorbance of each well at OD450 nm was read on ELISA reader and the mean value of the wells was calculated.

2.8 Determination of the Cut-off Value

Twenty negative serum samples were tested by peptide ELISA, the mean value and standard deviation (S) of OD450nm were calculated. Cut-off value=+3 S was used to calculate the cut-off value.

Sensitivity Analysis

Three positive sera of different antibody levels were diluted 1:100 to 1:500 and then detected by the peptide ELISA to analyze the sensitivity.

Specificity Analysis

Specificity of peptide ELISA was determined with sera of patients positive to HCV, HBV and HIV and HTLV-I negative serums.

Comparison of Peptide ELISA with ELISA in Clinical Serum Samples

A total of 40 different serum samples were subjected to the peptide ELISA and results were compared with results obtained from a commercial ELISA test.

Statistical Analyses

Statistical analysis were conducted using SPSS version 23 for windows (IBM Corp., Armonk, N.Y., USA). The significance difference of chimeric antigen and commercial kits was analyzed by Fisher's test. $p<0.05$ were considered significant.

RESULTS

Design of Chimeric Peptide

The epitopes of HTLV-I structural proteins gp-46-I and gp21-I were selected for the preset study. These linear epitopes were synthesized as separate (H1 and H2) or chimeric peptides (CP-1 and CP-2). The sequences of CP-1 and CP-2 are the same but in different order. The sequence of peptides used in this study is shown in Table 1.

Evaluation of Antigenecity of Peptides

To evaluate the antigenecity of peptides, peptides were coated in the same concentration (10 µg/mL) on the plate. Reactivity was determined with pooled sera consisting of 10 pooled HTLV-I infected sera. Figure 1 shows reactivity of synthetic peptides against pooled sera. The results indicate that CP-1 gives higher 450 nm absorbance when compared with CP-2 and single peptides. Thus, CP-1 was selected as a candidate antigen and used to develop peptide ELISA.

Optimization of ELISA Procedure

On the basis of results of OD450 nm and value of P/N, 100 mM $NaHCO_3$, pH 9.6 buffer was confirmed as coating buffer and 5% FBS was chosen as blocking buffer. According to results of matrix titration, the optimal dilution of reagents was determined at 10µg/Ml for peptides, 1:20 for sera, and 1:5000 for secondary rabbit anti-human IgG. Using these dilutions, the best signal to noise ratios were obtained and applied in the ELISA.

Table 1. List of peptides containing specific epitopes of HTLV-I glycoproteins tested as ELISA antigens

Peptide	Sequence	Protein
H1	AAQNRRGLDLLFWEQGGLCKALQEQC	gp21-I
H2	IWFLNTEPSQLPPTAPPLLPHSNLDHILEPSIPWK	gp46-I
CP-1	H1-GSGGSG-H2	gp21-I/gp46-I
CP-2	H2-GSGGSG-H1	gp21-I/gp46-I

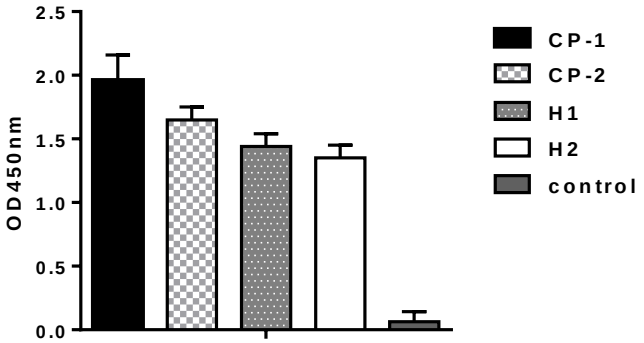

Figure 1.Comparison of OD450nm values of peptides tested with pooled human T-lymphotropic virus type I (HTLV-I) positive sera. The ELISA plates were coated as indicated with H1, H2, CP-1 and CP-2 and then incubated with 100 µLof infected sera and HRP-conjugated anti-Human IgG and TMB substrate as described in methods

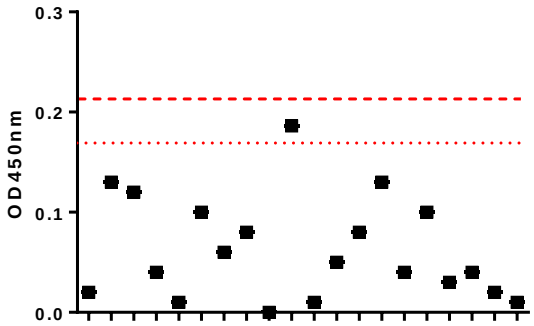

Figure 2.Plot of single mean of healthy blood donors. Dash line: cut-off above which those samples are regarded positive; dotted line: cut-off below which samples were regarded as negative. Samples showing OD450nm value between the lower and upper cut-off were considered questionable

Background Assessment and Cut-off Determination

Twenty negative sera were run in the CP-1 ELISA for background determinations. These sera had mean OD450nm 0.06±0.05. A cut-off point above which a sample was considered positive was calculated as the mean OD of negative population plus three standard deviations, so that cut-off value=(0.21) (Figure 2). Samples yield OD over 0.21were considered HTLV-I positive, while an OD450nm value below 0.2 was determined as HTLV-I negative.

Specificity of CP-1 ELISA

Specificity of CP-ELISA was assessed with the sera of positive to HBV, HCV, HIV and HTLV-I negative serum. The HBV, HCV, HIV and HTLV-I negative serums all verified negative by the CP-ELISA with

OD450 nm values of 0.157, 0.098, and 0.112, respectively. However, HTLV-I positive serums were detected to be IgG positive with OD450 nm value of 1.051 (Figure 3). Results showed that the CP-ELISA detected specifically HTLV-I positive serums.

Sensitivity of CP-ELISA

HTLV-I-infected serums were verified to be positive with a high titer of 1:700 using the CP-ELISA (cut-off=0.21) (Figure 4), indicating that the CP-1ELISA was highly sensitive.

Serological Validation of CP-1 ELISA

Twenty serum samples collected from the Blood Transfusion Center were subjected to commercial and CP-1 ELISA. HTLV-I-infected sera showed reactivity in CP-1 ELISA but health sera did not show any reactivity with CP-1 (Figure 5). The absorbance in CP-1 ELISA was compared with the results of commercial ELISA (Figure 6). There is no significant difference between OD450 nm absorption between the two ELISAs.

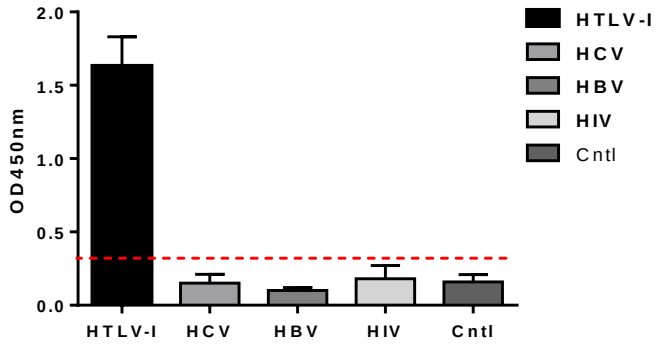

Figure 3. Specificity evaluation of CP-1 ELISA assay. Different sera collected from hepatitis C virus (HCV), hepatitis B virus (HBV) and human immunodeficiency virus (HIV)-infected patients tested by CP-1 ELISA. Each column represents a corresponding serum while the dashed line represents the cutoff value of CP-1 assay.

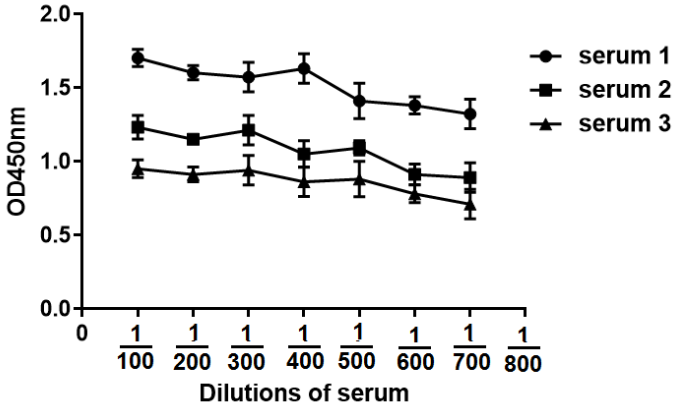

Figure4. Sensitive test of CP-1 ELISA. Different concentrations of the serum samples (1: 100 to 1: 700 dilution); the concentration of coatedCP-1 10µg/mL

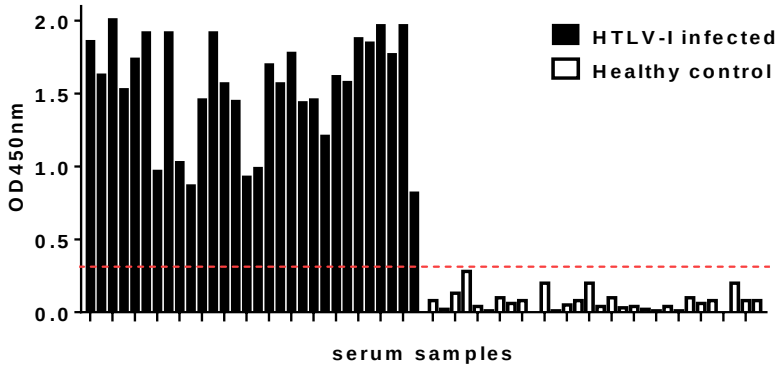

Figure 5.Evaluation of human T-lymphotropic virus type I (HTLV-I) infected and healthy control sera using CP-1 ELISA. Dash line is cut-off which above that serums considered HTLV-I positive.

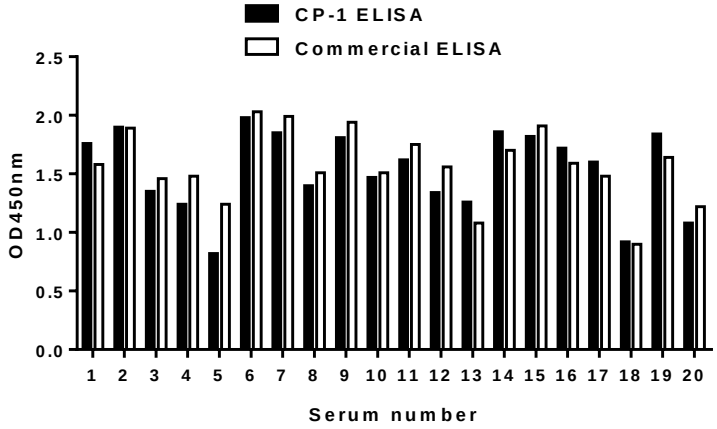

Figure 6.Comparison of OD450nm values between CP-1 ELISA and commercial ELISA kit.

DISCUSSION

The Yamada et al. report usage of the synthetic peptide for detection of HTLV-I antibodies.[11] They evaluated immunoreactivity different synthetic peptides from various proteins of HTLV-I protein with HTLV-I-infected sera. They suggested that peptide-derived C-terminal (100-130) of protein p19 has potent antigenicity, and antibody against this peptide is present in most HTLV-I-infected individuals. However, they found that 1 in 2000 infected sera had no antibody against the p19-derived peptide. They showed that if infected sera are evaluated simultaneously by p19 peptide and a peptide derived from a conserved region of gp46-I (190-213), all sera give a positive result.

Hernandez et al. design chimeric peptides using gp46-I, gp21-I and p19 proteins linear epitopes and a short amino-acid linker (sequence: GG) as spacer arm.[10,12] They showed that chimeric peptides could detect antibodies against more than one protein. Also, they showed chimeric peptides incorporating gp46-I and gp-21-I could detect all HTLV-I-infected sera. Also, they found antigenicity of chimeric peptides depends on epitope orientation.[12]Peptides with a gp46-I epitope in C-terminal showed more antigenicity than peptides with a gp46-I epitope in N-terminal. However, peptides designed by Hernandez et al were short and could not sufficiently absorb on a solid surface because short sequence form weak van der Waals interaction with the microplate surface which is a need to peptide absorption.[10] When they tried to increase the length of peptides to enhance absorption interaction, antigenicity

of peptides significantly decreased. They suggest that this may be due to epitope rupture or conformational hindrance resulting in antibody-antigen interaction being hindered.

It is presumed that GG amino-acid linker is not able to discriminate between two epitopes properly when the length of fragments increases, and the use of a flexible linker can avoid such difficulty. Therefore, we used a flexible linker (sequence: GSGGSG) as a spacer between epitopes to design chimeric peptides. In the previous study, we show this linker is able to discriminate epitopes in chimeric antigen properly.[13]We evaluated the antigenicity of chimeric peptide ELISA with pooled positive sera. The antigenicity of CP-1 when compared with chimeric peptides described previously indicated that the CP-1 yielded a higher OD450nm absorbance. This result indicates stronger antibody-antigen interaction and better accessibility of epitopes.

The antigenic activity of chimeric peptide was evaluated using 20 HTLV-I infected serum samples. According to Fig. 1, the chimeric peptide has high antigenicity and could detect all HTLV-I-infected sera. These results indicated that there is no significant difference between chimeric peptide and commercial kits that use recombinant protein for HTLV-I antibodies detection.

Finally, our results indicated that this peptide could be considered as a novel candidate for HTLV-I-infection screening, avoiding the cost of individual expression and purification of diagnostic antigen.

An indirect ELISA was established with the use of chimeric peptide as a coating antigen and a monoclonal HRP-conjugated anti-human IgG for the detection of antibodies to HTLV-I in human sera. The in-house ELISA shows good correlation to the commercial test based on recombinant proteins and western blot as confirmatory test. This assay shows high specificity and sensitivity and would be suitable for HTLV-I screening on a large scale in endemic regions.

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