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Protection of rabbits against HTLV-II infection with a synthetic peptide corresponding to HTLV-II neutralization region

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Summary. Rabbit immune sera raised against synthetic peptides of the HTLV-II envelope gp46 region were examined for HTLV-II neutralization ability by HTLV-vesicular stomatitis virus (VSV) pseudotype assay and syncytium inhibition assay. HTLV-II neutralization activity was detected in the sera against HTLV-II Env gp46, 80–103 but not in those to HTLV-II Env gp46, 171–196. Three rabbits immunized with the synthetic peptide of HTLV-II Env gp46, 80–103 and three non-immunized rabbits were challenged with intravenous inoculation of an HTLV-II-producing human cell line (MOT, 1×10^7 cells). The non-immunized rabbits showed seroconversion for HTLV-II after 2 weeks and maintained persistent infection but the immunized rabbits were protected from HTLV-II infection. Nested or repeated polymerase chain reaction revealed the presence of HTLV-II provirus sequences in the non-immunized rabbits but not in the immunized rabbits. These results suggest that peptide vaccination with a synthetic peptide corresponding to the HTLV-II neutralization region is useful for preventing HTLV-II infection.

Introduction

Human T-cell lymphotropic virus type II (HTLV-II) was initially isolated from a patient with a T-cell variant of hairy cell leukemia [9] and has subsequently been isolated only sporadically in malignancy [18] or in non-malignant maladies [10]. Recently, a high rate of HTLV-II infection has been noted among intravenous drug users [11] and patients with acquired immunodeficiency syndrome (AIDS) [16] and in some geographic areas [8]. HTLV-II infection has also been detected in a patient with large granular lymphocyte leukemia [12] and in one of nine patients with mycosis fungoides [22]. However, the etiological role of HTLV-II in these diseases remains obscure.

A number of neutralizing epitopes of HTLV-I proteins has been characterized [2, 17, 20], but only two HTLV-II neutralizing epitopes have been reported [17, 21]. Protection from HTLV-I infection in animals was achieved by immunization with HTLV-I envelope glycoproteins [14], and the protective effect of anti-HTLV-II antibody against HTLV-II infection was demonstrated in a rabbit model [13]. However, successful vaccination against HTLV-II using synthetic peptide has not been demonstrated. Here, we present evidence for the protective effect of synthetic peptide vaccine against HTLV-II infection in a rabbit model.

Materials and methods

Rabbits

Japanese White rabbits weighing 2.5–3 kg were purchased from Shimizu Laboratory Supplies (Kyoto, Japan).

Virus

MOT, an HTLV-II-producing human cell line, was purchased from the American Type Culture Collection (Rockville, MD, USA). HTLV-II from MOT cells belongs to the subtype of HTLV-IIa [4].

Preparation of antibodies for neutralization of vesicular stomatitis virus pseudotypes

Both HTLV-II-specific polyclonal (#A, #B) [5] and monoclonal antibodies (N5–4) [15] were developed using a synthetic peptide which corresponds to amino acids 171–196 of HTLV-II Env gp46 [19]. Polyclonal rabbit antibodies against HTLV-II Env gp46, 80–103 (#71 ~ #73) were also developed by the same method described elsewhere [5]. Briefly, three rabbits were each immunized with keyhole limpet hemocyanine (KLH)-conjugated peptide emulsified in Freund's complete adjuvant (Difco, Detroit, MI, USA). The rabbits received one subcutaneous injection every 2 weeks for a total of four injections (1 mg at the first immunization and 500 μg thereafter). Serum was obtained before immunization and every 2 weeks after the third injection. Polyclonal antibodies obtained 2 weeks after the fourth injection were used for vesicular stomatitis virus (VSV) pseudotype assay and syncytium inhibition assay.

Neutralization of VSV pseudotypes

VSV pseudotypes bearing envelope antigens of HTLV-I or -II were prepared as described elsewhere [7]. Briefly, human T cells or adherent cells productively infected with HTLV-I or -II, which were superinfected with VSV (Indiana strain) in culture media with an MOI of 0.1, were harvested 12–15 h later, clarified by low-speed centrifugation, then aliquoted and stored at −110 °C until use. For neutralization assay, 100–200 pfu of VSV pseudotype were mixed with twofold serial dilutions of heat-inactivated (56 °C for 30 min) HTLV-II-immune rabbit sera in the presence of rabbit anti-VSV antiserum for 1 h at 37 °C. 8C cells, seeded 1 day earlier at 10^6 cells/35-mm Petri dish, were inoculated with virus-serum mixtures and incubated for 1 h, and monolayers were overlaid with 1% agar prepared in Eagle's minimum essential medium (EMEM). Plaques were counted 24 h later, and neutralizing antibody titers are expressed as the reciprocal of the maximum serum dilution that resulted in the reduction of the number of plaques by $\geqq 80\%$.

Syncytium inhibition assay

An HTLV-II-mediated syncytium inhibition assay was performed according to the HTLV-I-mediated syncytium inhibition assay as described [20]. Briefly, Wil-2/43 cells were suspended in RPMI-1 at 1×10^6 cells/ml, and aliquots (50 μl/well) were incubated with 100 μl of heat-inactivated monoclonal antibody, rabbit sera or medium alone in wells of U-bottom 96-well plates (Corning 25850, Rochester, NY) at 37 °C for 15 min, and then 50 μl of Molt-4 cell suspension (1×10^6 cells/ml) was added into each well. After incubation at 37 °C for 16 h in a 5% CO_2 incubator, each well was examined for giant multi-nuclear cells under an inverted microscope. In some experiments, the number of the giant multi-nuclear cells in the cultures were counted under a microscope, using a hemocytometer. Inhibition titers were expressed as reciprocals of the antibody dilution at which syncytium formation was inhibited completely.

Enzyme-linked immunosorbent assays

Titers of polyclonal antibodies to the synthetic peptides were examined by enzyme-linked immunosorbent assay (ELISA). Each well of polystyrene microplates (96-well) (Corning, Rochester, NY, USA) was coated with 0.1 ml of the non-conjugated or KLH-conjugated synthetic peptide in 0.01 M phosphate-buffered saline (PBS), pH 7.4 (5 μg/ml) at 4 °C overnight. After discarding the peptide solution, each well was quenched with 5% bovine serum albumin for 1 h, washed with PBS, and then incubated with 0.1 ml of the serially diluted immune or preimmune rabbit sera (diluted tenfold from 1:10 to $1{:}10^5$) for 2 h. All wells were washed with PBS containing 0.05% Tween20 (PBS-Tween) and incubated with peroxidase-labeled goat anti-rabbit IgG (Cappel, Durham, NC, USA, 1:500, 0.1 ml/well) for 30 min. After washing with PBS-Tween, the enzyme reaction was developed with 0.1 ml/well of substrate solution composed of PBS containing 3, 3′-diaminobenzidine 4HCl (20 mg/100 ml) and 0.005% H_2O_2 for 10–30 min.

Indirect immunofluorescence (IF test)

Cytosmears of HTLV-II-infected MOT or Si-IIA cells and HTLV-I-infected MT-2 cells were fixed with acetone for 5 min at room temperature and incubated with the rabbit antibodies to the peptide or preimmune sera as primary antibodies (1:100 to 1:1000) for 1 h at room temperature. The smears were then reacted with fluorescein-5-isothiocyanate (FITC)- or phycoerythrine (PE)-labeled goat anti-rabbit IgG as a secondary antibody (Cappel, 1:100) for 30 min at room temperature. These samples were observed with a Zeiss fluorescence microscope.

Western blot analysis

The ability of the immune sera to recognize native viral protein associated with HTLV-II was examined by Western blot analysis. The rabbit sera before and after immunization and human sera from an HTLV-II-infected patient and from an HTLV-negative healthy adult were reacted with the whole virus lysates of HTLV-II or HTLV-I using the Nova Path ATL Immunoblot Assay Kit (Nippon Bio-Rad Laboratories, Tokyo) or HTLV BLOT 2.3 strips including recombinant envelope protein of HTLV-II Env gp46 (Diagnostic Biotechnology, Singapore). The strips were blocked with 10% skimmed milk for 1 h at room temperature, and subsequently incubated with each serum at dilutions of 1:20 ~ 1:100 for 1 h at room temperature. After the unbound serum was washed with 10% skimmed milk three times for 30 min, the strips were incubated with anti-rabbit or anti-human IgG antibody conjugated with alkaline phosphatase for 30 min at room temperature and then washed three times. The

bound antibody was detected by the addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solution.

Serologic tests of sera from the challenged rabbits

Serial serum samples were obtained from the peptide immunized rabbits (#71 ~ #73) or from the non-immunized rabbits (#40 ~ #42) which were inoculated intravenously with MOT cells (1×10^7). These sera were examined for the presence of anti-HTLV-I antibody by particle agglutination (PA) test (Serodia HTLV-I, Fujirebio, Tokyo) according to the manufacturer's instructions. This test can also detect anti-HTLV-II antibodies because of antigenic cross-reactivity due to the high amino acid sequence homology between HTLV-I and HTLV-II.

Nested or repeated polymerase chain reaction

The nested or repeated polymerase chain reaction (PCR) was done repeatedly on genomic DNA extracted from rabbit peripheral blood leukocytes (PBL) or spleens. MOT cells were used as the positive control and an HTLV-uninfected cell line (TALL-1) was used as the negative control. Extracted DNAs were subjected to 35 cycles of PCR amplification, using HTLV-II *pol*-specific primer pair SK58 and SK59 as described previously [6] and HTLV-II *pX* region primer pairs SK43 and SK44 [13] for the first amplification. The second amplification was carried out with 10 µl of the first amplified products and the primer pair SK58 and SK59 for HTLV-II *pol* region (repeated PCR) or an inner position primer pair SSK431

(7263–7282, 5′CGTGTTTGGCGATTGTGTAC3′)
and SSK442 (7332–7313, 5′ATGTAGGCGGGTGGAACATA3′)

for HTLV-II *pX* region (nested PCR). The inner position primers SSK431 and SSK442 were synthesized according to the complete sequence of HTLV-II [19]. Sensitivity of the repeated PCR and nested PCR was 50 pg and 10 pg of MOT cell DNA, respectively. The amplified products were electrophoresed in a 3% NuSieve gel and visualized with 0.5 µg/ml ethidium bromide.

Results

HTLV-II-specific neutralization activity was demonstrated by HTLV-VSV pseudotype neutralization assay only in anti-HTLV-II Env gp46, 80–103 sera with low titers (#71, 1:8; #72, 1:2; #73, 1:14) (Fig. 1) but not in pre-immune sera, anti-HTLV-II Env gp46, 171–196 sera or N5–4 monoclonal antibody (data not shown).

HTLV-II neutralization was also detected by syncytium inhibition assay in anti-HTLV-II Env gp46, 80–103 sera (#71, 1:32, #72, 1:4, #73, 1:8) but not in pre-immune sera, anti-HTLV-II Env gp46, 171–196 polyclonal and monoclonal antibodies.

ELISA revealed that the rabbit antisera raised against the oligopeptide of HTLV-II Env gp46, 80–103 showed an elevation of antibody titers of more than 1:10 000. The titers of polyclonal and monoclonal antibodies against the oligopeptide of HTLV-II Env gp46, 171–196 were also confirmed by ELISA.

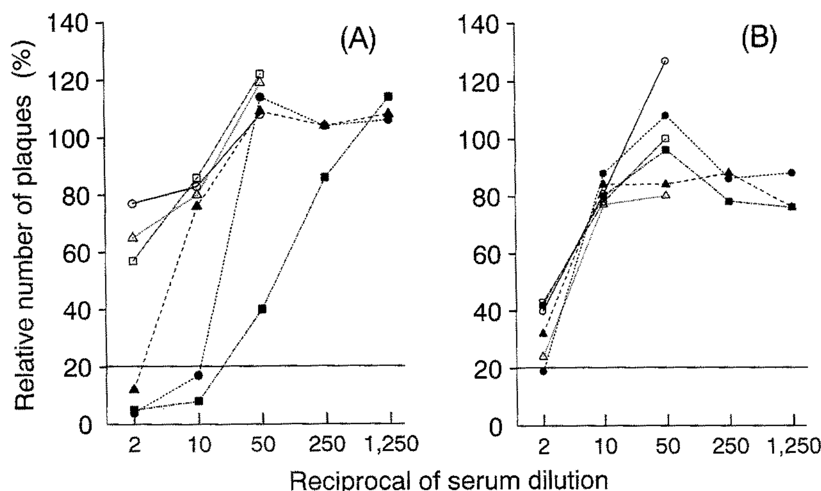

Fig. 1. Neutralization of vesicular stomatitis virus (VSV) pseudotype (**A** HTLV-II; **B** HTLV-I) by rabbit sera. Symbols: #71 rabbit (○, ●), #72 rabbit (△, ▲), #73 rabbit (□, ■). ○, △, □ Pre-immune sera, ●, ▲, ■ immune sera. Neutralizing antibody titers are expressed as the reciprocal of the maximum serum dilution that inhibited plating of VSV pseudotypes by ≧80% (solid horizontal line)

Reactivity of these antibodies to HTLV-II-infected or HTLV-I-infected cells was examined by the IF test. Both HTLV-II-infected cells (MOT cells or Si-IIA cells) and HTLV-I-infected cells (MT-2 cells) were reacted with polyclonal antibodies to the HTLV-II Env gp46, 80–103 peptide, whereas only HTLV-II-infected cells were reacted with the polyclonal and monoclonal antibodies to HTLV-II Env gp46, 171–196 peptide [5, 15].

Western blot analysis showed that the polyclonal antibodies to the HTLV-II Env gp46, 80–103 peptide reacted with neither gp46 of HTLV-II nor gp46 of HTLV-I while the polyclonal and monoclonal antibodies to HTLV-II Env gp46, 171–196 peptide reacted with gp46 of HTLV-II but not with gp46 of HTLV-I, as had been reported previously [5, 15].

The rabbits immunized with the peptide of HTLV-II Env gp46, 80–103 were boosted with an injection of 500 μg of peptide, and then challenged 7 days later by intravenous inoculation of 10^7 MOT cells. Serial determination of serum antibodies by the PA test showed that non-immunized rabbits challenged with 10^7 MOT cells produced significantly higher titers of anti-HTLV-II antibodies 2 weeks after the challenge than the immunized rabbits. The antibody titers of non-immunized rabbits reached 1:2048 and persisted until at least 12 weeks, whereas the immunized rabbits maintained the lower antibody response. Antibody response patterns representative of both immunized and non-immunized rabbits are shown in Fig. 2.

HTLV-II DNA sequences were detected in the positive control (MOT cells) and in PBL from the non-immunized rabbits inoculated with MOT cells but not in PBL and spleens from the immunized rabbits by the first PCR using

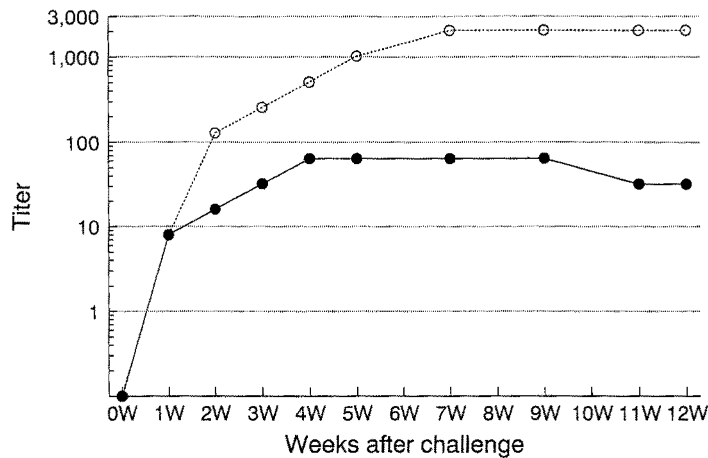

Fig. 2. Serial determination of rabbit antibodies to HTLV by the particle agglutination test. The immunized rabbits with synthetic oligopeptide of HTLV-II Env gp46 (●) showed lower titers than the non-immunized rabbits (○) after the inoculation of 10^7 MOT cells

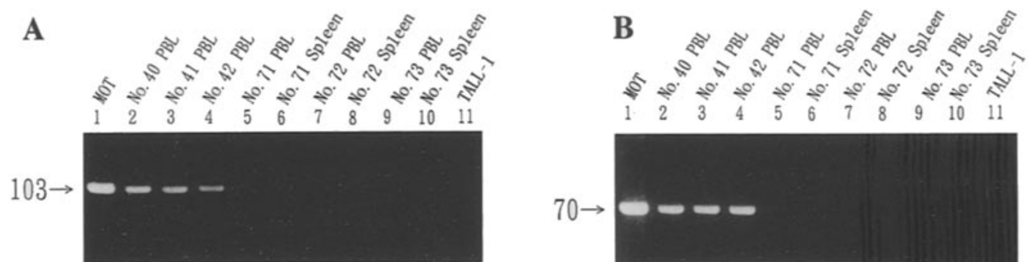

Fig. 3. Detection of HTLV-II provirus genome by repeated PCR using a primer pair of SK58 and SK59 (**A**) and nested PCR using a primer pair, SK43 and SK44 and the inner position primers, SSK431 and SSK442 (**B**). A positive band is seen at 103 bp (**A**) or 70 bp (**B**) in the positive control (*1*) and non-immunized rabbits inoculated with MOT cells (*2–4*), but not in the rabbits inoculated with MOT cells after immunization with the peptide (HTLV-II Env gp46, 80–103) (*5–10*) and the negative control (*11*)

HTLV-II-*pol* specific primers SK58 and SK59, and HTLV-II *pX* region primers SK43 and SK44. HTLV-II DNA was not amplified in PBL and spleens from the immunized rabbits inoculated with MOT cells except the positive control and PBL from the non-immunized rabbits even by both the repeated PCR using HTLV-II *pol* specific primers SK58 and SK59 (103bp amplified product) (Fig. 3A) and the nested PCR using HTLV-II *pX* region primers SSK431 and SSK442 (70bp amplified product) (Fig. 3B). The absence of HTLV-II infection in the immunized rabbits was confirmed by repeating the nested or repeated PCR at 4–11 weeks after inoculation with MOT cells.

Discussion

The present study showed that a synthetic peptide corresponding to one of the HTLV-II neutralization region, HTLV-II Env gp46, 80–103, evoked a protective

effect against HTLV-II infection in rabbits. The envelope glycoprotein of retroviruses including HTLV-I have been shown to induce humoral immunity that protects the host against viral infection and the antisera to HTLV-I had the effects of inhibition of syncytium formation, complement-dependent cytotoxicity on HTLV-I-carrying cell lines and neutralization of HTLV-I-VSV-pseudotype [14]. Especially the inhibition of syncytium induction by HTLV-I is most important for protection against HTLV-I infection [14]. HTLV-II Env gp46 is also expected to elicit host anti-viral immunity. Syncytium inhibition or/and neutralization of HTLV-II-VSV pseudotype were also demonstrated in the antisera to HTLV-II Env gp46 peptides in in vitro experiments [17, 21]. We are aware of only two reports on neutralization epitopes of HTLV-II [17, 21], while many neutralization epitopes of HTLV-I envelope gp46 protein have been characterized [2, 17, 20]. The first neutralization region of HTLV-II Env gp46 (amino acids 82–97) was determined by syncytium inhibition assay using goat antisera. Recently, Tanaka et al. discovered the second HTLV-II neutralization epitope (HTLV-II Env gp46, 186–192) using a monoclonal antibody [21]. The rabbit serum immunized with a synthetic peptide consisting of HTLV-II Env gp46, 182–199 also showed HTLV-II-specific neutralization activity in syncytium inhibition assay [21]. In this study using both HTLV-VSV pseudotype assay and syncytium inhibition assay, we also confirmed the HTLV-II type-specific neutralization activity in the rabbit antisera raised against HTLV-II Env gp46, 80–103, which contained the first neutralization epitope of HTLV-II Env gp46, 82–97. However, HTLV-II specific neutralization was not demonstrated in the polyclonal and monoclonal antibodies against HTLV-II Env gp46, 171–196, which contained the second neutralization epitope of HTLV-II Env gp46, 186–192. This discrepancy on the neutralization may be partially explained by difference of three-dimensional structures between the peptides of HTLV-II Env gp46, 171–196 and 186–192.

Polyclonal or monoclonal antibodies raised against HTLV-II Env gp46, 171–196, which specifically stained fixed HTLV-II-infected cells [5, 15], did not show HTLV-II neutralization activity in HTLV-VSV pseudotype assay and syncytium inhibition assay although monoclonal antibody developed with HTLV-II Env gp46, 186–192 was HTLV-II-specific in both serologic reactivity and neutralization ability. On the other hand, the polyclonal antibodies against HTLV-II Env gp46, 82–97 [17] or HTLV-II Env gp46, 80–103 used in this study had HTLV-II type-specific neutralization ability although these HTLV-II Env gp46 regions are almost homologous to those of HTLV-I (Fig. 4) and are cross-reactive antigenically to HTLV-I [17]. Palker et al. [17] showed that

HTLV-II *env* gp46, 80–103 L F P H W I K K P N R Q G L G Y Y S P S Y N D P

HTLV-I *env* gp46, 84–107 L F P H W T K K P N R N G G G Y Y S A S Y S D P

Fig. 4. Alignment of HTLV-I and HTLV-II Env gp46 neutralization regions, amino acids 80–103 and 84–107, respectively. Screened amino acid HTLV-II Env gp46 amino acids are different from those in HTLV-I Env gp46

amino acid differences between homologous HTLV-I and HTLV-II envelope sequences at HTLV-I amino acids 95 (N to Q) and 97 (G to L) determined the type specificity of these neutralizing sites.

Infectious transmission of HTLV-II in rabbits has already been reported [1], and protection from HTLV-II infection in animal models was accomplished for the first time by Morishita et al. in 1994 [13]. They demonstrated prevention against HTLV-II infection in rabbits with hyperimmune human anti-IgG to HTLV-II. In this study using the synthetic peptide of HTLV-II Env gp46 as a vaccine, the results of both single PCR and nested or repeated PCR indicate that HTLV-II DNA was not present in the PBL from the peptide-immunized rabbits, whereas it was detected in the PBL of non-immunized rabbits. These findings suggest that the synthetic peptide used confers protection against HTLV-II continuous infection. The rabbits immunized with the peptide responded to challenge with MOT cells with low levels of antibodies without being infected with HTLV-II. A similar low antibody response was also observed in the experiment of rabbits immunized with human immunoglobulin to HTLV-II [13]. This is postulated to have been elicited against extracellular HTLV-II virions from MOT cells used for challenge and does not imply infectious transmission of HTLV-II [13].

In this in vivo experiment, not only the inhibitory effect of plasmas from the immunized rabbits and neutralization against HTLV-II but cytotoxic T-lymphocyte activity on HTLV-II-infected cells may be also considered to contribute to protection against HTLV-II infection of rabbits. In the future, identification of target antigens on HTLV-II infected cells recognized by cytotoxic T-lymphocytes is very important for the development of potential vaccines.

It is known that HTLV-II has two closely related, but distinct molecular subtypes (HTLV-IIa and HTLV-IIb) [3, 4]. However, it has been estimated that the amino acid sequences of these two subtypes are the same, according to the data of nucleotide sequences of the *env* genes of HTLV-II isolates [3, 19, 21]. This fact is very important for developing peptide vaccines against both subtypes of HTLV-II; the peptide vaccine HTLV-II Env gp46, 80–103 used in this study against HTLV-IIa infection, can be expected to provide protection against HTLV-IIb infection.

The present study on protection of rabbits against HTLV-II infection with a peptide vaccine is a first basic step in development of an HTLV-II vaccine.

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