Epitope Mapping of Bovine Leukemia Virus Transactivator Protein Tax

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ABSTRACT. The immunogenicity of the bovine leukemia virus (BLV) transactivator protein (tax) was studied by mapping its B-cell and T-cell epitopes. Peptides (18 to 20-mer) overlapping by 10 amino acids, spanning whole amino acid sequence of BLVtax were synthesized. Recombinant BLV tax protein was used to immunize two different strains of mice, C57BL/6 and BALB/c. B-cell and T-cell epitopes of recombinant BLVtax protein was determined by screening all the 30 synthetic peptides, against immune serum in ELISA for antibody reactivity, and against immune spleen cells in lymphocyte proliferation assay for T-cell stimulation. Peptides with amino acids at position 111–130 and 131–150 were T-cell epitopes for C57BL/6 and BALB/c mice immune cells, respectively. B-cell epitope was mapped to amino acid sequence at 261–280 in both strains of mice. These results imply that BLVtax protein contains some of BLV-immunodominant epitopes and this information may be applied for designing an effective peptide vaccine capable of inducing neutralizing antibodies as well as cellular immunity. — KEY WORDS: bovine leukemia virus, epitope mapping, transactivator protein.

Retrovirus infections cause serious problems in humans and animals. Bovine leukemia virus (BLV) causes enzootic bovine leukosis. Although many vaccine trials have been undertaken [15], but effective vaccines have not yet been developed against these viruses including BLV. So far viral envelope glycoprotein (env) and major core protein (gag) have been studied for the development of subunit BLV vaccine [1, 7, 12, 23]. It was reported that the effective BLV vaccine should induce not only neutralizing antibodies but also virus specific cell mediated immunity (CMI) [21, 22]. It was found that some components of retroviruses suppress the host immune function [8]. From these reasons, it is considered that peptide vaccine which can induce CMI and can minimize side effects should be a candidate, and epitopes of BLV structural proteins have been hardly studied [3, 6, 18]. Lately, functional proteins, tax or tat, are being considered vaccine candidates in human retrovirus infection, human T-cell leukemia virus type I (HTLV-I) and immunodeficiency virus (HIV) because these molecule induce protective immunity [9, 14, 29].

In this study epitope mapping of BLVtax was undertaken to determine its immunogenicity. Mapping was performed in mice using synthetic peptides covering the whole BLVtax amino acid sequence.

MATERIALS AND METHODS

Recombinant BLVtax protein derived from recombinant baculovirus: A recombinant baculovirus bearing BLVtax gene was kindly supplied by Dr. D. Portetelle, Faculty of Agronomy, Gembloux, Belgium. High Five cells (Invitrogen, Carlsbad, California) were infected with the recombinant baculovirus at a m.o.i. of 10. The infected cells were harvested at 3 to 4 days after the infection, and lysed with 10 mM Tris-HCl (pH7.9) buffer containing 1% (v/v) Triton X-100 on ice for 30 min. After centrifugation, the supernatant was removed, and the pellet was collected.

Expression of recombinant BLVtax in E. coli: PCR-derived full-length cDNA from BLVtax was inserted into a plasmid vector, pGEM-T (Promega Corp., Madison, Wisconsin). This plasmid was digested by Nco I and inserted in frame into an expression vector, pET32a (Novagen Inc., Madison, Wisconsin) to yield the pET32a-BLVtax plasmid. The pET32a-BLVtax plasmid was introduced into E. coli (strain DH5α). Expression of recombinant BLVtax in E. coli was induced by stimulation with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6-9 hr. The resultant recombinant protein was purified using the Ni2+-nitrilo-triacetic acid (Ni-NTA) column following the manufacturer’s instructions. The fractions containing BLVtax were determined by SDS-PAGE and immunoblot analysis as described below.

Peptides: Based on the reported sequence of BLVtax [28], a series of 18- to 20-mer peptides overlapping by 10 amino acids were synthesized by PSSM-8 peptide synthesizer (Shimazu, Kyoto, Japan), or 9050 Plus (Millipore Corp., Bedford, Massachusetts) as shown in Fig. 1.

Immunization: Mice (4-5 weeks old) were divided into 3 groups, each of which consists of 3 mice. The first group of mice was inoculated with BLV-producing fetal lamb kidney (FLK) cells (4 × 10⁶ cells/200 µl/head) intraperitoneally. The second group of mice was intradermally immunized with 20 µg of taxTRX protein in 200 µl PBS emulsified in either complete or incomplete Freund’s adjuvant. The third group of mice was immunized with the TRX protein as a control. The immunization was performed once a week at least 3 times.

SDS-PAGE and immunoblots: Expression of the recombinant BLVtax protein was analyzed by SDS-PAGE and immunoblot as described previously [17]. The samples...
were fractionated on 12.5% acrylamide gels and transferred onto PVDF membranes (Millipore Corp.). These membranes were incubated with anti-BLVtax monoclonal antibody (kindly supplied by Dr. D. Portetelle, Belgium) for 1 hr at room temperature. After washing with PBS containing 0.05% Tween 20 (PBST), the membranes were soaked with peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno Research). The recombinant protein was visualized by using substrate solution (0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.03% cobalt chloride hexahydrate, 0.02% H₂O₂ in PBST).

**Lymphocyte proliferation assay:** Spleen was obtained from the immunized mice at one week after the last immunization, and single cell suspensions were prepared in complete RPMI1640 medium (GIBCO BRL, Gaithersburg, Maryland, containing 10% heat-inactivated fetal calf serum and 5 × 10⁻⁵ M 2-mercaptoethanol). These cells were cultured in triplicate wells (each 2 × 10⁵ cells per well) of 96-well microplate with the BLV virion antigen (50 µg/ml), taxTRX (5 µg/ml), or peptides (1 to 100 µg/ml) for 3 days at 37°C in 5% CO₂. Sixteen hours before harvesting the cells, 1.0 µCi of [³H]-thymidine was added to each well. Then, the cells were harvested onto glass-fiber filters and the incorporation of [³H]-thymidine into the cells was measured by liquid scintillation counter. Results were presented as stimulation indices (SI=cpm in test sample/cpm in control ones).

**Enzyme-linked immunosorbent assay (ELISA):** Antibodies against BLVtax-peptides were detected by ELISA. Peptides were absorbed to duplicate wells of 96 well microplates (Xenobind; Xenopore, Saddie Brook, New Jersey) in 0.5 M carbonate buffer (pH 12.5). After washing with PBST, the wells were coated with 5% skim milk solution for 1 hr at 37°C, and incubated with the immunized mouse serum diluted 1/100 for 1 hr at 37°C. Then, each well was washed and incubated with peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno Research). After washing, substrate solution (ABTS, Sigma, St. Louis, Missouri) was added and incubated for 30 min at 37°C. Optical density at 405 nm was measured by using an automatic ELISA reader.

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**Fig. 1. Sequences of 30 peptides covering BLVtax.** Peptides were all synthesized by peptide synthesizer (PSSM-8; Shimazu or 9050 Plus; MILLIPORE) automatically. Each of the 18–20 mer peptide contained the 10 mer-overlapped sequence before and behind.
Expression of BLVtax protein: The expression of a recombinant BLVtax protein was detected by SDS-PAGE and immunoblot analysis. The BLVtax protein derived from a recombinant baculovirus was detected as a 34-kDa band, and confirmed by using anti-BLVtax monoclonal antibody (Fig. 2A). This preparation was used as the baculovirus-derived recombinant BLVtax (HFtax) antigen. In addition, the BLVtax protein expressed by E. coli was detected as a
57-kDa band. This protein was expressed as a fusion protein with the plasmid-derived TRX protein (taxTRX) (Fig. 2B).

Immune responses against BLVtax protein in the immunized mice: We examined the antibody and lymphocyte proliferation responses to know whether BLVtax proteins can induce a specific immune response against BLV antigens in BALB/c mice (Table 1). In the immunoblot analysis, we used HFtax as an antigen to detect tax-specific antibody. Tax-specific antibodies were detected only in the taxTRX-immunized but not FLK-immunized mice. Lymphocyte proliferative responses against BLVtax were detected in both taxTRX- and FLK-immunized mice though control TRX- or non-immunized mice did not show any specific response. These results indicate that both tax-specific antibody and lymphocyte proliferation responses are induced in taxTRX-immunized mice.

Mapping of the B cell epitopes in mice: Mice immunized with the recombinant BLVtax protein produced specific

Table 1. Specific immune response to BLV protein in the BALB/c mice

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Antibody(^a)</th>
<th>Lymphoproliferation(^b) (SI)</th>
<th>BLV virion stimulate(^c)</th>
<th>tax stimulate</th>
</tr>
</thead>
<tbody>
<tr>
<td>taxTRX</td>
<td>+</td>
<td>3.1</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>FLK(^d)</td>
<td>–</td>
<td>2.9</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>TRX</td>
<td>–</td>
<td>1.7</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>–</td>
<td>0.7</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Antibody against HFtax were detected by immunoblot. \(^b\) Values of lymphoproliferation were shown as stimulation index (SI). SI of tax stimulate was shown as follows; SI of taxTRX stimulated/those of TRX stimulated. \(^c\) BLV virion was concentrated from the FLK cell culture supernatant. \(^d\) FLK cells: Fetal lamb kidney cells, permanently producing BLV.
antibodies against tax protein. To identify the B cell epitope of the BLVtax protein, sera from taxTRX immunized mice were tested for their reactivities to series of synthetic peptides covering whole amino acid sequences of the tax protein by ELISA. As shown in Fig. 3, the sera from both BALB/c and C57BL/6 mice reacted and showed relatively higher responses against the peptides 14 (131–150) and 27 (261–280). Peptides 5 (41–60), 15 (141–160), and 16 (151–170) reacted weakly with the sera from BALB/c mice, and peptides 11 (101–120) and 20 (191–210) also showed weak reaction with the sera from C57BL/6 mice. No specific reaction was detected in sera from the control mice. These results indicate that BLVtax contains B cell epitopes, and these epitopes (located in the peptides 14 and 27) were common for the two strains of mice examined.

**DISCUSSION**

For vaccine development against BLV, structural proteins such as env and gag gene products have been used as immunogens. Recently, it has been shown that cell-mediated immunity (CMI) including cytotoxic T lymphocyte (CTL) activity plays an important role in the protection against retrovirus infection [21, 22, 31]. Various studies have revealed that HTLV-I tax protein contains several CTL epitopes [9, 14, 16, 20, 25, 26] and induces strong CTL responses against HTLV-I-infected patients [11, 13, 24]. In the present study, we analyzed the immune responses and epitopes for BLVtax to know whether BLVtax can induce cellular and humoral immune responses in mice.

Immune responses against BLVtax were examined in mice immunized with two kinds of antigens, recombinant tax protein (taxTRX) and FLK cells which produce BLV virion. The taxTRX-immunized mice showed both specific antibody- and lymphoproliferative responses against BLVtax antigen, while mice immunized with control protein did not. FLK-immunized mice showed lymphoproliferative responses but did not produce any antibodies against BLVtax protein. Since FLK cells produce a relatively low amount of BLVtax protein (data not shown), these mice might not have been able to produce sufficient antibodies. It has been reported that CMI responses were induced by stimulation with a low dose of antigen [10, 19]. Thus, the
BLVtax protein was expressed enough to induce CMI but not antibody responses in the FLK-immunized mice in the present study.

This study showed that BLVtax peptides 14 (131–150) and 27 (261–280) contained B cell epitopes in BALB/c and C57BL/6 mice. Previously, several B cell epitopes as referred to regions A to H were identified in the BLV env protein [2]. Regions F, G and H were not only functional for viral infection, but also contained the neutralizing epitopes [4]. Because BLVtax acts as a viral transcriptional factor and plays an important role in the disease progression, it is expected that the antibody against BLVtax could inhibit viral replication. In case of HIV infection, antibodies against the HIVtat protein inhibited viral replication and prevent the disease progression [27, 29].

Peptides 14 (131–150) and 12 (111–130) contained T cell epitopes for BALB/c and C57BL/6 mice, respectively. Peptide 14 contained both B and T cell epitopes for BALB/c mice. It has been reported that some epitopes overlap or closely located in a restricted part [5, 30]. In the case of BLV, it was also reported that a peptide from BLV env 98–117 contained both neutralizing and Th epitopes for BLV-infected cows and BALB/c mice [4]. Peptide 14 of BLVtax also contained the multiple epitopes for BALB/c mice. In the preliminary experiments of sheep system, we found that there were different T cell epitopes for the individual sheep tested. In one sheep which exhibited the strongest immune responses against whole BLVtax protein, we could identify B cell epitope (position 51–70) and T cell epitope (position 181–200) which differed from those for mice. This result would be useful informations for the development of vaccine targeting BLVtax protein.

In this report, we found that BLVtax can induce specific immune responses, and that it contains B and T cell epitopes for mice like as previously reported in BLVenv and gag proteins [4, 6, 7, 18]. It is important to investigate whether BLVtax can induce specific immune response in natural hosts in applying BLVtax protein to the vaccine development. We are now studying the effects of the immunization with the recombinant BLVtax protein against BLV challenge in sheep.

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REFERENCES


