FULL PAPER *Immunology*

Protective Effects of Vaccination with Bovine Leukemia Virus (BLV) Tax DNA Against BLV Infection in Sheep

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(Received 24 April 2003/Accepted 22 July 2003)

ABSTRACT. A DNA vaccination trial was performed on sheep to determine whether vaccination with bovine leukemia virus (BLV) transactivator Tax DNA is effective against BLV infection. Immunization was carried out with cationic liposomes containing the Tax-expressing plasmid DNA and subsequently all sheep were challenged with BLV. BLV titers in peripheral blood mononuclear cell (PBMC) determined by syncytium formation assay and BLV provirus load detected by genomic PCR analysis showed higher levels of virus titers in control sheep than those in Tax-vaccinated sheep. Higher levels of IFN-γ mRNA expression have been demonstrated in vaccinated sheep after the challenge. These results suggested that Th1 type immune response induced by Tax DNA vaccine inhibited BLV propagation in vaccinated sheep at the early phase of infection.

KEY WORDS: BLV tax, DNA vaccine, real time PCR.

J. Vet. Med. Sci. 65(11): 1201–1205, 2003

Many vaccine trials, including recombinant vaccinia virus [20, 21], epitope peptide [10, 19] and a weakened or killed virus [13, 32], have been undertaken against bovine leukemia virus (BLV) infection. The targets for most of these vaccines are structural proteins such as envelope glycoprotein (Env) gp51 because this glycoprotein locating on the outermost layer of the virus is a major antigen recognized by the host immune system [14]. These vaccines can partially protect animals from the primary BLV infection, but it may be difficult to either eliminate infected cells or prevent from progression of disease, since retrovirus including BLV forms provirus in the host genome and is generally spread as cell-associated viruses. It was reported that effective BLV vaccine should induce not only neutralizing antibodies but also virus-specific cell-mediated immunity (CMI) [20, 21].

Recently, viral transactivator protein, Tax, is being considered the most potential vaccine candidates in human retrovirus infection, human T-cell leukemia virus type 1 (HTLV-I), because this molecule can induce protective immunity [12]. Tax protein is thought to play an important role on disease progression [2]. Moreover, various studies have revealed that Tax protein contains several epitopes for cytotoxic T lymphocytes (CTL) and can induce strong CTL responses [5, 11, 12, 23].

DNA immunization can mimic the effects of live attenuated vaccines through their ability to express immunogen in transfected host cells to induce CMI responses [4]. The DNA vaccination promises to be safer than the use of traditional live vaccines because it eliminates the risks associated with possible contamination of vaccines, and its immune response induced will be more long-lived. The DNA vaccines are also known to efficiently prime the immune responses in hosts to generate strong antibody and cell-mediated responses when hosts are boosted with recombinant antigens. Such prime-boost strategies have proven to be more effective than the use of virus-encoded antigens [25, 29].

More recently, it was demonstrated that the vaccination of HTLV-I Tax-coding DNA induced Tax-specific CTL responses and that the vaccine-induced T cells were capable of suppressing the HTLV-I induced tumor in a rat adult T-cell leukemia (ATL) model [18]. Only a few reports are available on DNA vaccine trials against BLV infection in sheep or cattle, and they used BLV Env-coding DNA similar to traditional vaccines [1, 13]. Thus, nothing has been reported on the protective effects of BLV Tax DNA against BLV infection.

In this study, sheep were vaccinated with BLV Tax-coding DNA and challenged with BLV to evaluate the protective effects of the BLV Tax DNA vaccination using the prime-boost strategies. BLV Tax DNA vaccine is an interesting attempt for the development of BLV vaccines in the point of its inhibition effect on the disease progression.

MATERIALS AND METHODS

Plasmids: Construction of Tax-expressing plasmid, pMEBLVtax has been described previously [30]. Briefly, the full length of *tax* gene was introduced into the downstream of the SRα promoter in mammalian cell expression plasmid pME18Neo.

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Plasmid construction (10 μg) was transfected into 3×10^6 of CC81 cells (feline cell line transformed with mouse sarcoma virus) by electroporation using the Electroporator II (Invitrogen) operated at 1,000 μF and 300 V. The expression of the 34 kDa Tax protein in cell lysate was confirmed by Western blot using specific antibody.

Animals, immunization and BLV challenge: In the preliminary experiment, Tax-expressing plasmid was inoculated into mice to evaluate the immune responses induced by the plasmid. Four- to 5-week-old C57BL/6 female mice were purchased from Nihon SLC Corporation (Shizuoka, Japan). Three mice were immunized 3 times at weekly intervals with 100 μg of pMEBLVtax entrapped in cationic liposomes by intramuscular injection into the quadricep muscles [31]. Control mice (n=3) were immunized with 100 μg of pME18Neo. For Tax-specific proliferation analysis, all mice were sacrificed, and spleen cells were harvested at 1 week after the last immunization.

To evaluate the protective effects of the DNA vaccine, sheep were injected with the pMEBLVtax plasmid, and challenged with BLV. Six male 2-year-old sheep (Suffolk) were purchased from Japan Lamb Company (Hokkaido, Japan). Three sheep were injected 3 times at weekly intervals and once at 12 weeks after the first immunization with the Tax DNA vaccine (500 μg) entrapped by cationic liposomes. Three control sheep were injected with liposome-entrapped pME18Neo. At 13 weeks after the first immunization, Tax-vaccinated sheep were injected with the recombinant Tax protein (500 μg/5 m*l*) mixed with an equal volume of incomplete Freund's Adjuvant [26]. At 1 week after the booster, all sheep were challenged with 7×10^6 PBMC obtained from BLV-infected sheep. This inoculation dose of PBMC produced 500 syncytia in the indicator CC81 cells. All injections were done intramuscularly into each quadricep muscles. The sheep were used for experiments after at least one month of acclimatization post castration.

Cell proliferation assay in BLV Tax DNA-immunized mice: Single cell suspensions in RPMI1640 medium containing 10% FCS were prepared from splenocytes of immunized mice and tested individually. Splenocytes (2×10^5 cell) were incubated with 50 μg/m*l* of the freeze-thaw treated lysates of pMEBLVTax-transfected CC81 cells, those of pME18Neo-transfected CC81 cells, or medium alone on 96-well culture plates. After 48 hr, the viability of proliferative cells was assessed by adding the MTT solution (5 mg/m*l* in PBS, 10% of the final volume) to the culture. Formazan crystals formed after 4 hr incubation with MTT were solubilized by adding 100 μ*l* of 0.04 N HCl in isopropanol. The amounts of formazan produced were determined by measuring the absorbance of the solutions at 570 nm.

The stimulation index was calculated as follows: Stimulation index (S.I.) = A_{570} of test cells/A_{570} of control cells. Control cells were incubated in medium alone. Results were expressed as mean S.I. ± standard deviation (SD) obtained from triplicate experiments.

Analysis of Virus titer and provirus load: Syncytium assay was performed according to the procedure described previously [22]. Briefly, PBMC (2×10^5) isolated from sheep blood were co-cultured with 10^5 indicator CC81 cells/well in 24-well culture plates at 37°C for 48 hr. Then cells were fixed and stained with the Giemsa solution, and the syncytia were counted. Results were represented as means ± SD obtained from triplicate wells each of three sheep.

Quantification of BLV-positive cells was performed by genomic PCR analysis as described previously [15]. Genomic DNA was extracted from sheep PBMC by the phenol/chloroform method. The long terminal repeat (LTR) region of the BLV genome was amplified from 100 *n*g of total cellular genomic DNA by using primers BLV-LTR1 (5'-TGTATGAAAGATCATGCCGAC-3') and BLV-LTR533 (5'-AATTGTTTGCCGGTCTCTC-3'). We also amplified the ovine β-actin in each sample using primers β-actin F (5'-ACCAACTGGGACGACATGGAG-3') and β-actin R (5'-GCATTTGCGGTGGACAATGGA-3') to monitor the concentrations of template DNAs. The amplified products were subjected to electrophoresis on a 2% agarose gel. Densities of the BLV-LTR- and β-actin-specific bands were determined quantitatively by Fluor-S MultiImager (BIO-RAD), and the ratio of densities of BLV-LTR to those of β-actin was calculated. Total DNA from fetal lamb kidney (FLK)-BLV cells, which harbor six copies of the BLV genome, was serially diluted with total DNA from FLK cells, and these samples were used as the standard template for calculation of the percentage of BLV-positive cells. PCR was performed in a thermal cycler (System 9700; Applied Biosystems).

Quantification of cytokine mRNA by real-time PCR: To determine the cytokine mRNA levels in sheep PBMC, we used a system for real-time quantitative PCR (LightCycler™; Roche Diagnostics, Germany). Total RNA was extracted with the TRIZOL™ reagent (Invitrogen) from sheep PBMC stimulated with Concanavalin A (5 μg/m*l*) for 24 hr. Five μg of total RNA were used for cDNA synthesis as described previously [9]. The mRNA of IFN-γ, interleukin (IL)-2, IL-4 or IL-10 was amplified and quantified from the cDNA template in the presence of LightCycler-DNA Master SYBR Green I (Roche Diagnostics) and primers. Each cytokine mRNAs were amplified by using the following specific primer sets: IFN-γ, 5'-ATAACCAGGTCATTCAAAGG-3' (forward, F), 5'-ATTCTGACTTCTCTTCCGCT-3' (reverse, R); IL-2, 5'-TTTTACGTGCCCAAGGTTAA-3' (F), 5'-CGTTTACTGTTGCATCATCA-3' (R); IL-4, 5'-CAAAGAACGCAACTGAGCGTACTTGTA-3' (F), 5'-AGGTCTCTCAGCGTACTTGTA-3' (R); IL-10, 5'-TGCTGGATGACTTTAAGGG-3' (F), 5'-AGGGCAGAAAACGATGACA-3' (R). We also amplified the ovine β-actin gene in each samples, using a primer pair, 5'-CGCACCACTGGCATTGTCAT-3' (F) and 5'-TCCAAGGCGACGTAGCAGAG-3' (R) to monitor the concentrations of template cDNA.

RESULTS

Effect of vaccination with BLV Tax DNA: Prior to the evaluation of the BLV Tax DNA vaccine in sheep, we examined cell proliferative response against the Tax protein in mice inoculated with the DNA vaccine. Spleen cells from mice immunized with the Tax DNA vaccine showed a significantly higher proliferative response against Tax-containing cell lysate (S.I. = 2.247 ± 0.033) compared to control mice (S.I. = 1.411 ± 0.046; $p<0.05$). On the other hand, no significant differences were observed between Tax-vaccinated and control mice against pME18Neo transfected cell lysate ($p>0.05$).

Next, we examined the protective effects of pME-BLVTax as vaccine in sheep. Tax-vaccinated sheep showed detectable antibody response at 1 week post infection (p.i.) demonstrated by Western blot (data not shown). To evaluate the effect of Tax-vaccine on the propagation of BLV in infected sheep, we analyzed the BLV titers in PBMC by syncytium formation assay. Syncytia were detected in all sheep at 2 weeks p.i. (Fig. 1(A)); the number of syncytia reached plateau levels between 3–5 weeks p.i., and the highest number of syncytia between 3–5 weeks p.i. in 3 individual control sheep were 71, 330 and 119, respectively. In contrast, the number of syncytia in Tax-vaccinated 3 sheep between 3–5 weeks p.i. were less than 60. We also evaluated the proviral load in Tax-vaccinated sheep by analyzing the percentages of BLV-positive cells. As shown in Fig. 1(B), at 2 weeks p.i., the percentages ranged 7–9% (the mean 8.3%) in control sheep whereas the values were 3% in one and 0% in other 2 vaccinated sheep. At 4 and 8 weeks p.i., the mean percentages were increased to 13–15% in control sheep, whereas those of Tax-vaccinated sheep remained less than 6%.

Cytokine gene expression in Tax-vaccinated sheep: The cytokine mRNA expression profiles were assessed in vaccinated sheep after BLV challenge. As summarized in Table 1, the 3 Tax-vaccinated sheep showed higher IFN-γ mRNA expression compared to control sheep at 2, 4 and 8 weeks p.i. At 2 and 4 weeks p.i., IL-2 mRNA expressions showed comparatively higher in vaccinated than in control sheep. In contrast, there were no significant differences in IL-4 and IL-10 mRNA expression between vaccinated and control sheep.

DISCUSSION

We have shown that vaccination with BLV Tax-coding DNA induced protective effect against BLV infection, suppressing the growth of BLV in experimentally infected sheep. The characteristics of retrovirus infection, such as persistent infection and cell-associated spread, lead us to believe that vaccines against BLV should be able to prevent disease progression. Furthermore, it has been suggested that the Th1-cell response including CTL activity plays an important role in the protection against retrovirus infection [20, 21]. There were two reasons why the Tax protein is considered as the most potential vaccine candidates. One is that activation and proliferation of BLV-infected cells are triggered by the transactivating activity of Tax [2, 6], and

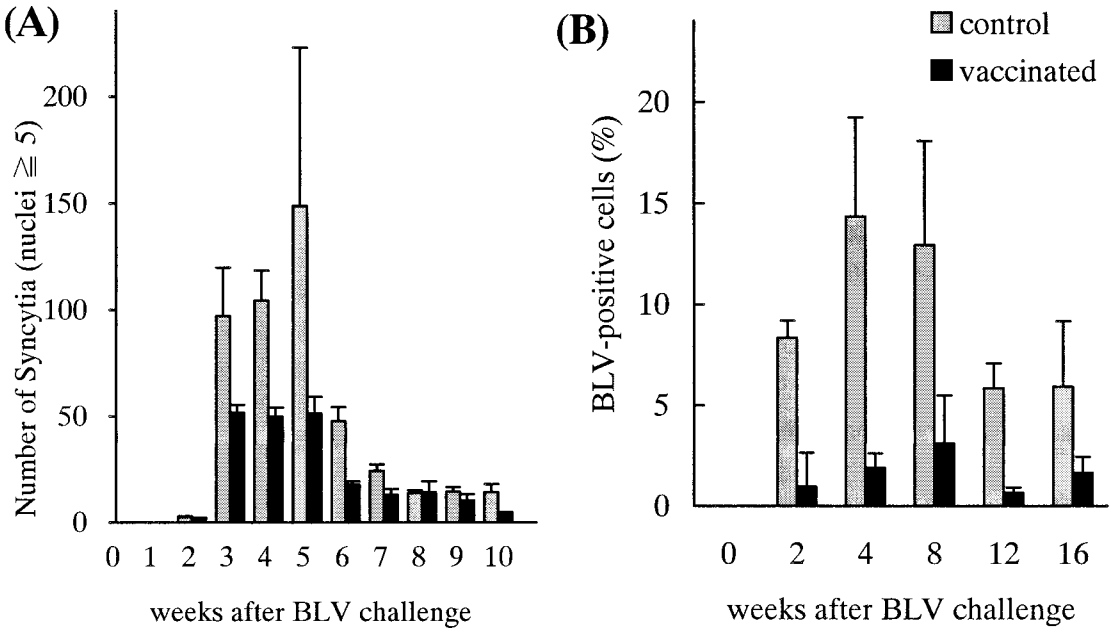

Fig. 1. Effect of immunization with BLV Tax DNA vaccine on the growth of BLV in PBMC of sheep. (A) Syncytium formation by PBMC (2×10^5) from Tax-vaccinated (filled bars) and control sheep (gray bars). Cells containing more than five nuclei were counted as syncytia. Each column and error bar represent the mean ± SD obtained from triplicate wells each of three sheep. (B)Semi-quantification of BLV provirus by genomic PCR analysis. Each column and error bar represent the mean ± SD of results from three sheep.

Table 1. Cytokine mRNA expression in PBMC from Tax-vaccinated sheep determined by real time RT-PCR[a)]

Sheep		IFN-γ			IL-2			IL-4			IL-10		
No.	Immunization	2w	4w	8w	2w	4w	8w	2w	4w	8w	2w	4w	8w
1	control	0.2	0.2	1.2	0.4	0.4	1.2	1.7	0.9	1.8	1.1	0.3	1.0
2		1.4	0.9	1.0	1.3	0.3	1.7	0.9	0.7	0.9	1.1	0.5	2.3
3		1.5	2.6	0.9	4.2	4.3	1.3	7.8	6.0	3.4	2.5	4.2	3.0
4	Tax-vaccinated	3.9	5.9	6.8	10.4	6.2	0.6	1.5	4.2	2.2	0.2	1.9	1.2
5		3.0	2.0	5.0	4.5	6.0	1.4	0.7	0.5	1.3	0.7	2.8	2.3
6		4.3	7.7	4.8	2.1	1.7	1.6	1.8	1.6	4.0	1.0	2.1	3.7

a) Results were expressed as fold increases in cytokine mRNA expressions compared to those at 0 week p.i.

the other is that the Tax protein contains several CTL epitopes and can induce strong CTL responses [5, 11, 12].

Recently, DNA vaccines have been shown to successfully induce protective cellular and humoral immune responses against various pathogens. One of the attractive aspects of DNA immunization is its capacity to generate complex immune response. Co-delivering genes encoding cytokines or co-stimulatory antigens enhances or biases the immune responses generated by the DNA vaccination [27]. DNA vaccines are known to efficiently prime the immune responses to generate strong antibody and cell-mediated responses when boosted with recombinant protein [27, 29]. Efficient delivery of DNA to target antigen presenting cells was also important because DNA injected intramuscularly appears to be degraded by extracellular DNases [17]. As a carrier of the genes, cationic liposomes were used in this study since cationic liposomes have many advantages as gene transfer vectors [3]. They evoke fewer inflammations or immune responses than naked DNA. It was demonstrated that cationic liposomes used in this study can protect the entrapped plasmids from enzymatic attack and are efficient vectors for gene transfer into cells [16, 31].

Based on the features of DNA vaccine as shown in other reports described above, the results shown in the present study suggest that mechanisms of protective effects observed in Tax-vaccinated sheep is probably due to cell-mediated responses primed by a series of DNA vaccines and booster with the protein antigen. Induction of cellular immunity may be indicated by the increased expression of IFN-γ mRNA because IFN-γ is strong indicator of the Th1 responses. PBMC from Tax-vaccinated sheep showed higher level of IFN-γ expression than IL-4 expression, indicator of the Th2 responses, at 2, 4 and 8 weeks p.i. (Table 1). IFN-γ is not only the indicator of the Th1 response but also has a suppressive activity against BLV replication *in vitro* [28].

Alternations in cytokine expression have been shown to be correlated with disease progression in chronic retroviral infections including BLV infection [7, 8]. For example, the production of Th1 cytokines such as IL-12, IL-2 and IFN-γ was known to be promoted in BLV-infected but healthy cattle, while an increased expression of Th2 cytokines such as IL-10 was shown in cattle with persistent lymphocytosis and leukemia [24]. These suggest the importance of the Th1 dominance during the infection to prevent disease progression. In this study, the level of IL-2 mRNA expression was also higher at 4 weeks p.i. in vaccinated sheep, but there were no significant differences in the expressions of Th2 cytokines, IL-4 and IL-10.

In conclusions, this study shows the protective effect of Tax DNA vaccine on sheep in the early phase of infection. However, detailed mechanisms in which BLV propagation was inhibited in Tax-vaccinated sheep and whether Tax vaccination can inhibit disease progression are not to be defined. Furthermore, the roles of cytokines in BLV infection remain to be elucidated. To modulate Tax DNA vaccine and to reveal the roles of cytokines in BLV infection, we are currently investigating the effects of co-delivery of cytokine genes with DNA vaccine on enhancement of cellular immunity and stronger protection by using sheep as an experimental model.

ACKNOWLEDGMENT. This work was partly supported by Recombinant Cytokine's Projects from the Ministry of Agriculture, Forestry and Fisheries, Japan (RCP-1997–2330).

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