Protection against Bovine Leukemia Virus Infection by Use of Inactivated Vaccines in Cattle

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ABSTRACT. The protective effects of vaccines made from the viral materials of LK₁₅ cell line (LK₁₅ vaccine) or bat lung cell line (Bat₂cl₁; Bat vaccine) infected with bovine leukemia virus (BLV) were examined in cattle. Twelve cattle were vaccinated twice at 4-week interval then challenged 4 weeks after the second inoculation. Nine cattle vaccinated with the LK₁₅ vaccine produced antibody to BLV-specific glycoprotein (gp), and the titers ranged from 1:16 to 1:64 by the agar gel immunodiffusion test. Four cattle challenged with 100 μ l (70 to 100 syncytia) of cow blood persistently infected with BLV were protected from infection. However, of the remaining 5 cattle challenged with 500 μ l of infected blood, only 2 were protected. Of the three cattle vaccinated with the Bat vaccine, gp antibody titers ranged from 1:8 to 1:64. Two of them were protected against the challenge with 100 μ l of infected blood. Two cattle protected against the challenge were rechallenged 32 weeks after the first vaccination and not protected. On the other hand, 2 animals protected against the challenge were revaccinated with the LK₁₅ vaccine 32 weeks after the first vaccination. They protected against rechallenge. The results show that all cattle which had gp antibody titers of 1:16 or above were protective against challenge with 100 μ l of the infected blood.—**KEY WORDS:** BLV, protection, vaccination.

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Bovine leukemia virus (BLV) causes enzootic bovine leukosis (lymphosarcoma) in cattle. Serologic surveys have indicated that the prevalence of BLV infection is relatively high in the enzootic areas of Japan [8, 18]. It seems that BLV infection in cattle increase gradually, mainly as a result of a characteristic of lifelong persistent infection of BLV.

To eradicate BLV positive animal, slaughter or segregation of infected cattle from antibody negative cattle is the most effective means [13, 16, 22, 24]. Control by vaccination has been tried by several investigators [12, 14, 15, 19–21], and the effects were confirmed experimentally. In these studies, the fetal lamb kidney cells persistently infected with BLV (FLK) were commonly used as a virus source [23]. However, it is now known that the FLK is contaminated with bovine viral diarrhea-mucosal disease virus [6]. Therefore, we used as a virus source two cell lines infected with BLV, which were free from such contaminant.

The purpose of the present study was to elucidate the possibility of protection against BLV infection in cattle immunized with inactivated vaccine.

MATERIALS AND METHODS

Cells persistently infected with BLV: Two kinds of

cell lines (LK₁₅ cells and Bat₂cl₁ cells) persistently infected with BLV were used. The LK₁₅ cell was established by the authors using co-cultivation method of fetal bovine lung cells and lymphosarcoma cells obtained from cattle suffering from bovine leukosis [unpublished data, S. Fukuyama *et al.*]. The Bat₂cl₁ cells was originated from bat lung cells and established by Graves and Ferrer [5], kindly supplied by Dr. Nakajima, National Institute of Animal Health, Tsukuba, Japan. These cells were grown in Dulbecco's modified Eagle minimum essential medium (Nissui Co., Ltd., Japan) containing 5% fetal calf serum (CSL Co., Ltd., Australia).

Preparation of vaccine: Culture fluid from LK₁₅ cells was centrifuged at 4,000 g at 4°C for 30 min. The supernatant was mixed with 8% polyethylene-glycol (molecular weight; 6,000), stirred at 4°C overnight on a magnetic stirrer and centrifuged at 4,000 g at 4°C for 30 min. The precipitate was dissolved in a minimal volume of phosphate-buffered saline at pH 7.2 and concentrated to 1/200 of the volume of the original culture fluid. The concentrate was mixed and inactivated with 0.1% Triton X-100 or 0.1% formalin at 4°C for a week as LK₁₅ immunogen. It contained BLV-glycoprotein (gp) antigen and BLV-protein (p) antigen, and the antigenic titers was 1:16 and 1:32, respectively, when measured by agar gel immunodiffusion

(AGID) test with bovine positive serum.

Concentrate of culture fluid of Bat₂cl₁ cells was obtained by the same method as above. The Bat immunogen was inactivated with 0.1% formalin. The antigenic titers of gp and p were 1:16 and 1:64, respectively.

Before use, these immunogens were tested for the presence of infectious BLV by means of intravenous (i.v.) inoculation with 10 doses of each immunogen into 2 cattle. Neither immunological response nor evidence of infection was found by syncytium induction assay (SIA) at 4 months after inoculation.

Furthermore, the immunogens did not form syncytia when inoculated into indicator cat cells infected with mouse sarcoma virus (cc81 cells). The immunogens were mixed with oil adjuvant (Montanide ISA-70) at a ratio of 1:2 before use, and the mixtures were used as LK₁₅ vaccine and Bat vaccine, respectively.

Cattle: Twenty-two cattle (Holstein) negative for BLV antibodies, aged 4 to 15 months old at the time of trials were used. Twelve of them were vaccinated and the remaining 10 were used as controls without vaccination.

Vaccination and challenge exposure: Twelve animals were injected intramuscularly (i.m.) twice at 4-week interval with 2 ml of vaccine. Nine were vaccinated with the LK₁₅ vaccine and 3 with the Bat vaccine. Four weeks later, all of the vaccinated and control cattle were challenged i.v. or subcutaneously (s.c.) with 100 or 500 μ l of heparinized blood of a donor cow infected with BLV persistently, which contained approximately 3×10^5 lymphocytes / 100 μ l and formed 70–100 syncytia by SIA. The donor cow was 6 years old, and inoculated experimentally with lymphosarcoma cells from a cow with spontaneous bovine leukosis, and showed BLV-gp and -p antibody titer of 1:32 and 1:4 by the AGID test. The leukocyte counts were approximately 5,500–6,500/ μ l.

Among 4 cattle which were protected against challenge exposure, 2 were rechallenged by the same method as described above. The remaining 2 were revaccinated with 2 ml of the LK₁₅ vaccine 32 weeks after the first vaccination and challenged again 4 weeks later in the same manner as described above.

Detection of BLV antibody: Serum antibody titers were determined by the AGID test with gp and p antigen of BLV [10]. The antibody titer was expressed as the highest dilution of the positive

serum.

Detection of BLV in lymphocytes: BLV was detected by SIA as described previously [2, 3, 11]. Briefly, 5×10^4 of cc81 cells which were used as indicator cells [4, 7, 9], were suspended in 0.5 ml of medium containing 10% fetal calf serum and seeded in a multi-well plate (in 17 mm). Immediately, the cells were inoculated with 1×10^6 , 1×10^5 and 1×10^4 of lymphocytes isolated from peripheral bovine blood by the Ficoll-Paque gradient density method and incubated at 37°C. The cells were stained 4 days after co-cultivation, and cells with 11 or more nuclei were counted as syncytial cells. To assay BLV *in vivo*, BLV-free cattle aged 2 and 7 months old were inoculated i.v. with 20 ml of the peripheral blood of test cattle and examined by the AGID test and SIA over 3 months.

RESULTS

Antibody production and protection from BLV infection in the vaccinated cattle: In 4 cattle vaccinated with 2 ml of the LK₁₅ vaccine treated with formalin, antibodies to gp and p antigen (gp and p antibodies) appeared at very low titers 4 weeks after the first inoculation, but they increased markedly after the second inoculation. The titers ranged from 1:16 to 1:64 for the gp antigen and from 1:16 to 1:32 for the p antigen 4 weeks after the second inoculation. The cattle were challenged i.v. or s.c. with 100 μ l whole blood of a BLV-infected donor cow. BLV was not isolated by SIA from 0 to 24 weeks after the challenge exposure (Tables 1 and 5).

In 3 cattle vaccinated with the formalin treated Bat vaccine, antibody titers ranged from 1:8 to 1:64 for the gp antigen and from 1:16 to 1:128 for the p antigen at the time of the challenge exposure. After i.v. challenge with 100 μ l of infected blood, in 2 of the 3 vaccinated cattle, no BLV was detected by both SIA and *in vivo* assay. In a cattle with a gp antibody titer of 1:8 at the challenge exposure, however, the titer was elevated and BLV was detected. Antibody responses and protective effects in cattle vaccinated with Bat vaccine were similar to those in LK₁₅ vaccine.

On the other hand, the 5 unvaccinated cattle became seropositive for gp and p antigens, and BLV was detected continuously in all cattle 2 or 4 weeks after the challenge exposure (Tables 1 and 5).

In 5 cattle vaccinated with the LK₁₅ vaccine treated with Triton X-100, the gp and p antibody

Table 1. Antibody titers in cattle immunized with LK₁₅ and Bat vaccines treated with formalin, and virus isolation after challenge exposure with 100 μ l whole blood of a BLV-infected cow

Exp. group	Cattle No.	Antibody titers at weeks after the first vaccination										Detection of BLV after challenge	
		Antigen	0	4	8	12	16	20	24	28	32		
Vaccinated and challenge exposed	LK ₁₅ Vaccine	gp		vac. ↓	vac. ↓	chal. ^{a)} ↓							
			<1	1	64	32	16	8	8	4	4	— ^{b)}	
		p	<1	1	32	16	8	8	4	4	4		
			gp	<1	<1	32	16	8	8	4	2	2	—
		p		<1	<1	16	8	8	4	4	2	1	
			gp	<1	1	16	32	16	8	4	4	2	—
		p		<1	2	32	16	8	4	4	2	2	
			gp	<1	2	32	32	16	8	8	8	8	—
	p	<1		1	16	16	8	4	4	4	4		
		Bat Vaccine	gp	<1	2	64	32	16	8	8	2	2	—
<1	2			128	64	8	8	4	2	2			
p	<1		<1	8	8	32	128	64	64	32	+		
	<1		<1	16	4	4	4	2	4	4			
Unvaccinated and challenge exposed	gp	<1	1	32	32	8	4	4	2	2	—	—	
		<1	<1	32	32	16	8	2	4	4			
	p			<1	1	4	8	8	8	16	+		
		<1	<1	<1	<1	4	4	4	4	2			
	gp			<1	4	32	32	32	16	16	+		
		<1	<1	<1	<1	2	2	2	2	2			
	p			<1	<1	32	16	32	32	16	+		
		<1	<1	<1	<1	1	1	1	1	2			
	gp			<1	2	16	64	64	64	32	+		
		<1	<1	<1	<1	4	4	4	4	2			

a) Cattle were challenged intravenously (No. 155, 156, 148, 150, 153, 158 and 164) or subcutaneously (No. 159, 166, 151, 154, and 169).

b) Tested by syncytium induction assay from 0 to 24 weeks after challenge. — : Not detected. + : Detected.

c) Tested by *in vivo* assay.

titers rose in the same manner as that of the formalin treated vaccine. They were also challenged s.c. with 500 μ l of infected blood. BLV was detected in 3 of the 5 vaccinated cattle, of which gp antibody titers were maintained after the challenge exposure. BLV was undetected in the remaining 2 cattle, of which gp and p antibody titers decreased gradually after the challenge exposure. BLV was also detected in the 2 unvaccinated cattle, which became seropositive 4 weeks after the challenge exposure (Tables 2 and 5).

Protection against rechallenge exposure: Two cattle vaccinated with LK₁₅ or Bat vaccine which were protected against the first challenge exposure were rechallenged with 100 μ l of infected blood 24 weeks later, showed gp antibody titers of 1:2 and 1:8. Elevation of the gp and p antibody titers was

observed 4 weeks after the rechallenge exposure. BLV was isolated from the 2 cattle by SIA 2 or 4 weeks after the rechallenge exposure. Antibodies and BLV were detected in the 2 unvaccinated cattle (Tables 3 and 5).

Revaccination and protection from BLV infection: Two cattle vaccinated with LK₁₅ or Bat vaccine which were protected against the first challenge exposure were revaccinated i.m. with 2 ml of the LK₁₅ vaccine 32 weeks after the first vaccination, when the antibody titers were 1:2 and 1:4 for the gp antigen and 1:4 in each for the p antigen. The antibody titers in the two cattle reached 1:16 and 1:32 for the gp antigen and 1:32 and 1:128 for the p antigen at 4 weeks after revaccination, when they were rechallenged s.c. with 100 μ l of infected blood. In these cattle, the antibody titers decreased gra-

Table 2. Antibody titers in cattle immunized with LK₁₅ vaccine treated with Triton X-100, and virus isolation after challenge exposure with 500 μ l whole blood of a BLV-infected cow

Exp. group	Cattle No.	Antibody titers at weeks after the first vaccination										Detection of BLV after challenge
		Antigen	0	4	8	12	16	20	24	28	32	
			vac. ↓	vac. ↓	chal. ^{a)} ↓							
Vaccinated and challenge exposed	172	gp p	<1 <1	2 1	64 16	64 4	32 8	16 4	8 4	4 2	4 2	— ^{b)}
	174	gp p	<1 <1	1 2	64 64	128 32	128 8	128 8	128 8	64 8	64 4	+
	179	gp p	<1 <1	1 <1	32 32	16 16	16 4	8 4	4 4	2 1	2 1	—
	180	gp p	<1 <1	<1 <1	16 32	32 16	128 16	64 16	64 16	64 16	32 8	+
	176	gp p	<1 <1	1 <1	32 64	32 32	128 16	64 4	64 4	64 4	32 4	+
	Unvaccinated and challenge exposed	171	gp p			<1 <1	8 1	32 4	32 4	32 4	16 4	NT ^{c)} NT
	181	gp p			<1 <1	4 <1	16 1	64 2	64 2	32 2	NT NT	+

a) Cattle were inoculated subcutaneously.

b) See footnotes to Table 1.

c) Not tested.

Table 3. Antibody production and virus isolation in immunized cattle after rechallenge exposure with 100 μ l whole blood of a BLV-infected cow 24 weeks after the first challenge

Exp. group	Cattle No.	Antigen	Antibody titers at weeks after rechallenge					Detection of BLV after rechallenge
			0	2	4	8	12	
Vaccinated and rechallenge exposed	148	gp	2	4	32	32	NT ^{b)}	+ ^{b)}
		p	2	1	8	8	NT	
	166	gp	8	8	128	256	512	+
		p	4	2	128	128	256	
Unvaccinated and challenge exposed	157	gp	<1	<1	4	16	NT	+
		p	<1	<1	<1	<1	NT	
	167	gp	<1	<1	1	64	128	+
		p	<1	<1	<1	2	8	

a) Not tested.

b) + : Detected.

dually after the rechallenge exposure, and the antibody titers for the p antigen were relatively higher than those for the gp antigen during the observation period. BLV was not isolated from any revaccinated cattle (Tables 4 and 5).

Isolation of BLV from the tested cattle: The virus titers of BLV were examined in the lymphocytes after the challenge exposure in all of the tested cattle. BLV was detected by SIA in 1×10^6 lympho-

cytes. In positive cattle, BLV was detected continuously from 2 or 4 weeks after the challenge exposure and reached a peak in about 8 weeks. This result correlated with the gp antibody titers after the challenge shown in Tables 1–4. The pattern of BLV detection was quite similar both in the non-protected and challenged control cattle (Table 5).

Changes in the ratio of gp to p average antibody titers in the vaccinated cattle after challenge exposure:

Table 4. Antibody production and virus isolation in cattle reimmunized with LK₁₅ vaccine and rechallenged with 100 μ l whole blood of a BLV-infected cow 32 weeks after the first vaccination

Exp. group	Cattle No.	Antibody titers at weeks after revaccination							Detection of BLV after rechallenge
		Antigen	0	4	8	12	16	20	
Revaccinated and rechallenge exposed	151	gp p	vac. ↓	chal. ↓					
			2	32	8	8	8	4	— ^{a)}
	4	128	32	32	16	8			
	155	gp p	4	16	16	16	8	4	—
			4	32	32	16	16	16	
	Unvaccinated and challenge exposed	161	gp p		<1	32	32	32	NT ^{b)}
				<1	<1	2	2	NT	

a) Tested by syncytium induction assay from 0 to 20 weeks after revaccination. - : Not detected. + : Detected.

b) Not tested.

Table 5. Virus isolation in vaccinated and unvaccinated control cattle after challenge exposure with whole blood of a BLV-infected cow

Challenge dose	Exp. group	Cattle No.	No. of syncytia (log/ 1×10 ⁶ lymphocytes) at weeks after challenge							
			0	2	4	8	12	16	20	24
100 μ l	Vaccinated	155	0	0	0	0	0	0	0	0
		156	0	0	0	0	0	0	0	0
		166	0	0	0	0	0	0	0	0
		159	0	0	0	0	0	0	0	0
		148	0	0	0	0	0	0	0	0
		150	0	0	2.8	3.5	3.1	3.6	4.3	3.2
		151	0	0	0	0	0	0	0	0
		148 ^{a)}	0	0.6	2.9	3.7	2.8	NT ^{c)}	NT	NT
		166 ^{a)}	0	0	3.5	4.2	4.0	NT	NT	NT
		151 ^{b)}	0	0	0	0	0	0	NT	NT
		155 ^{b)}	0	0	0	0	0	0	NT	NT
	Unvaccinated	158	0	0	3.3	4.2	4.0	NT	2.1	1.7
		164	0	1.6	2.2	4.2	3.5	NT	2.5	2.7
		169	0	0.9	3.7	4.0	2.1	1.5	0.6	0.5
		153	0	0	1.5	3.5	1.3	1.0	1.4	1.3
		154	0	0	2.9	3.3	3.4	3.9	3.3	NT
		157	0	0.5	3.3	3.5	2.7	NT	NT	NT
		167	0	0	4.0	4.1	4.4	NT	NT	NT
		161	0	0	2.8	3.3	4.5	NT	NT	NT
500 μ l	Vaccinated	172	0	0	0	0	0	0	0	0
		174	0	0	4.1	3.8	4.5	4.3	2.8	NT
		176	0	1.1	3.9	2.4	1.9	0.8	1.0	NT
		179	0	0	0	0	0	0	0	0
		180	0	0	3.8	4.0	3.8	3.4	2.5	NT
	Unvaccinated	171	0	1.8	4.7	3.3	3.1	2.8	1.4	NT
		181	0	0.8	3.9	3.9	4.2	4.4	4.0	NT

a) Rechallenged at 24 weeks after the first challenge.

b) Rechallenged after the revaccination.

c) Not tested.

Fig. 1. The changes in gp and p antibody titers in the vaccinated cattle after challenge exposure. The points indicate the average titer of antibodies. 1) Challenged with 100 μ l or 500 μ l whole blood of a BLV-infected cow. ●: gp antibody titers. ○: p antibody titers.

Table 6. The ratio of gp to p average antibody titers in syncytium induction assay (SIA)-positive and -negative cattle after challenge exposure

Challenge dose	Exp. group	SIA result	No. of cattle	Ratio of gp/p average antibody titer	
				Pre-challenge	Post-challenge ^{a)}
100 μ l	Vaccinated	—	8	0.83	0.87
		+	3	0.99	4.07
	Unvaccinated	+	8	—	10.45
500 μ l	Vaccinated	—	2	1.95	2.0
		+	3	1.00	8.1
	Unvaccinated	+	2	—	8.2

a) Tested at the last days (12 to 24 weeks) of observation periods after challenge.

Table 7. Protective effect against challenge exposure with whole blood of a BLV-infected cow in vaccinated and unvaccinated control cattle with various serum gp antibody titers

Challenge dose	gp Antibody titer	No. of tested cattle	No. of BLV-infected cattle	Protection rate (%)
100 μ l	64	2	0	100
	32	4	0	100
	16	2	0	100
	8	2	2	0
	2	1	1	0
	<1	8	8	0
500 μ l	64	2	1	50
	32	2	1	50
	16	1	1	0
	<1	2	2	0

In 10 protected and 4 non-protected cattle, changes in the ratio of gp to p antibody titers were examined until 20 weeks at 4-week intervals after the challenge exposure. In the protected group, the average antibody titers were 1:32 to both of gp and p antigens, and decreased gradually with time. On the other hand, in the non-protected group that had average antibody titers of 1:23 for the gp antigen and 1:27 for the p antigen, the gp antibody titers increased from 4 weeks after the challenge exposure, although the p antibody titers decreased with time. The titers were elevated in the control groups (Fig. 1).

In comparison of the average antibody titers of gp and p after challenge, they were parallel in the protected group, but in the non-protected group the gp titers were higher about 4-fold than p titers as observed in the challenged control group (Table 6).

Relationship between gp antibody titer and protective effect: The relationship between serum gp antibody titers and protective effect in all cattle used in the experiment was summarized in Table 7. All cattle that had gp antibody titers of 1:16 or more and challenged with 100 μ l of infected blood, and half of the cattle that had gp antibody titers of 1:32 or 64 and challenged with 500 μ l of infected blood protected against BLV infection (Table 7).

DISCUSSION

The results of the present experiments indicate that the amounts of challenge inoculum strongly influence the protection levels in cattle immunized with BLV vaccine. Miller *et al.* [14, 15] reported that in a preliminary experiment, 3 of 4 vaccinated cattle were protected against small challenge doses (approximately 1 to 4 infective doses) of BLV. However, in the second experiment, of 14 vaccinated cattle challenged with at least 100 infective doses, only 2 were protected, judging from the results of the sheep bioassay. Approximately 10 infective doses were contained in 100 μ l whole blood used for challenge in the present studies.

In the field, cattle are usually infected with BLV during summer and autumn when blood-sucking insects are active [1, 17]. However, detail mode of establishment of BLV infection remains unknown. One bite of blood-sucking insects with BLV may not establish an infection in a new host. It is possible that cattle are infected with BLV in the field by repeated bites of many blood-sucking insects carrying BLV from infected cattle. Therefore, to evaluate the effectiveness of the vaccine, the challenge doses may be a most important factor. It is estimated that the levels of antibody required for protection may be lower than those of suggested by previous investigations [12, 14, 15, 19–21].

From the results of our experiments, the gp antibody titer of 1:16 was sufficient to protect against BLV infection with 100 μ l blood of an infected donor cow. However, this titer was ineffective against challenge with 500 μ l of infected blood. On the other hand, Kono *et al.* [12] reported that a gp antibody titer of 1:64 is required for protection against BLV infection in sheep. This discrepancy might be due to differences in the methods used for the challenge exposure or in the animal species used.

In general, BLV infection in the vaccinated cattle

is confirmed by sheep bioassay, since this method is the most sensitive indicator of infection [12, 15]. Miller *et al.* [15] described the difficulties in obtaining positive SIA results in vaccinated but non-protected cattle. However, our present study demonstrated that the SIA was a sensitive indicator of infection, since the syncytia were detected at high titers or not. Also, no case of negative transition from positive syncytium was observed throughout the study. Therefore, we used the SIA to judge the establishment of infection. Furthermore, the gp antibody titers in the vaccinated but non-protected cattle became higher 4 and 8 weeks after the challenge exposure, and thereafter, they remained at comparatively high levels during the observation period. In contrast, those of the vaccinated and protected cattle decreased gradually.

The antibodies induced by natural infection may be distinguished from those induced by vaccination in the ratio of gp to p antibody titers. The gp antibody titers in the naturally infected cattle are consistently higher than those of the p antibody [10] and the gp and p antibody titers in the vaccinated and protected cattle decreased gradually in parallel.

To this time, there are some trials of BLV vaccination due to inactivated vaccine for protection [12, 14, 15, 19–21], and in this paper, we can suggest the possibility of protection against BLV infection by the vaccination.

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