Immune Effect of Toxoplasma Lysate Antigen (TLA) on Cattle against *Theileria sergenti* Infection

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ABSTRACT. Six Holstein calves 6–13 months old which had never been grazed on pasture were inoculated with 1,000 μg of toxoplasma lysate antigen (TLA) two or three times. They were placed on a pasture contaminated with ticks as a vector of *Theileria sergenti* 7 weeks after the initial inoculation with TLA emulsion. The lowest RBC was $649 \times 10^4/\mu\text{l}$ for the group of TLA emulsion inoculation and $229 \times 10^4/\mu\text{l}$ for a control group in grazing. The highest and average rates of parasitized erythrocytes were 2.6% and 0.6%, respectively, for the former group and 10.0% and 3.2% for the latter. When examined for production of antibody against TLA, all the inoculated calves showed an indirect hemagglutination (IHA) antibody titer higher than 1:6 after the initial inoculation with TLA emulsion, except one of three calves inoculated twice with this antigen. The uninoculated control calves also exhibited an increase in IHA antibody titer against TLA after they were placed on pasture. On the other hand, the complement fixation and the indirect immunofluorescence antibody titers against *T. sergenti* became detectable in one calf inoculated three times with TLA emulsion and the uninoculated control calves 1–5 weeks after the beginning of grazing. No calves possessed serum antibody against *Babesia ovata* or *Anaplasma centrale*.—**KEY WORDS:** cattle, *Theileria sergenti*, toxoplasma adjuvant.

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Inoculation with infected blood is one of the methods used for the prevention of bovine theileriosis. It is efficient enough to prevent cattle from manifesting clinical symptoms in the case of spontaneous infection. It is expected to present the same side effects as blood transfusion [12]. In consequence, another method is tried for the prevention of bovine babesiosis, in addition to the virulent blood method [19]. In it, cattle are sensitized with antigen extracted from *Babesia* protozoa by radioactive irradiation or from infected erythrocytes by thawing treatment [9, 10, 13–15, 17, 26, 32–34, 36].

A premedication of nonspecific antigen, such as bacillus Calmette-Guérin (BCG) killed *Brucella abortus* (strain 19) or *Corynebacterium parvum* [1, 2, 3, 8, 18] is another method for prevent of rodent and bovine babesiosis, and bovine theileriosis. Vaccination with killed specific antigens had been

attempted to prevent infection with *Theileria parva* and *T. annulata* [25, 35].

No attempts have been made, however, to prevent clinical infection with *T. sergenti* in cattle by using specific antigen. This may be because it is difficult to prepare pure antigen from *T. sergenti*, since this organism is smaller in size than the *Babesia* protozoa in the erythrocytes and has exhibited a rather low rate of infection [4–7, 16, 20, 27]. Therefore, it seems practically impossible to carry out inoculation with specific antigen, or a method of prevention with inactivated vaccine, in a number of cattle, at least for the time being.

On the other hand, Omata *et al.* [23, 24], and Ogawa *et al.* [21] reported that *toxoplasma* lysate antigen (TLA) had an effect of preventing mice from dying of babesia or plasmodium infection. In the present experiment, cattle were inoculated with TLA,

which is nonspecific antigen, to determine whether or not the antigen had an effect of preventing cattle from spontaneous clinical infection with bovine theileriosis during the period of grazing on pasture.

MATERIALS AND METHODS

Cattle: Nine Holstein calves 6–13 months old were used. They had not been grazed on pasture before. Six were inoculated intramuscularly with TLA and three used as uninoculated controls. One of the controls had already been grazing before the inoculated calves were placed on pasture. As it was found to have been infected clinically with *T. sergenti* one week after these calves were placed on pasture, it was added to the control group.

Preparation and inoculation of TLA: TLA was prepared by the method of Igarashi *et al.* [11], which was a modification of Jacobs and Molton's method. The six calves were divided equally into two groups. TLA was inoculated three times at two-week intervals in one group (the three-dose group) and twice at four-week intervals in the other (the two-dose group). For sensitization 1,000 μg per head of TLA was emulsified in Freund's incomplete adjuvant (FIA) so that it might be in a state of water in oil. The dose of TLA emulsion used was 2 ml per head. It was inoculated into the masseter muscle on both sides for the first time and into the gluteal muscle on both sides for the second and third time. The last dose of TLA inoculated was 500 μg per head in both groups.

Infection with *T. sergenti*: The calves were placed on a pasture contaminated with ticks as a vector of *T. sergenti*, so that they might be exposed to spontaneous infection with this organism. The pasture was that for common use which was located in Town "A" in the northeastern part of Hokkaido and in which most grazing cattle had been infected clinically with *T. sergenti* every year. The grazing

period extended over 9 weeks, beginning with 3 weeks after inoculation with the last dose of TLA emulsion.

Blood sampling and test: Blood samples amounting to about 15 ml each were collected from the jugular vein of each calf before inoculation on the day of TLA emulsion inoculation and 1, 5 and 9 weeks after the beginning of grazing. They were used for the estimation of red and white blood cell (RBC and WBC) counts and hematocrit (Ht) and hemoglobin (Hb) values. RBC was counted by a Sysmex platelet counter (Model PL-110, Toa Med. Elec. Co., Kobe). Ht value was calculated by a microhematocrit tube. Hb value was determined with a kit of Hb estimation (Wako pure Chem. Co., Ltd., Tokyo). To collect clinical data, the external appearance at large was examined, as well as the color tone of the visible mucous membranes, in every calf at the time of blood sampling. Serum was separated from each blood sample and subjected to the estimation of antibody titer and precipitating antibody against TLA. Antibody titer was estimated by the indirect hemagglutination (IHA) test [29], which is outlined as follows. Sheep red blood cells (SRBC) were treated first with formalin and then with tannic acid. They were allowed to adsorb TLA. After that, 1% suspension of these cells was prepared with phosphate buffer solution with the addition of 0.5% rabbit serum (0.5% NRS-PBS). Antiserum was inactivated at 65°C for 20 minutes. Serial dilutions, extending from 1:2 to 1:256, were prepared from 0.025 ml of inactivated antiserum. They were placed in the wells of a microplate. Add 0.05 ml of antigen-SRBC suspension to each well by dropping. The microplate was held at room temperature (18°C) for 24 hours to allow the reaction to take place. Finally, the results of the reaction were judged. Precipitating antibody was detected by Ouchterlony's method [22]. On the other hand, the same serum samples as examined by the author were sent to Dr. Shingo

Table 1. Response of inoculated calves exposed to *T. sergenti* infection

Group	No. of calves	Weeks of challenge exposure	Erythrocytes ^{a)} ($\times 10,000/\mu\text{l}$)		Hematocrit ^{a)} (%)		Max. parasitemia ^{a)} (%)		Max. IHA ^{b)} titer	No. of chemotherapy
			min.	max. (Average)	min. ^{c)}	max. ^{d)} (Average)	min.	max. (Average)		
Treated with 3 doses of TLA	3	9	649— (722 $\pm$ 43)	864	27—37 (31)		1.04— (1.8)	2.6	1:512	0
Treated with 2 doses of TLA	3	9	662—1033 (868 $\pm$ 67)		29—38 (32)		0.1 — (0.5)	1.3	1:512	0
Untreated control	3	9 to 17	229— (547 $\pm$ 211)	906	19—34 (28)		3.9 — (6.0)	10.0	1: 48	1

Remarks. Interval between inoculations: 2 weeks for the three-dose group and 3 weeks for the two-dose group. Calves were exposed to infection by grazing for 3 weeks after the last inoculation.

a) In the same grazing team.

b) Indirect hemagglutination test.

c) Minimum.

d) Maximum.

Ito, Department chairman, and Dr. Tetsuro Minami, of the National Institute of Animal Health, to estimate antibodies against *Anaplasma centrale*, *Babesia ovata*, and *T. sergenti* contained in these samples. In this manner a double check was performed on spontaneous infection with these protozoa among the experimental calves.

The rate of infection with *T. sergenti* was calculated from the number of parasitized erythrocytes (PE) per 1,000 RBC counted in a thin-film smear preparation stained by May-Gruenwald-Giemsa double staining.

RESULTS

Clinical findings: The site of inoculation with TLA emulsion was swollen in four calves, or two calves of each of the two-dose and three-dose groups. The swollen site of inoculation was noticed in the gluteal muscle, as well as in the masseter muscle. In the two calves of the two-dose group, induration persisted at the site of inoculation for about 2 and 3 months, respectively. In one calf of the three-dose group, anorexia was seen for 3 days after the initial inoculation. Nothing particularly abnormal was found in general health conditions in any other calf of this

group.

Hematological findings: Table 1 shows the summarized results of the hematological examination performed in the TLA emulsion inoculation groups and the control group. The lowest RBC was $649 \times 10^4/\mu\text{l}$ for the former groups and $229 \times 10^4/\mu\text{l}$ for the latter. The control calf showing the lowest RBC and suffering from severe anemia was treated by intramuscular injection with oleaginous primaquine.

The lowest of the Ht values estimated 1, 5 and 9 weeks after grazing was 19% for the control, 27% for the three-dose, and 29% for the two-dose group.

The mean of the highest rate of PE was 6.0% for the control, 1.8% for the three-dose, and 0.5% for the two-dose group.

The antibody titer against TLA determined by IHA was 1:512 for the TLA emulsion inoculation groups. It was 1:48 for the calf infected clinically with *Theileria* of the control group.

Figure 1 shows changes in RBC, Ht value, and parasitemia in each individual of every group. RBC was much lower in the control group than in the TLA emulsion inoculation groups during the grazing period. The calf of that group (control No. 2) which was affected

Fig. 1. Change in number of erythrocytes (RBC), hematocrit (Ht) and parasitemia (PE) of inoculated and control calves.

↓ : Inoculation with TLA ▽ : Exposure to infection by grazing
 † : Died by accident ↓ : Treatment
 ●—● : three-dose group ○—○ : Two-dose group
 ---- : Control

seriously with theileriosis, showing as RBC of $229 \times 10^4/\mu l$, was treated with oleaginous primaquine. As a result, its RBC returned to a level of $634 \times 10^4/\mu l$ on the day of the last hematological examination, or 9 weeks after grazing.

A calf of the three-dose group fell in a swamp and died 3 days after grazing.

Ht value presented the same rise and fall as RBC during the grazing period. It was distinctly smaller in the control group than in the

TLA inoculation groups. In the calf (control No. 2) of the control group suffering from severe theileriosis, Ht value continued to increase gradually during the grazing period of 9 weeks as a result of treatment.

The individual rate of PE, or that of parasitemia, was 10% in a calf (control No. 2) of the control group one week after grazing of the experimental groups. This calf was treated so successfully that its parasitemia was alleviated by 9 weeks after grazing, when that

Table 2. Serum IHA titer against TLA in inoculated and control calves after exposure to infection

Group	Calf No.	Weeks after grazing					
		-7	-5	-3	1	5	9
Treated with 3 doses of TLA	1	0	1:12	*1:512 ^{a)}	*1:256	*1:512	1:512
	2	0	1:6	:12	ND ^{b)}	1:6	1:12
	3	0	1:6	1:12 ^{c)}			
Treated with 2 doses of TLA	1	0	1:6	*1:96	*1:196	:192	1:96
	2	0	1:6	1:12	*1:152	1:192	1:12
	3	0	0	0	1:48	1:48	1:24
Untreated control	1	0	0	0	1:6	1:12	1:48
	2	ND	ND	ND	1:48	1:6	1:6

a) Agar gel diffusion test was also done. A precipitation line was seen in agar gel diffusion between collected serum and *Toxoplasma* lysate antigen (TLA).

b) Not done.

c) Died by accident.

Table 3. Serum CF and IFA titer for *T. sergenti* in inoculated and control calves

Group	Calf No.	Weeks after grazing			
		-7	-3	1	5
Treated with 3 doses of TLA	1	0	0	0	10 ^{a)}
		0	0	0	1000 ^{b)}
	2	0	0	ND ^{c)}	0
Treated with 2 doses of TLA	1	0	0	0	0
		0	0	0	0
	2	0	0	0	0
Untreated control	1	0	0	ND	10
		0	0		5000
	2	ND	ND	40	20
				5000	5000

a) Complement fixation antibody titer.

b) Indirect fluorescent antibody titer.

c) Not done.

rate was 0.8%. At the same time the rate was 3.9 and 4.2% in the other two calves of the control group, respectively. In the two calves of the three-dose group, the rate was 0.01 and 0.2%, respectively, 5 weeks and 1.0 and 2.6% 9 weeks after grazing. In the three calves of the two-dose group, it was 0.01, 0.01 and 0.1%, respectively, 5 weeks and

0.3, 1.3 and 0.1% 9 weeks after grazing. In brief, the individual rate of parasitemia was relatively lower in the TLA inoculation groups than in the control group.

Table 2 presents a rise and fall of serum antibody titer against TLA sensitization. In a calf (No. 1) of the three-dose group, IHA antibody titer was 1:12 2 weeks after the first inoculation (5 weeks before the beginning of grazing). It was 1:512 in this calf after the third inoculation up to 9 weeks after grazing. It ranged from 1:6 to 1:12 in the other two calves of this group over a period from 2 weeks after the first inoculation to 9 weeks after grazing. In two calves of the two-dose group, it was 1:6 on the day of the second inoculation and increased rapidly to 1:192–1:512 by one week after grazing. In the other calf of this group, it was undetectable on the day of the second inoculation and 1:48, 1:48 and 1:24 1, 5 and 9 weeks, respectively, after grazing. In the control group, it ranged from 1:6 to 1:48 over a period from 1 to 9 weeks after grazing.

In the gel precipitation reaction, a precipitation line was induced by serum samples collected from one calf of the three-dose group on the day of the third inoculation and 1 and 5 weeks after grazing. It was also

induced by serum samples collected from one calf of the two-dose group on the day of the second inoculation and from two calves of this group one week after grazing.

The results of the complement fixation (CF) test and the indirect fluorescent antibody (IFA) test were carried out to detect serum antibodies against *T. sergenti* (Table 3), *A. centrale* and *B. ovata*. No serum antibodies against *A. centrale* or *B. ovata* were detected from any grazing calf. Serum antibody against *T. sergenti* demonstrable by CF and IFA tests was detected from one calf of the three-dose group 5 weeks after grazing and from the calves of the control group.

DISCUSSION

Antigen is inoculated into cattle for the prevention of piroplasma infection. Various methods and sites have been used for the antigen inoculation [9, 14, 32]. Taylor *et al.* [34] injected cattle subcutaneously or intravenously with a freeze-thawed substance of blood cells infected with *B. divergens*. As a result, they pointed out that the substance displayed a greater effect on the prevention of clinical infection when injected subcutaneously than when injected intravenously. Kuttler and Johnson [14] administered cattle with mixture of soluble antigen of *B. bigemina* and Freund's complete adjuvant (FCA) and found that the mixture had an effect of preventing infection. On the prevention of bovine theileriosis and babesiosis by the mode of non-specific immunity, BCG had not effects of preventing infection of *T. parva* [3] and *B. divergens* [1], but of *T. annulata* [18].

The author and his associates made an attempt to confer immunity to cattle by the inoculation with a small amount of antigen. They injected cattle with a mixed emulsion of TLA and FIA into the masseter or gluteal muscle. As a result, swelling and other tissue reactions were noticed persistently at the site of injection for more than one month. Anti-

body titer tended to be high in cattle exhibiting such persistent tissue reactions. The dose of TLA contained in the mixed emulsion was rather small, or 1,000 μg per head. It was considered to be sufficient, since the cattle injected with this emulsion produced antibody against TLA.

In the present experiment, two-dose and three-dose groups were set up. There was, however, no difference in the effect of preventing infection between the two groups. Taylor *et al.* [33] inoculated cattle with a single dose of irradiated *B. divergens* and began to graze the cattle 3 weeks after inoculation to expose them to spontaneous infection with protozoa. Such being the case, further studies should be made on the number of inoculations and the interval between inoculations with this antigen for the prevention of infection.

It has been reported that bovine piroplasmosis is caused by *B. ovata* and *T. sergenti* in the mainland of Japan and that a mixed infection of these species is present in some part of the country [7, 28, 30]. In the present experiment, this mixed infection was negatived on the basis of serum antibody detected and the morphology of protozoan parasites. Therefore, the calves were considered to have been infected with *T. sergenti* alone. It is generally known that cattle are prevented most effectively from clinical infection with *T. sergenti* by the inoculation of live organisms. At present, however, it is very difficult to collect a large amount of these protozoa, which are rather small in size and infect cattle at a relatively low rate. Therefore, the present experiment was carried out to determine whether nonspecific antigen or heterogenetic antigen with cross-immunogenicity was available for the prevention of cattle from infection with *Theileria*.

There is much to be studied on the mutual immunological relationship among protozoan species. Sibinovic *et al.* [29] reported that no IHA had been induced between equine babe-

sia antigen and antibody against *Babesia* in dogs and mice. Ristic *et al.* [27] found, however, that a cross immunological reaction took place between *B. canis* and *B. gibsoni* in dogs. Homogenetic and heterogenetic antigens have been used for the prevention of bovine and rodent babesiosis [5, 8, 25, 30, 36], but heterogenetic antigen has not been used against infection with *T. sergenti* in cattle. In their experiment with mice, Omata *et al.* [23, 24] demonstrated that TLA presented nonspecific resistance to plasmodium infection in mice. The author and his associates [21] pointed out that inoculation with TLA had an effect of preventing dogs and mice from dying of babesia infection. They clarified that its defense mechanism consisted in the activation of macrophages by the sensitization with TLA and in the production of lymphokines from sensitized T lymphocytes in the animals. At present, it is considered that humoral and cell mediated immunity may participate independently or jointly in the defense mechanism of the animal against *T. sergenti* infection [6, 30, 31].

In the present experiment, there was a rapid increase in antibody titer against TLA after inoculation with this antigen and after infection with *T. sergenti*. This result makes it possible to presume that a part of toxoplasma antigen may possess cross-immunogenicity when examined with *T. sergenti*. It is unknown, however, what specific substance will confer a resistance to cattle infected with this protozoon and inoculated with TLA. If these problems are solved basically by studies to be made in future, it will be of practical value to prevent a number of grazing cattle from clinical infection with *T. sergenti* by inoculation with TLA.

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要 約

トキソプラズマ溶解抗原による *Theileria sergenti* 感染に対する牛の免疫効果: 佐藤基佳・堀 次郎¹⁾・広瀬恒夫・鈴木直義²⁾ (帯広畜産大学 獣医臨床放射線学教室, ²⁾家畜生理学教室, ¹⁾堀動物病院)——放牧牛の *Theileria sergenti* 感染による小型ピロプラズマ症の軽減あるいは予防を目的としてトキソプラズマ溶解抗原 (TLA) 投与による免疫賦与効果を検討した。放牧経験のない6～13カ月齢のホルスタイン種雌牛6頭にそれぞれ TLA 1,000 μg を2～3回投与し, 初回投与後7週に小型ピロプラズマ媒介ダニ汚染牧野に放牧, 放牧後9週まで観察した。放牧後の TLA 投与群および対照群の赤血球数最低値は, それぞれ $649 \times 10^4/\mu\text{l}$ および $229 \times 10^4/\mu\text{l}$ であった。最高原虫寄生率は, TLA 投与群では 2.6% (平均値 0.6%), 対照群では 10% (平均値 3.2%) を示した。6例中5例では, TLA 1回投与後に TLA に対する間接血球凝集 (IHA) 抗体価は 1 : 6 以上を示した。TLA 非投与牛においても放牧後には IHA 抗体価が上昇した。一方, *T. sergenti* に対する補体結合抗体価および蛍光抗体法による抗体価は, TLA 3回投与群の1例と非投与対照牛で放牧後1～5週に検出された。いずれの牛にも *B. ovata* および *A. centrale* に対する抗体は認められなかった。