Analyses of Antigenic and Genetic Diversities of *Theileria sergenti* Piroplasm Surface Proteins

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ABSTRACT. Monoclonal antibodies (MoAbs) against *Theileria sergenti* Fukushima stock, an intraerythrocytic protozoan parasite of cattle, were produced. The MoAbs reacted with 32 kDa or 23 kDa piroplasm surface protein (p32 or p23) in Western blot analysis. Using a panel of the MoAbs, antigenic analysis of the stocks and field isolates distributed in Japan was carried out. The results revealed antigenic diversity of *T. sergenti* isolates, but diversity did not appear to be correlated with the geographical region of the isolates. N-terminal amino acids sequences analysis revealed substitutions of a few amino acids between the p23 of two *T. sergenti* stocks. The sequences couldn't be found in amino acid sequence of the p32 deduced from cDNA, which indicated that the p23 is not a proteolytic product of the p32. Genetic diversity of *T. sergenti* isolates was also observed by Southern blot analysis using a cDNA of the p32 as a probe.—**KEY WORDS:** antigenic diversity, cDNA probe, monoclonal antibody, N-terminal amino acid sequence, *Theileria sergenti*.

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Cattle in Japan have been found to be infected with *Theileria sergenti*, an intraerythrocytic parasite transmitted by ticks (*Haemaphysalis longicornis*). Due to the economic losses caused by the disease, it is urgent to develop effective methods for controlling the disease.

Identification and characterization of parasite antigens are required to develop effective serodiagnostic methods, and are a critical step in the development of a vaccine. Studies on piroplasm antigens of *T. sergenti* using immune sera from infected cattle and monoclonal antibodies suggested that the 32 and 23 kDa piroplasm proteins (p32 and p23) are targets of humoral immunity [4, 7, 10] and p32 is a possible candidate for protective antigen [23]. Previous studies on other *Theileria* species including *T. parva* and *T. annulata* revealed antigenic diversities among parasite stocks [1–3, 17, 18]. In contrast, there are no systematic investigations on antigenic characteristics of Japanese *T. sergenti*.

In this study, we produced monoclonal antibodies against *T. sergenti* Fukushima stock. Using these MoAbs together with the MoAbs previously produced against another stock [10] and a cDNA probe [13, 14], we analysed the antigenic and genetic properties of *T. sergenti* piroplasms distributed in Japan.

MATERIALS AND METHODS

Parasites and experimental infection: The Fukushima stock [16], which had been maintained by blood passages, was used to infect splenectomized cattle by inoculation of parasitized erythrocytes. Infected erythrocytes were used

for preparations of *T. sergenti* antigen and blood smears. Chitose, Ikeda, Shintoku and Takahara stocks [12, 13] and field isolates of *T. sergenti* were also used for antigenic analysis. Fukushima and Ikeda stocks were kindly supplied by Dr. K. Fujisaki, National Institute of Animal Health, Tsukuba.

Purification and antigen preparation of piroplasms: Parasites were purified from bovine erythrocytes infected with Fukushima stocks when parasitemia reached more than 5% by using procedure described previously [10]. Piroplasm of other stocks and isolates were purified as previously described [21].

For preparation of enzyme-linked immunosorbent assay (ELISA) antigen, purified parasites were treated with 4% Triton X-100 in phosphate-buffered saline (PBS, pH 7.2) at 4°C for 3 hr. After centrifugation at 2,000 × g for 10 min, the supernatant was used for ELISA according to the method by Shimizu *et al.* [20].

Preparation of monoclonal antibodies: Eight weeks old BALB/C mice were subcutaneously immunized with 100 µg protein/0.25 ml of sonicated piroplasms of *T. sergenti* Fukushima stock emulsified with the same volume of Freund's complete adjuvant. They were boosted with the same amount of the antigen two weeks after the first injection. The spleen was removed 3 days after the second injection. The spleen cells were fused with myeloma cells (P3U1). Hybridoma supernatants were screened by ELISA and an indirect immunofluorescence assay (IFA). The antibody-producing hybridomas were cloned twice by the limiting dilution method in a 96 well plastic microplate. MoAbs 23C11, 23B7 and 23B10 against Chitose stock of *T. sergenti* [10] were also used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

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carried out using the procedure of Laemmli [11]. Parasites were solubilized with 10 volumes of SDS sample buffer, 10 mM Tris-HCl (pH 6.8) containing 2% SDS and 5% 2-mercaptoethanol at 100°C for 2 min and separated electrophoretically in a 10% polyacrylamide gel. The proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane sheet (Immobilon, Millipore Corporation, Bedford, MA) as described by Towbin *et al.* [25]. The sheet was blocked with 5% skim milk, and probed with MoAb (concentration: 10 µg/ml) followed by peroxidase-conjugated anti-mouse IgG diluted in 1:1,000 (Miles Laboratories, Naperville, IL). 3,3'-diaminobenzidine tetrahydrochloride was used for color development.

Enzyme linked immunosorbent assay (ELISA): ELISA was performed according to the method described previously [20, 26]. Microplates were coated with 0.6 µg piroplasm proteins/0.1 ml in 0.05 M carbonate/bicarbonate buffer (pH 9.6) per well. For color development, 0.1 ml of substrate (0.2 mM azino-di-[3-ethylbenzthiazoline] sulfonic acid) was used. The plates were read at 405 nm in a spectrophotometer (Corona Electric Co., Ltd., Tokyo). The end point of ELISA was estimated from the dose-response curve and the antibody titer was expressed as the reciprocal of the highest dilution of supernatant showing an absorbance over 0.1.

N-terminal amino acid sequencing: For N-terminal amino acid sequencing, p23 was immunoprecipitated with MoAb 13F5 from the piroplasm lysates of Shintoku stock. For Chitose stock, whole piroplasms were solubilized by SDS sample buffer. Immunoprecipitated p23 or piroplasm proteins were separated by SDS-PAGE, electrophoretically transferred to PVDF membrane (Immobilon SQ, Millipore) and stained by Commassie blue R-250. A band of p23 was cut out, and its N-terminal amino acid sequence was determined using a pulsed-liquid automated protein sequencer (477A, Applied Biosystems, CA, U.S.A.) as described by Matsudaira [15].

Southern blot hybridization: Southern blot hybridization of *T. sergenti* genomic DNA was carried out as described by Matsuba *et al.* [12]. Genomic DNA of *T. sergenti* isolates were digested with *Pst*I, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose filters. A cloned p32 cDNA was used as a probe [14].

RESULTS

Characterization of the monoclonal antibodies: Supernatants of hybridomas, which reacted with the antigen of Fukushima stock in ELISA, were selected. These hybridoma secreting antibodies reacted with the parasites in infected erythrocytes, but not with normal bovine erythrocytes in IFA assay (data not shown). The antibody titers determined by IFA and ELISA, and their isotypes are shown in Table 1.

Immunoblotting was carried out for further determination of the specificity of MoAbs and for the identification of the molecules recognized by these MoAbs. As shown in

Table 1. Characterization of monoclonal antibodies against piroplasm of *Theileria sergenti* Fukushima stock

MoAbs	IFA titer (C.S.)	ELISA titer (A.W.)	Subclass	Immunoblotting (MW: kDa)
C9	50	10,000	IgG1	32 ^{a)}
B6	50	10,000	IgG1	32
13F5	50	10,000	IgG2b	23
E3	200	20,000	IgG1	23
D11	100	20,000	IgG1	23
1B3	100	10,000	IgG1	23
C3	50	10,000	IgG1	23
C8	4	1,000	IgG1	NT
C12	4	NT	IgG1	NT
F5	4	NT	IgG1	NT

C.S.: Culture supernatant. A.W.: Ascitic water. NT: Not tested. a) Molecular weight (kDa) of polypeptide recognized by MoAbs.

Fig. 1. Detection of piroplasm antigen with MoAbs. Solubilized proteins were separated in 10% SDS-polyacrylamide gels, transferred to PVDF membrane sheets. The blots were probed with MoAbs. Lanes: 1. MoAb C9; 2. B6; 3. E3; 4. 1B3; 5. C3; 6. 13F5; 7. D11. MW marker: kDa.

Fig. 1, 7 out of 10 monoclonal antibodies reacted with the piroplasm antigen of homologous stocks in immunoblotting. C9 and B6 reacted with a polypeptide of 32 kDa, and the other 5 monoclonal antibodies, E3, 1B3, C3, 13F5 and D11 recognized a polypeptide of 23 kDa.

Cross-reactivity among stocks of *T. sergenti*: Using MoAbs produced against two stocks of *T. sergenti*, analysis of the cross-reactivity among 4 stocks was performed by immunoblotting. MoAb B6 reacted with homologous Fukushima stock and heterologous Chitose stock, but not with Ikeda stock. MoAb 13F5 reacted only with homologous Fukushima stock and not with Chitose and Ikeda stocks (Fig. 2). MoAbs E3, D11, 1B3, C9 and C3, and MoAbs against Chitose stock, 23C11, 23B7 and

Fig. 2. Detection of cross-reactivity of *T. sergenti* stocks with MoAbs. Solubilized proteins were separated in 10% SDS-polyacrylamide gels. The separated proteins were transferred to PVDF membrane sheets. The blots were probed with MoAbs B6 and 13F5 respectively.

Table 2. Reactivities of MoAbs against four *T. sergenti* stocks determined by Western blot

MoAbs	Stocks of <i>T. sergenti</i>			
	Fukushima	Chitose	Ikeda	Takahara
Against Fukushima				
C9	32 ^{a)}	32	32	32
B6	32	32	—	—
13F5	23	—	—	—
E3	23	23	23	23
D11	23	23	23	23
1B3	23	23	23	23
C3	23	23	23	23
Against Chitose				
23C11	32	32	32	32
23B7	32	32	32	32
23B10	23	23	23	23

a) Molecular weight of polypeptide recognized by MoAb (kDa).

—: No reaction.

23B10 showed cross-reactivity with piroplasm antigens prepared from Fukushima, Chitose and Ikeda stocks (Table 2). The Takahara stock showed an identical MoAb profile to that of the Ikeda stock.

Analysis of antigenic diversity of geographical isolates of T. sergenti in Japan: To characterize antigenic properties of *T. sergenti* distributed in Japan, we collected infected blood from different regions and purified piroplasms. Six MoAbs were selected to test the reactivity with the field

Table 3. Analysis of antigenic diversity of Japanese isolates of *Theileria sergenti*

Isolates	Monoclonal antibodies					
	C9	B6	13F5	23B10	23C11	23B7
Tokushima	32 ^{a)}	32	23	23	32	32
Fukuoka 106	32	32	23	23	32	32
Fukuoka 93	32	32	23	23	32	32
Fukuoka 99	32	32	23	23	32	32
Niigata 1	32	—	23	23	—	32
Kagoshima 2	32	32	23	23	32	32
Kagoshima 4	32	32	23	23	32	32
Aomori 15	32, 67	32	23, 67	23, 67	32	32
Aomori 19	32, 67	32	23, 67	23, 67	32	32
Miyagi 28	32	—	23	23	—	32
Nagano	32	—	23	23	—	32
Yamanashi	32	NT	23	23	—	32
Yamagata	32	32	23	23	32	32
Iwate	32	NT	23	23	32	NT
Kanagawa	32	NT	23	23	32	NT
Hokkaido						
Hiyama	—	NT	23	23	NT	32
Oshima	32	NT	—	23	NT	32

—: No reaction. NT: Not tested.

a) Molecular weight of polypeptide recognized by MoAbs (kDa).

isolates (Table 3). MoAb 23B10 and 23B7 reacted with all the isolates tested, but other MoAbs failed to react with some of the isolates. Besides polypeptides of 23 kDa and 32 kDa, a 67 kDa polypeptide of Aomori isolates was also

detected by MoAbs C9, 13F5 and 23B10. The extent of the diversity did not appear to be correlated with the region of the isolates. For example, two isolates from Hiyama and Oshima districts in Hokkaido prefecture showed different reaction patterns, but the isolates from Fukuoka and Yamagata prefectures, which are geographically separated, had the same reaction profiles.

N-terminal amino acid sequences of p23: N-terminal amino acid sequences of p23 of *T. sergenti* Chitose and Shintoku stocks were determined (Fig. 3). In 11 amino acids, which were successfully identified in 13 N-terminal amino acids, 9 were shared by both of the stocks. These sequences couldn't be found in the deduced amino acid sequences of p32 from Ikeda or Chitose stock [8, 14].

Genetic diversity of T. sergenti isolates: To determine genetic diversity of *T. sergenti* in Japan, a cloned p32 cDNA probe was used for Southern blot analysis (Table 4). The results revealed genetic diversity of *T. sergenti* isolates distributed in Japan. But there were no relationships between genotypes and phenotypes determined by MoAbs. For example, 3 isolates, Niigata, Fukuoka 93 and Kagoshima 2 had identical Southern blot hybridization patterns, but showed different MoAb reactivities (Table 3).

DISCUSSION

In this study, we have demonstrated antigenic diversities of *T. sergenti* distributed in Japan by using a panel of MoAbs which recognize two different polypeptides on the parasite surface.

All of MoAbs we have produced were directed either to the 32 or the 23 kDa polypeptide. These molecules are exposed on the surface of the piroplasm stages of the parasite as revealed by immunoelectron microscopy [19].

As the 32 kDa molecule is the most abundant protein on the parasite surface [7, 22], hosts infected with the parasites or animals immunized with the piroplasm materials may recognize predominantly these molecules.

In malaria parasites, some of the surface molecules are produced by proteolysis of higher molecular weight precursor proteins, and share common epitopes [9]. The N-terminal amino acid sequence of 23 kDa protein didn't show any homology with the deduced amino acid sequence from a cloned 32 kDa cDNA [8]. This indicates that the former polypeptide is not a proteolytic product of the 32 kDa molecule. MoAbs reaction indicated that there are no common epitopes between these two surface molecules. In contrast, MoAb C9 detected the 32 and 67 kDa polypeptides in field isolates from Aomori (Table 3). Polypeptides of 23 and 67 kDa from the same isolates were detected by MoAbs 13F5 and 23B10. Further studies are required to determine the molecular relationships among these 3 polypeptides.

As the 32 kDa protein is a target of host humoral immunity, parasite may exhibit antigenic polymorphisms as one of evasion mechanisms from host immune response. Recent studies by our group have shown that there are mixed parasite populations with different p32 gene structure, and population changes could be detected during the course of infection [14]. However, the comparison of the results obtained by DNA probing and MoAb profiles revealed no apparent correlations between genotypes and phenotypes. Further studies are needed to determine whether the antigenic polymorphism detected by MoAbs involved in parasite evasion from host immune responses. Amino acid sequence analysis also suggested minor polymorphism in p23 at the N-terminal end using piroplasms from two stocks. It should be determined whether the amino acid substitutions affect the antigenicity of p23.

Antigenic polymorphism is one of obstacles in developing effective protective methods against protozoan parasite infection. *T. parva* shows different cross immunity patterns among different parasite stocks [5]. As p32 of *T. sergenti* is one of candidates for protective antigen, identification of B cell-epitopes is essential. MoAbs 23C11, 23B7, C9, and B6 recognize four different epitopes, one of which has carbohydrate moiety [26]. Amino acid sequences, which bear the B cell epitopes for these MoAbs are being determined.

Parasites phenotypically and genotypically related to *T. sergenti* are distributed in European countries and Australia known as *T. orientalis* and *T. buffeli* [6-7, 22]. In other Asian countries, benign *Theileria* species are also distributed.

Table 4. Southern hybridization patterns using p32 cDNA probe

	11.5	4.1	3.6	0.5 ^{a)}
Aomori 19	+	-	-	-
Miyagi 28	+	+	-	-
Kanagawa	+	+	-	-
Niigata 1	+	+	+	+
Fukuoka 93	+	+	+	+
Kagoshima 2	+	+	+	+
Nagano	+	-	-	-
Hokkaido				
Hiyama	+	+	+	+

a) Length of *Pst*I digested genomic cDNA fragments (Kb).

	N													C	
Chitose stock	T	X	I	D	X	D	K	D	G	K	F	T	D		
Shintoku stock	T	X	I	D	X	I	K	D	G	K	F	A	D		

Fig. 3. N-terminal amino acid sequences of p23. X indicates an unidentified residue.

buted, and *Theileria* species in Korea is identified as *T. sergenti* recently by using polymerase chain reaction [24]. The MoAb panel developed in this study may be a useful tool for the characterization of these parasites. Interestingly, *Theileria* species in the tropics, *T. annulata* and *T. mutans*, express an immunodominant molecule which is considered to be homologous to 32 kDa polypeptide of *T. sergenti* [3, 22]. To understand the evolutionary relation of bovine species of *Theileria*, comparison of these molecules by genetical and protein chemical method are urgent.

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