VACCINE DEVELOPMENT AGAINST *THEILERIA* PARASITE

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Abstract. Bovine piroplasmosis caused by *Theileria sergenti* is a major cause of economical loss in grazing cattle in Japan. We found that parasite stocks and isolates consist of genetically and antigenically mixed population. To differentiate parasite populations bearing 2 allelic forms of p32, an immunodominant piroplasm surface protein, 2 sets of oligonucleotide primers were designed to amplify either of the 2 alleles by polymerase chain reaction (PCR). By using this allele-specific PCR, we found that the majority of *T. sergenti*-infected calves in Japan harbored mixed parasite populations with C and I type parasites. Amino acid sequence of p32 contains Lys-Glu-Lys (KEK) motif which is one of tripeptide necessary for malaria parasite to invade erythrocytes. We produced 2 vaccine candidates, recombinant baculovirus p32 and synthetic peptide containing KEK motif. Immunization of either recombinant p32 or synthetic peptide containing a KEK sequence with adjuvant resulted in low parasitemia and reduced the clinical symptoms compared to control calves. Interestingly, parasites with a p32 allelic form corresponding to one used as the immunogen were suppressed. Therefore, a cocktail vaccine containing KEK peptides derived from C and I type parasites is desired for control *Theileria* parasite infection in Japan.

INTRODUCTION

There are several species of *Theileria* recorded in cattle. The most pathogenic species are *T. parva* and *T. annulata.* The former has a limited distribution. being confined to Africa, however, the latter is more widely distributed, extending from Southern Europe to Southern Asia. The less pathogenic species of *Theileria, T. sergenti* is widely distributed in Eastern Asia. Bovine theileriosis caused by *T. sergenti* is a source of major economic losses in grazing cattle in Japan. The life cycle of *T. sergenti* involves two hosts, cattle and tick *(Haemaphysalis longicornis).* Infected calves show chronic anemia as intraerythrocytic piroplasms and occasionally die in severe cases. Parasites show persistent infection with up and down parasitemia. The erythrocytic stage of the parasites express a 32 kDa piroplasm major surface protein (p32), which shows significant diversity among isolates (Shirakata *et al,* 1989; Matsuba *et al,* 1993 a, b). To control *T. sergenti* infection, we analyzed p32 antigen because passive immunization of anti-p32 monoclonal antibody resulted in the inhibition of the progress of the disease (anemia) (Tanaka *et al,* 1990). Thus we considered p32 as a candidate vaccine. In the paper, we will introduce our recent work on the genetic and antigenic diversity of *T. sergenti* and vaccine trial.

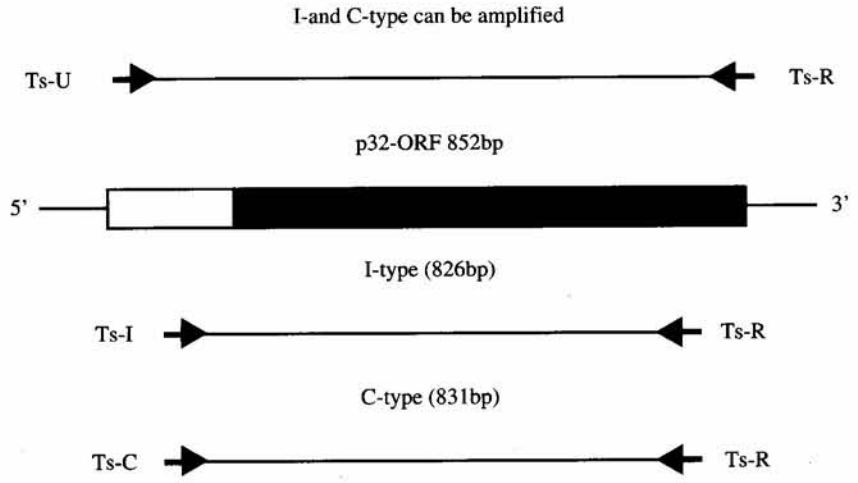
MATERIALS AND METHODS

Parasite stocks: Parasite stocks used in this study were Chitose, Ikeda (Matsuba *et al,* 1993 a, b) and Shintoku (Matsuba *et al,* 1992) of *T. sergenti.* The Shintoku stock had been maintained by blood or tick passages by *Haemaphysalis longicornis* ticks in splenectomized calves (Matsuba *et al,* 1993a,b).

Allele-specific PCR: In order to differentiate two allelic forms of p32, we designed allele-specific PCR primers for Chitose (C) type and Ikeda (I) type as shown in Fig 1. Parasite DNA was prepared from purified piroplasms as described previously (Tanaka *et al,* 1993). The oligonucleotide primers (20-25 mers) for PCR, Ts-I, Ts-C and Ts-reverse (Ts-R) primers were described previously (Kubota *et al,* 1995). For the reaction, 50 µl of a mixture consisted of 5-50 ng of parasite DNA as a template primers (1 µM each), deoxynucleotide triphosphates (200 µM each) and 1.25 U of *Taq* polymerase (Gibco BRL Life Technologies, Inc USA) in 1x PCR buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl, 2.0 mM $MgCl_2$) were used. When samples from infected carrier cattle were tested, the amount of DNA included in the reaction was adjusted according to the estimated extraction efficiency of parasite DNA.

The reactions proceeded in a programed temperature control system (Model PC-700 Astec Co, Ltd, Japan) for 35 cycles. Each cycle consisted of 1minute of denaturation at 94ºC, (4 minutes for the first cycle), 1 minute of annealing at 57ºC, and 1 minute of polymerization at 73°C , with an additional 3 minutes at 73°C, after the last cycle. After amplification, 5-10 µl of each smaple were subjected to agarose gel electrophoresis with or without restriction enzyme digestion. After staining with etidium bromide, the density of the stained bands were analyzed by using a computer-assisted image analysis system and an image software, and relative intensities between the bands with Ts-I or Ts-C primers were represented as boxes.

Antigens used for vaccination. We produced 2 candidates for a vaccine ; recombinant baculovirus p32 antigen derived from C type parasite (Matsuba *et al,* 1995) and synthetic peptides contaning a Lys-Glu-Lys (KEK) motif found in amino acid sequences of p32. The sequences of the peptides used are as follows ; C type parasite p32 (EEKKE-AAKADEKKDL and KEKKESKDLD) and I type parasite p32 (EEKK-DAKAEEKKDL and KEKKE-VKDLD) (Fig 2). Using F-moc chemistry, peptides were synthesized directly onto a branching lysine core with 8 copies of these peptides being linked to core by the COOH-terminal acid by a peptide synthesizer (PSSM-8, Shimadzu Co, Ltd, Japan).



5′ primer Ts-U:5′-CACGCTATGTTGTCCAAGAG-3′ (CAC+position 167-183 of L9-1)
Ts-I:5′-AAGGATCCGTCTCTGCTACCGCCGC-3′ (AAGGATC + position 126-143 of TSI-10)
Ts-C:5′-GCGGATCCTCATCGTCTCTGCAACT-3′ (GCGGA + position 213-232 of L9-1
Underline: *Bam*HI restriction site

3′ primer Ts-R:5′-TGTGAGACTCAATGCGCCTA-3′
(position 1,019-1,038)

Fig 1 - Schematic representation of the genes coding p32.
Blanking area represents region which coding mature p32 and stippled area represents signal peptide coding region. The position of the oligonucleotied primers used for PCR amplification are indicated by arrows.

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1'   MLSKRSFNVLCLGYFLIVSATAEEKKEAAKADEKKDLALEVNATQAENFTVNATNANDVV
     ***** ********** * *  *** ***** *****  ** * * * ******
1"   MLSKRTFNVLCLGYFLIVSATAAEEKKDAKAEEKKDLTLEVNATAAEHFKVDASNANDVV

61'  FTANEGYRIKTLKVGDKTLYTVDTSKFTPTVAHRLKHAEDLFLKLDLSHAKPLLFKKKSD
     *** ********** ********** *** ** ********** *
61'  FTAEEGYRIKTLKVGDKNLYTVDTSKFTPTVAHRLKHADDLFFKLNLSHAKPLLFKKKTD

121' KEWVQFSFAQYLDEVLWKEKKESKDLDASKFAEAGLFAPDAFGTGKVYDFVGNFKVTKVK
     * ********** ****** ********* *****  ****** * * ***** **
121" KDWVQFSFAQYLDEVVWKEKKEVKDLDASKFADAGLFAAEAFGTGKLYNFIGNFKVKKVM

181' FEDKEVGDSKKAKYTAVKVYVGTDDKKIVRLDYFYTGDERFKEVYFKLVDGKWKKLEQSD
     ** * **** ********** * ** ********** *** ***
181" FEEKDVGDSNKAKYTAVKVYVGSDEKKVVRLDYFYTGDERFKEVYFKLVDGKWKKVEQSE

241' ANKDLHAMNNAWPLDYKPLVDKFSPLAVLSAVLIALLAVSYYL
     ********* *** ********** *** ***
241" ANKDLHAMNSAWPSDYKPLVDKFSPLAVLSAVLIASLAVFYYL
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Fig 2 - Amino acid sequence of p32 gene. Upper lines (1', 61', 121'...) and lower lines (1", 61", 121"...) are amino acid sequence of C-type and I-type p32, respectively.
* indicates the same amino acids.
[ ] indicates the peptides used for vaccine.

Immunization and sporozoite challenge : Nine one-month-old calves were used in this experiment. Three (Nos. 1 to 3) and one (No. 4) calves were immunized with peptides derived from C type p32 (5mg or 1mg) and I type p32 (1mg), respectively. One calf (No. 4) was immunized with recombinant p32 (5mg). The synthetic peptides or recombinant p32 were mixed with Freund's complete adjuvant (FCA). The immunogens were injected intramuscularly 4 or 5 times every 2 weeks. Control calves (Nos. 6, 7, 8 and 9) were inoculated with Saline mixed with FCA. Table 1 shows the vaccine schedule and the results of vaccine effect. Two weeks after the last immunization, all calves were splenectomized and then challenged with sporozoites which contain I and C type parasites. After the challenge, clinical and hematological findings were observed. Allele-specific PCR was conducted to determine the type of parasites.

RESULTS AND DISCUSSION

In order to develop a reliable and useful vaccine, our attention was focused on the following 2 points; 1) Analysis of major surface antigens of piroplasm which are recognized by host immune responses and 2) antigenic and genetic variations of the surface antigens of parasites isolated from different districts of Japan.

1. Analysis of major surface antigens of piroplasm

Analysis of the structural proteins of *T. sergenti* showed 9 antigens in piroplasm. To conduct a more precise antigen analysis, we attempted to produce monoclonal antibodies against piroplasm of *T. sergenti.* Thirteen monoclonal antibodies against intraerythrocytic merozoites (piroplasms) of *T. sergenti,* were prepared for analysis of their structural proteins. All monoclonal antibodies stained the piroplasm but not uninfected erythrocytes by indirect fluorescent antibody test. Western blot analysis showed that 6 hybridomas tested recognized polypeptides with molecular weights of 32,000 (32K) or 23,000 (23K) (Kobayashi *et al,* 1987). Monoclonal antibodies which recognized 32K polypeptides were used to clarify the localization of the peptides on piroplasms. Protein A-colloidal gold was used as an electron-dense label to detect binding of the monoclonal antibodies. Monoclonal antibodies which recognized 32K and 23K polypeptides were both reacted only to surface but

not to intracellular antigens of merozoites (Shirakata *et al,* 1989). Since 32K protein(p32) is major piroplasm surface protein, we analyzed the epitopes of p32. The results of a competitive binding assay between monoclonal antibodies indicated that there were at least three epitopes in this protein. The presence of repeated epitopes was suggested by using two-site enzyme- linked immunosorbent assay. The protein was partit-ioned into detergent phase of Triton X-114 extractes, indicating that p32 is an integral membrane protein. Results of periodate treatment of p32 implies that one of the epitopes recognized by monoclonal antibody has a carbohydrate moiety (Zhuang *et al,* 1993).

Since p32 is located on the merozoite surface and is the major antigen recognized by host, we analyzed the protective effect against *T. sergenti.* infection by passive transfer of the monoclonal antibody that recognizes p32. As a result, the protective effect against piroplasms of *T. sergenti* infection in calves was demonstrated by passive transfer of the monoclonal antibodies (Tanaka *et al,* 1990). Since the result suggested that p32 was one candidate for a vaccine, we had started the molecular cloning of this gene. The gene for the p32 of *T. sergenti* was cloned into λgt 11 and its nucleotide sequence was determined. The gene encodes a protein of 283 amino acids as deduced from its nucleotide sequence with a 22 residue N-terminal signal peptide. Using this cDNA as a probe we have isolated another two clones from a cDNA library with a CDM8 vector system derived from the same parasite stock. Comparison with three cDNA clones revealed differential polyadenylation and difference in sequences of non-coding regions. Wtihin the coding regions, there were nucleotide transitions which affected the *Pst* I-restriction site, and one of the transitions was also accompanied by an amino acid substiution (Ala to Gly). Southern blot analysis showed hybridization pattern changes among the parasites isolated from individual calves at different times after infection. From the parasites we conclude that at least 3 genetically different parasite populations may coexist, and that transition to predominant parasite populations might occur during persistent infections in a host, possibly to evade the host immune responses (Matsuba *et al,* 1993 a).

2. Antigenic and genetic analyzes of *T. sergenti* p32

As injection of parasitized erythrocytes as live vaccine occasionally does not induce protective immunity in calves from different locations, the existence of antigenically different strains of *T. sergenti* in Japan is suggested. Identification and characterization of different stocks is fundamental to understanding the nature of variation. Restriction fragment length polymorphisms (RFLPs) of *T. sergenti* DNA were analyzed using probes of a genomic DNA fragment pTs2, which contains a 6.8Kb fragment of *T. sergenti* genomic DNA (Kajiwara *et al,* 1990; Hirano *et al,* 1991). RFLP analysis of 18 different isolates from 14 different locations of Japan by using pTs2 as a probe showed that polymorphisms were observed among stocks from different locations. However there was no correlation between the patterns of hybridization bands and the locations where parasites were collected (Matsuba *et al,* 1992). DNA from parasites isolated from the same calves at different points in the time after infection showed the alterations in hybridization patterns, the similar patterns found in field isolates. This result suggests that *T. sergenti* stocks or field isolates used were mixed parasite population (Matsuba *et al,* 1993a). The genomic DNA which we used for RFLP analysis consists 9 exons with short open reading frames coding for 23 to 109 amino acids but not coding surface antigens (Tanaka *et al,* 1992), thus we planned to use the gene encoding major surface protein, p32 for RFLP analysis. For analyzes of antigenic and genetic diversities of *T. sergenti* p32, we produced monoclonal antibodies against the other stocks. Using a panel to the monoclonal antibodies against p32 and p23, 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. Genetic diversity of *T. sergenti* isolate was also observed by Southern blot analysis using a cDNA of the p32 as a probe (Zhuang *et al,* 1994, 1996). As the p32 is a target of host humoral immunity, the parasite may exhibit antigenic polymorphisms as one of the evasion mechanisms from host immune response. Our studies have shown that there are mixed parasite populations with different p32 gene structure, and population changes could be detected during course of infection (Matsuba *et al,* 1993 a, b). In order to differentiate parasite populations bearing 2 allelic forms of p32, two oligonucleotide primers were designed to amplify either of the two allele by PCR (Kubota *et al,* 1995).

3. Control of *T. sergenti* infection by vaccination

p32 is an immunodominant antigen which is expressed abundantly on the surface of piroplams (Shirakata *et al,* 1989). Passive immunization of a calf with a monoclonal antibody against p32 produced

partial protection against parasite challenge with reduced clinical symptoms (Tanaka *et al,* 1990). Since p32 was considered to be one of the vaccine candidate, we attempted the molecular cloning of this gene. The nucleotide sequence of the cDNA predicts a molecular mass of 32,129 Da consisting of 283 amino acids with 3 possible N-linked glycosylation sites (Matsuba *et al,* 1993 a). In order to obtain high amounts of recombinant protein, we expressed p32 using an insect baculovirus vector system. This expression system is suitable not only for the production of high amounts of recombinant proteins but also for the analysis of post-translational modifications including glycosylation in eukaryotes.

Previous studies detcted a single amino acid substitution (Ala196 to Gly196) between cDNA clones encoding p32 of *T. sergenti* (C type) obtained from a persistently infected calf. Two different recombinant baculoviruses (pAc/p32-Ala^{196} and pAc/p32-Gly^{196}) were constructed for the expression of p32. Molecular masses of the polypeptides produced in *Spodoptera frugiperda* cells infected with the recombinant baculoviruses were the same as that of authentic p32. pAc/32-Ala^{196} produced additional polypeptides, with molecular masses higher than 32 kDa, which resulted from differential N-glycosylation as revealed by endo N-glycosidease treatment (Matsuba *et al,* 1995). We used pAc/p32-Ala^{196} for the production of recombinant p32.

The amino acid sequence of p32 contained several Lys-Glu motifs (Fig 2; Matsuba *et al* , 1993 a; Kawazu *et al,* 1992) which are reported to be potential protective epitopes of *Plasmodium falciparum,* the precursor of the major merozoite surface antigen, MSA-1 (Molano *et al,* 1991). Additionally, p32 contained a Lys-Glu-Lys motifs which is one of the tripeptide motifs for human erythrocyte binding (Molano *et al,* 1991). Surface localization of p32 and the presence of Lya-Glu and Lys-Glu-Lys motifs indicates that this molecule may play a crucial role in the interaction with the host red blood cell surface. Thus we used synthetic peptides as shown boxes in Fig 2 for the vaccine.

After 4 and 5 times vaccination with either recombinant p32 or synthetic peptides, calves were challenged with sporozoites. Antibody response against p32 or synthetic peptides was detemined by ELISA to analyze the immune response against vaccination. Vaccine effect was considered as the allele-specific inhibition of parasites by PCR, lower parasitemia level and the protection of clinical symptoms when compared to that of control calves. The summary of the vaccine effect is shown in Table 1. Calves vaccinated with either high amounts of peptides (Nos. 1 and 4) or recombinant p32 (No. 5) showed the lower parasitemia level (Fig 3) allele-specific inhibition of parasites (Fig 4) and no clinical symptoms when compared to that of control (Nos. 6-9, Table 1). As shown in Fig 4 control calf (No. 6) died at 46 days after challenge, however vaccinated calves with either recombinant p32 (No. 5) or synthetic peptides (No. 1) derived from C type p32 resulted in surviving and showing an allele-specific inhibition of the parasite. Since *T. sergenti* distributed in Japan is a mixed parasite populations with I and C type, for the control of *T. sergenti* - infection in Japan, a cocktail vaccine containing I and C type p32 antigens in desired.

Table 1

Summary of vaccine schedule and vaccine effect.

Calf No.	Antigens	*Amount* of antigen in mg	Times	Antibody response	Vaccine effect
1.	Peptide derived from C-type parasite	5	4	++	+++
2.	Peptide derived from C-type parasite	1	5	+	+
3.	Peptide derived from C-type parasite	1	5	+	-
4.	Peptide derived from I-type parasite	5	4	+	+++
5.	Recombinant p32 of C-type parasite	1	4	++	+++
6.	FCA only	0	5	-	-
7.	FCA only	0	4	-	-
8.	FCA only	0	4	-	-
9.	FCA only	0	4	-	-

FCA:Freund's complete adjuvant, vaccine effect is described in the text

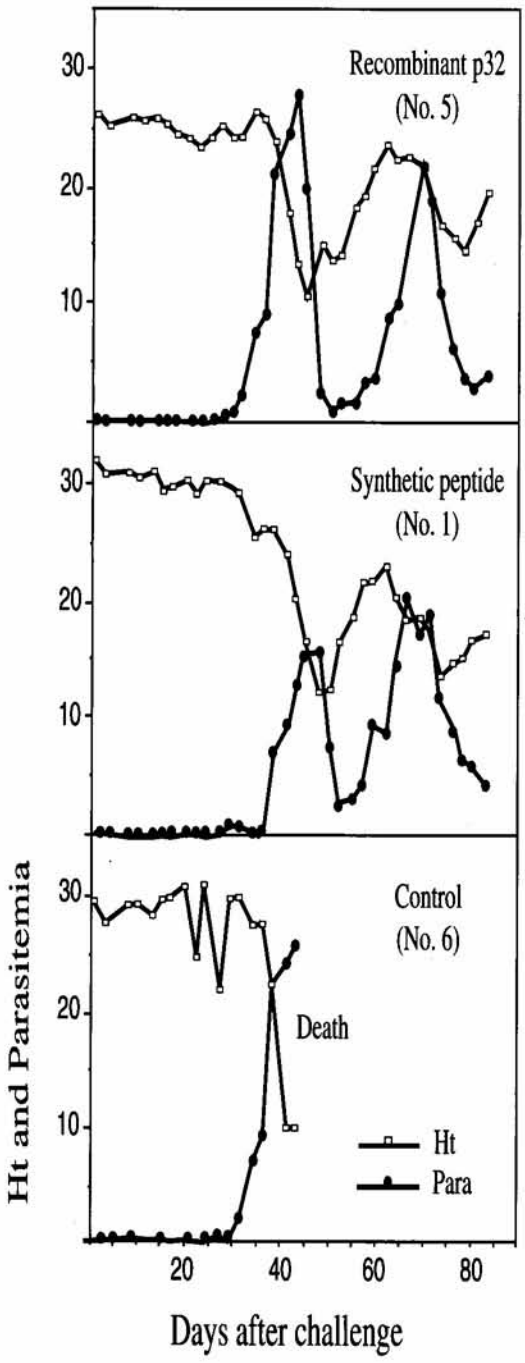

Fig 3 - Analysis of parasitemia level in vaccinated and control calves.

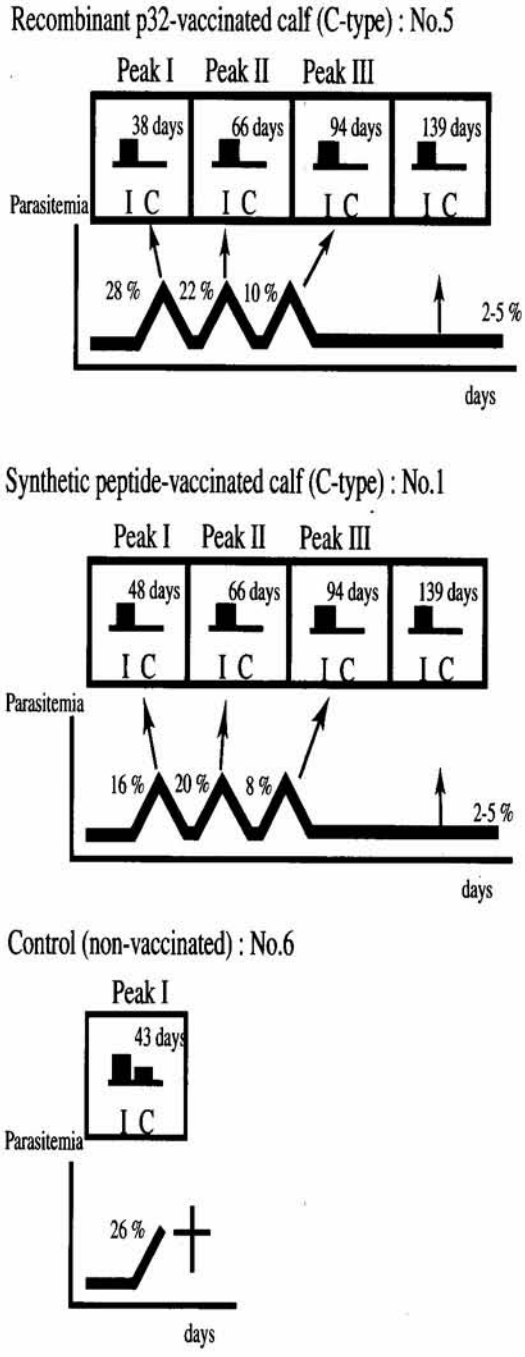

Fig 4 - Analysis of parasite population appeared in vaccinated and control calves. I, C : I type and C type parasites determined by allele-specific PCR. Calves vaccinated with antigens derived from C type p32 were found only. I type parasites but not C type parasites.

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