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Immunogenicity and protective efficacy of solubilized merozoite-enriched *Theileria sergenti* immunogens III. Characterization of immunodominant peptides

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Abstract: Immunoblot analysis utilizing bovine sera from naturally or experimentally infected with *Theileria sergenti* were used to determine the immunodominant polypeptides of *T. sergenti* (Korean isolate). The previously recognized major bands, 18 kDa, 29 kDa, 34 kDa and 45 kDa, were excised after electrophoresis and transfer to PVDF membrane. The individual bands were sequenced. The 34 kDa polypeptide which was the most antigenic and immunogenic peptide was observed in the Western blot. However, Chou-Fasman prediction sites (antigenic site) for antigen determinants of the 45 kDa, 34 kDa, 29 kDa and 18 kDa polypeptide were 6, 4, 2 and 0, respectively. However, the 45 kDa polypeptide showed no reaction with anti-*T. sergenti* hyperimmune serum.

Key words: *Theileria sergenti*, amino acid sequence, synthetic peptide, predicted antigenic value

INTRODUCTION

Bovine theileriosis caused by *Theileria sergenti* has well been established in most regions of South East Asia, and Korea (Park *et al.*, 1963; Han, 1971; Chang, 1974; Jeon, 1978; Kim, 1984; Baek *et al.*, 1990 a, b). At present the causative agent in Korea is *T. sergenti* recognized identified by its the antigenicity (Baek *et al.*, 1990 a, b), the vector (*Haemophysalis longicornis*) (Kang *et al.*, 1988; Kang & Jang 1989), and the characteristic ultrastructure (Baek *et al.*, 1990b). Baek *et al.* (1991 & 1992) demonstrated that *T. sergenti* merozoites have several antigenic polypeptides, 116, 105, 80, 77, 66, 60, 56, 49, 45, 38, 35, 34, 29 and 18 kDa. and 105, 66, 60, 34 , 29 kDa and 18 kDa polypeptide among them were found immunologically dominants.

Further evidences demonstrated that purified merozoites of *T. sergenti* fortified with adjuvant conferred significant protection against the reinfection of the parasite. This strongly suggests that *Theileria* immunogens are a feasible vaccine (Baek *et al.*, 1991, 1992). However, the production and standardization of the vaccines are should be optimized and standardized. Constraints include low yield, reliance on live animal donors and relatively low immunogenicity. Nevertheless, it seems reasonable to hypothesize that non-viable vaccines against *T. sergenti* could be developed because the above circumvented constraints

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should be eliminated.

Numerous attempts have been concentrated to develop immunization against a similar disease caused by *T. parva* (Brown *et al.*, 1971; Burridge *et al.*, 1972; Hashemi-Fesharki *et al.*, 1973; Radley *et al.*, 1975a, b; Dolan *et al.*, 1980; Pipano, 1988; Mutugi *et al.*, 1991). The purpose of this study is to introduce an alternative method based on the concept of synthesizing proteins of predictable antigenicity and immunogenicity. This approach would minimize the constraints. The proteins, therefore, could be produced on demand and made available in a standardized form and would be entirely independent of *in vitro* cultivation and mammalian or invertebrate hosts. The first step involved identification of the most immunodominant polypeptides, we performed NH_2 termini amino acid sequences and synthesized them. Also, we determined the hydrophilic sites of the respective proteins through this study.

MATERIALS AND METHODS

1. Preparation *Theileria sergenti*

The *T. sergenti* strain used in this study was isolated from Korean cattle reared in the Chonju area (Baek *et al.*, 1990a, b). Stabilates were prepared and cryopreserved in liquid nitrogen (Love, 1972).

2. Preparation of the merozoite immunogen

At peak parasitemia, percent parasitized erythrocytes (PPE) of 40%, the infected splenectomized calves were exsanguinated, and erythrocytes were separated aseptically from heparinized blood. Erythrocytes were washed in glycine buffer (0.1 M, pH 3.0) to remove adherent autoantibodies. Following a subsequent wash in phosphate buffered saline pH 7.4 (PBS), supplemented with phenylmethylsulfonyl fluoride (PMSF) and 0.02% sodium azide (Sigma, St. Louis, MO), hemolysis was achieved with lysis buffer (0.005 M Tris, 0.001 M EDTA, 0.0001 M PMSF, pH 8.0) at 4°C (Dodge *et al.* 1983). The lysate was centrifuged at 20,000 g for 30 minutes at 4 °C to pellet the merozoites. The supernatant was discarded. Purified merozoites were resuspended in PBS (1:5, v/v), sonicated at 20 kilocycles at a flow rate of 40 ml/minute. The sonicate was centrifuged at 20,000 g for 1 hour and the supernatant was saved. Resulting supernatant was used as a merozoite fraction. Negative control antigen was prepared similarly from a calf blood confirmed free from any hemotropic disease. Protein concentration was determined according to the standard procedure (Lowry *et al.*, 1951), and was adjusted to 0.5 mg/ml.

3. Partial characterization of the immunogen

The immunogen was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5-20% reducing condition (Laemmli, 1970) and transfer blotted to nitrocellulose paper and probed with the infected serum (Towbin *et al.*, 1979). The hyperimmune serum used in western blot was obtained from a infected splenectomized calf which gave an IFA titer of 1:10, 240. The immune serum recognized the 29, 34, and 105 kDa bands.

4. Determination of partial NH_2-termial amino acid sequence

The NH_2-terminal amino acid sequencing was carried out as described by Matsudaira (1989). Briefly, the merozoite preparation was electrophoresed on a 7.5-20% polyacrylamide gel. The gel was then soaked in 100 ml of 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid buffer containing, 10% methanol (pH 11.0) for 5 minutes. Upon completion of transfer blot, the membrane was removed, rinsed with distilled water, and then rinsed again for a few seconds with methanol, and stained with 0.1% Coomassie brilliant blue R-250 in 1% acetic acid and 40% methanol. The paper was then destained in 50% methanol in distilled water 1 minute for 3 times and rinsed thoroughly in distilled water. The specific target bands were excised individually and applied to a protein sequencer (Biosystems 120A). The PTH derivatives were separated by reverse phase HPLC and analyzed to obtain the sequence.

5. Amino acid synthesis

Peptides were synthesized on an Applied Biosystems model 430A peptide synthesizer using tertiary-butyloxycarbonyl (t-boc) solid phase synthesis by a modification of the Marrifield technique as described by Matsudaira (1987). Briefly, t-boc was used to protect the alpha amino group of the amino acids. The resin support for peptide synthesis was washed and then treated with diisopropylethylamine prior to couple the first amino acid. The t-boc amino acid was then activated with dicyclohexyl-carbodiimide. Following the coupling of each amino acid, acetic anhydride was added and the reaction was terminated. Finally, the t-boc protecting group was protected. Upon coupling of the final amino acid, hydrogen fluoride was added to remove any side chain protecting groups (deprotection). The final product was cleaved from the resin.

RESULTS

1. Amino acid sequence of immunodominant peptides

The amino acid sequence of the 18 kDa, 29 kDa, 34 kDa and 45 kDa amino acid were found as Fig. 1.

2. Predicted epitopes of the immunodominant polypeptides

The major immunodominant polypeptides of 18 kDa, 29 kDa, 34 kDa and 45 kDa are synthesized and isolated to homogeneity on HPLC. The corresponding hydrophobicity/hydrophilicity plots showed the distribution and localization of antigenic determinants corresponding to an antigenic index of $\leq$ to be 1.2. The number of predicted epitopes of 29 kDa, 34 kDa and 45 kDa was shown as 6, 4 and 2, respectively (Fig. 2, A, B, C). No epitopes were found in the 18 kDa polypeptides (Fig. 2, D).

DISCUSSION

Our goal is to make the best opportunity to the promising candidate proteins. Until now it is possible for us to locate the following immunodominant polypeptides: 116, 105, 80, 77, 66, 60, 56, 53, 49, 47, 38, 35, 34, 29 and 18 kDa (Baek *et al.*, 1992). The above polypeptides were available only in small amount and cutting the bands was highly labor-intensive. However, it was possible to obtain sufficient peptides isolated and purified on HPLC found subsequent amino acid sequence as exemplified. It was also possible derive the Kyte and Doolittle (1982) hydropathicity indices that correlated well with our immunogenicity data for some peptides such as the 34 and 45 kDa.

Our success in synthesizing peptides that expressed the relevant protein moieties perhaps depended on the fact that we adhered to rigorous criteria stipulated by various workers (Chou and Fasman, 1973; Houghten 1985; Rodriguez *et al.*, 1990). Accordingly, this study utilized the sequences obtained from the NH_2 termini of the various peptides which were expected to be more accessible to solvents and other conditions including the immune system of the natural host. In preparing the immunogen it was considered necessary to increase the charge characteristics of the peptides to enhance their immunogenicity, particularly with respect to proper antigen presentation of T-cell epitopes (Tam and Lu, 1989). The latter approach also guaranteed that the peptide had adequate charge for solubility and ease of emulsification and administration to the host.

The peptides used in this study were generally between 15 and 20 amino acid

	1	6	11	16	21
18 kDa:	Ala-Arg-Thr-Lys-Gln-	Thr-Ala-Arg-Met-Ser-	Thr-Gly-Gly-Lys-Ala-	Gln-Val-Val-Val-Leu-	Ala-Ala-Gln-Tyr-Ala.
29 kDa:	Ala-Leu-Glu-Phe-Lys-	Asp-Ser-Phe- Ile-Lys-	Arg-Ala-Val-Asp-Ser-	Asp-Ser-Asp-Arg-Asp.	
34 kDa:	Ala-Glu-Glu-Lys-Lys-	Glu-Pro-Ala-Lys-Ala-	Glu-Glu-Lys-Lys-Asp-	Leu-Ala-Leu-Glu-Val-	Asn-Ala-Thr-Gln-Asn.
45 kDa:	Ser-Val-Phe-Lys-Phe-	Glu-Ala-Met-Ala-Ile-	Val-Asp-Lys-Val-Val-	Ala-Arg-Asp-Pro-Phe.	

Fig. 1. The partial NH_2-terminal amino acid sequences of the major merozoite immunogens.

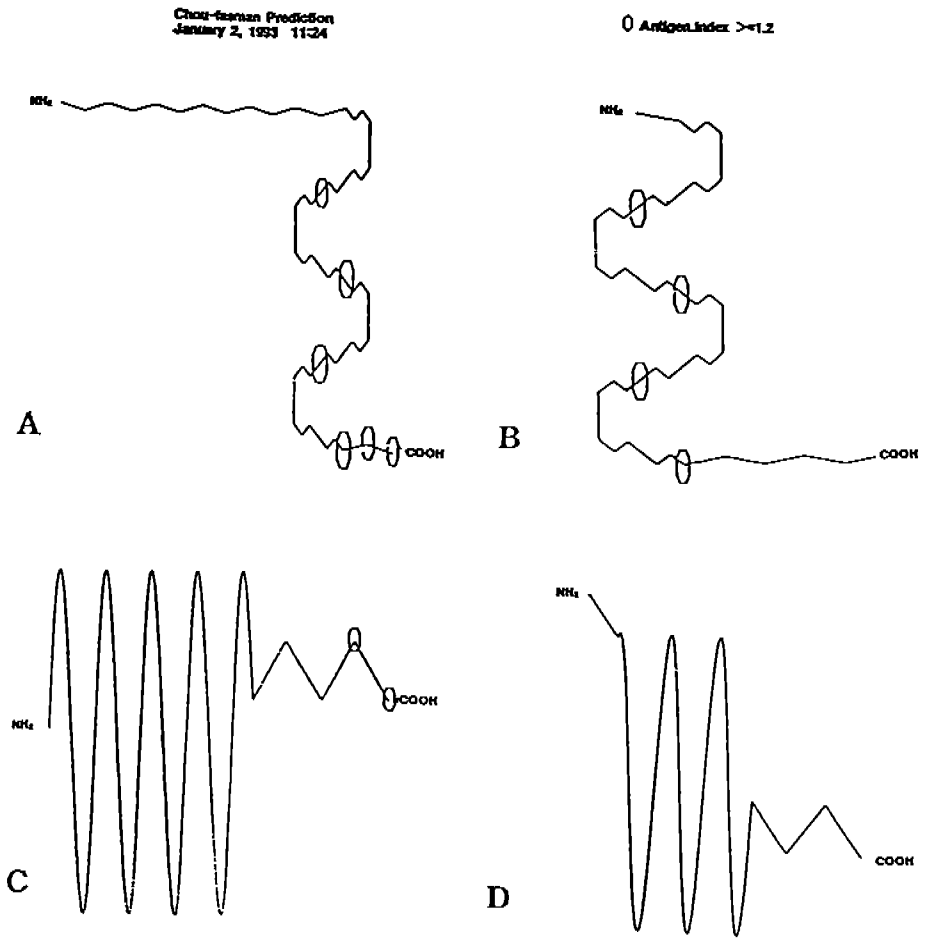

Fig. 2. Two-dimentional graphs of *T. sergenti* immunodominant peptide using Chou-Fasman parameter. Predicted antigenic sites in plotstructure of amino acid analyzed was sequenced from -NH_2 to -COOH. **A.** 6 hydrophilic regions (antigenic sites) are observed in 45 kDa major. **B.** 4 antigenic sites are observed in 34 kDa major. **C.** 2 antigenic sites are observed in 29 kDa major. **D.** No hydrophilic region is observed in 18 kDa major.

residues, thus falling within the generally acceptable critical size for immunogenicity. It is generally suggested that cysteine residues at either the carboxy or amino terminus facilitates crosslinkage. In the present study, these residues were not consistently found at the termini and, therefore, we decided to crosslink the peptides to themselves by a process of facilitated polymerization.

Based on our present data, it can be concluded that the synthetic peptides and the concept of predicting antigenicity are useful complementary approaches to development of vaccine of *T. sergenti* and may have long-term benefits in immunoprophylaxis against this disease in Korean cattle and may have potential for application in other diseases, as has been shown for malaria (Murillo *et al.*, 1991; Amador *et al.*, 1992; Rocha *et al.*, 1992).

The most important and decisive data will be prepared after detailed field tests are completed using natural homologous and heterologous challenges from the putative invertebrate host or other mechanical conduits. Another critical test of these "designer" vaccines is the short and long-term cost-benefit advantage (Mukhebi *et al.*, 1992), which have yet to be determined in the case of *T. sergenti* vaccines. In any case, it seems reasonable to speculate the proposed approach will certainly have a number of advantages over whole blood vaccines and provides an

alternative to the use of acaricides.

Clearly, a synthetic vaccine would be environment-friendly and could be designed to anticipate or accommodate any antigenic heterogeneity, shift or variation that could potentially occur with any given organism.

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=국문초록=

Theileria sergenti merozoite수용성 항원의 항원성과 면역성
III. 면역성 항원 peptide의 특성

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우리나라 소에 유행하고 있는 *Theileria sergenti*의 예방을 위한 연구의 일환으로 *T. sergenti* merozoite 수용성 항원중에서 면역원성 물질로 보고한 바 있는 34, 29 및 18 kDa와 항원성 물질인 45 kDa에 대하여 Peptdie 구성 특성을 밝히기 위하여, 이들 물질에 대한 아미노산 서열을 결정하였다. 이를 생합성하여 Chou-Fasman prediction법에 의해 항원 결정기를 확인하였던 바, 45 kDa, 34 kDa, 29 kDa 그리고 18 kDa의 polypeptide는 6, 4, 2 그리고 0개의 항원 결정기를 갖고 있었다. 그런데 45 kDa 물질은 항 *T. sergenti*혈청에 대하여 면역원성이 인정되않았던 물질이었다.

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