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Immunogenicity and Protective Efficacy of Solubilized Merozoite-enriched *Theileria sergenti* Immunogens I: Protection against Homologous Stabilate Challenge

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Abstract: *Theileria sergenti* were isolated from infected erythrocytes by hypotonic lysis, and soluble merozoite antigens were purified by sonication and differential centrifugation. The preparation contained 29, 34, 35 and 105 kD immuno-dominant polypeptides. The soluble antigens (0.5 mg/ml) were prepared and fortified with Freund's adjuvant. Five month old naive Korean calves were subcutaneously inoculated with the preparation and a booster dose was administered 4 weeks later. Nine weeks after the booster dose, vaccinates and controls were challenged with a homologous stabilate (5.6×10^6 RBC/dose, 40% Parasitemia). All animals were monitored for hematocrit, total erythrocyte count, parasitemia and for the specific antibody by Western immunoblot (WB) and indirect immuno-fluorescent antibody(IFA) test. By 18 weeks after vaccination (6 weeks after the challenge), vaccinated cattle had an average IFA titer of 1 : 10,240 compared with 1 : 1,280 of the controls. The vaccinates showed negligible change in hematocrit and total RBC count whereas control animals showed significant ($p<0.05$) hematological changes and associated anemia. After vaccination and challenge, the antibody responses demonstrated that vaccination had induced significant production of antibody to the 29 and 35 kD polypeptides. The latter polypeptide was much more strongly recognized by the vaccinated animals, and thus it may be a potential candidate for the vaccine.

Key words: *Theileria sergenti*, merozoites, soluble polypeptide, immunogenicity, cattle

INTRODUCTION

Tropical theileriosis, a tick-borne protozoal disease caused by *Theileria sergenti* (Chang, 1974; Han, 1971; Kang *et al.*, 1989), is an important hemotropic disease of cattle in Korea (Kim *et al.*, 1983 & 1984). It is characterized by progressive anemia, high morbidity and low mortality in native Korean cattle and a higher mortality among exotic breeds. Most researches on the Korean isolates have concentrated on morphological and immunological aspects (Baek *et al.*, 1990 a & b; Kang *et al.*, 1988; Suh *et al.*, 1972). Possible control measures include tick control, treatment of clinical cases, chemoprophylaxis and immunoprophylaxis. None of these either individually or collectively have been shown to be

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effective in the control of theileriosis. However, immunoprophylaxis appears to offer the best long term solution to this disease.

Current immunoprophylactic methods against theileriosis include infection and treatment or immunization with virulent or less virulent *Theileria* sp. (Brown *et al.*, 1971; Burridge *et al.*, 1972; Suh *et al.*, 1972; Young *et al.*, 1973; Cunningham *et al.*, 1974; Radley *et al.*, 1975a, b, c & d; Dolan *et al.*, 1980; Uilenberg *et al.*, 1981; Robinson *et al.*, 1982; Mutugi *et al.*, 1991). Recently, however, Baek *et al.* (1991) successfully utilized crude preparation of *T. sergenti* merozoites and was able to confer significant protection against a virulent challenge.

These research efforts have primarily been directed to East Coast Fever caused by *T. parva* in East and Central Africa and very little research has been carried out on control of the disease caused by *T. sergenti* in Korea or in other areas of Asia.

In the present study, we report the first data on the development of a merozoite-derived preparation evaluated for antigenicity, immunogenicity, safety and protective efficacy against a homologous needle challenge with a virulent *T. sergenti* stabilate isolated from Korea.

MATERIALS AND METHODS

Experimental cattle: Young animals born during the winter months were used in order to minimize *in utero* and neonatal infection with *T. sergenti* as suggested by Hubbert *et al.* (1975). A total of 7 native Korean cattle, aged 5 months (3 vaccinates, 4 controls), were used. Additional calves were splenectomized and used to isolate or reactivate *T. sergenti*. The criteria for selecting the animals included complete physical health, lack of parasitological or serological evidence of exposure to *T. sergenti* or any other tick-borne disease. All the animals came from and were maintained under tick-free conditions. The animals were allowed access to feed and water *ad libitum*.

Preparation of stabilate: The *T. sergenti* strain used in this study was isolated from Korean cattle reared in Chonju area and was identified as *T. sergenti* (Baek *et al.*, 1990b). The stabilate was cryopreserved in liquid nitrogen according to published procedures (Love, 1972).

Preparation of immunogen: At peak parasitemia, percent parasitized erythrocytes (PPE) of 40%, splenectomized calves were exsanguinated, and erythrocytes were aseptically separated from heparinized blood. Erythrocytes were washed in 0.1 M glycine, pH 3.0, to remove any adherent autoantibodies (James *et al.*, 1985). Following a subsequent wash in phosphate buffered saline (PBS), pH 7.4, supplemented with phenyl methyl sulfonyl 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 according to a modification of the method of Dodge *et al.* (1983). The lysate was centrifuged at 20,000 *g* for 30 minutes at 4°C to isolate the merozoites. The supernatant was discarded. The presence of merozoites was confirmed by light and electron microscopic examination (Baek, 1990b). 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 to fulfill the criterion of solubility and the supernatant was collected. Negative control antigen was prepared similarly from blood of a calf 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.

Partial characterization of the immunogen: The immunogen was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli (1970) and Western (Towbin *et al.*, 1979). Hyperimmune serum used in Western blot was obtained from a naturally infected splenectomized calf with an IFA titer of 1 : 10,240. The immune serum recognized the bands of 29, 34, 35 and 105 kD.

Immunization procedure: The solubilized antigen was fortified with an equal volume of Freund's complete adjuvant (primary inoculation) or incomplete Freund's adjuvant in the booster dose. The primary inoculum, 100 mg/animal of immunogen, was administered subcutaneously, and another dose of 100 mg/animal was boosted 4 weeks later.

Challenge: Vaccinates and controls were challenged 13 weeks post-vaccination with a virulent stabilate (5.63×10^6 RBC/animal, 40% PPE).

Collection and storage of sera: All sera were separated at room temperature on the same day of collection, aliquoted and stored at −70°C until tested.

Parameters for monitoring experimental animals: Animals were monitored for clinical condition, hematocrit, total RBC count, parasitemia and specific antibody response measured by IFA (Montenegro-James *et al.*, 1985) and Western blot.

RESULTS

1. Antigenicity

The results of electrophoretic profiles by SDS-PAGE revealed that major antigenic moieties of *T. sergenti* soluble merozoite polypeptides comprised of 116, 105, 80, 77, 66, 60, 56, 53, 49, 46, 38, 35, 34, 29 and 18 kD (Fig. 1). In the Western blot using hyperimmune bovine anti-*T. sergenti* serum, the 105, 66, 60, 35, 34 and 29 kD polypeptides were recognized. None of these bands were present in normal bovine erythrocyte extracts or normal bovine serum. Fig. 2 summarizes the SDS-PAGE and corresponding western blot profiles. Unique antigens were determined by exclusion of any peptides recognized by normal bovine serum or recognized by immune serum reacted with a semi-purified merozoite enriched preparation. By these criteria the immunodominant polypeptides were the 105, 35, 34 and 29 kD.

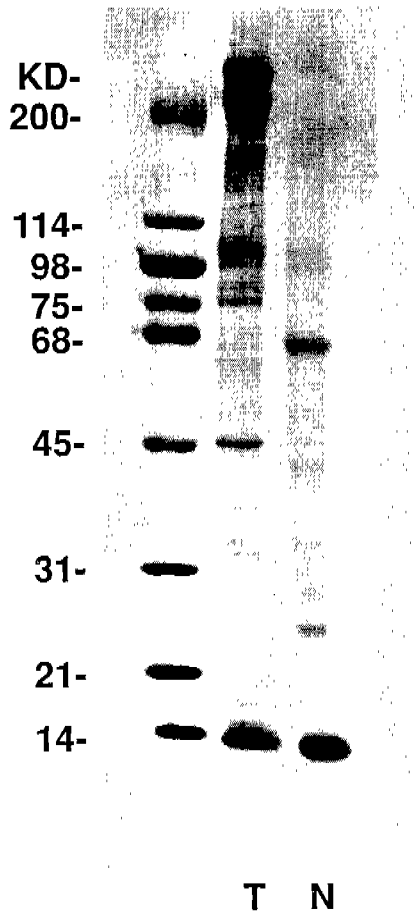

Fig. 1. SDS-PAGE results on *T. sergenti* antigen (Coomassie stain).
Key: KD=Molecular Weight(M.W) Kilodaltons
T=*T. sergenti* merozoite antigen
N=Normal bovine erythrocyte extract

2. Immunogenicity

IFA test: At the first week after vaccination, the vaccinates showed a mean IFA titer of 20, while the controls remained negative (Fig. 3). By 4 weeks the titer rose to 80. At 4 weeks following a booster injection the titer reached at 640 and at week 13 the titer dropped to 320 (Fig. 4). Four weeks post challenge, the titer rose to 5,120 in the vaccinates (Fig. 3), whereas in the control group a titer of 20 was observed at 1 week post challenge reaching a peak of 1,280 3 weeks later. A significant difference ($p < 0.05$) between vaccinates and controls was

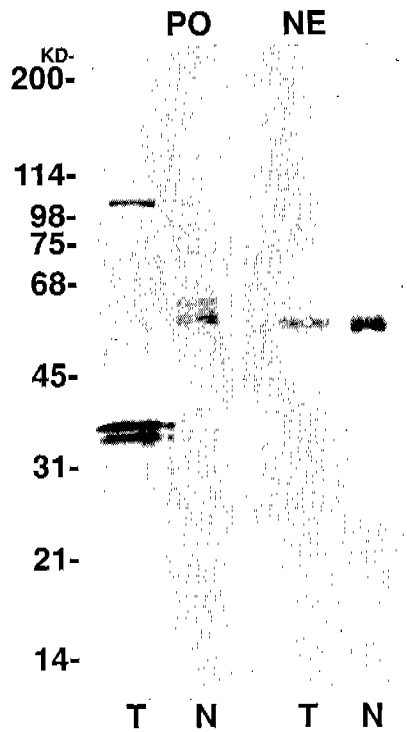

Fig. 2. Western immunoblot analysis of *T. sergenti* antigens.
Key: PO=Positive serum from a cow experimentally infected with *T. sergenti*.
NE=Normal pre-infection serum

observed at challenge and thereafter.

Western blot: The major observations were that the vaccinates recognized the 105, 35, 34 and 29 kD polypeptides post vaccination and a significant increase in the intensity of the bands was observed 2 weeks post challenge. The controls showed weak responses to the immunodominant polypeptides following challenge.

3. Clinical hematology

The average hematocrits in vaccinates and controls were 34 and 36 respectively prior to challenge. Four weeks post challenge, the hematocrit decreased to 33 and 26 in vaccinates and controls respectively. Anemia was grossly evident in the controls. The hematocrit of the vaccinates stabilized between 33 and 34 at 4 and 8 weeks post challenge, but the hematocrit of the controls progressively lowered to 22, at 8 weeks post challenge. Corresponding difference in total erythrocyte counts at significant levels between the controls and vaccinates ($p<0.05$) was observed (Fig. 5). Hematological changes correlated with severe deteriorating conditions in the vaccinates.

4. Parasitemia

The vaccinates and control animals showed no differences in the prepatent period and the kinetics of PPE in peripheral blood. Both groups

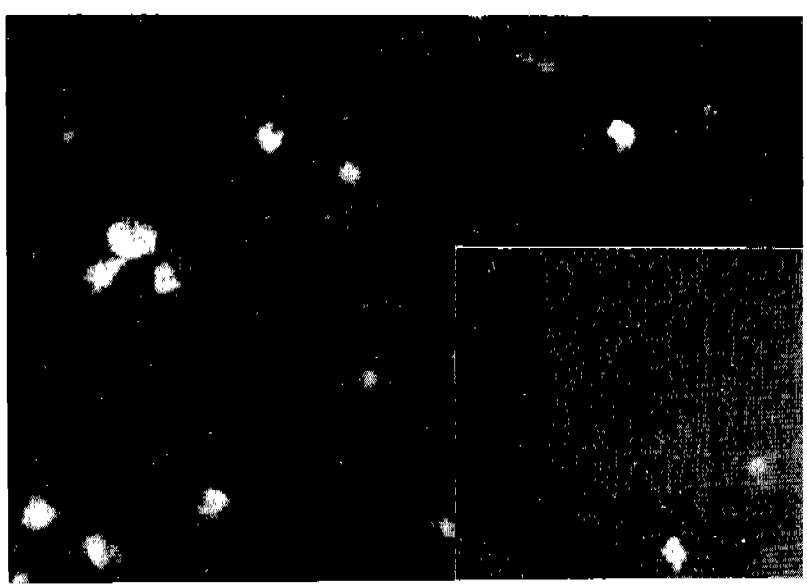

Fig. 3. Intensity of immunofluorescence with immune anti-*T. sergenti* serum (insert shows background for negative bovine serum).

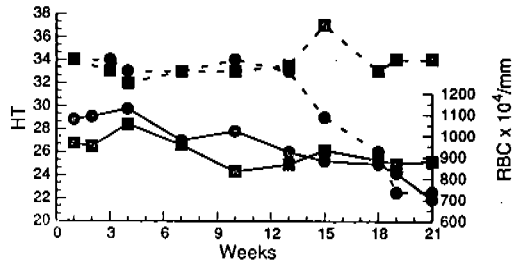

Fig. 4. IFA response in vaccinated(-▪-) and control(-•-) animals.

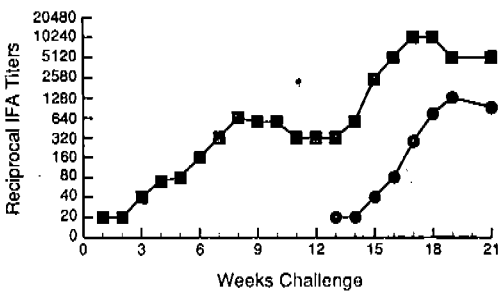

Fig. 5. Total RBC(——) and HT(--) profile in vaccinated(-▪-) control(-•-) animals.

showed peak parasitemia 8 weeks post challenge. The controls showed a mean peak parasitemia of 9.6% compared to 3.5% in the vaccinates ($p<0.05$).

DISCUSSION

Our previous experiments (Baek *et al.*, 1990a & b) purified and characterized intact merozoites of *T. sergenti*, and the present study revealed that the purified merozoites were immunogenic to Korean cattle. Our results were consistent with data reported by Ongitani *et al.* (1987). In addition we confirmed the reports of Kobayashi *et al.* (1987) and Sugimoto *et al.* (1991a & b). The current data demonstrated that it was possible to use a relatively small dose of purified and solubilized antigen to immunize cattle. This procedure, however, necessitated the use of Freund's adjuvant which is not readily acceptable in food producing animals. In the absence of a better adjuvant, we have shown at least that with optimal adjuvanticity the soluble merozoite antigens confer statistically significant protection against a virulent needle challenge with a homologous *T. sergenti* stabilate. The major indices of protection were clinical and hematological changes which paralleled the kinetics of parasitemia. Unvaccinated animals generally had a poor antibody response and suffered from drastic drops in total erythrocyte counts and coinciding severe anemia. The vaccinated animals generally maintained hematological and biochemical profiles within normal ranges (Schalm *et al.*, 1975; Kaneko, 1990). Recently, Baek *et al.* (1991) used crude extract of *T. sergenti* merozoites and succeeded to induce significant protection against homologous challenge. Our present results confirm the finding. However, in this report we have used relatively purified and solubilized merozoite polypeptides. We consider this as a promising step in producing standardized *T. sergenti* vaccines. As for the further development, Tanaka *et al.* (1991) have shown a preliminary datum that anti-idiotype antibody induced significant level of antibody to *T. sergenti* merozoites although no significant protection was observed against clinical disease.

The protective mechanisms in tropical theileriosis are not well understood. However, our results indicate that specific antibodies might play a role in limiting the levels of parasitemia. Asaoka *et al.* (1991) showed that *T. sergenti* activated peripheral blood macrophages and Yasotomi *et al.* (1991) showed that the organism stimulated proliferation of lymphocytes in infected calves. Those suggest a potential role of cell-mediated immunity(CMI). It seems likely, therefore, that protection induced by merozoite immunogens may be due to combinated function of CMI and specific antibody response. The successful use of Freund's adjuvant which is a potent stimulator of both CMI and humoral antibody leads us to suggest that a search for the most appropriate adjuvant requires special priority. Our results also indicate that 105, 35, 34 and 29 kD polypeptides appear to be candidates for the vaccine.

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=국문초록=

Theileria sergenti merozoite 수용성 항원의 항원성과 면역성

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T. sergenti merozoite 수용성 항원을 *T. sergenti* 감염적혈구로부터 분리하고자 저삼투압액으로 용혈, 조직분쇄기로 분쇄한 후에 고속 원심분리하여 수용성 항원을 얻었으며, SDS-PAGE와 Western blot의 방법으로 29, 34, 35 그리고 105 kD가 함유된 항원을 본 예방접종실험의 항원성 polypeptide로 정하였다. 본 수용성 항원(0.5 mg/ml)을 준비, Freund's adjunant를 이용하여 한우(5개월령)에 경피 접종하였으며, 다시 4주 후에 추가접종하였다. 추가접종 9주 후에 예방 접종군과 대조군에 동종의 병독충주(5.6×10^6RBC/dose, 40% 기생율)을 접종시킨 후에 적혈구용적비, 총적혈구수, 기생율, western blot에 의한 특이항체 그리고 간접형광항체(IFA) 등을 관찰하였던 바, 예방접종 후 18주(충 접종 6주 후)에 있어서 예방접종군의 IFA는 10,240 이었으나, 대조군은 1,280이었다. 예방접종군에 있어서의 충접종 전후에 있어서의 총적혈구소와 적혈구용적비는 유의적 차이 ($p<0.05$)를 나타내지 않았지만, 대조군에 있어서는 적혈구용적비와 총적혈구수에서 있어서 빈혈 소견을 관찰하였다 ($p<0.05$). 예방접종군의 충접종 후에 있어서의 western blot 반응에서는 29, 34, 35 그리고 105 kD polypeptide의 물질이 면역반응을 잘 나타내고 있어, 이들 polypeptide는 앞으로 vaccine 제조에 활용 가능성이 충분함을 예견할 수 있었다.

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