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Evidence for a common protective antigenic determinant on sporozoites of several *Theileria parva* strains

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Summary. The antigenic relationship amongst sporozoites of several *Theileria parva* strains isolated from different parts of East Africa was investigated. It was found that antibodies to *T. parva* (Muguga) sporozoites neutralized the infectivity of homologous sporozoites as well as those of *T. parva* (Uganda), *T. parva lawrencei*, *T. parva* (Kiambu 5), *T. parva* (Kabete 1) and *T. parva* (Marikebuni). The results correlated with those obtained from an *in vivo* challenge, in that cattle immunized against *T. parva* (Muguga) were either solidly resistant or developed only a transient parasitosis when challenged with *T. parva lawrencei* or *T. parva* (Uganda). The immunity exhibited by these animals paralleled the level of anti-sporozoite antibodies, such that animals with high titres were completely protected whereas those with low levels developed transient parasitosis. Furthermore, monoclonal antibodies made against sporozoites of *T. parva* (Muguga) were also capable of neutralizing the infectivity of sporozoites of other strains. These results indicate that the outcome of cross-challenge may depend on the level and avidity of the anti-sporozoite antibodies at the time of challenge. In addition, these findings show that there is a common protective antigenic determinant on sporozoites of all the *T. parva* strains investigated in this study.

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INTRODUCTION

Theileria parva, a protozoan parasite, causes one of the most economically important tick-borne diseases of cattle in East Africa. The disease, East Coast fever (ECF), is often fatal and is characterized by two intracellular stages of the parasite in the vertebrate host: a schizont stage within lymphoblastoid cells and the piroplasm stage within erythrocytes. In the vector, *Rhipicephalus appendiculatus*, the infective stage to the mammalian host (the sporozoite) is found in the salivary glands and is introduced into the mammalian host by the tick during feeding. The parasites appear to develop mainly in a subpopulation of T lymphocytes (Pinder & Roelants, 1981) although recent evidence suggests that a wider range of cells, including B lymphocytes and macrophages, can be invaded (Don Fawcett, personal communication).

Cattle that recover, spontaneously or by treatment, acquire resistance against re-infection with the same *T. parva* strain. It is thought that the immunity that develops in these animals is cell-mediated, since it can be transferred adoptively between twins using thoracic duct leukocytes from the immune partner (Emery, 1981). In addition, cytotoxic leucocytes with activity restricted to target cells carrying the autologous genotype have been detected in these animals (Eugui & Emery, 1981). Recently, however, an alternative mechanism which involves antibodies was proposed (Musoke *et al.*, 1982). These authors postulated that,

since neutralizing antibodies to sporozoites are detectable in sera from immune cattle, these antibodies may act in concert with cell-mediated responses—the antibodies being the first line of defence at the site of the tick bite. Any parasites escaping this attack would then be eliminated by cellular mechanisms.

The observation that animals which recover from ECF resist subsequent homologous challenge (Radley *et al.*, 1975b) suggests that vaccination against the disease is possible. It is thought, however, that antigenic diversity amongst *T. parva* strains is a major obstacle to the development of such a vaccine (Radley *et al.*, 1975b; Irvin *et al.*, 1983). We present here evidence that the sporozoites of several *T. parva* strains possess a common antigenic determinant which induces protective humoral responses against this stage, indicating that it may be possible to circumvent the reported antigenic diversity by using sporozoite antigen(s) for vaccination against ECF.

MATERIALS AND METHODS

Animals

Cattle (6- to 12-month-old crosses between Zebu and Hereford) were born and raised on ILRAD premises to avoid exposure to ECF and other diseases. They were screened for, before use, and found to have no antibodies against *T. parva*, *Babesia bigemina* or *Anaplasma marginale* (Burridge, 1971).

BALB/c mice aged between 2 and 3 months and weighing approximately 20 g were obtained from the ILRAD colony.

Parasites

Six *T. parva* strains were used in this study: *T. parva* (Muguga), *T. parva* (Kiambu 5), *T. parva* (Marikebuni), and *T. parva* (Kabete 1), all isolated from different parts of Kenya (Blocklesby, Barnett & Scott, 1961; Irvin *et al.*, 1974; Irvin *et al.*, 1983); *T. parva* (Uganda) isolated from North West Uganda (Uilenberg *et al.*, 1982); and *T. parva lawrencei* isolated from a buffalo in Serengeti National Park, Tanzania (Young & Purnell, 1973). They had all been maintained in our laboratory in *R. appendiculatus* and cattle.

Immunization of cattle against T. parva *sporozoites*

A group of 12 steers was immunized against *T. parva* (Muguga) by the infection and treatment method (Radley *et al.*, 1975a). Each animal was given booster doses thrice by attaching 1000 adult infected ticks to the ears at intervals of 2 weeks. Immune sera were collected from the animals 4 weeks after the last exposure. Antisera against sporozoites of two more strains, *T. parva lawrencei* and *T. parva* (Uganda), were produced in a similar manner. These sera were later tested for the presence of antibodies against homologous as well as heterologous sporozoites using an *in vitro* neutralization assay.

Four weeks after the last exposure to *T. parva* (Muguga), six of the 12 *T. parva* immunized cattle were challenged using a stabilate (Cunningham *et al.*, 1973) of *T. parva* (Uganda) sporozoites while the other six were challenged similarly using *T. parva lawrencei*. Six control animals (three per group) fed upon by uninfected ticks and treated with long-acting tetracyclines (Radley *et al.*, 1975a) were also challenged.

Derivation of monoclonal antibodies to T. parva *sporozoites*

Nymphs of *R. appendiculatus* were fed on cattle with patent piroplasmaemia. Whole salivary glands from 200 ticks containing about 12,000 infected acini were dissected out of the resultant adults and slightly macerated, using a grinder, in 10 ml of Roswell Park Memorial Institute medium 1640 (RPMI-1640) containing 10% foetal bovine serum (FBS). The suspension was centrifuged at 500 ***g*** for 5 min at 4° to remove salivary gland tissue and the supernatant passed through 5 μm and 2 μm cellulose acetate filters (Millipore Corp., U.S.A.) Stagg *et al.*, 1981). The filtrate was centrifuged at 3500 ***g*** for 15 min and the sediment containing sporozites was washed twice with 0·01 M phosphate-buffered saline (PBS). This enriched sporozoite preparation was reconstituted into 2 ml of PBS and inoculated intraperitoneally into five BALB/c mice.

The mice received two further inoculations of similarly prepared sporozoites at weekly intervals. Four days after the last inoculation, one mouse was killed and the spleen fused to NS1 myeloma cells (Galfre *et al.*, 1977). Antibody activity in culture fluids from the fusion was detected by indirect immunofluorescence on sporozoites applied onto microscope slides as follows. Fifty microlitres of a sporozoite suspension were delivered in a well of a Wellcome slide and the suspension immediately withdrawn using a 50 μl pipette and transferred to the next well, leaving behind a thin sporozoite layer. This procedure was repeated until all the 10 wells on the slide had received the sporozoite suspension. The slides were then left to dry at 37° and indirect immunofluorescence performed

following a described method (Nantulya *et al.*, 1979). Culture fluids were also checked for antibodies against *T. parva* macroschizonts by indirect immunofluorescence (Burridge & Kimber, 1972).

From this fusion, antibody activity against sporozoites was detected in culture fluids from seven wells. The seven culture fluids did not react with the macroschizonts. Hybrids from two of the fusion wells that had shown anti-sporozoite antibody activity were selected for cloning (Pearson *et al.*, 1980) on the basis of high intensity of immunofluorescent staining. Several positive clones were identified by indirect immunofluorescence. Culture fluids from four of the clones, and another clone from a different fusion (TpM 12/18.15.6) selected randomly, were concentrated 20-fold and the Ig class of each monoclonal antibody determined. All the five monoclonal antibodies were of IgM class. The five concentrated culture fluids and an IgM fraction isolated from monoclonal antibody TpM 10/3.25.3 (100 μg to 10 μg per well) were then tested for their capability to neutralize the infectivity of sporozoites of three strains: *T. parva lawrencei*, *T. parva* (Uganda) and *T. parva* (Muguga). Controls consisted of 20-fold concentrated Tb 7/8.1.48, an IgM monoclonal antibody against *Trypanosoma burcei* procyclics, and tissue culture medium.

Neturalization of infectivity of T. parva *sporozoites*

The neutralization of infectivity of sporozoites was carried out as follows. Fibroblast-like cells initiated from a bovine embryo thymus (BE5/81T) were grown in 75 cm^2 (275 ml) plastic T-type tissue culture flasks (Costar, Cambridge, MA, U.S.A.) in growth medium which consisted of RPMI-1640 containing 20% heat-inactivated FBS, 20 mM HEPES, 10 mM sodium bicarbonate and 2 mM L-glutamine. Confluent cell monolayers were then trypsinized, washed once in growth medium and cryopreserved in the same medium containing 10% dimethylsulphoxide. Forty-eight hours before the neutralization assays were performed, frozen fibroblast-like cells were thawed at 37°, washed once in growth medium and diluted in the same medium to give a concentration of 2×10^4 cells/ml. Five millilitres of the cell suspension were then added into each well of the six-well tissue culture plates (Cluster^6, Costar) and incubated at 37° in a 5% CO_2/air mixture. Growth medium was removed completely from the wells and replaced with 500 μl of the monoclonal antibody or immune sera. For each antibody the assay was set up in triplicate and each experiment repeated two or three times. After the addition of the antibodies into the wells, 100 μl of the sporozoite suspension isolated from infected tick salivary glands were added to each well and the tissue culture plates incubated for 30 min at 37° in 5% CO_2/air mixture. The plates were then removed from the incubator and 500 μl of a peripheral blood lymphocyte (PBL) suspension, containing 5×10^6 cells/ml, and 500 μl of growth medium added. The plates were incubated at 37° in 5% CO_2/air mixture. After 24 hr, 1 ml of growth medium was removed from each well and replaced by 5 ml of fresh growth medium containing 1% mycostatin and 0·1% gentamycin. Thereafter, the medium was changed every 3–4 days, while feeder layer cell culture plates were changed every 6 days. After 1 week, mycostatin was excluded from the growth medium.

Starting from 5 days after initiating the cultures, samples of PBL from each culture well were separately centrifuged onto slides in a cytospin (Cytospin 2, Shandon Southern Products Ltd., Cheshire, U.K.), stained with Giemsa and examined for the presence of intra-lymphocytic schizonts. The cultured PBL were screened every 5 days for 50 days, and if no schizonts were detected in any of the test wells, then neutralization of infectivity was regarded as having occurred.

Enzyme-linked immunosorbent assay (ELISA)

To determine whether the monoclonal antibodies generated recognized the same or different antigenic determinants on sporozoites, competitive inhibition ELISA was used. Forty Pristane-treated BALB/c mice were inoculated intraperitoneally with 1×10^7 hybrid cells of one clone (TpM 10/3.25.3) to induce ascitic fluids. The IgM fraction was prepared from ascites by filtration through Sepharose 6B and labelled with horseradish peroxidase (Wilson & Nakane, 1978). One hundred microlitres of a suspension of *T. parva* (Muguga) sporozoites were introduced into the wells of microtitre plates, and incubated at 37° overnight. The plates were washed twice before serial dilutions of unlabelled anti-sporozoite monoclonal antibodies and the control (IgM monoclonal antibody against *T. brucei* procyclics) were added. After incubation at room temperature for 1 hr, the plates were washed, the labelled monoclonal antibody added, and left at room temperature for 1 hr before further washing. The peroxidase substrate (0-phenylenediamine) was added and incubated for 30 min at room temperature, before reading the optical densities at 405 nM.

RESULTS

In vivo cross-challenge of *T. parva* (Muguga)-immunized cattle

A group of 12 steers immunized with *T. parva* (Muguga) were challenged with *T. parva lawrencei* (six animals) or *T. parva* (Uganda) (six animals). The six animals challenged with *T. parva lawrencei* were completely resistant to infection whereas the non-immunized controls died of ECF within 18 days (Table 1). Of the other group of six challenged with *T. parva* (Uganda), three animals were completely protected, two showed a transient parasitosis in the regional lymph node lasting 1 day, and one animal, B 750, developed ECF but recovered spontaneously (Table 1). The non-immunized controls for this group died of ECF within 24 days post-challenge (Table 1). The breakthrough parasite population from B 750 was transmitted through *R. appendiculatus* and sporozoites serotyped by the neutralization assay using pre-challenge sera to determine whether the ensuing sporozoites were antigenically different from those in the challenge stabilate.

In vitro neutralization of *T. parva* sporozoites

To examine the above findings in detail, it was necessary to utilize an *in vitro* assay for neutralization of infectivity of sporozoites because, quite apart from the expense involved in using cattle, the variability of immune responses in cattle is enormous due to outbreeding. Moreover, such an *in vitro* assay could allow an assessment of the level of humoral response against sporozoites prior to challenge.

Using this assay, the prechallenge sera from the 12 animals were examined for their ability to neutralize the infectivity of sporozoites of *T. parva* (Uganda), *T. parva* (Muguga) and *T. parva lawrencei*. It was found that all the prechallenge sera had neutralization titres of 1:64 or greater, except the serum from Animal B 750 which had a titre of only 1:8 (Table 2).

Interestingly, the breakthrough parasite population from animal B 750, when transmitted through *R. appendiculatus*, gave rise to sporozoites which were antigenically identical to those of the infecting stabilate, in that they were neutralized by the 12 prechallenge sera.

A further series of experiments was carried out to extend these observations to other *T. parva* strains. Antisera to sporozoites of two more strains, *T. parva lawrencei* and *T. parva* (Uganda) made in cattle were also tested for neutralizing antibody activity against sporozoites of *T. parva* (Marikebuni), *T. parva* (Kiambu 5), *T. parva* (Kabete 1) and *T. parva* (Muguga). The test and control (bovine anti-tick saliva) sera were used at dilutions of 1/2, 1/4 and 1/8. It was found that the antisera against *T. parva lawrencei* and *T. parva* (Uganda) neutralized the infectivity of sporozoites of the five strains at all these dilutions. We concluded that the sporozoites of all the strains studied here were either completely identical or possessed a common protective antigenic determinant.

To determine which of these alternatives applied, it was necessary to derive monoclonal antibodies against sporozoites of at least one of the strains.

Table 1. Immunity of *Theileria parva* (Muguga)-immunized cattle to challenge with *T. parva* (Uganda) or *T. parva lawrencei*

Experimental group*	Number of cattle	Mean time to appearance of macroschizonts in local drainage lymph node (days)	Mean time to spread to any other lymph node (days)	Number of animals with generalized infection	Mean time to death (days)
I	6	0	0	0	0
II	3	7·5	8	3	18
III	6	15 (3)†	17 (1)†	1	0
IV	3	9	12	3	24

* Group I animals were challenged with *T. parva lawrencei*, and group II animals were the controls. Group III animals were challenged with *T. parva* (Uganda), while Group IV served as controls.

† Three animals in this group developed parasitosis in the local drainage lymph node, and one of the three animals developed the disease but recovered spontaneously.

Table 2. *In vitro* neutralization of *T. parva* (Uganda) sporozoites using anti-*T. parva* (Muguga) sera

Source of antisera (Cattle number)	Serum dilution tested*					
	1:2	1:8	1:16	1:64	1:128	1:256
B 748	6/6	6/6	6/6	6/6	6/6	6/6
B 749	6/6	6/6	6/6	6/6	6/6	6/6
B 750	6/6	6/6	0/6	0/6	ND	ND
B 751	6/6	6/6	6/6	6/6	0/6	0/6
B 752	6/6	6/6	6/6	6/6	6/6	0/6
B 753	6/6	6/6	6/6	6/6	6/6	6/6

* For each serum dilution the assay was set up in triplicate and each experiment repeated once. All pre-immunization sera had no neutralizing activity.

ND = not done.

Reactivity of monoclonal antibodies with *T. parva* sporozoites

The five concentrated culture fluids and an IgM fraction isolated from monoclonal antibody TpM 10/3.25.3 (ranging from 100 μg, to 10 μg per well) were tested for their capability to stain, by immunofluorescence, and neutralize the infectivity of sporozoites of three strains: *T. parva lawrencei*, *T. parva* (Uganda) and *T. parva* (Muguga). Controls consisted of 20-fold concentrated Tb 7/8.1.48, an IgM monoclonal antibody against *Trypanosoma brucei* procyclics, and tissue culture medium. The five monoclonal antibodies against *T. parva* (Muguga) stained (Fig. 1) the sporozoites of the three strains. The neutralization results (Table 3) showed that each monoclonal antibody was able to neutralize the infectivity of sporozoites of the three *T. parva* strains.

To determine whether the monoclonal antibodies recognized the same or a different antigenic determinant on sporozoites of the strains tested, a competitive inhibition ELISA was used. It was found that each unlabelled antibody blocked totally the reactivity of the labelled monoclonal antibody, unlike the control which did not block the reaction, indicating that all the monoclonal antibodies were directed against the same antigenic determinant.

DISCUSSION

The work presented here has provided evidence for the existence, on sporozoites of several *T. parva* strains, of a common antigenic determinant which is capable of inducing neutralizing antibodies. The results of *in vivo*

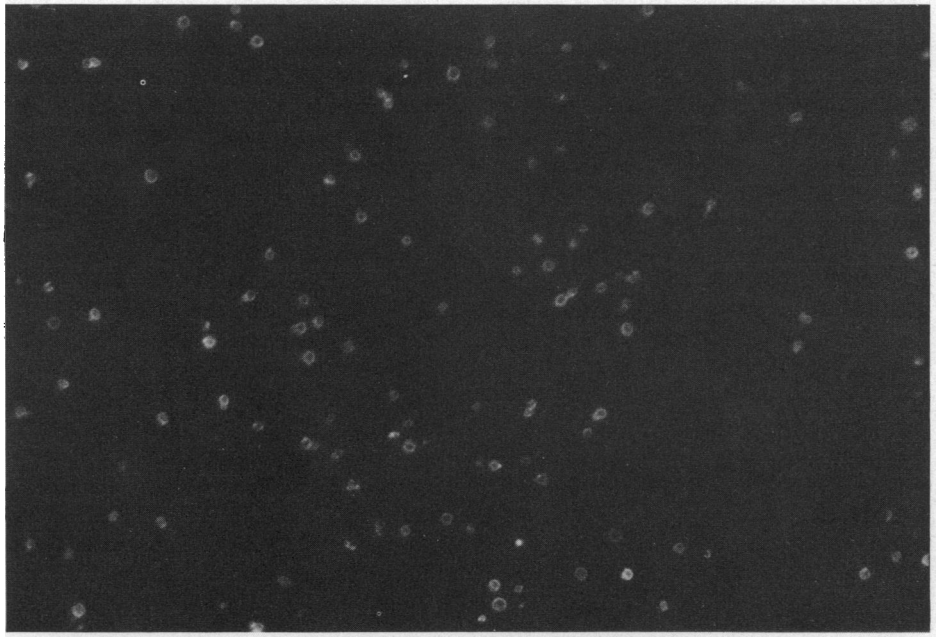

Fig. 1. Indirect immunofluorescent staining of *T. parva* (Muguga) sporozoites using monoclonal antibody TpM 10/3.25.3. Other monoclonal antibodies stained *T. parva* sporozoites similarly. (Magnification × 1000.)

Table 3. Neutralization of sporozoites of three *Theileria parva* strains using monoclonal antibodies

Monoclonal antibody	*T. parva* (Muguga)	*T. parva* (Uganda)	*T. parva lawrencei*
TpM 10/3.12.13	9/9	9/9	9/9
TpM 10/3.25.3	9/9	9/9	9/9
TpM 10/3.33.2	9/9	9/9	9/9
TpM 10/14.19.23	9/9	9/9	9/9
TpM 12/18.15.6	6/6	6/6	6/6
TpM 10/3.25.3 (enriched IgM fraction)			
100 μg	3/3	3/3	3/3
50 μg	3/3	3/3	3/3
10 μg	0/3	0/3	0/3
TB7/8.1.48	0/9	0/9	0/9
RPMI-1640 medium	0/9	0/9	0/9

* IgM monoclonal antibody against *T. brucei* procyclics.

challenge were consistent with those of neutralization assays in that animals immunized with *T. parva* (Muguga) were resistant to subsequent challenge with *T. parva lawrencei* or *T. parva* (Uganda). Immunity in these animals correlated with levels of anti-sporozoite antibodies, such that the one animal, B 750, which developed disease on challenge, was the one that had low titres of neutralizing antibodies.

The cross-protection observed against *T. parva* (Uganda) and *T. parva lawrencei* was quite unexpected inasmuch as these strains are reported to override the immunity induced by *T. parva* (Muguga) (Uilenberg *et al.*, 1982; Radley *et al.*, 1975b). The discrepancy in results may be attributed to the level of immunity against sporozoites induced by the immunization method. The method of immunization employed in the present study favours the production of antibodies to the sporozoites due to the fact that the immune system is presented with fewer antigens as compared to the previous procedure that involved inoculation of ground-up ticks into animals (Radley *et al.*, 1975a). Indeed, in a preliminary study, animals immunized in this manner had little or no neutralizing antibodies against sporozoites in their sera (unpublished observation).

In a recent report (Musoke *et al.*, 1982), we postulated that in immunized animals the first line of defence against *T. parva* infection is at the level of the sporozoite, and that any parasites escaping this attack were eliminated by other mechanisms, including cell-mediated responses. It is, therefore, possible that in animals lacking sufficient quantities of high avidity antibodies to sporozoites, the first line of defence is ineffective, leading to the development of macroschizonts which, if not completely controlled by the second line of defence, would result in disease. This could be the explanation for the observations reported here and elsewhere (Radley *et al.*, 1975b) that some immunized animals may develop a transient parasitosis or even disease on homologous challenge.

There are currently two methods of immunization against ECF, namely, infection and treatment developed by Radley *et al.* (1975b), and the use of *in vitro* cultured macroschizonts (Brown *et al.*, 1978; Dolan, Njuguna & Stagg, 1982). Although both methods produce long-lasting immunity, their major drawback resides in the fact that an infection has to be established in the host before solid immunity develops. Also, in the case of the macroschizont method, a large number of cells is required to immunize allogeneic animals. In some instances severe disease and occasionally deaths have been associated with the two methods of immunization, particularly the macroschizont method (Brown *et al.*, 1978; Dolan *et al.*, 1982).

The observation, therefore, that all the *T. parva* strains in the present study possess a common protective antigenic determinant is of major interest with regard to prospects for the development of an alternative approach to vaccination against ECF. Since the strains so far studied were isolated from widely separated areas, it is hard to escape the conclusion that this antigenic determinant could be present on sporozoites of other strains. Thus it may be possible to circumvent the reported antigenic diversity in *T. parva*

by the use of a sporozoite antigen as a vaccine. A vaccine based on sporozoite antigens would eliminate the requirement for establishing infection and also circumvent the problem of histoincompatibility (Spooner & Brown, 1980) associated with the macroschizont vaccine. In malaria, *Plasmodium* infection, which bears close resemblence to ECF, it has been reported that vaccination against the disease using sporozoite antigens (Nussenzweig, 1980; Cochrane *et al.*, 1982) or antigens cloned in bacteria (Ellis *et al.*, 1983) is possible.

The biochemical nature of the *T. parva* sporozoite antigen(s) has not been determined, although preliminary results indicate that recovery serum recognizes at least two antigens of molecular weight 105,000 and 70,000 (unpublished observations). These antigens appear to be sporozoite-specific as they are absent in uninfected tick salivary gland extracts. It is not, however, clear whether any of these two major antigens is responsible for the induction of protective immunity. Further studies are currently being pursued to characterize these antigens biochemically, as this would determine the approach to *in vitro* synthesis of the antigen in bulk for immunization purposes.

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