A 32 kDa surface antigen of *Theileria parva*: characterization and immunization studies

R. A. SKILTON, A. J. MUSOKE, C. W. WELLS, Y. YAGI†, V. NENE, P. R. SPOONER, J. GACHANJA, J. OSASO, R. P. BISHOP *and* S. P. MORZARIA*

International Livestock Research Institute (*ILRI*), *P.O. Box 30709, Nairobi, Kenya*

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SUMMARY

Previous studies using monoclonal antibody (mAb) 4C9 specific for a 32 kDa antigen (p32) of *Theileria parva* demonstrated expression of the antigen on the surface of the sporozoite, making it a potential antigen for sporozoite neutralization. A full-length cDNA encoding the major merozoite/piroplasm surface antigen (mMPSA) of *T. parva* was cloned and expressed in bacteria. The expressed product reacted strongly with mAb 4C9, demonstrating identity between the p32 and mMPSA of *T. parva*. Using immunoblot analysis and immunoelectron microscopy with mAb 4C9 it was shown that the mMPSA is a major antigen of the merozoite and piroplasm at the cell surface, while lower levels of antigen are expressed in the sporozoite and schizont stages. Upregulation of the mMPSA occurs at merogony and can be induced by culturing schizont-infected lymphocytes at 42 °C. Recombinant mMPSA of *T. parva* induced high titres of specific antibodies in cattle but failed to confer protection against a *T. parva* sporozoite stabilate challenge. The pre-challenge sera also failed to neutralize infectivity of sporozoites in an *in vitro* assay. Possible reasons for the lack of parasite neutralization *in vivo* and *in vitro* are discussed.

Key words: *Theileria parva*, surface antigen, subunit vaccine.

INTRODUCTION

East Coast fever (ECF) is an acute and frequently lethal disease of cattle that is caused by the sporozoan parasite, *Theileria parva*. The current method for inducing immunity to ECF is by infecting cattle with cryopreserved sporozoites and simultaneous treatment with long-acting tetracycline (Norval, Perry & Young, 1992). Immunity develops from a schizont parasitosis and is mediated by cytotoxic T-lymphocytes specific for schizont-infected lymphocytes (Morrison, Taracha & McKeever, 1995). Although infection and treatment can induce life-long protection, immunity is often strain-specific. There are also practical difficulties associated with delivering cryopreserved parasites to the field.

An experimental *T. parva* subunit vaccine based upon a 67 kDa sporozoite surface antigen (p67) can induce protection in approximately 65% of vaccinated cattle against challenge by heterologous cattle-derived *T. parva* (Musoke *et al*. 1992; Nene *et al*. 1995, 1996). Although the mechanism of immunity induced by p67 is unclear, antibodies to p67 can neutralize sporozoite infectivity *in vitro* (Musoke *et al*. 1982, 1992; Nene *et al*. 1992). It is envisaged that the p67 will form a major part of a multivalent subunit vaccine against ECF in combination with other antigens, particularly those that induce cytotoxic T-lymphocytes. However, additional antigens with potential to augment sporozoite neutralization are also worth evaluating.

A 32 kDa antigen (p32) is also expressed on the *T. parva* sporozoite surface, as demonstrated by surface-labelling experiments (Y. Yagi & V. Nene, unpublished data). The p32, therefore, has potential to induce sporozoite-neutralizing responses. In this paper we report on the stage expression of p32 and show that it has identity with the major merozoite/piroplasm surface antigen (mMPSA) which is a member of a highly conserved immunodominant antigen family within the genus *Theileria* (Katzer *et al*. 1998*b*). We evaluated recombinant p32 for its capacity to neutralize *T. parva*. Although recombinant p32 of *T. parva* is highly immunogenic in cattle, vaccination with it was unable to prevent the development of ECF or reduce piroplasm parasitaemia. In addition, the pre-challenge sera from these cattle did not neutralize infectivity of sporozoites *in vitro*. Possible reasons for the lack of parasite neutralization by the p32 antigen are discussed.‡

MATERIALS AND METHODS

Parasite materials and antibodies

Sporozoites of *T. parva* (Muguga stock; Brocklesby,

* Corresponding author: Tel: +254 2 630 743. Fax: +254 2 631 499. E-mail: s.morzaria@cgiar.org
† Present address: Hokkaido Research Station, National Institute of Animal Health, 4-Histujigaoka, Toyohira, Sapporo 062-0045, Japan.
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Barnett & Scott, 1961) were isolated from infected salivary glands of *Rhipicephalus appendiculatus* as described (Iams *et al*. 1990). *T. parva* (Muguga) schizont-infected lymphocyte cell line (clone H12; Kurtti *et al*. 1981) was cultured as described (Iams *et al*. 1990). Piroplasms of *T. parva* were isolated from infected bovine blood as described (Conrad *et al*. 1987) and piroplasm DNA was purified as described (Iams *et al*. 1990). MAb 4C9 (IgG_1 isotype) was derived from a mouse inoculated with a lysate of *T. parva* piroplasms (Marikebuni stock; Irvin *et al*. 1983). MAb 4C9 purified from hybridoma culture medium was used as the source of antibody. Mouse myeloma IgG_1 (MOPC21) (Sigma, St Louis, MI, USA) was used as an isotype control antibody. Bovine antiserum E99 was raised against a lysate of *T. parva* sporozoites and has a sporozoite neutralization titre of $>1:1000$ (A. J. Musoke, unpublished data).

Isolation of the cDNA encoding the mMPSA of T. parva

Degenerate oligonucleotide primers IL3041 (forward primer: 5′-CGY TTC AAG ACN CTY AAG GYN GG-3′) and IL3046 (reverse primer: 5′-GGI GAG AAC TTG TCG ACM RBH GGY TTG TAR TC-3′) were designed from conserved sequences within mMPSA genes of *Theileria* (Kawazu *et al*. 1992; Shiels *et al*. 1995; Katzer *et al*. 1998*b*). These primers were used in a polymerase chain reaction (PCR) to amplify a DNA fragment with a predicted size of approximately 590 bp from *T. parva* piroplasm DNA. The PCR product was used to screen a *T. parva* (Muguga) piroplasm cDNA library in λgt11 using standard methods (Sambrook, Fritsch & Maniatis, 1989). An insert from a positive plaque was subcloned into pUC-18 and sequenced on both strands by *fmol*™ cycle sequencing (Promega Corp., Madison, WI, USA). Nucleotide and deduced amino acid sequences were analysed using DNASIS V2.1 for Windows (Hitachi Software Engineering America Ltd, San Bruno, CA, USA). Internet sites of the Center for Biological Sequence Analysis, Denmark, were used for signal peptide prediction (http://genome.cbs.dtu.dk/services/SignalP/) and for the prediction of transmembrane helices in proteins (http://www.cbs.dtu.dk/services/TMHMM-1.0/).

Bacterial expression of the T. parva (*Muguga*) *mMPSA*

Bacterial recombinant antigens were generated as *N*-terminal fusion proteins with glutathione S-transferase (GST) and 6 × histidine tag (HisTag) using the pGEX (Smith & Johnson, 1988) and pQE (Qiagen GmbH, Hilden, Germany) expression systems, respectively. A DNA fragment encoding the *T. parva* (Muguga) mMPSA, corresponding to amino acids A^{22}–L^{280}, was generated by PCR from cDNA clone p25-5 and cloned into pGEX1λT (Pharmacia Biotech, Uppsala, Sweden) and pQE-30. GST-mMPSA was produced in *E. coli* strain XL1-Blue (Stratagene Cloning Systems, La Jolla, CA, USA) and purified by affinity chromatography on glutathione–Sepharose (Pharmacia) according to the manufacturer's instructions. HisTag-mMPSA was produced in *E. coli* strain M15[pREP4] and purified by agarose/Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography in the presence of 8 M urea according to the Qiagen instructions. Parent GST was produced from non-recombinant pGEX1N (Smith & Johnson, 1988).

Analysis of T. parva *mMPSA mRNA expression by reverse transcription-PCR* (*RT–PCR*)

Total RNA was purified using the method of Xie & Rothblum (1991) from uninfected and *T. parva* (Muguga) sporozoite-infected salivary glands of *R. appendiculatus*, *T. parva* (Muguga) schizont-infected lymphocyte cell line (clone H12), G6 uninfected-lymphocyte blast cells (Brown & Grab, 1985) and *T. parva* (Muguga) piroplasms. cDNA was synthesized from total RNA with M-MLV reverse transcriptase (Gibco-BRL Life Technologies, Paisley, UK) as described (Sambrook *et al*. 1989). cDNA was PCR-amplified using an oligo-dT anchor primer (5′-AGA GCG GCC GCT_{19}-3′) and a *T. parva* mMPSA gene-specific primer (5′-GGT TGA AGG AGG TCT ACT TCG-3′). PCR products were separated by agarose gel electrophoresis, transferred to a nylon membrane (Hybond-N; Amersham International, Aylesbury, UK) and hybridized to a ^{32}P-labelled, nested mMPSA gene probe that was generated by PCR. As a control, RT–PCR was performed as described except RT was omitted from cDNA synthesis reactions.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (*SDS–PAGE*), *immunoblotting and enzyme-linked immunosorbent assay* (*ELISA*)

Total cell lysates were separated by SDS–PAGE under reducing conditions according to standard protocols (Harlow & Lane, 1988). For immunoblotting, proteins were transferred to nitrocellulose (Hybond-C) (Amersham) and processed as described (Harlow & Lane, 1988). Goat anti-mouse IgG alkaline phosphatase conjugate (Promega) was used as the detection conjugate and the blots were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate/chromogen solution. Antibody capture ELISA

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   1    A GTT ATT GGT CTT TTT CCA TAA CAT TTA TCC GGT TAT TTA ATT TTT AAT ATT TGG ATC

  59  GCT AAC TAG TCT GCC TTT TGT TCG GTT TTT ATA ATA TAA TTA TTT GAG ATG TTG TCC AGA
   1                                                                 M   L   S   R

 119  AAT ACC CTC AAG TTC TTA TAT TTG AGT TTC TTC GTT ATC TCT TGC GTT AAT GCC GCA AAA
   5    N   T   L   K   F   L   Y   L   S   F   F   V   I   S   C   V   N   A▲A   K

 179  GAA GAA GAG AAG AAG AAG GAG AAA AAG GAG GAT CTT ACA GTT GAT GTT ACC CTT TCG TCA
  25   E   E   E   K   K   K   E   K   K   E   D   L   T   V   D   V   T   L   S   S

 239  TGG GAA AAT GTT ACT TCT ACT CCC GAG GCT GGC GGT ACA TTA TTG AAA GCC AAT GAA GGT
  45    W   E   N   V   T   S   T   P   E   A   G   G   T   L   L   K   A   N   E   G

 299  TAC CGT TTC AAG ACA CTT AAG GTC GGC GAC AAG ACT TTG TAC AAC GTT GAC ACC TCA AAA
  65    Y   R   F   K   T   L   K   V   G   D   K   T   L   Y   N   V   D   T   S   K

 359  TAC GAT GCA GTA CAC CTA TAC AAA CTT ACC CAT GAT TCT GAT GAA TGG CTA AAG CTC CTT
  85    Y   D   A   V   H   L   Y   K   L   T   H   D   S   D   E   W   L   K   L   L

 419  CTC CAC CCA GCC AAG CCA GTG ATG TTC AAG AAG AAG TCA GAC AAG GAA TAT TCC GAA GTC
 105   L   H   P   A   K   P   V   M   F   K   K   K   S   D   K   E   Y   S   E   V

 479  AAA TTC GAA ACC TAC TAT GAT GAT GTC TTG TTC AAG GGG AAA TCA GCC AAG GAA CTC GAT
 125   K   F   E   T   Y   Y   D   D   V   L   F   K   G   K   S   A   K   E   L   D

 539  GCT TCC AAG GTC ACT GAT ACT GGC TTG TTT ACC CAA GAG AGC TTC GGC ACT GGG AAG AAG
 145   A   S   K   V   T   D   T   G   L   F   T   Q   E   S   F   G   T   G   K   K

 599  TAC ACC TTC AAC AAT AGC TTC AAA CCT TCC AAG GTC TCA TTC GAC AAA AAA GAT GTT GGA
 165   Y   T   F   N   N   S   F   K   P   S   K   V   S   F   D   K   K   D   V   G

 659  AAG CCC GAC AAG GCC AAG TTC CTC GAC GTT TTC GTC TAT GTC GGC TCT GAT GAC AAG AAG
 185   K   P   D   K   A   K   F   L   D   V   F   V   Y   V   G   S   D   D   K   K

 719  GTT GTT AGG CTC GAC TAC TTC TTT GGT GGT GAC TCA AGG TTG AAG GAG GTC TAC TTC GAG
 205   V   V   R   L   D   Y   F   F   G   G   D   S   R   L   K   E   V   Y   F   E

 779  CTT AAA GAC GAC AAG TGG GTC AAA ATG GAA CAG AAT GAC GCA AAC AAG GCA TTG CAT GCC
 225   L   K   D   D   K   W   V   K   M   E   Q   N   D   A   N   K   A   L   H   A

 839  ATG AGC GAT TCA TGG AAA TTG GAC TAC AAA CCA GTC GTC GAC AAG TTC TCT CCC CTT GCA
 245   M   S   D   S   W   K   L   D   Y   K   P   V   V   D   K   F   S   P   L   A

 899  GTC CTC GCC TCA GTA CTC ATC GTC GCC GCT TCA GTC TTT TAC AAC CTT TAA AAC CCA TGT
 265   V   L   A   S   V   L   I   V   A   A   S   V   F   Y   N   L   *

 959  GCG TAA CAA CTT ATC AAC GTT TAA AAC AAT ATT GAT AAT TTG TAT ACA ATT ACA GCA GCT
1019  AAC TAA CTT AAG TCA TTT TAT GCC ACT TAA TTT CAT ACC CTT CGA AAA AAA A
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Fig. 1. cDNA and deduced amino acid sequence of the *Theileria parva* (Muguga) mMPSA. Several predicted features of the deduced amino acid sequence are indicated: site of signal sequence cleavage (arrowhead), highly charged region (bold italics), transmembrane helix (underline), *N*-linked glycosylation site (N). A stop codon is denoted by '*'.

was performed essentially as described (Harlow & Lane, 1988). Immuno–Polysorp ELISA plates (Nunc, Kamstrup, Denmark) were coated with 500 ng antigen/well, and goat antimouse IgG/alkaline phosphatase and recombinant protein-G/alkaline phosphatase (Calbiochem, San Diego, CA, USA) were used as detection conjugates for mouse and bovine antibodies, respectively. The wells were developed with *p*-nitrophenyl phosphate (*p*-NP) substrate solution (Sigma).

Immunofluorescence and immunoelectron microscopy (immunoEM)

T. parva (Muguga) schizont-infected lymphocyte cells were cytospun onto glass slides, air dried and fixed in cold methanol. The cells were incubated with mAb 4C9 diluted to 1:100, washed and then incubated with goat anti-mouse IgG–FITC (Sigma). Slides were viewed by differential interference contrast (DIC) illumination and epifluorescence using a Zeiss Axiophot 1 microscope. For immunoEM, Lowicryl K4M-embedded specimens were prepared as described (Burleigh *et al*. 1993). Ultrathin (60 nm) sections were incubated with mAb 4C9 diluted to 1:50 and then labelled with goat anti-mouse IgG conjugated to 5 nm gold (Biocell, Cardiff, UK). Sections were stained with 2% aqueous uranyl acetate and Reynolds lead citrate and then examined in a Zeiss EM10A electron microscope. Control EM sections were incubated with an unrelated mAb, the mAb replaced with BSA, antibody to GST or with mAb 4C9 which had been pre-incubated for 30 min with GST–mMPSA.

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Immunization and challenge of cattle

Four to five-month-old Boran (*Bos indicus*) calves were used in this study. The animals were maintained under strict tick control and were negative for antibodies to *T. parva*, *T. mutans*, *Babesia bigemina* and *Anaplasma marginale*. Two groups of 7 animals were given 3 inoculations with 450 μg of recombinant mMPSA (GST–mMPSA or HisTag-mMPSA), administered at monthly intervals. The antigens were emulsified in RWL, a proprietary adjuvant from SmithKline Beecham, PA, USA. A group of 7 non-immunized Boran cattle of a similar age group served as controls for sporozoite challenge. Fourteen days after the final mMPSA inoculation all animals were challenged with 1 LD_{70} dose of *T. parva* (Muguga) sporozoite stabilate 4133. Lymph node and blood smears were sampled and processed as described (Musoke *et al*. 1992). Clinical reactions were classified as previously described (Anon, 1989).

Sporozoite neutralization assay

Assays to measure neutralization of sporozoite infectivity *in vitro* were performed as described previously (Musoke *et al*. 1982). Briefly, a suspension of sporozoites was incubated for 30 min with pre- or post-immunization sera at a final dilution of 1:50, and then incubated with bovine peripheral blood lymphocytes. Cultures were examined on days 6, 10 and 14 and assessed for the percentage of infected lymphocytes.

RESULTS

Cloning and sequence analysis of the cDNA encoding the T. parva (*Muguga*) *mMPSA*

A *T. parva* (Muguga) piroplasm cDNA library in λgt11 was screened with a PCR product encoding a fragment of the *T. parva* mMPSA gene. The insert from 1 positive clone was subcloned in pUC-18 to produce a plasmid designated p25-5. In Fig. 1 the complete nucleotide sequence of the 1070 bp cDNA insert from plasmid p25-5 is shown together with the deduced amino acid sequence. Stop codon analysis revealed an open reading frame encoding a protein of 280 amino acids, beginning with an ATG translation initiation site at bp 107 and ending with a TAA stop condon at bp 947. The predicted molecular mass of this protein is 32045 Da. The N-terminal amino acid sequence has a putative secretory signal sequence with cleavage occurring between A^{22} and A^{23} (Neilsen *et al*. 1997) and the predicted molecular mass of the protein less the signal sequence is 29426 Da. The N-terminus of the mature protein (K^{24}–E^{34}) is composed entirely of the charged amino acids lysine and glutamine. Overall, the *T. parva* mMPSA is very rich in lysine and glutamine, and 23% of the mature protein is composed of these amino acids. The hydrophobic C-terminus (F^{260}–V^{276}) is a predicted transmembrane helix and there are 2 potential sites of *N*-linked glycosylation at N^{47} and N^{168}. A partial length genomic sequence encoding the *T. parva* Tpms1 antigen (Shiels *et al*. 1995) has complete identity with nucleotides 53–874 of the *T. parva* (Muguga) mMPSA DNA sequence reported here, except for a silent nucleotide substitution (T for C) at base 874.

Production of recombinant mMPSA of T. parva

cDNA encoding amino acids A^{22}–L^{280} of the *T. parva* (Muguga) mMPSA was cloned into pGEX-1λT and pQE-30 and expressed as fusion proteins with GST and HisTag, respectively. The fusion proteins were analysed by SDS–PAGE (Fig. 2A). The majority of purified GST-mMPSA appeared as a protein with an apparent molecular mass of 53 kDa (Fig. 2A). The HisTag-mMPSA was a stable fusion protein with an apparent molecular mass of 32 kDa (Fig. 2A).

The T. parva *mMPSA has identity with the 32 kDa antigen* (*p32*) *that is recognized by mAb 4C9*

In the current and previous studies (Y. Yagi & V. Nene, unpublished data) mAb 4C9 was shown to recognize a *T. parva* 32 kDa antigen (p32). MAb 4C9 also showed strong reactivity to the *T. parva* mMPSA expressed as GST and HisTag fusion proteins (Fig. 2B), demonstrating identity between the mMPSA and p32 antigen. There was no significant reactivity by mAb 4C9 with parent GST or by mouse myeloma IgG_1 control antibody with any of the recombinant proteins (Fig. 2B).

Stage expression of the T. parva *mMPSA*

Immunoblots of total proteins from *T. parva* (Muguga) sporozoites, schizont-infected lymphocytes and piroplasms were probed with mAb 4C9. This revealed expression of a 32 kDa antigen (p32/mMPSA) in each of these stages (Fig. 3A). Expression of this antigen was notably higher in piroplasms compared to that in sporozoites and schizont-infected lymphocytes. Mouse myeloma IgG_1 antibody showed no reactivity with control immunoblots of sporozoites, schizont-infected lymphocytes and piroplasms (data not shown).

Non-quantitative analysis of *T. parva* mMPSA mRNA expression was undertaken by RT–PCR. mMPSA mRNA expression was detected in sporozoite-infected *R. appendiculatus* salivary glands, schizont-infected lymphocytes and piroplasms (Fig. 3B). No mMPSA RT–PCR product was detected in

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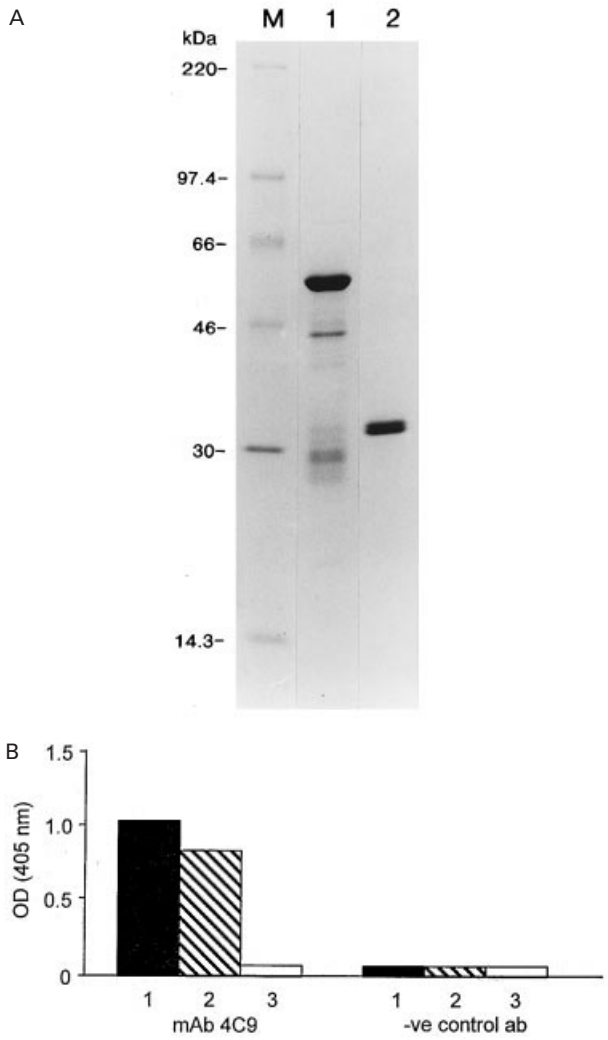

Fig. 2. SDS–PAGE and mAb 4C9 immunoreactivity of recombinant mMPSA of *Theileria parva*. (A) SDS–PAGE and Coomassie Blue staining of purified GST-mMPSA (lane 1) and HisTag-mMPSA (lane 2). Molecular weight markers for sizes indicated (lane M). (B) ELISA showing reactivities of mAb 4C9 (1:8100 dilution) and a mouse myeloma IgG_1 negative control antibody (125 ng/ml) with GST-mMPSA (1), HisTag-mMPSA (2), and parent GST (3).

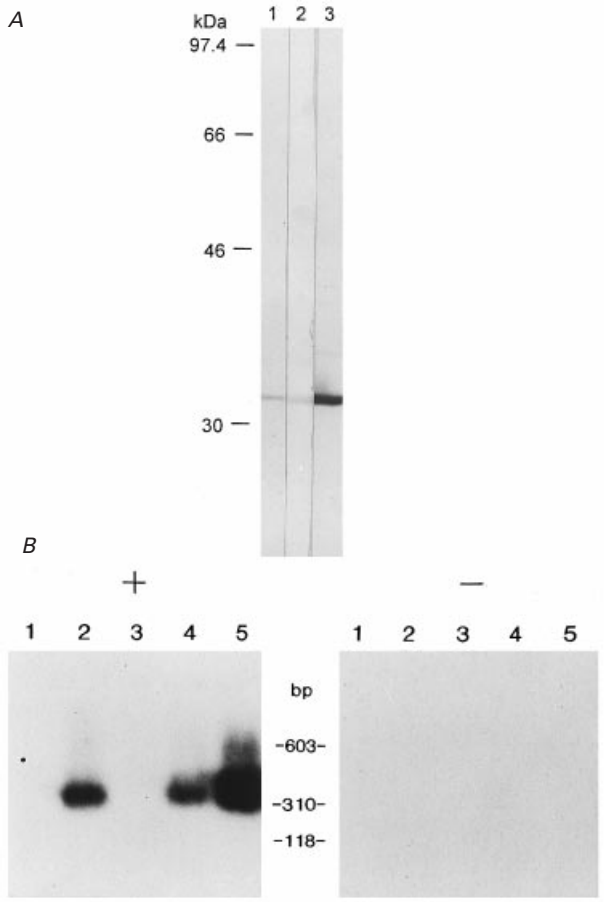

Fig. 3. Stage expression of *Theileria parva* (Muguga) mMPSA protein and mRNA. (A) Immunoblot analysis of lysates from life-cycle stages of *T. parva* with mAb 4C9. Lane 1, sporozoites purified from infected salivary glands of *Rhipicephalus appendiculatus*; lane 2, schizont-infected lymphocyte cell line; lane 3, purified piroplasms. Approximately 8 μg total protein was electrophoresed in each lane. Lane M, molecular weight markers for sizes indicated. (B) mMPSA mRNA expression detected by RT–PCR. A blot is shown of RT–PCR products hybridized with a radio-isotope labelled, nested probe. Panel '+': lane 1, uninfected salivary glands of *R. appendiculatus*; lane 2, sporozoite-infected salivary glands of *R. appendiculatus*; lane 3, uninfected lymphocyte blast cells; lane 4, schizont-infected lymphocyte cell line; purified piroplasms (lane 5). Panel '–': control reactions, as for panel '+' but with reverse transcriptase omitted from the reactions. Positions of DNA size markers in base pairs are indicated in the middle of the figure.

uninfected *R. appendiculatus* salivary glands, uninfected lymphocyte blast cells or in any of the control reactions where RT was omitted (Fig. 3B).

ImmunoEM localization of the T. parva *mMPSA*

MAb 4C9 was used to localize the mMPSA in life-cycle stages of *T. parva* by immunogold EM. No significant mAb 4C9 reactivity was detected in sporozoites or schizont-infected lymphocytes during schizogony (data not shown). However, in the parasite at an advanced stage of merogony (merozoite budding) the mMPSA was clearly localized to the surface of the newly formed merozoites and to the cytoplasm of the parasite residual body (Fig. 4A). The mMPSA was also localized to the surface of the subsequent life-cycle stage, the intra-erythrocytic piroplasm (Fig. 4B). There was no significant immunogold labelling in any of the control sections (data not shown).

Incubation of T. parva *schizont-infected lymphocytes at 42 °C can upregulate mMPSA protein expression*

When grown at 37 °C, occasional schizonts (< 1 %) in an infected lymphocyte culture labelled weakly

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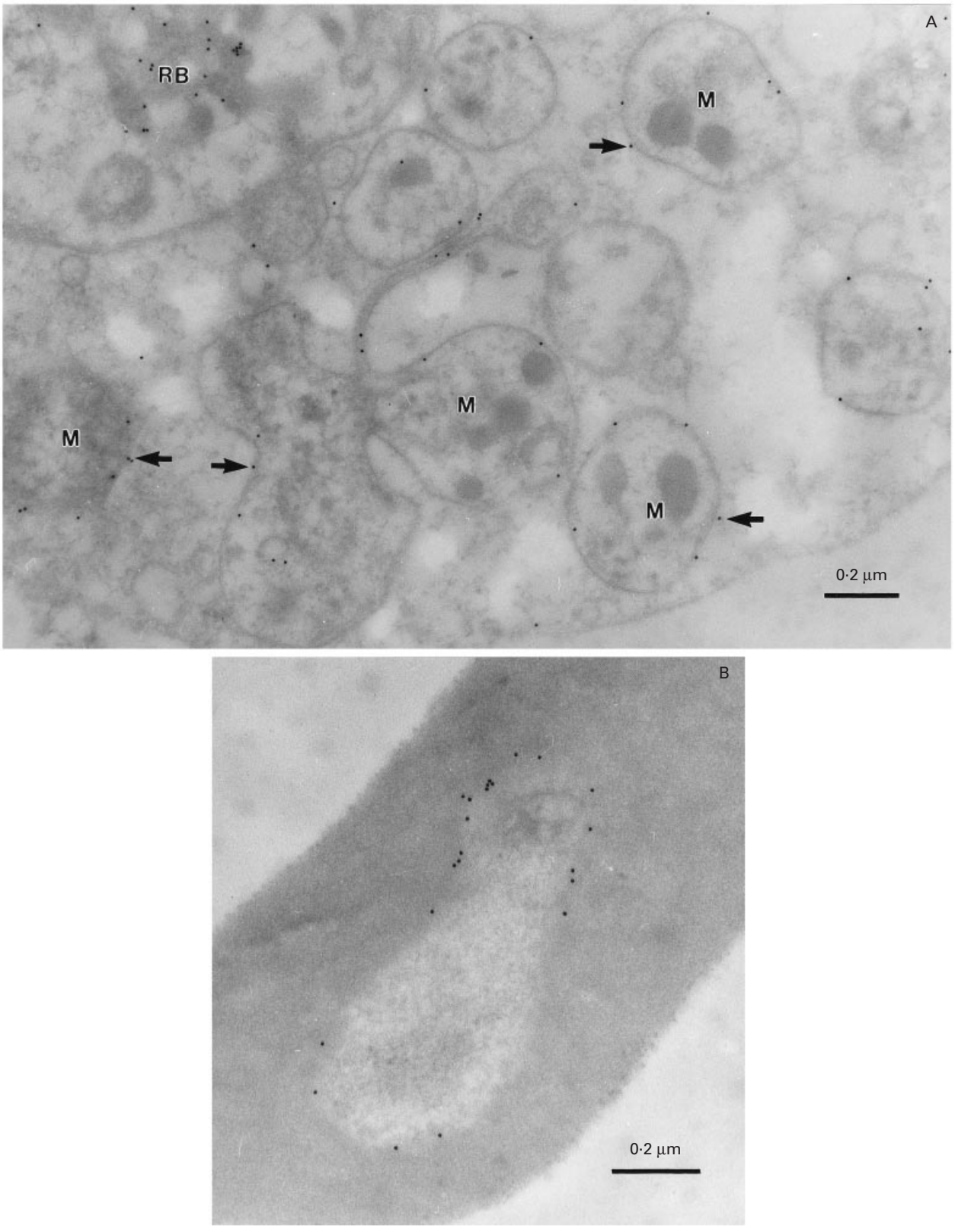

Fig. 4. Localization of the *Theileria parva* mMPSA in the merozoite and piroplasm by immunogold-EM with mAb 4C9. (A) Micrograph of a *T. parva*-infected bovine lymphocyte showing the parasite undergoing merogony. Merozoites (M) appear to have budded, while some are in the process of budding, from a central residual body (RB). The mMPSA is localized to the surface of the merozoites and also to the cytoplasm of the residual body of the parasite. (B) Micrograph of a piroplasm-infected bovine erythrocyte. The mMPSA is localized to the surface of the piroplasm.

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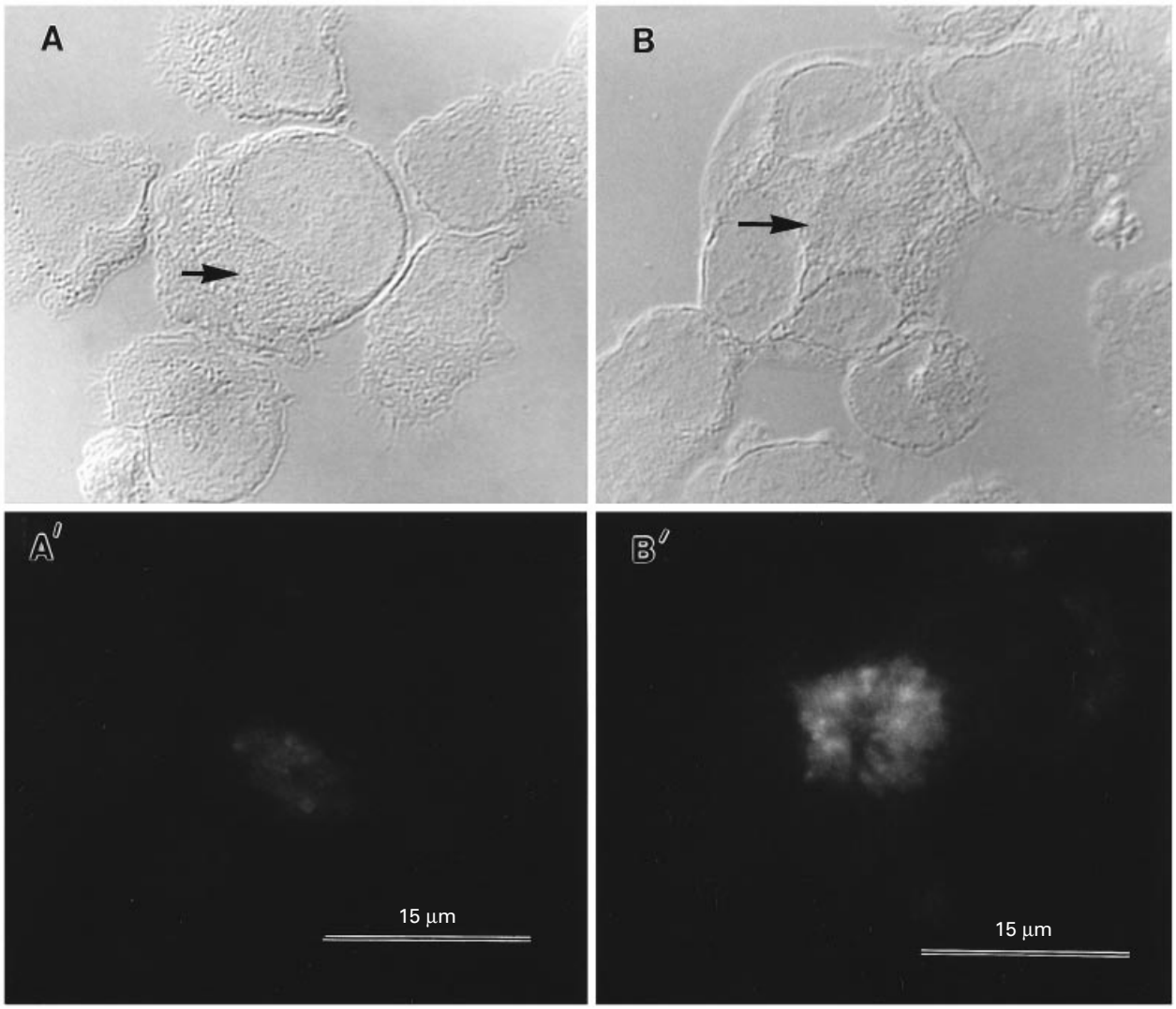

Fig. 5. Heat shock induction of mMPSA expression in *Theileria parva* schizonts. Schizont-infected lymphocytes cultured at 37 °C, imaged by DIC microscopy (A), showed occasional (< 1 %) schizonts labelling weakly with mAb 4C9 when examined by immunofluorescence (A′). Schizont-infected lymphocytes heat shocked at 42 °C for 7 h, followed by recovery for 18 h at 37 °C, imaged by DIC microscopy (B), showed approximately 5 % of schizonts with strong immunofluorescence when labelled with mAb 4C9 (B′). Schizonts in DIC images are indicated by an arrow.

with mAb 4C9 as shown by immunofluorescence (Fig. 5A). After incubation at 42 °C for 7 h, followed by recovery at 37 °C for 18 h, approximately 5 % of the parasites exhibited strong labelling with mAb 4C9 (Fig. 5B).

Analysis of sera from mMPSA-immunized cattle

Analysis of sera from the 14 calves immunized with recombinant mMPSA prior to *T. parva* sporozoite challenge showed ELISA antibody titres to HisTag-mMPSA of between 1:81000 and 1:243000 (Table 1). Although there was considerable variation between post-inoculation sera to neutralize sporozoites in an *in vitro* assay (between −13 and +10 change in percentage lymphocyte parasitosis compared to pre-inoculation sera) the mean change in percentage lymphocyte parasitosis for the post-inoculation sera was a very small increase (+0·3 %). Therefore, there was no evidence of sporozoite neutralization *in vitro* (Table 1). The positive control serum E99 against *T. parva* sporozoites showed complete neutralization of sporozoites.

T. parva *sporozoite stabilate challenge of cattle immunized with mMPSA*

Fourteen calves immunized with recombinant mMPSA were challenged with an LD_{70} dose of *T. parva* sporozoites. Seven naïve calves served as controls for the sporozoite stabilate. Of the 14 immunized calves, 3 (21 %) exhibited mild ECF reaction and 11 (79 %) exhibited moderate or severe ECF reactions (Table 2). Of the controls, 2 (29 %) showed mild or mild/moderate ECF reactions and 5 (71 %) showed moderate or severe ECF reactions

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Table 1. Antibody analysis of sera from cattle immunized with recombinant mMPSA

Group and calf number		Sporozoite neutralization assay: percentage parasitosis			ELISA titre†
		Pre-immunization sera	Post-immunization sera*		
HisTag-mMPSA immunized					
BN441		31	25	(−6)‡	243000
BP97		16	23	(+7)	243000
BP100		27·5	23	(−4·5)	243000
BP101		30·8	30	(−0·8)	243000
BP102		30	20·5	(−9·5)	243000
BP104		27·8	29	(+1·2)	243000
BP110		25	25·6	(+0·6)	243000
GST-mMPSA immunized					
BN442		24	25	(+1)	243000
BP99		18·7	23·5	(+4·8)	81000
BP103		16	27	(+11)	243000
BP105		29	35·5	(+6·5)	81000
BP107		24	34	(+10)	81000
BP109		29	16	(−13)	243000
BP111		31	27	(−4)	243000
	Total	359·8	364·1		
	Mean	25·7	26	(+0·3)	
Positive control§		0			
Negative control¶		28			

* Sera collected prior to sporozoite challenge.
† Reciprocal of the ELISA titre of antibody to HisTag-mMPSA.
‡ The difference in percentage parasitosis between pre- and post-immunization sera.
§ Positive control: E99 bovine serum against *T. parva* sporozoites.
¶ Negative control: L15 culture medium.

(Table 2). There appeared to be no overall reduction in the level or duration of piroplasms between the immunized and control cattle. These results demonstrate that inoculation of cattle with recombinant mMPSA, using the immunization regime described, offers no protection against ECF following *T. parva* sporozoite challenge and does not prevent the development of piroplasms.

DISCUSSION

Using mAb 4C9 it was shown that *T. parva* expresses a major merozoite/piroplasm antigen of 32 kDa (p32). Given its size and stage expression it was considered likely that this antigen was homologous to conserved 30–34 kDa major merozoite/piroplasm surface antigens (mMPSA) that have been sequenced and characterized from several *Theileria* species (Kawazu *et al*. 1992; Shiels *et al*. 1995; Knowles, Kappmeyer & Perryman, 1997; Katzer *et al*. 1998*b*). In this study we have described the cloning and expression of a full length cDNA encoding the mMPSA of *T. parva* (Muguga). The expressed product was shown to react specifically with mAb 4C9, confirming identity between the mMPSA and p32 antigens of *T. parva*. The amino acid sequence of the *T. parva* (Muguga) mMPSA reported here has identity to the partial-length sequence of the *T. parva* (Muguga) Tpms1 antigen described by Shiels *et al*. (1995). The *T. parva* mMPSA sequence is most closely related to that of *T. taurotragi*, but also has strong homology with mMPSA of *T. annulata* and *T. lestoquardi* (Katzer *et al*. 1998*b*).

Being a major component of the surface of an invasive stage, the merozoite, mMPSAs have been implicated in the events of parasite recognition /invasion of host erythrocytes. It has been suggested that the mMPSA binds to the host erythrocyte through KE and KEL charged amino acid motifs within the antigen (Kawazu *et al*. 1992; Knowles *et al*. 1997). In relation to this, it is worth noting that a region of 11 amino acids towards the N-terminus of the *T. parva* mMPSA (K^{24}–E^{34}) is composed entirely of the charged amino acids lysine and glutamine. The real significance of KE and KEL motifs within mMPSA remains to be elucidated given that one or both of these motifs also occur in other *Theileria* proteins including p150, p104, spm1, HSP70, cysteine protease and sporozoite surface antigens.

By immunoblotting with mAb 4C9 and RT–PCR we have shown that expression of the *T. parva* mMPSA protein and mRNA occurs within the sporozoite, schizont and piroplasm stages. Immunoblot analysis demonstrated that sporozoites and schizont-infected lymphocytes expressed the mMPSA at lower levels relative to the piroplasm stage. We were unable to detect mMPSA expression in the sporozoite and schizont-infected lymphocytes by immunoEM with mAb 4C9, indicating that

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Table 2. ECF reactions in recombinant mMPSA-immunized cattle following sporozoite challenge

Group and calf number	Days to pyrexia*	Days to schizonts†	Days to piroplasms‡	Maximum piroplasms§ (duration)¶	Severity of disease‖
HisTag-mMPSA immunized					
BN441	10	7	15	< 1/1000 (4)	SR
BP097	11	10	15	14/1000 (11)	SR
BP100	10	8	14	52/1000 (8*)	SR
BP101	10	6	15	28/1000 (7*)	SR
BP102	9	10	—	NPS	MR
BP104	9	6	14	40/1000 (8*)	SR
BP110	10	8	15	6/1000 (6)	MR
GST-mMPSA immunized					
BN442	12	10	15	250/1000 (7*)	SR
BP099	11	10	15	8/1000 (10)	SR
BP103	9	7	14	20/1000 (8*)	SR
BP105	9	10	—	NPS	MR
BP107	10	9	14	6/1000 (6)	MOD
BP109	10	8	15	2/1000 (10)	MOD
BP111	10	9	16	140/1000(6*)	SR
Controls					
BP003	8	10	16	< 1/1000 (8)	MOD
BP011	9	8	13	8/1000 (12)	MOD
BP018	9	8	13	180/1000 (9*)	SR
BP029	10	7	14	6/1000 (7)	MOD
BP113	7	7	13	108/1000 (9)	SR
BP120	4	7	15	4/1000 (10)	MR/MOD
BP122	4	7	15	< 1/1000 (1)	MR/MOD

* Number of days taken to develop pyrexia, and to the initial detection of schizonts in lymph node biopsies† and piroplasms in blood smears‡.
§ Maximum piroplasm parasitaemia.
¶ Duration of piroplasm parasitaemia in days: *duration of piroplasm parasitaemia prior to animal being euthanised *in extremis*.
‖ Severity of disease following challenge. NR, no reaction; MR, mild reaction; MOR, moderate reaction; SR, severe reaction. Reactions were classified according to Anon. (1989).
NPS, No piroplasms seen.

within these parasite stages the mMPSA is expressed at insufficient levels to be detected by this method. In a previous study, however, immunoprecipitation of a biotinylated 32 kDa antigen from surface-labelled sporozoites by mAb 4C9 demonstrated that the antigen is expressed on the surface of this stage (Y. Yagi & V. Nene, unpublished data). Using immunoEM we have clearly shown that the mMPSA is expressed on the parasite surface during an advanced budding stage of merogony and also in piroplasms, indicating that *T. parva* mMPSA up-regulation occurs at merogony.

We demonstrated that *T. parva* schizonts can be induced to upregulate mMPSA protein expression *in vitro* by incubating a schizont-infected lymphocyte culture at an elevated temperature of 42 °C followed by a recovery period at 37 °C. These results are further evidence that upregulation of the *T. parva* mMPSA occurs at merogony because heat induction has been shown previously to induce merogony in *T. parva* schizont-infected lymphocyte cultures (Hulliger, Brown & Wilde, 1966). Using a similar high temperature culture method it was shown that Tams1, the mMPSA of *T. annulata*, is also upregulated at merogony (Shiels *et al*. 1994). Like the *T. parva* mMPSA, Tams1 is also expressed at low levels in the sporozoite and schizont (Shiels *et al*. 1994). Tams1 has been localized to the surface of the differentiating schizont surface prior to merozoite budding (Shiels *et al*. 1998). We observed < 1 % *T. parva* schizonts in lymphocyte culture at 37 °C that expressed low levels of mMPSA, as demonstrated by weak immunofluorescent labelling with mAb 4C9. The lack of strongly immunofluorescent schizonts in culture at 37 °C, as opposed to those cultured at 42 °C, suggested that weakly labelled schizonts may represent the parasite at an early, uncommitted stage of the schizont–merozoite differentiation pathway. This is in agreement with Shiels *et al*. (1994) who have shown that a small, reversible increase in Tams1 expression by the *T. annulata* schizont can occur prior to a commitment to high level Tams1 expression and differentiation to the merozoite.

Members of the mMPSA family have proved useful in serodiagnosis of *T. mutans* and *T. equi* infections (Katende *et al*. 1990; Knowles *et al*. 1992). mMPSAs have also shown potential in stimulating protective immune responses in cattle against *T. annulata* and *T. sergenti* infections (d'Oliveira *et al*. 1997; Onuma, Kakuda & Sugimoto, 1998). In these

examples protection is assumed to be against the merozoite. Immunization against the merozoite would have no benefit for the host infected with *T. parva* since ECF is associated with schizont stage rather than the erythrocytic stages (Lawrence, de Vos & Irvin, 1994), although it could offer the possibility of a transmission-blocking component in a multivalent vaccine against ECF. Expression of the mMPSA on the surface of the *T. parva* sporozoite (Y. Yagi & V. Nene, unpublished data), the stage infective for the host, indicated that this antigen may have potential in protection through sporozoite neutralization.

We immunized cattle with recombinant mMPSA of *T. parva*. Although the mMPSA generated a strong humoral response, sera from these animals were unable to neutralize sporozoite infectivity *in vitro*. Furthermore, the immunized cattle were not protected against a *T. parva* sporozoite stabilate challenge and there was no reduction in the level of piroplasms, indicating a lack of sporozoite and merozoite neutralization *in vivo*. There are several possibilities as to why sporozoite/merozoite neutralization and protection against ECF were not induced. Bacterial recombinant mMPSA used in this study may lack neutralizing epitopes that in the native antigen are formed by post-translational modifications or secondary conformation. There is evidence that mMPSAs from other species of *Theileria* undergo post-translational modifications, including glycosylation (Katende *et al*. 1990; Knowles *et al*. 1991; Dickson & Shiels, 1993; Zhuang *et al*. 1993), although it is unknown whether or not these are involved in protective responses. It is also possible that the recombinant mMPSA used in this study directed the immune response to non-protective epitopes on the molecule. In support of this, studies with the mMPSA of *T. mutans* have shown that cattle immunized with either recombinant or native mMPSA appear to generate antibody responses to different regions of the molecule (R. A. Skilton, unpublished data). Because mMPSA of *T. sergenti* and *T. annulata* exhibit intraspecies antigenic polymorphisms between alleles (Zhuang *et al*. 1994; Katzer *et al*. 1998*a*) it is possible that the products of other *T. parva* mMPSA alleles may induce improved protection compared to the one tested here. In addition, *T. parva* mMPSA immunization regime used in this study may have been suboptimal. Alternative methods of immunization with *T. annulata* and *T. sergenti* mMPSA have produced some protection against these parasites in cattle. Immunization with recombinant mMPSA incorporated into ISCOMS or naked DNA encoding Tams1 induced limited protection against *T. annulata* piroplasm-infected blood challenge (d'Oliveira *et al*. 1997), while immunization with baculovirus-expressed recombinant mMPSA or synthetic mMPSA peptides of *T. sergenti* resulted in lower piroplasm parasitaemia and less severe clinical symptoms following sporozoite challenge (Onuma *et al*. 1998).

Although alternative methods of *T. parva* mMPSA immunization may stimulate merozoite neutralization and thereby reduce the level of piroplasms, it may still be difficult to generate a protective response against the sporozoite given the comparatively low level of mMPSA expression by this stage and the observed lack of sporozoite neutralization *in vitro* by high titre antibodies. Even though the mMPSA is implicated in the events of merozoite recognition/invasion of host erythrocytes, this antigen may not play a major role in mediating sporozoite infection of host lymphocytes so that effective neutralization of this molecule may have little effect upon sporozoite infectivity.

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