Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 514–518, January 1992
Immunology

A recombinant sporozoite surface antigen of *Theileria parva* induces protection in cattle

(East Coast fever/neutralizing antibodies/vaccine/fusion protein)

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Communicated by Don W. Fawcett, October 11, 1991

ABSTRACT **At present immunization against *Theileria parva* is by infection with live sporozoites and simultaneous treatment with a long-acting oxytetracycline. This method has major limitations in that live organisms are used and the immunity engendered is parasite stock specific. In an attempt to develop an alternative immunization procedure, the gene encoding p67, a major surface antigen of sporozoites, has been expressed by using the plasmid expression vector pMG1. The gene, which has been characterized previously, encodes 709 amino acid residues, contains a single intron of 29 base pairs, and is only transcribed during sporogony. The recombinant p67 sequences were fused to the first 85 amino acid residues derived from a nonstructural gene (NS1) of influenza virus A. Immunization with a partially purified recombinant antigen emulsified in 3% saponin induced sporozoite neutralizing antibodies in cattle and provided protection in six of nine animals on homologous challenge with *T. parva* sporozoites. This recombinant antigen is therefore a candidate for development of a vaccine against *T. parva*.**

Over 20 million cattle in eastern, central, and southern Africa are at risk of contracting theileriosis caused by the protozoan parasite *Theileria parva* (1). The disease is of major economic importance because of the high morbidity and mortality it causes and the expensive measures used to control the tick vector (1, 2). *T. parva* causes East Coast fever (ECF) and is transmitted by the three-host tick *Rhipicephalus appendiculatus*. The sporozoite develops in the salivary glands of the vector and is introduced into the mammalian host during tick feeding. Sporozoites rapidly enter lymphocytes by a receptor-mediated process (3, 4) and differentiate into schizonts. After several cycles of multiplication, a proportion of schizonts undergo merogony to produce merozoites, which invade erythrocytes and develop into piroplasms, the infective stage for ticks. The disease is characterized by pyrexia, generalized lymphadenopathy, and leukopenia (5).

At present the only practical method of immunization against ECF is by infection with live sporozoites and simultaneous treatment with a long-acting oxytetracycline (6). Since the immunity engendered is parasite stock specific, the method has serious limitations. In addition, the infrastructure in ECF risk areas is seldom capable of supporting the efficient delivery of cryopreserved sporozoites to the field.

The infection and treatment method produces a transient schizont parasitosis, which results in generation of schizont-specific and major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTLs) (7, 8). This response is believed to be the major protective mechanism in ECF immune cattle (7, 8), since immunity can be transferred adoptively between twins by using thoracic duct lymphocytes from the immune donor (7). Furthermore, CTLs specific for autologous cells bearing schizonts have been detected during recovery from ECF (9) and antibodies to schizonts do not confer protection (10). However, the possibility of antibodies to the sporozoite stage mediating protection is suggested by the observation that sera from cattle hyperimmunized with lysates of *T. parva* sporozoites or from animals in ECF endemic areas neutralize sporozoite infectivity *in vitro* (11). Monoclonal antibodies (mAbs) that recognize a 67-kDa stage-specific surface antigen (p67) of sporozoites also neutralize sporozoite infectivity (11–14).

Characterization of p67 with mAbs has indicated that the B-cell epitopes on the antigen are conserved among the sporozoites of different parasite stocks (11, 13). Hence, a vaccine based on this antigen may circumvent the stock specificity of the responses engendered by the infection and treatment method of immunization (6). In addition, an anti-sporozoite vaccine may only need to reduce the sporozoite challenge in order to confer protection, since the severity of disease is dose dependent (15).

Here we report a major advance in immunization against ECF based on a recombinant form of the p67 sporozoite surface antigen, which has potential as an alternative ECF vaccine.

MATERIALS AND METHODS

Cattle. Boran cattle (*Bos indicus*) 6–9 months old were used. They were born on an ECF-free farm and at the age of 1–2 weeks were brought to the International Laboratory for Research on Animal Diseases, where they were maintained under strict acaricidal control. The animals were screened for antibodies to *T. parva* before experimentation by the indirect immunofluorescent antibody test (IFAT) (16), immunoblotting (17), ELISA (18), and neutralization of sporozoite infectivity (11) and were found to be negative. All cattle were of different MHC phenotype.

Parasites. The *T. parva* Muguga stock (19) used in these studies was originally obtained from the East African Veterinary Research Organisation, Muguga; it was maintained by tick/cattle passage until cryopreserved as sporozoite stabilate 10 (20). This stabilate was passaged twice through tick/cattle before the bulk stabilate 3087 was prepared from infected adult *R. appendiculatus* ticks.

Purification of *T. parva* Sporozoites. Sporozoites for neutralization assays were harvested in RPMI 1640 medium (Flow Laboratories) by homogenizing dissected salivary glands from 4-day-fed infected adult ticks. The homogenate was centrifuged at 150 × *g* for 5 min to remove salivary gland debris, and the supernatant was used without further purifi-

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Abbreviations: ECF, East Coast fever; NS1, nonstructural protein 1 of influenza virus A; CTL, cytotoxic T lymphocyte; IFAT, indirect immunofluorescent antibody test; MHC, major histocompatibility complex; mAb, monoclonal antibody.

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cation. Sporozoites for use as antigen in ELISA and immunoblotting were purified further by passing the above supernatant over a DEAE-cellulose column (Pharmacia) following a described method (21). The sporozoites were lysed in 50 mM Tris·HCl (pH 8.0) containing 1.0% Nonidet P-40 and protease inhibitors (5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N^{α}-(*p*-tosyl)lysine chloromethyl ketone, and 1 mM leupeptin; Sigma). The p67 antigen contained in this preparation is referred to as native p67.

Expression of *T. parva* (Muguga) p67 by Using pMG1. A cloned 2.3-kilobase *Bam*HI DNA fragment, containing the gene encoding *T. parva* (Muguga) p67, was extracted from agarose gels with a Geneclean kit (Bio 101, La Jolla, CA) and repaired with the Klenow fragment of DNA polymerase I to produce blunt ends. This DNA was ligated to *Hpa* I-digested, dephosphorylated pMG1 (M. Gross, personal communication; refs. 14 and 22) and *Escherichia coli* strain MM 294 (cI^+) was transformed with the ligation mixture. Recombinants containing insert DNA in the correct orientation were identified and the DNA sequences at the junctions of pMG1 and the insert were determined to ensure that the constructs were as predicted. Recombinant plasmids were then transferred into the expression host AR 58 (*cI857*). All DNA manipulations were carried out by standard methods as described (23). The recombinant p67 is produced as a fusion protein with the first 85 amino acid residues derived from a nonstructural gene (NS1) of influenza virus A.

Antigen Preparation. The antigen for immunization was prepared from bacteria derived from a 20-liter fermentation. One gram (wet weight) of cells was disrupted in 10 ml of glycine buffer (50 mM glycine/2 mM EDTA, pH 8.0). After centrifugation at 13,000 × *g* for 30 min to clear the lysate, the pellet, which contains the majority of the fusion protein, was washed six times with glycine buffer, and the fusion protein was extracted from the pellet with 10 ml of glycine buffer containing 0.1% Nonidet P-40 (Sigma) at 4°C for 1 hr. The supernatant, recovered after centrifugation, was designated as recombinant p67 and was stored at −80°C until used for immunization. A control antigen was prepared in an identical manner from *E. coli* expressing NS1 alone (24).

Competitive Radioimmunoassays. The quantity of NS1–p67 fusion protein in the recombinant p67 preparation was determined by competitive radioimmunoassay (25), using NS1–p67 extracted from SDS/polyacrylamide gels by electroelution and a mAb raised against recombinant p67, which binds to both NS1–p67 and native p67 (see Fig. 2*E*). The protein concentration of the electroeluted NS1–p67 was determined by the BCA protein assay reagent kit (Pierce) and it was labeled with ^{125}I (Amersham) by the chloramine-T method (26).

Immunization of Cattle. The immunization regimen consisted of five inoculations in 2-ml vol containing 1 mg of recombinant p67 emulsified in 3% saponin (Merck) and administered subcutaneously at monthly intervals. Two immunization experiments were performed. In the first experiment, two immunized and two nonimmunized control animals were challenged 10 days after the final inoculation of recombinant p67. In the second experiment, seven animals were immunized with recombinant p67 and four were immunized with 1 mg of an *E. coli* preparation containing NS1 alone. Both immunized groups were challenged 10 days after the final inoculation along with four nonimmunized control animals. In both experiments, the nonimmunized controls served to test the infectivity of the sporozoite stabilate.

ELISA and Sporozoite Neutralization Test. Serum antibody titers to NS1–p67 and native p67 were estimated (18) by a sandwich ELISA using a mAb raised against recombinant p67 to trap NS1–p67 or native p67, while antibodies to NS1 alone were detected by direct ELISA to purified NS1 (24).

The neutralization of sporozoite infectivity assays were performed following a slight modification of a described method (11). A sporozoite suspension containing 5×10^4 sporozoites in RPMI 1640 medium with 20% fetal bovine serum and various dilutions of pre- or postimmunization sera were added to each well of a 24-well microtiter plate. After a 30-min incubation at 37°C in a mixture of 5% CO_2/95% air, 1×10^6 bovine peripheral blood mononuclear cells isolated from uninfected bovine blood were added to each well and the plates were maintained under the same conditions. Giemsa-stained cytospin smears prepared from each well were examined for the presence of schizonts. Four hundred cells were counted and the percentage bearing schizonts was determined. The dilution of serum at which >90% inhibition was achieved at day 14 was considered the neutralization titer of the serum.

Immunoblotting of Parasite Antigens. The specificity of sera from immunized cattle to NS1–p67 and native p67 was assessed by immunoblotting. The antigens were separated by SDS/PAGE using a gradient gel of 7.5–17.5% (27) and transferred onto nitrocellulose membranes as described (17).

The nitrocellulose filters were blocked with 10% skimmed milk and probed with representative sera from animals immunized with recombinant p67 or NS1 preparations. The bound antibodies were detected by autoradiography using ^{125}I-labeled protein G (Amersham).

Challenge and Sampling of Cattle. Cattle were challenged with 1 ml of a 1:100 dilution of *T. parva* stabilate 3087. This dilution had previously been shown to produce an LD_{68} in cattle of the same breed and background as that used in the present study (T. T. Dolan and S.M., unpublished data). The stabilate was diluted in Eagle's minimal essential medium (GIBCO) containing 3.5% (wt/vol) bovine plasma albumin and 7.5% (vol/vol) glycerol and was inoculated subcutaneously over a parotid lymph node (28).

All the experimental cattle were monitored daily for changes in rectal temperature and other clinical manifestations of ECF (5). Total leukocyte counts were determined three times weekly starting 1 week before sporozoite inoculation. Needle biopsy smears from the parotid lymph node adjacent to the site of sporozoite inoculation were taken 7 days after inoculation, stained with Giemsa's stain, and examined for schizonts. Smears from the contralateral prescapular lymph nodes and blood smears were taken from cattle showing schizonts. ECF reactions were classified as mild, moderate, or severe as described (29). Animals were euthanized if they exhibited severe ECF reactions and had a total leukocyte count of <2000 per μl.

Detection of Parasite DNA by PCR. Needle biopsies from the parotid lymph node adjacent to the site of sporozoite inoculation were collected from nonreacting and control animals in 1 ml of Dulbecco's phosphate-buffered saline 8–10 days after the sporozoite challenge. The biopsies were stored at −20°C until required. For the PCR, 10-μl samples were processed for the preparation of DNA as described (30) and DNA amplification of a 390-base-pair sequence was carried out with primers IL194 and IL197 derived from a *T. parva*-specific repetitive DNA sequence (31).

RESULTS

Expression of Recombinant p67. The sporozoite antigen p67 has been expressed (14) in *E. coli* strain JM 109 as C-terminal fusion proteins with the *Schistosoma japonicum* antigen Sj-26. However, these fusion proteins were found to be highly unstable (14). For expression of p67, the plasmid pMG1, a derivative of the pAS expression vector system (22), was found to be more suitable than the pGEX system. This plasmid directs synthesis of C-terminal fusion proteins with the NS1 protein of influenza virus A. Transcription of the

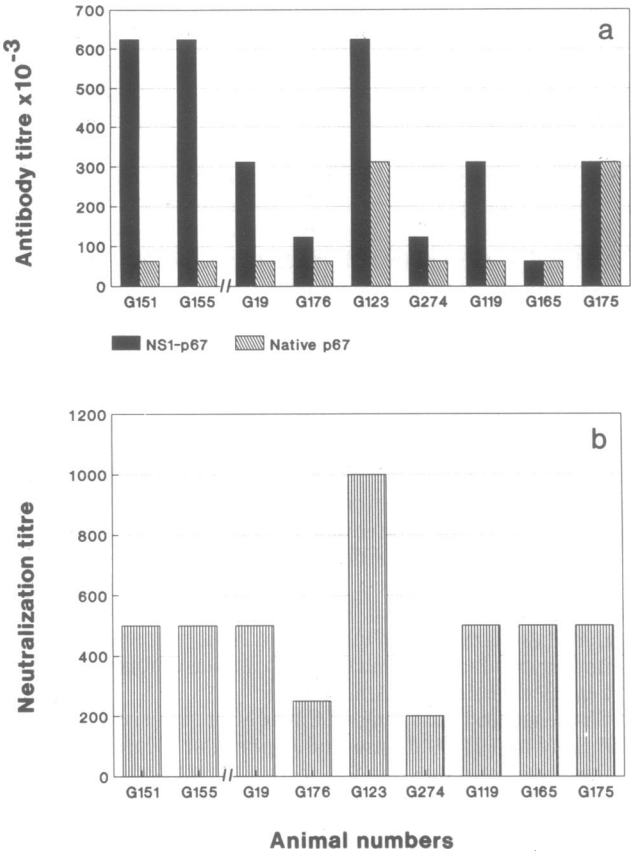

FIG. 1. Antibody responses in sera from cattle immunized with recombinant p67 taken before challenge. (*a*) Reciprocal titers of antibodies against NS1–p67 and native p67 as determined by sandwich ELISA. (*b*) Reciprocal neutralization titers of sporozoite infectivity. The cattle are arranged in order of severity of the disease response (see Table 1).

hybrid gene is regulated by a temperature-sensitive mutant of the *c*I repressor gene of bacteriophage λ and is driven by the P_L promoter of λ. NS1–p67 is produced as an insoluble fusion protein and it contains the first 85 amino acid residues of NS1, two residues encoded by a DNA linker sequence, and all 709 residues of p67. The fusion protein was partially purified as described and the preparation was shown by competitive radioimmunoassay to contain 60% NS1–p67.

Serological Analysis. Analyses of sera from all nine NS1–p67 immunized animals before sporozoite challenge showed sandwich ELISA antibody titers of >1:62,000 to purified NS1–p67 and native p67 (Fig. 1*a*). The ELISA antibody titers to NS1 alone ranged between 1:500 and 1:2500 in all immunized animals (data not shown), while antibody titers were >1:200 in *in vitro* neutralization of the sporozoite infectivity assay (Fig. 1*b*). Sera from animals immunized with NS1 alone failed to neutralize sporozoite infectivity (data not shown). By immunoblotting analysis, the sera from animals immunized with recombinant p67 recognized native p67, the NS1–p67 antigen, and several *E. coli* antigens (Fig. 2*A*). The sera from the four animals inoculated with NS1 alone did not recognize native p67 (Fig. 2*C*; animal BH105). Antischizont antibodies were not detected in the sera of any of the experimental cattle by IFAT before sporozoite challenge.

ECF Reactions of Immunized Cattle on Challenge with *T. parva* Sporozoites. Ten days after the last immunizing dose, the experimental animals were challenged with 1 ml of a 1:100 dilution (LD_{68}) of *T. parva* (Muguga) sporozoite stabilate 3087.

In the first experiment, both animals immunized with recombinant p67 were immune to challenge, showing no parasites or signs of disease, while the control animals underwent severe reactions (Table 1). In the second experiment, of the seven animals immunized with recombinant p67, two were nonreactors, two exhibited a mild but transient clinical disease, and three underwent a severe reaction (Table 1). Hence, six of the nine cattle were immune to ECF. The mean duration of pyrexia and schizont parasitosis in the mild reactors was 5.5 and 6.0 days, respectively (Table 1), and the mean minimum total leukocyte count during the ECF reaction was 4.9×10^3 per μl. The mean prechallenge leukocyte count of the experimental animals was 9.7×10^3 per μl.

In contrast, all 10 control animals—4 immunized with NS1 alone and 6 nonimmunized animals—underwent severe ECF; 7 were euthanized *in extremis* and 3 recovered after undergoing a prolonged clinical disease. The animals that recovered had a mean duration of pyrexia and schizont parasitosis of 10.7 and 14.7 days, respectively, and a mean minimum leukocyte count of 2.2×10^3 per μl.

Sixty days after challenge, the four animals that showed no reaction on challenge did not have antibodies to *T. parva* schizonts by IFAT, while both mild reactors and the three severe reactors that recovered developed antibodies to schizonts. PCR was performed on lymph node biopsy samples from two nonreacting animals (G19 and G176) and one control (G272) to screen for subclinical infection not detected by microscopy or serology. Only the control animal, which had a schizont parasitosis of 1 parasite per 1000 lymphocytes, contained a detectable PCR product (Fig. 3).

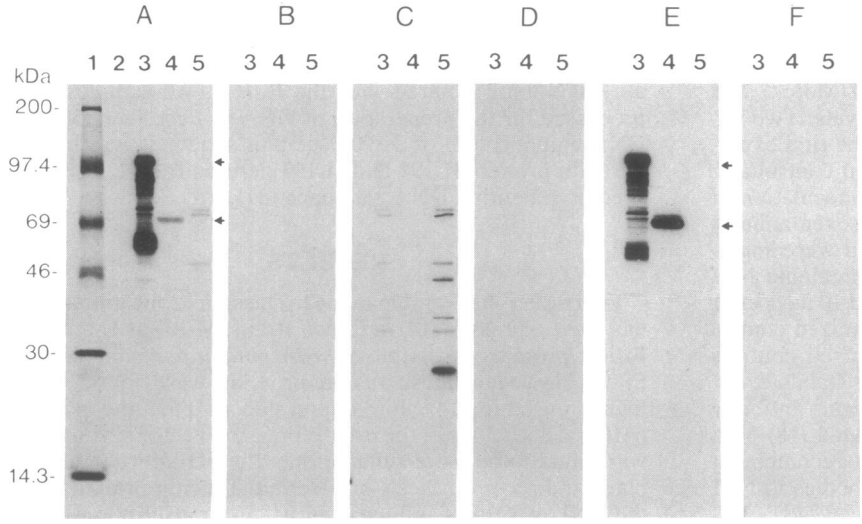

FIG. 2. Immunoblotting analysis of representative sera from cattle inoculated with recombinant p67 or NS1 antigen preparations. Lanes: 1, molecular size markers; 2, blank; 3–5, recombinant p67, sporozoite lysate, and NS1 antigen preparations, respectively. (*A*) Probed with serum from G123 immunized with recombinant p67. (*C*) Probed with serum from BH105 inoculated with the NS1 preparation. (*B* and *D*) Probed with preinoculation serum from G123 and BH105, respectively. (*E*) Probed with a mAb raised against the recombinant p67. (*F*) Probed with a nonrelated mAb raised against a *Babesia bigemina* merozoite antigen (J. M. Katende, personal communication). The mobility of the NS1–p67 protein in SDS/PAGE is much slower than expected from the calculated molecular mass of the fusion protein [110 kDa (upper arrow) as opposed to 85 kDa]. Lower arrow marks the position of native p67 in sporozoite lysates.

DISCUSSION

The results described clearly show that immunization with the p67 recombinant surface antigen of *T. parva* sporozoites induced protection in six of nine cattle. The immunized animals had high ELISA antibody titers to purified NS1–p67 and native p67 (Fig. 1) but very low titers to NS1 alone. The higher antibody titers to NS1–p67 as compared to native p67 may be attributed, at least in part, to differences in conformation of the two molecules. Since the *E. coli* NS1-inoculated cattle were susceptible to ECF (Table 1), the protection observed in the six animals must be assumed to be induced by p67.

The isolation of the single copy gene encoding p67 has been described (14). The gene encodes 709 amino acid residues, contains a single intron 29 base pairs long, and is only transcribed during sporogony. Rat antisera raised against two nonoverlapping regions of p67 expressed in *E. coli* by using the pGEX expression vectors neutralized sporozoite infectivity (14). However, the instability of these soluble fusion proteins resulted in poor yields of affinity-purified protein. The use of pMG1 to express p67 has resulted in high yields of more stable but insoluble recombinant fusion protein. The stability of NS1–p67 can be ascribed to the NS1 sequences since expression of p67 in AR 58 using a pMG1 construct lacking the NS1 sequences results in rapid degradation of p67 to fragments of ≈20 kDa (data not shown). A second important advantage of the pMG1 system for vaccine development is that the NS1 sequence contains potent T-helper cell epitopes that stimulate the production of high antibody titers to the fusion protein (32).

Table 1. Summary of ECF reactions and immune status after sporozoite challenge

Animal	Days to schizont	Duration of schizont	Days to pyrexia	Duration of pyrexia	Severity of disease
			Immunized		
Exp. 1					
G151	—	—	—	—	NR
G155	—	—	—	—	NR
Exp. 2					
G19	—	—	—	—	NR
G176	—	—	—	—	NR
G123	10	6	10	5	MR
G274	9	6	9	6	MR
G119	11	10	11	10	SR
G165	8	15	8	14	SR
G175	8	14	11	10	SR
			Controls		
Exp. 1					
G171	10	10	10	11	SR
G174	9	14	11	10	SR
Exp. 2					
G20*	8	14	9	12	SR
G170*	9	15	9	9	SR
G272*	8	7	9	6	SR
BH105*	7	15	7	15	SR
G365	9	12	10	10	SR
G366	9	8	11	6	SR
G367	8	14	8	14	SR
G368	7	15	9	11	SR

Immunized and control animals were challenged with 1 ml of a 1:100 dilution (LD_{68}) of *T. parva* (Muguga) sporozoite stabilate 3087. Classification of ECF reactions was based on clinical and parasitological examination (29). The nonreactors (NR) and mild reactors (MR) were classified as immune animals and the severe reactors (SR) were classified as susceptible.

*Cattle immunized with the NS1 antigen alone. They are placed together with the nonimmunized controls since both groups were equally susceptible to ECF.

Three disease categories were noted on challenge of recombinant p67 immunized cattle with an LD_{68} of *T. parva* sporozoites. If all the immunized cattle had a similar neutralizing antibody effect on sporozoites, then variation in disease reactions could be explained by differences in their infectivity thresholds (33). However, the antibody titers in the immunized cattle were not similar nor did they correlate with disease reactions, suggesting that the quality of antibody may also be important. The severity of disease reaction is dose dependent (15, 34), and this will be influenced by the number of sporozoites exceeding the infectivity threshold and the capacity of the animal to acquire immunity. In animals in which infection did not establish, it may be assumed that the neutralizing effect reduced the sporozoite dose to below the infectivity threshold or eliminated it. In cattle that reacted severely, the sporozoites surviving the neutralizing effects exceeded the infectivity threshold and were in sufficient numbers to cause severe disease. In cattle with mild disease reactions, the sporozoite dose exceeded the infectivity threshold but was reduced to within a range in which the development of the parasite was controlled by other immune mechanisms, probably a cell-mediated immune response (see below).

The mechanism of the protective immunity induced by the NS1–p67 is unclear. However, antibody responses appear to have played a role in limiting the establishment of the sporozoites as evidenced by the lack of schizont parasitosis and schizont-specific antibodies in the four nonreacting animals. Also two of the nonreactors showed no parasite DNA on PCR amplification, again indicating a failure of parasite establishment in these animals. There are several ways in which NS1–p67-specific antibody may contribute to sporozoite neutralization and clearance *in vivo*. First, it may enhance phagocytosis of sporozoites by opsonization; it is known that bovine IgG1 and IgG2, both of which were generated during immunization (data not shown), induce phagocytosis by homologous neutrophils and macrophages (35). Second, the antibody may operate through antibody-dependent cell-mediated cytotoxicity (ADCC). There is ev-

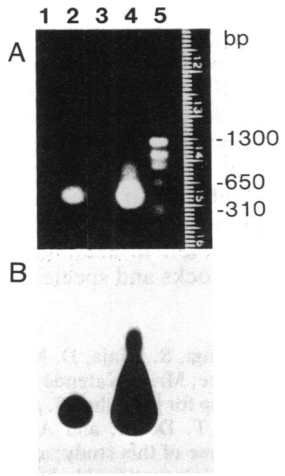

FIG. 3. Detection of *T. parva* DNA in lymph node biopsies by PCR. (*A*) Ethidium bromide-stained gel of samples of amplification reaction mixtures electrophoresed through a 0.8% agarose gel (31). (*B*) Southern blot of the gel probed with a nick-translated *T. parva* (Muguga)-specific probe. Samples of the amplification reaction mixtures obtained from DNA isolated from G19, G272, G176, and *T. parva* piroplasms were loaded in lanes 1–4, respectively. The 390-base-pair (bp) PCR product seen in the positive control (lane 4) was only detected in animal G272 (lane 2), which had a schizont parasitemia of 0.1%.

idence that ADCC plays a role in the clearance of *Trypanosoma theileri* infection in cattle (36). Finally, although complement is not essential for the *in vitro* neutralization of sporozoite infectivity (11), it may contribute to the elimination of the sporozoites *in vivo*.

In Plasmodia, which are closely related to *Theileria* (37), vaccine strategies based on generation of high titers of antibody to the circumsporozoite protein have yielded equivocal results (38). Indeed, observations in humans immunized with fragments of the circumsporozoite antigen of *P. falciparum* fused to NS1 were similar to those described here in revealing no obvious correlation between antibody levels and protection (32). It is possible therefore that cell-mediated responses induced by NS1–p67 could also have contributed to protection. The manner in which the NS1–p67 was delivered to the animals in the present study is likely to have biased the immune system toward the generation of $CD4^+$ rather than $CD8^+$ T lymphocytes (39). In view of the high titers of antibodies against NS1–p67, it is likely that potent T-helper cell responses were elaborated. In addition to producing lymphokines and providing help for antibody synthesis, $CD4^+$ T lymphocytes are known to mediate class II MHC-restricted cytotoxicity (40) and indeed such cells have been shown to confer protection against murine malaria (41). Since p67 remains associated with the lymphocyte surface after sporozoite entry (12), it is possible that p67 is taken up by the newly infected cell and expressed in association with class II MHC molecules. Such cells would be susceptible to killing by $CD4^+$ T lymphocytes. Further clarification of these issues, together with the effect of lymphokines on the establishment of sporozoites, will await results of ongoing studies of T-cell responses in NS1–p67-immunized animals.

Cattle that recover from *T. parva* primary infection either spontaneously or after treatment develop a potent class I MHC-restricted CTL response directed against the schizont-infected cell (7, 8). The two immunized animals that underwent mild ECF reactions in this study generated a CTL response against schizont-infected cells (E. Innes, G. Lamb, and A.M., unpublished data) and were immune on subsequent challenge with a lethal dose (LD_{100}) of *T. parva* (Muguga) sporozoites. This feature is desirable in a vaccine strategy directed at *T. parva* since it allows vaccinated animals to develop immunity against both the sporozoite and schizont stages of the parasite.

The immunization protocol used here failed to induce protection against ECF in all cattle. However, by modifying antigen preparation, dose, frequency of application, and adjuvant systems, a more efficient protocol could be developed. A more critical question is whether the window of protection can be widened to anticipate the most likely primary challenge in the field since infection rates in ticks are known to vary greatly (42). In addition, protection against heterologous parasite stocks and species remains to be evaluated.

We thank Messrs. J. Ngugi, S. Minja, D. Mwaengo, and D. Lugo for their technical assistance; Mr. J. Katende for serological analysis of the sera; Dr. R. P. Bishop for providing *T. parva* primers for PCR; Drs. D. J. McKeever, T. T. Dolan, and A. S. Young for useful discussions during the course of this study; and Ms. M. Mbaabu for typing the manuscript. The International Laboratory for Research on Animal Diseases (ILRAD) Tick Unit provided the *T. parva* (Mugaga) sporozoites. The help of SmithKline Beecham is gratefully acknowledged, in particular Drs. A. R. Shatzman and M. Rosenberg for providing the pAS vector system and Drs. T. J. Miller and M. Gross for discussions and advice. This is ILRAD publication no. 988.

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