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Delivery of the p67 Sporozoite Antigen of *Theileria parva* by Using Recombinant *Salmonella dublin*: Secretion of the Product Enhances Specific Antibody Responses in Cattle†

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The p67 sporozoite antigen of *Theileria parva* has been fused to the C-terminal secretion signal of *Escherichia coli* hemolysin and expressed in secreted form by attenuated *Salmonella dublin aroA* strain SL5631. The recombinant p67 antigen was detected in the supernatant of transformed bacterial cultures. Immunization trials in cattle revealed that SL5631 secreting the antigen provoked a 10-fold-higher antibody response to p67 than recombinant SL5631 expressing but not secreting p67. Immunized calves were challenged with a 80% lethal dose of *T. parva* sporozoites and monitored for the development of infection. Two of three calves immunized intramuscularly with the p67-secreting SL5631 strain were found to be protected, whereas only one of three animals immunized with the nonsecreting p67-expressing SL5631 strain was protected. This is the first demonstration that complete eukaryotic antigens fused to the C-terminal portion of *E. coli* hemolysin can be exported from attenuated *Salmonella* strains and that such exported antigens can protect cattle against subsequent parasite challenge.

The tick-borne protozoan parasite *Theileria parva* causes an economically important disease of cattle in eastern, central, and southern Africa known as East Coast fever (ECF). Infection with *T. parva* is initiated by inoculation of sporozoites into the mammalian hosts during tick feeding. Sporozoites enter lymphocytes through a receptor-mediated process and rapidly differentiate into schizonts, an event that is associated with transformation of the infected cell to a state of uncontrolled proliferation. Subsequent invasion of nonlymphoid tissues by parasitized cells and the associated immunopathological effects usually result in death of the animal within 3 weeks of infection (19). Animals that recover from the disease are solidly immune to homologous challenge and exhibit both neutralizing antibodies against the sporozoite stage and cell-mediated responses to the schizont-infected cell (5, 9, 20). Although it is believed that the latter mechanisms are largely responsible for protection in recovered animals, it has been shown that immunization of naive cattle with a recombinant form of p67, the major surface antigen of *T. parva* sporozoites, can give rise to protective immunity (21). We have recently evaluated attenuated *Salmonella* strains as live delivery vehicles for p67 in cattle (12). Strong specific antibody responses were observed in all immunized animals, and the level of protection was similar to that observed with recombinant antigen. However, three booster inoculations were required to achieve protection.

We reasoned that intracellular expression of vaccine antigens in sufficient amounts for immunization may compromise the viability of attenuated bacteria such that they are incapable of optimal stimulation of desired immune responses (1). Transport of the antigen from the cytoplasm into the extracellular medium could circumvent this problem.

A very powerful method for expression and secretion of protein antigens in *Salmonella* is the use of the *Escherichia coli* hemolysin transport system, which has been recently shown to function equally well in *Salmonella* and in *E. coli* (6, 7, 10, 30, 32). Hemolysin (HlyA) is produced by certain *E. coli* strains (mainly uropathogenic isolates) and is transported across both membranes of the gram-negative *E. coli* cell. Hemolysin synthesis and secretion is genetically determined by an operon consisting of four genes in the order *hlyC hlyA hlyB hlyD*. The export mechanism is based on a secretion signal within the C-terminal 50 to 60 amino acids of HlyA and a translocation complex composed of the two inner membrane proteins, HlyB and HlyD, and the outer membrane protein TolC (33, 34). This specific translocation apparatus, the prototype of the type I secretion system (26), allows the secretion of hemolysin or hemolysin fusion proteins directly to the medium without a periplasmic intermediate (8, 18).

In this report, we describe the expression of recombinant p67 antigen as a fusion with the C terminus of HlyA and its resulting export to the extracellular medium. The fusion protein has been expressed in the *Salmonella dublin aroA* vaccine delivery strain SL5631. Immunization of cattle with the recombinant SL5631 strain provoked specific antibody responses to p67, and a proportion of the immunized animals were protected against challenge with *T. parva* sporozoites.

MATERIALS AND METHODS

Experimental animals. A group of 12 unrelated Boran (*Bos indicus*) calves, 1 month of age, was selected for the study and housed in tick-proof isolation facilities. All were seronegative for *T. parva* sporozoite and schizont antigens prior to experimentation as determined by enzyme-linked immunosorbent assay (ELISA).

Parasites. The Muguga stock of *T. parva* was used for the study and was originally obtained from the East Africa Veterinary Research Organization, Muguga, Kenya. The bulk stabilate 4133 was prepared as described previously (21) from adult *Rhipicephalus appendiculatus* ticks infected with the stock and stored in liquid nitrogen.

Bacteria and plasmids. *S. dublin aroA* mutant SL5631 was kindly provided by B. A. D. Stocker (Stanford University School of Medicine, Stanford, Calif.). Low-copy-number plasmid pJFF224XN (RSF1010 ori) was the gift of J. Frey (Institute of Bacteriology, University of Berne, Berne, Switzerland). The p67

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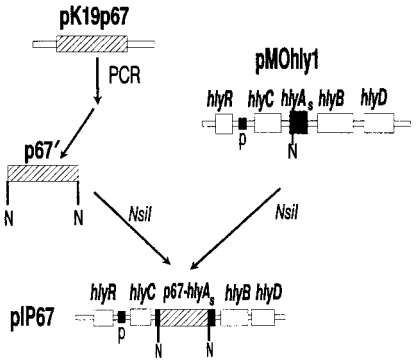

FIG. 1. Construction of recombinant plasmid pIP67. The p67 gene of *T. parva* was amplified by PCR using the pUC19-based plasmid pK19p67 as a template. Both primers (see Materials and Methods) contained *Nsi*I sites. The PCR product was then ligated into the *Nsi*I site of pMOhly1. The resulting plasmid was designated pIP67.

gene (encoding amino acids 20 to 709) was inserted together with the *tac* promoter in the *Hin*dIII/*Xho*I sites of pJFF224XN, resulting in plasmid pJF67. Medium-copy-number plasmid pGEX67 (pBR322 ori) was provided by V. Nene (International Livestock Research Institute, Nairobi, Kenya) and gives rise to the expression of p67 (amino acid 1 to 709) as a fusion protein with glutathione *S*-transferase of *Schistosoma japonicum* (24). High-copy-number plasmid pMc622 (ColE1 ori) resulted from an insertion of a part of the p67 gene (encoding amino acids 20 to 622) in the vector pMc5-19 and was a gift of J. Steyaert (International Livestock Research Institute). This construct incorporates a phosphatase signal sequence that causes the recombinant antigen to be transported to the periplasmic space of transformed bacteria (28).

Plasmid pMOhly1 incorporates a copy of the *E. coli* operon in which all sequences of the *hlyA* gene except those encoding the N-terminal 34 and the C-terminal 61 residues have been deleted; an intervening *Nsi*I insertional cloning site allows replacement of deleted sequences with foreign genes (Fig. 1). Sequences encoding residues 21 to 699 of the p67 gene were cloned into pMOhly1 in frame with *hlyA* sequences as described previously (6, 7). Briefly, a 2,034-bp fragment was amplified from pK19p67 (24) by using two p67-specific primers containing *Nsi*I sites (p67-5′ [5′ GCAGGGGATGCATTGCCTACGGAG 3′] and p67-3′ [5′ GATGCTGATAATTATGTATGCATTTGGTATCAT 3′]; *Nsi*I sites are underlined) and blunt ended before cloning into the *Sma*I site of pUC19. The resulting plasmid was digested with *Nsi*I to release the p67 insert. This fragment was subcloned in *Nsi*I-digested pMOhly1 (Fig. 1), and positive clones were sequenced to confirm appropriate orientation and frame of the insert with respect to the *hlyA* gene flanking sequences. The resulting plasmid was designated pIP67.

In vitro stability studies. *S. dublin* SL5631 containing plasmids pIP67 and pMC622 was grown overnight at 37°C with appropriate antibiotic selection. Bacteria were then subcultured at a dilution of $1:10^6$ on successive days and allowed to reach stationary phase in the absence of antibiotics. On each day, the approximate number of generations was calculated and the percentage of cells carrying plasmids was determined by plating dilutions of the culture in the presence or absence of antibiotics.

Immunoblotting of parasite antigens. Expression of p67 in attenuated *Salmonella* was characterized by immunoblotting. Cell lysates and supernatants of recombinant SL5631 cultures were prepared as described previously (6, 12) and separated in a sodium dodecyl sulfate–10% polyacrylamide gel before being transferred to nitrocellulose. Filters were blocked with 5% skimmed milk and probed with the p67-specific monoclonal antibody 22.7 (22). Bound antibodies were detected by autoradiography using a horseradish peroxidase-labeled anti-mouse antibody and the Amersham enhanced chemiluminescence detection system.

Immunization of cattle. The 12 calves were divided in four groups of three. Group 1 was immunized intramuscularly (i.m.) with SL5631 secreting p67, while group 2 received the same strain orally. Group 3 was inoculated i.m. with an equal mixture of three recombinant SL5631 strains transformed with pMC622, pGEX67, and pJF67. The rationale of this approach was to combine the advantages of high- and medium-copy-number plasmids (enhanced expression of recombinant p67) with that of low-copy-number plasmids (higher stability in *Salmonella*). It has been shown previously that animals immunized with a mixture of p67-expressing recombinants seroconverted to p67. Group 4 served as a naive control to confirm the infectivity of the sporozoite challenge. The immunization regimen consisted of three inoculations of recombinant SL5631 on days 0, 7, and 28. Group 2 received 1×10^{10} to 2×10^{10} CFU of recombinant SL5631 on day 0, 4×10^{10} CFU on day 7, and 10^{11} CFU on day 28 in milk replacer as described previously (27). Groups 1 and 3 were inoculated with 1×10^9 to 2×10^9 CFU of recombinant SL5631 in a 2-ml volume of phosphate-buffered saline on day 0, 4×10^9 CFU on day 7, and 8×10^9 CFU on day 28. Numbers of bacteria for i.m. immunization have been determined in previous experiments (data not shown).

ELISA. Serum antibody titers (immunoglobulin G1 [IgG1]) to recombinant p67 were determined by using a direct ELISA as described previously (15). IgA titers to p67 were quantitated by using a specific mouse monoclonal antibody subsequently detected by an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin and the Sigma Fast pNPP (*p*-nitrophenyl phosphate) substrate tablet kit.

Challenge and sampling of cattle. Cattle were challenged by subcutaneous inoculation of 1 ml of stabilate 4133, diluted 1:80 in Eagle's minimal essential medium (GIBCO) containing 3.5% (wt/vol) bovine plasma albumin and 7.5% (vol/vol) glycerol, into the drainage area of the right parotid lymph node. All animals were monitored daily for changes in rectal temperature and other clinical manifestations of ECF. Giemsa-stained smears were prepared daily from needle aspirates of the draining node from day 5 onward and examined for the presence of schizonts. ECF reactions were classified as mild, moderate, or severe as described previously (25). Severe reactors with total leukocyte counts below 2,000 per ml were euthanized.

RESULTS

Expression of a p67-HlyA fusion protein by recombinant *S. dublin* SL5631. *S. dublin* SL5631 was transformed with pIP67 and characterized for the expression of recombinant protein. Expression was compared with that of previously constructed recombinant SL5631 expressing p67 (Fig. 2). SL5631 bacteria transformed with the high-copy-number plasmid pMC622 expressed large amounts of recombinant p67, but the antigen was present only in cell lysates and was not detectable in culture supernatant (lanes 1 and 2). Recombinants transformed with the medium- and low-copy-number plasmids pGEX67 and pJF67 expressed less protein, none of which was present in the culture medium (lanes 3 to 6). In contrast, the HlyA-p67 fusion protein was entirely transported into the medium (lane 8), with no antigen being detected in the cell lysate (lane 7). The size of the dominant species observed in the culture supernatant corresponded with the predicted molecular mass of the HlyA-p67 fusion protein, 78 kDa. The amount of fusion protein secreted into the medium was approximately 200 times less than that found in cell lysates of SL5631 transformed with the high-copy-number plasmid pMC622.

In vitro stability studies. Plasmid pIP67 was evaluated for its stability in SL5631 in the absence of antibiotic selection. Approximately 30% of the bacteria had lost the plasmid after 18 generations, and the proportion of resistant bacteria decreased further after 36, 54, and 72 generations. However, 24% of the population remained resistant after 72 generations without selection. In contrast, high-copy-number plasmid pMC622 could be found in only 4% of the bacteria after 18 generations in culture without antibiotic selection. Medium-copy-number plasmid pGEX67 (68% positive colonies after 18 generations)

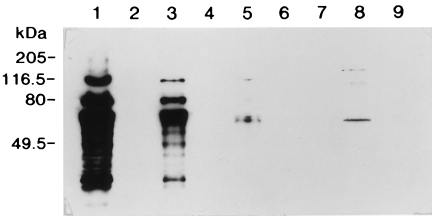

FIG. 2. Attenuated SL5631 strains expressing p67. *S. dublin* SL5631 was transformed with various plasmid constructs. Cell lysates and supernatants of the bacterial culture were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The filter was then probed with a monoclonal antibody raised against p67. Nonrecombinant SL5631 served as a negative control (lane 9). Lanes 1 and 2, SL5631(pMC622), cell lysate and supernatant; lanes 3 and 4, SL5631(pGEX67), cell lysate and supernatant; lanes 4 and 5, SL5631(pJF67), cell lysate and supernatant; lanes 7 and 8, SL5631(pIP67), cell lysate and supernatant. Molecular weight markers are indicated at the left.

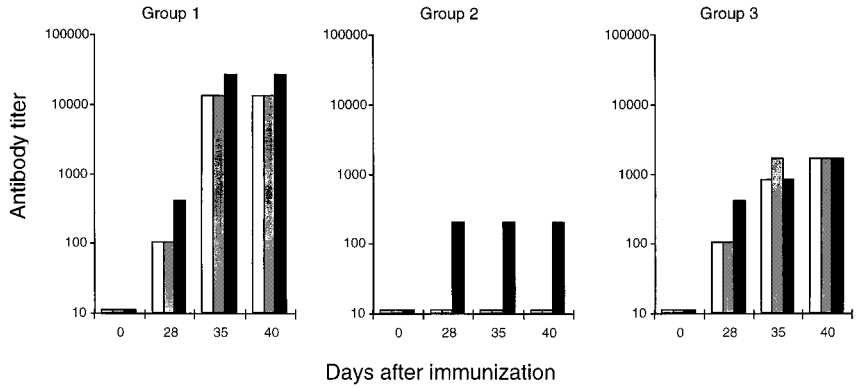

FIG. 3. Kinetics of p67-specific antibody responses in serum of animals immunized with recombinant SL5631 strains, as evaluated by ELISA. Animals were immunized with recombinant *S. dublin* SL5631 on days 0, 7, and 28. Group 1 received the p67-secreting strain i.m., while group 2 were inoculated orally. Group 3 were inoculated i.m. with a strain cocktail expressing p67 intracellularly. Bars represent titers of individual animals in each of the groups.

and low-copy-number plasmid pJF67 (90% positive colonies after 18 generations) were maintained relatively stably in SL5631 (12).

p67-specific antibody responses of cattle after oral and i.m. immunization with recombinant SL5631 expressing p67. Specific antibody responses were monitored after oral or i.m. inoculation of calves with different recombinant SL5631 *aroA* strains. None of the animals developed signs of acute salmonellosis following administration of the first rather low-dose inoculum of recombinant SL5631, and no anti-p67 antibody response was detected 1 week later (data not shown). The animals were then boosted with a higher dose of bacteria and reevaluated for specific antibody responses to p67 on day 28. Both groups of calves inoculated i.m. with p67-expressing SL5631 were found to be positive for specific IgG, although the intensity of the response remained low, with a maximum titer of 1:200 (Fig. 3). After a second boost with a higher dose of recombinant bacteria on day 28, all animals became febrile for 2 to 4 days but continued feeding and showed no signs of acute salmonellosis. On day 35 after primary inoculation, all immunized calves had seroconverted to p67, with the titers being about 10-fold higher in animals immunized with SL5631 secreting p67 than in those receiving SL5631 expressing the antigen intracellularly (Fig. 3; Table 1). Specific IgG was detected in only one of the orally inoculated animals, and titers did not exceed 1:200 (Fig. 3). None of the animals immunized orally or i.m. developed a significant IgA serum response specific for p67.

Challenge with *T. parva* sporozoites. The calves were challenged 2 weeks after the final inoculation with a 80% lethal dose of stabilated *T. parva* sporozoites. This challenge has been shown to provoke a severe ECF reaction in all naive animals challenged to date (data not shown). In line with this observation, the three unimmunized calves included in the experiment developed severe disease and were euthanized 18 days after challenge (Table 1). In contrast, two of the animals immunized with SL5631 secreting HlyA-p67 developed only mild and moderate reactions. However, the third calf developed severe signs of ECF similar to those observed in the control animals. Two of the orally immunized calves developed severe disease and were euthanized on days 18 and 20 after challenge; the third survived the challenge after moderate reaction. Of the calves immunized i.m. with the cocktail of SL5631 expressing p67 intracellularly, one (calf BP87) was completely protected. This animal did not become febrile, and no parasite-infected cells could be detected in lymph node biopsies. However, the draining lymph node of BP87 was found to be active for several days, and the body temperature of the animal increased from 38.3 to 39.2°C in response to the sporozoite challenge. The two other calves of this group developed severe disease and were euthanized on day 20 after challenge (Table 1).

TABLE 1. Prechallenge immune status and ECF reactions of all animals after inoculation with sporozoite stabilate

Vaccine	Animal	Antibody titer to p67	Days of[a]		Day		ECF reaction[b]
			Pyrexia	Schizonts	Euthanized	Recovery	
SL5631 secreting p67 (i.m.)	BP78	1/12,800	>10	>11	18		Severe
	BP79	1/12,800	8	9		22	Moderate
	BP80	1/25,600	2	2		15	Mild
SL5631 secreting p67 (oral)	BP58	0	6	11		22	Moderate
	BP59	0	>10	>12	18		Severe
	BP86	1/200	>10	>13	20		Severe
SL5631 expressing p67 (i.m.)	BP81	1/1,600	>10	>10	20		Severe
	BP76	1/1,600	>11	>12	20		Severe
	BP87	1/1,600	0	0			None
None	BP77	0	>11	>11	18		Severe
	BP90	0	>9	>12	18		Severe
	BP88	0	>10	>13	18		Severe

[a] The temperature of the animals and the appearance of schizonts in the draining lymph node were evaluated daily.
[b] Classification was based on clinical and parasitological examination.

DISCUSSION

This is the second report of protection of cattle against *T. parva* challenge by immunization with recombinant SL5631 expressing the p67 antigen. In a previous report (12), we described the use of a cocktail of SL5631 recombinants transformed with three different p67 expression constructs. This immunization regimen gave rise to moderate to high specific antibody titers and a significant degree of protection against severe disease. However, four inoculations were required for adequate seroconversion, and a proportion of immunized calves were not protected.

To improve the viability of the *Salmonella* carrier strain, we constructed an additional SL5631 strain secreting a chimeric Hly-p67 recombinant antigen. In vitro stability studies revealed that the expression plasmid was retained by the majority of transformed bacteria after 18 generations in the absence of antibiotic selection. Given that *aro*-deleted bacteria are known to undergo only limited replication in vivo (13), it can be assumed that most of the inoculated recombinants retain the plasmid and secrete the antigen until eliminated by the host.

All calves immunized i.m. seroconverted to p67, although, in line with previous observations (12), those immunized with the cocktail of recombinant strains had rather low titers of specific IgG1 after three inoculations. In contrast, those immunized by i.m. inoculation of secreting recombinant SL5631 had high prechallenge titers. The same recombinant SL5631 strain delivered orally did not provoke specific antibody titers in two immunized calves. Only one animal seroconverted with very low titers to the recombinant p67 antigen.

The results of the challenge experiment, although derived from small numbers of animals, suggest that immunization with p67-secreting recombinant SL5631 by the i.m. route can provide a degree of protection similar to that observed with recombinant p67 formulated in adjuvant (21). The importance of this observation is enhanced by the fact that the calves used in the trial were only 1 month old at the time of the first inoculation. Younger calves are known to be more susceptible to *T. parva* infection (25) but were selected for the trial to allow optimal delivery of the oral inoculum to the gut rather than the rumen. The capacity to induce protection at this age is an attractive feature for a *T. parva* vaccine, since it is in young calves that the greatest mortality occurs.

It is not clear whether the weaker protection in the orally immunized group was related to ineffective delivery of the recombinant bacteria to the gut immune tissue or to the inappropriate induction of immune responses to challenge at the periphery. In this regard, it is interesting that we could not detect a significant IgA response to p67 in animals immunized orally, which suggests that mucosal priming may not have been effective. In either event, i.m. inoculation appears to have engendered more effective immunity with substantially fewer recombinant bacteria. In addition, because of the negligible risk of environmental contamination and recombination with wild-type *Salmonella* in vivo, the i.m. route is far more attractive for widespread use in the field.

Because protection in animals immunized with recombinant p67 formulated in adjuvant does not correlate with titer, avidity, isotype, or peptide specificity of the antibody response, it has been suggested that cell-mediated mechanisms play an important role, possibly through the elaboration of cytokines (23). A characteristic feature of p67 immunizations has been the failure to consistently detect specific T-cell proliferation in immunized cattle. Although we have previously observed p67-specific T-cell proliferation in a proportion of calves immunized with recombinant SL5631 expressing but not secreting p67 (12), we failed to detect proliferation in the present study (data not shown). Nonetheless, the nature of the antibody response observed in this and previous studies (9, 21, 23) is consistent with effective T-cell help. It is possible that T-cell populations are involved in the protection observed in this study but do not proliferate efficiently in vitro. Under this scenario, the more effective induction of specific immunity by the p67-secreting SL5631 could be related to the efficiency of antigen processing and presentation in vivo. Processing of the recombinant p67 antigen produced by intracellularly expressing recombinants for T-cell recognition is likely to require phagocytosis and degradation of the bacteria by macrophages. This would inevitably result in competition between bacterial and p67-derived peptides for major histocompatibility complex binding. In contrast, the soluble secreted p67 recombinant would be accessible to professional antigen-presenting cells such as veiled cells and dentritic cells, which are known to have poor phagocytic function. In addition, presentation of the soluble antigen by macrophages after phagocytosis would not require degradation of the bacteria and so would not result in high levels of antigenic competition.

This possibility is supported by the results of a study that compared the immunogenicity of attenuated *Salmonella* strains producing *Listeria monocytogenes* p60 or listeriolysin in secreted or intracellular form (11). Vaccination with these *S. typhimurium aroA* recombinant strains effectively protected mice against a challenge with a virulent *L. monocytogenes* strain when the antigen was produced in secreted form. In contrast, protection was either low or absent when the corresponding antigens remained intracellularly in the vaccine strain (11).

The apparent enhancement of specific humoral responses through the use of the p67-secreting recombinant SL5631 has additional significance in the context of environmental acceptability. Because extrachromosomal DNA can be exchanged between bacteria through conjugation events, plasmid-based bacterial antigen delivery systems may be undesirable for use in the field. Chromosomal integration of foreign genes would be more acceptable in this regard (14, 29) and has the additional advantage of recombinant stability. However, this system has been considered less attractive because of the low levels of expression that have been associated with it (2). In light of our observation that, at least in vitro, SL5631 secretes minimal amounts of recombinant antigen, it is possible that expression of a secreted recombinant antigen from a chromosomal site would result in satisfactory immunization. Nonetheless, the plasmid expression system described herein has considerable potential for improvement. A number of reports have described the enhancement of in vivo immune responses by the administration of recombinant cytokines (16, 17, 31), and a *Salmonella* recombinant expressing murine interleukin-4 has been shown to enhance the humoral response of mice to *Salmonella* antigens (3). It may be possible to construct a dual recombinant SL5631 expressing both p67 and bovine interleukin-4 as HlyA chimeras and so enhance the immunity already observed with the antigen alone. This possibility is now being explored.

The results of more than 150 cattle immunizations with recombinant p67 formulated in adjuvant indicate that approximately 30% of vaccinates are not protected against severe disease (21, 22). Our observations in cattle immunized with p67-recombinant *Salmonella*, in this and a previous trial (12), are consistent with this pattern. For this reason and because variation has been observed among cattle in the dose of sporozoites required to produce severe disease (4), it is possible that complete herd protection will not be achieved through immu-

nization with p67 alone. It is known that animals that recover from ECF are protected by virtue of class I major histocompatibility complex-restricted cytotoxic T lymphocytes specific for schizont-infected cells (19). An intensive search for the parasite antigens that provoke these responses is under way, with a view to the development of a second-generation subunit vaccine with broader protective capacity.

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Ivo Gentschev and Ines Glaser contributed equally to this publication.

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