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FINAL REPORT

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Development of a peptide vaccine for protection against bovine anaplasmosis

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Table of Contents

	Section	Pages
I.	Cover Sheet	1
II.	Table of Contents	2
III.	Executive Summary	3
IV.	Research Objectives	4-9
	Statement of the Problem	4-7
	Research Strategy	7-9
V.	Methods and Results	9-23
VI.	Impact, Relevance, and Technology Transfer	24-30
	Scientific Impact	24-25
	Research Capacity Building	25-30
VII.	Project Activities/Outputs	30-34
VIII.	Project Productivity	34-35
IX.	Future Work	35-36
X.	Literature Cited	36-38

III. Executive Summary

Production of food, fiber, and traction, improved management of grazing lands, and societal stability are all impacted by the level of livestock health in rural sub-Saharan Africa. The primary arthropod-borne infectious diseases remain a primary constraint to the improvement of livestock health in tropical lesser developed countries. Anaplasmosis, caused by the rickettsia *Anaplasma marginale*, is the most prevalent of these diseases in sub-Saharan Africa. Current control is based on continual acaracide application to cattle, a method that is poorly sustainable due to economics, development of resistance in the arthropod vectors, and environmental concerns. In contrast, immunization would provide a sustainable means of protecting cattle from anaplasmosis. This PSTC project has focused research on development of effective immunoprophylaxis against bovine anaplasmosis. The primary outputs of the project have been: 1] primary research, published in international journals, demonstrating protective immunization against *Anaplasma marginale*; 2] characterization of a Zimbabwe strain of *A. marginale* that allows comparison with similar molecular based research at other institutions worldwide; 3] enhancement of the research capacity of the Zimbabwe Veterinary Research Laboratory by collaborative research and laboratory development resulting in technology transfer; 4] longterm capacity building by training staff scientists in immunology and microbial pathogenesis; 5] development of a longterm research collaboration with the Veterinary Research Laboratory that has resulted in training of an additional Zimbabwe staff scientist using African-American Foundation funding; and 6] inclusion of the Zimbabwe scientists and Veterinary Research Laboratory into a research based network on hemoparasitic diseases including both U.S.-Africa linkages and inter-African linkages.

IV. Research Objectives

Statement of the Problem: Production of food, fiber, and traction, improved management of grazing lands, and societal stability are all impacted by the level of livestock health in rural sub-Saharan Africa. The primary arthropod-borne infectious diseases (anaplasmosis, babesiosis, heartwater, theileriosis, and trypanosomiasis) remain a primary constraint to improvement of livestock health in tropical lesser developed countries[1]. Anaplasmosis, caused by the rickettsia *Anaplasma marginale*, is the most prevalent of these diseases in sub-Saharan Africa. Although present in many nations with sub-tropical and temperate climates, the impact of the disease is borne predominantly by lesser developed nations in Africa, Asia, and Latin America[2]. Morbidity and mortality rates are highest in animals brought into an enzootic area[2]. This susceptibility is an impediment to genetic improvement of local livestock by crossbreeding with animals imported into the enzootic region. Emphasis on upgrading the health and genetics of cattle is part of an integrated plan to de-emphasize inefficient, large number herds which contribute heavily to overgrazing and do not optimize production of meat, milk, and fiber[1].

Following transmission of *Anaplasma marginale* by biological or mechanical vectors, there is a prepatent period of 20-40 days during which there is a low but increasing percentage of parasitized erythrocytes[3,4]. The prepatent period, during which cattle are clinically normal, is followed by an acute phase during which the parasitemia increases dramatically and severe hemolytic anemia occurs[3]. Dramatic weight loss (average 86 kg per animal), abortion (24% of clinical cases), and death (36% of clinical cases) can occur during the acute phase[5] Cattle recovered from acute disease remain persistently infected with *A. marginale* and may serve as a reservoir for transmission[3,4]. These recovered cattle are fully resistant to challenge with the

homologous strain, however, they remain susceptible to challenge with certain heterologous strains[6].

Current control measures include 1] control of arthropod transmission; 2] use of antibiotics to prevent or treat *A. marginale* infections; and 3] vaccination of susceptible cattle. Control of arthropod transmission by regular use of acaricides is expensive and creates a highly susceptible cattle population[7]. The economic expense (foreign currency requirement) is often cited as a disadvantage to acaricide programs, as well there is a less often stated concern about the environmental and public health aspects of acaricide use. Paradoxically, efficient acaricide treatment increases the risk of disease epizootics by creating a susceptible animal population which undergoes high morbidity and mortality when exposed to *A. marginale* due to development of acaricide resistance or breakdown in the treatment program[7,8]. The economic and environmental concerns combined with the detrimental effects on population immunity are not consistent with our longterm goal of promoting low input sustainable agriculture.

Reliance on antibiotic prophylaxis by continual feeding of tetracyclines results in herds vulnerable to program disruptions. The expense involved to ensure that all cattle receive a minimum daily intake of antibiotic is not feasible in lesser developed countries. In spite of the efficacy of tetracyclines in treatment and chemoprophylaxis of anaplasmosis[9], antibiotic based control programs have found only limited acceptance in the United States and virtually none in other countries[2].

In contrast, immunization provides a low input method to protect cattle against severe morbidity and mortality due to *A. marginale* infection. Prophylaxis through vaccination can be incorporated into existing government veterinary services and killed vaccines can be safely stored

and delivered in remote regions. The protection engendered by recovery from *Anaplasma* infection clearly indicates that immunoprophylaxis is a realistic and achievable goal[6,10]. Vaccination provides a means to efficiently and economically protect cattle both within enzootically stable and unstable areas as well as susceptible adult cattle to be imported into these areas. Premunization (vaccination by deliberate infection) with live *A. centrale* (currently the only available vaccine in Zimbabwe) provides protection against severe clinical disease following challenge with most *A. marginale* strains[10-13]. However, there are reports of severe clinical disease in cattle premunized with *A. centrale* following challenge with certain *A. marginale* strains[13-14]. The greatest impediment to the success of these infectious vaccines is their requirement for standardized production and distribution of a cold-chain (liquid nitrogen) dependent live organism. Inoculated cattle must become infected with the organism, yet severe morbidity avoided[6]. Morbidity is more severe in older animals and therefore the use of live vaccines to immunize highly susceptible adult cattle is precluded[15]. In addition, the risk of transmitting other infectious agents (known and unknown viral, bacterial, and protozoal pathogens) in the cryopreserved inoculum cannot be completely avoided.

Immunization with killed whole organism vaccines induces partial protection against challenge with heterologous *A. marginale* strains[16]. Vaccination usually reduces the severity of the clinical disease, however, significant parasitemia, anemia, and weight loss occur[16,17]. Challenge with virulent strains, notably the Florida strain, results in severe anemia and high mortality similar to that seen in unvaccinated cattle[16]. In addition, because the vaccine requires an oil adjuvant and is composed of whole *A. marginale* admixed with erythrocyte stroma, immunized cattle develop anti-erythrocyte isoantibodies which can cause isoerythrolysis in calves

ingesting these antibodies in colostrum[18-19]. These drawbacks have limited the overall deployment and effectiveness of killed whole organism vaccines against anaplasmosis[1,2].

Although premunization has been practiced for 75 years and commercially available killed whole organism vaccines have been used for nearly 20 years, the need remains to develop a widely cross-protective, economical vaccine[1]. The severe constraints posed by anaplasmosis to livestock production in sub-Saharan Africa, including Zimbabwe, and in many other subtropical and tropical countries, combined with the inadequacy of current immunoprophylaxis led to a recommendation by the U.S. National Research Council that high priority be given to development of an effective vaccine[1].

Research Strategy: The failure to develop effective immunoprophylaxis against anaplasmosis results from the complexity of the disease, including persistence of the parasite and from lack of application of current research technology to the disease.

We identified, in 1986, a neutralization-sensitive epitope on an *Anaplasma marginale* surface protein of 105,000 kD apparent molecular mass[20]. This protein, designated MSP1a (major surface protein 1a), was purified from the Florida strain of *A. marginale* using immunoaffinity chromatography and shown to protect immunized cattle from challenge with the homologous strain[20]. In collaborative research with scientists in Israel (Kimron Veterinary Institute) and Kenya (Kenya Agricultural Research Institute, Kabete and the International Laboratory for Research on Animal Diseases), we demonstrated that the neutralizing monoclonal antibodies bound a common epitope on 12 strains from the Americas, Africa, and the Middle East[21,22]. We cloned the gene for *Anaplasma marginale* MSP1a with the objective of deriving

the primary structure of the native polypeptide. Determination of the structure of MSP1 and mapping of the neutralization-sensitive epitope would provide a basis to examine structural variation relevant to crossprotective immunity. Specifically, this research at the molecular level could be extended to strains of *A. marginale* from sub-Saharan Africa to identfy coomon and unique antigens relevant to immunity. In addition, the neutralization-sensitive epitope could be tested as a synthetic peptide immunogen in cattle. Synthetic peptide immunogens have several unique properties which may be critical to effective use of a subunit vaccine: 1] the protection-inducing peptide can be efficiently synthesized in vitro without the requirement for large scale fermentation followed by purification of the single expressed protein from *E. coli* host cells; 2] the presentation of the key protection-inducing epitopes, especially if linked in tandem repeats, may focus the immune-response more effectively on this isolate-common epitope than vaccines containing extraneous proteins; 3] the peptide based vaccine carries no risk of reversion to virulence or as a threat to immunodeficient animal caretakers. The demonstration that MSP1 is capable of inducing protection in immunized cattle and bears an invariant peptide epitope are key developments in anaplasmosis vaccine research.

The collaborative research in this PSTC project was designed to identify the MSP1a neutralization-sensitive epitope, extend the molecular comparison to a Zimbabwe strain of *A. marginale* (to be isolated during the project), and to test vaccines incorporating the MSP1a. Concomitantly, our goals were to develop research capacity in molecular approaches to vaccine development in the Veterinary Research Laboratory. The inclusion of a Zimbabwe strain would ensure that research and vaccine development appropriate to Zimbabwe could be tied into ongoing research efforts worldwide. It is important to emphasize that research by the U.S.

institutions (Washington State University and the University of Florida) on vaccine development is also funded by other federal granting agencies. The co-funding by other agencies is consistent with the goals of the PSTC program and was critical is providing the technical base for U.S. research on anaplasmosis vaccine development. The PSTC funds are committed 100% for collaborative research support and training in the U.S. and for technology transfer and program development in Zimbabwe.

Co-funding from other research organizations for US Laboratories.

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Co-funding from other research organizations for Zimbabwe Laboratories.

Ministry of Lands, Agriculture, and Rural Resettlement, Government of Zimbabwe

V. Methods and Results

The research in this project focused on identification of the MSP1a neutralization-sensitive epitope, isolation and characterization of a Zimbabwe strain of *A. marginale*, definition of crossprotective immunity and epitope conservation among strains, and development of novel immunogens including synthetic peptide analogs of the neutralization-sensitive epitope. As requested in the instructions for the Final Report, we have enclosed reprints of primary research publications resulting from this project. The research accomplishment is briefly described in the text below and then referred to the appropriate manuscript. The project research is presented as the following seven specific sub-projects:

1] Molecular size variations in an immunoprotective protein complex among strains of *Anaplasma marginale*.

2] Ability of MSP1 to induce protective immunity against structurally variant strains.

3] Isolation of a Zimbabwe strain of *Anaplasma marginale* and testing of crossprotective immunity with the Florida strain.

4] Antigenic characterization of the Norton Zimbabwe strain with monoclonal antibodies to surface exposed polypeptides.

5] Molecular basis for MSP1a size polymorphisms among strains of *Anaplasma marginale*.

6] Immunization of cattle using synthetic peptide analogs of the neutralization-senstive MSP1a epitope.

7] Induction of protective immunity against the Norton, Zimbabwe strain of *Anaplasma marginale* using characterized outer membranes incorporating MSP1a.

Note that in the early phases of the PSTC project, as we developed the collaborative research linkage, funding from the U.S. portion of the project budget was used for partial support of ongoing research in U.S. laboratories because this research generated basic information on the molecular basis of strain variability. This strain variability is directly applicable to development of vaccines effective against Zimbabwe strains of the organism and was critical to project progress in Zimbabwe. Publications from this early research are authored (as is appropriate) by U.S. scientists and the support of the PSTC project was acknowledged. This success, which is the capstone of several years of research, should not be interpreted as a lack of full commitment to the primary goal of the PSTC project--technology transfer and

capacity building in Zimbabwe.

We place a high priority upon publication in high quality, refereed journals as demonstrated by our publication record. The criteria for success in technology transfer should not be limited to co-authorship of scientific papers by LDC scientists but should be primarily the research completed in the host country and published by the LDC scientist. The necessity for first author publications prevents the LDC scientist from having a field assistant or merely technical role. Our requirement that primary research be done in the host country ensures that the investigator will be effective in the relevant host country laboratory rather than a developed country laboratory. In turn, this ensures that capacity building and technology transfer occur. We have published two manuscripts, with Dr. Ntando Tebele as first author, based on project research done 100% at the Veterinary Research Laboratory in Zimbabwe. The lag in time between project initiation (for 7.384, project initiation was July 1987, with budgetary concurrence by the Ministry of Lands, Agriculture, and Rural Resettlement delayed until May 1988) and publication of research accurately reflects the time needed to initiate and complete quality, independent research in an LDC laboratory.

Molecular size variations in an immunoprotective protein complex among strains of *Anaplasma marginale*.

Rationale: MSP1 had previously been shown to induce protective immunity against a homologous strain challenge and to bear a neutralization-sensitive epitope common among diverse strains. However, very little was known about the structural and antigenic conservation

in MSP1 among different strains. We compared MSP1a molecular size and peptide polymorphism among 5 strains of *A. marginale*. The strains tested were all from the U.S. because we had not successfully isolated a Zimbabwe strain at that time.

Summary: The MSP1a surface protein was demonstrated to be part of a heterodimeric complex and to be markedly polymorphic among strains with molecular size differences ranging from <70kD to 105kD. Despite this marked variation, surface exposed peptides were shown to be conserved among the strains. This research, partially funded by the PSTC grant, was significant for the PSTC project in identifying strain polymorphism and raising the question of variation in B and T lymphocyte epitopes among strains. These results directly led to our early testing of cross-protective immunity between a U.S. strain and our Zimbabwe field isolate of *A. marginale* (reported below). In addition, this report had impact worldwide as it allowed investigators to identify MSP1a and MSP1b in their own strains of *A. marginale*.

Methods and Results reported in: Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. Infection and Immunity 56:1567-1573, 1988.

Ability of MSP1 to induce protective immunity against structurally variant strains.

Rationale: Following the identification of MSP1a size polymorphism among strains in the research described above, we tested the ability of MSP1 immunization to protect cattle against a structurally variant, heterologous strain of *A. marginale*.

Summary: Cattle were immunized with MSP1 affinity purified from the Florida strain of *A. marginale* and then challenged with either the nomologous Florida strain or the Washington-O strain. The Washington-O strain (isolated in 1982) was shown to be structurally variant from the Florida strain (MSP-1a was shown to be 14kD smaller) and antigenically different as assessed by reactivity with a panel of monoclonal antibodies. Despite these differences, MSP1 immunization was shown to provide significant cross-protective immunity against the Washington-O strain. This research, partially funded by the PSTC grant, was significant for the PSTC project in demonstrating that cross-protective immunity was possible despite MSP1 polymorphism. The results raised the question about the feasibility of cross-protection against more geographically distinct strains such as those from sub-Saharan Africa. These results directly led to our early testing of cross-protective immunity between a U.S. strain and our Zimbabwe field isolate of *A. marginale* (reported below).

Methods and Results reported in: Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. Infection and Immunity 57:3666-3669, 1989.

Isolation of a Zimbabwe strain of *Anaplasma marginale* and testing of crossprotective immunity with the Florida strain.

Rationale: The ability of purified MSP1 to induce protection against challenge provided the basis for development of an inactivated vaccine containing protection-inducing antigens. Research on subunit vaccine development had focused on the Florida strain with limited cross-protection experiments. The Florida strain had been shown to induce crossprotective immunity against other *A. marginale* strains in North and South America. The objectives of this study was to: 1] isolate a Zimbabwe strain of *A. marginale*; and 2] determine if the Florida strain, used as the prototype in recent U.S. vaccine development studies, would induce protective immunity in cattle against challenge with the Zimbabwe strain. Despite the practical drawbacks which have limited its effectiveness as a vaccine against anaplasmosis, immunization by infection with live *A. marginale* induces solid immunity against homologous challenge. The ability of live vaccines to induce protection against heterologous challenge depends on the strain used to immunize the cattle and the challenge strain. The efficacy of the Florida strain in inducing widely crossprotective immunity has been the basis for our isolation and characterization of protective antigens using this strain. Crossprotective immunity has been supported by identification of conserved neutralization-sensitive epitopes on a molecular basis. Prior to initiating antigenic characterization of the Norton, Zimbabwe strain (isolated by Dr. Tebele as a project aim), we used immunization with live organisms to determine if protective antigens were conserved between the two stocks.

Summary: The 1988 isolation of the Norton, Zimbabwe strain by our project collaborators is described in this paper. The efficacy of live Florida strain immunization in this trial was confirmed by the strong protection against homologous challenge. Although protected relative to non-immunized cattle, the Norton, Zimbabwe challenged cattle were only partially protected relative to the protection observed with homologous Florida challenge. This research was significant in that it indicated that induction of complete protection against the Norton, Zimbabwe strain may require an immune response to a unique epitope not found on the Florida strain. It indicated that development of subunit vaccines based on the Florida strain alone may not be sufficient for use in Zimbabwe and possibly in many regions where strains bear critical, different epitopes. Alternatively, the inability of live Florida strain immunization to induce complete heterologous protection may reflect a relatively poor response to epitopes common between the stocks. Complete protection against multiple heterologous strains including the Norton, Zimbabwe strain may require antigen presentation that focuses the immune response to the relevant common epitopes.

Methods and Results reported in: Crossprotective immunity between the Florida and a Zimbabwe stock of *Anaplasma marginale*. Tropical Animal Health and Production, 1991.

Antigenic characterization of the Norton Zimbabwe strain with monoclonal antibodies to surface exposed polypeptides.

Rationale: We hypothesized that the variation in protection (described in the previous section) was due to strain variation in surface exposed membrane epitopes. Previously we had shown epitope variation among different strains of *A. marginale*[21-22] and had, in 1989, documented cyclic rickettsemia in persistently infected cattle, an observation consistent with intrastrain antigenic variation[23]. These observations, combined with the incomplete crossprotective immunity seen in our project, led us to more fully characterize the membrane polypeptides and exposed epitopes of the Norton, Zimbabwe strain of *A. marginale* (research done 100% in Zimbabwe).

Summary: [see Table 1 and research publications described below] This research confirmed conservation of epitopes on *A. marginale* membrane polypeptides MSP-1a (including the six residue neutralization-sensitive epitope on a 65kD polypeptide), MSP-1b, MSP-2, and MSP-3 (Table 1).

Methods and Results reported in: Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infection and Immunity 59:3199-3204, 1991.

TABLE 1. Conserved surface polypeptide epitopes between the Florida and Norton Zimbabwe strains of *A. marginale*

Monoclonal antibody	Polypeptide bound[a]	Strain Reactivity	
		U.S. strains	Norton Zimbabwe strain
Ana 22B1	MSP-1a	9/9[b]	+
AmR R38A6	MSP-1b	1/1[c]	+
Ana F19E2	MSP-2	9/9[b]	+
43/19.3.5	MSP-3	1/1[c]	-
43/23.2.8	MSP-3	1/1[c]	+
Ana R76A1	AmF 31	1/1[c]	+
Ana F16C1	AmF 19	1/1[c]	+
Tryp 1E1[d]	*T. brucei*	0/9[b]	-

[a] See attached reprint.
[b] Strains used are Florida, Idaho, Kansas, Missouri, North Texas, Oklahoma, Virginia, Washington-C, and Washington-O.
[c] Only the Florida strain tested to date.
[d] Monoclonal antibody to a *Trypanosoma brucei* variable surface glycoprotein used as a negative control.

Molecular basis for MSP-1a size polymorphisms among strains of *Anaplasma marginale*.

Rationale: The hypothesis was that synthetic peptides representing the neutralization-sensitive epitope of MSP1a would induce protective immunity in immunized cattle. Determination of the structure of MSP1a and subsequent identification of the neutralization-sensitive epitope was necessary for development of synthetic peptide analogs for immunization of cattle.

Summary: The cloned MSP1a gene from the Florida, South Idaho, Virginia, and North Texas strains of *A. marginale* (Norton, Zimbabwe strain had not been isolated at time of project initiation) was sequenced and the primary amino acid structure derived. By comparison of the sequences from different strains (known to share the neutralization-sensitive epitope) and radioimmunoassay we were able to precisely locate the neutralization-sensitive B cell epitope in the MSP1a surface protein. To summarize our findings: i) the neutralization-sensitive epitope on MSP1a polypeptide was localized to the tandemly repeated sequence: S-A-G-G-Q-Q-Q-E-S-S-V-S-S-Q-S-D(EA)-Q-A-S-T-S-S-Q-L-G-A-D-S-S; ii) the precise monoclonal binding site was localized within this sequence to a six amino acid peptide, Q(E)ASTSS; iii) this sequence, originally identified in the Florida strain, is completely conserved among three additional strains, Virginia, South Idaho, and North Texas; iv) the MSP1a molecular size polymorphism among strains is due to differences in number of 29-mer repeats; and v) strain variable regions in the MSP1a (not in the tandem repeat region) were identified.

Methods and Results reported in: Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proceedings of the National Academy of Sciences USA, 87:3220-3224, 1990.

Immunization of cattle using synthetic peptide analogs of the neutralization-senstive MSP1a epitope.

Rationale: The initial hypothesis in this project was that synthetic peptides representing the neutralization-sensitive epitope of MSP1a would induce protective immunity in immunized cattle. We used synthetic peptide polymers of the MSP1a repeat incorporating the neutralization-sensitive epitopes.

Summary: We determined that carbodiimide polymerized D-S-S-S-A-G-G-Q-Q-Q-E-S-S-V-S-S-Q-S-D-Q-A-S-T-S-S-Q-L-G-A peptide retained the neutralization-sensitive epitope, as demonstrated by immunoblot reactivity with the neutralizing monoclonal antibody. In an initial immunization trial in five calves, carbodiimide polymerized peptide induced a significant antibody response. Immunization of five calves with polymerized 29-mer (500 μg/calf/injection, initially w/ complete Freund's adjuvant, boosted 3x w/ incomplete Freund adjuvant) resulted in antibody titers of 10^3 to 10^4 against both the 29-mer and the native MSP1a. Significantly, one of the five calves did not respond to the 29-mer, likely reflecting a genetic restriction. All five calves immunized on an identical schedule with the native MSP1a developed antibody titers of 10^4. We repeated the immunization with the measurement of both antibody responses and lymphoproliferation, followed by challenge with the Florida strain of *A. marginale*. Three groups of cattle were immunized: Group 1 (synthetic peptide); Group 2 (native MSP1); Group 3 (adjuvant control). Groups 1 and 2 developed significant antibody titers and lymphocyte responses (see Table 2) while control animals were unresponsive. Upon

challenge, 4/5 Group 1 synthetic peptide immunized cattle were protected and 5/5 native MSP-1 immunized cattle were protected as assessed using peak rickettsemia responses compared to control immunized cattle. In summary, the synthetic peptide immunized cattle were protected similar to the protection induced in cattle immunized with native MSP-1a, as assessed by reduction in peak rickettsemia.

Table 2: Immune responses and protection induced in synthetic peptide immunized cattle

Group	Peak Rickettsemia	Ab Titer[1]	Ab Titer[2]	LSI[3]
Peptide[4]	1.8	10^3	10^4	7.1
MSP-1	1.4	10^3	10^3	16.2
Control[5]	5.0	0	0	1.0

[1]Ab titer to polymerized 29mer peptide
[2]Ab titer to native MSP-1
[3]Lymphocyte Stimulation Index vs. MSP-1
[4]n=4, the fifth animal was not protected.
[5]Immunized with adjuvant only on an identical schedule

Methods and Results reported in: manuscript in preparation; published manuscript will acknowledge project funding and reprints will be sent to the USAID Office of Research.

Induction of protective immunity against the Norton, Zimbabwe strain of *Anaplasma marginale* using characterized outer membranes.

Rationale: As noted above, in Zimbabwe we have identified and isolated a local field strain of *Anaplasma marginale* for molecular characterization and challenge of vaccinates. The isolation and characterization of this strain was completed solely at the Veterinary Research Laboratory

based on collaborative project design occurring during Dr. Palmer's 1988 research visit in Zimbabwe. This strain, designated the Norton, Zimbabwe strain, is currently maintained in the Veterinary Research Laboratory. As described above, Dr. Ntando Tebele, Research Officer at the Veterinary Research Laboratory, completed an initial animal trial in which cattle infected with the prototype Florida strain (vaccine work to date is based on this strain as it has been shown to be widely cross-protective based on strains isolated in the Americas) were challenged with either the Florida strain or the recent Zimbabwe strain. Significantly, cattle infected with Florida *A. marginale* were protected against homologous challenge but only partially protected against heterologous Zimbabwe strain. This finding is dissimilar to the protection seen with heterologous Virginia challenge of Florida infected cattle. This may reflect a divergence in protection-inducing epitopes between the Florida and the Zimbabwe strains.

We hypothesized that the variation in protection is due to strain variation in surface exposed membrane epitopes. Previously we have shown epitope variation among different strains of *A. marginale* and documented cyclic rickettsemia in persistently infected cattle, an observation consistent with intrastrain antigenic variation. Therefore, upon recommendation from the National Research Council BOSTID review of our project, we extended our characterization of the Norton Zimbabwe strain of *A. marginale* to include testing of outer membranes as protective immunogens and identification of immunogenic polypeptides.

Summary: In Zimbabwe, we characterized the membrane polypeptides and exposed epitopes of the Norton, Zimbabwe strain of *A. marginale*. In addition to conserved epitopes on MSP1a, MSP1b, MSP2, and MSP3 (Table 1), several membrane antigens were described for the first time using the Norton, Zimbabwe strain including 55, 31, 30, 28, 26.5, and 25 kD antigens.

Immunization of cattle with a membrane preparation containing these polypeptides solidly protected cattle against the Norton, Zimbabwe strain (Table 3). All outer membrane immunized calves were protected from clinical disease as demonstrated by significantly lower decreases in

TABLE 3. Responses of immunized and non-immunized calves to challenge with the Zimbabwe strain of *A. marginale*

Immunogen	Mean[a] decrease in PCV (%)	Mean[a] decrease in Hb (%)	Peak rickettsemia (%)
Fraction 1 immunized	29.7±16.4*	32.3±16.5*	1.3 ± 2.0*[b]
Fraction 2 immunized	23.2±5.9*	25.12±6.3*	0.5 ± 1.0*[c]
Saponin control	52.0±4.9	54.7±4.9	11.8±4.6
Non-immunized	49.8±6.9	51.2±6.4	12.1±5.5

[a] Mean ±SD.
[b] Only 2 out of 5 calves developed rickettsemias.
[c] Only 1 out of 5 calves developed a rickettsemia.
* All means with an asterisk were significantly different from saponin control and non-immunized calves.

PCV and Hb when compared to non-immunized and saponin immunized calves. Decreases in PCV and Hb are a measure of the severity of anemia which is a prominent clinical sign of anaplasmosis. Significantly, all immunized calves with antibody titers $\geq 10^4$ against outer membrane polypeptides were completely protected from microscopically detectable rickettsemia. Diluted sera from calves immunized with outer membranes recognized six polypeptides (55,36,31,30,25,19 kD), two of which share epitopes with surface exposed polypeptides of the Florida strain. A 31 kD polypeptide was common to two membrane preparations that induced protection. Protection in membrane immunized calves could be due to responses to antigens

unique to the Norton, Zimbabwe strain, antigens conserved among strains (such as MSP-2 and AmF19), or a combination of unique and common antigens. Further fractionation of membranes and immunization trials will help identify those Norton, Zimbabwe strain polypeptides which are critical to inducing protective immunity.

This research demonstrated a significant relationship between increase in antibody titer and protection against anemia (Table 4). Coefficient of determination values indicate that 81%-95% of protection from anemia can be predicted by antibody titer. All calves with antibody titers greater than 10^3 against membrane antigens were completely protected from rickettsemia.

TABLE 4. Correlation of antibody titers and protection in *A. marginale* outer membrane immunized calves

Animal number	Antibody titer[a]	Peak rickettsemia (%)	Decrease in PCV (%)[b]	Decrease in Hb (%)[c]
3	10^6	0	20.6	21.6
15	10^6	0	17.6	19.8
7	10^5	0	22.6	25.8
11	10^5	0	21.9	22.6
22	10^3	2.3	33.3	35.6

[a] Pre-challenge antibody titers against Norton Zimbabwe strain initial bodies.
[b] Increase in antibody titer has a negative linear relationship with change in PCV ($r = -0.97$; $r^2 = 0.95$, $P < 0.05$).
[c] Increase in antibody titer has a negative linear relationship with change in Hb ($r = -0.96$; $r^2 = 0.94$, $P < 0.05$).

Based on this correlation, we plan to determine the effector role of antibody using immunoglobulin transfer from membrane immunized calves.

Methods and Results reported in: Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infection and Immunity 59:3199-3204, 1991.

VI. Impact, Relevance, and Technology Transfer

Scientific Impact: The most objective measure of scientific impact is publication in international, refereed journals (see Section VII). A second measure of impact is the influence our findings have on current and future research in vaccine development against *A. marginale* and other rickettsial pathogens. The research done in this project has directly led to the following ongoing experiments: i) development and testing of recombinant expressed MSP1a encoding either the repeats only (bearing the neutralization-sensitive epitope) or the non-repeat sections (joint University of Florida-Washington State University project funded by USAID); ii) use of the epitope-specific assays to measure antibody responses against MSP1a (included in USDA-BARD funded project 1561-88); iii) cloning and expression of the gene encoding the 19kD surface protein identified as an outer membrane protein in our studies (USDA Agricultural Research Service project); iv) fractionation of the outer membranes polypeptides for identification of protective subunit immunogens (Zimbabwe Veterinary Research Laboratory project); v) use of outer membranes of U.S. strains to induce protective immunity (joint research at Oklahoma State University and Washington State University); and vi) determination of the mechanism of immunity by passive transfer of antibody from membrane immunized, protected cattle (Zimbabwe Veterinary Research Laboratory project).

The scientific impact on the host country research priorities and capabilities is indicated by their 100% support (salaries, goods and services, equipment) for continuation of vaccine research in Zimbabwe. Specifically, the Veterinary Research Laboratory has initiated further research to identify which outer membrane polypeptides are responsible for the protective immunity induced by immunization with whole outer membranes. These additional

immunization trials will include cattle immunized with the synthetic peptide polymer for direct comparison of efficacy between the polymer and polypeptides unique to the Zimbabwe strain. Importantly, the isolation and characterization of the Norton, Zimbabwe strain has provided basis for comparison to vaccine research worldwide and for detection of variant strains in Zimbabwe. It is important to stress that the research in Zimbabwe, funded 100% by the PSTC project, initiated a new approach to anaplasmosis vaccine development that without project support would not have occurred.

Research Capacity Building: The Veterinary Research Laboratory in Harare, Zimbabwe is the sole institution responsible for animal health research and training in Zimbabwe. This PSTC project enhanced the innovative research capability in animal health by providing advanced training in molecular biology to Veterinary Research Laboratory staff members and support for development of an independent research laboratory. This training and support is essential to the development of research capability applicable to the needs of veterinary services in Zimbabwe and throughout sub-Saharan Africa. Specifically, the project enhanced capability for innovative research by:

1] **Focusing research on a disease of high priority to Zimbabwe with generation of novel immunogens and molecular characterization of local pathogen strains.** Anaplasmosis remains a severe problem in Zimbabwe as the most prevalent of the arthropod-borne hemoparasitic diseases. The reliance on acaricide application for control of the vector and subsequent transmission has become unsustainable due to the high foreign currency requirement for acaracide importation. The priority is to develop a vaccine effective against southern African strains of *A. marginale*. Project funded

research provided a molecular based comparison between a Zimbabwe strain and characterized strains, demonstrated synthetic peptide induced protection based on a conserved epitope, and identified a novel immunogen based on characterization of the Zimbabwe strain. The commitment and priority of the host country is indicated by their 100% support (salaries, goods and services, equipment) for continuation of vaccine research in Zimbabwe.

2] Providing specific training in molecular approaches to vaccine development to staff of the Veterinary Research Laboratory. Specifically, we accomplish technology transfer through training of an individual scientist with a commitment to the host institution in Zimbabwe. As the host country project develops, this results in additional on-site training of new and existing staff. Clearly for effective, longterm transfer, the initial scientist must be in a position to develop and maintain a research program in Zimbabwe. This requires the longterm support of the host country laboratory and government. Dr. Ntando Tebele, a Zimbabwean citizen and a permanent scientist (Veterinary Research Officer) at the Veterinary Research Laboratory in Zimbabwe was selected by joint agreement between project investigators, including Dr. W.N. Madzima, Deputy Director of Veterinary Services. The joint investment by the U.S. institution (Dr. Tebele completed her M.S. at Washington State University with thesis research in Zimbabwe) and the Zimbabwe institution (she is a full-time scientist in a laboratory with severe manpower limitations, her assignment to the project was not taken lightly) ensures that the commitment to training and subsequent transfer is made. The cornerstone of the technology transfer is that while Dr. Tebele completed coursework for her M.S. in the U.S., all thesis research was done in Zimbabwe based on the local strain of the

organism. During this period she worked closely with technical staff in the Veterinary Research Laboratory, providing a transfer of technology. The Veterinary Research Laboratory is currently (January 1992) recruiting a second permanent Research Officer for this research unit. Dr. Tebele will be responsible for training of this individual.

Briefly we would like to summarize the training program for Dr. Tebele. She has completed her M.S. with a focus on microbial immunology/molecular biology at Washington State University in Spring 1991. [grade point average 4.0/4.0; 13 of 19 graded graduate course semester hours focused either directly on molecular biological techniques (Micro 462, Micro 590) or on the research application of molecular techniques to solving problems in animal disease (VPa 544, VPa 545, VPa 548). All M.S. research was done in Zimbabwe where she has a permanent appointment as a Veterinary Research Officer in the Veterinary Research Laboratory. This program emphasizes on site research in order to facilitate technology transfer and to encourage independent development.

During a PSTC supported visit by Dr. Madzima to Washington State University, we had the opportunity to review progress in technology transfer. Dr. Madzima met with scientists in the College of Veterinary Medicine with an emphasis on biotechnology research in vaccine development. Although the visit was short-term due to his administrative responsibilities in Zimbabwe, the updating is significant due his responsibility for enhancing the research capacity of the Veterinary Research Laboratory.

As a result of PSTC sponsored collaboration between the investigators at the two institutions and as an outgrowth of Dr. Madzima's support for the training mechanism, we were able to arrange for a second Research Officer from the Veterinary Research

Laboratory to begin M.S. training at Washington State University. Dr. Tendai Ushe, Research Officer at the Veterinary Research Laboratory, was awarded an AFGRAD Fellowship from the African-American Institute. She began graduate coursework in experimental pathology at Washington State University in January 1991 and will return to Zimbabwe for thesis research early in 1992. Although no USAID-PSTC funds were used to support this training, the development of the program was a direct result of the PSTC collaboration. Significantly, this program extends the benefits of the PSTC program to additional collaboration with co-funding.

3] **Placing a high priority upon first author publication in refereed journals by the principal host country scientist.** We place a high priority upon publication in high quality, refereed journals as demonstrated by our publication record. The criteria for success in technology transfer should not be limited to co-authorship of scientific papers by LDC scientists but should be primarily the research completed in the host country and published by the LDC scientist. The necessity for first author publications (needed to complete the graduate degree) prevents the LDC scientist from having a field assistant or merely technical role. Our requirement that thesis research be done in the host country ensures that the investigator will be effective in the relevant host country laboratory rather than a developed country laboratory. In turn, this ensures that capacity building and technology transfer occur. Dr. Tebele has published two first author full-length primary manuscripts resulting from her project research done 100% at the Veterinary Research Laboratory in Zimbabwe. The lag in time between project initiation (for 7.384, project initiation was July 1987, with budgetary concurrence by the Ministry of Lands, Agriculture, and Rural Resettlement delayed until May 1988) and publication

of research accurately reflects the time needed to initiate and complete quality, independent research in an LDC laboratory.

*Tebele, N. and Palmer, G.H.: Crossprotective immunity between the Florida and a Zimbabwe stock of *Anaplasma marginale*. Tropical Animal Health and Production. 1991.

*Tebele, N., McGuire, T.C. and Palmer, G.H.: Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infection and Immunity 59:3199-3204, 1991.

Note that Dr. Madzima was not a co-author on these manuscripts. While his support and effort has been critical in the project development and mangement, his increasing administrative duties have precluded an investigator role in the experiments.

4] **Preparing trainees to develop as independent scientists.** The longterm goal of our training program is to prepare a individual to perform as an independent and collaborative scientist in the host country. Briefly to summarize the progress in this program: i) to broaden Dr. Tebele's exposure to research in the U.S., she attended the Eighth National Veterinary Hemoparasite Disease Conference in St. Louis during April 11-12, 1989; ii) in designing her program, we included a course on research proposal preparation (EdAd596 Grant Proposals) with the goal of facilitating her development as an independent scientist competitive for research funding independent of a U.S. collaborator; iii) subsequently, her research in Zimbabwe was developed in a proposal format and followed through with periodic written updates; iv) she conducted project funded collaborative research with scientists at the Kenya Agricultural Research Institute and at ILRAD in Kabete, Kenya; v) to enhance training in molecular approaches, she

attended the advanced program (USAID/National Research Council, Sept. 1990) on use of the polymerase chain reaction in biotechnology research in lesser developed countries; and vi) presented project research at the Fourth International Congress on Malaria and Babesiosis in Rio de Janeiro (August 1991).

5] **Research laboratory enhancement.** The Veterinary Research Laboratory had previously committed a laboratory to immunology research in anaplasmosis. This laboratory was sufficiently equipped for clinical research and basic laboratory procedures. The project equipping of the laboratory was guided by the specific research to be done in Zimbabwe. The project significantly enhanced capability by providing equipment and reagents for polyacrylamide and agarose gel electrophoresis, continuous gradient centrifugation, microscale centrifugation, immunofluorescence, protein quantitation, and non-radioactive immunoblotting. In addition, the project provided a microcomputer used for manuscript preparation, statistical analysis of data, and graphics for data presentation. The effective transfer of technology with the instrumentation is shown by its use in the 2 research manuscripts based 100% on research done in Zimbabwe.

VII. Project Activities/Outputs

Publications resulting from project:

1. Tebele, N., McGuire, T.C. and Palmer, G.H.: Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infection and Immunity 59:3199-3204, 1991.

2. Tebele, N. and Palmer, G.H.: Crossprotective immunity between the Florida and a Zimbabwe isolate of *Anaplasma marginale*. Tropical Animal Health and Production, 1991.

3. Allred, D.R., McGuire, T.C., Palmer, G.H., Leib, S.R., Harkins, T.M., McElwain, T.F., and Barbet, A.F.: Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proceedings National Academy of Sciences, USA, 87:3220-3224, 1990.

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5. Oberle, S.M., Palmer, G.H., Barbet, A.F. and McGuire, T.C.: Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. Infection and Immunity 56:1567-1573, 1988.

6. Palmer, G.H.: Anaplasma Vaccines. In Veterinary Protozoan and Hemoparasite Vaccines, Wright, I.G., Ed. pp. 1-29. CRC Press, Boca Raton. 1989.

Theses resulting from project:

1. Ntando Tebele, Master of Science, Washington State University, 1991. Thesis title: Induction of protective immunity by using *Anaplasma marginale* outer membranes.

Training enhancement:

1. Dr. Ntando Tebele attended The Eighth National Veterinary Hemoparasite Disease Conference in St. Louis, Missouri. March 1989. This meeting had international

participation with a focus on control of veterinary hemoparasitic diseases. Dr. Tebele did not present at this meeting as it was early in her training period.

2. Dr. Ntando Tebele was an invited participant at the BOSTID/USAID sponsored Workshop on polymerase chain reaction (and selected topics in recombinant DNA technology) held September 24-28, 1990. Center for Advanced Training in Cell and Molecular Biology, Catholic University, Washington, D.C.

3. Dr. W.N. Madzima was a short-term exchange participant to Washington State University in May-June 1990. In addition to emphasis on new techniques in infectious diseases research, Dr. Madzima met with the Vice-Provost for International Programs regarding development of additional cooperative projects. Dr. Madzima completed his exchange with a 2 day visit at the Centers for Disease Control in Atlanta.

4. Dr. Ntando Tebele conducted joint research with Dr. Fred Rurangirwa at the Kenya Agricultural Research Institute in Kabete, Kenya and with Dr. Antony Musoke at the International Laboratory for Research in Animal Diseases in Kabete, Kenya. This joint research, conducted in August 1990, was critical for project success in Zimbabwe (provided ultracentrifuge and electron microscopy facilities) and important in developing collaborative linkages among scientists within sub-Saharan Africa.

5. Dr. Ntando Tebele was an invited speaker at the IV International Congress on Malaria and Babesiosis in Rio de Janeiro in August 1991. This invitation, based on her project research, provided an opportunity to present her results at an international forum and learn current developments in vaccine research in babesiosis, a co-endemic hemoparasitic disease.

Conference proceedings resulting from project:

1. Tebele, N.: Induction of protective immunity using *Anaplasma marginale* membranes. In Proceedings of the IV International Congress on Malaria and Babesiosis, no. 5.29. Rio de Janeiro, 1991.

2. Palmer, G.H., Barbet, A.F., Davis, W.C., Oberle, S.M., McGuire, T.C.: Characterization of *Anaplasma marginale* surface protein MSP-2 (36 kD) as a protective immunogen. In Proceedings of the Eighth National Veterinary Hemoparasite Disease Conference, R.J. Hidalgo, Ed. pp. 491-498. Louisiana State University, 1989.

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International network communications resulting from project:

1. Harrington, R.D., Palmer, G.H., and Knowles, D.P.: *Anaplasma* and *Babesia* vaccines. In Anaplasmosis and Babesiosis Network Newsletter, Volume 1, No. 3, pp.4-5. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.

2. Kieser, S.T., Knowles, D.P., and Palmer, G.H.: Virus vectors: a new era in vaccine technology. In Anaplasmosis and Babesiosis Network Newsletter, Volume 1, No. 4, pp.2-3. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.

3. Kieser, S.T., Palmer, G.H., and Knowles, D.P.: Mechanisms of immunity in hemoparasitic diseases. In Anaplasmosis and Babesiosis Network Newsletter, Volume 2, No. 1, pp.3-4. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.

4. Trueblood, E.S. and Palmer, G.H.: Anaplasmosis: a review of diagnostic techniques. In Anaplasmosis and Babesiosis Network Newsletter, Volume 2, No. 3, pp.2-4. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.

5. Raymond, R.J., Palmer, G.H., and Knowles, D.P.: The polymerase chain reaction: a new tool for detecting hemoparasitic diseases. In Anaplasmosis and Babesiosis Network Newsletter, Volume 2, No. 4, pp.1-2. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.

VIII. Project Productivity

The collaborative research in this PSTC project was designed to identify the MSP1a neutralization-sensitive epitope, extend the molecular comparison to a Zimbabwe strain of *A. marginale* (to be isolated during the project), and to test vaccines incorporating the MSP1a. Concomitantly, our goals were to develop research capacity in molecular approaches to vaccine

development in the Veterinary Research Laboratory. All goals stated in the proposal were met although the specific methodology was updated as new techniques became available. In addition, research early in the project identified important antigenic differences between the prototype Florida strain and the Norton, Zimbabwe strain isolated by our project. This identification led to an enhanced emphasis on characterization of the surface antigens of the Zimbabwe strain and testing defined outer membranes as protective immunogens. This approach, supported by a prior BOSTID project review, initiated a new direction in anaplasmosis vaccine research and identified new immunogens that are being characterized in ongoing research. This new approach would not have been initiated in absence of PSTC support.

IX. Future Work

The research in this project has had a significant impact on ongoing research in vaccine development against *A. marginale* both in Zimbabwe and other laboratories worldwide. The research done in this project has directly led to experiments based on our findings: i) development and testing of recombinant expressed MSP1a encoding either the repeats only (bearing the neutralization-sensitive epitope) or the non-repeat sections (funded by USAID); ii) use of the epitope-specific assays to measure antibody responses against MSP1a (included in USDA-BARD funded project 1561-88); iii) cloning and expression of the gene encoding the 19kD surface protein identified as an outer membrane protein in our studies (USDA Agricultural Research Service project); iv) fractionation of the outer membranes polypeptides for identification of protective subunit immunogens (Zimbabwe Veterinary Research Laboratory project); v) use of outer membranes of U.S. strains to induce protective immunity (joint research

at Oklahoma State University and Washington State University); and vi) determination of the mechanism of immunity by passive transfer of antibody from membrane immunized, protected cattle (Zimbabwe Veterinary Research Laboratory project). Specifically, the Veterinary Research Laboratory has initiated further research to identify which outer membrane polypeptides are responsible for the protective immunity induced by immunization with whole outer membranes. These additional immunization trials will include cattle immunized with the synthetic peptide polymer for direct comparison of efficacy between the polymer and polypeptides unique to the Zimbabwe strain. Importantly, the isolation and characterization of the Norton Zimbabwe strain has provided basis for comparison to vaccine research worldwide and for detection of variant strains in Zimbabwe. It is important to stress that the research in Zimbabwe, funded 100% by the PSTC project, initiated a new approach to anaplasmosis vaccine development that without project support would not have occurred.

In addition to research on anaplasmosis, the success of the approach to identifying protective immunogens using outer membrane preparations has led to collaboration with the Kenya Agricultural Research Institute using a similar approach in research on the rickettsia *Cowdria ruminantium*.

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Induction of Protective Immunity by Using *Anaplasma marginale* Initial Body Membranes

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Anaplasma marginale initial bodies of the Norton Zimbabwe strain were disrupted and separated into two membrane fractions banding at 1.15 and 1.22 g/cm^3 by sucrose density centrifugation. The membrane fractions differed in their morphology and polypeptide composition. Membranes banding at 1.22 g/cm^3 shared epitopes with surface-exposed polypeptides of the Florida strain of *A. marginale*, confirming the outer membrane location of these polypeptides. Immunization of cattle with either membrane fraction induced protection against homologous challenge, as demonstrated by significantly less anemia and lower peak rickettsemia values compared with those of adjuvant-immunized and nonimmunized calves. Protection correlated with antibody titer to membrane polypeptides. Although both membrane fractions induced protection, a 31-kDa polypeptide was the only common antigen to both fractions, as shown by reactivity of immune sera. Identification of membrane antigens capable of inducing protective immunity should facilitate development of vaccines against anaplasmosis suitable for use in Zimbabwe.

Anaplasmosis is a tick-borne disease widespread in sub-Saharan Africa with a 50 to 75% infection prevalence in Zimbabwe (16). The disease is caused by the rickettsia *Anaplasma marginale* and is characterized by intraerythrocytic rickettsemia, anemia, weight loss, and death of infected cattle (24, 31). Recovered animals remain carriers of anaplasma and act as reservoirs of infection for tick transmission to susceptible cattle (3, 8, 33). Anaplasmosis control in Africa is based on intensive application of acaricides to kill the tick vectors and prevent transmission of disease (14, 15). The purchase of acaricides requires the expenditure of foreign capital, which is not always available in developing countries. Prolonged use of acaricides creates a cattle population prone to epizootics when acaricide application is disrupted or acaricide resistance occurs in ticks (9). Consequently, in Zimbabwe and throughout Africa there is a focus on development of immunization strategies for more sustainable disease control.

Animals that recovered from infection with *A. marginale* have immunity against challenge with the homologous strain (25). This immunity is the basis for current use of infection (premunition) and treatment using live strains of *A. marginale* and *Anaplasma centrale* to protect cattle against anaplasmosis (25). Protection induced by premunition is effective against homologous challenge and challenge with less virulent strains. However, when challenged with a more virulent field strain, animals immunized by premunition are susceptible to infection and severe disease (19). Such incomplete protection was observed in cattle recovered from infection with the Florida strain and challenged with the Norton Zimbabwe strain and indicated the presence of both conserved and strain-specific protection-inducing antigens (29).

In developing control strategies based on immunization, we have evaluated membrane proteins of *A. marginale*. These proteins may be capable of generating protective immune responses which include direct initial body lysis, receptor blockade, or antibody-mediated phagocytosis (19). Since bacterial outer membranes contain surface-exposed polypeptides, our hypothesis is that immunization with an outer membrane fraction of *A. marginale* would induce protective immunity. Testing an outer membrane preparation will help identify specific antigens required for protection against challenge with Zimbabwe strains of *A. marginale*. Inclusion of these antigens in the development of subunit vaccines may be needed if inactivated vaccines are to be effective in Zimbabwe. In testing this hypothesis, we isolated an outer membrane fraction, identified antigens recognized by immune sera from protected cattle, and demonstrated that immunization with membrane fractions induced protection against homologous challenge.

MATERIALS AND METHODS

Organisms. *A. marginale* isolated in 1988 from a holstein steer with acute clinical anaplasmosis was designated the Norton Zimbabwe strain after the geographic area of isolation (29). The strain was maintained as a cryopreserved stabilate (10). Cryopreserved infected erythrocytes were thawed in a 37°C water bath, and initial bodies (individual organisms) were isolated by ultrasonic disruption and differential centrifugation as previously described (22). Protein concentration was determined by using the bicinchoninic acid assay (Pierce, Rockford, Ill.) with a standard curve based on bovine serum albumin in the identical buffer as described previously (27).

Immunoblotting. Initial bodies were solubilized and denatured by boiling for 3 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.025 mM Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 15% glycerol, 2.5% [vol/vol] β-mercaptoethanol, 0.02% bromophenol blue), electrophoresed in a 12% polyacrylamide gel with SDS (20 μg of protein per lane), and transferred to a 0.45-μm-pore-size nitrocellulose filter by electroblotting at 70 V for 3 h (30). The filters were blocked in Tris-Tween 20 solution (0.05% Tween 20, 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5% nonfat dry milk) overnight at 4°C (7). Sera diluted in blocking solution were incubated with filters

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TABLE 1. Conserved surface polypeptide epitopes between the Florida and Norton Zimbabwe strains of *A. marginale*

Monoclonal antibody	Polypeptide bound	Strain reactivity	
		U.S. strains (no. positive/total no.)	Norton Zimbabwe strain
Ana 22B1	MSP-1a[a]	9/9[b]	+
Am R38A6	MSP-1b	1/1[c]	+[d]
Ana F19E2	MSP-2	9/9[b]	+
43/19.3.5	MSP-3[e]	1/1[c]	−
43/23.2.8	MSP-3[e]	1/1[c]	+
Ana R76A1	AmF31	1/1[c]	+[d]
Ana F16C1	AmF19	1/1[c]	+
TRYP 1E1[f]	*T. brucei VSG*	0/9[b]	−

[a] See references 13, 21, and 23.
[b] Strains used were Florida, Idaho, Kansas, Missouri, North Texas, Oklahoma, Virginia, Washington-C, and Washington-O (21).
[c] To date, only the Florida strain has been tested.
[d] Positive only by immunofluorescence assay.
[e] See reference 12.
[f] Monoclonal antibody to a *T. brucei* variable surface glycoprotein (VSG), which was used as a negative control.

overnight at 4°C. The filters were then washed in blocking solution and incubated with biotin-labelled goat anti-bovine immunoglobulin G at a final concentration of 1 μg/ml in blocking solution for 1 h at room temperature. Avidin conjugated to alkaline phosphatase (diluted 1:1,000 in Tris-Tween 20) was added to the filters and incubated for 30 min (4). The enzyme solution was then discarded, and filters were washed three times in Tris-Tween 20. Antigen bands were developed following the addition of substrate solution (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM MgCl, 0.325 mg of nitroblue tetrazolium per ml, 0.165 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml) and incubation at room temperature (4). Filters were incubated in 20 mM Tris-HCl (pH 8.0) with 5 mM Na_2EDTA for 30 min to stop the reaction (4).

To compare the Norton Zimbabwe strain (29) with the Florida strain (25), immunoblotting and immunofluorescence assay were performed as previously described (17, 21), using seven monoclonal antibodies directed against surface polypeptides of the Florida strain (Table 1). This panel includes several antibodies reactive with epitopes conserved among U.S. strains (21). TRYP 1E1, a monoclonal antibody to the WaTat 1.1 *Trypanosoma brucei* variable surface glycoprotein (1), was used as the negative control.

Membrane isolation. *A. marginale* initial body membranes were isolated by using sucrose density gradient centrifugation (5, 6). Briefly, purified initial bodies (75 mg) were suspended in 2 ml of 20% sucrose in 10 mM *N*-2-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer with DNase and RNase A (50 μg/ml each). The suspension was sonicated for 6 min at 250 W and centrifuged at 1,000 × *g* for 15 min to pellet any residual initial bodies. The supernatant was layered on a sucrose step gradient consisting of 2 ml (each) of 52, 48, 44, 38, and 32% sucrose and centrifuged at 82,000 × *g* for 20 h at 4°C. After centrifugation, only two visible bands at 1.15 and 1.22 g/cm^3 were observed and they were designated fractions 1 and 2, respectively. The two fractions were individually collected by inserting a 12-gauge cannula attached to a 10-ml syringe into the top of the gradient. Fractions were individually suspended in cold 10 mM HEPES buffer (pH 7.4) and centrifuged at 177,000 × *g* at 4°C for 1 h. Pellets were collected and washed twice in cold 10 mM HEPES buffer by centrifugation at 177,000 × *g* for 1 h.

Each fraction was fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) overnight and processed for electron microscopy (11). Individual fractions were solubilized and denatured by boiling in SDS-PAGE buffer and electrophoresed on a 12% polyacrylamide gel containing SDS as previously described (22). Polypeptide bands were visualized by staining with Coomassie blue. Detection of membrane fraction antigens following electrophoresis on SDS-containing polyacrylamide gels was done by using monoclonal antibodies directed against Florida strain surface polypeptides in immunoblots as previously described (17).

Animal immunization and challenge. Twenty male holstein calves purchased when they were 1 to 4 days old were reared in fly- and tick-proof pens. Before immunization, blood from each calf was examined weekly for hemoparasites, packed cell volume (PCV), and hemoglobin concentration (Hb). During the 7-month observation period, no hemoparasites were detected on weekly microscopic examination of Giemsa stained blood smears from each calf, and PCV and Hb values were within normal ranges (2). Sera collected when the calves were 7 months old were tested for antibodies to *A. marginale* by immunoblotting. All calves were seronegative. The calves were then randomly assigned to four groups of five animals each. Each calf in the different groups received the following subcutaneous inoculation in 2 ml of phosphate-buffered saline: group I received 100 μg of membrane protein from fraction 1 in 6 mg of saponin; group II received 100 μg of membrane protein from fraction 2 in 6 mg of saponin; and group III received 6 mg of saponin. Group IV did not receive any inoculations. Identical booster inoculations were given to all calves in groups I, II, and III at 2, 6, and 10 weeks following initial immunization. Saponin was selected as the adjuvant on the basis of its ability to induce high antibody titers and T-lymphocyte responses to membrane antigens in cattle with minimal adverse tissue reaction (26, 32). Blood from all calves was monitored daily for hemoparasites, PCV, and Hb until challenge.

Serum samples from all calves were collected at 2-week intervals. After immunization, serum antibody titers to initial body polypeptides were determined by immunoblotting. Antibody titers were reported as reciprocals of the last serum dilution that resulted in one or more visible protein bands.

Seventy-two days after the first immunization, all calves were challenged with an intravenous inoculation of 4.8×10^4 live *A. marginale* parasitized erythrocytes of the Norton Zimbabwe strain in 2 ml of Hanks balanced salt solution free of calcium and magnesium (HBSS-CMF). To obtain live *A. marginale* parasitized erythrocytes, freshly collected blood from a splenectomized donor calf with 2% rickettsemia was diluted in HBSS-CMF and immediately inoculated. The calves were monitored daily for percent rickettsemia; PCV and Hb were monitored for a period of 90 days postchallenge. Differences between the highest prechallenge and lowest postchallenge PCV and Hb were determined for each calf and expressed as percentage decreases. The means and standard deviations for decrease in PCV, decrease in Hb, and peak rickettsemia values were determined for each group. An analysis of variance and a Fisher-protected least significant difference using square root-transformed data were used to determine significant differences between means (18). Correlation coefficients (r) and coefficients of determination (r^2) were calculated for antibody titer versus

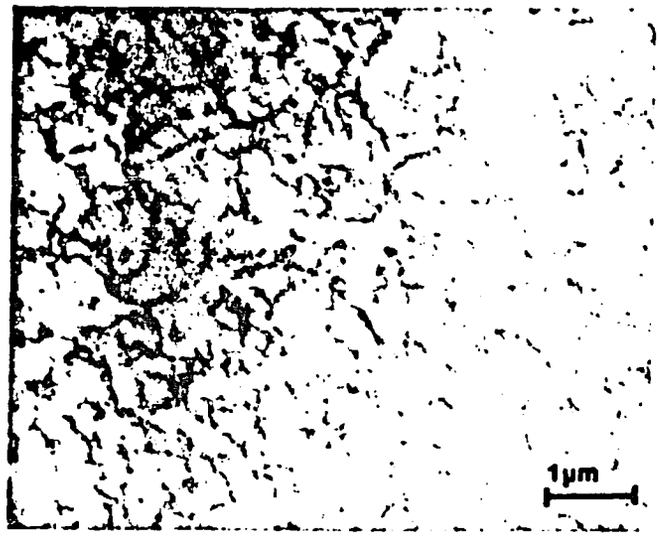

FIG. 1. Electron micrograph of membrane fraction 1 (1.15 g/cm^3) of the Norton Zimbabwe strain of *A. marginale*.

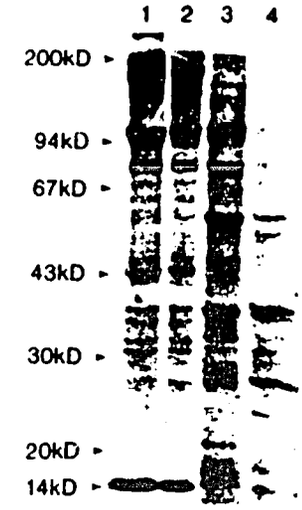

FIG. 3. Polypeptide composition of membrane fractions 1 and 2 of the Norton Zimbabwe strain of *A. marginale*. Membrane fractions were electrophoresed on a 12% polyacrylamide gel containing SDS, and protein bands were visualized by staining with Coomassie blue as previously described (22). Infected erythrocyte ghosts (lane 1), initial bodies (lane 2), membrane fraction 2 (lane 3), membrane fraction 1 (lane 4). Arrowheads mark the migration of indicated molecular size markers (in kilodaltons).

percent decrease in PCV and antibody titer versus percent decrease in Hb.

RESULTS

Isolation of membrane fractions. Sucrose density gradient centrifugation of disrupted initial bodies produced two visible bands at 1.15 and 1.22 g/cm^3. The bands were individually harvested as fractions 1 and 2, respectively. When examined by electron microscopy, both fractions consisted of membranes and no intact initial bodies were present (Fig. 1 and 2). Morphologically, fraction 1 consisted of small fragments of membranes and fraction 2 consisted of membrane vesicles. The fractions had different polypeptide profiles when evaluated on Coomassie blue-stained polyacrylamide gels containing SDS (Fig. 3). The polypeptide profiles of the membrane fractions were distinctly different from either purified initial bodies or erythrocyte ghosts containing initial bodies. The profile of infected erythrocyte ghosts is similar to that of purified initial bodies as a result of the presence of multiple initial bodies per erythrocyte ghost.

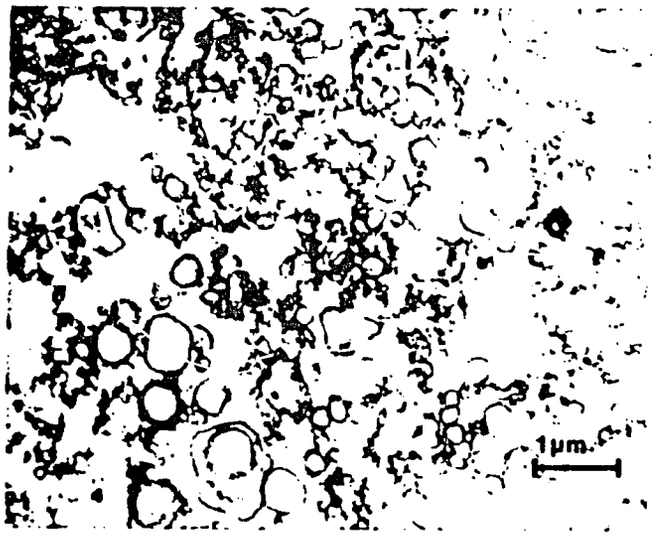

FIG. 2. Electron micrograph of membrane fraction 2 (1.22 g/cm^3) of the Norton Zimbabwe strain of *A. marginale*.

Antigenic characterization of Norton Zimbabwe strain initial bodies and membrane fractions. Monoclonal antibodies directed against surface polypeptides MSP-1a, MSP-1b, MSP-2, MSP-3, AmF31, and AmF19 of the Florida strain recognized epitopes on the Norton Zimbabwe strain (Fig. 4; Table 1). Characterization of membrane fractions with the same panel of monoclonal antibodies revealed that fraction 2 included polypeptides bearing surface-exposed epitopes conserved on MSP-1a, MSP-2, MSP-3, and AmF19 on the Florida strain (Fig. 5). No conserved epitopes were present in polypeptides of fraction 1 (data not shown).

Fraction 1—responses of calves to immunization and challenge. Serum immunoblot antibody titers from fraction 1-immunized calves ranged from 10^2 to 10^5 (Table 2). Three Norton Zimbabwe initial body polypeptides of 31, 28, and 26.5 kDa were recognized by immune sera diluted 1:50 from all membrane fraction 1-immunized calves (Fig. 6, lanes 11 to 15). The 26.5-kDa polypeptide was immunodominant in all five calves (data not shown). Sera from nonimmunized and saponin-immunized calves did not recognize any polypeptides on immunoblotting and had no antibodies to *A. marginale* (Fig. 6, lanes 1 to 5 and 16 to 20, respectively). All five fraction 1-immunized calves were protected from anemia, as demonstrated by significantly ($P < 0.05$) lower decreases in both PCV and Hb compared with the values for the five saponin-immunized calves and the five nonimmunized calves (see Table 4). The three calves with serum antibody titers of $\geq 10^4$ to these polypeptides were completely protected from microscopically detectable rickettsemia (Table 2). Decreases in PCV and Hb observed in the three completely protected calves were not significantly ($P < 0.05$) different from decreases due to daily variation observed in normal uninfected calves of the same age group reared under the same conditions (data not shown). The two fraction

41

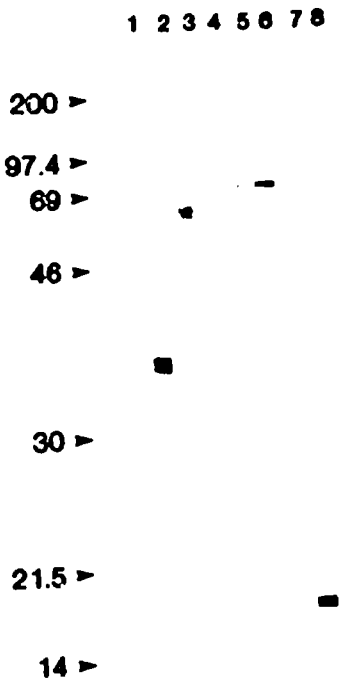

FIG. 4. Identification of epitopes conserved between the Norton Zimbabwe and Florida strains of *A. marginale*. Monoclonal antibodies directed against surface proteins of the Florida strain were reacted with Norton Zimbabwe strain initial body antigens separated by SDS-PAGE (20 μg per lane) and transferred to nitrocellulose filters as previously described (17). Conserved epitopes were identified by Ana F19E2 (lane 2), Ana 22B1 (lane 3), 43/23.2.8 (lane 6), and Ana F16C1 (lane 8). Monoclonal antibodies Am R38A6 (lane 4), 43/19.3.5 (lane 5), and Ana R76A1 (lane 7) did not recognize any epitopes when tested by this method. Monoclonal antibody TRYP 1E1 (lane 1) directed against a variable surface glycoprotein of *T. brucei* (1) was used as a negative control. Arrowheads mark the migration of indicated molecular size markers (in kilodaltons).

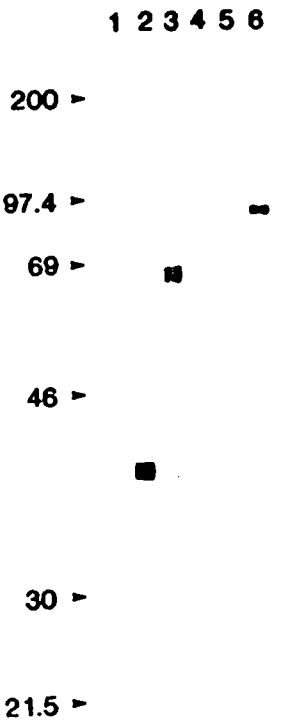

FIG. 5. Identification of epitopes conserved between membrane fraction 2 of the Norton Zimbabwe strain and the Florida strain of *A. marginale*. Monoclonal antibodies directed against surface proteins of the Florida strain were reacted (17) with membrane fraction 2 antigens separated by SDS-PAGE (20 μg per lane) and transferred to nitrocellulose filters. Conserved epitopes were identified by Ana F19E2 (lane 2), Ana 22B1 (lane 3), 43/23.2.8 (lane 6), and Ana F16C1 (data not shown). Monoclonal antibodies Am R38A6 (lane 4) and Ana R76A1 (lane 5) did not recognize any epitopes when tested by this method. Monoclonal antibody TRYP 1E1 (lane 1) directed against a variable surface glycoprotein of *T. brucei* was used as a negative control. Arrowheads mark the migration of indicated molecular size markers (in kilodaltons).

1-immunized calves with serum antibody titers of 10^3 and 10^2 developed mild anemia with peak rickettsemias of 1.7 and 4.8%, respectively (Table 2). The mean peak rickettsemia value of 1.3% for all five immunized calves was significantly ($P < 0.05$) lower than the mean rickettsemia values of 11.8% for saponin-immunized calves and 12.1% for nonimmunized calves (see Table 4). Within fraction 1-immunized calves, increase in antibody titer had a negative linear relationship with change in PCV ($r = -0.90$, $P < 0.05$) and in Hb ($r = -0.95$, $P < 0.05$). Decreases in PCV and Hb could be predicted by antibody titers, as shown by coefficients of determination of 0.81 and 0.90, respectively.

Fraction 2 (outer membrane fraction)-responses of calves to immunization and challenge. Four fraction 2-immunized calves had serum immunoblot antibody titers ranging from 10^5 to 10^6 and the fifth had a titer of 10^3 (Table 3). Sera from all calves recognized six major initial body polypeptides of 55, 36, 31, 30, 25, and 19 kDa on the Zimbabwe strain (Fig. 6, lanes 6 to 10), with the 55- and 36-kDa polypeptides being immunodominant in all calves. Two polypeptides, the 19- and 36-kDa polypeptides, share epitopes with AmF19 and MSP-2 of the Florida strain, respectively (Table 1). All calves were protected from anemia, as demonstrated by significantly ($P < 0.05$) lower decreases in PCV and Hb compared with those for saponin-immunized and nonimmunized calves (Table 4). The four membrane fraction 2-immunized calves with serum antibody titers of $\geq 10^5$ were completely protected from microscopically detectable rickettsemia (Table 3). Decreases in PCV and Hb observed in the completely protected calves were not significantly ($P < 0.05$) different from decreases due to daily variation observed in normal uninfected calves of the same age group

TABLE 2. Correlation of antibody titers and protection in *A. marginale* membrane fraction 1-immunized calves

Animal no.	Antibody titer[a]	Peak rickettsemia (%)	Decrease in PCV[b] (%)	Decrease in Hb[c] (%)
2	10^5	0	20.0	19.3
14	10^4	0	14.3	20.0
20	10^4	0	22.8	23.7
10	10^5	1.7	36.1	41.3
6	10^2	4.8	55.5	57.1

[a] Prechallenge antibody titers against Norton Zimbabwe strain initial bodies.

[b] Increase in antibody titer has a negative linear relationship with change in PCV ($r = -0.90$; $r^2 = 0.81$; $P < 0.05$).

[c] Increase in antibody titer has a negative linear relationship with change in Hb ($r = -0.95$; $r^2 = 0.90$; $P < 0.05$).

42

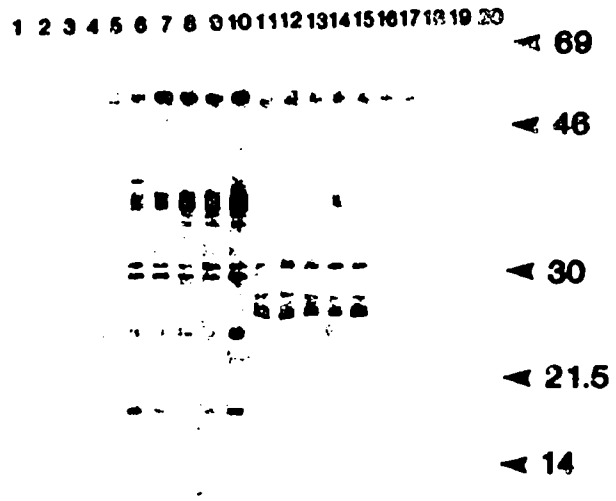

FIG. 6. Initial body polypeptides of the Norton Zimbabwe strain of *A. marginale* recognized by serum from membrane fraction-immunized calves. Sera were collected from all calves 72 days after initial immunization (prior to challenge), diluted 1:50, and reacted (20) with Norton Zimbabwe strain electrophoresed initial body antigens (20 μg per lane) in immunoblots. Sera from nonimmunized calves (lanes 1 to 5), saponin-immunized calves (lanes 16 to 20), fraction 1-immunized calves (lanes 11 to 15), and fraction 2-immunized calves (lanes 6 to 10). Arrowheads mark the migration of indicated molecular size markers (in kilodaltons).

reared under the same conditions (data not shown). The fifth calf, with a serum antibody titer of 10^3, developed mild disease, with a peak rickettsemia value of 2.3%. The mean peak rickettsemia value of 0.5% for fraction 2-immunized calves was significantly ($P < 0.05$) lower than the mean peak rickettsemia values of 11.8% for saponin-immunized calves and 12.1% for nonimmunized calves (Table 4). Within fraction 2-immunized calves, increase in antibody titer had a negative linear relationship with change in PCV ($r = -0.97$, $P < 0.05$) and in Hb ($r = -0.96$, $P < 0.05$). Decreases in PCV and Hb could be predicted by antibody titers, as demonstrated by coefficients of determination of 0.95 and 0.94, respectively.

TABLE 3. Correlation of antibody titers and protection in *A. marginale* membrane fraction 2-immunized calves

Animal no.	Antibody titer[a]	Peak rickettsemia (%)	Decrease in PCV[b] (%)	Decrease in Hb[c] (%)
3	10^6	0	20.6	21.6
15	10^6	0	17.6	19.8
7	10^5	0	22.6	25.8
11	10^5	0	21.9	22.6
22	10^3	2.3	33.3	35.6

[a] Prechallenge antibody titers against Norton Zimbabwe strain initial bodies.
[b] Increase in antibody titer has a negative linear relationship with change in PCV ($r = -0.97$; $r^2 = 0.95$; $P < 0.05$).
[c] Increase in antibody titer has a negative linear relationship with change in Hb ($r = -0.96$; $r^2 = 0.94$; $P < 0.05$).

TABLE 4. Responses of immunized and nonimmunized calves to challenge with the Zimbabwe strain of *A. marginale*

Immunogen	Mean ± SD[a] (%)		
	Decrease in PCV	Decrease in Hb	Peak rickettsemia
Fraction 1	29.7 ± 16.4*	32.3 ± 16.5*	1.3 ± 2.0*[b]
Fraction 2	23.2 ± 5.9*	25.12 ± 6.3*	0.5 ± 1.0*[c]
Saponin (control)	52.0 ± 4.9	54.7 ± 4.9	11.8 ± 4.6
None (nonimmunized)	49.8 ± 6.9	51.2 ± 6.4	12.1 ± 5.5

[a] All values with an asterisk were significantly different ($P < 0.05$) from values for saponin-immunized (control) and nonimmunized calves.
[b] Only two of five calves developed rickettsemias.
[c] Only one of five calves developed rickettsemia.

DISCUSSION

We have demonstrated that two membrane fractions can be resolved by sucrose density gradient centrifugation of disrupted *A. marginale* initial bodies. The evidence that the two fractions represented unique membrane preparations consists of the following: (i) morphologic differences, as demonstrated by electron microscopy; (ii) banding at different specific gravities, i.e., 1.15 g/cm^3 for fraction 1 and 1.22 g/cm^3 for fraction 2, which correlates with banding of *Rickettsia prowazekii* inner membranes at 1.19 g/cm^3 and outer membranes at 1.23 g/cm^3; (iii) distinctly different polypeptide profiles, as evaluated by Coomassie blue-stained SDS polyacrylamide gels; (iv) distinctly different reactivities with monoclonal antibodies to surface-exposed epitopes of the Florida strain; and (v) different antigenic specificities of antibodies produced by calves immunized with the two fractions. In view of the similarities in banding with characterized outer membranes of *R. prowazekii* (28) and the presence of surface-exposed polypeptides, fraction 2 appears to contain outer membranes of *A. marginale*.

Immunization with fraction 2 outer membranes induced protection against virulent homologous challenge with the Norton Zimbabwe strain. All outer membrane-immunized calves were protected from clinical disease, as demonstrated by significantly lower decreases in PCV and Hb compared with values for nonimmunized and saponin-immunized calves. Decreases in PCV and Hb are measures of the severity of anemia, which is a prominent clinical sign of anaplasmosis. It is significant that all immunized calves with antibody titers of $\geq 10^5$ against fraction 2 outer membrane polypeptides were completely protected from microscopically detectable rickettsemia. Similarly, calves immunized with fraction 1 membranes were protected from anemia and three of these calves were completely protected from rickettsemia.

Antibodies elicited by immunization with fraction 1 had different antigenic specificities from those elicited by the fraction 2 outer membranes. Diluted sera from calves immunized with outer membranes recognized six polypeptides, at least two of which share epitopes with surface-exposed polypeptides of the Florida strain. Only three polypeptides, including a 31-kDa polypeptide common to both membrane fractions, were recognized by sera from fraction 1-immunized calves. Protection in membrane-immunized calves could be due to responses to the 31-kDa polypeptide antigen common to both membrane fractions, antigens unique to each fraction, or a combination of unique and common antigens. Further fractionation of membranes and immunization trials will help identify those Norton Zimbabwe strain

43

polypeptides which are critical to inducing protective immunity.

We have shown a significant relationship between increase in antibody titer and protection against anemia. Coefficient of determination values indicate that 81 to 95% of protection from anemia can be predicted with antibody titers (18). All calves with antibody titers greater than 10^3 against membrane antigens were completely protected from rickettsemia. On the basis of this correlation, we plan to determine the effector role of antibody by using immunoglobulin transfer from membrane-immunized calves.

ACKNOWLEDGMENTS

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44

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CROSSPROTECTIVE IMMUNITY BETWEEN THE FLORIDA AND A ZIMBABWE STOCK OF *ANAPLASMA MARGINALE*

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SUMMARY

Cattle immunised by infection with the Florida stock of Anaplasma marginale were protected against subsequent homologous challenge, as demonstrated by complete prevention of microscopically detectable parasitaemia. Identically immunised cattle were partially protected against challenge with the Norton, Zimbabwe stock of A. marginale as determined by the significant prolongation of the prepatent period, a significantly lower peak parasitaemia, and a significantly lower percentage drop in haemoglobin concentration when compared to non-immunised calves challenged identically. The partial protection induced by live Florida stock immunisation demonstrates that while protection-inducing epitopes are shared between the two stocks, induction of complete immunity against a Zimbabwe stock may require alternative presentation of Florida stock epitopes or inclusion of additional Zimbabwe stock epitopes in the immunogen.

CONTD/

INTRODUCTION

020 Anaplasmosis, an arthropod-borne disease caused by the rickettsia *Anaplasma marginale*, is a constraint to agricultural development throughout livestock production areas within tropical and subtropical regions worldwide. Anaplasmosis is enzootic in much of sub-Saharan Africa including Zimbabwe (Norval, Fivaz, Lawrence and Brown, 1984). Zimbabwe cattle herds in both commercial and communal-land farms most commonly have an infection prevalence of 50 to 75% (Norval *et al.*, 1984). This prevalence indicates that infected carrier cattle and susceptible cattle are in contact, a situation resulting in clinically affected cattle during seasons of high transmission.

021 Anaplasmosis control in Zimbabwe has focused on reducing transmission by regular application of acaricides (Norval, 1979). The efficacy of acaricides as a control measure for anaplasmosis in Zimbabwe has been questioned (Norval, 1983). Prolonged acaricide treatment results in a highly susceptible cattle population subject to epizootics in the event of a breakdown in dipping services or development of acaricide resistance in ticks (Norval, 1979; Lawrence, Foggin and Norval, 1980). Vaccination has been recommended to control clinical anaplasmosis within both low and high prevalence regions in Zimbabwe (Norval *et al.*, 1984). Unfortunately, the current method of vaccination, inoculation with live *A. centrale*, provides variable protection and creates the risk of transmitting other infectious diseases (Rogers, Dimmock, de Vos and Rodwell, 1988). Consequently, *A. centrale* vaccine is not widely utilised in Zimbabwe (Lawrence and Norval, 1979). The above problems associated with current control measures indicate the need for an effective vaccine that will provide protection against anaplasmosis in enzootic areas.

022 The ability of purified *A. marginale* proteins to induce protection against challenge provides a basis for development of an inactivated vaccine containing protection-inducing antigens (Palmer, Barbet, Davis and McGuire, 1986; Palmer, Oberle, Barbet, Davis, Goff and McGuire, 1988a; Palmer, Barbet, Cantor and McGuire, 1989). To date, research on subunit vaccine development has focused on the Florida stock of *A. marginale*. This stock has been shown to induce crossprotective immunity against other *A. marginale* stocks in North and South America (Vizcaino, Corrier, Terry, Carson, Lee, Kuttler, Ristic and Trevino, 1980; Kuttler, Zaugg and Johnson, 1984; Palmer, 1989). *A. marginale* stocks from sub-Saharan Africa have antigenic differences from the Florida stock (Palmer, Barbet, Musoke, Rurangirwa, Katende, Pipano, Shkap, Davis and McGuire, 1988b). The objective of this study was to determine if the Florida stock, used as the prototype in recent vaccine development studies, will induce protective immunity in cattle against challenge with a recently isolated Zimbabwe stock.

MATERIALS AND METHODS

023 The Norton, Zimbabwe stock was obtained by intramuscular inoculation of 20 ml of blood from a steer with acute clinical anaplasmosis into a splenectomised Friesian calf. After the development of 20% parasitaemia, parasitised erythrocytes were washed and cryopreserved in dimethylsulfoxide as previously described (Love, 1972). The Florida stock, originally obtained by Ristic, was maintained as a dimethylsulfoxide cryopreserved stabilate (Ristic and Carson, 1977).

024 Sixteen Friesian calves, obtained at one to seven days of age, were kept in fly- and tick-free isolation pens. No haemoparasites were detected on daily microscopic examination of blood smears obtained from each calf and the calves had packed cell volume (PCV) and haemoglobin concentration (Hb) values within the normal range. At age three months, weight and gender matched calves were assigned to one of three groups. Groups I and II each had five calves while group III contained six calves. The groups of calves were kept in separate buildings throughout the experiment. All calves in groups I and II were immunised by intramuscular inoculation with 2×10^{10} Florida stock parasitised erythrocytes obtained directly from cryopreserved stabilate. Group III calves received no inoculum. PCV, Hb, and percent parasitaemia, using microscopic examination of Giemsa stained blood smears, were determined daily for all calves for 63 days. The means and standard deviations for decrease in PCV, decrease in Hb, days to development of 0·1% parasitaemia, days to development of peak parasitaemia and peak parasitaemia were determined for each group. Statistical significance between means was determined using Student's pooled t test with a $P < 0{\cdot}05$ (Steel and Torrie, 1980).

002 On day 64, group I calves were challenged by intramuscular inoculation with 2×10^{10} stabilate-derived parasitised erythrocytes of the Florida stock. Group II calves were similarly challenged by intramuscular inoculation with 2×10^{10} stabilate-derived parasitised erythrocytes of the Norton, Zimbabwe stock. Calves in group III, which had no previous exposure to *A. marginale*, were challenged with 2×10^{10} stabilate-derived parasitised erythrocytes of the Norton, Zimbabwe stock. PCV, Hb, and parasitaemia were determined daily for 63 days post-challenge. Data were analysed as described above for the responses to immunisation.

003

RESULTS

004 **Responses to immunisation with live organisms**

All calves in group I and II inoculated with the Florida stock of *A. marginale* developed acute anaplasmosis as demonstrated by development of microscopically detectable parasitaemia and anaemia (Table I). As expected, parasitised erythrocytes were not seen in group III calves which remained clinically normal. There were no statistically significant differences (at $P < 0{\cdot}05$) between group I and group II responses to live immunisation using the Florida stock. All calves in groups I and II recovered from acute infection as determined by the return of PCV and Hb to preinoculation levels.

005 **Responses to challenge**

The group I Florida stock immunised calves were protected from subsequent homologous challenge with the Florida stock as indicated by the absence of microscopically detectable parasitaemia (Table II). Decreases in PCV and Hb were significantly less following challenge compared to the response to the identical inoculum given for live immunisation (Tables I and II). Calves in group II also immunised with the Florida stock were partially protected against heterologous challenge with the Norton, Zimbabwe stock (Table II). The mean prepatent period and mean interval to peak parasitaemia were significantly longer and the peak parasitaemia significantly lower compared to the identically challenged but non-immunised group III calves (Table II). All group III calves developed parasitaemia on challenge. In contrast one of the group II calves did not develop any microscopically detectable parasitaemia after challenge with the Norton, Zimbabwe stock. The drop in Hb was significantly less in the group II calves compared to group III calves (Table II). However, there was no significant difference (at $P < 0{\cdot}05$) in the mean decrease in PCV between groups II and III. Protection against Zimbabwe stock heterologous challenge was not as complete as against Florida stock homologous challenge as indicated by the development of microscopically detectable parasitaemia in four of the five group II calves. In addition, the PCV and Hb were significantly decreased in group II calves on challenge compared to group I calves. No haemoparasites other than *A. marginale* were detected microscopically in the blood of any of the calves during the 63 day post-challenge observation period.

CONTD/

006 DISCUSSION

A. marginale stocks can be distinguished on the basis of morphology (Kreier and Ristic, 1963), protein structure (Barbet, Anderson, Palmer and McGuire, 1983; Oberle, Palmer, Barbet and McGuire, 1988), antigenic composition (McGuire, Palmer, Goff, Johnson and Davis, 1984), and recently, variation in genomic sequence (Allred, McGuire, Palmer, Leib, Harkins, McElwain and Barbet, 1990). Critically important to the development of widely applicable vaccines is induction of crossprotective immunity against heterologous stocks. In this study, we have isolated and initiated antigenic characterisation of an *A. marginale* stock, designated Norton, Zimbabwe.

007 Despite the practical drawbacks which have limited its effectiveness as a vaccine against anaplasmosis, immunisation by infection with live *A. marginale* induces solid immunity against homologous challenge. The ability of live vaccines to induce protection against heterologous challenge depends on the stock used to immunise the cattle and the challenge stock (Kuttler *et al.*, 1984). The efficacy of the Florida stock in inducing widely crossprotective immunity has been the basis for our isolation and characterisation of protective antigens using this stock (Palmer, 1989). Crossprotective immunity has been supported by identification of conserved neutralisation-sensitive epitopes on a molecular basis (Palmer *et al.*, 1986; Palmer *et al.*, 1988b, Allred *et al.*, 1990). Two different Florida stock surface polypeptides, MSP1 (105 kD) and MSP2 (36 kD) have been shown to induce protective immunity against challenge with either the homologous stock or a heterologous stock (Washington-O) that bears common epitopes on these surface polypeptides (Palmer *et al.*, 1988a; Palmer *et al.*, 1989). Prior to initiating antigenic characterisation of the recently isolated Norton, Zimbabwe stock, we used immunisation with live organisms to determine if protective antigens were conserved between the two stocks. Previously, Kuttler (1967) has used this method to demonstrate crossprotection between African (East African) and American (Beltsville and Nevada) stocks of *A. marginale*. The efficacy of live Florida stock immunisation in this trial was confirmed by the strong protection against homogous challenge. Under identical conditions, the significant protection against challenge with the Norton, Zimbabwe stock demonstrates that protection-inducing epitopes are shared between the two stocks. This observation provides a basis for further antigenic characterisation of the two stocks on a molecular level.

008 Although protected relative to non-immunised cattle, the Norton, Zimbabwe challenged cattle were only partially protected relative to the protection observed with homologous Florida stock challenge. Induction of complete protection against the Norton, Zimbabwe stock may require an immune response to a unique epitope not found on the Florida stock. If further antigenic characterisation confirms that a unique epitope is necessary, development of subunit vaccines based on the Florida stock alone will not be sufficient for use in Zimbabwe and possibly in many regions where isolates bear critical, different epitopes. Alternatively, the inability of live Florida stock immunisation to induce complete heterologous protection may reflect a relatively poor response to epitopes common between the stocks. Cattle immunised with MSP-1 as a subunit antigen emulsified in adjuvant developed antibody titers to a neutralisation-sensitive epitope that are 10^2 to 10^3 higher than titers induced by infection (Palmer *et al.*, 1989; unpub). These MSP-1 immunised animals were completely protected against challenge with the heterologous Washington-O stock. Complete protection against multiple heterologous stocks including the Norton, Zimbabwe stock may require antigen presentation that focuses the immune response to the relevant epitopes conserved among stocks.

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-43

11 pts only

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026 IMMUNITE CROISEE ENTRE UN STOCK *d'Anaplasma marginale* DE FLORIDE ET DU ZIMBABWE

027 **Résume** – Du bétail immunisé après une infection avec le stock Floride d'*Anaplasma marginale* a été protégé d'un risque d'infection homologue ultérieur, comme l'a révélé la prévention complète d'une parasitémie décelable au microscope. Du bétail immunisé dans des conditions analogues s'est trouvé partiellement protégé d'une infection par le stock Norton du Zimbabwe. Confirmation en a été donnée par l'allongement significatif de la période prépatente, un pic significantivement plus bas de la pourcentage de la concentration en hémoglobine, par comparaison avec des veaux non immunisés et soumis au même risque. Alors que les épitopes qui engendrent la protection sont partagés entre les 2 stocks, la protection partielle induite par l'immunisation à l'aide du stock vivant Floride montre que la création d'une immunité complète contre un stock Zimbabwe peut exiger soit la présence alternative

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TABLE I

Responses of calves to live immunisation with the Florida stock of A. marginale

Parameter[1,2]	Group I	Group II
Mean drop in PCV (%)	50·1 ± 8·1	40·8 ± 8·6
Mean drop in Hb (%)	48·3 ± 12·1	41·8 ± 7·4
Mean days to 0·1% parasitaemia	18·6 ± 1·3	20·6 ± 3·7
Mean days to peak parasitaemia	26·2 ± 1·5	27·6 ± 2·1
Mean peak parasitaemia (%)	9·9 ± 7·9	2·9 ± 1·9

[1] Means with standard deviations were calculated for each parameter.
[2] There were no significant differences in any parameter between group I and II calves at $P < 0·05$.

TABLE II

Responses of immunised and non-immunised calves to challenge with the Florida stock or Norton, Zimbabwe stock of A. marginale

Parameter[1,2]	Group I	Group II	Group III
Mean drop in PCV (%)	20·2 ± 4·4	43.6 ± 11·0	52·2 ± 3·8
Mean drop in Hb (%)	21·8 ± 1·6	43·1 ± 7·3	53·5 ± 3·5
Mean days to 0·1% parasitaemia	—[3]	21·0 ± 1·8[4]	14·2 ± 1·0
Mean days to peak parasitaemia	—[3]	26·5 ± 1·6	21·8 ± 1·5
Mean peak parasitaemia (%)	—[3]	2·6 ± 2·6	6·6 ± 1·7

[1] Means with standard deviations were calculated for each parameter.
[2] Significant statistical differences between groups are described in the text.
[3] None of the five group I calves developed microscopically detectable parasitaemia.
[4] One of the group II calves did not develop microscopically detectable parasitaemia; means and standard deviations for days to 0·1% and peak parasitaemia were determined using the four parasitaemic calves.

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Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*

(tick-borne diseases/rickettsia/gene structure/tandem repeats)

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ABSTRACT Anaplasmosis is one of several tick-borne diseases severely constraining cattle production and usage in many parts of the world. Cattle can be protected from anaplasmosis by immunization with major surface protein 1, a surface protein of *Anaplasma marginale* carrying a neutralization-sensitive epitope. Marked size polymorphisms exist among different isolates of *A. marginale* in the AmF105 subunit of major surface protein 1, yet all isolates still contain the neutralization-sensitive epitope. To clarify the basis for these observations, the *mspIα* gene encoding AmF105 was cloned from four isolates and sequenced. The encoded polypeptides share a high degree of overall homology between isolates but contain a domain with various numbers of tandemly repeated sequences and three regions of clustered amino acid substitutions outside the repeat domain. The polypeptide size differences are completely explained by the variations in the numbers of tandem repeat units. We have mapped the neutralization-sensitive epitope to a sequence that is present within each repeat unit. These results identify a basis for size polymorphisms of the surface polypeptide antigen concomitant with B-cell epitope conservation in rickettsiae.

Anaplasmosis, a hemoparasitic disease of cattle caused by the rickettsia *Anaplasma marginale*, is devastating to the production, utilization, and movement of cattle. A half-billion cattle are at risk worldwide, primarily in tropical and subtropical areas, restricting particularly the advancement of lesser-developed countries. Annual losses due to anaplasmosis total more than $100 million in the United States (1), where animal husbandry practices limit the effects of the disease.

A. marginale is transmitted through the bite of infected ticks or by contaminated needles or fomites (2, 3) and invades only circulating erythrocytes (4). Antibody-mediated immunity to anaplasmosis is likely to be particularly important (5), due to a lack of parasite stages susceptible to direct cell-mediated cytotoxicity. One target of humoral immunity is the immunoprotective (5) *m*ajor *s*urface *p*rotein 1 (MSP-1) (6). A subunit of this heterodimeric protein (5, 7, 8), AmF105, exhibits apparent size polymorphisms of up to 50% among isolates (6), yet all isolates tested from the United States, Israel, and Kenya carry an epitope sensitive to neutralization by mouse monoclonal antibody Ana22B1 (mAb Ana22B1) (7, 9, 10). To understand the molecular basis for these observations, we cloned and sequenced the gene (*mspIα*)¶ for this subunit from four isolates. The epitope recognized by mAb Ana22B1 was then mapped to determine its involvement in the size variation.

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MATERIALS AND METHODS

Cloning of the *mspIα* Gene. DNA was isolated from purified *A. marginale* initial bodies as described (5, 8). Plasmid pAMT1 was constructed by partial *Sau*3A digestion of Florida isolate (FL) genomic DNA, C-tailing, ligation (11) into G-tailed pUC9 plasmid, and transformation of *Escherichia coli* JM83 (12). pAMT1, which expresses a 56-kDa product, was isolated by expression screening (13) with mAb Ana22B1 and ^{125}I-labeled protein A (8). To obtain the complete gene, *Nco* I linkers were added to FL genomic DNA random-sheared by sonication, with ligation into the expression vector pKK233-2. Transformants were screened with mAb Ana22B1 as above, yielding plasmid pKAna420. The insert of pKAna420 was subcloned into the *Sma* I site of plasmid pGEM-4 after filling-in the *Nco* I overhangs (11), yielding plasmid pFL10. pVA1 was cloned as a *Kpn* I fragment, whereas pID6 and pWA1 were cloned as *Kpn* I–*Pst* I fragments in pGEM-4. pVA1, pID6, and pWA1 were isolated by colony hybridization screening (14) with ^{32}P-radiolabeled (15) pAMT1 sequences.

Immunoblot Analysis of Recombinants. Recombinants were analyzed by SDS/polyacrylamide gel electrophoresis (16) on 7.5–17% (wt/vol) polyacrylamide gradient gels and by immunoblotting with mAb Ana22B1 and ^{125}I-labeled protein A (5).

Nucleic Acid Analyses. DNAs were isolated, restriction mapped, and compared by Southern blot analysis (11). Plasmid inserts were sequenced as double-stranded DNA (17, 18), using Sequenase (United States Biochemical) as recommended by the manufacturer. SP6 and T7 promoter-specific primers were used in the initial sequencing reactions, and then new oligonucleotide primers were synthesized (19) based on the sequences obtained ("primer-walking"). RNA was isolated from FL initial bodies (20) and sequenced by a modification (21) of the method of Inoue and Cech (22). The primer was the reverse complement of bases 147FL to 166FL (for bases 147 to 166 of the FL isolate sequence; all sequence numbering hereafter is given relative to FL).

Computer Analyses of Sequence Data. Sequence homology searches were performed using the FASTN program (23) (Cyborg Database Manager, International Biotechnologies). Probable transcription termination sites, structural characteristics, and hydropathy of AmF105 were predicted as described (24–26).

Abbreviations: ORF, open reading frame; mAb, monoclonal antibody; MSP-1, major surface protein 1.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M32868–M32872).

Mapping of the Neutralization-Sensitive Epitope. The epitope recognized by mAb Ana22B1 was mapped by assaying antibody binding to synthetic oligopeptides containing overlapping portions of the AmF105 tandem repeat unit B form. Antibody binding was assayed by a solution-phase inhibition radioimmunoassay using ^{125}I-labeled AmF105 (27) and was confirmed by an ELISA (28) and by immunoblots (8), using peptides linked to the solid phase with glutaraldehyde.

RESULTS

Cloning of the *mspla* Gene. To characterize the *mspla* gene (named for inclusion of its encoded product as a subunit of MSP-1) among *A. marginale* isolates, we chose the following isolates for analysis: Florida (FL) and Virginia (VA) because they express the largest and smallest polypeptides, respectively, of the isolates tested to date (6); Idaho (ID) because it appeared the most variable by restriction endonuclease analysis; and Washington-O (WA-O) because cattle immunized with FL MSP-1 complexes were protected from challenge with the WA-O isolate (29). After cloning of the *mspla* genes, the fidelity of all four cloned fragments with the chromosome was confirmed by Southern blot restriction analysis. A single copy of the *mspla* gene was detected at the same chromosomal locus in each isolate (Fig. 1). The expression of full-sized immunoreactive products by each transformant gave further evidence that the recombinant constructs represent the *mspla* gene (Fig. 2).

Definition of *mspla* Gene Structure. Plasmid pAMT1 and portions of each of the four cloned DNA inserts were sequenced and the *mspla* genes were defined to determine how the isolates differ (Figs. 1 and 3). One long open reading frame (ORF) was present in each, encompassing the same region of each sequence. Sequencing of total cellular RNA from FL initial bodies with a primer complementary to a region near the 5′ end of the long ORF identified base 1FL as the transcription start site (Figs. 3 and 4). The transcription termination site is predicted (24) to be at base 2458FL, shortly after the stop codon at 2429FL.

The position of base 1FL within the cloned fragments suggests that transcription of the *mspla* gene is under control of the *mspla* promoter, an assertion supported by the expression of AmF105 at comparable levels when the gene is placed in either orientation in a promoterless vector (data not shown). The presumptive *mspla* promoter was identified by its location relative to the transcription start site and by its similarity with *E. coli* promoter consensus sequences and structures (30) (Fig. 3). The spacings of the −35, −10 and start sites of all four *mspla* genes exactly match those of the *E. coli* consensus sequence (30). The *mspla* alleles have apparently untranslated leaders of 127 (FL, WA-O, and VA) or 71 (ID) bases defined by the start of transcription and the start methionine codon at position 128FL (Fig. 3). Despite large differences in this region, the FL, WA-O, and ID genes are all expressed at comparable levels by *E. coli* (DH5α) recombinants (Fig. 2), indicating a lack of effect on translational control.

The probable start of translation is the methionine codon at position 128FL for the following reasons. (*i*) The only long ORF in this gene begins 24 base pairs (bp) upstream of this codon. (*ii*) The upstream methionine codon at base 45FL is not in the same reading frame as the long ORF and is absent

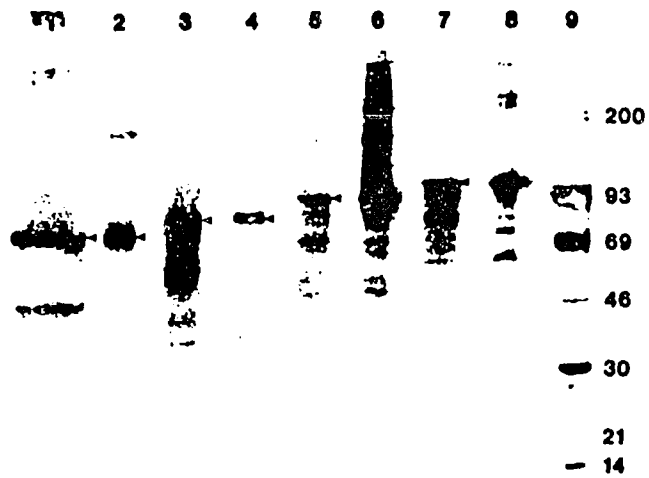

FIG. 2. Expression of immunoreactive products by *E. coli* recombinants and *A. marginale* initial bodies. The full-sized polypeptide bands recognized by mAb Ana22B1 are indicated by arrowheads. The major immunoreactive product expressed by each recombinant matches in size the native polypeptide from the corresponding isolate initial bodies. The lower molecular mass products may be breakdown products or may reflect the use of adventitious ribosome binding sites by *E. coli* (see *Discussion*). Lanes: 1, 3, 5, and 7, recombinants pVA1, pWA1, pID6, and pFL10, respectively; 2, 4, 6, and 8, VA, WA-O, ID, and FL isolate initial bodies, respectively; 9, ^{14}C-radiolabeled molecular mass standards; apparent molecular masses are indicated in kDa.

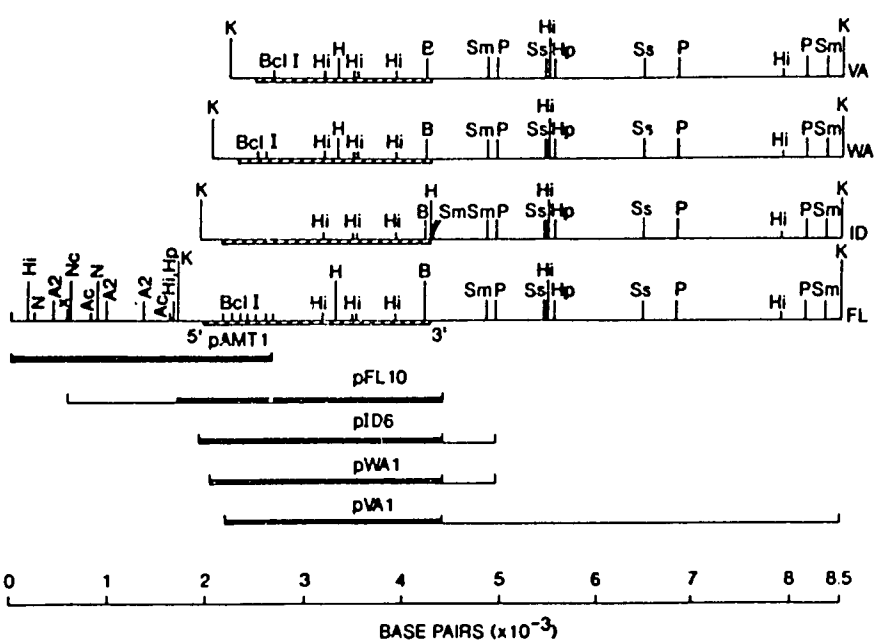

FIG. 1. Restriction maps of *A. marginale* chromosomal DNAs and the recombinant plasmids carrying the *mspla* gene. The nearly identical restriction maps of each isolate demonstrate that the *mspla* genes occupy the same physical locus on the chromosome. The cross-hatched boxes on the genomic maps indicate the coding region of each *mspla* allele; the solid boxes on the plasmid maps are the regions that were sequenced. Isolate chromosomes: FL, Florida; ID, Idaho; WA, Washington-O; VA, Virginia. Ac, *Acc* I; A2, *Ava* II; B, *Bam*HI; BclI, *Bcl* I (multiple sites in WA and FL isolates); H, *Hin*dIII; Hi, *Hin*cII; Hp, *Hpa* I; K, *Kpn* I; N, *Nsi* I; Nc, *Nco* I; P, *Pst* I; Sm, *Sma* I; Ss, *Sst* I; X, *Xma* III.

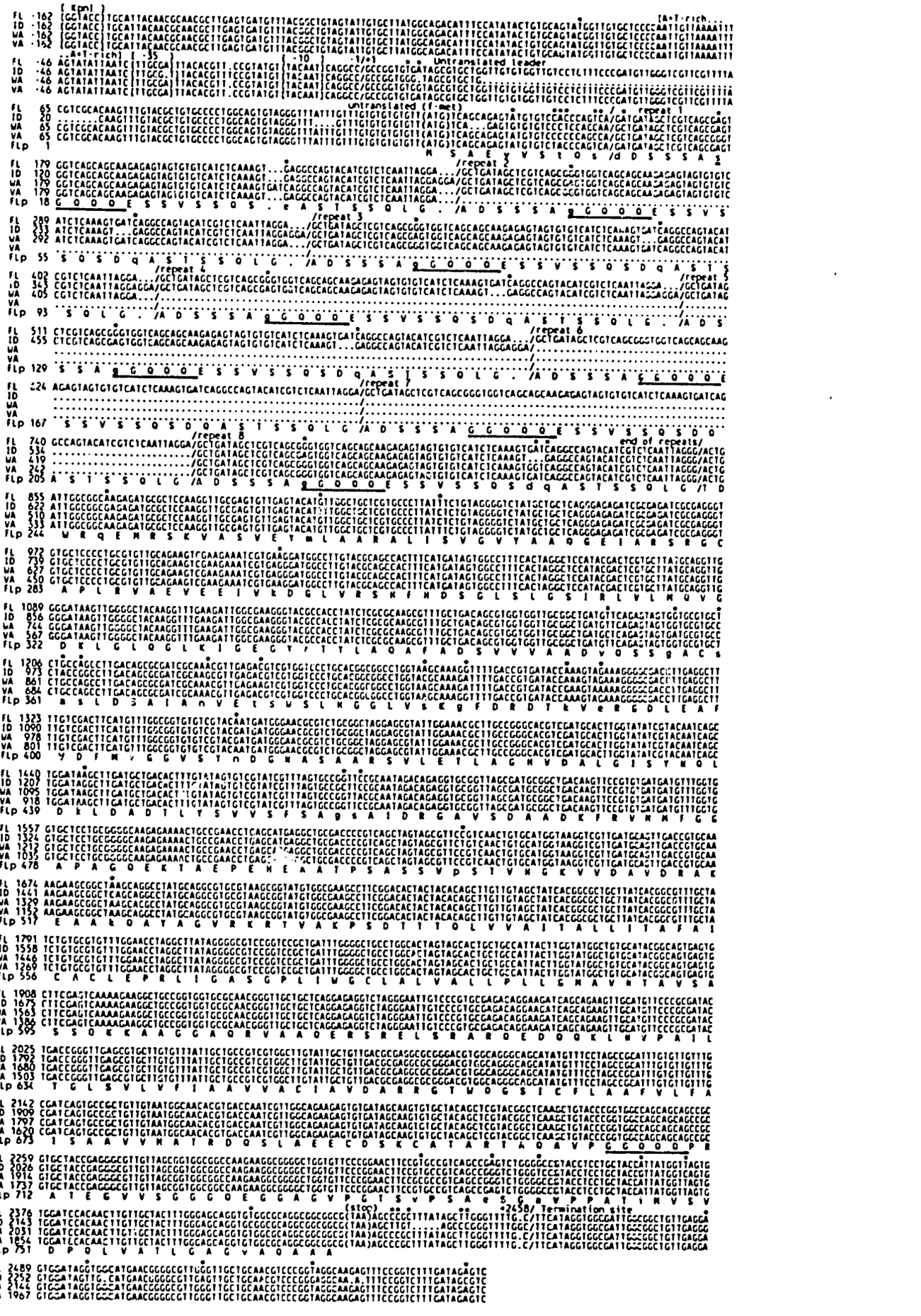

FIG. 3. *(Legend appears at the bottom of the opposite page.)*

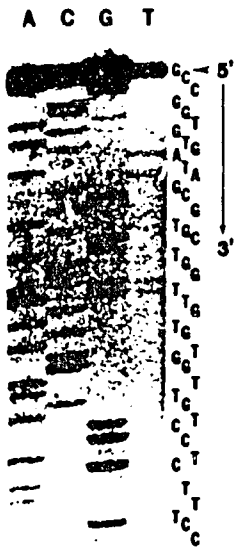

FIG. 4. Start of transcription from the FL isolate *mspla* gene. Total cellular RNA from FL initial bodies was sequenced to identify the 5' end of the mRNA (defined as base 1FL). The sequence of the mRNA is given on the right side, reading from 5' to 3'; this is the reverse complement to the sequence read directly from the gel.

altogether in ID. (*iii*) There are no other methionine codons in the ORF until a point beyond that contained in plasmid pAMT1, which expresses a fragment of the polypeptide. (*iv*) mAb Ana22B1 binds to a synthetic oligopeptide encoded only by this reading frame. In each isolate, the long ORF extends to a stop codon at base 2429FL (Fig. 3). This results in coding sequences and polypeptide lengths of 2301 bp and 767 amino acids in FL, 1779 bp and 593 amino acids in VA, 1956 bp and 652 amino acids in WA-O, and 2124 bp and 708 amino acids in ID (Fig. 3).

The most notable feature of the *mspla* genes is a series of 84- or 87-bp sequences (i.e., 28 or 29 amino acids) that are tandemly repeated two (VA), four (WA-O), six (ID), or eight (FL) times (Fig. 3; Table 1). The tandem repeats immediately follow a short variable region at the N-terminal ends of the polypeptides. Among the four isolates, five forms of the tandem repeats are present (Table 1; forms A–E). The repeat sequences vary minimally, with 25 amino acid residues completely conserved in all five forms (Table 1). The variations in the number of tandem repeats in each isolate can completely explain the size polymorphisms. Even so, the polypeptides migrate anomalously during electrophoresis, appearing much larger than the encoded size, a common effect among proteins containing tandem repeats (31, 32).

The identity of these genes as *mspla* variants is confirmed by the high degree of homology throughout their coding regions, including a 639-bp region from bases 1686FL to 2324FL that is completely conserved. However, there are three regions of clustered variability in the coding sequence. In the first 30 bp of the coding sequence FL, VA, and WA-O each have three differences, whereas ID has only 27 bp in this region, of which five differ. This region is thus 10 or 9 amino acids long, with 3 substitutions between isolates, of which 2 are nonconservative. Base substitutions at the 3' end result in 5 amino acid differences among the isolates in the final 35 residues. Finally, between bases 1184FL and 1303FL, 11 base changes result in the substitution of 11 of 40 amino acids (Fig. 3). Eight of the 11 substitutions are nonconservative.

Mapping the Epitope Sensitive to Antibody-Mediated Neutralization. The neutralization-sensitive epitope recognized by mAb Ana22B1 was mapped because of its potential importance to immunity. The minimum structure necessary to bind mAb Ana22B1 was found by ELISA to be the 6-amino acid sequences Gln-Ala-Ser-Thr-Ser-Ser and Glu-Ala-Ser-Thr-Ser-Ser (Table 1), found in the tandem-repeat domain. Conformation may influence binding, as the IC_{50} measured by inhibition RIA with native MSP-1 as antigen was 70- to 100-fold less for a 29-amino acid polypeptide representing a B repeat (Asp-Ser-Ser-Ser-Ala-Gly-Gly-Gln-Gln-Gln-Glu-Ser-Ser-Val-Ser-Ser-Gln-Ser-Asp-Gln-Ala-Ser-Thr-Ser-Ser-Gln-Leu-Gly-Ala) compared with the 6-mers (41 pmol for the 29-mer versus 3900 pmol or 2800 pmol for Gln-Ala-Ser-Thr-Ser-Ser or Glu-Ala-Ser-Thr-Ser-Ser, respectively). The 5-amino acid oligopeptides, Gln-Ala-Ser-Thr-Ser and Ala-Ser-Thr-Ser-Ser, did not bind detectable amounts of antibody.

Table 1. Tandem repeat forms present in the FL, VA, WA-O, and ID variants of the *mspla*-encoded polypeptides

Form	Sequence	Number in allele FL	VA	WA	ID
A	DDSSSASGQQQESSVSSQS · (EASTSS)QLG ·	1	1	0	0
B	ADSSSAGGQQQESSVSSQSD(QASTSS)QLG ·	7	1	3	0
C	ADSSSAGGQQQESSVSSQSG(QASTSS)QLG ·	0	0	1	0
D	ADSSSASGQQQESSVSSQS · (EASTSS)QLGG	0	0	0	5
E	ADSSSASGQQQESSVSSQS · (EASTSS)QLG ·	0	0	0	1

The epitope recognized by mAb Ana22B1 is in parentheses; residues common to all five forms are underlined. Deletions are indicated by dots. The number of each repeat form in each isolate is given on the right. In each isolate, repeat forms are present in alphabetical order relative to the N-terminal end (e.g., in FL there is one A form followed by seven B forms). The single-letter amino acid code is used.

Predicted Structure of the AmF105 Polypeptide. Although highly charged, the repeat domain contains no positive amino acids and is predicted (25) to be comprised almost solely of coil/turn segments, consistent with presentation of short hydrophilic epitopes (33). This contrasts with the remainder of the polypeptide that is predicted to have a high overall helical content. In addition, a hydropathy plot (26) of the predicted polypeptide revealed five major hydrophobic stretches: amino acids 255FL to 270FL, 541FL to 557FL, 567FL to 585FL, 631FL to 650FL, and 662FL to 678FL—the last four of which are sufficient in length and hydrophobicity to serve as transmembrane domains. Since there is no obvious N-terminal signal sequence, one of the internal regions may be an uncleaved internal signal sequence (34) for localization of AmF105 in the outer membrane.

DISCUSSION

These studies on *mspla*, encoding a major surface polypeptide, MSP-1, of *A. marginale*, revealed four important findings. (*i*) The large size variations of the *mspla*-encoded polypeptides among *A. marginale* isolates are explained by the presence of a domain containing various numbers of tandem repeats. Although size differences among isolates in immunologically cross-reactive antigens have been observed in other rickettsia (35, 36), the basis for this was unknown. (*ii*) The neutralization-sensitive epitope recognized by mAb Ana22B1 is defined and is present in every tandem repeat unit of each isolate. (*iii*) In the polypeptides there are three

Fig. 3 (*on opposite page*). DNA sequences of the *mspla* genes obtained from FL, VA, WA-O, and ID isolates of *A. marginale*. The DNA sequences are given from the 5' *Kpn* I site of each clone to the same point corresponding to the 3' end of the FL isolate cloned insert. The predicted sequence of the FL *mspla*-encoded polypeptide (FLp) is indicated beneath the DNA sequences, the single-letter amino acid code being placed beneath the first base of each codon. Annotated above the sequences are the *Kpn* I site, features of the promoter region, the transcription start and predicted termination sites, the start and stop codons of the presumed coding sequences, and the tandem repeat units. Variant bases are indicated by superior asterisks, variant amino acids are in lower-case letters, and insertions/deletions are indicated by dots. The 3' region homologous with the repeat region (see *Discussion*) is double-underlined there and in the repeats.

54

regions of clustered variability, including the N-terminal end, perhaps representing immunologic targets. (*iv*) The rickettsial *mspla* gene uses promoter structures similar to the *E. coli* consensus promoter (30).

One significant difference emerged between *A. marginale* and *E. coli* gene structure. Although mspla mRNA is expressed in *E. coli*, no obvious ribosome binding site was detected in the untranslated leader. The sequence GTGTGTG, found in the −11 to −5 position (relative to the ATG codon), may allow ribosome binding (the sequence of the *E. coli* 16S rRNA is 5'-GAUCACCUCCUUA-3') (37), as a sequence from *Rickettsia rickettsii* with the same pattern of alternating guanine bases, AGAGAGA, also enables expression in *E. coli* (38). This may reflect a difference in the ribosome binding sites used by rickettsiae as compared with other Gram-negative bacteria. In addition, in each repeat there is a GTG codon preceded by a GAGAG sequence 5–9 bases upstream. These sequences may serve as alternative start sites in *E. coli* and may explain some of the lower apparent molecular mass bands found in recombinants expressing the *mspla* gene.

Repeat structures, such as those in *mspla*, are thought to develop by unequal homologous recombination (39), slipped-strand mispairing during replication (40), or both. The involvement of entire repeat units during these events could explain the presence of various repeat numbers, as in the group A streptococci where unequal homologous recombination provides antigenic variation (21) or in *Neisseria gonorrhoeae* where slipped-strand mispairing controls phase variation of the P.II surface protein gene (41). Sequences sharing significant homologies with a 42-bp region of the repeats (236FL to 277FL) are seen at other sites within (bases 2240FL to 2254FL) and outside the *mspla* coding sequence. That same 42-bp sequence also shares sequence similarities with a number of invasive or mobile DNAs, including avian sarcoma virus, Fujinami sarcoma virus, and the maize transposable elements, activator and dissociation (71%, 68%, and 69% similarity, respectively) (42–45). An upstream region containing this sequence, centered around base −1300FL, is surrounded by interspersed direct and inverted repeats (data not shown), a common characteristic of mobile elements. Should a mobile element have invaded the *A. marginale* chromosome, sequences may have been retained upon its exit, giving rise to the repeats.

It is enigmatic that a surface-exposed neutralization-sensitive epitope encoded by sequences of potentially high genetic plasticity remains constant despite immune pressure. The ubiquity of tandemly repeated epitopes in the surface proteins of taxonomically distant parasites (21, 31, 32, 41, 46–49) suggests that such domains fulfill essential functions or impart selective advantages. These data on the structure and variability of a rickettsial surface protein gene and its encoded product should aid in dissection of the immune response to these pathogens, their potential mechanisms of immune evasion, and the development of vaccines.

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55

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Immunization of Cattle with the MSP-1 Surface Protein Complex Induces Protection against a Structurally Variant *Anaplasma marginale* Isolate

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The *Anaplasma marginale* surface protein complex MSP-1 of the Florida isolate is composed of a 105-kilodalton (kDa) polypeptide, which bears a neutralization-sensitive epitope, and a 100-kDa polypeptide. Antigenically similar polypeptides in the Okanogan, Wash. (Washington-O), isolate MSP-1 are 86 and 100 kDa, respectively. Immunization of cattle with Florida isolate MSP-1 induced antibody titers to both MSP-1 polypeptides and protected cattle against homologous and heterologous challenge.

Rickettsial pathogens vary antigenically and structurally among strains and consequently may vary in their ability to induce cross-protection against heterologous strains (3, 5, 7, 8, 19). In bovine anaplasmosis, caused by the intraerythrocytic rickettsia *Anaplasma marginale*, cattle that have recovered from acute infection are protected against homologous challenge but are usually susceptible to infection with heterologous isolates (7, 8). Significantly, the Florida isolate of *A. marginale* appears to induce postinfection immunity against heterologous isolates (8, 17, 20). Consequently, we have used the Florida isolate to identify surface proteins for vaccine development (14). Immunization of cattle with a 100- to 105-kilodalton (kDa) surface protein complex, identified in the Florida isolate with neutralizing antibodies, induces protection against challenge with a homologous isolate of *A. marginale* (12). The ability of the 100- to 105-kDa surface complex, designated major surface protein-1 (MSP-1), to induce heterologous protection has not been tested.

The Florida isolate MSP-1 is composed of two noncovalently linked polypeptides of 105 and 100 kDa (2). The 105-kDa polypeptide (referred to in reference 2 as 105U) bears surface-exposed epitopes, including a neutralization-sensitive epitope conserved among *A. marginale* isolates (12, 13, 16). The 100-kDa polypeptide, previously referred to as 105L, also has surface-exposed epitopes (2). Although MSP-1 is conserved as a bimolecular complex in different *A. marginale* isolates, the molecular size of the polypeptide components varies markedly among isolates (11). The polypeptide bearing the conserved neutralization-sensitive epitope is 105 kDa in the Florida isolate MSP-1 complex and is 70 to 100 kDa in the complexes of the five isolates characterized to date (11). The second component of the MSP-1, 100 kDa in the Florida isolate, varies by approximately 3 kDa among the five isolates (11).

The Okanogan, Wash. (Washington-O), isolate differs antigenically, morphologically, and in protein composition from the Florida isolate (1, 6, 7, 9). Despite the antigenic differences, the neutralization-sensitive epitope on the MSP-1 is conserved (9, 12). Comparison of MSP-1 between the Florida and Washington-O isolates was done by using immunoprecipitation and immunoblotting with antibodies previously defined against each of the Florida MSP-1 polypeptides (2). Following radiolabeling of *A. marginale* proteins with [^{35}S]methionine during short-term in vitro cultivation of each isolate (1), 10^5 cpm (acid precipitable) were reacted with either monoclonal antibody ANA 22B1 (specific for the 105-kDa polypeptide) or rabbit antibody R911 (specific for the 100 kDa polypeptide). Bound complexes were precipitated with protein A-bearing *Staphylococcus aureus*, and the specifically bound polypeptides were identified by polyacrylamide gel electrophoresis and fluorography. As previously demonstrated (11), immunoprecipitation of Florida isolate proteins with either monoclonal antibody ANA 22B1 (Fig. 1) or rabbit antibody R911 (data not shown) precipitated both the 105-kDa polypeptide and the 100-kDa polypeptide. The Washington-O isolate polypeptides precipitated by either antibody were 100-kDa and 86-kDa components (Fig. 1). Therefore, as expected, the bimolecular nature of MSP-1 is conserved in the Washington-O isolate.

The antigenic identity of each Washington-O MSP-1 polypeptide was determined by immunoblotting. Approximately 100 μg of solubilized whole-organism antigen (Washington-O isolate) per lane was electrophoresed in 5% polyacrylamide gels containing 4 M urea to separate the MSP-1 polypeptides. The antigens were electrophoretically transferred to 0.45-μm-pore-size nitrocellulose and reacted with either monoclonal antibody ANA 22B1 or rabbit antibody R911, and antibody binding was detected with ^{125}I-protein A. Monoclonal antibody 22B1, which binds the Florida isolate 105-kDa polypeptide, bound the 86-kDa component but not the 100-kDa component of the Washington-O MSP-1 (Fig. 2). Rabbit antibody R911, which recognizes the Florida isolate 100-kDa polypeptide, bound only to the 100-kDa component in the Washington-O MSP-1 (Fig. 2). Therefore, the polypeptide bearing the neutralization-sensitive epitope is approximately 19 kDa smaller in the Washington-O isolate than in the Florida isolate. In contrast, the size of the second polypeptide, shown to have minor size variation among other characterized isolates, is similar in both the Washington-O and Florida isolates.

The ability of MSP-1 to induce antibody to each polypeptide component and to induce cross-protective immunity was assessed by immunization of cattle with Florida isolate MSP-1. MSP-1 was purified from the Florida isolate *A. marginale* by using monoclonal immunoaffinity chromatography as previously described (12). Seronegative cattle were

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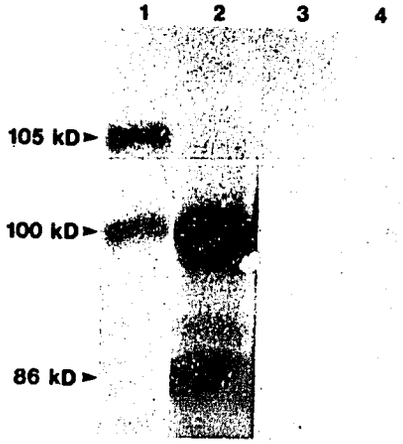

FIG. 1. Comparison of MSP-1 complexes between the Florida and Washington-O isolates of *A. marginale*. Organisms from each isolate were radiolabeled with [^{35}S]methionine, detergent disrupted, and immunoprecipitated with monoclonal antibody ANA 22B1, which recognizes a conserved MSP-1 epitope. Immunoprecipitates were identified by polyacrylamide gel electrophoresis with fluorography. Florida isolate proteins were immunoprecipitated with monoclonal antibody ANA 22B1 (lane 1) or control monoclonal antibody TRYP 1E1 (lane 3). Washington-O isolate proteins were immunoprecipitated with ANA 22B1 (lane 2) or TRYP 1E1 (lane 4). Arrows in the left margin designate the apparent molecular masses, in kilodaltons, of the polypeptides.

immunized with 50 μg of MSP-1 emulsified in complete Freund adjuvant for the initial immunization and in incomplete adjuvant for three subsequent immunizations at 3-week intervals. Control seronegative cattle were immunized with 50 μg of ovalbumin emulsified in identical adjuvants and boosted on an identical schedule. Following the last immunization, antibody titers to each MSP-1 component were determined by endpoint titration by using serial dilutions of sera in immunoblots (4, 15). All cattle immunized with

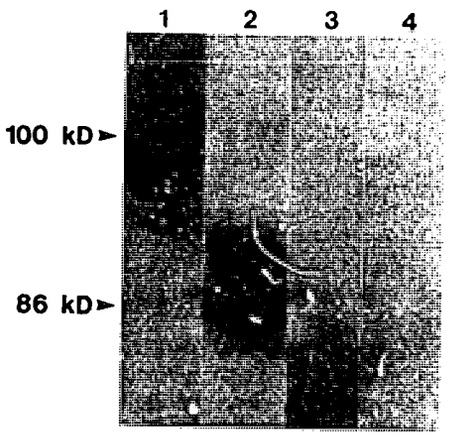

FIG. 2. Antigenic identity of MSP-1 polypeptides in the Washington-O isolate of *A. marginale*. Washington-O isolate antigens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Antibody binding was detected by reaction with ^{125}I-protein A, followed by autoradiography. Lanes: 1, rabbit antibody R911 (defined against the Florida isolate 100-kDa polypeptide); 2, monoclonal antibody ANA 22B1 (defined against the Florida isolate 105-kDa polypeptide); 3, control rabbit antibody (against *E. coli*); 4, control monoclonal antibody TRYP 1E1.

TABLE 1. Protection of cattle immunized with Florida isolate MSP-1 against challenge with the *A. marginale* Florida isolate

Immunogen and animal no.	Titer of antibody to: 105-kDa polypeptide	Titer of antibody to: 100-kDa polypeptide	DPC to 1% rickettsemia[a]	Peak rickettsemia (%)	Low PCV (%)[b]
MSP-1					
B209	256,000	128,000	23	4.5	22.5
B211	256,000	128,000	Uninfected	0.0	29
B218	256,000	256,000	26	2.2	25.5
B226	128,000	64,000	Uninfected	0.0	29
B227	128,000	128,000	26	4.0	24.5
Ovalbumin					
B219	—[c]	—	16	25.6	13
B220	—	—	18	8.1	23
B222	—	—	25	9.7	22
B224	—	—	16	8.9	23.5
B225	—	—	15	10.1	26

[a] Rickettsemia was determined by daily microscopic examination of Wright-stained blood smears for 75 DPC.
[b] PCV, Packed-cell volume.
[c] —, Sera from all ovalbumin-immunized cattle were unreactive with *A. marginale* polypeptides, including MSP-1, at the lowest dilution tested, 1:500.

MSP-1 developed antibody to both polypeptide components (Tables 1 and 2). On the basis of responses of all MSP-1-immunized cattle, there was no significant difference (P = 0.68) in the titers of the MSP-1 components, as evaluated with the paired t-test (18). The range of titers was from 1:64,000 to 1:256,000 against each polypeptide. Sera from all ovalbumin-immunized cattle were unreactive against *A. marginale* antigens, including both MSP-1 polypeptides (Tables 1 and 2).

Five MSP-1-immunized cattle and five ovalbumin-immunized cattle were challenged by intramuscular inoculation of 10^{10} Florida isolate-infected erythrocytes derived from cryopreserved stabilate (homologous challenge). All five ovalbumin-immunized cattle developed microscopically detectable rickettsemia and had 1.0% infected erythrocytes in a mean of 18 days postchallenge (DPC) (Table 1). Cattle in this

TABLE 2. Protection of cattle immunized with Florida isolate MSP-1 against challenge with the *A. marginale* Washington-O isolate

Immunogen and animal no.	Titer of antibody to: 105-kDa polypeptide	Titer of antibody to: 100-kDa polypeptide	DPC to 1% rickettsemia[a]	Peak rickettsemia (%)	Low PCV (%)[b]
MSP-1					
B187	64,000	128,000	Uninfected	0.0	36
B194	64,000	128,000	Uninfected	0.0	35
B196	128,000	128,000	Uninfected	0.0	33
B203	64,000	64,000	Uninfected	0.0	35
B208	128,000	128,000	Uninfected	0.0	33
Ovalbumin					
B189	—[c]	—	25	4.8	27.5
B195	—	—	25	3.6	25
B197	—	—	29	3.0	27.5
B207	—	—	22	5.5	26.5
B217	—	—	25	4.6	26

[a] Rickettsemia was determined by daily microscopic examination of Wright-stained blood smears for 75 DPC.
[b] PCV, Packed-cell volume.
[c] —, Sera from all ovalbumin-immunized cattle were unreactive with *A. marginale* polypeptides, including MSP-1, at the lowest dilution tested, 1:500.

control immunized group developed a mean peak of 12.5% infected erythrocytes. The mean low packed-cell volume, a measure of the anemia characteristic of acute anaplasmosis, was 21.5%. In contrast, two of the five MSP-1-immunized cattle did not develop detectable rickettsemia (Table 1). The three MSP-1 vaccinates that were infected upon challenge were partially protected as determined by the significant prolongation of the prepatent period (DPC to 1% infected erythrocytes) as compared with the ovalbumin-immunized cattle ($P < 0.025$, pooled t-test). As a group, the MSP-1-immunized cattle had significantly lower peak rickettsemia (mean of 2.1%) ($P < 0.025$) and were significantly less anemic (mean packed cell volume of 26%) ($P = 0.055$) than ovalbumin-immunized cattle.

Cross-protective immunity induced by MSP-1 immunization was tested by heterologous challenge with intramuscular inoculation of 10^{10} Washington-O isolate-infected erythrocytes derived from cryopreserved stabilate. The Washington-O isolate was less virulent than the Florida isolate, as judged by challenge infections in the control cattle (Table 2). The Washington-O isolate caused significantly less severe disease on the basis of all three parameters: DPC to 1.0% infection, peak rickettsemia, and minimum packed-cell volume ($P < 0.03$ for all parameters by the pooled t-test) (18). All five ovalbumin-immunized cattle challenged with Washington-O isolate *A. marginale* developed microscopically detectable rickettsemia in a mean of 25 DPC (Table 2). The ovalbumin-immunized cattle reached a mean peak of 4.3% infected erythrocytes and a mean low packed-cell volume of 26.5%. In contrast to the challenge infections in the ovalbumin-immunized cattle, none of the five MSP-1-immunized cattle challenged with the Washington-O isolate developed microscopically detectable infection (Table 2). The packed cell volumes were unchanged from prechallenge levels (data not shown).

Protection against antigenically and structurally variant isolates of *A. marginale* is a primary requirement for development of an improved vaccine (10). The identification of isolates structurally variant in the MSP-1 raised the possibility that, despite conservation of a neutralization-sensitive epitope, the MSP-1 may not induce significant protection against challenge with a heterologous isolate (11). The demonstration that immunization of cattle with the Florida isolate MSP-1 induced complete protection against challenge with the Washington-O isolate indicates that MSP-1 epitopes relevant to cross-protection are conserved. Determination of the extent of conservation among isolates and the ability of MSP-1 immunization to induce widely cross-protective immunity requires challenge with additional isolates. The contribution of each MSP-1 polypeptide to the protective immunity is unknown. Both polypeptides have surface-exposed epitopes and are immunogenic when presented in the MSP-1 complex. The requirement for each polypeptide in a vaccine will be determined by using individual purified recombinant-derived polypeptides for immunization.

The complete protection afforded the five calves challenged with the Washington-O isolate versus the complete protection in only two Florida isolate-challenged calves is probably the result of the Washington-O being significantly less virulent. Complete protection of only a proportion of the MSP-1-immunized cattle following Florida isolate challenge was similar to the results of immunization experiments previously reported (12). The basis for the differences in protection is not clear; there were no significant differences in antibody responses to either MSP-1 component between completely protected cattle and partially protected cattle. Similarly, there were no obvious differences between the two groups in the ability of antibody to promote *A. marginale* opsonization (data not shown). Although in vitro incubation of antibody with *A. marginale* neutralizes infectivity (12, 14), the mechanism of neutralization in MSP-1-immunized cattle is unknown. Identification of the basis for neutralization in vivo is needed to understand the difference between complete and partial protection following virulent challenge.

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Molecular Size Variations in an Immunoprotective Protein Complex among Isolates of *Anaplasma marginale*

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A major surface protein complex from the Florida isolate of *Anaplasma marginale* has been previously shown to induce protection in immunized cattle and has been proposed as the basis of a subunit vaccine against anaplasmosis. This complex in the Florida isolate is composed of two noncovalently associated polypeptides with molecular masses of 105 and 100 kilodaltons (kDa). The analogous protein complex from four geographically different isolates of *A. marginale* was immunoprecipitated and compared with the protein complex of the Florida isolate. The polypeptides of the complex varied in apparent molecular mass among the isolates. By using antibodies recognizing epitopes on each polypeptide of the Florida isolate, the antigenic identity of the polypeptides in the analogous complexes was determined. The polypeptides recognized by the neutralizing monoclonal antibody $22B_1$, which recognizes a 105-kDa polypeptide in the Florida isolate, ranged from 70 to 100 kDa in the other isolates. Those polypeptides recognized by rabbit antiserum R911, which recognizes a 100-kDa polypeptide in the Florida isolate, ranged from 97 to 100 kDa. The surface-exposed peptides in the complexes were compared by limited enzymatic digestion to assess structural homology among isolates. Despite the marked variations in molecular weight, there were conserved peptides between the $22B_1$-reactive polypeptides and between the R911-reactive peptides. Determination of the role of the conserved peptides in inducing immunity will be critical in the application of these polypeptides as the basis of a subunit vaccine for bovine anaplasmosis.

Bovine hemoparasite infections are a significant economic hindrance to the improvement of meat, milk, and fiber production in lesser-developed nations. The most prevalent of these diseases, anaplasmosis, is enzootic to nearly half the world's livestock production regions (18). Responsible for an estimated 100 million dollars per year in economic losses in the United States alone (16), anaplasmosis generates severe losses through mortality, reduced weight gains, and decreased milk production. Control of the causative rickettsia, *Anaplasma marginale*, is presently attempted by chemotherapy, vector control, and vaccination. However, current forms of vaccination, including live, attenuated strains (24) or killed, whole-organism preparations, have proven inadequate for widespread use, and the U.S. Agency for International Development has placed a high priority on the development of more effective immunization (18).

A promising approach towards immunoprophylaxis is a subunit vaccine. Monoclonal antibodies against an erythrocyte-stage surface protein of the Florida isolate of *A. marginale* neutralize infectivity (20). This major surface protein, designated MSP-1, is recognized by postinfection antisera from cattle immune to *A. marginale*, regardless of the isolate used to infect the cattle (21). MSP-1 has the ability to induce protection in immunized cattle against both a homologous (20) and a heterologous (G. H. Palmer, T. C. McGuire, and A. F. Barbet, unpublished data) *A. marginale* challenge and has been proposed as the basis of a subunit vaccine.

When purified from the Florida isolate of *A. marginale*, MSP-1 consists of a complex of two separate gene products (3), with apparent molecular masses of 105 and 100 kilodaltons (kDa), designated AmF105 and AmF100, respectively (by genus, species, isolate, and molecular mass in kilodaltons). The exact nature of the noncovalent association between AmF105 and AmF100 is unknown, but the polypeptides may be resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis under reducing conditions. AmF105 bears a neutralization-sensitive epitope common to all isolates of *A. marginale* examined in the United States, Israel, and Kenya (21a).

Demonstrated differences in morphology (10), antigenicity (17), virulence (11), tick transmissibility (27), protein structure (2), and ability to induce cross-protection (12) exist among the various isolates of *A. marginale*. Because an MSP-1-based subunit vaccine must protect against multiple isolates of *A. marginale* within a region, it is essential to determine whether differences exist between the polypeptides of the MSP-1 complex among various isolates. In this study, we examined these polypeptides from four antigenically distinct isolates and compared them with AmF105 and AmF100. The antigenic identity and apparent molecular mass of each polypeptide were determined, and the surface-exposed peptides were compared by partial proteolysis to assess structural homology among isolates.

MATERIALS AND METHODS

Source of *A. marginale* organisms. Five field isolates of *A. marginale* were used in this study. The isolates are designated by the original location of isolation (17): Florida (F), southern Idaho (I), northern Texas (T), Virginia (V), and Clarkston, Wash. (W). The isolates were collected (11) and stored in liquid nitrogen as an infected-blood stabilate (15) before being used to initiate infections. Thawed stabilate (20 ml) from each isolate was inoculated intramuscularly into splenectomized 4-month-old, male Holstein calves. Calves

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were monitored daily by determination of percent parasitemia and hematocrit.

^{35}S labeling of organisms. *A. marginale* initial bodies were radiolabeled during short-term erythrocyte culture, as described previously (2). Barbet et al. (2) demonstrated that the radiolabel is incorporated exclusively into the initial bodies during this procedure. Briefly, approximately 10 ml of blood was drawn from each calf when its parasitemia reached between 15 and 30%. The blood was washed three times with Hanks balanced salt solution without calcium or magnesium and then was washed once more with Eagle minimal essential medium containing Earle salts, 10% fetal bovine serum, 2 mM L-glutamine, 100 μg of streptomycin per ml, and 100 U of penicillin per ml. After each sterile wash, the buffy coat was removed. Washed erythrocytes were diluted 1:8 in minimal essential medium, and [^{35}S]methionine (125 μCi/ml) was added. The suspension was incubated at 37°C with 5% CO_2 in air for 48 h. Cultures were collected and washed four times in Hanks balanced salt solution. Organisms were lysed by dilution in a 50 mM Tris (pH 8.0) buffer containing 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-alpha-*p*-tosyl-L-lysyl-chloromethyl ketone (TLCK), 1% Nonidet P-40 (NP-40), and 0.1% SDS, and frozen at −70°C until used.

^{125}I surface labeling of organisms. *A. marginale* initial bodies were purified from infected-blood stabilates as previously described (22). The isolated organisms were suspended in 250 μl of phosphate-buffered saline (10 mM sodium phosphate, 14 mM NaCl [pH 7.4]). Approximately 5 × 10^8 isolated *A. marginale* initial bodies were labeled with ^{125}I (1 mCi) by the lactoperoxidase method (26). Free iodine was removed by G-50 column chromatography, and incorporation of the label was determined by trichloroacetic acid precipitation.

Antibodies. All antibodies were prepared and screened as previously described (7). $22B_1$ is a neutralizing monoclonal antibody recognizing an epitope of AmF105 common to multiple isolates of *A. marginale* (17, 20). T1E1 is a monoclonal antibody specific for the variable surface glycoprotein of *Trypanosoma brucei* and serves as a negative control. R911 is a polyclonal, monospecific rabbit antiserum recognizing the complete recombinant polypeptide, AmF100, as expressed in *Escherichia coli* (3). R907 is a rabbit serum against *E. coli* without the inserted AmF100 gene and serves as a negative control for R911. R767 and R865 are rabbit antisera recognizing all mouse antibody subclasses.

Immunoprecipitation and electrophoresis of surface proteins. Labeled *A. marginale* initial bodies were disrupted by detergent and sonication, centrifuged, and filtered, as previously described (22). Approximately 10^6 trichloroacetic acid-precipitable cpm of ^{35}S-labeled proteins or 10^7 trichloroacetic acid-precipitable cpm of ^{125}I-labeled proteins were diluted with TEN buffer (20 mM Tris, 5 mM EDTA, 10 mM NaCl [pH 7.6]) with 1% NP-40 and 0.1% SDS. Either 5 μg of $22B_1$ (or T1E1) or 10 μl of R911 (or R907) was added, and the solution was incubated at 4°C for 40 min. A 10-μl quantity of R767 was added to the $22B_1$ (T1E1) precipitations, followed by another 40-min incubation at 4°C. A 100-μl quantity of a 10% (wt/vol) suspension of protein A-bearing *Staphylococcus aureus* was added, and the solution was incubated for 30 min at 4°C. Pellets were washed six times with TEN with NP-40 and SDS; the final four washes were done with an additional 2 M NaCl. The pellets were suspended and washed two more times in TEN without detergent. The precipitates were suspended in 50 μl of sample buffer (2% SDS, 2.5% 2-mercaptoethanol, 25 mM Tris [pH 6.8], 15% glycerol, 0.002% bromophenol blue) and boiled, and the supernatants were either frozen at −20°C or used directly on 5% polyacrylamide gels containing 4 M urea under reducing conditions. ^{14}C-labeled proteins for molecular mass comparisons consisted of the following: myosin, 200 kDa; phosphorylase *b*, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa, trypsin inhibitor (soybean), 21.5 kDa; lysozyme, 14.3 kDa; cytochrome *c*, 12.5 kDa; aprotinin, 6.5 kDa; B chain of bovine insulin, 3.4 kDa; and A chain of bovine insulin, 2.3 kDa. The gels were fixed in 40% methanol for 30 min, and ^{35}S-labeled gels were processed for fluorography by immersion in En^{3}Hance (New England Nuclear Corp.) and then in deionized water. Vacuum-dried gels were exposed to Kodak XAR-5 X-ray film at −70°C. Gels containing iodine-labeled proteins were exposed to X-ray film with an intensifying screen.

Western blots (immunoblots). *A. marginale* initial bodies were isolated, suspended in electrophoresis sample buffer, and separated on a 5% polyacrylamide gel with 4 M urea. The polypeptides were transferred onto nitrocellulose (pore size, 0.2 μm) blocked with 1% bovine hemoglobin in a pH 7.6 buffer containing 17 mM NaCl, 10 mM Tris, and 0.1 mM phenylmethylsulfonyl fluoride. The nitrocellulose was incubated for 30 min with the above buffer plus 0.1% SDS, 1% Triton X-100, 1 mM EDTA, and either a 1:100 dilution of R911 or R907 or 2 μg of $22B_1$ or T1E1 per ml. The nitrocellulose was washed in the same buffer, and the $22B_1$ or T1E1 filters were incubated for an additional 30 min with a 1:2,000 dilution of R865, followed by four more washes. All nitrocellulose filters were then incubated for 30 min with 10^7 cpm of ^{125}I-labeled protein A and washed 10 times, the final 5 without hemoglobin. The nitrocellulose was dried, covered with Saran Wrap, and exposed to X-ray film, as above.

Antigenic identity of immunoprecipitated polypeptides. Autoradiographs were aligned with dried gels, and the major ^{125}I-labeled bands immunoprecipitated by $22B_1$ or R911 were excised. The gel was placed back on X-ray film to ascertain that the protein bands had been properly removed. The gel fragments were rehydrated in TEN buffer with NP-40 and SDS and electroeluted into dialysis tubing, with a molecular weight cutoff of 10,000, at 150 V and 25 mA for 3 h. The current was reversed for 10 min, and the gel fragments were pelleted by centrifugation. The supernatant was collected, and the eluted polypeptides were individually reimmunoprecipitated with $22B_1$ and R911, as described above.

Structural relationship of immunoprecipitated polypeptides. Autoradiographs were aligned with dried gels, and the ^{125}I-labeled polypeptides were cut out as described above. The gel fragments were then placed into the wells of the stacking gel and allowed to rehydrate in running buffer. Polypeptides were digested in the gel for 45 min with 1.5 μg of *S. aureus* V8 protease, as previously described (4). Polyacrylamide gels (10% polyacrylamide) were fixed, vacuum dried, and exposed to X-ray film, as described above.

RESULTS

Immunoprecipitation of *A. marginale* proteins. The major polypeptides precipitated by $22B_1$ have apparent molecular masses of approximately 105 kDa in the Florida, 97 kDa in the south Idaho, 89 kDa in the north Texas, 70 kDa in the Virginia, and 86 kDa in the Washington isolates (Fig. 1). Other bands were present with apparent molecular masses of 100 kDa in the Florida, Virginia, and Washington isolates, 97 kDa in the Idaho isolate, and 98 kDa in the Texas isolate.

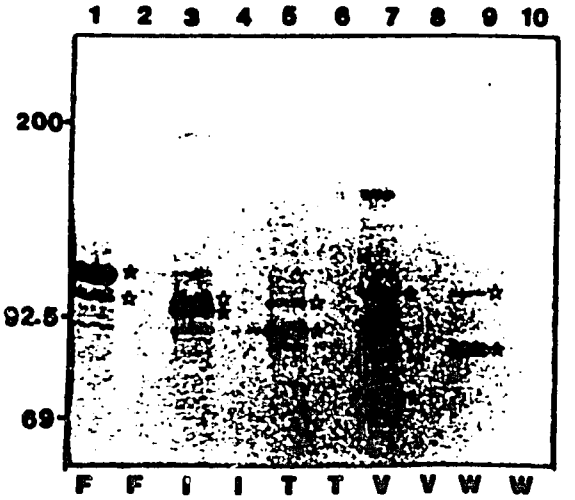

FIG. 1. Polyacrylamide electrophoresis of *A. marginale* polypeptides immunoprecipitated by monoclonal antibodies $22B_1$ (lanes 1, 3, 5, 7, and 9) or T1E1 (lanes 2, 4, 6, 8, and 10), as described in Materials and Methods. F, I, T, V, and W represent the Florida, south Idaho, north Texas, Virginia, and Clarkston, Wash., isolates of *A. marginale*, respectively. The polypeptides later demonstrated to be recognized by $22B_1$ (★) and polypeptides later demonstrated to be recognized by rabbit antiserum R911 (☆) (see text and Table 1) are indicated. The numbers to the left of the gel represent the positions of the molecular mass markers (in kilodaltons).

Polypeptides ranging from 82 to 92 kDa occasionally appeared in most isolates but were inconsistent. The major polypeptides precipitated by R911 have apparent molecular sizes of 100 kDa in the Florida, Virginia, and Washington isolates, 97 kDa in the Idaho isolate, and 98 kDa in the Texas isolate (Fig. 2). Similarly, fainter bands appeared with molecular masses correlating with the molecular masses of the major bands immunoprecipitated by $22B_1$ (i.e., 70 to 105 kDa). No proteins were precipitated by either T1E1 or R907.

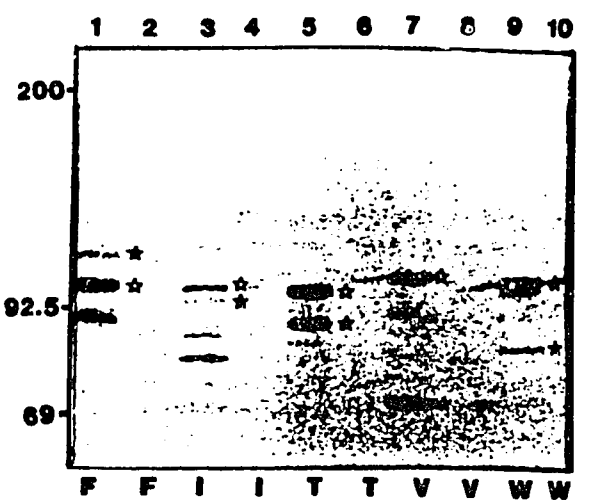

FIG. 2. Polyacrylamide electrophoresis of *A. marginale* polypeptides immunoprecipitated by rabbit antisera R911 (lanes 1, 3, 5, 7, and 9) or R907 (lanes 2, 4, 6, 8, and 10), as described in Materials and Methods. F, I, T, V, and W represent the Florida, south Idaho, north Texas, Virginia, and Clarkston, Wash., isolates of *A. marginale*, respectively. The polypeptides later demonstrated to be recognized by R911 (☆) and polypeptides later demonstrated to be recognized by monoclonal antibody $22B_1$ (★) (see text and Table 1) are indicated. The numbers to the left of the gel represent the positions of the molecular mass markers (in kilodaltons).

Western blots. The apparent molecular masses of the major polypeptides recognized by $22B_1$ in the Western blot were the same as t' ose precipitated by $22B_1$ above, i.e., 105, 97, 89, 70, and 86 kDa for the Florida, Idaho, Texas, Virginia, and Washington isolates, respectively (Fig. 3A). Similarly, the polypeptides recognized by R911 in the Western blot had the same molecular weights as the primary polypeptides immunoprecipitated by R911 (Fig. 3B). Upon increased exposure of the autoradiographs, lower-molecular-weight polypeptides infrequently appeared in blots with both antibodies, ranging in molecular mass from 85 to 92 kDa. No proteins were recognized by T1E1 or R907.

Antigenic identity of immunoprecipitated polypeptides. We decided to analyze which polypeptides in the complex contained epitopes that were recognized by each antibody, $22B_1$ or R911. ^{125}I-labeled initial bodies were immunoprecip-

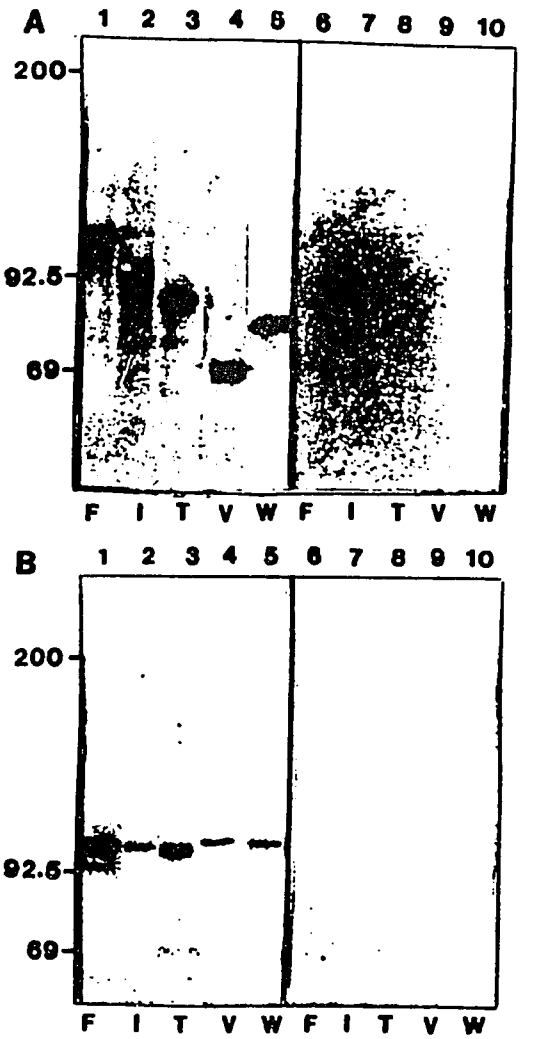

FIG. 3. Western blot analyses of *A. marginale* initial body proteins separated by SDS-polyacrylamide gel electrophoresis and probed with (A) monoclonal antibodies $22B_1$ (lanes 1 through 5) or T1E1 (lanes 6 through 10), or (B) rabbit antisera R911 (lanes 1 through 5) or R907 (lanes 6 through 10), as described in Materials and Methods. F, I, T, V, and W represent the Florida, south Idaho, north Texas, Virginia, and Clarkston, Wash., isolates of *A. marginale*, respectively. The numbers to the left of the gel represent the positions of the molecular mass markers (in kilodaltons).

62

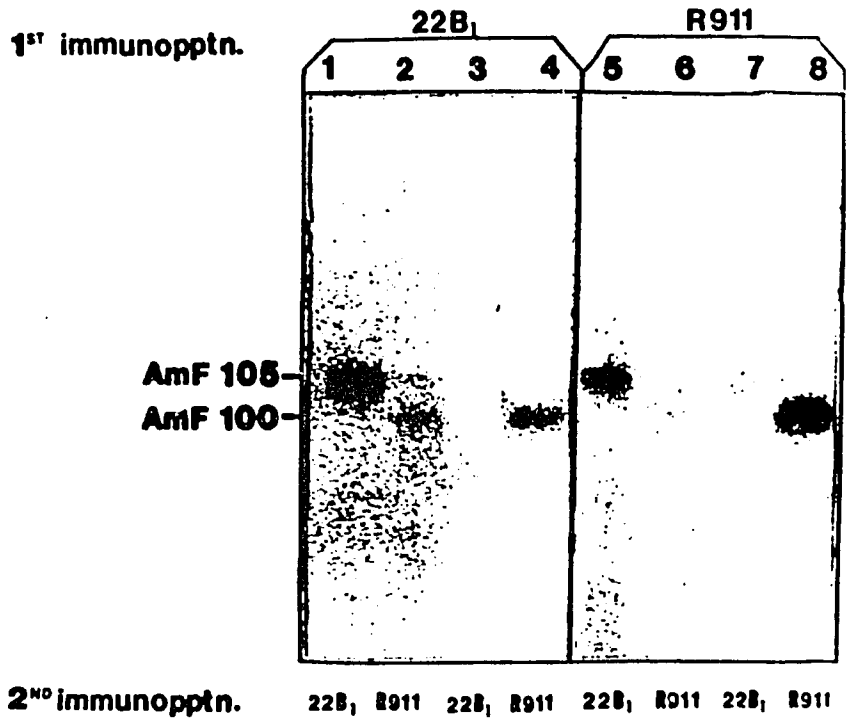

FIG. 4. Antigenic identity of polypeptides of the immunoprotective complex of *A. marginale*. The complex was immunoprecipitated with $22B_1$ (lanes 1 through 4) or R911 (lanes 5 through 8) and the individual polypeptides were excised, as described in Materials and Methods. The higher-molecular-mass polypeptide of each complex was reprecipitated by either $22B_1$ (lanes 1 and 5) or R911 (lanes 2 and 6). Similarly, the lower-molecular-mass polypeptide of each complex was reprecipitated by either $22B_1$ (lanes 3 and 7) or R911 (lanes 4 and 8). The identity of the polypeptides of the Florida isolate of *A. marginale* is demonstrated above; the results for the south Idaho, north Texas, Virginia, and Clarkston, Wash., isolates are not shown but are summarized in Table 1. immunopptn., Immunoprecipitation.

itated with either $22B_1$ or R911 and electrophoresed, and the individual polypeptides were excised. The polypeptides were eluted from the gel fragments, and each separated polypeptide was reimmunoprecipitated individually with R911 and $22B_1$. The results of the Florida isolate are depicted in Fig. 4; the polypeptides of the Idaho, Texas, Virginia, and Washington isolates were identically reprecipitated (results not shown).

Within an isolate, the size of the complex component containing the neutralization-sensitive epitope recognized by $22B_1$ did not differ, whether the complex was originally precipitated with $22B_1$ or R911. Similarly, the size of the complex component recognized by R911 was identical when initially precipitated by either antibody. These results confirmed that the complex of two polypeptides precipitated by $22B_1$ contained the same two polypeptides as that precipitated by R911. The antigenic identities of the polypeptides in the complex for all five isolates are summarized in Table 1. The complex components recognized by $22B_1$ had molecular masses of 105, 95, 89, 70, and 86 kDa for the Florida, Idaho, Texas, Virginia, and Washington isolates, respectively, while the polypeptides recognized by R911 had molecular masses of 100, 98, 97, 100, and 100 kDa for the same isolates.

Structural relationship of immunoprecipitated polypeptides. The polypeptides of the complex were individually examined by partial proteolysis to analyze structural polymorphisms between isolates (Fig. 5A and B). The $22B_1$-reactive polypeptides all exhibited cleavage peptides of apparent sizes of 58, 38.5, 27.5, 16.5, and 11.8 kDa. Isolate-specific peptide bands could be identified between 15 and 20 kDa in most isolates. The polypeptides recognized by R911 were cleaved into major fragments with approximate molecular masses of 29, 23, and 10.8 kDa in all five isolates. In addition, several fragments between 16 and 19 kDa were present in every isolate, and a few isolate-specific peptides were visible between 32 and 45 kDa in most isolates.

DISCUSSION

Despite severe losses and widespread regions enzootic for the causative rickettsia, *A. marginale*, effective immunoprophylaxis for anaplasmosis has not been developed. Immunization of cattle with a complex of two initial body surface polypeptides (MSP-1) induces protection against virulent *A. marginale* challenge (20). Comparison of the polypeptides of MSP-1 among isolates is essential to ensure that a subunit vaccine based on this complex will protect cattle against all the different isolates of *A. marginale* within a region.

The polypeptides of MSP-1 from the five isolates examined exhibit striking variations in molecular mass, differing by as much as 35 kDa between the Florida and Virginia isolates. Extensive size polymorphisms of proteins have been similarly discovered in other organisms, including the

TABLE 1. Antigenic identity of the polypeptides in the immunoprotective MSP-1 complex of *A. marginale*

Major complex component	Apparent molecular mass (kDa) in isolate[a]:				
	F	I	T	V	W
Recognized by $22B_1$	105	95	89	70	86
Recognized by R911	100	98	97	100	100

[a] Isolates: F, Florida; I, south Idaho; T, north Texas; V, Virginia; W, Clarkston, Washington.

62

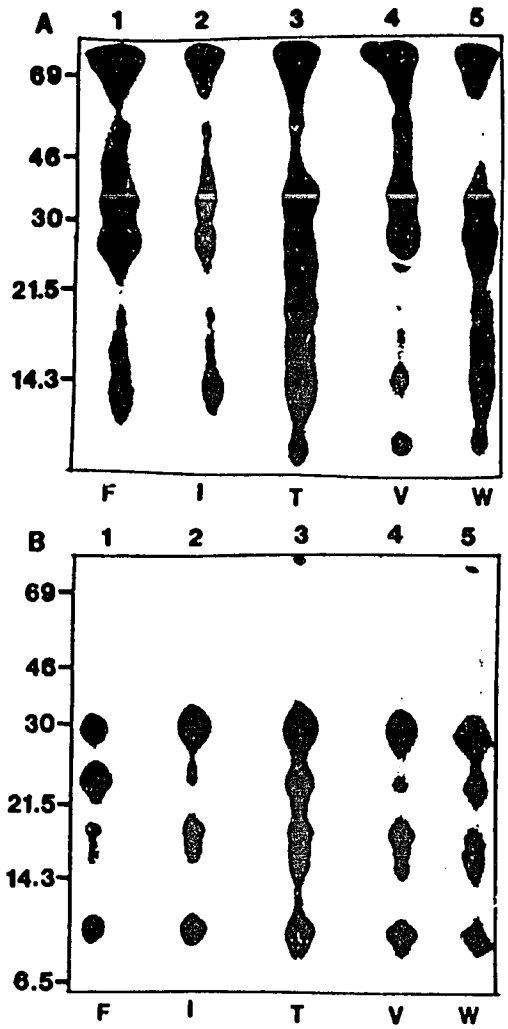

FIG. 5. Structural identity of polypeptides of the immunoprotective complex of *A. marginale*. Polypeptides were excised and partially digested as described in Materials and Methods. F, I, T, V, and W represent the Florida, south Idaho, north Texas, Virginia, and Clarkston, Wash., isolates of *A. marginale*, respectively. (A) $22B_1$-reactive polypeptides. Lanes: 1, AmF105; 2, AmI95; 3, AmT89; 4, AmV70; 5, AmW86. (B) R911-reactive polypeptides. Lanes: 1, AmF100; 2, AmI98; 3, AmT97; 4, AmV100; 5, AmW100. The numbers to the left represent the positions of the molecular mass markers (in kilodaltons).

M protein of group A streptococci (8), the variant-specific surface glycoprotein of *Trypanosoma vivax* (9), and the circumsporozoite (6, 30) and S-antigens (5) of *Plasmodium falciparum*. Considerable protein size differences among rickettsial species have been reported less frequently; only slight protein size variations were found between strains of *Rickettsia rickettsii* (1), although the 110-kDa protein of the Karp isolate of *Rickettsia tsutsugamushi* appears to vary by as much as 20 kDa in the Kato and Gilliam strains (19, 28).

Wide divergence among the isolate proteins could be critical to the application of the polypeptides of the Florida isolate MSP-1 as the foundation of a subunit vaccine. However, the Florida MSP-1 polypeptides have been demonstrated as capable of inducing protection not only against the Florida isolate of *A. marginale*, but also against a heterologous Washington (Okanogan) isolate (20). All of the isolates used in this study, as well as others from the United States, Israel, and Kenya, contain the neutralization-sensitive epitope recognized by the monoclonal antibody $22B_1$ (21a), supporting the possibility of an AmF105-based vaccine. The Florida polypeptides are the largest of all the isolates examined, suggesting potential determinants essential for protection have not been lost or deleted, as is possible with a vaccine based on the lower-molecular-weight polypeptides of the other isolates. Besides being one of the more virulent isolates (12, 23–25, 29), the Florida isolate is also the most widely cross-protective of all the isolates tested so far (13, 24, 29). This may imply that there may be more conserved determinants on the polypeptides of the Florida isolate.

One unusual feature of MSP-1 is that the polypeptides are integrally associated, despite the large size variations between isolates. The reimmunoprecipitations clearly demonstrate that the polypeptides precipitate as a complex, regardless of which polypeptide is initially recognized by the antibody. The polypeptides tend to dissociate during immunoprecipitation, probably due to the stringent washing conditions used (3). As a result, more of the polypeptide containing the epitope initially recognized is precipitated, as demonstrated by the greater intensity of this polypeptide band.

The MSP-1 appears to consist of just the two major polypeptides, although lower-molecular-weight polypeptides were inconsistently precipitated as well. Excision and reimmunoprecipitation of some of these fainter polypeptides confirmed these polypeptides as being either recognized by the rabbit antiserum, R911, or not precipitated at all (results not shown). These polypeptides may be breakdown products of the higher-molecular-weight proteins, which either occur naturally or result from handling during the procedures described above. Alternately, they may be unrelated proteins which are also complexed but dissociated to a greater degree by the washing steps of the immunoprecipitation protocol. Another possibility is that the smaller, R911-reactive polypeptides are the products of one of the other two to four copies of the gene for this protein (3); this seems less likely, as the polypeptides varied in size, even among identically treated immunoprecipitations of the same isolate, and there is presently no evidence that the other gene copies are expressed. Only one copy of the gene for the $22B_1$-reactive polypeptide has been detected by hybridization experiments (D. R. Allred, A. F. Barbet, G. H. Palmer, and T. C. McGuire, unpublished data).

Upon extremely long exposures of the autoradiographs of the $22B_1$ immunoprecipitations or the Western blots using $22B_1$, some proteins of very high molecular mass became visible at approximately 150 to 225 kDa. The different sizes of these high-molecular-mass proteins correspond to the sizes of the major $22B_1$-reactive complex polypeptides; that is, the largest of these high-molecular-weight proteins is found in the Florida isolate, the next largest is found in Idaho, the smallest is found in Virginia, etc. These high-molecular-weight proteins may be precursors or dimers of the $22B_1$-reactive polypeptides, or, like the *N*-acetyl-D-glucosamine-containing bands seen in the precipitations of the M protein of *Streptococcus pyogenes* (8), may contain noncovalently attached glycolipids, other complexed proteins, or cell wall components.

The polypeptides in the MSP-1 complex appear to be generally homologous in structure. Despite size variations as large as 35 kDa, there are several conserved peptides between the $22B_1$-reactive polypeptides. Similarly, the pattern of fragments resulting from a digestion of the R911-reactive polypeptides is very similar among isolates, suggesting regions of structural homology. Identification of any role for

- 64

these conserved peptides in inducing protection may be important for determining the mechanisms of immunity and of size variation.

Proteins containing multiple repeats may migrate anomalously in SDS-polyacrylamide gels (14); therefore, apparent molecular mass differences observed here may not be exact. The best way to precisely determine structural differences in MSP-1 between isolates is to define the primary structure via the gene sequence. Currently, research is in progress to complete the DNA sequence for the AmF105 gene; data suggest that the AmF105 gene contains a region of 87 bases tandemly repeated seven times or more (A. F. Barbet, G. H. Palmer, T. M. Harkins, D. A. Allred, and T. C. McGuire, unpublished data), and like the circumsporozoite genes of *P. falciparum* (6, 30), the $22B_1$-reactive polypeptides in other isolates may differ in the length, sequence, or number of repeats.

Repeated sequences may be important in determining the rate and processes of variation. While the polypeptides have not been observed to alter in size during several passages in cattle, the exact rate of the recombination, deletion, or duplication events which may alter these proteins is not known. Similarly, the relationship of these repeated sequences and the protein heterogeneities described to the induction of protective immunity remains to be established. Defining conserved immunoprotective determinants and the mechanism of structural variation will be critical to the application of MSP-1 in a subunit vaccine for bovine anaplasmosis.

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65

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66

Veterinary Protozoan and Hemoparasite Vaccines

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Chapter 1

ANAPLASMA VACCINES

Guy H. Palmer

TABLE OF CONTENTS

I. Introduction 2

II. Postinfection Immunity 4

III. Antigens Relevant to Protection 5
- A. Isolate-Restricted and Isolate-Common Antigens 5
- B. Antigenic Variation 7
- C. Antigenic Differences Between *Anaplasma* Species 9
- D. Antigenic Differences Between Tick Stages and the Intraerythrocytic Stage 11

IV. Current Methods of Immunization 12
- A. Premunization Using *Anaplasma*-Parasitized Erythrocytes 12
  - 1. Premunization with Virulent *A. marginale* Followed by Antibiotic Treatment 13
  - 2. Premunization with Minimal Infective Doses of *A. marginale* 14
  - 3. Premunization with *A. centrale* 14
  - 4. Premunization with Attenuated *A. marginale* 15
- B. Immunization with Killed Whole *A. marginale* Organisms 15

V. Development of Antigenically Defined Vaccines 16
- A. AmF 105 as a Protective Immunogen 18
- B. AmF 36 as a Protective Immunogen 23
- C. Progress in Subunit Vaccine Development 24

Acknowledgments 24

References 25

I. INTRODUCTION

Arthropod-borne hemoparasitic diseases are enzootic in half of the livestock production areas of the world and remain a severe constraint to agricultural development in the tropics.[1] Currently these diseases are controlled primarily by reducing or eliminating transmission using regular application of acaricides to cattle. Paradoxically, efficient arthropod control results in a highly disease-susceptible cattle population and sets the stage for devastating epizootics if breaks in transmission control occur.[2] Increased hemoparasite transmission caused by use of blood-contaminated fomites between cattle, development of acaricide resistance in ticks, and disruption in dipping programs caused by currency or labor shortages are continual threats to animal health in the tropics.[2,3] This is well illustrated by the loss of over 1 million cattle to hemoparasitic diseases in Zimbabwe when highly efficient tick control was disrupted by war in the late 1970s.[2] In contrast, vaccines may be used to protect cattle in regions with either high- or low-transmission levels as well as fully susceptible cattle to be imported into an enzootic area. Vaccines against bovine anaplasmosis have been used successfully to prevent severe morbidity and mortality in susceptible cattle, and research to develop more effective vaccines continues. In this chapter, the development of vaccines against *Anaplasma* infections is reviewed with focus upon recent advances in antigenically defined vaccines.

Anaplasmosis is an arthropod-borne hemoparasitic disease of cattle and wild ruminants caused by the rickettsia *Anaplasma marginale* or the less virulent *A. centrale*.[4] Anaplasmosis occurs worldwide and, along with babesiosis, cowdriosis, theileriosis, and trypanosomiasis, remains the greatest obstacle to meat, milk, and fiber production in tropical, lesser developed nations.[1,2,3] Infection results from inoculation of cattle with either a tick stage of *Anaplasma* (following transstadial or intrastadial replication in the midgut epithelium of a variety of ixodid ticks) or the intraerythrocytic stage (after mechanical transmission on the mouth parts of biting flies or by blood-contaminated fomites, i.e., syringes or dehorning instruments).[5,6] Following transmission there is a prepatent period of 20 to 40 d, during which there is a low but increasing percentage of parasitized erythrocytes.[5,6a] The prepatent period, during which infected cattle are clinically normal, is followed by an acute phase, during which the parasitemia increases dramatically and severe hemolytic anemia occurs.[5] Dramatic weight loss, abortion, and death frequently occur during the acute phase.[7] Cattle recovered from acute disease remain persistently infected with a low-level parasitemia for long periods and serve as a reservoir for transmission of the organism.[8] These cattle are fully resistent to challenge with the homologous isolate; however, they remain susceptible to infection from certain heterologous isolates of *Anaplasma*.[9]

Subtropical and tropical regions enzootic for anaplasmosis correspond to the distribution of the arthropod vectors including 29 species of ticks and numerous hematophagous flies and mosquitos shown experimentally to transmit the disease.[10,11] Several of these vectors also transmit other hemoparasites; as a result, anaplasmosis frequently exists coenzootically with babesiosis, cowdriosis, and theileriosis.[12] Due to this complex of hemoparasitic diseases, the economic impact strictly attributable to anaplasmosis is difficult to estimate in many of the nations with enzootic regions. Within the U.S., where anaplasmosis occurs exclusive of the other major hemoparasitic diseases, the annual loss of 50,000 to 100,000 head of cattle has been estimated at $300 million (1986 $U.S.).[13] Recent epidemiologic data from Texas, with approximately 30% enzootic regions, attributed severe losses to anaplasmosis, including death in 36% of clinical cases, abortion in 24% of clinical cases in pregnant cows, an average weight loss of 86 kg during acute infection, and increased veterinary and management costs of $52 and $30 per head, respectively.[7] Severe production losses attributed to prolonged subclinical anemia in persistently infected cattle, especially in regions where anaplasmosis and babesiosis occur together, have recently been recognized and add dra-

matically to the already severe constraint upon livestock production in enzootic regions.[14]

In enzootic regions with a high level of transmission an "enzootically stable" livestock population may develop if no control measures are instituted. Calves are uniformly infected at an early age and usually suffer only mild clinical disease, presumably due to partial protection from colostral-derived antibody.[15] The calves upon recovery are protected from severe morbidity and mortality when subsequently challenged. A high level of transmission ensures continual reexposure to boost immunity. Allowing an enzootically stable herd to develop may be considered desirable; however, many regions thought to be stable may actually be relatively unstable as transmission levels vary greatly seasonally and annually depending upon climatic conditions being favorable for arthropod vectors.[16-18] Prolonged levels of low transmission allow some calves to mature without becoming infected and to coexist within a herd with carrier cattle.[18] A return to high levels of transmission following climatic changes may result in disastrous epizootics. In addition, recent epidemiologic data demonstrates that the persistently infected *Anaplasma* carriers that create enzootic stability suffer from prolonged subclinical anemia and poor weight gain, especially in regions endemic for both anaplasmosis and babesiosis.[14] The risk of devastating losses due to instability created by climatic changes and the production losses resulting from chronic anemia may combine to make a goal of enzootic stability undesirable for nations dependent upon efficient livestock production and with the infrastructure to develop anaplasmosis control programs.

Current control measures include (1) control of arthropod transmission, (2) premunization or vaccination of susceptible cattle, and (3) use of antibiotics to treat or prevent *Anaplasma* infections. Commonly a combination of these measures is employed, and the prevailing control programs vary markedly between regions and nations depending upon principal means and seasonality of transmission, presence of other major arthropod-borne hemoparasitic diseases, livestock management systems used, availability and economics of antibiotics, acaricides, and vaccines, and the goals of the program, i.e., eradication vs. control, and the level of control desired.[19] Despite the availability of these programs, the distribution of anaplasmosis has not been reduced, and the severe losses continue on five continents, clear evidence that current control programs are not sufficient.[1,3,12] Control of arthropod transmission by regular use of acaricides is labor intensive, expensive (frequently requiring a significant percentage of foreign capital expenditure by lesser developed nations), and creates a highly susceptible cattle population. This population is vulnerable to disruptions in the control program, development of acaricide resistance in ticks, and any non-arthropod mechanisms of transmission.[2,3,19] Similarly, reliance on antibiotic prophylaxis by continual feeding of tetracyclines results in herds vulnerable to program disruptions. The labor and expense involved to ensure that all cattle receive a minimum daily amount of antibiotic is not amenable to most tropical livestock systems. In spite of the efficacy of tetracyclines in treatment and chemoprophylaxis of anaplasmosis, antibiotic-based control programs have found only limited acceptance in the U.S. and virtually none in other regions. The effect of pending U.S. legislation regarding low-level antibiotic feeding to cattle upon this control method is not presently clear.

The protection engendered by recovery from *Anaplasma* infection clearly indicates that immunoprophylaxis is a realistic and achievable goal. Vaccination provides a means to efficiently and economically protect cattle both within enzootically stable and unstable areas as well as susceptible adult cattle to be imported into these areas. Although premunization (deliberate infection of cattle to induce immunity) has been practiced for 75 years and commercially available killed and live whole organism vaccines have been used for nearly 20 years, the need remains to develop a widely cross-protective, economical vaccine suitable for use in tropical regions.[20]

II. POSTINFECTION IMMUNITY

Cattle recovered from acute infection with *Anaplasma marginale* are protected from severe morbidity and mortality when subsequently challenged with a homologous isolate of *A. marginale*.[9] Parasitemia upon challenge is very low and frequently undetectable. This postinfection immunity is not dependent upon the animal remaining persistently infected, as cattle chemotherapeutically cleared of infection retain immunity for at least 8 months.[21] Chemical immunosuppression or splenectomy abrogates this immunity as would be expected; however, the cellular and molecular mechanisms responsible for immunity are not clear.[22-24] Tenable mechanisms include (1) antibody blockade of the erythrocyte binding site on the *A. marginale* initial body surface; (2) direct lysis of initial bodies by antibody or complement; and (3) phagocytosis and intracellular killing of initial bodies or parasitized erythrocytes coated with antibody or complement. To date no significant data to verify or exclude any of these mechanisms in postinfection immunity has been presented. Effector antibody in any of the three mechanisms requires a defined epitope specificity and affinity that need to be identified. Correlative data between the level of protection and antibody titer to crude *A. marginale* antigens from serologic tests have not been helpful in understanding immune mechanisms.[25] Previously a correlation has been made between the level of postinfection immunity and the lymphocyte migration inhibition test.[26-28] Subsequent inability to identify a discrete migration inhibition factor (MIF) has made it difficult to interpret this finding. Several lymphokines, notably the interferons, have some MIF-like activity. This suggests that the previously noted correlation with immunity most likely reflects macrophage and/or T lymphocyte (including helper functions) activation. Complete understanding of the mechanistic basis of postinfection immunity will be significant in designing rational strategies to develop improved vaccines.

The presence of postinfection immunity provides a strong basis for vaccine development. Initial "vaccines" attempted to mimic the immunity engendered by natural field exposure while avoiding the severe morbidity and mortality of virulent infections. This method, premunization, has provided a method to partially protect cattle from field infections (both homologous and more virulent heterologous isolates) and remains in use in several countries (advantages and disadvantages of premunization will be discussed in Section IV). Importantly, both experimental and field experience with premunization have provided information regarding cross-species and cross-isolate protection, the likelihood of antigenic variation, and immunity to tick challenge vs. challenge with parasitized erythrocytes. Infection of cattle with *A. centrale* (a less virulent *Anaplasma* species decribed by Theiler and originally confined to Africa) does not prevent challenge infection by *A. marginale*, but decreases the severity of the clinical signs with most A. marginale isolates.[29,30] Other *A. marginale* isolates produce severe morbidity in *A. centrale*-premunized cattle.[31,32] In contrast, *A. marginale*-premunized cattle are uniformly protected against *A. centrale* or homologous *A. marginale* challenge, but clinical signs of variable severity result upon challenge with heterologous isolates of *A. marginale*.[9,33,34] This variation in disease upon heterologous challenge ranges from mild anemia (25% decrease in packed cell volume [PCV]) to severe anemia (75% decrease in PCV) with parasitemias greater than 20%. These experiments indicated for the first time that antigenic differences critical to protection existed between *A. centrale* and *A. marginale* and between different isolates of *A. marginale*, and that effective vaccines must protect against the different organisms. A second potential obstacle to effective immunoprophylaxis is antigenic variation. Although there is no evidence for cyclical emergence of antigenic variants in anaplasmosis, the ability of the parasite to persist in spite of an immune response and the occurrence of recrudescent parasitemias in carrier cattle indicates that a complex host-parasite relationship develops. The role of antigenic variation in this relationship and, therefore, its effect on immunization efficacy is unknown.[35,36] A third consideration

Table 1
REACTIVITY OF ISOLATE-RESTRICTED MONOCLONAL ANTIBODIES TO GEOGRAPHICALLY DISTINCT *A. MARGINALE* ISOLATES[a]

	Ab Reactivity[b]									
Monoclonal Ab	FL	ID	TX	VA	WA-C	WA-O	ISNT	IST	KKb	KKp
Tryp 1E1[c]	–	–	–	–	–	–	–	–	–	–
Ana F22A4	+	–	–	–	–	–	–	–	–	–
Ana 011C2	–	–	–	–	+	+	–	–	+	+
Ana 012B5	–	–	–	–	+	+	–	–	+	+
Ana 023D5	–	+	+	+	+	+	–	+	–	–
Ana 024D5	–	+	+	+	+	+	–	+	–	–
Ana 070A2	+	+	+	+	+	+	+	+	–	+
Ana R19A6	–	–	–	+	–	–	–	–	+	+
Ana R94C1	–	–	–	+	–	–	–	–	+	+
Ana R17A6	–	–	–	+	–	–	–	–	–	–
Ana R83B3	–	–	–	+	–	–	–	–	–	–

[a] Isolate abbreviations: FL (Florida, U.S.), ID (South Idaho, U.S.), TX (North Texas, U.S.), VA (Virginia, U.S.), WA-C (Clarkston, Washington, U.S.), WA-O (Okanogan, Washington, U.S.), ISNT (Israel-non-tailed), IST (Israel-tailed), KKb (Kabete, Kenya), KKp (Kapiti, Kenya).

[b] Reactivity was determined using indirect immunofluoresence on acetone-fixed smears of parasitized erythrocytes. Monoclonal antibodies were unreactive with *Anaplasma ovis*, *Babesia bigemina*, *Babesia bovis*, and *Trypanosoma brucei*.

[c] Tryp 1E1 is an anti-*Trypanosoma brucei* monoclonal antibody used as a negative control.

is the relative roles of intraerythrocytic stages and tick stages of *A. marginale* in inducing protective immunity and the stage-specificity of the immune response. These influences upon vaccine development will be addressed in the following section.

III. ANTIGENS RELEVANT TO PROTECTION

A. Isolate-Restricted and Isolate-Common Antigens

Cross-protection experiments in premunized cattle established that antigenic differences among geographically distinct *A. marginale* isolates existed.[9-34] Structural and antigenic differences among isolates have been established by (1) identification of morphologicaly distinct isolates;[37] (2) demonstration of protein structural variation among isolates;[36] and (3) presence of isolate-common and isolate-restricted epitopes.[39-42] Morphologically distinct isolates were identified using phase contrast and fluorescence microscopy initially using Oregon and Florida isolates of *A. marginale*.[37] The Oregon isolate was shown to contain an appendage lacking in the Florida isolate. Numerous isolates of either morphology have been subsequently described within the U.S. and in other countries. The morphologic type remains constant within an infection and between passages in splenectomized and nonsplenectomized cattle.[43-45] Adsorption of polyclonal antisera made to appendaged *A. marginale* with the Florida isolate demonstrated that the appendage bears specific antigens not represented on the initial body itself.[33] That antigenic differences are not limited to the appendage has been shown by cross-adsorption experiments using two-appendaged isolates.[41] In our laboratories, we have used a panel of ten monoclonal antibodies (MAbs) that recognize isolate-restricted epitopes to discriminate among *A. marginale* isolates from the U.S., Israel, and Kenya (Table 1).[40,45] Using this panel, we have shown that both within a morphologic type or between morphologic types, there are multiple epitopes that differ between *A. marginale* isolates.[40,45]

Despite the identification of isolate-restricted epitopes, the majority of antigens are conserved between isolates.[40-42] Resolution of parasite-specific proteins using two-dimensional

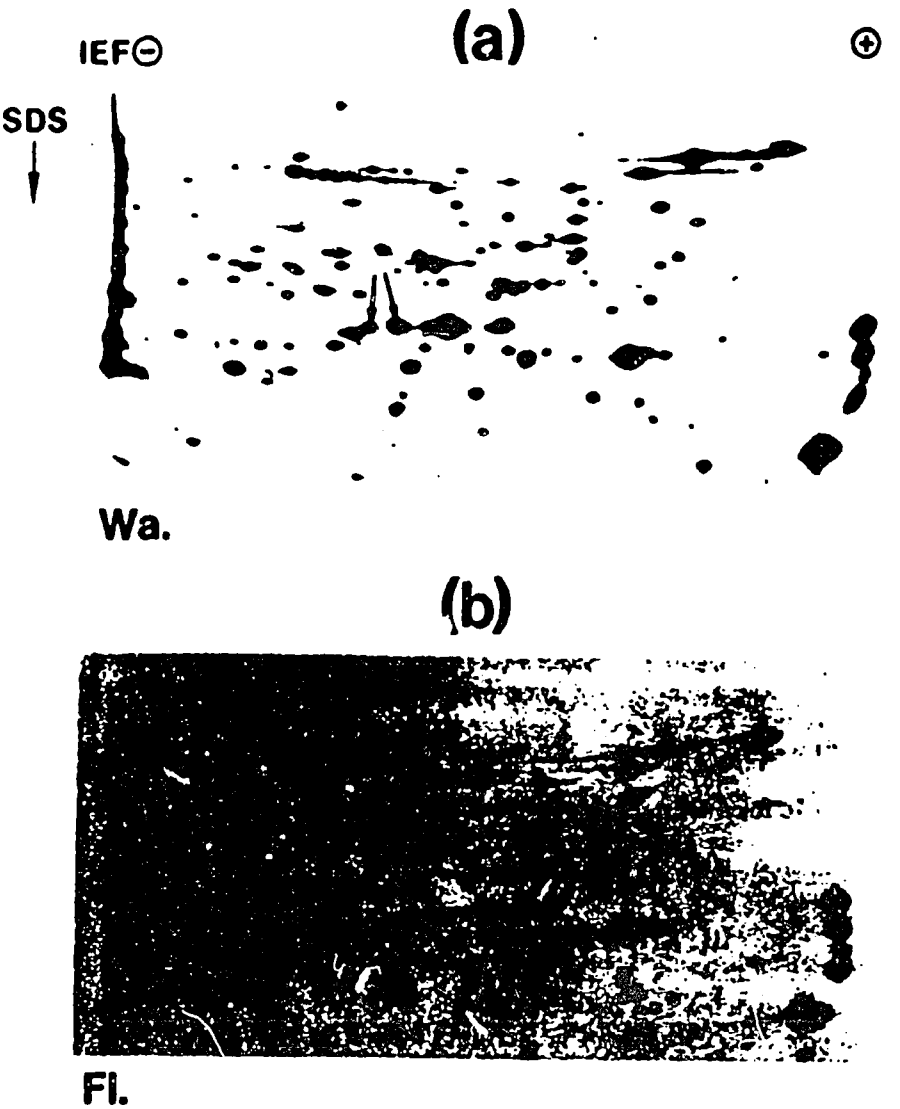

FIGURE 1. Comparison of ^{35}S-radiolabeled proteins synthesized by the Washington-O and Florida isolates of *A. marginale*.[38] *A. marginale* initial bodies were radiolabeled with ^{35}S-methionine during short-term *in vitro* erythrocyte culture. Barbet et al.[38] previously demonstrated that the radiolabel incorporated exclusively into the *A. marginale* initial body using this procedure. Radiolabeled initial bodies were solubilized and subjected to two-dimensional electrophoresis followed by detection using fluorography.[38] (A) Washington-O isolate and (B) Florida isolate. The majority of the proteins are conserved in molecular weight and isoelectric point between the two isolates; however, three major radiolabeled proteins appeared unique to either the Washington-O or Florida isolate (arrows).[38] Comparison of ^{3}H-radiolabeled proteins (using a mixture of ^{3}H-radiolabeled amino acids not including methionine) from the two isolates confirmed the presence of these three unique proteins.[38]

gel electrophoresis allowed direct structural comparison of two morphologically distinct isolates (Figure 1).[38] The majority of proteins are common to both isolates, as would be expected for organisms of the same species and equally dependent upon intraerythorcytic invasion and replication. However, several major proteins were found to vary in molecular weight and isoelectric point, indicating primary protein structural differences between isolates (Figure 1).[38] Additional work comparing proteins from the Florida and Illinois (an appendaged isolate) identified variant high-molecular-weight antigens that shared common epitopes

and one Florida isolate antigen that may bear only isolate-restricted epitopes.[40] The critical question relevant to cross-protective immunity and vaccine development is whether the protection-inducing epitopes are common or variant. Epitopes capable of generating protective immune responses (by direct initial body lysis, receptor blockade, or antibody-mediated phagocytosis) must be surface exposed. We have identified a panel of seven MAbs that recognize highly conserved epitopes on initial body surface proteins (Table 2).[45] The epitopes recognized by the seven MAbs are limited to two of the major initial body surface proteins, a protein with an apparent molecular weight of 105 kDa in the Florida isolate, AmF 105, and a 36-kDa protein, AmF 36.[40,45-47] The MAbs directed against epitopes on AmF 105 (15D2, 22B1, F34C1) recognize 100% of the organisms within parasitized erythrocytes regardless of the isolate.[40,45] This 100% binding is constant throughout the cycle of infection from <1% parasitemia through peak parasitemia with hemolytic crisis.[40] This striking epitope conservation exists despite antigenic, morphologic, and virulence differences among these 13 *A. marginale* isolates.[38-42,45] In contrast, MAbs against AmF 36 epitopes (F19E2, 050A2, 058A2, and 066A2) bind only 65 to 75% of the organisms in parasitized erythrocytes in all isolates.[40,45] This approximately 70% binding is constant between passages and regardless of the level of parasitemia.[40] The explanation for this limited epitope expression and its influence on cross-protective immunity remains unknown. There are undoubtedly additional conserved epitopes on AmF 105 and AmF 36 as well as on other surface-exposed initial body proteins that should be identified as potentially cross-protective immunogens.

A single, apparently immunodominant epitope on AmF 105 is recognized by MAbs 15D2 and 22B1 (Table 2).[47,48] Both antibodies, produced against a mixed inoculum of Virginia and Washington-O *A. marginale*, can neutralize 100% of the infectivity of the antigenically, morphologically, and structurally distinct Florida isolate (Table 3).[47] The high degree of conservation of this neutralization-sensitive epitope provided rationale for testing of native AmF 105 bearing this epitope and recombinant or synthetic constructs of this epitope for efficacy as a widely cross-protective immunogen (Section V).

B. Antigenic Variation

The differential reactivity of the various *A. marginale* isolates with a panel of isolate-restricted MAbs demonstrates that mechanisms to generate antigenic variants are present.[40,45] The limited cross-protective immunity seen with certain isolates in premunization or using a killed whole organism vaccine indicates that some of these antigenic variants are important in protection.[9,34] Critically relevant to development of effective immunoprophylaxis is whether this variation can be generated rapidly enough to avoid a neutralizing host response. While cyclical episodes of high parasitemia analagous to African trypanosomiasis are not seen, the persistence of *Anaplasma* in an immunologically hostile host and the occurrence of relapse parasitemias may result from emergence of antigenic variants. Systematic investigations of this phenomenon at the molecular level have not been reported for anaplasmosis. We have used panels of isolate-restricted and isolate-common MAbs to test for variation in a limited number of epitopes.[49] Constant patterns of reactivity using 18 MAbs on six U.S. isolates of *A. marginale* (Florida, Idaho, North Texas, Washington-C, Washington-O, and Virginia) were observed from <1% parasitemia through peak parasitemia with hemolytic crisis.[40] In addition, the antigenic profile of the Florida isolate was invariant after 18 months persistent infection in six calves.[49] These observations were limited to a relatively few epitopes and would not detect variation in other epitopes. Importantly, however, seven of these MAbs recognize surface protein epitopes, and that variation did not occur in these epitopes may indicate that cyclical variation will not be a severe impediment to immunoprophylaxis.[45] The highly cross-isolate-conserved AmF 105 epitope recognized by MAbs 15D2 and 22B1 was among the invariant epitopes and may represent an epitope required for erythrocyte invasion, metabolism, replication, or erythrocyte exit that is essential for *A. marginale*

Table 2

RECOGNITION OF *A. MARGINALE* INITIAL BODY SURFACE PROTEIN EPITOPES HIGHLY CONSERVED AMONG ANTIGENICALLY DISTINCT ISOLATES[a]

Monoclonal Ab	Surface protein reactivity	Ab reactivity[b]									
		FL	ID	TX	VA	WA-C	WA-O	ISNT	IST	KKb	KKp
Tryp 1E1[c]	—	–	–	–	–	–	–	–	–	–	–
Ana 15D2	AmF 105	+	+	+	+	+	+	+	+	+	+
Ana 22B1	AmF 105	+	+	+	+	+	+	+	+	+	+
Ana F34C1	AmF 105	+	+	+	+	+	+	+	+	+	+
Ana F19E2	AmF 36	+	+	+	+	+	+	+	+	+	+
Ana 050A2	AmF 36	+	+	+	+	+	+	+	+	+	+
Ana 058A2	AmF 36	+	+	+	+	+	+	+	+	+	+
Ana 066A2	AmF 36	+	+	+	+	+	+	+	+	+	+

[a] Isolate abbrevations are as in Table 1.

[b] Reactivity was determined by indirect immunofluorescence on acetone-fixed smears of parasitized blood smears. All Ana monoclonal antibodies were unreactive with other hemoparasites as in Table 1.

[c] Tryp 1E1 is an anti-*Trypanosoma brucei* monoclonal antibody used as a negative control.

Table 3
NEUTRALIZATION OF *A. MARGINALE* INITIAL BODY INFECTIVITY BY MONOCLONAL ANTIBODIES Ana 15D2 AND Ana 22B1[a]

	Number infected/challenged			
A. Monoclonal Ab	10^7	10^8	10^9	10^{10}
Tryp 1E1	3/3	3/3	3/3	7/7
Ana 15D2/22B1	0/4[b]	4/4	4/4	9/10
	Mean number of days between inoculation and 1% parasitemia			
B. Monoclonal Ab	10^7	10^8	10^9	10^{10}
Tryp 1E1	34	30	28	25
Ana 15D2/22B1	>75[b]	37	35	33
P[c]	ND[d]	≤0.01	≤0.01	≤0.01

[a] Graded numbers of initial bodies were purified from Florida isolate-parasitized erythrocytes and mixed with ascitic fluid from mice bearing Tryp 1E1 hybridomas (anti-*Trypanosoma brucei*) or Ana 15D2/22B1 hybridomas (anti-*A. marginale* reactive with a single epitope on AmF 105). The mixture was incubated at 20°C for 45 min and then inoculated intramuscularly into splenectomized calves. Blood samples were collected daily for 75 d postinoculation in order to determine packed cell volume and presence of parasitized erythrocytes.

[b] No parasitized erythrocytes were seen through 75 d postinoculation.

[c] The mean number of days between inoculation and 1% parasitemia were calculated for all infected cattle in each challenge group and compared between Tryp 1E1 and Ana 15D2/22B1 groups using the pooled t test.

[d] ND: not determined.

survival. The requirement that *Anaplasma* invade and adapt to intracellular existence undoubtedly limits the variation tolerable in certain surface epitopes. Determination of proteins and epitopes involved in these functions may be a fruitful strategy to identify invariant antigens for incorporation in vaccines. Nonetheless, the persistent nature of *Anaplasma* infection and occurrence of relapse parasitemias indicate a complex host-parasite relationship. Understanding the molecular basis of persistence and relapses will be necessary to develop optimal vaccines that completely prevent *Anaplasma* infection and the accompanying carrier state.

C. Antigenic Differences Between *Anaplasma* Species

The classification of a second *Anaplasma* species, *A. centrale*, was based upon the observation that the organism differed in position in parasitized erythrocytes and that this centrally located organism caused a less severe hemolytic disease.[29,30,31] Theiler noted in 1912 that although *A. marginale* challenge of *A. centrale*-premunized cattle resulted in establishment of an *A. marginale* infection, the clinical disease was less severe than in nonpremunized cattle.[29] (Subsequent work has shown the level of protection to be variable and is reviewed in Section IV.) The partial protection afforded by *A. centrale* premunization suggested that both species-common and species-specific epitopes related to protection were

Table 4
REACTIVITY OF ANTI-*A. MARGINALE* MONOCLONAL ANTIBODIES WITH *A. CENTRALE*

Panel reactivity w/*A. marginale*	Reactivity w/*A. centrale*[a]
Panel 1: isolate restricted[b]	–
Panel 2: isolate common[c]	–
Panel 3: isolate common, reactive w/AmF 36[d]	+
Panel 4: isolate common, reactive w/AmF 105[e]	+

[a] Reactivity was determined by indirect immunfluorescence on acetone-fixed smears of *A. centrale* paratized erythrocytes.
[b] Includes all ten Ana monoclonal antibodies in Table 1.
[c] Monoclonal antibody Ana F35A1.
[d] Monoclonal antibodies Ana F19E2, Ana 050A2, Ana 058A2, and Ana 066A2.
[e] Monoclonal antibodies Ana 15D2, Ana 22B1, and Ana F34C1.

likely to be present.[29,50-52] Comparisons between capillary-tube agglutination test antigens of *A. marginale* (East African and USDA isolates) and of *A. centrale* demonstrated that the organisms were highly cross-reactive, but that *A. centrale* had significant antigenic differences from the two *A. marginale* isolates.[53] From a taxonomic viewpoint it is unclear whether the speciation is justified on other than a historical basis. The pathophysiologic changes in infection with either are indistinguishable, and the severity varies as greatly between *A. marginale* isolates as between *A. centrale* and *A. marginale*.[9,31,50,54] The validity of the present speciation will most likely be resolved by antigenic, protein structural, and genomic comparisons involving numerous *A. marginale* isolates and *A. centrale*. More important is whether the antigenic differences are relevant to cross-species protection. Cross-protection experiments using *A. centrale*-premunized cattle (experiments which originally supported the speciation) do not differ significantly from similar experiments using various heterologous isolates of *A. marginale*.[9,31,55-57] Briefly, the severity of disease seen upon *A. marginale* challenge of *A. centrale*-premunized cattle does not differ significantly from *A. marginale* challenge of cattle premunized with an *A. marginale* isolate of mild virulence. Conversely, the mild reaction seen upon *A. centrale* challenge of *A. marginale*-premunized cattle does not differ significantly from that seen with mild virulence *A. marginale* challenge. On the basis of these experiments, there is little evidence to indicate the *A. centrale-A. marginale* differences are greater than differences among *A. marginale* isolates. Molecular approaches to understanding antigenic differences between the two species have only recently begun. The panels of MAbs produced against *A. marginale* and used to characterize different *A. marginale* isolates have been used to search for species-common epitopes on the Israel isolate of *A. centrale*.[40,45] Four different patterns of reactivity were seen (Table 4): (1) none of the ten *A. marginale* isolate-restricted MAbs reacted with *A. centrale;* (2) a MAb that reacted with all 13 *A. marginale* isolates did not react with *A. centrale* and was tentatively considered species specific; and MAbs directed against either (3) the AmF 36 or (4) the AmF 105 surface proteins of *A. marginale* reacted with *A. centrale*. These latter groups reacted with *A. centrale* in the same pattern as with *A. marginale* — the anti-AmF 105 MAbs bound 100% of the *A. centrale* within parasitized erythrocytes, while the anti-AmF 36 MAbs bound 70%.[45] The lack of *A. centrale* reactivity with the panel of *A. marginale* isolate-restricted MAbs and the putative *A. marginale*-specific MAb may indicate that true species differences occur; however, the relevance of the observation to protection is not clear. The reactivity of the MAbs to AmF 105 and AmF 36 with *A. centrale* is particularly significant because of the surface location of these proteins. The reactivity of the anti-AmF 105 MAbs with *A. centrale* included the antibodies (15D2/22B1) demonstrated to neutralize 100% of *A. mar-*

ginale infectivity. We are currently identifying the *A. centrale* protein that bears this epitope, its surface topography, and its role in protection. The reactivity of MAbs against key epitopes on the AmF 105 *A. marginale* surface protein with *A. centrale* suggests that cross-species as well as cross-isolate protection is feasible. The ability of AmF 105 or other relevant proteins to provide protection against anaplasmosis is addressed in Section V.

D. Antigenic Differences Between Tick Stages and the Intraerythrocytic Stage

Erythrocyte-to-erythrocyte transmission of the initial body stage of *Anaplasma* has been demonstrated *in vitro* and is responsible for the pathologic effects of the infection *in vivo*.[58-60] The infection is initiated by either direct inoculation of parasitized erythrocytes or by ixodid tick transmission of an infective tick stage.[5-9] Because morbidity is mediated through the intraerythrocytic stage (the initial body) and this stage is a common pathway regardless of the mode of transmission, development of vaccines and antigenic studies have focused upon the initial body. This strategy appears sound, as many regions have primary transmission by mechanical inoculation of parasitized erythrocytes rather than by tick transmission.[61,62] Within the U.S., anaplasmosis in the southeast region does not depend upon tick transmission which appears to be significant in the western U.S.[10,63-65] Recent research identified two U.S. isolates (Florida and Illinois) as non-tick transmitted using *Dermacentor andersoni* (Florida and Illinois tested) or *D. variablis* (Illinois only tested).[66] If a significant number of field isolates are not tick transmitted, then immunization against initial body stages would be required to provide acceptable protection to field challenge. Therefore, a goal of developing a tick stage-based vaccine for sole use is not recommended. However, the most effective vaccine may encompass both tick stage and initial body antigens.

While premunization and other initial body-based immunization experiments demonstrated that anti-initial body immunity generated protection against disease, corresponding studies examining immunity against tick stages have been few. Whether anti-tick-stage immunity would provide protection against tick-stage challenge similar to the antisporozoite immunity in hemoprotozoan infections has not been fully investigated. Identifying which of the morphologically distinct tick stages of *A. marginale* is the infective stage is needed to progress on these objectives. Current emphasis is on the salivary gland stage of *Anaplasma* and determination of its infectivity and antigenic structure.[67]

In addition, emphasis has been placed upon identification of tick stage antigens that cross react with initial body antigens.[46] Antibodies produced against *A. marginale* midgut tick stages in *Dermacentor andersoni* were used to immunoprecipitate *A. marginale* initial body proteins (Figure 2).[46] The results demonstrated that multiple initial body proteins in the range of >14 to 200 kDa shared at least one epitope with tick stage *Anaplasma*. The stage-common antigens included epitopes on several of the initial body surface proteins, AmF 105, AmF 86, AmF 61, and AmF 36 (Figure 3).[46] The significance of these shared-surface epitopes is potentially threefold: (1) if the epitopes shared with the initial body stage are present on the surface of the infective tick stage, immunization with initial body surface antigens may protect *directly* against tick stage challenge as well as through the common pathway of the intraerythrocytic stage of *A. marginale* (2) immunization with tick stage antigens may protect against both tick stage and initial body challenge; (3) the shared epitopes, even if not surface exposed on the infective tick stages, would serve to boost anti-initial body immunity in areas where repeated tick challenge occurs and, therefore, extend the duration of immunity. This mechanism will be of significance only if the shared epitopes on the initial body surface proteins are those relevant in protection. Whether these stage-common epitopes include the AmF 105 epitope shown to be capable of inducing neutralizing antibodies is unknown, but is presently being determined using colloidal gold-labeling studies in sections of tick-stage organisms in tick midgut and salivary gland epithelium.[68]

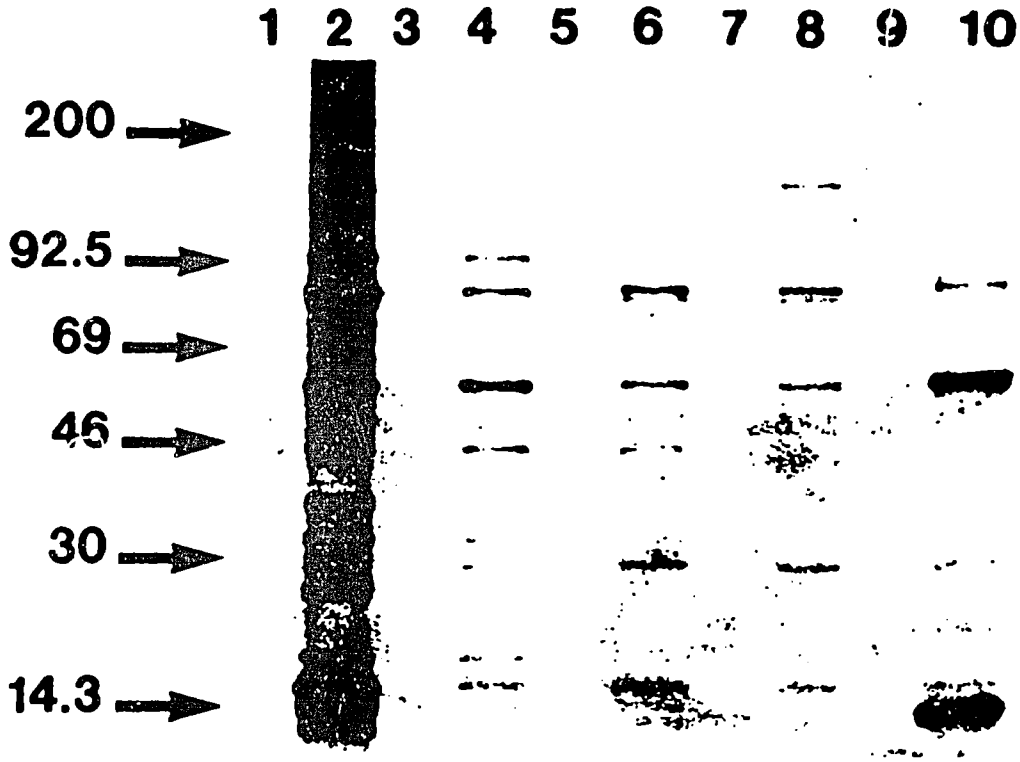

FIGURE 2. Identification of *A. marginale* antigens common to both the erythrocyte stage (initial body) and tick stages.[46] *A. marginale* initial bodies were radiolabeled with ^{35}S-methionine during short-term *in vitro* erythrocyte culture and immunoprecipitated with sera from cattle infected with parasitized erythrocytes (preinfection sera, Lane 1; postinfection sera, Lane 2) or sera from cattle immunized with killed tick-stage *A. marginale* (preimmunization sera, Lanes 3, 5, 7, and 9; postimmunization sera, Lanes 4, 6, 8, and 10).[38,46] Immunoprecipitated proteins were separated by SDS polyacrylamide gel electrophoresis followed by detection using fluorography. ^{14}C molecular weight standards are in thousands (arrows at left margin). The precipitation of numerous initial body proteins (in the molecular weight range of <14 to 200 kDa) by cattle sera exposed only to tick-stage *A. marginale* demonstrates that multiple epitopes are common to both vertebrate and invertebrate stages.[46]

IV. CURRENT METHODS OF IMMUNIZATION

Current methods of immunization are principally divided into two strategies: first, premunization using *Anaplasma* parasitized erythrocytes or, second, immunization with killed whole initial body antigens. These methods have been available for years, and specific data regarding their effectiveness under different field conditions as well as experimental use has been reviewed.[5,31,56,69-72] In this chapter, the antigenic basis, the host response, and the primary advantages and disadvantages of the different immunization methods will be discussed primarily as guideposts in developing strategies for improved vaccines.

A. Premunization using *Anaplasma*-Parasitized Erythrocytes

Induction of immunity by premunization requires that the individual develop a patent primary infection.[9] Following resolution of the acute infection, the animal remains persistently infected and develops solid immunity to homologous isolate challenge. To date, this overall strategy remains the most effective available method to induce protective immunity against anaplasmosis. Primary disadvantages are apparent: (1) all inoculated cattle must receive sufficient live organisms to cause a primary infection — this requires either an uninterrupted cold-chain if a standardized dose is used or use of a nonstandardized inoculum from a local carrier animal; (2) the immunity is dependent upon development of an acute

FIGURE 3. Identification of *A. marginale* initial body surface protein epitopes common to tick-stage *A. marginale*.[46] Initial bodies purified from *A. marginale*-parasitized erythrocytes were surface radiolabeled with ^{125}I and immunoprecipitated with sera from cattle immunized with killed tick-stage *A. marginale* (preimmunization sera, Lanes 2, 4, 6, and 8; postimmunization sera, Lanes 3, 5, 7, and 9).[46,104] ^{14}C molecular weight standards are in thousands (Lane 1). Precipitation of initial body surface proteins, AmF 105, 86, 61, and 36 by sera following tick-stage immunization demonstrates that stage-common epitopes include those on protection-inducing (AmF 105 and AmF 36) and potential protection-inducing (AmF 86 and AmF 61) surface proteins.[46,47,104,114]

infection, yet the severe morbidity and mortality frequently seen in acute infections must be avoided; (3) the premunized animals are persistently infected and are subject to relapse to acute disease during periods of stress; (4) persistently infected cattle may be less productive, especially in areas with poor nutrition and coenzootic with babesiosis; (5) persistently infected cattle maintain the organism in the population for transmission to susceptible cattle; and (6) although homologous immunity appears solid (except during stress-induced relapses), the degree of heterologous immunity varies widely depending on the premunizing isolate.[8,9,14,33,34] The paradox created by the noted disadvantages has limited the overall effectiveness of premunization — the more highly virulent isolates usually induce the most widely cross-isolate protection, but are also most likely to cause morbidity or mortality.[9,54] The virulent isolates also cause the more severe stress-induced relapses, carrier-associated production losses, and disease when transmitted to susceptible cattle. The particular methods of premunization developed share the disadvantages of this paradox to different degrees.

1. Premunization with Virulent A. marginale Followed by Antibiotic Treatment

This method most closely resembles the immunity engendered by natural infection in enzootic areas. Cattle, preferably calves partially protected by colostral antibody, are inoculated with a virulent local isolate and monitored clinically to detect early signs of illness which, if severe, can be treated with imidocarb or tetracyclines.[56,73] Comprehensive trials in Colombia have demonstrated that, given good veterinary supervision, this method is effective, particularly if cattle are reexposed periodically by natural challenge.[74] The primary disadvantage is the reliance upon prompt detection and treatment of animals undergoing severe premunizing infections. This requires skilled animal health personnel and close sur-

veillance not available or amenable to production systems in many tropical regions. The premunizing isolate selected needs to protect against all local isolates or isolates to be brought in by new additions to the herd. The usual source of the inocula is a persistently infected carrier cow. This obviates the need for a prolonged cold-chain, but results in variation in infective doses and, therefore, makes the efficacy of establishing a premunizing infection unreliable. Failure to establish premunition in an individual within a herd of persistently infected cattle will frequently result in severe morbidity or mortality during the subsequent vector season. In addition, direct use of carrier blood as a premunizing inocula carries the risk of transmitting unsuspected infectious agents, including other hemoparasites. Alternatively, inocula can be standardized and maintained in liquid nitrogen at a regional animal health laboratory. While ensuring more consistent premunition, this again requires a prolonged cold-chain frequently unavailable in the tropics.

Improvement of the basic method has been attempted by antibiotic treatment at set intervals in order to reduce the need for continual supervision.[56] Unfortunately, variation in prepatent period among individuals can result in severe morbidity in some, while others are treated with antibiotics prior to developing immunity and remain fully susceptible.[56,73] In summary, the variability in the response to the premunizing inocula and the need for detailed veterinary supervision are severe limitations on the overall effectiveness of this method.

2. Premunization with Minimal Infective Doses of A. marginale

The severity of acute infection and prepatent interval have been shown to be related to the number of organisms inoculated.[47,75] Based upon these findings, several investigators have attempted to use a minimal infective dose that would uniformly infect (premunize) all recipients, but avoid serious morbidity and mortality associated with higher numbers of organisms of the same isolate.[56,74] The obvious advantage would be to avoid the need for veterinary surveillance of premunized cattle. Unfortunately, the dose response varies widely depending upon the isolate of *A. marginale*, the length of storage in liquid nitrogen, route of inoculation, and the age, breed, and nutritional status of the inoculated cattle.[54,74,76-79] Although experimental work showed that the principle was sound, the number of variables make the method unfeasible for field use.[56]

3. Premunization with A. centrale

This method of protecting cattle against anaplasmosis was first demonstrated by Theiler in 1912, when he showed that premunization of cattle with the less virulent *A. centrale* prevented severe morbidity upon *A. marginale* challenge.[29] *A. centrale* premunition has subsequently been used extensively in Israel and Australia and throughout Africa.[80-82] Although conflicting reports are common, the efficacy of this method under ideal conditions has been demonstrated in Israel.[81] Within Israel, a well-coordinated program of *A. centrale* premunization exists. Government veterinarians are responsible for producing and standardizing the inocula, maintaining a liquid nitrogen cold-chain, inoculating the cattle, and ensuring that a premunizing infection occurs without significant morbidity.[81,82] The effectiveness of the method as practiced in Israel certainly reflects upon the available veterinary intrastructure as much as on the particular method. In contrast, very few other subtropical or tropical nations, including regions in the U.S., have livestock production systems and regulated veterinary services amenable to the *A. centrale* program as practiced in Israel.[19] Disadvantages include the cold-chain requirement and the need for veterinary supervision to recognize and treat severe clinical disease following premunition with vaccine isolates of *A. centrale*.[32,56,71,83] Whether these vaccine isolates causing severe morbidity have varied antigenically from the Israel vaccine *A. centrale* isolate is unknown. As well, the efficacy of *A. centrale* premunization has been shown to vary depending upon the *A. marginale* isolate used in challenge.[31,57] Infection, but not severe disease, results from challenge with

Israel isolates of *A. marginale*, while severe morbidity has been reported upon challenge with other *A. marginale* isolates.[51,57] The effect upon production in cattle harboring both the premunizing *A. centrale* infection and *A. marginale* isolates following natural challenge infection has not been described in *Babesia* coenzootic regions. In summary, although the *A. centrale* program in Israel demonstrates the potential of the method, its applicability in most tropical regions is limited, and its use has been reported to be diminishing.[56]

4. Premunization with Attenuated A. marginale

The search for naturally occurring *A. marginale* isolates of low virulence that could be used to premunize cattle without morbidity and protect against challenge with virulent isolates has been largely unrewarding. In contrast, laboratory attenuation of a virulent *A. marginale* isolate (Florida isolate) using irradiation combined with passages through deer and sheep resulted in an isolate meeting these criteria.[69,84] Premunization of young cattle with a standardized dose of attenuated Florida isolate usually results in low parasitemia and mild anemia, but no or minimal clinical disease including effect on weight gain.[54,69,84,85] Premunization of older bulls and cows may cause severe morbidity, abortion, or mortality.[86] In addition, severe anemia accompanied by lethargy and depression has been reported to occur even in yearlings.[86] Upon challenge, cattle develop a low parasitemia, but are protected against severe clinical disease.[55,69,87] The immunity includes protection against several heterologous isolates upon both experimental and field challenge.[55,69,84,88] There is one report of failure to protect against challenge with a Colombian field isolate.[89] Premunization of fully susceptible cattle prior to introduction into an enzootic-susceptible region in Colombia provided solid protection against disease and production losses.[55] In addition, the attenuated *A. marginale* is not tick transmissible and, therefore, allows greater control over its spread to fully susceptible older animals.[90] The attenuated organism is within ovine erythrocytes and does not induce significant titers of isoantibodies in recipients, thus avoiding hemolytic isoerythrolysis in calves ingesting colostral antibodies.[91]

The primary disadvantages of this method of premunization is its reliance on a cold-chain in the tropics. Although reversion to virulence has been reported following 12 passages in splenectomized calves, the significance of this potential problem is not clear.[92] The occurrence of severe premunizing infections and the failure to protect against challenge with certain heterologous isolates can be serious drawbacks to the efficacy of the method, even if infrequent.[86,89] Nevertheless, the standardization of the inoculum and the use of an attenuated *A. marginale* isolate make this method the most effective form of premunization available.

B. Immunization with Killed Whole *A. marginale* Organisms

Immunization with nonliving antigens would be highly desirable as it would avoid the necessity for a prolonged cold-chain in the tropics, the morbidity of premunizing infections, and the introduction of premunizing infections into disease-free but susceptible herds. As well, the risk of transmitting other infectious agents in a cryopreserved inoculum and production losses from the induced carrier status would be abrogated. The challenge in developing a killed vaccine is to induce solid, cross-protective immunity — a goal more readily achieved in most infectious diseases by a live vaccine due to the repeated antigenic stimulus. Presentation of key protection-inducing antigens, avoiding isoantibody formation, and adjuvant selection are all critical criteria in developing an effective vaccine. To date, the available killed vaccines have been limited in their effectiveness by their inability to protect against virulent, heterologous isolates and by the induction of isoantibodies which, upon colostral transfer, cause hemolytic isoerythrolysis in calves.[34,93]

Immunization with the Anaplaz® vaccine (Fort Dodge Laboratories, Fort Dodge, IA) induces partial protection against challenge with heterologous isolates.[34] The severity of the

clinical disease is usually reduced compared to nonvaccinated controls; however, significant parasitemia, anemia, and weight loss occur.[54,55] Challenge with virulent isolates, notably the Florida isolate, has been reported to cause severe anemia and high mortality similar to that in unvaccinated cattle.[34] Cattle which have recovered from acute infection remain persistently infected and serve as reservoirs for transmission to unvaccinated cattle, as well as remaining susceptible to relapses and production losses associated with the carrier state.[69] In addition, because the vaccine requires an oil adjuvant and is composed of whole *Anaplasma* organisms admixed with erythrocyte stroma, immunized cattle develop antierythrocyte isoantibodies which can cause isoerythrolysis in calves ingesting these colostral antibodies.[93-95] The overall incidence of isoerythrolysis is low; however, morbidity and mortality can be significant within individual, especially purebred herds.[96] Efforts to reduce neonatal isoerythrolysis have included alteration of the immunization schedule, production of the isolate in cattle with less common blood groups or in ovine erythrocytes, and conjugation of the immunogen to lipids to diminish the isoantibody response.[97-100] While this vaccine induces strong antibody titers to whole organism antigens and *Anaplasma*-specific lymphocyte/macrophage responses, the significance of these responses in protection and which epitopes induce protection have not been described.[69]

Immunization of cattle using related whole organism preparations (also containing erythrocyte stroma) from other *A. marginale* isolates provided partial protection against challenge similar to that seen with the Anaplaz® vaccine.[101,102] The severity of morbidity upon challenge is reduced in most, but not all, vaccinates, and significant parasitemia and anemia developed in all vaccinated cattle upon challenge.[101] The results again indicate that more basic research in epitope identification and presentation is needed to develop a more effective vaccine.

V. DEVELOPMENT OF ANTIGENICALLY DEFINED VACCINES

The lack of progress in developing effective, widely used *Anaplasma* vaccines results from the complexity of the organism's invertebrate vector and vertebrate stages, antigenically variant isolates, and their ability to persist in the host. Yet, the protection-inducing ability of current methods of immunoprophylaxis, despite their other drawbacks, clearly indicate that effective immunization is feasible. Advances in the molecular biology of infectious agents have provided the opportunity to unravel the complexity of the host-parasite relationship to identify conserved *Anaplasma* epitopes capable of inducing cross-protection. Once identified, protective epitopes can be efficiently produced and presented to the host immune system using genetically engineered viral vectors carrying key *Anaplasma* epitopes.[103] This approach focuses the immune response on these key epitopes, does not incorporate erythrocyte antigens, and, by using a live viral vector, overcomes the traditional poor efficacy of killed vaccines.[20,56,69,103] Erythrocyte culture of *Anaplasma* and purification of initial bodies are important tools in identifying protective antigens, but are unlikely to be competitive as a source of vaccine due to erythrocyte contamination, poor efficacy of killed vaccines, and high cost of production.[56,59,104-106] The current emphasis in anaplasmosis, as well as in other hemoparasitic diseases, is to clone and express genes coding for protective epitopes.

Our strategy is to identify and isolate initial body-surface proteins using a combination of biochemical and immunological techniques. Cattle immunized with individual surface proteins are challenged with homologous and heterologous isolates of *A. marginale*. Proteins that induce partial or complete protection are selected as candidates for incorporation in a genetically engineered vaccine. Next, full-length genes coding for the proteins or gene sequences coding only for the isolate-common, protective epitopes are cloned, and either the recombinant protein is retested as an immunogen or the gene is inserted directly into vaccinia for testing as a recombinant viral vector.

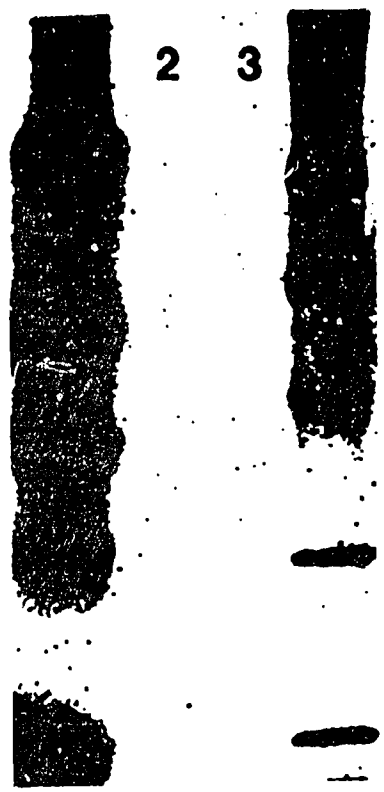

FIGURE 4. Identification of *A. marginale* initial body surface proteins recognized by neutralizing antibody.[104] Initial bodies purified from Florida isolate *A. marginale*-parasitized erythrocytes were surface radiolabeled with ^{125}I, detergent disrupted, and immunoprecipitated with either preimmune antibody (Lane 3) or antibody capable of neutralizing the infectivity of 10^{10} initial bodies (Lane 1).[104] Immunoprecipitated proteins were separated by SDS polyacrylamide gel electrophoresis and detected by autoradiography. Surface proteins of apparent molecular weights 105, 86, 61, 36, and 31 kDa were specifically recognized by neutralizing, but not preimmune sera (Lanes 1 and 3).[104] Uninfected erythrocyte membranes radiolabeled with ^{125}I under identical conditions were not recognized by the neutralizing antibody (Lane 2).[104] ^{14}C molecular weight standards are 200, 92.5, 69, 46, 30, and 14.3 kDa (Lane 4).

We selected the Florida isolate of *A. marginale* as the principal isolate for our studies, due to its high virulence and ability to induce widely cross-protective immunity.[34,55,69,84,88] The latter indicated that the Florida isolate may express more cross-protective epitopes than other isolates and increased our chances of detecting these epitopes. Surface radioiodination of purified initial bodies, followed by immunoprecipitation with a neutralizing antibody, identifies five major surface proteins with apparent molecular sizes of 105, 86, 61, 36, and 31 kDa (designated with the prefix AmF to indicate organism and isolate) (Figure 4).[104] The antibody was produced against purified, infective, and intact initial bodies and was shown to completely neutralize the infectivity of 10^{10} Florida isolate initial bodies for splenectomized calves (Table 5).[104] Our results clearly established that initial body epitopes capable of inducing protective antibody were present and that these five surface proteins most likely bear the epitopes responsible for the induction of the antibody. The key questions raised by this initial experiment: which protein or proteins bear the critical epitopes, were the epitopes shared by other isolates and *A. centrale*, and could cattle immunized with individual proteins mount a protective response? We have isolated, characterized, and tested both AmF 105

Table 5
ANTIBODY-MEDIATED NEUTRALIZATION OF VIRULENT *A. MARGINALE* INITIAL BODIES[a]

Ab reactivity[b]	No. infected/challenged	No. dead/challenged
Preimmune	4/4	4/4
Anti-initial body	0/6	0/6

[a] Initial bodies were purified from Florida isolate-parasitized erythrocytes and incubated for 45 min at 20°C with antisera diluted 1:1 in RPMI 1640.[104] The mixture was inoculated intramuscularly into splenectomized calves.[104] Blood samples were collected daily for 75 d postinoculation in order to determine packed cell volume and presence of parasitized erythrocytes.

[b] Immune sera was produced by repeated immunization with 5×10^8 purified initial bodies in either complete or incomplete Freund's adjuvant.[104] This sera had an indirect immunofluorescent antibody titer of 1:16,000 to initial bodies. Preimmune sera was unreactive.[104]

and AmF 36 as protective immunogens. AmF 86, AmF 61, and AmF 31 are currently being isolated in our laboratories and have not been tested as immunogens to date.

A. AmF 105 as a Protective Immunogen

We have identified a neutralization-sensitive epitope on AmF 105 which was subsequently shown to be highly conserved among *A. centrale* and *A. marginale* isolates from Israel, Kenya, and the U.S. (Tables 2 to 4, reviewed in Section III).[40,45,47] The presence of this conserved epitope on 100% of initial bodies in all stages of acute infection provided strong rationale for testing AmF 105 as a cross-protective immunogen.[40,45] In addition, AmF 105 shares cross-reactive epitopes with tick midgut stages and is recognized by high-titer antibodies from effectively premunized cattle.[46,107] To test whether this subunit could induce protection in immunized cattle, we isolated AmF 105 by immunoaffinity chromatography on a MAb 15D2-Sepharose® 4B column (Figure 5).[47] Calves immunized with the purified protein developed high titers of antibody to AmF 105 and were significantly protected from challenge with either 10^8 purified Florida isolate initial bodies (Table 6) or 10^{10} Florida isolate-parasitized erythrocytes compared to identically challenged, ovalbumin-immunized calves.[47,108] Two of the AmF 105 immunized calves in each group did not show any parasitized erythrocytes in Wright's stained blood smears, while the other six developed only a transient parasitemia following a significantly prolonged prepatent period.[47,108] To test the ability of AmF 105 to induce cross-protective immunity, we challenged a third group of immunized calves with 10^{10} Washington-O-parasitized erythrocytes.[108] We chose the Washington-O isolate because it is a recent field isolate with clearly demonstrated antigenic, morphologic (Washington-O bears an appendage, Florida does not), and protein structural differences.[38,40] None of the five AmF 105-immunized calves developed parasitemia upon challenge, while all ovalbumin-immunized control calves were infected.[108] The more complete protection seen with heterologous challenge vs. homologous challenge with the Florida isolate is most likely due to the Washington-O isolate being less virulent, even in control calves, and therefore less likely to "breakthrough" in AmF 105-immunized calves.[108] These experiments clearly demonstrated that AmF 105 had strong potential as a protective immunogen and that production of AmF 105 via recombinant DNA expression was a necessary next step towards more complete characterization and testing.

Initial characterization of AmF 105 indicated that the affinity-purified immunogen occurred as a doublet on sodium dodecyl sulfate polyacrylamide gels (Figure 5).[47] Although true glycosylation would be highly unlikely in a prokaryote, the presence of strong noncovalent

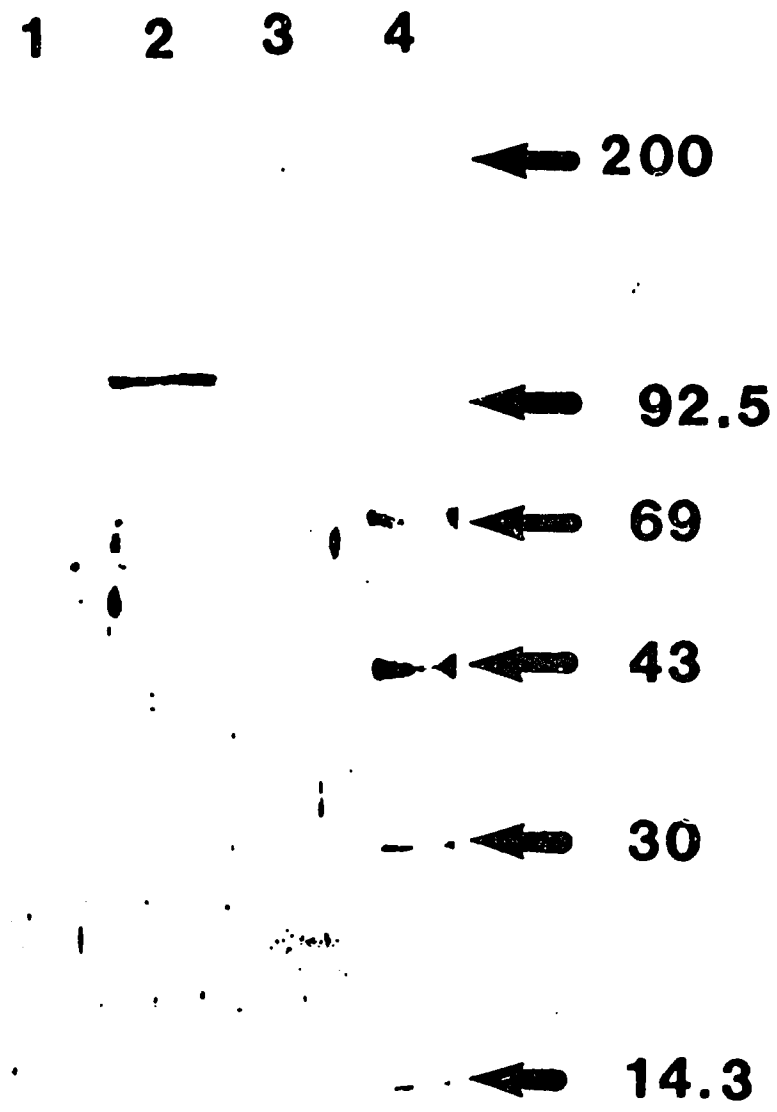

FIGURE 5. Purification of AmF 105 by immunoaffinity chromatography.[47] 10^{12} Florida isolate initial bodies were purified from parasitized erythrocytes, detergent disrupted, and bound to a MAb 15D2-Sepharose® 4B affinity column.[47] Following extensive washing, AmF 105 was eluted using 0.5% deoxycholate and 2 *M* KSCN.[47] Initial body proteins were separated by SDS polyacrylamide gel electrophoresis and detected by silver staining. Lane 2 contains initial body proteins prior to chromatography, Lane 3 contains purified AmF 105 following elution and dialysis.[47] The "doublet" composition of native AmF 105 comprising AmF 105U and AmF 105L can be noted in Lane 3.[109] Purified AmF 105 was subsequently tested as a protective immunogen in cattle.[47] Lane 1 was not loaded in order to control for silver staining artifacts. Lane 4 contains molecular weight standards (indicated in kilodaltons in the right margin).

interactions between surface membrane proteins and polysaccharides raised the possibility that binding of carbohydrate to the protein surface could account for the molecular weight heterogeneity and that neutralization may be directed against a carbohydrate moiety.[48,109] Examination of AmF 105 for bound carbohydrate residues has been uniformly negative using several different methods: periodic acid Schiff staining of purified AmF 105, metabolic

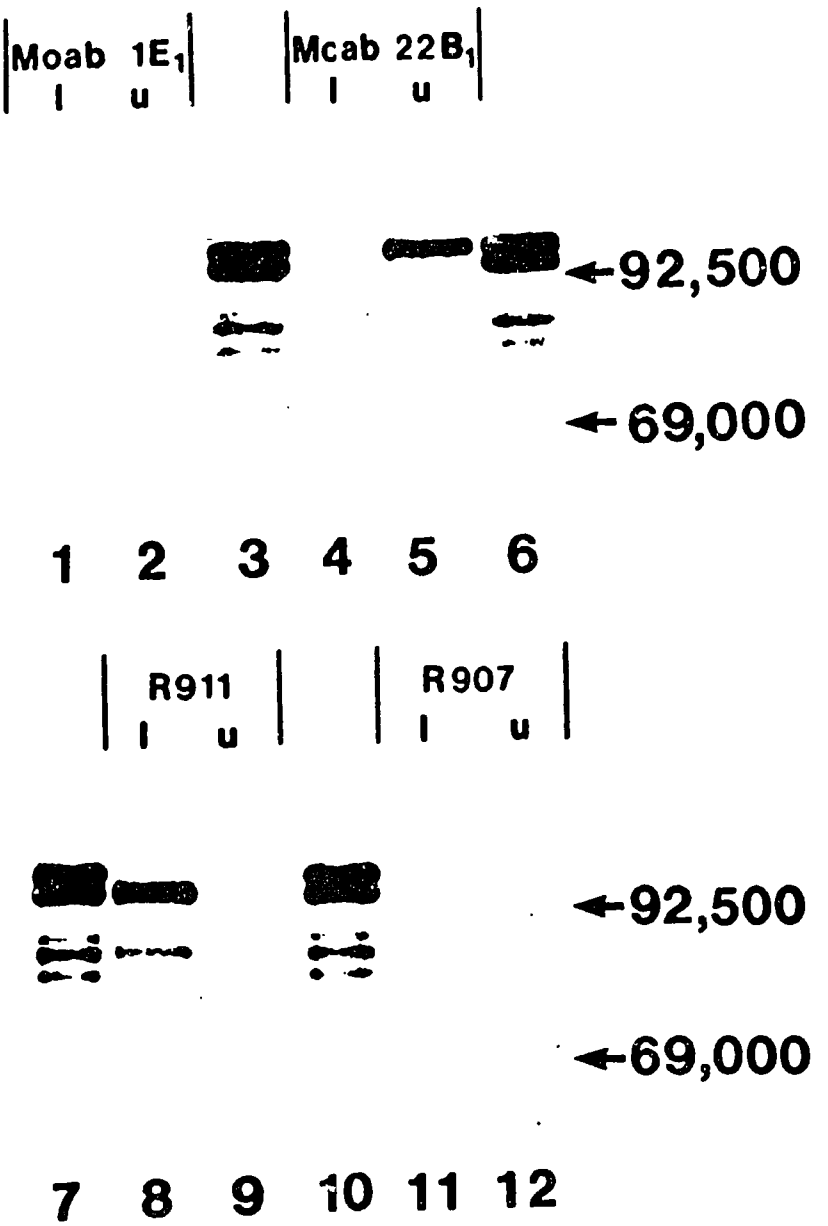

FIGURE 6. Antigenic dissimilarity of AmF 105U and AmF 105L. *A. marginale* initial bodies were radiolabeled with ^{35}S-methionine during short-term *invitro* erythrocyte culture, immunoprecipitated with MAb 22B1, and the precipitated AmF 105U + L detected by SDS polyacrylamide gel electrophoresis with fluorography (Lanes 3, 6, 7, 10).[38,110] Individual AmF 105U or AmF 105L bands were then excised, electroeluted, and reimmunoprecipitated with different antibodies. The reimmunoprecipitates were separated by electrophoresis on 7.5% polyacrylamide SDS gels containing 4 *M* urea and detected by fluorography.[110] AmF 105L was reimmunoprecipitated with MAb 1E1 (an unrelated MAb against *Trypanosoma brucei*, Lane 1); MAb 22B1 (neutralizing MAb to *A. marginale*, Lane 4); R911 (rabbit antibody made to recombinant *E. coli* expressing AmF 105L); and R907 (preimmune rabbit antibody).[110] AmF 105U was reimmunoprecipitated with MAb 1E1 (Lane 2), MAb 22B1 (Lane 5), R911 (Lane 9), and R907 (Lane 12).[110] These results demonstrate that AmF 105U and AmF 105L are antigenically dissimilar and that only AmF 105U bears the neutralization-sensitive epitope recognized by MAb 22B1.[110]

Table 6
AmF 105 IMMUNIZATION INDUCES PROTECTION AGAINST ANAPLASMOSIS[a]

Group	No. infected/challenged	Mean days to 1% parasitemia (range)	Mean peak parasitemia	Mean low PCV
Nonimmunized	4/4	29(26—31)	4.2	23
Ovalbumin	5/5	33(31—35)	5.4	24.5
AmF 105	3/5[b]	b	<0.01[c]	31[c]
p[d]	ND[e]	≤0.01	≤0.01	≤0.01

[a] Calves were immunized with 100 μg of AmF 105 or 100 μg of ovalbumin emulsified in complete Freund's adjuvant and boosted three fold at 2-week intervals with 100 μg of antigen in incomplete Freund's adjuvant.[47] The AmF 105 vaccinates developed a titer of 10^4 to 10^5 as determined by radioimmunoassay with ^{125}I-AmF 105.[47] The calves and four nonimmunized control calves were challenged by intramuscular injection with 10^8 purified Florida isolate *A. marginale* initial bodies.[47] Blood samples were collected daily for 100 d postchallenge in order to determine packed cell volume and presence of parasitized erythrocytes. The mean number of days postchallenge to 1% parasitemia was calculated for all infected calves in each group.

[b] Two of the five AmF 105-immunized calves did not become infected and remained hematologically normal.[47] The three AmF 105 vaccinates that became infected developed a transient parasitemia of <0.01% on day 39 and did not develop clinical disease.[47]

[c] These hematologic values are based upon the three AmF 105 vaccinates with mild infection to demonstrate protection against disease afforded by AmF 105 immunization.[47]

[d] p values are calculated with the pooled t test to compare the responses of the AmF 105 and ovalbumin-immunized cattle.

[e] ND: not determined.

incorporation of radiolabeled carbohydrate precursors into AmF 105 during short-term *in vitro* cultivation of parasitized erythrocytes, carbohydrate-specific surface radiolabeling, and binding of AmF 105 to various lectins.[48] The lack of carbohydrate residues on AmF 105 suggested that the neutralizing MAbs are recognizing a peptide epitope.[48] Further indication that the AmF 105 neutralization-sensitive epitope is composed of amino acids was its sensitivity to complete protease digestion using pronase, proteinase-K, or trypsin.[48] Given that carbohydrate differences were not likely responsible for the AmF 105 doublet, we considered two possibilities: (1) both proteins were derived from one gene and bear the neutralization-sensitive epitope reactive with MAbs 15D2/22B1 and were derived by differences in processing, or (2) the proteins were separate gene products with only one bearing the neutralization-sensitive epitope and the second protein being noncovalently bound to the first and, therefore, copurifying.[110] Complete separation of the two proteins — designated AmF 105U (upper) and AmF 105L (lower) — on 7.5% polyacrylamide gels containing 4 *M* urea followed by elution and reimmunoprecipitation with MAbs 15D2 or 22B1 indicated that AmF 105U bore the relevant epitope, while AmF 105L did not (Figure 6).[110] Peptide mapping using partial proteolysis of AmF 105U and AmF 105L demonstrated that the two proteins were structurally dissimilar and were most likely separate gene products (Figure 7).[110] Although only AmF 105U bears the demonstrated neutralization-sensitive epitope, we have shown that both AmF 105U and AmF 105L have surface-exposed epitopes, and therefore either or both could be responsible for protection in affinity-purified AmF 105-immunized cattle.[110]

Our approach to dissecting the antigenic basis of AmF 105 induced protection is to clone and express full-length genes coding for each protein in *Escherichia coli* and to test the recombinant-produced protein as a protective immunogen. The testing of full-length recombinant AmF 105U (rAmF 105U) and rAmF 105L homologous to native AmF 105U and L will allow determination of which components are required for a vaccine: rAmF 105U,

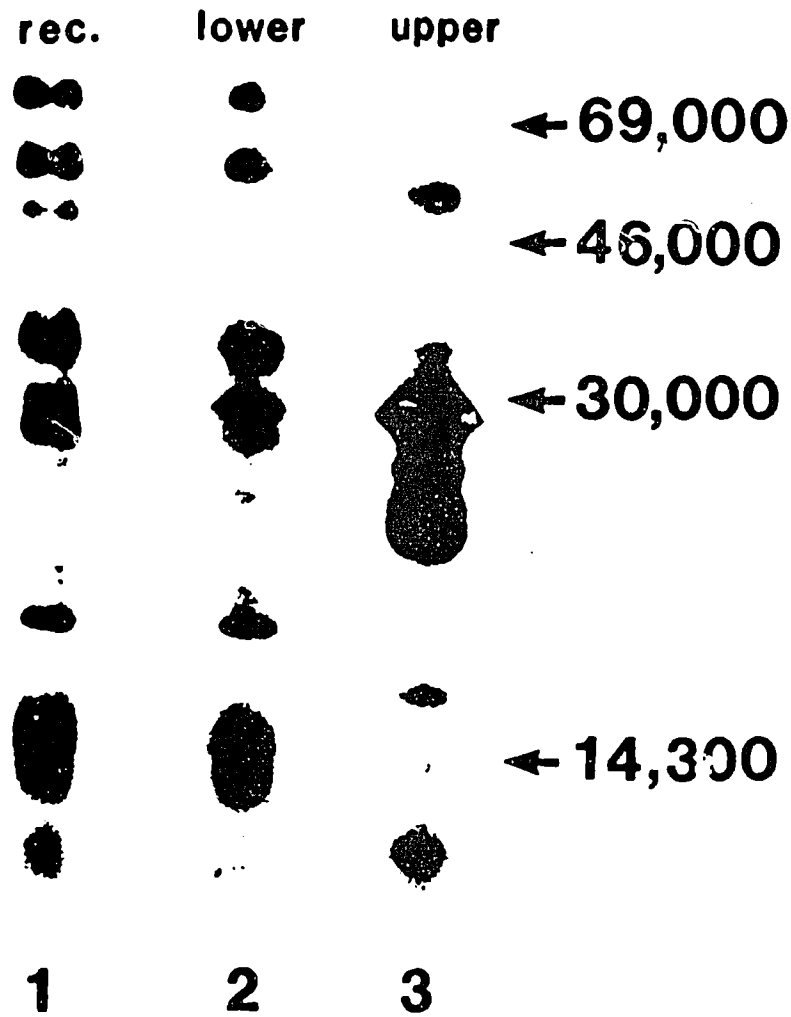

FIGURE 7. Structural dissimilarity between AmF 105U and native or recombinant AmF 105L.[110] Individual ^{35}S-radiolabeled AmF 105U, AmF 105L, or recombinant AmF 105L bands were excised, electroeluted, and subjected to digestion with 0.025 μg *Staphylococcus aureus* V8 protease.[110] The resulting fragments were separated on a 15% polyacrylamide gel and visualized by fluorography. Digestion patterns of recombinant AmF 105L (Lane 1) and native AmF 105L (Lane 2) demonstrate substantial homology, but are markedly dissimilar from AmF 105U (Lane 3).[110] Molecular weight markers are indicated by arrows (right margin).

rAmF 105L, or rAmF 105U + L. In the latter instance it may be critical to restore the noncovalent interaction between the integral membrane proteins.

We have cloned and expressed the AmF 105L gene in *E. coli* using pBR 322.[110] Full-length rAmF 105L is structurally and antigenically homologous with native AmF 105L and dissimilar to AmF 105U (Figure 7).[110] Recombinant AmF 105L is stablely produced as approximately 1% of the *E. coli* proteins and appears to be expressed under its own promoter, as inserts in either orientation are equally expressed.[110] Production of antibody against rAmF 105L has enabled us to antigenically characterize AmF 105L independent of AmF 105U. The agglutination of initial bodies incubated with rabbit antibody to rAmF 105L confirms that AmF 105L has surface-exposed epitopes and meets criteria for a potential immunogen.[110] In addition, this antibody recognizes epitopes on 100% of initial bodies in parasitized erythrocytes throughout acute infection with any of the eight *A. marginale* isolates yet

tested.[110] This demonstrates that similar to the conservation of the AmF 105U epitope detected by neutralizing MAbs 15D2 and 22B1, AmF 105L bears widely conserved epitopes. Unlike AmF 105U, the ability of these conserved epitopes to induce neutralizing antibody is unknown. Sera from the protected calves immunized with native AmF 105U + L have a high titer of antibody to rAmF 105L, indicating that the surface epitopes were immunogenic, but not necessarily protective in cattle.[111] The role of AmF 105L in inducing protective immunity and its effectiveness as an immunogen have not yet been determined. A pilot experiment in which 10^8 Florida isolate initial bodies were incubated with rabbit anti-rAmF 105L polyclonal antibody did not result in significant neutralization.[111] We are currently isolating rAmF 105L using monoclonal antibody affinity chromatography in order to test its efficacy as a protective immunogen in cattle.

Evidence to date indicates that very likely AmF 105U is important in inducing protective immunity and may be largely responsible for the protection seen in native AmF 105-immunized cattle.[47,110] AmF 105U bears the isolate- and species-conserved, neutralization-sensitive epitope, and this epitope is specifically recognized by high-titer antibody from the protected calves.[47,49] This epitope is conserved despite marked molecular size variation in the parent molecule among different isolates.[112] We have recently obtained expression of the AmF 105U gene in *E. coli* using pKK-233-2.[113] The protein is structurally identical to native AmF 105U and includes the neutralization-sensitive epitope recognized by MAbs 15D2 and 22B1.[113] In addition, sera from the native AmF 105-immunized cattle reacts strongly with recombinant Amf 105U protein.[113] Our efforts are presently directed at determining the role of AmF 105U in protective immunity and examining isolate and species differences in this surface protein and its coding gene. Testing recombinant-derived AmF 105U alone and with rAmF 105L as a protective immunogen remains the highest priority. In addition, we are determining the primary structure of the neutralization-sensitive epitope by DNA sequencing and synthetic peptide constructions. This will allow presentation of the neutralization-sensitive epitope to the host immune system in order to focus the response upon this epitope. If protective, this strategy could be used in viral vectors to generate an effective vaccine.

Critical to further development of AmF 105 as a vaccine component, is to understand the mechanism of protection in the native AmF 105 immunized cattle. We have determined that antisera from these protected cattle mediate initial body phagocytosis *in vitro* using bone marrow-derived macrophages.[108] Generation of recombinant AmF 105U and AmF 105L and their testing as protective immunogens will enable us to determine which component induces opsonizing antibody and how well opsonization correlates with protection. Other possible mechanisms of neutralization have not been closely examined in AmF 105U-immunized cattle. Understanding the mechanism of protection in subunit immunized cattle may facilitate selection and incorporation into a vaccine of only those epitopes that induce protective antibody.

B. AmF 36 as a Protective Immunogen

AmF 36 fulfills several criteria of a potential protective immunogen: (1) the protein has surface-exposed epitopes as demonstrated by lactoperoxidase-mediated radioiodination (Figure 4), (2) epitopes are conserved among *A. marginale* isolates in the U.S., Israel, and Kenya and *A. centrale* (Tables 2 and 4), and (3) AmF 36 epitopes are present in tick midgut stages of the organism (Figures 2 and 3). In addition, high titers of antibody to AmF 36 are present in cattle effectively premunized with different *A. marginale* isolates.[107] AmF 36, purified by monoclonal antibody affinity chromatography, elicits high titers of specific antibody in immunized calves, and these calves are significantly protected against challenge with either 10^8 Florida isolate initial bodies or 10^{10} Washington-O-parasitized erythrocytes compared to ovalbumin-immunized controls.[114] Two of the AmF 36-immunized calves in

each challenge group did not become infected with *A. marginale*, and those AmF 36-immunized calves that became infected had significantly prolonged prepatent periods compared to control calves.[114] These experiments demonstrate that AmF 36 can induce protective immunity in cattle and is a candidate for inclusion in an antigenically defined vaccine. We are now screening gene libraries for expression of AmF 36 in *E. coli* in order to further characterize the molecule and test the ability of recombinant AmF 36 to induce protection in immunized cattle.

C. Progress in Subunit Vaccine Development

The current effort in hemoparasitic diseases is primarily to identify antigens relevant to protective immunity and to develop effective methods to present these antigens to the host. It is important to remember that testing of individual antigens as protective immunogens is quite different from testing an end-product vaccine. Testing of individual components is a critical but early step in vaccine development — the eventual vaccine may incorporate epitopes from several different surface proteins in order to achieve maximal efficacy. In anaplasmosis, two proteins, AmF 105 and AmF 36, have been identified as bearing protection-inducing epitopes.[47,108,110,114] In addition, at least three other initial body surface proteins, AmF 86, AmF 61, and AmF 31, are potential vaccine candidates.[104,107] Our strategy is to clone and express full-length genes coding for these surface proteins and to test recombinant-derived proteins as protective immunogens in cattle. Concomitantly, it is essential to identify isolate- and species-conserved epitopes and to determine the role of these epitopes in the induced protection.[45,47] Increased understanding of antigenic variation in key epitopes, the differences between tick-stage and erythrocyte-stage challenge, and mechanisms of immunity will be instrumental in selecting epitopes for improved anaplasmosis vaccines.

Although the steps remaining to develop, test, and deploy an effective anaplasmosis vaccine are numerous, they are becoming more clearly defined due to advances in the molecular biology of complex hemoparasites including *Anaplasma*. Similarly, the successes and failures of current *Anaplasma* vaccines indicate the requirements any vaccine must meet to be widely effective: (1) safe (unable to induce morbidity in vaccinates including antierythrocyte antibody); (2) induce strong protection; (3) induce cross-protection, requiring conserved epitopes; (4) not reliant upon a prolonged cold-chain; and (5) economical.[103] In addition, because different hemoparasites frequently share tick vectors and are coenzootic, to be truly effective and replace acaracide use as a principal means to control hemoparasitic disease, a vaccine should incorporate protective antigens for all regionally important hemoparasites. The availability of replicating viral vectors with large capacities for foreign genes and a wide host range provide an ideal vehicle to develop a multidisease vaccine.[103] Live recombinant vaccinia viruses expressing foreign genes are highly immunogenic in cattle, economically produced in tissue culture or animal skin, stable in freeze-dried form (continuous refrigeration is unnecessary), and simply administered by skin scratch — fulfilling all criteria for an effective vaccine in the tropics.[103,115,116] These breakthroughs in vaccine technology combined with advances in identifying protective *Anaplasma* immunogens makes development of an economical, widely cross-protective vaccine for anaplasmosis a realistic goal for the first time. Similar advances in identifying candidate vaccine antigens in the coenzootic arthropod-borne hemoparasites provide a promising future for integrated control over the devastating hemoparasitic diseases of cattle in the tropics.

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