INFECTION AND IMMUNITY, June 1988, p. 1526–1531
0019-9567/88/061526-06$02.00/0

Vol. 56, No. 6

Immunization of Cattle with a 36-Kilodalton Surface Protein Induces Protection against Homologous and Heterologous *Anaplasma marginale* Challenge

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Received 3 December 1987/Accepted 5 March 1988

Immunization of cattle with a purified *Anaplasma marginale* major surface protein, AmF36, induced protection against homologous challenge with the Florida isolate. Similarly, immunized cattle were protected from challenge with the antigenically and structurally distinct Washington-O isolate of *A. marginale*. The degree of protection in AmF36-immunized cattle varied from complete prevention of rickettsemia to significant delay in the onset of rickettsemia compared with control immunized cattle. A single AmF36 vaccinate was not protected against homologous challenge despite development of a strong antibody response. Immunoprecipitation of *A. marginale* proteins with a monoclonal antibody to AmF36 identified minor molecular size heterogeneity in this protein from different isolates, including the Florida and Washington-O isolates. The apparent molecular size of this surface protein in the Florida isolate was 36 kilodaltons, whereas the analogous proteins in Washington-O and four other isolates of *A. marginale* from the United States had molecular masses of 33 to 34 kilodaltons. Significantly, the surface-exposed peptides of these proteins appear to be conserved among the different isolates. These results demonstrate the potential of AmF36 as a subunit immunogen for bovine anaplasmosis and indicate a structural basis for its cross-protective ability.

Anaplasmosis, the most prevalent of the major livestock hemoparasitic diseases, remains without effective control and therefore is a severe impediment to efficient livestock production in tropical regions worldwide (22, 30). The disease is characterized by severe anemia caused by intraerythrocytic infection with *Anaplasma marginale* (32). The rickettsia is transmitted either via infected ixodid ticks or mechanically on blood-contaminated surgical instruments, needles, or the mouthparts of biting flies (29). Following a prepatent period of 20 to 40 days, there is a rapid increase in rickettsemia resulting in anemia, dramatic weight loss, abortion, or death (2, 29). Cattle recovered from acute infection are solidly protected from challenge with homologous isolate organisms and partially protected from heterologous challenges (7, 13, 14, 16).

A. marginale isolates differ in antigenic composition, morphology, protein structure, tick transmissibility, and virulence (3, 12, 13, 15, 16, 18, 31, 33). Therefore, development of improved vaccines must identify protective immunogens that are conserved among the numerous different isolates. We have previously identified five proteins of erythrocyte-stage *A. marginale* (Florida isolate) that have surface-exposed epitopes recognized by neutralizing antibody (27). At least two of these proteins, AmF36 and AmF105 (designated by abbreviations for genus, species, isolate, and apparent molecular mass in kilodaltons), bear epitopes highly conserved among *A. marginale* isolates from Israel, Kenya, and the United States (24, 25a, 28). In addition, epitopes from both surface proteins are shared with tick stages of *A. marginale* within *Dermacentor andersoni* ticks (26). Our strategy in vaccine development is to identify one or more antigens that induce protective immunity and to produce these antigens by recombinant DNA expression. Both AmF36 and AmF105 fulfill criteria for testing as protective subunit immunogens in cattle. Immunization of cattle with AmF105 (a complex of two surface-exposed polypeptides) has been shown to induce protection against *A. marginale* challenge, and we have recently cloned the genes for both polypeptides of the complex (4; T. C. McGuire, unpublished data). Although immunization with AmF105 alone induces protection against experimental challenge, construction of an optimal vaccine capable of protecting all cattle against all isolates may require incorporation of additional surface-exposed antigens. In this paper, we report the effectiveness of AmF36 in inducing protection to homologous and heterologous challenge and demonstrate conservation of surface-exposed peptides among different *A. marginale* isolates.

MATERIALS AND METHODS

Immunization of cattle with purified AmF36. AmF36 was isolated from Florida isolate *A. marginale*-infected erythrocytes by using monoclonal immunoaffinity chromatography as previously described (26). Briefly, 10^{12} organisms were purified from erythrocytes and detergent-soluble proteins were extracted in a 50 mM Tris buffer (pH 8.0) containing 1.0% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and 0.1 M *N*-α-*p*-tosyl-L-lysyl-chloromethylketone. The detergent-soluble proteins were applied to an affinity column composed of a monoclonal antibody against AmF36 (ANAO-58A2) coupled to Sepharose 4B (26).

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Unbound proteins were removed by washing in 100 volumes of Tris buffer, and AmF36 was eluted with 0.5% deoxycholate and 2 M potassium thiocyanate. Following extensive dialysis against phosphate-buffered saline, the purity of the eluted AmF36 was confirmed by silver staining of SDS-containing polyacrylamide gels (19, 26). Cattle were immunized with 100 μg of AmF36 emulsified in complete Freund adjuvant for the initial immunization and in incomplete adjuvant for three subsequent immunizations at 2-week intervals. Control cattle were immunized with 100 μg of ovalbumin emulsified in identical adjuvants and boosted on an identical schedule. Prior to immunization, all cattle were seronegative to *A. marginale* and were unreactive with AmF36 as determined by an enzyme-linked immunosorbent assay (ELISA) (17, 26). Following immunization, antibody titers to AmF36 were determined by endpoint titration in the ELISA, using 10-fold dilutions of serum (26). The specificity of the immune response to AmF36 was demonstrated by immunoblotting postimmunization sera on detergent-solubilized *A. marginale* antigen. Approximately 100 μg of Florida isolate antigen per lane was electrophoresed on 10% polyacrylamide gels containing SDS, electrophoretically transferred to 0.45-μm nitrocellulose, and reacted with cattle sera diluted 1:1,000. The blocking, reaction, washing, and detection methods with ^{125}I-*Streptococcus* protein G have been previously described (1, 5).

Homologous and heterologous isolate challenge. Immunized cattle were challenged by intramuscular inoculation of 10^8 Florida isolate organisms purified as previously described (homologous challenge) or 10^{10} Washington-O isolate-infected erythrocytes (heterologous challenge) (24, 27). Rickettsemia was determined by daily microscopic examination of Wright-stained blood smears for 75 days postchallenge (DPC). The number of days between challenge and 1% rickettsemia (or detectable rickettsemia in calves that did not develop 1.0% rickettsemia) was calculated for all infected calves in each group. Protection was determined by significant prolongation of the prepatent interval in AmF36-immunized individuals, as compared with the mean of the prepatent intervals in the ovalbumin-immunized cattle, using the one-sample (unpaired) *t* test (6). Individual cattle that did not develop any microscopically detectable rickettsemia were screened for low-level rickettsemia by dot hybridization using a sensitive ^{32}P-labeled DNA probe to test for *A. marginale* DNA in 5×10^6 erythrocytes collected at 36 DPC. The construction of this probe (a 2-kilobase fragment derived from the Am105L gene), its radiolabeling by nick translation, and hybridization conditions have been previously detailed (4). This *A. marginale*-specific probe is capable of detecting 250 parasitized erythrocytes in 5 μl of whole blood, equivalent to 0.001% rickettsemia (10a). This level of detection is approximately 10 to 100 times the reproducible sensitivity obtained by microscopic examination of blood smears. This probe was developed subsequent to the homologous challenge of AmF36-immunized cattle and therefore was used to confirm results from microscopic examination following heterologous challenge only.

Molecular size comparisons of analogous proteins from different isolates. Relative molecular weights of proteins analogous to AmF36 from different isolates were compared to determine whether protein size was largely conserved or, similar to AmF105, markedly variant (23a). *A. marginale* isolates (Florida, south Idaho, Missouri, north Texas, Virginia, and Washington-O), previously shown to be antigenically and structurally distinct, were radiolabeled with [^{35}S]methionine during in vitro short-term erythrocyte culture as previously described (3, 18). This technique has been shown to incorporate radiolabel exclusively into the rickettsiae (3). Following removal of unincorporated radiolabel, infected erythrocytes were solubilized in a 50 mM Tris buffer containing 1.0% Nonidet P-40, 0.1% SDS, and the proteolytic inhibitors described above. The analogous protein in each *A. marginale* isolate was identified by immunoprecipitation with monoclonal antibody ANAO-58A2 followed by electrophoresis on polyacrylamide gels containing SDS. The detailed immunoprecipitation and electrophoresis methods have been previously described (26). ^{14}C-molecular-weight-standard proteins were electrophoresed on lanes flanking the immunoprecipitates to confirm that uniform migration occurred and for apparent molecular weight determination.

Comparison of surface-exposed peptides among analogous proteins from different isolates. Organisms from each of six *A. marginale* isolates were purified from infected erythrocytes and surface radiolabeled with ^{125}I by using lactoperoxidase as previously described (27). Radiolabeled initial bodies were solubilized in 50 mM Tris buffer containing 1.0% Nonidet P-40 and 0.1% SDS and immunoprecipitated with ANAO-58A2, and the protein analogous to AmF36 was excised from dried polyacrylamide gels following electrophoresis. Each radiolabeled protein was partially digested with 1.5 μg of *Staphylococcus aureus* V8 protease for 45 min as previously described (8). Peptides produced by this limited proteolysis were separated on a 10 to 20% polyacrylamide-SDS gel, and surface-exposed peptides were detected by using autoradiography.

RESULTS

Homologous challenge of AmF36-immunized cattle. All five AmF36-immunized cattle developed a high antibody titer (>1:10,000) to the immunogen. The antibody response was AmF36 specific as demonstrated by immunoblotting (Fig. 1A). All ovalbumin-immunized cattle developed 1% rickettsemia in a mean of 33 days following challenge with 10^8 Florida isolate organisms (Table 1). Two of the five AmF36-immunized cattle did not develop any microscopically detectable rickettsemia during the 75-day observation period (Table 1). In two additional AmF36 vaccinates, there was significant prolongation of the prepatent interval (DPC to 1% rickettsemia) compared with the ovalbumin-immunized controls. The remaining AmF36-immunized animal was not protected.

Heterologous challenge of AmF36-immunized cattle. Similar to the cattle in the homologous challenge experiment, all AmF36-immunized cattle developed high antibody titers (>1:10,000) specific to the immunogen (Fig. 1B). All ovalbumin-immunized control cattle developed 1% rickettsemia in a mean of 17 days following challenge with 10^{10} Washington-O isolate organisms (Table 2). Two of the five AmF36 vaccinates did not develop rickettsemia during the observation period (Table 2) and were confirmed negative at 36 DPC by failure of the DNA probe to hybridize to 5×10^6 erythrocytes from these animals (Fig. 2). The three remaining AmF36-immunized cattle had significant prolongation in the number of DPC to detectable rickettsemia compared with control cattle. None of the AmF36-immunized cattle developed 1.0% rickettsemia during the 75-day observation period, whereas all three ovalbumin-immunized animals did (Table 2). The DNA probe hybridized to infected erythrocytes from the three infected AmF36 vaccinates and all ovalbumin vaccinates at 36 DPC. The rickettsemia was <0.01% in all infected cattle at this time.

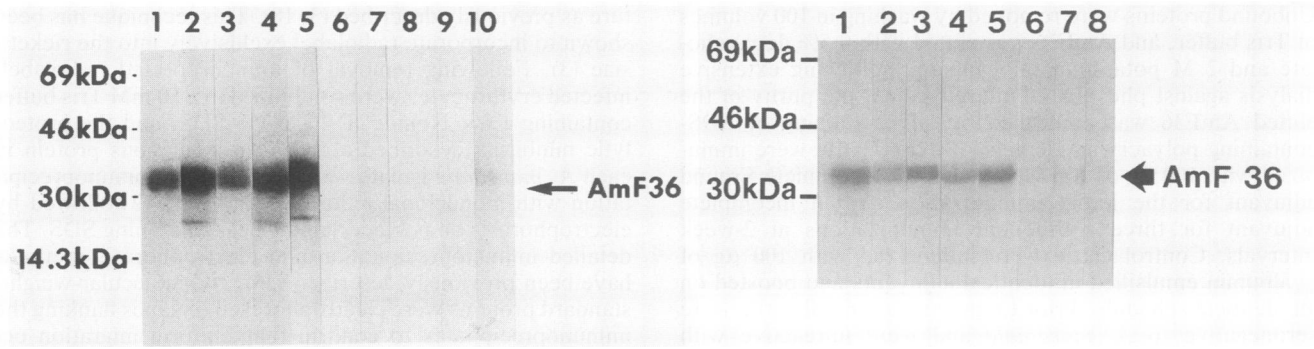

FIG. 1. Demonstration of the specificity of the immune response in AmF36-immunized cattle. Sera from cattle after immunization with AmF36 but before homologous (A) or heterologous (B) challenge were reacted with *A. marginale* Florida isolate antigens separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Antibody binding was detected by reaction with ^{125}I-labeled streptococcal protein G followed by autoradiography. (A and B) Lanes 1 to 5, sera from individual animals immunized with AmF36 before challenge; lanes 6 to 10 (A) and 6 to 8 (B), sera from individuals immunized with ovalbumin. Molecular size markers are indicated in the left margins.

Structural comparison of analogous proteins from different isolates. ANAO-58A2-reactive proteins from all isolates migrated with a relative molecular size in the range of 33 to 36 kilodaltons (kDa) (Fig. 3). Direct comparison on the same gel consistently demonstrated small differences in migration of the proteins from the different isolates. The molecular size variation was minor but reproducible on 10% polyacrylamide gels (Fig. 3) or on gels ranging from 7.5 to 17.5% polyacrylamide (data not shown). The following molecular sizes were calculated for the ANAO-58A2-reactive protein in each isolate: Florida, 36 kDa; south Idaho, 33 kDa; north Texas, 34 kDa; Virginia, 34 kDa; and Washington-O, 33 kDa. A sixth isolate, Missouri, migrated with a molecular size of 34 kDa (data not shown). Comparison of limited digestion peptide maps of each isolate demonstrated that major surface-exposed peptide fragments of approximately 30, 26, and 13 kDa were present in all six isolates examined (Fig. 4). A 20-kDa fragment was clearly present in all isolates except Missouri, in which the peptide was faintly present only after long exposures (Fig. 4).

DISCUSSION

Development of improved vaccines against hemoparasitic infections by using molecular approaches requires accurate identification of protective immunogens. In vitro correlates of immunity are frequently poor predictors for efficacy of subunit immunogens, and total reliance upon such correlates in antigen identification may focus efforts on inappropriate molecules (9, 10). Our strategy in development of a vaccine against anaplasmosis is to identify surface antigens on the erythrocyte stage of *A. marginale* that are recognized by immune sera and to test these isolated antigens for ability to induce protection in immunized cattle. Surface-exposed epitopes of AmF36 are strongly recognized by sera from recovered cattle and by neutralizing rabbit antibody (25, 27). Immunization with AmF36 induced significant protection against homologous Florida isolate challenge in four of five cattle. The reason for the failure of the fifth AmF36 vaccinate to be protected is unknown. The calf developed a high titer of antibody to AmF36 that was indistinguishable from the response of the protected calves. However, there may be significant differences in the response to key epitopes sensitive to neutralization. Variations in protection among individuals may also reflect differential activation of macrophages for phagocytosis of *A. marginale*, a role shown to be significant in other rickettsial diseases and to vary among individuals (20, 21). Since AmF36 has been identified as a vaccine candidate by in vivo protection, identification of the mechanism of AmF36-induced immunity may allow antigen

TABLE 1. Protection of cattle immunized with AmF36 against challenge with the *A. marginale* Florida isolate

Immunogen and animal no.	DPC to 1% rickettsemia[a]	Peak rickettsemia (%)
AmF36		
B117[b]	>75 (uninfected)	0.0
B155[b]	40	2.0
B157	32	4.5
B159[b]	>75 (uninfected)	0.0
B160[b]	43	1.0
Ovalbumin		
B113	32	2.0
B151	32	2.5
B153	33	2.0
B154	33	5.0
B156	33	11.0

[a] Rickettsemia was determined by daily microscopic examination of Wright-stained blood smears for 75 DPC.
[b] Protected individuals as determined by significant prolongation of the prepatent interval compared with the mean prepatent interval in the ovalbumin-immunized cattle, using the one-sample (unpaired) *t* test.

TABLE 2. Protection of cattle immunized with AmF36 against challenge with the *A. marginale* Washington-O isolate

Immunogen and animal no.	DPC to detectable rickettsemia (DPC to 1% rickettsemia)[a]	Peak rickettsemia (%)
AmF36		
B380[b]	22 (did not reach 1%)	<1.0
B382[b]	22 (did not reach 1%)	<1.0
B387[b]	>75 (uninfected)	0.0
B388[b]	>75 (uninfected)	0.0
B390[b]	22 (did not reach 1%)	<1.0
Ovalbumin		
B366	14 (17)	1.3
B368	14 (16)	2.3
B377	17 (19)	1.0

[a] Rickettsemia was determined by daily microscopic examination of Wright-stained blood smears for 75 DPC.
[b] Protected individuals as determined by significant prolongation of the prepatent interval compared with the mean prepatent interval in the ovalbumin-immunized cattle, using the one-sample (unpaired) *t* test.

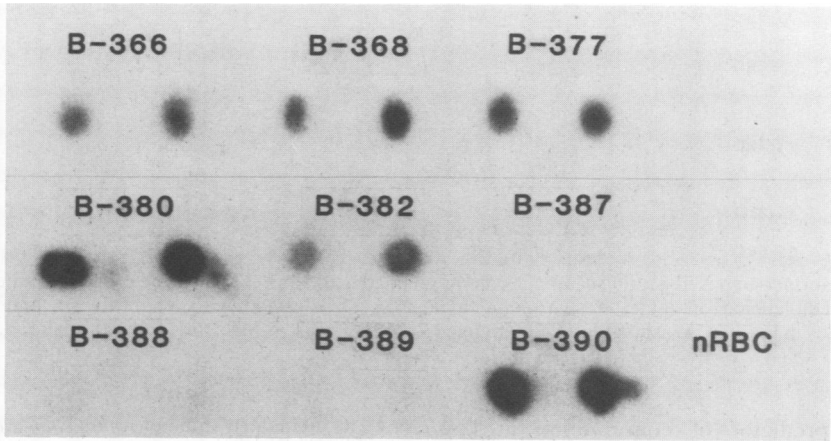

FIG. 2. Confirmation of complete protection from Washington-O challenge by using DNA hybridization. Erythrocytes (5×10^6) collected at 36 DPC were dotted on nitrocellulose in a volume of 1 μl and reacted with a 2-kilobase DNA probe derived from the gene coding for AmF105L. B366, B368, and B377 were immunized with ovalbumin. B380, B382, B387, B388, and B390 were immunized with AmF36. B389 was a previously infected calf subsequently cleared of the infection by using oxytetracycline and served as a negative control. Erythrocytes from an unexposed calf (nRBC) were also used as a negative control.

presentation to be optimized to provide more uniform protection.

Cross-isolate protection is a critical issue in vaccine development against most hemoparasitic and rickettsial diseases, including anaplasmosis. *A. marginale* isolates have significant antigenic, cross-protective, protein structural, and virulence differences (3, 12, 13, 15, 16, 18, 31). We have selected the Florida isolate as the source for antigen identification and isolation because it is the most widely cross-protective of all *Anaplasma* isolates examined (16, 31). The Washington-O isolate was selected for heterologous challenge of AmF36-immunized cattle because, unlike the Florida isolate, it is a recent field isolate (18). The Washington-O isolate differs from the Florida isolate antigenically when a panel of isolate-restricted monoclonal antibodies is used, morphologically because Washington-O bears an "appendage" that the Florida isolate lacks and structurally in several proteins as demonstrated by two-dimensional electrophoresis (3, 18). The Washington-O isolate is also less virulent than the Florida isolate. However, to test the effectiveness of AmF36 as a cross-protective immunogen, we challenged immunized cattle with 10^{10} Washington-O-parasitized erythrocytes. This challenge, approximately 100 times the number of organisms used in the homologous challenge, resulted in rickettsemia in control cattle and allowed assessment of protection in immunized cattle. The protection of all five AmF36 vaccinates against heterologous challenge demonstrated that protective epitopes on AmF36 were conserved between these two isolates. Whether these protective epitopes include any of the widely cross-isolate-conserved AmF36 epitopes recognized by a panel of five monoclonal antibodies is not known (18, 25a).

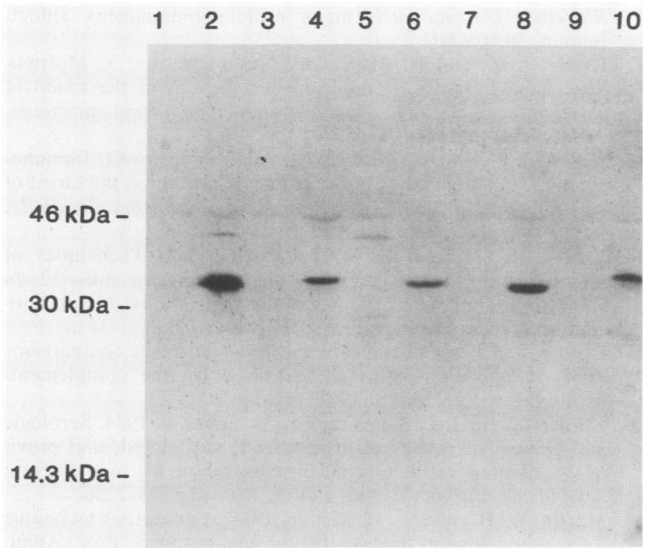

FIG. 3. Molecular size heterogeneity among proteins analogous to AmF36 from different *A. marginale* isolates. ^{35}S-labeled *A. marginale* isolates were immunoprecipitated with an isolate-common monoclonal antibody (ANAO-58A2) directed against AmF36. The apparent molecular size of the precipitated protein from each isolate was determined by electrophoresis on 10% polyacrylamide gels containing SDS with detection by fluorography; immunoprecipitation was with ANAO-58A2 (lanes 2, 4, 6, 8, and 10) or TRYP-1E1, a control monoclonal antibody against *Trypanosoma brucei* (lanes 1, 3, 5, 7, and 9). Isolates in lanes: 1 and 2, Washington-O; 3 and 4, Virginia; 5 and 6, north Texas; 7 and 8, south Idaho; 9 and 10, Florida.

The structural basis for the molecular size differences in this protein from the different isolates is unknown. In contrast to the >30-kDa molecular size variation in the AmF105 surface protein complex of *Anaplasma* (23a), the variation between AmF36 and the analogous proteins from other isolates is minor. Similar major and minor size variations have been observed in, respectively, the 110- and the 56-kDa immunodominant surface proteins of the Karp strain of *Rickettsia tsutsugamushi* when compared with the Kato and Gilliam strains (11, 23). The role of these *R. tsutsugamushi* proteins in inducing immunity and the functional relevance of the variation have not yet been reported. In *A. marginale*, the size difference between AmF36 and the analogous protein in the Washington-O isolate does not involve variation in antigens needed for neutralization. Whether these antigens are conserved among the other isolates and whether AmF36 will induce widely cross-protective immunity requires testing. However, if the conservation of surface-exposed peptides following partial proteo-

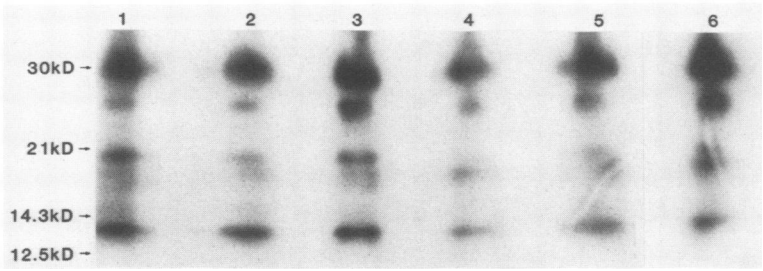

FIG. 4. Comparison of surface-exposed peptides among proteins analogous to AmF36. Surface-exposed peptides of the analogous protein from each isolate were radiolabeled on intact *A. marginale* by using ^{125}I, digested with V8 protease, and separated on 10 to 20% polyacrylamide gels. Isolates in lanes: 1, Washington-O; 2, Virginia; 3, north Texas; 4, Missouri; 5, south Idaho; 6, Florida. kD, Kilodaltons.

lysis is an accurate predictor of conservation in key epitopes, AmF36 immunization may be expected to induce protection against at least the additional four isolates from the United States examined here.

We are presently screening Florida isolate genomic libraries for expression of the gene coding for AmF36. Expression and purification of recombinant-derived AmF36 will allow us to more widely test AmF36 as a protective immunogen by using different methods of antigen presentation and in combination with recombinant AmF105 polypeptides. In addition, expression cloning will allow us to identify defined regions of AmF36 capable of inducing protection in cattle. Definition of these epitopes will facilitate understanding of the mechanism of protective immunity and development of AmF36-based vaccines capable of inducing uniform, widely cross-protective immunity.

ACKNOWLEDGMENTS

This work was supported by U.S. Department of Agriculture competitive research grants 83-CRSR-2-2194, 85-CRCR-1-1908, 86-CRCR-1-2247, and 87-CRSR-2-3106 and by United States-Israel Binational Agricultural Research and Development Fund grant US-846-84.

We thank Jan Carlson, Teresa Harkins, Pam Kaylor, and Lynn Peck for technical assistance.

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