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FINAL REPORT

PROJECT NO. US-1561-88

Protection of Cattle Against Anaplasmosis: Vaccine Development on a Molecular Basis

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1993

14-02

DATE: February 11, 1993

BARD
P.O. BOX 6
Bet Dagan, ISRAEL

BARD project No. US-1561-88

Title:

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FINAL SCIENTIFIC REPORT BARD: US-1561-88

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C. Abstract

Anaplasmosis is an arthropod-borne hemoparasitic disease of cattle caused by the rickettsia *Anaplasma marginale*. The disease occurs worldwide and remains an impediment to efficient livestock production within both Israel and the United States. The efficacy of live blood-based vaccines clearly indicates that immunoprophylaxis against anaplasmosis is an achievable goal. However, technical problems limit the use of current live and inactivated vaccines. We identified an *A. marginale* surface protein complex, composed of MSP-1a and MSP-1b subunits. MSP-1a was shown to bear a tandemly repeated neutralization-sensitive epitope conserved among all 16 *A. marginale* strains examined, including two field isolates from Israel, and *A. centrale*. Immunization of cattle with MSP-1 induced high antibody titers against the neutralization-sensitive oligopeptide epitope and opsonizing antibody for macrophage phagocytosis. *A. marginale* MSP-1a and MSP-1b genes were overexpressed in pGEX2T followed by affinity purification based on the glutathione-S-transferase fusion sequence and on-column thrombin cleavage. The structural and antigenic relationship of these polypeptides to the native polypeptides was established and the recombinant polypeptides used to immunize cattle. Immunization of cattle with recombinant MSP-1a,b or a synthetic polymer based on the MSP-1a neutralization-sensitive epitope induced statistically significant protection against rickettsemia and anemia following homologous strain challenge as compared to sham-immunized control cattle. Although significantly protected on a group basis, protection among MSP-1 vaccinates was not uniform and included completely protected, partially protected, and unprotected individuals. Additional immunization trials using different adjuvants did not significantly improve uniformity of protection. Cattle were not significantly protected against heterologous challenge with the Israel-NT strain. Failure to consistently induce immunity, resulting in partially protected and unprotected vaccinates, represents a significant constraint to vaccine development based on a single protein.

In contrast to lack of uniform protection induced by single antigens, immunization of cattle with a multivalent immunogen composed of purified *A. marginale* outer membranes was shown in collaborative studies to induce uniform protection against virulent challenge. Importantly, the level of protection was comparable to that induced by premunition. Our BARD research identified three outer membrane surface polypeptides (MSP-2, MSP-3, MSP-5) with epitopes conserved among Israel strains, U.S. strains, and the protection-inducing outer membrane immunogen. MSP-2 is the immunodominant *A. marginale* membrane protein recognized by sera from *A. centrale* premunized cattle protected against Israel strain *A. marginale* challenge. MSP-3 was identified as an outer membrane polypeptide with epitopes widely conserved among strains and MSP-5 was shown to be highly conserved among strains and with Israel *A. centrale*. MSP-3 and MSP-5 have been cloned and sequenced in our collaborative research. In summary, our BARD supported research has contributed substantially to development of a vaccine against anaplasmosis by testing and rejecting the hypothesis that recombinant MSP-1 alone can induce protective immunity and by identifying, cloning, and sequencing outer membrane polypeptides incorporated in a protection-inducing multivalent vaccine. The observation that a multivalent immunogen induces solid immunity, the observation that the membrane polypeptides have epitopes common to Israel and U.S. strains, and the current availability of the recombinant polypeptides for insertion in immunogenic membrane complexes provides a new approach for vaccine development against anaplasmosis.

D. Objectives of the Original Proposal

Anaplasmosis is an infectious, arthropod-borne disease of cattle caused by the rickettsia, *Anaplasma marginale*. The disease is characterized by intraerythrocytic parasitism leading to severe anemia. Dramatic weight loss (average 86kg per animal), abortion (24% of the clinical cases), or death (36% of clinical cases) may occur during acute infection¹. The severity of the production losses in tropical and subtropical regions, including Israel and the United States, combined with the inadequacy of current immunoprophylaxis make development of a safe, effective vaccine a high priority in both countries². We have identified an *A. marginale* surface protein complex (composed of two subunits, 105kD and 100kD) that bears a tandemly repeated neutralization-sensitive epitope³⁻⁵. This surface protein complex has been designated MSP-1 (Major Surface Protein-1) to be consistent with nomenclature for bacterial proteins⁶. The two subunits have been designated MSP-1a (105kD) and MSP-1b (100kD)^{5,6}. This repeated epitope is conserved among all 16 *A. marginale* strains examined, including two field isolates from Israel, and *A. centrale*⁷. Immunization of cattle with the purified surface complex bearing this epitope induces protection against the homologous Florida strain using challenge with either purified *A. marginale* initial bodies or parasitized erythrocytes^{3,8}. The neutralization-sensitive epitope is contained on the MSP1a polypeptide^{4,5}. The objective of our proposed project is to develop and test a subunit vaccine based on recombinant derived polypeptide against challenge with Israel and U.S. strains.

Hypothesis: Recombinant-derived MSP1 will provide protection against challenge with homologous or heterologous strains of *A. marginale*.

The specific aims to test this hypothesis are:

- 1] Establish that native MSP-1a immunization induces protection against challenge with Israel strains of *Anaplasma marginale*.
- 2] Obtain high-level expression of the gene coding for MSP-1a.
- 3] Test the effectiveness of recombinant MSP-1a as a protective immunogen against challenge with either Israel or U.S. strains of *Anaplasma marginale*.
- 4] Identify additional polypeptides with conserved epitopes for inclusion in a recombinant vaccine.

The project was extended to include Specific Aim 4 based on the reviewers' recommendation that complete protection may require additional polypeptides. This recommendation was accepted and was approved during review of the year 1 annual report.

E. Detailed Description of Research Activities and Results

We have completed each specific aim and tested the hypothesis. Briefly, we accomplished the following: i) development of a challenge protocol using live *A. marginale* that consistently induced clinical disease; ii) development of an immunization protocol that

consistently induced statistically significant protection against homologous challenge; iii) development of assays that measure immune responses against the critical neutralization-sensitive MSP1a epitope; iv) demonstration that antibody against MSP-1a promoted macrophage phagocytosis of *A. marginale*; v) immunization of cattle with native MSP1/2 polypeptides followed by heterologous Israel strain challenge; vi) recombinant MSP1a and MSP1b expression in pGEX2T; vii) immunization of cattle with recombinant MSP1 followed by heterologous Israel strain challenge; viii) identification of MSP-2 and MSP-3 as conserved polypeptides on Israel strains of *A. marginale*; ix) identification of MSP-2 and MSP-3 as immunodominant surface polypeptides with conserved epitopes between *A. centrale* and *A. marginale*; x) identification of MSP-5 as a genus common polypeptide (*A. centrale*, *A. marginale*, *A. ovis*); xi) identification of antibody against MSP-5 in cattle premunized with *A. centrale* and protected against *A. marginale*; and x) cloning and sequencing the single copy gene encoding MSP-5.

Specific Aim 1: Establish that native MSP-1a immunization induces protection against challenge with Israel strains of *Anaplasma marginale*.

Strategy: Immunization with MSP-1 purified from Florida strain *A. marginale* significantly protected cattle from clinical disease upon challenge with the homologous Florida strain as determined by significant prolongation of the prepatent period, diminished peak rickettsemia, and decreased severity of anemia.^{3,8} MSP-1 vaccinates were also protected against clinical disease following challenge with the Washington-O strain⁸. The Washington-O strain, isolated from an acutely infected bull in 1982, differs antigenically, morphologically, and structurally from the Florida strain^{7,9,10}. Despite these differences, the Washington-O strain contains the tandemly repeated neutralization-sensitive epitope^{7,9}. We identified this epitope on two field isolates of *A. marginale* from Israel⁷. These strains, designated Israel-T and Israel-NT, differ structurally and antigenically from each other and from all U.S. strains previously characterized.²⁸ The cross-protection data suggested that MSP-1 immunization should protect against challenge with either Israel NT or T. However this indication was limited by the single heterologous challenge completed to date and assumed that the presence of the MSP1a neutralization-sensitive epitope was responsible for the observed cross-protection.

Results: Prior to initiating the immunization trial using the Israel *A. marginale* strains for heterologous challenge, we addressed four critical questions related to MSP1a immunization: i) defining whether the immunogen must contain both MSP1a and MSP1b subunits; ii) defining the immunization protocol that consistently induces protection against homologous strain challenge; and iii) development of assays that measure immune responses against the critical epitope. Points i-iii were addressed using the Florida strain as the challenge strain because this is the homologous challenge and serves as a positive control for MSP-1 immunization efficacy.

i) Definition of the immunogen: MSP-1 contains two subunits, MSP1a and MSP1b. Previous successful immunization experiments utilized MSP-1 as a bimolecular complex^{3,8}. To determine if both subunits were required, MSP-1a was purified by affinity chromatography using washing and elution conditions that resulted in the isolation of native MSP-1a. Absence of contaminating

MSP-1b was shown by silver staining of 5% polyacrylamide gels and by the failure of immunization to induce an antibody response to MSP-1b. Cattle were immunized by four intramuscular injections with 500 μ g of MSP1a emulsified in saponin. Control cattle were immunized on an identical schedule with saponin alone. Antibody responses to MSP-1a and MSP-1b were determined by immunoblotting as previously described. Cattle were challenged by intravenous inoculation of 10³ live *A. marginale* parasitized erythrocytes (Florida strain). Challenge responses were monitored as previously described³.

Shown below are three tables: Tables 1 and 2 demonstrate antibody and challenge responses in cattle immunized with native MSP-1 containing both polypeptides; Table 3 shows the results utilizing native MSP-1a devoid of MSP-1b. Note: the challenge was 10¹⁰ stabilate-derived parasitized erythrocytes (Tables 1 and 2) while the current challenge was the more relevant 10³ live *A. marginale* parasitized erythrocytes (Table 3). All experiments subsequent to those described in Tables 1 and 2 utilized the live *A. marginale* challenge.

These experiments demonstrate two significant points relative to protective immunization and Specific Aim 1: first, protective immunization can be induced by the native MSP-1a polypeptide alone; and second, the immunization is protective against challenge with live *A. marginale*.

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:		DPC to 1% rickettsemia ^a	Peak rickettsemia (%)	Low PCV (%) ^b
	105-kDa polypeptide	100-kDa polypeptide			
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.

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:		DPC to 1% rickettsemia ^a	Peak rickettsemia (%)	Low PCV (%) ^b
	105-kDa polypeptide	100-kDa polypeptide			
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.

TABLE 3: *Protection of cattle immunized with Florida strain MSP-1a against challenge with the *A. marginale* Florida strain.

Immunogen and Animal No.	Antibody titer to MSP1a ^a :	DPC ^b to 1% rickettsemia	Peak rickettsemia ^c	Low PCV
MSP-1a				
B525	62,500	24	4.9	32
B537	10 ⁶	uninfected	0.0	28
B541	10 ⁶	uninfected	0.0	27
B560	250,000	----	0.4	26
mean ($\pm$ standard deviation)			1.3 $\pm$ 2.3	28 $\pm$ 2.6
Saponin				
B535	<500 ^d	30	2.6	22
B544	<500	27	2.1	22
B553	<500	26	4.3	19
B554	<500	28	7.2	22
B578	<500	24	7.3	18
mean ($\pm$ standard deviation)			4.7 $\pm$ 2.5	20 $\pm$ 1.9

*MSP-1a immunized cattle were protected as demonstrated by the absence of detectable rickettsemia in two vaccinates, less than 1.0% rickettsemia in a third vaccinee, and significant differences between MSP-1a immunized and sham immunized cattle with respect to minimum PCV and % PCV reduction.

^aMeasured by immunoblot.

^bDPC, days post-challenge.

^cRickettsemia was determined by daily microscopic examination of Wright stained blood smears for 75 DPC.

^dSera from all saponin immunized cattle were unreactive with *A. marginale* polypeptides, including MSP-1a and MSP-1b, at the lowest dilution tested, 1:500.

ii) **Definition of the immunization protocol that consistently induces protection against challenge:** The protective immunization (Table 3) demonstrated that the substitution of saponin (an acceptable adjuvant for eventual field use) for Freund's complete and incomplete adjuvant (previously used in all protective immunization trials) was acceptable. This substitution was tested because while Freund's adjuvant is a strong immunostimulator, it is unacceptable for eventual field use of an improved vaccine against anaplasmosis. In addition, the trial reported in Table 3 indicated that the challenge dose of 10³ live *A. marginale* parasitized erythrocytes induced clinical disease in control cattle (mean PCV decrease was 33%). This challenge dose, consisting of live organisms, is more relevant than the previously used stabulate challenges, which contain a low percentage of live organisms (data not shown). The challenge with live

parasitized erythrocytes mimics natural challenge by biting flies or blood contaminated fomites [challenge with infected ticks will be done when recombinant immunized cattle have been shown to be protected against challenge with live *A. marginale* parasitized erythrocytes].

The remaining variable to be tested prior to the heterologous challenge with the Israel NT strain of *A. marginale*, was the amount of MSP-1a to be used as the immunogen. The experiment detailed in Table 3 utilized 500 μ g of affinity purified MSP-1a emulsified in saponin. In contrast, prior immunization experiments with native MSP-1 (both polypeptides) emulsified in Freund's complete (first immunization) and incomplete (subsequent immunizations) adjuvants utilized 50-100 μ g of combined polypeptides. The immunization trial described for Table 3 was repeated utilizing four injections with 50 μ g of MSP-1a. As noted in Table 4, the antibody responses to MSP-1a were lower and protection was not achieved when 50 μ g of MSP-1a was used as compared to 500 μ g of immunogen. Repetition of the 500 μ g immunization trial confirmed that the high dose immunization induces protective immunity. These are the immunization conditions used in the heterologous protection trial using the Israel NT strain of *A. marginale*.

TABLE 4: *Immunization of cattle with low dose of Florida strain MSP-1a and challenge with the *A. marginale* Florida strain.

Immunogen and Animal No.	Antibody titer to MSP1a ^a :	DPC to 1% rickettsemia	Peak rickettsemia	Low PCV
MSP-1a				
B557	100,000	31	4.4	17
B588	100,000	27	7.6	20
B592	100,000	36	1.3	27
B593	100,000	33	5.5	21
B595	100,000	27	4.6	18
mean ($\pm$ standard deviation)		31 $\pm$ 3.9	4.7 $\pm$ 2.2	21 $\pm$ 3.9
Saponin				
B543	---- ^b	27	5.6	20
B599	----	27	3.2	24
B597	----	30	9.1	19
B602	----	29	11.5	13
B606	----	26	18.1	15
mean ($\pm$ standard deviation)		28 $\pm$ 1.6	9.5 $\pm$ 5.8	18 $\pm$ 4.3

^aMeasured by immunoblot.

^bSera from all saponin immunized cattle were unreactive with *A. marginale* polypeptides, including MSP-1a and MSP-1b, at the lowest dilution tested, 1:500.

iii) **Development of assays that measure immune responses against the critical epitope:** We demonstrated that neutralizing monoclonal antibodies bound a six residue peptide contained within the larger tandemly repeated 29-mer in MSP-1a. In order to better assess the efficacy of immunization with either native or recombinant polypeptides, we have developed a direct ELISA for reactivity of bovine sera to the native antigen, the 29-mer and an inhibition ELISA for reactivity to the 6-mer. Peptides were synthesized based on the translated sequence of the Florida *msh-1a* gene [29 mer-DSSSAGGQQQESSVSSQSDQASTSSQLGA]. The 29-mer ELISA was performed as described¹² except that the peptide was fixed to BSA coated Immulon-I wells using glutaraldehyde. The inhibition ELISA is done by using dilutions of bovine sera to inhibit the binding of neutralizing monoclonal antibody ANA22B1 to the 29-mer. This assay was used retrospectively to test the sera of the high dose MSP-1a immunized cattle described in Table 3 for binding to the critical epitope. Table 5 includes the epitope reactivity and the challenge results from Table 3. Note that cattle protected against infection and the individual with a 0.4% peak rickettsemia developed titers of 10,000 against the 29-mer as compared to the less protected vaccinate (B525). These assays have been published in refereed journals and are listed in Section H.

TABLE 5: *Responses to the neutralization-sensitive epitope in cattle immunized with Florida strain MSP-1a and protected against challenge with the *A. marginale* Florida strain.

Immunogen and Animal No.	Antibody titer to 29-mer repeat ^a :	DPC to 1% rickettsemia	Peak rickettsemia	Low PCV
MSP-1a				
B525	1,000	24	4.9	32
B537	10,000	uninfected	0.0	28
B541	10,000	uninfected	0.0	27
B560	10,000	----	0.4	26

*MSP-1a immunized cattle were protected as demonstrated by the absence of detectable rickettsemia in two vaccinates, less than 1.0% rickettsemia in a third vaccinate, and significant differences between MSP-1a immunized and sham immunized cattle with respect to minimum PCV and % PCV reduction.

^aMeasured by 29-mer ELISA.

In addition to serologic assays, we have adapted a T lymphocyte proliferation assay to measure T_{helper} responses to defined *A. marginale* antigens in immunized cattle. Briefly, lymphocytes are isolated by buoyant gradient centrifugation and 1.5x10³ lymphocytes/well cultured in 96 well plates containing Hepes buffered Eagles's minimal medium with 10% heat-inactivated serum. Each well contains either *A. marginale* (negative control is uninfected erythrocytes) or purified recombinant-derived polypeptide. Cells are incubated at 37°C for 5-7 days and incorporation of methyl³H thymidine determined. All wells are tested in triplicate and significant differences between groups determined using a paired t-test.

iv) **Demonstration that MSP-1 immunized cattle develop opsonizing antibody:** Antibody against the whole organism¹³ and specific antibody against the major surface protein complex-1 (MSP-1)³ has been shown to neutralize *A. marginale* infectivity for splenectomized calves. However the mechanism by which antibody contributes to protective immunity is unknown. Postulated mechanisms for antibody mediated immunity include direct antibody and complement mediated killing, antibody blockade of organism attachment and penetration into host erythrocytes, antibody-dependent cellular cytotoxicity by non-MHC restricted lymphocytes, and antibody mediated opsonization for phagocytosis. A role for antibody mediated opsonization followed by killing by activated macrophages in immunity to *A. marginale* is supported by several observations. Phagocytosis occurs within splenic macrophages in vivo and splenomegaly develops during acute infection^{20,21}. Secondly, cattle splenectomized prior to infection develop a higher peak rickettsemia, more severe clinical disease, and have a higher case fatality rate than non-splenectomized cattle²². Thirdly, persistently infected carrier cattle recrudesce and develop microscopically detectable rickettsemia within one to two weeks following splenectomy or treatment with immunosuppressive drugs^{23,24}. Recrudescence within this brief period during which antibody levels would decline only minimally indicates a suppressible cellular function is involved. We tested the hypothesis that antibody specific for MSP-1 promotes macrophage phagocytosis of *A. marginale*.

The Florida strain of *A. marginale* was used in all experiments. Sera for opsonization studies were obtained from cattle immunized with either affinity-purified MSP-1 (5 individuals) or sham immunized on an identical schedule with ovalbumin (5 individuals). The sera used for these opsonization studies was collected from the animals after immunization and before challenge. The immunization of these cattle, determination of antibody titers to the MSP1a and MSP1b polypeptides that form the MSP-1 complex, and challenge with *A. marginale* have been described⁸. Bovine macrophages were derived from neonatal undifferentiated bone marrow stem cells and were cultured by growing bone marrow cells in supplemented growth medium containing 10% fetal bovine serum, 5% equine serum, and 15% fibroblast-conditioned medium, as the source of macrophage-colony stimulating factor (M-CSF)²⁵. Initial bodies were incubated with sera diluted 1:30 and then added to macrophages at a ratio of 50:1. The number of initial bodies ingested by macrophages was significantly greater when initial bodies were pre-incubated with anti-MSP-1 serum antibodies (pooled from 5 MSP-1 immunized cattle), as compared with those pre-incubated with anti-ovalbumin serum antibodies (pooled from 5 ovalbumin immunized cattle) ($p < 0.01$; Fig. 1a and 1b). The mean number of MSP-1 antibody treated initial bodies ingested per macrophages was 10.4, and the median was 16. The percentage of macrophages which had ingested 2 or more initial bodies was 80%. By contrast, the mean number of ovalbumin antibody-treated initial bodies ingested per macrophages was 0.8, and the median was 0. Only 18% of the macrophages ingested 2 or more initial bodies.

Initial bodies were pre-treated with antibody from each of the ten individual animals. Pretreatment with serum antibody from each of the five MSP-1 immunized animals resulted in more phagocytosis than pretreatment with antibody from each of the five ovalbumin-immunized animals ($p < 0.05$) (Table 6). There were also statistically significant differences in the ability of antibodies from different MSP-1 immunized animals to promote phagocytosis (Table 6). In this study, we demonstrated that antibody from MSP-1 immunized cattle promotes phagocytosis of *A. marginale* initial bodies by bovine macrophages. Demonstration of

opsonization by MSP-1 antibody, from cattle which were shown to be protected against challenge, suggests that this may be a significant protective mechanism *in vivo*.

Increased phagocytosis of *A. marginale* opsonized with MSP-1 antibody. Initial bodies in each of 50 macrophages were counted. The experiment was repeated 5 times with similar results.

TABLE 6: Increased phagocytosis of *A. marginale* opsonized with MSP-1 antibody: effect of serum antibodies from individual animals.

Immunogen and Animal No.	Antibody titer to MSP1a ^a :	Antibody titer to MSP1b:	No. of initial bodies per 100 macrophages
MSP-1			
B194	64,000	128,000	1303
B203	64,000	64,000	754 ^a
B187	64,000	128,000	850 ^{a,b}
B208	128,000	128,000	460 ^b
B196	128,000	128,000	258
Ovalbumin			
B195	-----	-----	233 ^c
B217	-----	-----	155 ^{c,d}
B207	-----	-----	125 ^{d,e}
B189	-----	-----	86 ^{d,e}
B197	-----	-----	53 ^c

Each of the groups incubated with MSP-1 antibody is statistically greater than those incubated with ovalbumin antibody, based on the Mann-Whitney two-sample test ($P < 0.05$). Identical superscripts indicate no significant differences ($P = 0.05$) based on the Mann-Whitney two-sample test.

v) **Immunization of cattle with MSP1/MSP2 for challenge with the Israel-NT strain:** As a group cattle immunized with either MSP-1 or MSP-1a are significantly protected against infection and disease as compared to control cattle. However, not all individuals are completely protected upon challenge with the virulent Florida strain. Based on reviewer suggestions, we extended Specific Aim 1 to include definition of additional surface polypeptides with epitopes conserved among U.S. and Israel strains of *A. marginale* that could be included with MSP-1a in a subunit vaccine (immunization with MSP-1 alone was done in Specific Aim 3). Research in year 1 demonstrated that MSP-2 was the major immunodominant surface polypeptide shared between *A. marginale* and *A. centrale*. The successful use of *A. centrale* live vaccine to protect cattle against Israel *A. marginale* suggested that MSP-2 was a candidate protein involved in immunity against Israel strains. The decision to use MSP-2 was submitted in the Year 1 Annual Report.

TABLE 7. Israel NT strain challenge of cattle immunized with Florida strain MSP-1/MSP-2.

Immunogen and Animal No.	Antibody titer to Israel NT ^a	DPC to 1% rickettsemia	Peak fever (°C)	Peak rickettsemia	Low PCV
MSP-1/2					
B55	1024	-- ^b	38.7	< 1	27
B57	4096	27	40.2	13	22
B58	1024	25	40.3	8	18
B276	4096	27	40.6	10	18
B277	4096	23	39.7	7	19
<i>mean (±standard deviation)</i>		<i>25.5±1.9</i>	<i>39.9±0.74</i>	<i>7.6±4.8</i>	<i>208±38</i>
Saponin					
B56	< 64 ^c	23	40.1	16	18
B63	< 64	25	39.6	14	21
B64	< 64	27	39.9	4.5	23
B278	< 64	18	38.9	8.5	17
B279	< 64	21	40.3	9	15
<i>mean (±standard deviation)</i>		<i>22.8±3.5</i>	<i>39.8±0.5</i>	<i>10.4±4.6</i>	<i>188±32</i>

^a Measured by indirect immunofluorescence.

^b Only rare organisms seen.

^c Sera from all saponin-immunized cattle were unreactive with *A. marginale* at the lowest dilution tested, 1:64.

Cattle were assigned randomly to two groups, principals and controls, each composed of 5 animals. This is a reduction from the 10/group originally proposed for the trial due to the decreased budget. Cattle were seronegative and free from microscopically detectable

rickettsemia. MSP1 and MSP2 were purified from Florida strain organisms by monoclonal antibody affinity chromatography as previously described^{3,8,16}. Purified antigen was quantitated using the bichinonic acid assay as modified for membrane proteins, purity confirmed by silver staining of polyacrylamide gels containing approximately 10 μ g antigen, and immunologic reactivity confirmed by immunoblotting with monoclonal antibodies directed against MSP1a and MSP2^{3,8,16}. Each dose of combined MSP1/MSP2 was mixed with 3mg saponin. Cattle were immunized four times and then challenged with 10⁴ parasitized erythrocytes (Israel NT strain) intravenously. As noted in Table 7, one MSP1/MSP2 vaccinee was completely protected against Israel challenge; however on a group basis there was no significant protection afforded by MSP1/MSP2 immunization.

Specific Aim 2: Obtain high-level expression of the gene coding for MSP-1a.

Strategy: Development of a vaccine based on MSP-1a is currently limited by the amount of antigen that can be purified from *A. marginale* parasitized erythrocytes. The lack of a long-term in vitro cultivation method for *A. marginale* requires that splenectomized calves be infected and blood obtained at high rickettsemia in order to obtain organisms for the MSP-1a purification procedure. Reliance on an in vivo antigen source is unacceptably expensive and susceptible to variation in biologic and structural properties of the organism. Cloning of the gene coding for MSP-1a with high level expression in *E. coli* would provide a uniform source of antigen obtained by in vitro bacterial cultivation. Toward this goal, we cloned and expressed the full-length gene coding for MSP-1a in the plasmid vector pKK233-2⁵. The recombinant expressed MSP-1a is antigenically and structurally homologous to the native MSP-1a⁵. Although we have successfully purified recombinant MSP-1a from *E. coli* using monoclonal antibody affinity chromatography, the current level expression (<0.1% of bacterial proteins) is suboptimal for the larger scale purification needed for immunization trials.

We originally proposed to obtain high level expression by insertion of the MSP-1a gene into a pAS vector system (see Year 1 Annual Report). However, the availability of pGEX vectors resulted in a change in strategy to take advantage of the unique characteristics of this expression system (high level stable expression combined with affinity purification of the fusion protein and on-column cleavage with release of the purified protein of interest)^{17,18}. This vector system utilizes the carboxy terminus of the *Schistosoma japonicum* glutathione S-transferase gene under the control of the *tac* promoter¹⁸. In-frame cloning of the gene of interest results in expression of a fusion protein with affinity for glutathione. A thrombin cleavage site has been engineered into the vector immediately 5' to the multiple cloning site. Consequently, the fusion protein can be purified on a sepharose 4B-glutathione column and the protein of interest is cleaved free of the fusion partner using thrombin¹⁸. This vector system was used to provide an abundant source of recombinant protein for immunization trials.

Results: Full-length MSP-1a was cloned into the NcoI site of pKK233-2⁵. The expressed protein, detected immunologically with a monoclonal antibody against the repeated neutralization-sensitive epitope, co-migrates electrophoretically with native MSP-1a at an apparent molecular size of 105kD, and is structurally identical by one-dimensional peptide

mapping. Recombinant MSP-1a, purified by affinity chromatography, is antigenically and structurally intact as determined by its reactivity with the monoclonal antibody, its ability to inhibit native MSP-1a binding to antibody on an equimolar basis, and its ability to induce antibody in rabbits and cattle reactive with native MSP-1. Within the insert we have identified regulatory signals, the transcriptional and translational start sites, and have obtained the DNA sequence including the tandem repeats composing the neutralization-sensitive epitope⁵.

To express recombinant MSP-1a in pGEX2T, the pKK233-2 bearing MSP-1a was digested with KpnI to expose the 5' end of the gene. [Note: cloning of *msp1a* and *msp1b* into pGEX2T was done in collaboration with Dr. Tony Barbet, Department of Infectious Diseases, University of Florida] Following confirmation by sequencing of the reading frame, the *msp1a* sequence was ligated in frame with carboxy end of the glutathione-S-transferase gene. A similar strategy has been used to clone the expressed copy of the *msp1b* gene¹⁹ into pGEX2T. The *msp1a* and *msp1b* proteins are expressed >5% of total protein in the pGEX2T vector. For immunization of cattle for Specific Aim 3, *msp1a* and *msp1b* have been purified by affinity chromatography on a glutathione-sepharose 4B column. The antigenic characterization of the recombinant MSP1a and MSP1b are briefly described under specific aim 3.

Specific Aim 3: Test the effectiveness of recombinant MSP1 as a protective immunogen against challenge with Israel and U.S. strains of *Anaplasma marginale*.

Strategy: We proposed to test the hypothesis that recombinant expressed MSP1 was capable of inducing protection against challenge with homologous and heterologous strains of *A. marginale*. Demonstration of protection induced by recombinant MSP1 produced by high level expression would provide the basis for further testing and deployment of a reliable, stable source of immunogen. Although we have shown that MSP1a alone can induce significant protection against homologous challenge, the results of the heterologous challenge reported in Specific Aim 1 indicated that heterologous protection was not predictable based on homologous challenge results. Consequently, we decided to use a combined recombinant MSP1a/MSP1b immunogen. Our rationale was that MSP1 (the bimolecular complex of MSP1a and MSP1b) has been shown to induce heterologous protection against a Washington-O strain (not tested for MSP1a or MSP1b alone). A positive observation with recombinant MSP1a/MSP1b would allow us to subsequently determine if both components are required for protection against heterologous Israel strain challenge. Although MSP2 has been identified as a candidate cross-protective immunogen, recombinant MSP2 has not been incorporated into the recombinant trial under Specific Aim 3. The sole reason for exclusion of recombinant MSP2 is simply that we had not yet identified the expressed full-length copy of MSP2 at the time of the recombinant immunization.

Results: MSP1a and MSP1b were obtained from pGEX2T vectored recombinant bacteria and isolated by affinity chromatography on a sepharose 4B-glutathione column. Purified MSP1a and MSP1b were quantitated using the bichinonic acid assay as modified for membrane proteins, purity confirmed by silver staining of polyacrylamide gels containing approximately 10 μ g antigen, and immunologic reactivity confirmed by immunoblotting with monoclonal antibodies

directed against MSP1a and MSP1b^{3,8}. Each dose of combined recombinant MSP1a/MSP1b was mixed with 3mg saponin. Cattle (5 principals and 5 controls) were immunized as described previously and then challenged with 10⁴ parasitized erythrocytes (Israel NT strain) intravenously. As noted in Table 8 there was no significant protection afforded by recombinant MSP1 immunization.

TABLE 8. Israel NT strain challenge of cattle immunized with Florida strain recombinant MSP-1.

Immunogen and Animal No.	Antibody titer to Israel NT ^a	DPC to 1% rickettsemia	Peak fever (°C)	Peak rickettsemia	Low PCV
MSP-1					
B60	64	27	39.8	8	21
B65	64	30	39.0	4.5	29
B66	64	28	39.5	14	21
B70	256	25	40.0	15	23
B71	64	25	41.4	17	21
<i>mean (±standard deviation)</i>		<i>27±2.1</i>	<i>39.9±0.9</i>	<i>11.7±5.2</i>	<i>23±3.4</i>
Saponin					
B56	< 64 ^b	23	40.1	16	18
B63	< 64	25	39.6	14	21
B64	< 64	27	39.9	4.5	23
B278	< 64	18	38.9	8.5	17
B279	< 64	21	40.3	9	15
<i>mean (±standard deviation)</i>		<i>22.8±3.5</i>	<i>39.8±0.5</i>	<i>10.4±4.6</i>	<i>18.8±3.2</i>

^a Measured by indirect immunofluorescence.

^b Sera from all saponin-immunized cattle were unreactive with *A. marginale* at the lowest dilution tested, 1:64.

Specific Aim 4: Identify additional polypeptides with conserved epitopes for inclusion in a recombinant vaccine.

Strategy: As a group cattle immunized with either MSP-1 or MSP-1a are significantly protected against homologous strain infection and disease as compared to control cattle. However, not all individuals are completely protected upon challenge with the homologous virulent Florida strain. Cattle were not significantly protected against heterologous challenge with the Israel-NT strain. Failure to consistently induce immunity, resulting in partially protected and unprotected vaccinates, represents a significant constraint to vaccine development based on a single protein.

In contrast, a multivalent outer membrane polypeptide would induce a polyclonal immune response against multiple surface polypeptides and expand the number of fully responsive cattle. Recently, Tebele, in collaboration with investigators at Washington State University, demonstrated that *A. marginale* native outer membrane polypeptides, in a Quil A-membrane matrix, induced protective immunity against virulent challenge in all vaccinates²⁶. Ten control cattle (5 unimmunized and 5 sham immunized with Quil A) developed acute anaplasmosis while 4/5 outer membrane-Quil A matrix vaccinates were completely protected from microscopically detectable rickettsemia (the remaining vaccinee developed only mild anemia and low rickettsemia)²⁶.

From Tebele N, McGuire TC, Palmer GH: Induction of protective immunity using *Anaplasma marginale* membranes. Infection and Immunity, 59:3199-3204, 1991.

TABLE 9. Protection induced by immunization with native outer membrane polypeptides in Quil A matrix: Group Comparison Data

Immunogen	Peak Rickettsemia (%) ^a	Low PCV (%) ^a	Low Hb (g/dl) ^a
Membrane	0.5 ± 1.0 ^b	26±2.4 ^{*c}	8.3±1.0 ^{*c}
Quil-A	11.8 ± 4.6	16±1.9	5.1±0.8
control			
Non-immunized	12.1 ± 5.5	16±2.7	5.3±0.9

^a Mean ±SD; *All means with an asterisk were significantly different from saponin control and non-immunized calves.

^b Only 1 out of 5 calves developed a rickettsemia.

^c Mean PCV and Hb for membrane immunized animals was not significantly different than for age-matched, non-challenged cattle kept on site during the same time period.

Protection in outer membrane immunized cattle correlates ($r=0.97$, $P<0.05$) with antibody titer to these polypeptides (Table 10)²⁶. Importantly, this level of protection against the Norton strain of *A. marginale* (recently isolated from an acutely infected animal in the field and used following a single *in vivo* passage) is indistinguishable from the immunity induced by premunition²⁷. Significantly, protection against heterologous challenge (an Africa strain) appears to be uniform and complete²⁸. The successful induction of immunity similar to premunition represented a significant step in development of an effective inactivated vaccine. Based on these results reported in 1991, we examined the Israel and U.S. strains for the presence of the outer membrane polypeptides, MSP-1, MSP-2, MSP-3, MSP-4, and MSP-5.

Results: MSP-1 had previously been shown to be conserved among Israel and U.S. strains of *A. marginale*. In our BARD research we identified three surface polypeptides with strain-common epitopes: MSP-2, MSP-3, and MSP-5. MSP-2 has shown to be capable of inducing statistically significant protection against homologous and heterologous challenge and along with

TABLE 10: Protection induced by immunization with native outer membrane polypeptides in Quil A matrix: Individual Membrane Vaccinate Data

Animal number	Antibody titer ^a	Peak rickettsemia (%)	Low PCV (%) ^b	Low Hb (g/dl) ^b
3	10 ⁶	0	27	8.7
15	10 ⁶	0	28	9.3
7	10 ⁵	0	27	8.9
11	10 ⁵	0	25	8.3
22	10 ³	2.3	22	6.7

^a Pre-challenge antibody titers against outer membrane polypeptides.

^b Low PCVs and Hbs for animals 3,15,7,and 11 were within the range of 15 age-matched, on-challenged cattle kept on site during the same time period.

MSP-3 is the immunodominant *A. marginale* membrane protein recognized by sera from *A. centrale* premunized cattle protected against *A. marginale* challenge. MSP-5 has been recently identified, is highly conserved among *A. marginale* strains (including U.S. and Israel) and with *A. centrale*, and has been cloned and sequenced as part of our BARD project.

Msp-2 and Msp-3 To identify relevant shared antigens, we used two sources of antibody: 1] antibody from rabbits immunized with intact, purified organisms, a procedure previously demonstrated to induce neutralizing antibody highly reactive with surface epitopes¹³; and 2] antibody from cattle recovered from experimental infection, a procedure demonstrated to induce protection against homologous and heterologous challenge^{14,15}. Using these antibodies, a 36 kD molecule was shown to be shared between *A. marginale* strains from the U.S. and Israel and with the Israel vaccine strain of *A. centrale*. This molecule was shown to be immunodominant whether presented to the immune system by immunization with intact organisms or by infection. In addition, an 86 kD *A. marginale* molecule was recognized by high titers of antibody from cattle recovered from infection with either *A. marginale* or *A. centrale*.

The dominance of the 36 kD protein in inducing cross-reactive antibody against *A. marginale* and *A. centrale*, regardless of the mode of presentation, suggests that cross-protective immunity is based on relatively few epitopes. This would strongly support the incorporation of this antigen in improved vaccines against anaplasmosis. Previously, a 36 kD polypeptide (designated MSP-2) capable of inducing protective immunity has been identified on the surface of the Florida strain of *A. marginale*¹⁶. The conservation of molecular size suggested that the 36 kD molecule in the Israel strains of *A. marginale* and *A. centrale* is MSP-2. Binding of MSP-2 specific antibodies, including monoclonal antibodies to conserved epitopes, confirmed that the 36 kD molecule defined by cross-reactive antibody is indeed the surface polypeptide MSP-2.

The antigenic and molecular size conservation of MSP-2 between two Israel strains of *A. marginale*, seven characterized U.S. strains, and *A. centrale*, suggests that MSP-2 may

significantly contribute to induction of widely cross-protective immunity. MSP-2 does not show the striking molecular size variation shown in MSP-1a among different *A. marginale* strains⁶. In addition, peptide maps of MSP-2 from seven U.S. strains of *A. marginale* have demonstrated conservation of surface exposed peptides¹⁶. The current research, approached from an independent direction, supports the relevance of MSP-2 to induction of widely cross-protective immunity. Including the present work, the evidence supporting MSP-2 as a protective immunogen includes: 1] MSP-2 is a surface exposed polypeptide recognized by neutralizing antibody¹³; 2] MSP-2 is the immunodominant polypeptide recognized by cross-reactive antibody between *A. centrale* and *A. marginale* (this study); 3] MSP-2 bears epitopes conserved among all strains of *A. marginale* examined (Africa, Asia, North America, South America)^{7,9,16}; 4] immunization of cattle with affinity purified MSP-2 induces protection against challenge with the homologous or a heterologous strain of *A. marginale*¹⁶; 5] MSP-2 bears epitopes conserved between *A. centrale* and *A. marginale* (this study); and 6] surface exposed peptides are conserved among MSP-2 from different strains¹⁶. This evidence supports the incorporation of affinity purified MSP-2 with MSP-1a in immunization of cattle for challenge with Israel strains.

Msp-5 MSP-5 was identified as an outer membrane protein and shown to be immunogenic in immunized and protected cattle²⁶. To identify MSP-5 in the Israel-T and Israel-NT strains of *A. marginale* and in Israel vaccine strain of *A. centrale*, anti-MSP-5 MAb ANAF16C1 was reacted with immunoblots. As noted in Figure 2, MSP-5 was detected in all Israel and U.S. strains tested. *A. centrale* is used to protectively immunize cattle in Israel against challenge. Therefore, we examined sera from *A. centrale* premunized cattle to bind to MSP-5 using the competitive ELISA described in reference 29. As shown in Figure 3, sera from all 6 premunized cattle bound MSP-5. We have cloned and sequenced the *msh-5* gene during our BARD studies and have demonstrated that it is a single-copy gene and have derived the primary amino acid sequence (Figure 4)²⁹.

< < < Fig. 2

Fig. 3 > > >

FIG. (A) Immunoblot reacted with ANAF16C1 (lanes 1 through 6) and isotype control MAb (lanes 7 through 12) demonstrating conservation of MSP-5 among *Anaplasma* strains. Lanes: 1 and 7, lysates of uninfected erythrocytes; 2 through 5 and 8 through 11, 2.5×10^9 initial bodies of the Mississippi, Washington-O, North Texas, and Missouri strains, respectively; 6 and 12, affinity purified native MSP-5 (Florida strain). (B) Immunoblot containing equivalent lysates of stabilates with parasitemia and reacted with MAb ANAF16C1. Lanes: 2, 4, 6, 8, and 10, no sample; 1, uninfected erythrocytes; 3, Florida strain of *A. marginale* (56% parasitemia); 5, Israeli *A. centrale* lysate (44% parasitemia); 7, nontailed Israeli *A. marginale* strain (63% parasitemia); 9, tailed Israeli *A. marginale* strain (57% parasitemia); 11, Idaho *A. ovis* strain (12% parasitemia).

FIG. Demonstration of a conserved epitope on recombinant MSP-5. (A) Percent inhibition of immune sera against U.S. *A. marginale* strains. Columns (strains): 1, Florida; 2, Virginia; 3, southern Idaho; 4, northern Texas; 5, Washington-O; 6, preinfection sera. (B) Percent inhibition of immune sera against the following: 1, Norton (Zimbabwe) *A. marginale* outer membrane fraction; 2, Norton inner membrane fraction; 3, Food and Agricultural Organization reference sera; 4, bovine *A. centrale* postinfection sera; 5, rabbit anti-*A. centrale* serum.

Fig. 4

<p><i>AccI</i></p>			
1	GTATACTCAGTTGCCCTGGCGC	54	TTGACCAACCTGGGCATAGGTGCTACGATCG
55	CGCTCGTCGCTTTTGGCGTCCGGCAATGTGGCGCATTTTGTAGTGTTCGTTGGG	108	
109	GTGTGATAGATGAGAATTTCAAGATTGTGTCTAACCTTCTGCTGTTCTGTTGCT	162	
	M R I F K I V S N L L L F V A		
163	GCCGTGTTCTCTGGGGTACTCTATGTGAACAAGAAAGGCATTTTCAGCAAAATC	216	
	A V F L G Y S Y V N K K G I F S K I		
217	GGCGAGAGGTTTACCACCTCCGAAGTTGTAAGTGAGGGCATAGCCTCCGCGTCT	270	
	G E R F T T S E V V S E G I A S A S		
371	TTCAACAATTTGGTTAATCACGAGGGGGTCACCGTCAGTAGCGCGCATTTTGGC	324	
	F N N L V N H E G V T V S S G D F G		
325	GGCAAGCACATGTTGGTAATATTCGGCTTCTCAGCCTGTAAGTACACGTGCCCT	378	
	G K H M L V I F G F S A C K Y T C P		
379	ACCGAGTTAGGCATGGCTTCTCAGCTCCTAAGTAACTAGGCGACCATGCCGAT	432	
	T E L G M A S Q L L S K L G D H A D		
433	AAGTTGCAAGTTGTGTTCAATACTGTGTATCCGAAAAATGACACCGTAGCCAAG	486	
	K L Q V V F I T V D P K N D T V A K		
<p><i>EcoRI</i></p>			
487	CTTAAGAGTACCACAAGTCTTTTGTAGCGAGAATTCAGATGCTCAGAGCGGAA	540	
	L K E Y H K S F D A R I Q M L T G E		
541	GAAGCAGACATAAAGCGGTGGTTGAAAACTACAAGGTGTATGTGGCGACAAG	594	
	E A D I K S V V E N Y K V Y V G D K		
		<p><i>EcoRI</i></p>	
595	AAGCCAAGTGATGGTGATATCGACCACTCAACGTTTCATGTACCTCATCAATGGG	648	
	K P S D G D D H S T F M Y L I N G		
649	AAAGGCAGGTATGTCGGGCATTTTGGGCCAGATTTTAACGGCTCTGAGGGCCAA	702	
	K Q R Y V G H F A P D F N A S E G Q		
703	GGCGAGGAGCTGTTAAGTTTGTACGCGGTCACATGCTTAATTCTTAGTTAAGC	756	
	G E E L F K F V S G H M L N S *		
757	ATGGCAGTGGTACAGTTTCGTGTGTCGGTCGTCTTGTGAGGCAGTAGAAAGTA	810	
811	TGGGGCTTGGGGGCTTTTCTTTGTGGCGTTTGTGCGCTTGGCTTAGGAGCTG	864	
865	GGGCTGACCATGACGGGTGGTTGGCTCTCCACCGTGTCCCATTTATCTCTT	918	
919	CTGTTGCCGAAGAGTTTGGTAGATTCTCCGCTATAGAACCCCGTCATAGAGT	972	
973	CCGTGGGAAGTGGCATGGGCTTTAACATGTTTTCGCGTGGCAGCAGCAGTGATA	1026	
1027	CACCAGACATAGCCATGTCTTAGGCGCATCAAGGATGCAGAAGTCGAACCTT	1080	
1081	GCGGCATGAATGGCGTGAAGGACATGATCGAGATAGGTCTGGGCTACGACGGCA	1134	
1135	TAGCCCCGAATTC	1146	

FIG. Nucleotide and deduced amino acid sequences of the *msp5* gene. Within the flanking sequences, the predicted -35 and -10 loci, ribosomal binding site, and termination site are underlined, and selected restriction enzyme sites are indicated above the nucleotide sequence. The deduced amino acid sequence is indicated below the nucleotide sequence, and the stop codon is indicated with an asterisk.

F. Description of Cooperation

The investigators maintained a strong collaborative research relationship. This is best denoted by the collaborative publications noted in Section H, publications include authors from both institutions. Dr. Palmer travelled to Beit Dagan in March 1990 to complete experiments on MSP-2 as a conserved immunodominant antigen between Israel and U.S. strains of *A. marginale* (reference 30). Consequently a joint decision to incorporate MSP-2 into the immunization trials was made during this period. In addition, newly developed serologic assays to measure specific immune responses were transferred between institutions. Dr. Palmer travelled to Beit Dagan in October 1991 (on return trip from Kenya in order to save airfare) and in collaboration with Drs. Pipano and Shkap designed the experiments for Specific Aim 3. To improve collaboration on the recombinant DNA aspects of the proposal, Dr. Varda Shkap of the Kimron Veterinary Institute spent two months at Washington State University in 1990 and a second 6 week period in 1991. During this period she became proficient at restriction mapping, Southern and Northern hybridizations, transformation, and antibody and oligonucleotide screening procedures.

As a result of BARD sponsored collaboration between the investigators at the two institutions, we arranged for a senior veterinary student at Washington State University to spend three months in a supervised research externship at Kimron Veterinary Institute. Although no BARD funds were used to support this student exchange, the development of the exchange was a direct result of the BARD collaboration. Significantly, this exchange extends the benefits of the binational program to university undergraduate education.

G. Evaluation of the Research Achievement

Hypothesis and Specific Aims (p.13 original proposal):

Hypothesis: Recombinant-derived MSP1 will provide protection against challenge with homologous or heterologous strains of *A. marginale*.

- Specific Aim 1: Establish that native MSP-1a immunization induces protection against challenge with Israel strains of *Anaplasma marginale*.
- Specific Aim 2: Obtain high-level expression of the gene coding for MSP-1a.
- Specific Aim 3: Test the effectiveness of recombinant MSP-1a as a protective immunogen against challenge with either Israel or U.S. strains of *Anaplasma marginale*.
- Specific Aim 4: Identify additional polypeptides with conserved epitopes for inclusion in a recombinant vaccine.

We have completed each specific aim and tested the hypothesis. Briefly, we accomplished the following: i) development of a challenge protocol using live *A. marginale* that consistently induced clinical disease; ii) development of an immunization protocol that consistently induced statistically significant protection against homologous challenge; iii) development of assays that measure immune responses against the critical neutralization-sensitive MSP1a epitope; iv) demonstration that antibody against MSP-1 promoted macrophage phagocytosis of *A. marginale*; v) immunization of cattle with native MSP1/2 polypeptides followed by heterologous Israel strain challenge; vi) recombinant MSP1a and MSP1b expression in pGEX2T; vii) immunization of cattle with recombinant MSP1 followed by heterologous Israel strain challenge; viii) identification of MSP-2 and MSP-3 as conserved polypeptides on Israel strains of *A. marginale*; ix) identification of MSP-2 and MSP-3 as immunodominant surface polypeptides with conserved epitopes between *A. centrale* and *A. marginale*; x) identification of MSP-5 as a genus common polypeptide (*A. centrale*, *A. marginale*, *A. ovis*); xi) identification of antibody against MSP-5 in cattle premunized with *A. centrale* and protected against *A. marginale*; and x) cloning and sequencing the single copy gene encoding MSP-5.

Although we were disappointed that MSP-1 immunization did not induce significant protective immunity against heterologous challenge, this observation has been critical in guiding vaccine research towards presentation of surface antigens in a membrane context rather than as individual subunits. This observation, complete protection in a low % of vaccinates and partial protection in the remaining individuals, also follows immunization with either individual MSP-2 or MSP-3 and represents a considerable constraint to vaccine development based on a single protein in adjuvant. Consequently the research in the current BARD led us to hypothesize that a multivalent outer membrane polypeptide would induce a polyclonal immune response against multiple surface polypeptides and expand the number of fully responsive cattle. As described in Section D and reference 26, we now have substantial evidence that this approach induces solidly protective immunity.

During the BARD project, we have identified those outer membrane polypeptides with common epitopes among Israel and U.S. strains. Consequently we have a basis for selection

of the outer membrane polypeptides to be developed in a multivalent vaccine. High-level expressed MSP-1a and MSP-1b have been purified in our BARD research and we have collaboratively cloned and expressed genes encoding MSP-2 and MSP-5. MSP-3 has been cloned and expressed as part of a separate project at Washington State University. In summary, our collaborative research on vaccine development has led to new directions in vaccine research and has resulted in critical reagent development.

H. Publications

*Describe new assays to determine immunologic responses to immunized cattle and development of diagnostic reagents that can discriminate between infected cattle and cattle immunized with MSP1 or MSP1/MSP2.

BARD supported proceedings and abstracts:

Shkap, V., Pipano, E., Bin, H., Barbet, A.F., Davis, W.C., McGuire, T.C., Palmer, G.H.: Conservation of surface protein epitopes between *Anaplasma centrale* and *Anaplasma marginale*: a review of the basis for vaccine development. In **Proceedings of the Eighth National Veterinary Hemoparasite Disease Conference**, R.J. Hidalgo, Ed. pp. 499-506. Louisiana State University, 1989.

Barbet, A.F., Allred, D.R., McGuire, T.C., Palmer, G.H.: Analysis of genes for an immunoprotective protein of *Anaplasma marginale*. International Society for Parasitology, Paris, France, 1990.

Shkap, V., Palmer, G.H., Barbet, A.F., Pipano, E., Bin, H.: Protein Mr 36,000, a common immunodominant surface antigen of *Anaplasma marginale* and *A. centrale*. *Journal of Protozoology* (Supplement), 1989.

Palmer, G.H., Barbet, A.F., McElwain, T.F., Stephens, E.B., Allred, D.R. and McGuire, T.C.: Immunization strategies to focus immune responses against the MSP-1a neutralization-sensitive epitope of *Anaplasma marginale*. Parasitology and Immunoparasitology Section, Conference on Research Workers in Animal Disease; 71st Annual Meeting. 1990.

Visser, E.S., McGuire, T.C., Palmer, G.H., Davis, W.C., Knowles, D.P.: Expression of a 19kD *Anaplasma marginale* protein with a geographically conserved epitope. Parasitology and Immunoparasitology Section, Conference on Research Workers in Animal Disease; 72nd Annual Meeting. 1991.

BARD supported refereed publications:

Palmer, G.H., Barbet, A.F., Cantor, G.H., and McGuire, T.C.: 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.

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Shkap, V., Pipano, E., McGuire, T.C. and Palmer, G.H.: Identification of immunodominant polypeptides common between *Anaplasma centrale* and *Anaplasma marginale*. *Veterinary Immunology and Immunopathology*, 29:31-40, 1991.

Visser, E.S., McGuire, T.C., Palmer, G.H., Davis, W.C., Shkap, V., Pipano, E., Knowles, D.P.: The *Anaplasma marginale* *msp5* gene encodes a 19-Kilodalton protein conserved in all recognized *Anaplasma* species. *Infection and Immunity*, 60:5139-5144, 1992.

Cantor, G.H., Pontzer, C.H. and Palmer, G.H.: Opsonization of *Anaplasma marginale* mediated by bovine antibody against surface protein MSP-1. *Veterinary Immunology and Immunopathology*, in press.

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

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