77

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**Development of a Multivalent ISCOM Vaccine
against Anaplasmosis**

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Development of a multivalent ISCOM vaccine against anaplasmosis

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Table of Contents

Section	Description	Pages
A.	Cover Page	1
B.	Table of Contents	2
C.	Abstract	3
D.	Research Activities: Introduction and Objectives	4-5
E.	Detailed Description of Research Activities and Results	5-11
F.	Description of Cooperation	11-13
G.	Evaluation of the Research Achievement	13-14
H.	List of Publications	15-17
I.	References	17-22

C. Abstract for BARD project US-2238-92C "Development of a multivalent vaccine against anaplasmosis"

Anaplasmosis is an arthropod-borne disease of cattle caused by the rickettsia *Anaplasma marginale* and an impediment to efficient production of healthy livestock in both Israel and the United States. Our research focuses on development of a recombinant membrane surface protein (MSP) immunogen to replace current vaccines derived from the blood of infected cattle. The risk of widespread transmission of both known and newly emergent pathogens has prevented licensure of live blood-based vaccines in the U.S. and is a major concern for their continued use in Israel. Briefly, we accomplished the following in our BARD supported research: i) characterization of the intramolecular and intermolecular relationships of the native Major Surface Proteins (MSP) in the outer membrane; ii) expression, purification, and epitope characterization of the recombinant MSP-2, MSP-3, MSP-4, and MSP-5 proteins required to construct the recombinant ISCOM ; iii) demonstration that the outer membrane-Quil A induces CD4+ T lymphocytes specific for the outer membrane polypeptides; iv) identification of CD4+ T lymphocytes that recognize outer membrane polypeptide epitopes conserved among otherwise antigenically distinct strains; v) determination that immunization with the outer membrane-Quil A construct does not affect the ability of ticks to acquire or transmit *A. marginale*; and vi) demonstration that the outer membrane-Quil A construct induces complete protection against rickettsemia upon homologous challenge and significant protection against challenge with antigenically distinct strains, including tick transmission. Importantly, the level of protection against homologous challenge in the MSP vaccinates was comparable to that induced by live blood-based vaccines and demonstrates that development of a new generation of vaccines is feasible.

D. Research Activities: Introduction and Objectives

Introduction

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. Our research goal is the development of an inactivated vaccine that induces consistent protective immunity against acute anaplasmosis and circumvents the need for blood-derived antigen. We have demonstrated that *A. marginale* native outer membrane polypeptides, presented in a Quil A-membrane matrix, induce solid immunity against virulent challenge³. The multivalent antigen in membrane context induces immune responses against multiple *A. marginale* surface polypeptides and, importantly, avoids problems of non-responding animals which may occur following immunization with a single antigen. We have identified the polypeptide immunogens in the native outer membrane³ and cloned and expressed the genes encoding these polypeptides. Incorporation of recombinant outer membrane polypeptides provides a standardized immunogen without reliance on native *A. marginale*.

The objective of this proposal was to test the following hypothesis:

Immunization of cattle with a multivalent vaccine will induce protective immunity against anaplasmosis in cattle.

Our specific aims to test this hypothesis were:

- 1] Characterize the antigenic and structural composition of the native outer membrane polypeptide immunogen.
- 2] Develop and characterize an ISCOM bearing *A. marginale* polypeptides (MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, MSP-5).
- 3] Test the multivalent ISCOM as a protective immunogen against homologous and heterologous *A. marginale* challenge.

We have completed all three specific aims of the project (a no-cost extension was awarded to complete the final aim in early 1997). Briefly, we have now accomplished the following: i) characterization of the intramolecular and intermolecular relationships of the native Major Surface Proteins (MSP) in the outer membrane; ii) expression, purification, and epitope characterization of the recombinant MSP-2, MSP-3, MSP-4, and MSP-5 proteins required to construct the recombinant ISCOM ; iii)

demonstration that the outer membrane-Quil A induces CD4+ T lymphocytes specific for the outer membrane polypeptides; iv) identification of CD4+ T lymphocytes that recognize outer membrane polypeptide epitopes conserved among otherwise antigenically distinct strains; v) determination that immunization with the outer membrane-Quil A construct does not affect the ability of ticks to acquire or transmit *A. marginale*; and vi) demonstration that the outer membrane-Quil A construct induces complete protection against rickettsemia upon homologous challenge and significant protection against challenge with an antigenically distinct strain. These experiments and results are described in the next section and are presented in detail in both the previously submitted reprints and the attached reprint copies (Publication list is in section H).

E. Detailed Description of Research Activities and Results

Specific Aim 1: Characterize the antigenic and structural composition of the native outer membrane polypeptide immunogen.

1a. Determine the structural and antigenic distribution of polypeptides in the native membrane: The outer membrane-Quil A immunogen induces immunity superior to all previously reported inactivated anaplasmosis vaccines^{4,5}. The project goal is to circumvent the need for native antigen and membrane by developing a recombinant polypeptide ISCOM immunogen. To provide a basis for recreating the native immunogen, the structural and antigenic conformation of the native polypeptides in the membrane needs to be established. The structural organization of the MSPs in the native *A. marginale* membrane has now been elucidated. Under non-reducing conditions, MSP-2 and MSP-5 occur in the membrane as multimers. A large (>300 kDa) non-reduced membrane protein complex contained MSP-1a linked by disulfide bonds to MSP-1b and by non-covalent bonds to MSP-5. MSP-2 was also noncovalently bound to this complex. The nearest neighbor membrane proteins were identified by cross-linking reactions followed by immunoblotting with anti-MSP antibodies. A cross-linked aggregate retained in the stacking gel contained MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, and MSP-5. Collectively the data indicate that MSP-2 and MSP-5 occur as monomers and as disulfide bonded multimers. The MSP-1 complex occurs as both disulfide bonded and noncovalently associated MSP-1a and MSP-1b, and MSP-2 and MSP-5 are noncovalently associated with MSP-1. Also MSP-1, MSP-2, MSP-3, and MSP-4 are nearest neighbors and MSP-5 is non-covalently associated with this cross-linked complex. The results are described in detail in reference 9.

1b. Test the ability of the native multivalent outer membrane immunogen to induce protective immunity against heterologous challenge: The efficacy of premunition against both homologous and heterologous field challenge indicates that conserved epitopes critical to the induction of protective immunity are present and immunogenic in cattle⁶. We tested the hypothesis that outer membrane immunization would induce protection against an antigenically variant strain. Outer membranes were isolated from the Virginia strain of *A. marginale* using density gradient centrifugation, combined with saponin adjuvant, and used to immunize Holstein cattle. Immunized cattle developed high antibody titers (80,000-160,000) against outer membrane polypeptides including MSP-2 and MSP-5 in both the

homologous Virginia and heterologous Norton strains. Outer membrane immunized cattle were significantly protected following challenge with 10^4 Norton strain parasitized erythrocytes as demonstrated by significant differences in prepatent period and peak rickettsemia when compared to adjuvant immunized control cattle. The mean peak rickettsemia of outer membrane-Quil A immunized cattle [n=10] was 9.0 ± 5.9 while the mean peak rickettsemia of Quil A control immunized cattle [n=10] was 27.3 ± 9.2 . These parameters were significantly different at $p < 0.05$. One outer membrane immunized animal was completely protected against infection. However, there were no overall significant differences in severity of anemia between outer membrane immunized cattle and the control group, indicating that a significant reduction in rickettsemia does not necessarily result in less severe anemia. This demonstrates that outer membrane-Quil A immunization induces significant protection against rickettsemia and can prevent infection upon heterologous challenge. Optimization of outer membrane vaccines to induce complete protection in a higher percentage of vaccinates and prevention of concurrent anemia in partially protected cattle will be necessary for vaccine development based on this approach. The results are described in detail in reference 10.

A second heterologous challenge was done in a limited trial of eight cattle to test whether outer membrane immunization would protect against tick challenge. Cattle immunized with $100 \mu\text{g}$ outer membrane polypeptides developed a mean rickettsemia of 2.3% upon challenge with the *Dermacentor andersoni* ticks infected with the Virginia strain of *A. marginale*. Cattle immunized with adjuvant alone on the identical schedule developed a mean rickettsemia of 9.6%. Although the limited size of the trial precluded demonstrating statistical significance, the data suggest that protective immunity is induced against tick challenge. In a corollary experiment, we tested whether tick feeding upon immunized cattle affected subsequent development of *A. marginale* in the salivary glands. The propensity of male ticks to move from one animal to another during intermittent feeding raises the possibility that feeding upon an immunized animal may affect the ability of the tick to subsequently transmit infection. However there was no significant difference in the mean number of *A. marginale* infected salivary gland acini whether the infected ticks had fed on immunized versus non-immunized cattle. In addition, there was no apparent affect on the ability of these infected *D. andersoni* male ticks to subsequently transmit the infection. These results are described in reference 13.

Specific Aim 2: Develop and characterize an immune stimulating complex bearing recombinant *A. marginale* polypeptides (MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, MSP-5).

Incorporation of recombinant outer membrane polypeptides in a defined ISCOM will provide a standardized immunogen without reliance on native *A. marginale*. The recombinant outer membrane polypeptides to be inserted into the ISCOM are immunogenic in the native outer membrane preparation and protection in outer membrane immunized cattle correlates ($r=0.97$, $P < 0.05$) with antibody titer to these polypeptides³. All six polypeptides have epitopes conserved among strains of *A. marginale* from Israel and the U.S.^{7,38}. To construct a standardized multivalent recombinant immunogen, purified recombinant membrane polypeptides will be inserted into an ISCOM.

MSP-4 and MSP-2 were expressed during the first year of the project. MSP-5 and MSP-3 were expressed during project years 2 and 3, respectively. The description of the expression and characterization of the recombinant MSP-4, MSP-2, MSP-3 and MSP-5 is briefly summarized below

and is presented in detail in references 11, 12, 14, and 15. **MSP-4:** The *msp-4* gene was expressed in *E. coli* using the vector pGEX-2T and shown to present epitopes that mimicked the native protein. Sera from cattle immunized with the outer membrane-Quil A construct, and shown to be protected against challenge, bound the recombinant MSP-4. MSP-4 epitopes and molecular size have been shown to be conserved among eight strains that are otherwise antigenically and structurally unique. Similarly, the single copy gene appears to be, based on restriction enzyme mapping, conserved among these strains. The results are described in detail in reference 11. **MSP-2:** *Anaplasma marginale* Florida strain *msp-2* was cloned and expressed in *E. coli* using the vector pKK233-3. Pulse-field gel electrophoresis and Southern blot analysis revealed the presence of multiple *msp-2* gene copies that were widely distributed throughout the chromosome of all three strains examined. Genomic polymorphism among copies was greatest in the 5' end of *msp-2* but also occurred in 3' regions. The presence of gene copy specific epitopes was indicated by the reactivity of the cloned *msp-2* copy (designated pCKR11.2) with some, but not all monoclonal antibodies that bound native MSP-2. Multiple antigenically distinct MSP-2 molecules were expressed within a strain and were co-expressed by individual *A. marginale*. These results suggest that expression of polymorphic *msp-2* gene copies is responsible for the significant percentage of *A. marginale* organisms within a strain that do not react with individual anti-MSP-2 monoclonal antibodies. Sequence analysis revealed highly significant MSP-2 homology with two rickettsial surface proteins, *A. marginale* MSP-4 and *Cowdria ruminantium* MAP-1. Similar to MSP-2, immunization with MSP-4 has been shown to induce protective immunity. The results are described in detail in reference 12. In year 2, we subcloned the pCKR11.2 *msp-2* into a higher level expression vector, pMal. The MSP-2 fusion has been purified on a maltose column and cleaved with Factor Xa to release the purified MSP-2. In addition in year 2, we cloned and sequenced a second *msp-2* genomic copy. This copy, designated AR-1, has a defined region of amino acid polymorphism as compared to pCKR11.2 despite an overall amino acid identity of >90%. Using a cDNA library developed from *msp-2* mRNA expressed during acute rickettsemia (by reverse transcription of hybrid selected *msp-2* mRNA), we have confirmed that AR-1 is an expressed gene and identified a more markedly polymorphic (~70% identity) expressed *msp-2* gene designated AR-5. The regions that are polymorphic between AR-1 and AR-2 include distinct B cell epitopes which are expressed during acute rickettsemia. **MSP-3:** *A. marginale msp-3* was cloned from a library constructed by adapting *EcoRI* linkers onto mechanically sheared genomic Florida strain DNA and ligation into lambda ZAP phage. The initial cloned copy, designated *msp3-12*, was used as a probe to identify multiple polymorphic *msp-3* copies in the genome. These multiple partially homologous copies, reminiscent of *msp-2*, were shown to be distributed throughout the chromosome and sequencing three copies (*msp3-11*; *msp3-12*; *msp3-19*) revealed both conserved and copy-specific regions. Importantly, *msp-3* contains amino acid blocks related to the outer membrane protein MSP-2, also encoded by a polymorphic family. The current hypothesis is that these polymorphic MSP-2 and MSP-3 polypeptides are involved in antigenic variation during the cyclic rickettsemia of persistent infection and may not affect the ability of a multivalent outer membrane protein vaccine to induce protection against acute rickettsemia. This hypothesis is supported indirectly by the presence of B and T cells conserved among structurally distinct MSP-2 and MSP-3 and directly by the ability of the multivalent vaccine to induce protection against heterologous strains. These results are described in detail in references 3, 10, 12, 14, and 15. **MSP-5:** *A. marginale msp-5* has been subcloned into the

pMal fusion vector described for MSP-2. Using truncations of the pMal/MSP-5 we have shown that the retention of B cell epitopes is dependent upon retention of both conformation and especially intra-chain disulfide bonding. This is described in detail in a paper to be submitted in September 1997. The expressed protein has been isolated in high quantities using an amylose resin column and shown to bear the B and T cell epitopes presented on the native MSP-5.

Specific Aim 3: Test the multivalent ISCOM as a protective immunogen against homologous and heterologous *A. marginale* challenge.

3a. Determine whether immunization with the multivalent outer membrane protects against tick transmitted *A. marginale*: The ability of outer membrane immunization to protect against rickettsemia following tick transmission was tested using infected *Dermacentor andersoni* adult males fed on cattle immunized with either the multivalent vaccine or, as a control, adjuvant alone. Cattle were immunized using monophosphoryl lipid A-trehalose dicorynomycolate as an adjuvants. Infected *D. andersoni* males were allowed to feed on cattle for 7 days and the rickettsemia determined using examination of Giemsa stained blood films. Vaccinated non-splenectomized cattle developed a mean peak rickettsemia of 2.25% while adjuvant control cattle developed a mean rickettsemia of 9.6%¹³.

3b. Determine whether immunization with the multivalent outer membrane affects the ability of ticks to transmit *A. marginale*: The effect of antibodies induced by outer membrane immunization on *A. marginale* infection in ticks was tested using infected *Dermacentor andersoni* adult males fed on cattle immunized with either the multivalent vaccine or, as a control, adjuvant alone. Cattle were immunized using either Quil A (Trial 1) or monophosphoryl lipid A-trehalose dicorynomycolate (Trial 2; as reported in 3a above) as adjuvants. Infected *D. andersoni* males were allowed to feed on cattle for 7 days and the effect of antibodies on the *A. marginale* infection rate in the salivary glands determined by *msp-1b* RNA probe hybridization and light microscopy. Vaccine derived antibodies ingested with the blood meal did not alter the development of *A. marginale* in previously infected ticks. The infection rates in the salivary glands were not significantly different between groups of ticks fed on multivalent immunogen vaccinated cattle versus adjuvant control cattle. Similarly, there was no difference in the ability of the ticks to transmit *A. marginale* when the ticks were fed on susceptible cattle. These results are described in detail in references 13 and 16.

3c. Demonstrate that outer membrane-Quil A immunization induces CD4+ T lymphocytes specific for the outer membrane polypeptides: Cattle were immunized with the multivalent outer membrane protein complex and antigen-specific T cell responses determined using peripheral blood mononuclear cells (PBMC). In experiment 1, performed at Kimron Veterinary Institute, stimulation indices were determined following antigen presentation in vitro. As seen in Figure 1, all six immunized cattle developed *A. marginale* specific responses (URBCs = uninfected erythrocytes) and 5/6 responded with indices >10 against both the homologous strain (Florida strain, AmFl) and a heterologous strain from Israel (AmIsL). In experiment 2, performed at Washington State University in collaboration with Dr. Wendy Brown, PBMCs were similarly stimulated with the Florida strain *A. marginale* and used to develop T cell lines and clones.

Figure 1: Response of calves immunized with the multivalent immunogen to homologous (AmFL) and heterologous (AmIsL) antigen

T cell clones were developed from three immunized cattle (95B05, 95B06, 95B09) and shown to be CD4+, CD8- by flow cytometric analysis. Purified outer membrane proteins were presented using autologous irradiated PBMC as antigen-presenting cells and used to determine whether CD4+ T cells responded specifically to the individual MSPs characterized in specific aims 1 and 2. Clones were identified that responded to MSP-1, MSP-2, and MSP-3 (Table 1). These clones, shown to transcribe both IFN- γ and IL-4 (characteristic of bovine Th0 cells, data not shown), were used in 3c to test whether outer membrane protein T cell epitopes were conserved among strains. This data will be submitted for publication in October 1997.

Table 1: MSP specificity of T cell clones and strain, stage, and Anaplasma species reactivity

Th clone	MSP sp- ecificity	Tick stage	Am Florida	Am WA-C	Am WA-O	Am Idaho	Am Virginia	A ovis
9.4E3	MSP3	-	+	-	-	-	-	-
5.1D3	Unk	-	+	+	+	+	-	-
6.1E3	MSP2/3	+	+	+	+	+	+	-
9.4A11	Unk	+	+	+	+	+	+	+
9.4H4	Unk	+	+	+	+	+	+	+
5.1D11	MSP2	-	+	+	+	+	+	+
9.4E7	MSP2 [⊙]	+	+	+	+	+	+	+
6.1F11	MSP2 ^{⊙⊙}	+	+	+	+	+	+	+
9.3D3	MSP2	+	+	+	+	+	+	+
9.4G4	MSP3 [⊙]	+	+	+	+	+	+	+
9.4G8	MSP3	-	+	+	+	+	+	+

[⊙] Also proliferated in response to MSP1

^{⊙⊙} Also proliferated in response to MSP2

[⊙] Also proliferated in response to MSP3

Figure 2: Response of calves immunized with the multivalent immunogen to homologous *Anaplasma marginale* (AmFL) and *Anaplasma centrale* (AcIB's) antigen

3d. Demonstrate that the multivalent outer membrane immunogen includes Th epitopes conserved among *A. marginale* strains: In experiment 1 (Kimron Veterinary Institute), the PBMCs from immunized cattle were stimulated with different strains of *A. marginale* and with the vaccine strain of *A. centrale*. Figure 1 shows that immunized cattle respond to both the homologous strain (Florida strain) and a genetically and structurally distinct Israel strain. Figure 2 demonstrates the diminished but significant response to the vaccine strain of *A. centrale*. Figures 3a and 3b show the responses of two representative vaccinates, 247 and 249, which respond to the homologous Florida strain (AmFL) and two heterologous strains from Israel (AmIsL and AmIsUp). In addition Fig 3b shows the response of vaccinate 249 to *A. centrale* (AcIB's). Fig. 3c shows an adjuvant control animal, 257, that responded only to T cell growth factor (TCGF) and not to any of the control or *Anaplasma* antigens. In experiment 2 (Washington State University, in collaboration with Drs. Katherine Kocan and Edmour Blouin of Oklahoma State University), CD4+ T cell clones derived from outer membrane immunized cattle were tested for proliferation in response to genetically distinct strains of *A. marginale* and *A. ovis*. In addition, the response to *A. marginale* stages derived from in vitro culture in tick cells were determined for each clone. The responses are shown in Table 1. Together these experiments from Kimron Veterinary Institute and Washington State University provide the first demonstration that despite marked genetic and structural differences among *A. marginale* strains, outer membrane immunized cattle developed MSP specific responses that included conserved T cell epitopes. Furthermore, the responses to *A. centrale*, *A. ovis*, and the tick stages of *A. marginale* indicate that the multivalent immunogen induces immune responses conserved within the genus and among the mammalian and tick stages of the pathogen. These data, which support development of improved vaccines, will be submitted for publication in October 1997.

Figure 3a: Response of calf #247 immunized with the multivalent immunogen to homologous and heterologous *Anaplasma maginale* antigens

Figure 3b: Response of calf #249 immunized with the multivalent immunogen to homologous and heterologous *Anaplasma maginale* antigens

Figure 3c: Response of control calf #257 immunized with adjuvant alone to homologous and heterologous *Anaplasma maginale* antigens

3e. Demonstrate that the multivalent outer membrane immunogen induces protection against rickettsemia upon homologous and heterologus challenge: In experiment 1 (Washington State University), cattle were immunized with the multivalent outer membrane-Quil A immunogen. The MSPs, characterized in specific aims 1 and 2, were derived from the Florida strain. The T cell clones described above and in Table 1 were derived from these cattle. Multivalent immunogen-Quil A vaccinates and control cattle immunized on an identical schedule with Quil A were challenged with 10^2 live Florida strain *A. marginale* by intravenous inoculation. Rickettsemia and PCV (as a measure of anemia) were determined daily for all cattle. Vaccinates were protected against acute rickettsemia (all cattle developed <0.1% rickettsemia) and had a mean minimal PCV of 35%. Adjuvant control cattle developed a mean peak rickettsemia of 5.4% with a mean minimal PCV of 20%. Thus, vaccination protected against both high level rickettsemia and anemia. To determine if the vaccinates became persistently infected, nested *msp-5* PCR was done as described¹⁷ on blood collected 60 and 90 days post-challenge. All adjuvant control cattle were persistently infected while some (vaccinates 96B06 and 96B09) but not all vaccinates were clear of infection. The challenge with a genetically and antigenically distinct *A. marginale* strain, Israel-NT, was conducted at Kimron Veterinary Institute. The design was identical to the Washington State University trial with multivalent outer membrane protein-Quil A vaccinates and adjuvant controls. The T cell responses of these cattle were described in section 3b and 3c above; all vaccinated cattle developed specific antibody responses while control cattle remained seronegative (data not shown). Vaccinates developed a peak rickettsemia of 4.5% with two animals (251 and 252) developing <0.5% rickettsemia. This protection was statistically significant as compared to the 15.4% peak rickettsemia in adjuvant control cattle. The decrease in PCV was less severe in vaccinates (48% decrease) as compared to adjuvant controls (60% decrease) and the duration of fever during acute rickettsemia was significantly reduced in vaccinates. However, the peak febrile responses were not significantly different between the two groups of cattle.

F. Description of Cooperation

The BARD funding provided the opportunity to strengthen our research collaboration. This is most notable in the design and completion of the third specific aim: complementary approaches were used independently at Kimron Veterinary Institute and Washington State University to show the conservation of epitopes among genetically distinct strains and establish the induction of immunity against homologous and heterologous strain challenge. The investigators met at the Ninth International Veterinary Hemoparasite Conference in Merida, Yucatan, Mexico in October 1993 where they presented new data on the BARD supported research at the conference. This meeting provided an opportunity to further develop the research objectives for project years 1 and 2. The procedure for development of the outer membrane preparation was transferred to Kimron Veterinary Institute during year 2 of the project. Drs. Pipano and Shkap visited Washington State University in July 1995 in association with their attendance at the Fourth International Veterinary Immunology Symposium at the University of California at Davis. During this period, the investigators reviewed progress, planned immunization experiments for year 3, and planned an important corollary study (recommended by the reviewers) on examining the cell-mediated response following immunization. In addition, Dr. Shkap learned new techniques on measuring bovine T cell responses to defined immunogens in the laboratory

of Dr. Wendy Brown. In August 1996, Drs. Knowles (non-BARD funds) and Palmer (BARD funds) traveled to Israel to complete the coordination of specific aim 3 including the inclusion of U.S. strains in the experiments at Kimron Veterinary Institute and Israel strains in the experiments at Washington State University.

G. Evaluation of the Research Achievement

Our research goal is the development of a recombinant membrane surface protein (MSP) immunogen to replace current vaccines derived from the blood of infected cattle. The risk of widespread transmission of both known and newly emergent pathogens has prevented licensure of live blood-based vaccines in the U.S. and is a major concern for their continued use in Israel. In our BARD supported research, we have firmly established the immunologic basis for development of a vaccine targeted against the genetically diverse strains of *Anaplasma marginale*. In part 1 of our BARD supported research, we mapped the outer membrane composition of intact *A. marginale* to identify the intra- and inter-molecular relationships among the major surface proteins (MSP) and the adhesin function of surface exposed ligands. The multivalent MSP complex developed and tested in the BARD project incorporates the six major outer membrane proteins, MSP-1a, -1b, -2, -3, -4, and -5. With the completion of the cloning, sequencing, and expression of *msh-2* and *msh-3* during the current BARD period, all six MSPs have now been expressed and purified as recombinant proteins.

Immunization of cattle with the multivalent outer membrane-Quil A complex induces specific antibody against each MSP component and CD4+ Th0 cells against MSP1, MSP2, and MSP3. Most importantly, immunized cattle are solidly protected against acute rickettsemia as compared to adjuvant-control cattle upon challenge with the homologous strain. Each of the six MSP components, both as native proteins and as expressed recombinant proteins, bears epitopes common among 15 examined strains from the U.S. and Israel. Although we have identified MSP-2 and MSP-3 antigenic variants, this variation appears to be involved in persistence and is not an impediment to vaccination against acute rickettsemia. Consequently, we hypothesized that immunization with the multivalent MSP complex would induce protection against challenge with genetically distinct strains. We have now completed a total of three multivalent MSP immunization trials to test this hypothesis. In the first trial, immunized cattle were challenged with 10^4 live *A. marginale* infected erythrocytes using a freshly isolated, non-passaged heterologous strain (Norton). The immunized cattle ($n=10$, mean peak rickettsemia of $9.0 \pm 5\%$) were significantly protected ($p < 0.05$) as compared to adjuvant-control immunized cattle ($n=10$; $27.3 \pm 9.2\%$). However, the protection against rickettsemia was not uniform and varied from complete prevention of infection to lack of protection (defined as rickettsemia within the 95% confidence interval of levels in control cattle). A second trial of MSP complex immunized cattle using heterologous strain challenge confirmed the induction of protection in most, but not all vaccinates. *A. marginale* (VA strain) infected *Dermacentor andersoni* ticks were allowed to attach and feed on immunized and adjuvant-control cattle to test the vaccine efficacy against tick-transmitted stages. Similar to the first heterologous challenge trial, immunized cattle developed lower peak rickettsemia as compared to adjuvant control cattle but included both highly protected

and poorly protected individuals. In the third trial, reported as part of the third specific aim, cattle were again segregated into solidly and partially protected responders. Based on all trials to date, approximately 70% of the individual MSP vaccinates are significantly protected against acute rickettsemia, however the inability to protect the remaining individuals precludes replacement of current vaccines derived from infected cattle. However, the findings of this BARD study have provided a clear direction to enhance the uniformity of immunity and meet our goal of replacement of blood-based vaccines.

The successful induction of protection against heterologous strain challenges indicates that the MSP complex vaccine incorporates epitopes capable of inducing cross-protection against genetically and antigenically distinct strains. The level of protection in the fully protected MSP complex vaccinates, based on three separate immunization trials, is comparable to that induced by live blood-based vaccines and demonstrates that development of a new generation of vaccines is feasible. However, the non-uniform protection induced by the MSP complex vaccine remains a major constraint to replacement of blood-based vaccines. The current focus, resulting from our results, is to identify the MSP specific immune responses in fully-protected vaccinates and to enhance these responses in individual vaccinates using recombinant cytokines as defined adjuvants.

The cytokine environment at the time of vaccine antigen presentation is the key regulator of the type and magnitude of the immune response. Critically, this environment has been shown to determine the success or failure of vaccination to induce protective immunity¹⁸⁻²¹. Specifically, protective immunity against intracellular bacteria, including rickettsia, requires induction of type 1 immune responses²²⁻²⁵. Immunoregulatory type 1^{26,27} responses are characterized by commitment of T lymphocytes to the interferon- γ secreting Th1 phenotype, enhanced macrophage mediated microbial killing, and preferential production of the IgG2 antibody isotype in cattle²⁸⁻³¹. Unlike the strict type 1 versus type 2 immune response dichotomy in mice, cattle, like humans^{21,32,33}, respond to antigens within a continuum of cytokine patterns with type 1 and type 2 at opposite ends of the spectrum^{28,34-39}. Consequently, we hypothesize that cattle respond to MSP complex vaccination with a spectrum of different individual cytokine patterns and that induction of a predominant type 1 response will be associated with development of protective immunity.

Interleukin 12, a cytokine expressed by activated macrophages and B cells, is the prime initiator for type 1 responses⁴⁰⁻⁴⁴. As predicted from these properties, IL-12 enhances protection against intracellular bacterial and parasitic infections^{18,20,45-47}. Importantly, IL-12 induces type 1 responses in humans and outbred non-human primates in vivo^{44,47-50} and we have shown that, in vitro, IL-12 enhances interferon- γ production from both newly antigen stimulated bovine peripheral blood mononuclear cells (PBMC) and antigen-specific bovine CD4+T cell clones^{51,52}. Consequently, we now hypothesize that IL-12 can direct type 1 MSP responses in cattle in vivo and enhance the uniformity of protective immunity. In summary, our collaborative research on vaccine development has provided the basis for now dissecting the protective immune response and allows us and others to address the critical issue constraining replacement of blood-based vaccines in livestock.

H. List of Publications

Primary publications:

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*, 37:343-350, 1993. [Supported by US-1561-88; reported as in press in final report].

Oberle, S.M., Palmer, G.H., and Barbet, A.F.: Expression and immune recognition of the conserved *Anaplasma marginale* surface protein MSP-4. *Infection and Immunity*, 61:5245-5251, 1993. [Supported by US-1561-88; reported as submitted in final report].

Vidotto, M., McGuire, T.C., McElwain, T.F., Palmer, G.H., and Knowles, D.P.: Intermolecular relationships of major surface proteins of *Anaplasma marginale*. *Infection and Immunity*, 62:2940-2946, 1994.

Palmer, G.H., Eid, G., Barbet, A.F., McGuire, T.C. and McElwain, T.F.: The immunoprotective *Anaplasma marginale* major surface protein-2 (MSP-2) is encoded by a polymorphic multigene family. *Infection and Immunity*, 62:3808-3816, 1994.

McGarey, D.J., Barbet, A.F., Palmer, G.H., and Allred, D.R.: Putative adhesins of *Anaplasma marginale* major surface polypeptides (MSP) 1a and 1b. *Infection and Immunity* 62:4594-4601, 1994.

Palmer, G.H., Munodzana, D., Tebele, N., Ushe, T., and McElwain, T.F.: Heterologous strain challenge of cattle immunized with *Anaplasma marginale* outer membranes. *Veterinary Immunology and Immunopathology*, 42:265-273, 1994.

Eriks, I.S., Stiller, D., Goff, W.L., Panton, M., Parish, S.M., McElwain, T.F. and Palmer, G.H.: Molecular and biological characterization of a new isolated *Anaplasma marginale* strain. *Journal of Veterinary Diagnostic Investigation* 6:435-441, 1994.

Palmer, G.H. and McElwain, T.F.: Molecular basis for vaccine development against anaplasmosis and babesiosis. *Veterinary Parasitology*, 57:233-253, 1995.

Eid, G., French, D.M., Lundgren, A., Barbet, A.F., McElwain, T.F., and Palmer, G.H.: Expression of Major Surface Protein 2 antigenic variants during acute *Anaplasma marginale* rickettsemia. *Infection and Immunity*, 64:836-841, 1996.

Kocan, K.M., Blouin, E.F., Palmer, G.H., Eriks, I.S., Edwards, W.L., and Claypool, L.: Preliminary studies on the effect of *Anaplasma marginale* antibodies ingested by *Dermacentor andersoni* ticks (Acari: Ixodidae) with their bloodmeal on infections in salivary glands. *Experimental and Applied Acarology*, 20:297-311, 1996.

Alleman, A.R., Palmer, G.H., McGuire, T.C., McElwain, T.F., Lance E. Perryman, and Barbet, A.F.: *Anaplasma marginale* major surface protein-3 (MSP-3) is encoded by a polymorphic multigene family. *Infection and Immunity*, 65:156-163, 1997.

Torioni de Echaide, S., Knowles, D.P., McGuire, T.C., Palmer, G.H., Suarez, C.E., and McElwain, T.F.: Detection of cattle naturally infected with *Anaplasma marginale* in an endemic region using nested PCR and recombinant MSP5-cELISA. Submitted.

Invited review articles based on BARD project #US-2238-92C supported research:

Palmer, G.H. and McElwain, T.F.: Molecular basis for vaccine development against anaplasmosis and babesiosis. *Veterinary Parasitology*, 57:233-253, 1995.

Musoke, A.J., Palmer, G.H., McElwain, T.F., Nene, V., and McKeever, D.: Prospects for subunit vaccines against tick-borne diseases. *British Veterinary Journal*, 152:621-639, 1996.

Abstracts and Procedures:

Pipano, E.: Vaccines against hemoparasitic diseases in livestock. Proceedings of the Ninth International Veterinary Hemoparasite Disease Conference. Merida, Yucatan, Mexico. October 6-9, 1993.

Palmer, G.H.: Recombinant vaccines for hemoparasitic diseases in livestock. Proceedings of the Ninth International Veterinary Hemoparasite Disease Conference. Merida, Yucatan, Mexico. October 6-9, 1993.

Blouin, E.F., Bergman, D.K., Kocan, K.M., Wikel, S.K., Palmer, G.H., Ramachandra, R.N.: Antibodies produced in cattle in response to vaccination or natural infection with *Anaplasma marginale*. Parasitology and Immunoparasitology Section, Conference on Research Workers in Animal Disease; 74th Annual Meeting. 1993.

Kocan, K.M., Blouin, E.F., Edwards, W., Palmer, G.H., Ramachandra, R.N., Wikel, S.K.: Blood vs. tick challenge of splenectomized calves immunized with the erythrocytic stage of *Anaplasma marginale*. Parasitology and Immunoparasitology Section, Conference on Research Workers in Animal Disease; 74th Annual Meeting. 1993.

Palmer, G.H., McElwain, T.F., McGuire, T.C., Kappmeyer L., Davis, W.C., Stiller, D., Visser, E., Tebele, N., Ndung'u L., Pipano, E., Shkap, V., and Knowles, D.P.: Recent advances in serological diagnosis of anaplasmosis: development of the msp-5 competitive inhibition ELISA. In *Use of Applicable Biotechnological Methods for Diagnosing Haemoparasites*, G. Uilenberg, A. Permin, and J.W. Hansen, Eds. pp. 102-104. Food and Agriculture Organization of the United Nations, Rome, 1994.

Kocan, K.M., Blouin, E.F., Palmer, G.H., Eriks, I.S., and Edwards, W.L.: Strategies to interrupt the development of *Anaplasma marginale* in its tick vector: the effect of bovine derived antibodies. *Proceedings of the New York Academy of Sciences*, pp.157-165, 1996.

Brown, W.C., Shkap, V., McGuire, T.C., McElwain, T.F., Kocan, K.M., Blouin, E.F., and Palmer, G.H.: Diversity in *Anaplasma marginale* specific T helper cell specificities. Fourth Annual Meeting of the Society of Tropical Veterinary Medicine, Montpellier, France, May 1996.

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