77

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FINAL REPORT

**Development of a Recombinant DNA Derived
Vaccine Against *Babesia Bovis***

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Development of a recombinant DNA derived vaccine against

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TABLE OF CONTENTS

	Page
1. Cover Page.....	1
2. Table of Contents.....	2
3. Abstract.....	3
4. Objectives of Original Proposal.....	4
5. Research Activities and Results.....	4-8
6. Evaluation of Research With Respect to Original Objectives.....	8
7. Description of Cooperation.....	8
8. Publications.....	8-9

ABSTRACT

The observation that an immune response to blood stages of Babesia bovis protects animals challenged via the arthropod vector suggests strongly that a host immune response to appropriate blood stage antigens would be sufficient to protect cattle against natural Babesia infections. Our strategy towards the development of an improved vaccine against Babesia bovis was to identify and characterize recombinant analogues of antigens exposed on the surface of the merozoite. Using antibodies from cattle completely protected against clinical babesiosis and monoclonal antibodies to live merozoites, we characterized four immunodominant merozoite surface antigens having apparent molecular weights of 42, 55, 85, and 125 kDa in SDS-polyacrylamide gels. Bv42 was the surface antigen that elicited the highest antibody titer in immune animals. We described the molecular cloning, characterization and expression of the gene for the immunodominant Babesia bovis surface protein Bv42. The recombinant protein expressed in Escherichia coli (E. coli) was a faithful immunologic replica of Bv42 that could be used to induce antibodies against the native glycoprotein. Monospecific antisera against both the recombinant protein and native Bv42 neutralized merozoites in vitro, but immunization of cattle with recombinant Bv42 did not induce significant protection against clinical babesiosis after challenge. We have demonstrated that Mexico strain Bv42 contained variable epitopes that were not shared with several Israel strains of B. bovis, and that the Mexico and Israel strains are cross-protective, suggesting that Bv42 is not significantly involved in induction of protective immunity by premunition. Another surface protein, Bv60, appeared to be widely conserved. The gene coding for this protein was also cloned, characterized and expressed in E. coli.

OBJECTIVES OF ORIGINAL PROPOSAL

Our objectives as stated in the original proposal were:

1. Identification of Babesia bovis merozoite surface proteins recognized by immune serum and surface-reactive monoclonal antibodies.
2. Isolation of merozoite surface proteins and demonstration of their efficacy as protective immunogens.
3. Cloning of complementary DNA (cDNA) coding for relevant merozoite surface proteins.
4. High level expression of merozoite surface protein determinants.

The hypothesis was that specific surface epitopes of the merozoite stage of the protozoan are capable of inducing a protective immune response that prevents detectable parasitemia upon challenge. Cloning and expression of these antigens using recombinant DNA techniques would provide a convenient source of immunogen for testing in endemic areas.

DETAILED DESCRIPTION OF RESEARCH ACTIVITIES AND RESULTS (All project years)

Introduction

Cattle that recover from infection with an attenuated vaccine strain of Babesia bovis are protected against clinical disease induced by arthropod-borne sporozoite challenge. Infection with such an attenuated blood product has serious practical limitations, including variation in infective dose and the potential transmission of other blood borne diseases. However, the observation that an immune response to blood stages protects animals challenged via the arthropod vector suggests strongly that a host immune response to appropriate blood stage antigens would be sufficient to protect cattle against natural Babesia infections. Our strategy toward the development of an improved vaccine against Babesia bovis was to identify, isolate, and test antigens exposed on the surface of the merozoite. Merozoite surface proteins are accessible to the host immune system and play a functional role in erythrocyte invasion.

Surface reactive antibody that is relevant to protection will likely be present at high levels in immune animals to ensure antibody-merozoite binding during the parasite's short extracellular phase. In our first BARD report, using antibodies from cattle completely protected against clinical babesiosis and monoclonal antibodies to live merozoites, we described the characterization of four immunodominant merozoite surface antigens having apparent molecular weights of 42, 55, 85, and 125 kDa in SDS-polyacrylamide gels. Bv42 was the surface antigen that elicited the highest antibody titer in immune animals. This data was published (Project publication 2) and completed Objective 1 of the original proposal. We also realized that obtaining sufficient, pure protein for immunization of cattle by purification from the parasite (Objective 2) could not be readily achieved and decided to concentrate on a recombinant DNA approach directly. In our second BARD report we described the molecular cloning and expression of the gene for the immunodominant Babesia bovis surface protein Bv42. Also, that the recombinant protein expressed in Escherichia coli (E. coli) was a faithful immunologic replica of Bv42 that could be used to induce antibodies against the native glycoprotein (Objectives 3 and 4). We also demonstrated that

Bv42 contained variable epitopes that were not shared with an Israel isolate of B. bovis, whereas another surface protein, Bv60, appeared to be conserved. This information on shared and variable epitopes is described in the attached manuscript: "Strain variation of Babesia bovis merozoite surface exposed epitopes," (Project publication 7). In the final year of the proposal we focused on further characterization of these two proteins and their recombinant analogues, and their potential as immunogens.

Characterization of Bv42

The gene encoding Bv42 has now been cloned, sequenced and expressed in E. coli. Importantly, monospecific antisera against both the recombinant protein and native Bv42 neutralize merozoites in vitro (Fig. 1). The relevance to in vivo protection is supported by the observation that Bv42 is the immunodominant blood stage antigen recognized by antibodies from cattle protected against babesiosis. Antibody appears to be the most important effector mechanism in immune animals. Classic MHC-restricted cytotoxic T cells are less likely to play a role in protective immunity because Babesia parasites do not infect a host cell with the subcellular machinery or surface molecules required for antigen processing and presentation.

FIGURE 1: NEUTRALIZATION OF B. BOVIS MEROZOITES IN VITRO

	SERUM SPECIFICITY	PPE*
Exp. 1		
A	Pre-immune rabbit (n=4)	2.72 +/- 1.4
B	Rabbit anti-recombinant Bv42 (n=4)	0.33 +/- .21 @
C	Rabbit anti- <u>E. coli</u> (n=4)	1.73 +/- 1.3
Exp. 2		
D	Bovine anti-native Bv42 (n=5)	1.04 +/- 0.9 #
E	Bovine anti-ovalbumin (n=5)	3.69 +/- .47

* percentage parasitized erythrocytes (at 96 hr.) +/- standard deviation
 @ P = 0.017 when compared to A; P = .041 when compared to C
 # P > .001

However, the potential of Bv42 as an immunogen with application outside the New World is limited because the molecule is not conserved in all geographic strains. The surface exposed epitopes of Bv42 defined by monoclonal antibodies are present on merozoites from Mexico and Texas strains, and Bv42 is recognized by antibodies from cattle infected with Honduras and Brazil strains. In contrast, Australia and Israel strains express an immunologically distinct major merozoite surface protein which does not bear the surface exposed epitopes defined by our monoclonal antibodies to Bv42.

Bv42 appears to be a member of a multigene family. Hybridization of a Bv42 RNA probe to genomic DNA from a cloned Mexico strain of B. bovis reveals four major bands when the genomic DNA is digested with a restriction enzyme (EcoRI) that cuts outside the Bv42 sequence. The band pattern is identical to Southern blots probed with the gene encoding a 44 kDa merozoite surface protein designated Bo44. These genes are clearly related but not identical. EcoRI digested DNA probed with Bv42 and washed at higher stringency reveal only the two larger bands of 2.5 and 2.1 kb. Likewise, duplicate Southern blots probed with Bo44 and

washed at higher stringency reveals only the two smallest bands at 1.35 and 1.25 kb.

Sequence analysis using the FASTP program reveals that Bv42 and Bo44 (a) share a conserved 35 amino acid sequence at their carboxy-termini and (b) are related over the remaining portion of the protein which is less similar. A search of the NBRF database also reveals significant homology of Bv42 and Bo44 to a family of genes (designated BabR) previously identified in Australia strains of B. bovis. The BabR gene family, whose function has not previously been described, is notable because of its marked polymorphism among Australia strains. Importantly, the 3' nucleotide sequence encoding the conserved carboxy-terminus of Bv42 and Bo44 is also conserved in two BabR genes, including the only BabR gene (BabR 0.8) that is completely sequenced. However, this sequence conservation does not translate into amino acid homology because of a deletion in the Australia strain which leads to a change in reading frame. A subsequent insertion changes the reading frame again so that all three genes have termination signals at identical sites.

Bv42 RNA probes from the conserved 3' sequence and the variable 5' region of Bv42 have been hybridized to restriction enzyme digested genomic DNA from the Australia strains which express an antigenic variant of the major merozoite surface protein. The probes reveal a unique pattern of bands and confirm that the Australia strains contain homologous sequences. This data, together with the sequence and immunological analyses, suggests that these strains express related, but immunologically distinct surface glycoproteins. These potentially protective surface antigens are likely encoded by polymorphic members of the Bv42/BabR gene family.

In further experiments it appeared that Bv42 may be present on the surface of B. bovis-infected erythrocytes. Bovine erythrocytes infected with B. bovis parasites from Mexico and the United States (Texas) were assayed for the presence of new epitopes on the erythrocyte surface. Indirect immunofluorescence assays indicated that extensive antigenic cross-reactivity of the infected erythrocyte membranes exists between these isolates. Despite this, isolate-specific surface epitopes were detected by means of a whole-cell antigen capture assay; these epitopes were shown to be limited to the infected cells. Repertoires of parasite-synthesized antigens which varied in antigenic reactivity were identified on each isolate using a surface-specific immunoprecipitation technique to analyze infected erythrocytes that had been metabolically labeled with L-[³⁵S]-methionine. The antigen repertoire of the United States isolate included six polypeptides carrying isolate-specific epitopes whereas a cloned Mexico line repertoire included three such antigens. One cross-reactive antigen of Mr 42,000 was identified that carries one or more epitopes common to both isolates. Further control experiments are being performed to confirm the identity of this 42,000 molecule and its location on the erythrocyte surface. These data are described in the attached (draft) manuscript: "Isolate-specific, parasite-derived antigens of the Babesia bovis-infected erythrocyte surface," (Project publication 6).

To test whether the lack of epitope conservation of Bv42 among isolates affects their ability to induce heterologous protection, an immunization trial using live biologically cloned Israel and Mexico strains of B. bovis was conducted. Groups of 10 cattle each were preimmunized with either Israel or Mexico strain parasitized erythrocytes, and 21 days later received a homologous challenge to boost immunity. The groups were divided equally and were challenged with either homologous or heterologous strain parasitized erythrocytes. Two unimmunized control cattle were also challenged to verify the viability of the inoculum. There were no differences in the ability of either the Mexico or Israel strains to protect against homologous and heterologous challenge, while

all control calves developed fever and parasitemia. Interestingly, serum antibodies from calves prior to final challenge did not recognize Bv42 in the heterologous strain in either western immunoblots or immunoprecipitations. Thus, significant heterologous protection occurs in the absence of detectable antibody response to Bv42. A manuscript describing these results is in preparation.

Although an immune response against Bv42 may not be necessary for protection induced by premunition, its surface accessibility, immunogenicity, and conservation in some regions of the world suggest that Bv42 is an important potential immunogen against some isolates of B. bovis. To test this hypothesis, an immunization trial using E. coli produced recombinant Bv42 has been completed. Recombinant Bv42 (rBv42) was purified by immunoaffinity chromatography. The purity and specificity of isolated antigen was confirmed by silver-stained SDS-PAGE gels and western immunoblots using mAb against Bv42. No E. coli proteins could be detected in the antigen in ELISA assays using serum from cattle that had been hyperimmunized with E. coli containing an irrelevant recombinant protein from A. marginale. A group of 10 cattle was immunized four times with purified rBv42 in saponin adjuvant, the first three immunizations with 50 ug each, and the last with 100 ug each. Five control cattle were immunized with saponin alone. All immunized calves responded with serum antibodies against Bv42 as detected in fixed IFA assays. Titers ranged from $1-4 \times 10^3$ in indirect IFA. Two weeks after the final immunization, all cattle were challenged with 2×10^3 Texas strain infected erythrocytes freshly collected from a splenectomized donor calf. (Bv42 from Texas strain B. bovis appears identical to Bv42 from the Mexico strain.) The results are presented in the Table I. No significant protection against clinical babesiosis was induced by immunization with purified rBv42.

TABLE I
rBv42 Immunization Trial

Immunogen	n	% Decrease in PCV	Days Body Temperature Above 103°F	Days Detectable Parasitemia
rBv42	10	58+/-16*	8.4+/-2.2	3.3+/-1.3
saponin	5	64+/-8.3	8.0+/-1.9	2.8+/-1.8

The lack of conservation of Bv42, lack of recognition by serum antibodies from cattle protected against heterologous challenge, and inability of purified rBv42 to induce protective immunity all indicate that its potential as an immunogen is limited. Thus, we have turned our attention to Bv60, an apparently highly conserved polypeptide with surface exposed epitopes that also appears to be a constituent of the apical complex.

Characterization of Bv60

A clone expressing a surface exposed, conserved epitope of the 60 kD merozoite polypeptide was identified in a cDNA library constructed from a cloned Mexico strain of Babesia bovis. Sequencing of the 1.9 kb insert (pBv60) revealed an open reading frame encoding a 65 kD polypeptide with a signal peptide and a tandemly repeated region. Monoclonal antibody 23/56.156, which binds a surface exposed epitope on the native polypeptide, specifically immunoprecipitated [35 S]-methionine labeled polypeptides ranging from 60-30 kD from pBv60 directed transcription and translation. Antibodies raised in rabbits against recombinant

polypeptide reacted with the live merozoite surface in a polar immunofluorescence pattern, immunoprecipitated the native 60 kD polypeptide, and were used to deplete the polypeptide by adsorption from a preparation of native [³⁵S]-methionine labeled merozoite antigen. Restriction enzyme analysis indicated a single gene copy and the absence of introns. Hybridization demonstrated the presence of the gene in Mexico, Australia "L", and Texas strains of B. bovis, but not in Babesia bigemina. A slightly different hybridization pattern was present in uncloned Australia "L" B. bovis, indicating minor sequence diversity in the Bv60 gene among isolates. Cloning and structural analysis of pBv60 provides a source of defined antigen for determining the role of conserved merozoite surface epitopes in protective immunity against babesiosis. These data are described in the attached manuscript "Characterization of the gene encoding a 60-kd Babesia bovis merozoite protein with conserved and surface exposed epitopes," (Project publication 5).

EVALUATION OF THE RESEARCH WITH RESPECT TO ORIGINAL OBJECTIVES

In this project we have achieved objectives 1, 3 and 4 "Identification of B. bovis merozoite surface proteins recognized by immune serum and surface reactive monoclonal antibodies", "Cloning of cDNA coding for relevant merozoite surface proteins", and "High level expression of merozoite surface protein determinants". We have been unable to demonstrate protective immunity using native or recombinant merozoite surface proteins (objective 2), and have raised as a significant concern the antigenic variation in Bv42 for potential world-wide use in a vaccine. We are now concentrating on Bv60, a conserved merozoite surface protein that also appears to be part of the apical complex, and will test its efficacy as an immunogen. Additional apical complex antigens are being identified and characterized to test the hypothesis that blocking the second phase of merozoite entry into the erythrocytes, mediated at least in part by apical complex polypeptides, will neutralize infectivity.

DESCRIPTION OF COOPERATION

Collaboration between investigators on this proposal has enabled significant comparisons to be made between Israel and other isolates of B. bovis regarding conservation of surface proteins with potential for use in vaccines (see manuscript "Strain variation of B. bovis merozoite surface exposed epitopes" (Project publication 7) with authors from the USA and Israel.

LIST OF PROJECT PUBLICATIONS

1. Goff, W.L., Davis, W.C., Palmer, G.H., McElwain, T.F., Johnson, W.C., Bailey, J.F. and McGuire, T.C. (1988) Identification of Babesia bovis merozoites surface antigens by using immune sera and monoclonal antibodies. Infect. Immun. 56, 2363-2368.
2. Hines, S.A., McElwain, T.F., Buening, G.B. and Palmer, G.H. (1989) Molecular characterization of Babesia bovis merozoite surface proteins bearing epitopes immunodominant in protected cattle. Mol. Biochem. Parasitol 37, 1-10.

3. Goff W.L., McElwain T.F., Palmer G.H., McGuire T.C. (1989) Development of ELISA diagnostic tests for Babesia infections using highly immunogenic, species-specific, and strain-common surface glycoproteins. Proceedings of the Eighth National Veterinary Hemoparasite Disease Conference, St. Louis, Missouri, 353-376.
4. Hines S.A., McElwain T.F., Buening G.M., Goff W.L., Davis W.C., Reduker D.W., Perryman L.E., McGuire T.C., Palmer G.H. (1989) Identification and characterization of Babesia bovis merozoite surface glycoproteins recognized by cattle protected against babesiosis. Proceedings of the Eighth National Veterinary Hemoparasite Disease Conference, St. Louis, Missouri, 589-595.
5. Suarez, C.E., Palmer, G.H., Jasmer, D.P., Hines, S.A., Perryman, L.E., McElwain, T.F. (1991) Characterization of the gene encoding a 60-kd Babesia bovis merozoite protein with conserved and surface exposed epitopes. Mol. Biochem. Parasit. 46, 45-52.
6. Allred, D.R., Hines, S.A., Ahrens, K.P. Isolate-specific, parasite-derived antigens of the Babesia bovis-infected erythrocyte surface. (in preparation).
7. Palmer, G.H., McElwain, T.F., Perryman, L.E., Davis, W.C., Reduker, D.R., Jasmer, D.P., Shkap, V., Pipano, E., Goff, W.L., McGuire, T.C. Strain variation of Babesia bovis merozoite surface exposed epitopes (in press).

(Manuscripts 1-4 were submitted with previous reports, 5-7 are attached to this).