BARD

FINAL REPORT

PROJECT NO. US-2496-94

**Protection of Cattle against Babesiosis: Immunization
against *Babesia bovis* with and Optimized RAP-1/Apical
Complex Construct**

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X Yes

No

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Subunit vaccine, rhoptries, T cell epitopes, B cell epitopes, spherical bodies.

Abbreviations: aa, amino acids; HSP, heat shock protein; Ig, immunoglobulin; IL, interleukin; kb, kilobase; kD, kilodaltons; mAb, monoclonal antibody; MHC, major histocompatibility complex; *orf*, open reading frame; PBMC, peripheral blood mononuclear cells; RAP-1, rhoptry associated protein-1; rRAP-1, recombinant rhoptry associated protein-1; SBP, spherical body protein

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	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	1	12		13
Submitted, in review, in preparation		1		1
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Abstracts		7	2	9
Not refereed (proceedings, reports, etc.)			1	0

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				0
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Licensed				0

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	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	1	2	3	6
Sabbaticals				0
Postdoctorates				0

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

The nature of cooperation between U.S. and Israeli teams is best described both as synergistic and supportive. In vivo and in vitro experiments with the infectious agent have been primarily the responsibility of Israeli scientists, who have particular expertise and unique strains for this research. Molecular biological aspects of the project were the responsibility of U.S. scientists in whose laboratories unique expertise is located. Immunological aspects of the research were divided between U.S. and Israeli teams, since both possess capabilities for these experiments.

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Abstract

Previous research and current efforts at control of babesiosis fall short of meeting the needs of countries where the disease is endemic, such as Israel, as well as the needs of exporting countries and countries bordering on endemic areas, such as the U.S. Our long-term goal is to develop improved methods of immunization against bovine babesiosis based on an understanding of the molecular mechanisms of immune protection and parasite targets of a protective immune response. In our previous BARD project, we established the basis for focusing on rhoptry antigens as components of a subunit vaccine against bovine babesiosis, and for additional research to better characterize rhoptry associated protein-1 (RAP-1) as a target of protective immunity. In this continuation BARD project, our objectives were to [1] optimize the immune response against RAP-1, and [2] identify additional rhoptry candidate vaccine antigens.

The entire locus encoding *B. bovis* RAP-1 was sequenced, and the *rap-1* open reading frame compared among several strains. Unlike *B. bigemina*, in which multiple gene copies with variant domains encode RAP-1, the *B. bovis* RAP-1 locus contains only two identical genes which are conserved among strains. Through testing of multiple truncated constructs of rRAP-1, one or more immunodominant T cell epitopes were mapped to the amino terminal half of RAP-1. At least one linear and one conformational B cell epitope have been demonstrated in the same amino terminal construct, which in *B. bigemina* RAP-1 also contains an epitope recognized by neutralizing antibody. The amino terminal half of the molecule represents the most highly conserved part of the gene family and contains motifs conserved broadly among the apicomplexa. In contrast, the carboxy terminal half of *B. bovis* RAP-1 is less well conserved and contains multiple repeats encoding a linear B cell epitope potentially capable of inducing an ineffective, T cell independent, type 2 immune response. Therefore, we are testing an amino terminal fragment of RAP-1 (RAP-1N) in an immunization trial in cattle. Cattle have been immunized with RAP-1N or control antigen, and IL-12 with Ribi adjuvant. Evaluation of the immune response is ongoing, and challenge with virulent *B. bovis* will occur in the near future.

While no new rhoptry antigens were identified, our studies did identify and characterize a new spherical body antigen (SBP3), and several heat shock proteins (HSP's). The SBP3 and HSP21 antigens stimulate T cells from immune cattle and are considered new vaccine candidates worthy of further testing.

Overall, we conclude that a single RAP-1 vaccine construct representing the conserved amino terminal region of the molecule should be sufficient for immunization against all strains of *B. bovis*. While results of the ongoing immunization trial will direct our next research steps, results at this time are consistent with our long term goal of designing a subunit vaccine which contains only the epitopes relevant to induction of protective immunity. Parallel studies are defining the mechanisms of protective immunity.

Apicomplexan protozoa, including babesiosis and malaria, cause persistent diseases for which control is inadequate. The apical organelles are defining features of these complex protozoa, and have been conserved through the evolutionary process. Past and current BARD projects on babesiosis have established the validity and potential of exploiting these conserved organelles in developing improved control methods applicable to all apicomplexan diseases.

Achievements

Significance of achievements and potential for application

Apicomplexan protozoa cause a large number of acute and persistent diseases of livestock and humans, including babesiosis, coccidiosis, cryptosporidiosis, theileriosis, and malaria. One defining feature of these protozoa is the presence of an apical complex that consists of rhoptries, micronemes, dense granules, and in the babesias, spherical bodies. The apical complex is unique to this phylum, and its organellar structure has been strictly conserved through evolution, similar to subcellular organelles of eukaryotes. This conservation is the result of necessary organellar functions, such as host cell invasion, that if lost would be lethal. We reasoned that this conservation of function would result in genetic stability reflected in conservation of sequence, either in an entire apical complex molecule or more likely in smaller motifs. If this was true, the broad conservation of structure, function, and genetic sequence might be exploited to design widely applicable methods for improved disease control.

We first tested this hypothesis using the babesial rhoptry associated protein-1 (RAP-1) sequence. Consistent with the logic presented above, short stretches of identical or closely related sequences broadly conserved among the apicomplexa were identified. An understanding of the relationship of these conserved sequences to protective epitopes would have broad application in design of vaccines against protozoal agents. Our results in this project are an important step in understanding that relationship. We have found that the most conserved region of the RAP-1 family in the babesias (the amino terminal 300 amino acids) contains sequence encoding both B cell and T cell epitopes. In comparative studies with *B. bigemina*, a conformational epitope in the amino terminal half of RAP-1 bound by neutralizing antibodies includes sequence motifs that are strictly and broadly conserved in the babesias. Epitopes recognized by T cells also are present in the conserved region, but do not map exactly to the same sequence motifs that are very widely conserved among the erythroparasitic apicomplexan protozoa. Thus, while one cannot directly infer from our results that a conserved motif which encodes a T cell epitope in one molecule would do the same in another related molecule, one can postulate that regions containing motifs shared

among related protozoa are likely to encode immunologically important B cell conformational epitopes. For this reason the results from this project could have broad implications for enhanced control of this very important group of pathogens. Application of these results is likely medium term (5-10 years).

Impact/Economic Benefits

Bovine babesiosis caused by *Babesia bovis* occurs throughout tropical and subtropical areas, including Israel, Mexico and Central America, and the Caribbean basin.^{1,2} Approximately three-quarters of the world's domestic cattle population resides in tropical to sub-tropical regions where they are at risk for babesiosis. It remains a significant deterrent to livestock production in endemic areas, and is a threat to livestock in regions bordering endemic areas (including the United States) where climatic conditions are appropriate for the survival of vector *Boophilus spp.* ticks.^{1,2} Current control relies on the use of live vaccines in combination with acaricides for tick control, and chemotherapeutic agents for treatment of field or vaccine induced clinical disease.³ These methods have both biological and technical drawbacks.^{4,5} Thus, the development of an improved method of vaccination would have immediate and direct impact, and would allow economic growth through enhanced livestock production and use of livestock as draught animals.

Cooperation

Drs. Shkap, Pipano, McElwain, and Palmer met in Beit Dagan at the Kimron Veterinary Institute in August, 1996. All four scientists also attended the World Association of Veterinary Laboratory Diagnosticians Symposium in Jerusalem. The meeting afforded an opportunity for a round table discussion of current data, research problems, and experimental design for the next phase of the project. Israeli scientists presented and discussed their experimental results on the responses of cattle immunized with rRAP-1, and the development of a neutralizing mAb (E10.1). U.S. scientists shared their results on sequence variation of the *rap-1* locus and characterization of T cell clones against RAP-1. In 1997, Dr. Pipano attended the First International Veterinary Vaccines and Diagnostics Conference in Madison, Wisconsin.

Following the conference he visited Washington State University, providing an additional opportunity to discuss recent progress, problems, and plans for the upcoming year.

Drs. Shkap and Brown met in Onderstepoort, South Africa in February, 1998 as part of a USAID funded project on heartwater. Both scientists also met at the 5th International Veterinary Immunology Society meeting in Ludhiana, India, Nov. 8-13, 1998. These meetings afforded an opportunity to discuss progress on T cell responses to RAP-1, examine problems, and design the immunization trial to be conducted during the final phase of the project.

Drs. Shkap and Brown both attended the 5th Biennial Society for Tropical Veterinary Medicine meeting in Key West, Florida June 12-16, 1999. Dr. Shkap conducted a symposium in which Dr. Brown presented a talk. Following the STVM meeting, Dr. Shkap visited Washington State University (June 17-19, 1999) for a brief scientific exchange. Progress in the RAP-1N immunization trial was discussed and results to date for the entire project analyzed.

Success of a project in which comparison of strain-specific sequences and strain cross-reactivity is proposed relies heavily on cooperation of collaborating investigators. Collaboration was impeded somewhat in this project as a result of our inability to obtain USDA permission (first requested in March, 1977) for importation of *B. bovis* organisms, antigen, antibodies, or DNA from Israel into the United States. Fortunately, significant progress was still possible through the cooperative efforts of collaborating scientists and alternate approaches.

The nature of cooperation between U.S. and Israeli teams is best described both as synergistic and supportive. In vivo and in vitro experiments with the infectious agent have been primarily the responsibility of Israeli scientists, who have particular expertise and unique strains for this research. Molecular biological aspects of the project were the responsibility of U.S. scientists in whose laboratories unique expertise is located. Immunological aspects of the research were divided between U.S. and Israeli teams, since both possess capabilities for these experiments.

Background, scientific and agricultural relevance of the project

Our approach toward the development of an improved method of vaccination against babesiosis caused by *B. bovis* is to define the antigens necessary for the induction of protective immunity and to deliver them as recombinant DNA derived or chemically synthesized products in a subunit vaccine. Apical organelles, including rhoptries, micronemes, dense granules, and, in babesial organisms, the spherical bodies, are subcellular structures unique to apicomplexan parasites.^{6,7} While we do not understand all the functions of these organelles in the apicomplexa, their conservation through evolution emphasizes the critical role they must fulfill for survival of the parasite. Rhoptry and microneme antigens mediate several steps of erythrocyte invasion by merozoites, including attachment, receptor-ligand binding on the erythrocyte surface and invagination of the host cell membrane.⁶⁻¹⁹ These polypeptides are antigenically conserved, unlike many of the major babesial surface proteins, and in other apicomplexan parasites, including *Plasmodium spp.*, are able to induce at least a partially protective immune response.⁶⁻¹⁹

The rhoptry associated protein-1 (RAP-1) is a neutralization sensitive, rhoptry and merozoite surface-associated babesial antigen.^{6,20-25} It is capable of inducing partial, but significant immune protection in cattle.^{26,27} While its function is unknown, RAP-1 contains short peptide motifs that are broadly conserved not only among babesial species, but also in the related erythroparasitic protozoan *Plasmodium* species, suggesting conservation of function dependent upon these sequences.²⁸ Despite this remarkable conservation of oligopeptides, nucleotide sequence heterogeneity is present between RAP-1 loci within a parasite clone and among different strains.²⁹⁻³¹ In *B. bigemina*, RAP-1 sequence heterogeneity results in multiple gene copies within a population of parasites that contain unique combinations of variable domains at the amino and carboxy termini.³² Similar data characterizing *B. bovis rap-1* genes was not available at the initiation of this project. An understanding of the relationship of protective B- and T-cell epitopes, conserved motifs, and sequence heterogeneity would provide a molecular basis for optimization of the immune response against RAP-1.

Vaccine optimization may also require the inclusion of additional antigens. The logic underlying selection of rhoptry-associated polypeptides as candidate vaccine

antigens is strong.^{6,19,33,34} It is becoming increasingly clear that many of the broadly conserved molecules that are necessary for invasion of the host cell are derived from these organelles unique to the apicomplexa.^{6,34} In addition, we recently completed an immunization trial with collaborators in Brazil using gradient purified *B. bigemina* rhoptries, and found highly significant, and nearly complete protection against field challenge with infected ticks.³⁵ Consequently, inclusion of multiple rhoptry antigens is an important parallel approach to vaccine development.

Based on these observations, we hypothesize that the immune response induced by RAP-1 can be optimized by immunization with a construct containing only conserved RAP-1 B and T cell epitopes relevant to immune protection, either with or without additional rhoptry antigens. The objectives for optimizing the immune response against RAP-1 were to:

- R1. Define the sequence heterogeneity of RAP-1 gene copies within a cloned strain and among geographically defined strains.
- R2. Define the B cell and T cell epitopes of RAP-1 and the relationship of these epitopes to areas of sequence heterogeneity and conservation.
- R3. Based on the results from the above two objectives, design and test an optimized construct for inducing immune protection with RAP-1.

Objectives for selection of additional rhoptry candidate vaccine antigens were to:

- A1. Define rhoptry candidate vaccine antigens based on the presence of conserved, neutralization sensitive B cell epitopes, and conserved T cell epitopes.
- A2. Test additional candidate vaccine antigen(s) alone and in combination with the optimized RAP-1 construct determined in RAP-1 objectives R2 and R3 above.

Methodology, Research Results, Discussion

Research Progress, Objectives R1, R2, and R3:

[1] *Characterization of rap-1 locus (Objective R1)* - The genetic organization of the rap-1 locus in most babesial species appears to be highly complex. For example, in work related to this project and partially supported by BARD it was found that *B. bigemina* has at least four different *rap-1a* genes which encode unique proteins distinguished by

polymorphism in the amino and carboxy terminal ends.³² Both neutralization sensitive B cell epitopes and T cell epitopes map to the polymorphic regions (see below).^{41,43} Furthermore, the number and orientation of *rap-1a* genes encoding the polymorphic regions varies among *B. bigemina* strains. This complex multigene *rap-1a* organization occurs in most examined babesial species, including *B. ovis* and *B. divergens*. The complexity of the *rap-1* locus in *B. bigemina* is increased by the presence of two additional divergent groups of genes related to *rap-1a*, termed *rap-1b* and *rap-1c*.³⁶ The *rap1b* and *rap1c* genes are cis-linked to the *rap-1* locus and were only identified after direct sequencing of the entire *B. bigemina rap-1* locus. The expression of multiple *rap-1a* genes containing polymorphic regions and additional genes in the *rap-1* family, such as *rap-1b* and *rap-1c*, results in the production of diverse proteins which may affect the efficacy of individual gene products as immunogens.

Preliminary evidence suggests that the *B. bovis rap-1* locus is much simpler, consisting of two gene copies with a small amount of heterogeneity both between gene copies and within the population. However, to establish this with certainty and determine whether *rap-1b* and *rap-1c* equivalents are present in the *B. bovis* locus, the entire genomic fragment containing these genes was cloned and sequenced. Open reading frames from multiple *rap-1* loci in different strains were also sequenced to gain some appreciation of overall *rap-1* population heterogeneity. Interestingly, in contrast to *B. bigemina*, *B. divergens*, and *B. ovis*, the *B. bovis rap-1* locus in the cloned Mexico strain Mo7 contains only two tandemly arranged identical gene copies. These copies are separated by a 1 kb intergenic region that has sequence similarity to regions known to regulate gene expression (see below). No additional *rap-1* gene copies equivalent to *rap-1b* or *rap-1c* are present in this 11kb fragment. However, three additional open reading frames were identified flanking the *rap-1* genes. An *orf* located 2.6 kb downstream of *rap-1a* gene copy 2 was identified that is capable of encoding an approximately 44.6 kD protein with significant sequence homology to the DNA-J heat shock protein family. It is designated gene *hsp-40*. A second *orf* is located between *rap-1a* gene copy 2 and *hsp-40*. It encodes an acidic 35.8 kD protein highly homologous to a well conserved but uncharacterized *orf* previously identified in an intergenic region of nematodes, yeast, *Plasmodium falciparum*, and *Cryptosporidium parvum*, and designated *ig*. Finally,

upstream of *rap-1* gene copy 1 is an *orf* encoding a product highly similar to the enzyme phosphomannomutase from humans, yeast, and other species. A least two of these three genes contain introns, and all three are expressed, as verified using a combination of PCR and RT-PCR. Thus, the boundaries of the *B. bovis rap-1* locus are determined by these flanking genes, providing further evidence that the locus, unlike other babesial species, contains only two identical genes in this cloned strain. Further, the data indicate that *rap-1* is located in a transcriptionally active segment of the genome.

To more precisely compare the *rap-1* sequence among different strains of *B. bovis*, clones of both *rap-1* genes were PCR amplified and cloned from a second Mexico, two Argentina, one Uruguay, and one Brazil strain. There is a very limited degree of sequence variation, consisting of a pattern of four amino acid substitutions in both genes of the Brazil and Uruguay strains at identical positions. Both *rap-1* genes from the Texas strain T2B and gene 2 from the Argentina strain R1A are identical to the *rap-1* copies from the cloned Mexico strain Mo7. The most extensive variation between gene 1 and gene 2 from the same strain was observed for the attenuated Argentina strain R1A (9 substitutions). In the second Argentina strain, Uruguay, and Brazil strains, genes 1 and 2 differed by only two amino acids. Notably, nearly all changes occurred outside of the first 300 amino acids, the region of *rap-1* most highly conserved among babesial species. These results indicate that the heterogeneity previously found with Southern blot analysis of the same strains occurs outside the *orf*. These results are published in Suarez et al.³⁶

Collectively, the results suggest that very limited allelic and interstrain sequence variation is present in the *orf* of *rap-1*, particularly in the first 300 amino acids. As previously indicated, this is in striking contrast to other babesial species. However, the lack of polymorphism is encouraging for vaccine development, as a single *rap-1* gene copy may be sufficient for optimum immune protection. In addition, these results support the testing of a truncated RAP-1 construct including only the first approximately 300 amino acids, which is the most conserved part of the molecule, for its ability to induce immune protection.

[2] *Characterization of rap-1 intergenic region (Objective R1)* - With the long term goal of determining the significance of previously described broadly conserved RAP-1 peptide motifs in immune protection by transfection of parasites with mutated *rap-1*

genes, the regulatory potential of the intergenic region was investigated. First, the 1 kb non-coding region separating the two *rap-1a* genes of *B. bovis* was obtained by PCR amplification and sequenced. The region is AT-rich (67.5%) and contains multiple areas of similarity to known eukaryotic regulatory elements. Two separate intergenic regions of *B. bigemina* were also obtained and analyzed in a similar fashion. These regions contain 780 and 1,534 base pairs, and are less AT rich than in *B. bovis*. No long *orfs* were identified in any of these regulatory regions.

To identify genus common regulatory motifs in the intergenic regions, we performed multiple and pairwise alignments among the sequences of *rap-1* intergenic regions from *B. bovis*, *B. bigemina*, *B. canis*, and *B. ovis*. Overall, sequence conservation is relatively low. However at least three nucleotide boxes are well conserved among species. These areas are designated the -59, -36, and mRNA boxes. The mRNA box represents a 17 base pair sequence that is conserved in the untranslated 5' region of *B. bovis* and *B. bigemina rap-1* mRNA. The -59 box is located 59 nucleotides 5' from a possible *B. bovis rap-1* transcription start site identified by mRNA sequencing, and is perfectly conserved among all the babesial *rap-1* intergenic regions analyzed. The -36 box is a 9 base pair sequence located 36 base pairs 5' from the putative transcription start site. Seven of the 9 base pairs are perfectly conserved among the *B. bigemina* intergenic region 2, *B. bovis*, and *B. ovis* intergenic regions. The significance of these conserved regions is unknown at this time, but their strong conservation suggests a role in gene regulation.

Based on these observations, the intergenic region was subcloned in both directions into the vector pCAT to determine whether the region could promote transcription of chloramphenicol acetyltransferase in a cell free in vitro *B. bovis* nuclear transcription system developed for this purpose. Production of a CAT transcript was significantly enhanced in the construct containing the intergenic region in a 5'→3' orientation when compared to promoterless pCAT, pCAT positive and no DNA controls. The data indicate that the intergenic region is capable of promotion in an in vitro system, and support the possibility of transfection analysis as a means of understanding the significance of broadly conserved RAP-1 peptide motifs in immune protection. These results are published in Suarez et al.³⁷

[3] *Defining RAP-1 immunodominant, cross reactive T helper cell epitopes (Objective R2)* - In a continuation of work begun as part of the previous BARD grant, T lymphocyte lines were derived from animals immune to challenge with *B. bovis* by stimulation of peripheral blood mononuclear cells (PBMC) with either crude membrane antigen or recombinant RAP-1 (rRAP-1). Membrane antigen stimulated lines proliferated strongly against rRAP-1, demonstrating the immunodominant nature of this protein. rRAP-1 specific lines proliferated against *B. bovis* but not *B. bigemina* merozoites, indicating that the dominant T cell epitopes were not encoded by species-conserved peptide motifs. T cell clones derived from the lines were CD4+ and recognized all strains of *B. bovis* tested, including Mexico, Texas, Australia, and Israel strains. T cell clones strongly expressed gamma interferon, but expressed little or no IL-2, IL-4, or IL-10. The data indicate that RAP-1 contains species specific, strain common, immunodominant T-cell epitopes that can induce T cells with a type 1 phenotype. Results of these experiments are published in Brown et al.³⁸

Comparative studies with *B. bigemina* demonstrated that the T lymphocyte response in cattle immunized with native RAP-1 included a vigorous anamnestic response of CD4 T cells with a type 1 phenotype. The T cells responded to both native and recombinant RAP-1, indicating the vaccine potential of recombinant protein or epitopes derived therefrom to evoke specific recall responses after natural infection. Cells derived from draining lymph nodes of immunized cattle, when stimulated ex vivo, also expressed a type 1 cytokine profile with relatively high levels of IL-2 and gamma interferon, and low levels of IL-4 and IL-10. Finally, *B. bigemina* RAP-1 specific CD4+ T cell clones can provide help to autologous B cells and can enhance production of both IgG1 and IgG2 immunoglobulin isotypes. Importantly, cattle immunized with native *B. bigemina* RAP-1 were protected against challenge, corroborating earlier work which initially established the feasibility of using RAP-1 to induce protective immunity. The above observations are published in Rodriguez et al, Ruef et al, Brown et al, and Brown et al.³⁹⁻⁴¹

Cloned T cells specific for *B. bigemina* RAP-1 have variable responses to different *B. bigemina* strains. Since *B. bigemina* RAP-1 contains variable N and C terminal domains, it was possible that at least one T cell epitope was contained in a

variable region, or that antigenic variation resulted in a lack of strain cross-reactivity. To address this question, the epitopes recognized by two T cell clones were mapped. One epitope was contained within one of the three different C terminal domains identified in different *rap-1* gene copies. However, no sequence differences in the domain or epitope were found in genomic DNA among seven strains of *B. bigemina*. Thus, expression of an alternate C terminal domain is the most likely explanation for lack of strain cross-reactivity of this T cell clone. Importantly, the second T cell epitope was defined in a constant region within the amino terminal half of RAP-1 between amino acids 159-187. Again, the sequence defining this epitope was invariant among seven different strains of *B. bigemina*. These results, which are published in Hotzel et al and Brown et al,^{41,43} provide further support for testing the amino terminal half of RAP-1 as an enhanced immunogen.

To map the epitopes recognized by *B. bovis* RAP-1 T cell clones, two subclones of rRAP-1 expressing amino acids (aa) 6-317 and 316-565 as maltose binding fusion proteins were prepared in a pMAL-2C vector by PCR amplification and directed subcloning. The amino terminal fragment, designated RAP-1NT, was able to stimulate peripheral blood mononuclear cells from *B. bovis* immune cattle as well as whole RAP-1. In addition, RAP-1NT was able to induce proliferation of T cell lines derived by stimulation with crude *B. bovis* antigen and T cell clones specific to RAP-1. To further define the epitope(s) recognized in RAP-1NT, an additional truncated RAP-1 construct, RAP-1A (aa6-87) was prepared and expressed as a maltose binding fusion protein. RAP-1A was not able to stimulate T cell lines and clones that respond to RAP-1NT, suggesting that aa6-87 do not encode a T cell epitope recognized by these lines and clones. Collectively, these results and previous work demonstrating that RAP-1 is immunodominant for T cells of unrelated MHC haplotype indicate that aa88-317 of RAP-1 contain immunodominant T cell epitopes, and that these epitopes are immunogenic for unrelated outbred cattle (unpublished data)

[4] *Defining RAP-1 B cell epitopes (Objective R2)* – In previous work, several mAbs against *B. bovis* RAP-1 were derived and characterized. The epitopes bound by these mAbs include a linear epitope in the C terminal repeat region (aa386-396), a linear epitope in the amino terminal half of RAP-1 (aa254-265), and a conformational

epitope in the amino terminal half of RAP-1 (aa145-218). In our previous BARD project, an additional mAb designated E10.1 was found to be capable of neutralizing *B. bovis* growth in vitro. We initially planned to map the epitope bound by mAb E10.1, but were never able to obtain USDA permission for importing this mAb into the US. Therefore, we have relied on comparative data from *B. bigemina* to focus attention on the region(s) of RAP-1 encoding neutralization sensitive epitopes.

Several neutralizing mAbs against *B. bigemina* RAP-1 were available. A series of truncated constructs of rRAP-1 were prepared and tested for reactivity with these mAbs. In addition, the dependence of mAb binding on intact disulfide bonds was tested, since strictly conserved cysteine residues are a defining feature of the RAP-1 gene family. Four B cell epitopes, including two recognized by neutralizing mAbs previously shown to bind the same epitope, were contained within the amino terminal half of RAP-1. All of these B cell epitopes were conformationally dependent and required the presence of intact disulfide bonds. The conformational epitope recognized by neutralizing mAbs occurred between aa56-130. This region includes the strictly conserved 14mer sequence and cysteines which define the gene family and which are present in *B. bovis*. An additional conformational B cell epitope was present in the midsection of the protein. Only one mAb bound an epitope in the C terminal region. These data, which are published in Hotzel et al,⁴³ further support testing of a construct including only the amino terminal half of the molecule for enhanced ability to induce protective immunity.

[5] *Ability of rRAP-1 to induce cross-reactive neutralizing antibodies and T lymphocyte responses (Objectives R1 and R2)* - Calves were immunized with whole rRAP1 from Mexico strain *B. bovis* in Freund's adjuvant. All calves produced antibodies that were able to significantly inhibit growth of heterologous Israel strain *B. bovis* in culture from 14% - 60% when compared to pre-inoculation sera from the same animals. Additionally, peripheral blood mononuclear cells from all animals significantly responded to stimulation with Mexico strain rRAP-1 (homologous response) and crude membrane antigen containing native RAP-1 from Israel strain *B. bovis* (heterologous response). These results are compatible with the lack of sequence variation found among strains of *B. bovis*, and indicate that cattle respond to strain cross-reactive, neutralization

sensitive B lymphocyte and immunodominant T lymphocyte epitopes of rRAP-1 that are also present in native RAP-1 (unpublished data).

[6] Immunization of cattle with an optimized RAP-1 construct (Objective R3) - Collectively, the results of *B. bovis* and *B. bigemina* sequence analysis and T and B cell epitope mapping suggest that the amino terminal 317 amino acids may be the best portion of RAP-1 for inducing protective immunity. This fragment of the molecule is exclusive of the seven 23 aa peptide repeats (aa317-477) that comprise a large portion of the carboxy terminal half of the 565 aa protein. The repeats are immunodominant, linear B cell epitopes, and theoretically would be capable of inducing a T cell independent, type 2, non-protective immune response. Therefore, we have begun an immunization trial in cattle using rRAP-1N antigen (aa1-317) expressed as a fusion protein. Four immunizations with antigen, recombinant human IL-12 and Ribi adjuvant have been completed. We currently are determining antibody responses. PBMC responses were first positive after the third antigen inoculation and remain positive to date (Table 1, Appendix A). The plan is to measure antigen-specific proliferation and production of IL-2, IFN-gamma, IL-4 and IL-10 in the draining lymph node cells removed 3 days following a final antigen boost. Immunized calves will be challenged with a virulent Texas isolate and the level of immune protection compared with 5 control calves inoculated with RIBI adjuvant and IL-12 only.

Research Progress, Objectives A1 and A2:

[1] Identification of additional rhoptry antigens (Objective A1) – Rhoptries from *B. bigemina* are relatively easily isolated using gradient fractionation techniques. Similar techniques have been used to isolate *B. bovis* spherical bodies. However, attempted isolation of a relatively pure fraction of *B. bovis* rhoptries has been a frustrating and largely unrewarding experience. We have been unable to isolate *B. bovis* rhoptries using gradient fractionation techniques that were successful for *B. bigemina* rhoptries and *B. bovis* spherical bodies. Two gradient fractions that clearly contain organelles compatible with rhoptries were identified in one experiment, but we have been unable to repeat this work. Nevertheless, we immunized three mice each with one or the other of two fractions that contain rhoptries mixed with spherical bodies and a large amount of free membranes presumably derived from subcellular organelles, merozoites, and

erythrocytes. The sera from these mice have been tested for antibodies against rhoptries by indirect immunofluorescence and immunoblot assays. Unfortunately, no parasite specific response could be detected against rhoptries, merozoites or infected cells. Since we have been unable to obtain mAbs against additional rhoptry antigens, we have focused on existing mAbs with apical binding patterns (135 kD antigen), on newly identified *orfs* associated with the *rap-1* genomic locus (*hsp40*), and on other heat shock proteins.

[2] *Characterization of spherical body proteins* – Several monoclonal antibodies previously derived from mice immunized with a spherical body preparation or whole merozoites bind to merozoites in an apical pattern. One of these mAbs reacted with spherical bodies and the cytoplasmic side of the infected erythrocyte, characteristic of spherical body proteins SBP1 and SBP2. The mAb identifies an antigen of 135 kD, which has been termed SBP3. SBP3 was cloned, expressed as a maltose binding fusion protein, and sequenced. There was no significant sequence homology with SBP1 or SBP2. Results of these studies have been accepted for publication (Ruef et al).⁴⁴ Purified rSBP3 was able to induce significant stimulation of T cell lines derived from two different *B. bovis* carrier cattle after stimulation for 2-3 weeks with *B. bovis* crude membrane antigen. The data indicate that the 135 kD antigen contains T cell epitope(s) recognized by unrelated *B. bovis* immune cattle, and warrant testing of this polypeptide as an immunogen.

[3] *Characterization of heat shock proteins* – As indicated above, in sequencing the entire *B. bovis rap-1* locus, an *orf* was identified that encoded a heat shock protein designated HSP40. Since heat shock proteins from other prokaryotic and eukaryotic organisms are able to induce a strong T cell response, we evaluated whether the *B. bovis* HSP40 contained B cell or T cell epitopes recognized by immune cattle. For this purpose, a recombinant construct encoding HSP40 as a histidine fusion protein was developed and the recombinant protein purified. In initial experiments, this protein has not been able to induce significant stimulation of peripheral blood T cells or T cell lines and clones from *B. bovis* carrier cattle. Neither have we been able to detect HSP40 specific antibody in immune sera. However, mice immunized with the recombinant HSP40 produce antibodies that identify a 44 kD protein in immunoblots.

In a related collaborative project primarily funded by the National Institutes of Health, Wendy Brown and colleagues identified three additional HSP's by screening a *B. bovis* cDNA library with rabbit antiserum prepared against a BioRad Prep-Cell fraction. This serum identified a clone designated 621 that has been sequenced and shares minimal homology (33% identity) with a heat shock protein of *Toxoplasma gondii* designated bag-1. Purified recombinant fusion protein was used to immunize rabbits and the resulting serum reacted predominantly with a 21kDa band of *B. bovis* and *B. bigemina*. It also reacted with Mexico, Texas, Australia and Israel strains of *B. bovis*. T cell clones prepared from a *B. bovis* immune carrier respond to recombinant HSP21, and to membrane antigen from *B. bovis* strains from Mexico, Texas, Australia, and Israel. Several of these T cell clones also respond to *B. bigemina*, while others do not. This indicates the presence of 2 T cell epitopes that are conserved across multiple strains of *B. bovis*, with one also conserved in *B. bigemina*.

Two HSP's with homology to apicomplexan HSP70 and 90 proteins also were identified in the cDNA library with rabbit antiserum.⁴⁵ We currently are expressing recombinant HSP70 and 90 to test their reactivity with PBMC's from carrier cattle.

Scientific Implications and Potential Impact

The data allow several conclusions to be drawn. *First*, clearly the *rap-1* locus of *B. bovis* does not vary within the *orf* to the extent of *B. bigemina*, *B. canis*, or *B. ovis*. In addition, all cross-neutralization and stimulation data indicate that relevant B and T lymphocyte epitopes are conserved, consistent with the strict sequence conservation data obtained with all strains analyzed. This is encouraging for vaccine development, since it suggests that genetic variation of *rap-1* is constrained, and that a single vaccine construct may be capable of providing broad immune protection. *Second*, the presence of B and T cell epitopes in the most highly conserved amino terminal half of RAP-1 implies that this region alone may be capable of inducing immune protection. As noted above this region contains the most highly conserved sequences and motifs, and, at least in *B. bigemina*, contains a neutralization sensitive B cell epitope. The ability to induce protection with an amino terminal construct would allow immunization in the absence of the carboxy terminal repeat region, avoiding the possibility of inducing a type 2, T independent, non-protective immune response against the repeats. *Third*,

spherical body proteins contain epitopes recognized by T cells from immune cattle. Therefore these organelles unique to the babesias may contain more vaccine candidates. *Fourth*, the *B. bovis rap-1* locus is closely associated with at least one heat shock protein (HSP40). While babesial HSP's in general are not necessarily immunogenic, at least one, the HSP21 antigen, contains epitopes recognized by T cells from immune cattle, and thus also is a new vaccine candidate.

Overall, we conclude that a single RAP-1 vaccine construct representing the conserved amino terminal region of the molecule should be sufficient for immunization against all strains of *B. bovis*. While results of the ongoing immunization trial will direct our next research steps, results at this time are consistent with our long term goal of designing a subunit vaccine containing only the epitopes relevant to induction of protective immunity. Parallel studies are defining the mechanisms of protective immunity.

References – See Appendix D

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Patents:

No patents have been filed as a result of research on the current project.

Appendix A

Table 1

Babesia bovis RAP-1-specific proliferative responses of PBMC following intradermal immunization with RAP-1N in RIBI adjuvant and interleukin-12.

Table 1. *Babesia bovis* RAP-1-specific proliferative responses of PBMC following intradermal immunization with RAP-1N in RIBI adjuvant and interleukin-12.

Calf and week ^a		Proliferation (Stimulation Index) to these antigens:				
		URBC	<i>B. bovis</i> CM	MBP	RAP-1N	RAP-1
89	pre	1.6	1.0	0.8	NT	NT
	5	0.8	1.2	0.4	0.6	NT
	8	0.9	8.8	0.8	3.5	NT
	9	1.2	8.5	1.8	4.2	4.7
	14	3.9	10.7	1.8	5.4	7.9
90	pre	1.1	2.6	1.1	NT	NT
	5	1.0	1.0	0.5	1.0	NT
	8	1.7	2.5	1.4	3.2	NT
	9	1.2	11.8	3.6	4.9	6.2
	14	1.0	2.2	1.4	10.4	6.8
95	pre	0.9	3.0	1.0	NT	NT
	5	0.8	0.8	0.5	1.1	NT
	8	0.9	3.4	1.2	2.5	NT
	9	1.7	11.6	3.0	6.5	5.6
	14	1.1	2.5	1.2	2.2	2.3
97	pre	1.0	0.8	1.4	NT	NT
	5	0.9	0.9	0.5	1.3	NT
	8	1.0	4.0	0.4	3.6	NT
	9	1.7	21.4	2.8	7.5	2.7
	14	1.8	9.3	1.0	6.3	5.9
99	pre	0.8	0.8	1.8	NT	NT
	5	0.9	1.2	0.5	0.5	NT
	8	1.0	3.6	1.0	4.1	NT
	9	1.2	11.3	4.0	12.3	10.3
	14	1.9	4.0	1.9	5.1	4.6

a. Calves were immunized intradermally at week 0, 3, and 6 with 40 µg RAP-1N and 10 µg human rIL-12 mixed in RIBI adjuvant. Calves were boosted at week 12 with antigen and RIBI adjuvant without IL-12. PBMC were collected at the indicated weeks and assayed for proliferation following a 6 day culture of 2×10^6 cells with 25 µg antigen per ml in triplicate wells of 96-well plates. Antigens consisted of uninfected red blood cell membranes (URBC), *B. bovis* membranes (CM), maltose binding protein (MBP), the N-terminal portion of RAP-1 consisting of aa 1-317 fused to MBP (RAP1-N) and full length RAP-1 fused to MBP (RAP-1). Results are presented as the stimulation index, calculated as the mean cpm of PBMC cultured with antigen/mean cpm PBMC cultured with medium.