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BARD

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**Molecular Characterization and Function of Brucella
Immunodominant Proteins**

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BARD FINAL REPORT

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Molecular Characterization and Function of *Brucella* Immunodominant Proteins

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I. Abstract

The BARD project was a continuation of a previous BARD funded research project. It was aimed at characterization of the 12kDa immunodominant protein and subsequently the cloning and expression of the gene in *E.coli*. Additional immunodominant proteins were sought among genomic *B. abortus* expression library clones using T-lymphocyte proliferation assay as a screening method. The 12kDa protein was identified as the L7/L12 ribosomal protein demonstrating in the first time the role a structural protein may play in the development of the host's immunity against the organism. The gene was cloned from *B. abortus* (USA) and *B. melitensis* (Israel) showing identity of the oligonucleotide sequence between the two species. Further subcloning allowed expression of the protein in *E. coli*. While the native protein was shown to have DTH antigenicity its recombinant analog lacked this activity. In contrast the two proteins elicited lymphocyte proliferation in experimental murine brucellosis. CD4⁺ cells of the Th1 subset predominantly responded to this protein demonstrating the development of protective immunity (γ -IFN, and IL-2) in the host. Similar results were obtained with bovine *Brucella* primed lymphocytes. UvrA, GroEl and GroEs were additional *Brucella* immunodominant proteins that demonstrated MHC class II antigenicity. The role cytotoxic cells are playing in the clearance of brucella cells was shown using knock out mice defective either in their CD4⁺ or CD8⁺ cells. CD4⁺ defective mice were able to clear brucella as fast as did normal mice. In contrast mice which were defective in their CD8⁺ cells could not clear the organisms effectively proving the importance of this subtype cell line in development of protective immunity. The understanding of the host's immune response and the expansion of the panel of *Brucella* immunodominant proteins opened new avenues in vaccine design. It is now feasible to selectively use immunodominant proteins either as subunit vaccine to fortify immunity of older animals or as diagnostic reagents for the serological surveillance.

II. Objectives of the Research Proposal

- 1) Characterize the *B. abortus* and *B. melitensis* 12 kDa gene and the flanking nucleotide sequences.
- 2) Evaluate the role of the 12kDa protein in the development of *Brucella* humoral and cellular immunity in the host.
- 3) Locate and analyze the function of the 12 kDa protein on the bacterium.
- 4) Identify additional proteins encoded by *B. abortus* and *B. melitensis* genes that stimulate T lymphocyte proliferation.

III. Relevant Data, Methodology, Results and Discussion

1. Generality

This grant is a continuation of a previous BARD funded research grant aimed at identification and characterization of *Brucella* immunodominant proteins. Using Western blot-T lymphocyte proliferation assay a number of *Brucella* immunodominant proteins were identified. The 12 kDa protein was specifically found as a predominant immunodominant protein. The gene was cloned in the frame of the first BARD project. However, gene sequencing and analysis of the protein's function were proposed as part of the goals of the continued grant proposal. As a consequence of the successful identification of the 12 kDa protein we postulated that additional immunodominant proteins can be identified using similar approaches to those used during the first BARD project. Below we report on the results achieved in the second BARD project.

2. Methodology

2.1. Subcloning and expression in *E. coli* of the L7/L12 gene

Two approaches were used. In Israel the gene was subcloned into pQE30, a cloning vector that includes a 6 histidine tag in the 5' DNA sequence. Then, after transformation of *E. coli* SG13009 the recombinant protein was expressed by IPTG induction. Finally the protein was purified from the cytosolic extract using Ni-NTA column. To obtain the recombinant protein in the form of the natural L7/L12 protein (no histidine tag) a similar vector was used that includes also an enterokinase sequence between the histidine sequence and the L7/L12 gene sequence. The recombinant protein was digested with enterokinase and eluted through Ni-NTA column. Undigested protein was captured on the column and the protein with the natural amino acid sequence was eluted out.

In the USA brucella genes were subcloned into the expression vector pAML-c2 which encodes Maltose Binding Protein (MBP). *E. coli* DH5 α was transformed with the ligation reaction product and the cells were induced with IPTG to produce the protein. The fusion protein was purified from cytosolic lysates using rabbit anti-MBP serum. The vector also contains a coding sequence for the recognition site of the specific protease factor Xa, which allows MBP to be cleaved from the polypeptide of interest after protein purification. Recombinant proteins Ssb, UvrA, GroES and GroEL were similarly produced using sequences for *GroEL* and *GroES* provided by J. Mayfield, Iowa State University, Ames, IA.

To endow the L7/L12 recombinant protein with DTH antigenicity it was incubated with *B. melitensis* strain Rev1 lysate. Then, the protein was captured on a Ni-NTA

column. After elution the protein was re-purified by SDS-PAGE electroelution. To identify potential modifications, [γ - 32] ATP (3000Ci/mmol, 50 μ Ci per reaction) or [p,19(n)- 3 H] palmitic acid (51Ci/mmol, 1mCi per reaction) were added to 1.5 ml of the brucella lysate prior to incubation of the recombinant protein in the medium.

2.2. Immunological methods

2.2.1. DTH skin testing

Male Hartley guinea pigs (350g) were inoculated with 10^6 Rev1 cells. Three weeks later the guinea pigs were considered sensitized to the brucellergen allergic skin test. Brucellergen and brucella proteins were inoculated intradermally at 0.1 ml aliquots. Immediate response was evaluated 4hs. later and DTH response was tested 24 hs. after inoculation. Development of erythema and skin thickness were used to judge the amount of the skin reaction.

2.2.2. T-cell proliferation

Peripheral blood mononuclear (PBM) cells were isolated from cattle blood samples (density gradient centrifugation). Washed isolated PBM cells were cultured for 4 days (or as specified) in 96 well plates with the addition of the antigen of interest. Then, lymphocytes were pulsed with 1 μ Ci of [3 H]thymidine in each well. The cells were harvested onto glass fiber filters and the radioactivity was determined.

2.2.3. Murine T-cell proliferation

Single cell suspensions were prepared from the spleen of naive and infected animals. Splenocytes were isolated by density gradient centrifugation. CD4 $^+$ T cells were purified and the phenotypes were identified by flow cytometry using a panel of mAbs. CD4 $^+$ cells were stimulated with either recombinant protein or with γ -irradiated B. abortus cells. Syngeneic irradiated (2000 rads) spleen cells from naive animals were added as antigen presenting cells (APC) to the CD4 $^+$ T cells. The cells were cultured for 4 days and pulsed with [3 H]thymidine for 18hs. Then, cells were harvested and counted for radioactivity to measure proliferation.

2.2.4. Cytokine analysis

Proliferating T cells were simultaneously analyzed for cytokine expression. Reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis was used to determine β -actin, interleukin-2 (IL-2), IL-4, and INF- γ mRNA expression. Cytokines were directly detected in the supernatants of CD4 $^+$ T-cell cultures using ELISA to mouse IFN- γ , IL-2, and IL-4.

2.2.5. Knock out mice

Mice homozygous for the β 2-microglobulin mutation (β 2-m $^{-/-}$) - MHC class I-deficient mutants, were kindly provided by E. Balish and D. Muller (University of Wisconsin, Madison, WI) or purchased from the Jackson Laboratory (Bar Harbor, ME). The MHC class II deficient mutants (A β $^{-/-}$) were kindly provided by W.P. Weidanz (University of Wisconsin, Madison, WI). Female C57BL/6 were purchased from The Jackson Laboratory and used as controls. Male and female mutant mice were

used between 8-14 weeks of age. In all experiments, homozygous mutants were backcrossed to C57BL/6 mice, and F1 mice were used.

3. Results

Objective 1

After protein sequencing of the N-terminus amino acid sequence the corresponding gene oligonucleotide sequence was deduced. A gene probe was designed using homologous sequences to identify the genetic sequence on the brucella chromosome. In the USA *B. abortus* genomic library was used as target DNA while in Israel we used *B. melitensis* genomic DNA as target DNA. Using slightly different recombinant DNA technology both laboratories successfully cloned the gene encoding the 12 kDa protein (1,2). GenBank sequence analysis revealed that: a) the gene sequence in *B. melitensis* and *B. abortus* is identical; b) the gene encodes the *Brucella* L7/L12 ribosomal protein. Because the US laboratory cloned a larger DNA sequence than did the Israeli laboratory they also identified the L10 ribosomal protein's gene sequence on the *rplL* cistron.

Objective 2

Different approaches were used by the two laboratories for gene cloning and expression in *E. coli*. The US laboratory cloned the gene as a fusion protein between the MBP and the L7/L12 proteins using the MBP vector (3). This protein was isolated and purified from the cells using MBP specific monoclonal antibodies. Then, the intact L7/L12 protein was obtained by digestion of the fusion protein with protease factor Xa. The Israeli laboratory used a cloning vector that links a 6 histidine tag upstream to the cloned gene. Then, the recombinant protein was purified by elution through a nickel column that binds the protein via the histidine moiety. The intact protein (with no histidine tag) was obtained using a similar vector that also includes an Enterokinase sequence between the 5' sequence of the gene and the histidine tag sequence. Following expression of the recombinant protein in *E. coli* it was digested with Enterokinase to obtain the native structure.

The US laboratory tested activity of the recombinant protein by *Brucella* primed-lymphocyte proliferation assays analyzing FACS sorted T-cell subpopulations. Murine primed T-lymphocytes reacted by CD4⁺ subset and Th1 cytokine profile. Recombinant L7/L12 protein induced INF- γ while intact *B. abortus* cells also elicited IL-2 cytokine excretion (4). When testing bovine primed PBM cells rL7/L12 induced CD4⁺ cell proliferation (3). The Israeli laboratory tested activity of the protein in the brucellergen skin test. Using DTH-Western blot analysis we were able to demonstrate that the 12 kDa protein is a predominant fraction in brucellergen that elicits the skin response (unpublished data). Therefore, the recombinant protein was tested in sensitized guinea pigs for this response in comparison to the native protein. The data indicated that the recombinant protein lacks DTH antigenicity. In contrast, we showed that the purified L7/L12 protein from brucellergen is a predominant antigen in the DTH response. In addition, removal of the L7/L12 protein from brucellergen by anti-L7/L12 antibody affinity chromatography (produced against the recombinant protein) significantly reduced the DTH antigenicity of the brucellergen (5).

We also tested DTH antigenicity of the protein in sheep. Recombinant protein produced in the US laboratory and recombinant protein produced in the Israeli

laboratory were tested in the field in comparison to brucellergen. Sheep from an infected flock were used. The results conclusively suggested that irrelevant whether the recombinant protein was produced as an MBP fusion protein construct or was it tagged with the histidine moiety the recombinant proteins lacked DTH antigenicity. In contrast, brucellergen produced skin response in brucella infected sheep.

Objective 3

The fact that the recombinant protein lacked DTH activity while the native protein showed such antigenicity could not be simply explained. We hypothesized that brucellae cells confer DTH antigenicity on the protein due to post translation modification mechanism. To test this hypothesis recombinant protein was incubated in the presence of freshly prepared brucella cytosol. After treatment the protein was endowed with DTH activity. To demonstrate that the DTH activity was linked to the protein it was further purified by gel electrophoresis and elution of the band from the gel. This fraction maintained the DTH activity. The possibility of chemical modification of the protein was tested separately. The native protein from brucellergen did not stain by Schiff reagent suggesting it was not glycosylated. Possible phosphorylation modification of the protein was not proved as incubation of the protein in the brucella cytosol in the presence of P^{32} labeled ATP did not phosphorylate the protein. However, other brucellergen proteins were labeled proving the technical acceptability of the method. When tritium-labeled palmitic acid was added in the incubation mixture the L7/L12 protein was covalently labeled proving acylation modification of the protein in the presence of brucella cytosol (6, submitted for publication).

Objective 4

In light of the success achieved in identification, characterization, gene cloning and expression in *E. coli* of the L7/L12 protein we sought identification of other *Brucella* immunodominant proteins. To achieve this goal we screened genomic phage expression library of *B. abortus* as the testing antigen with brucella primed lymphocyte proliferation assay. Using this approach *Ssb*, *UvrA*, *GroEL* and *GroES* were identified, cloned and sequenced (7,8). The genes were then subcloned and expressed in *E. coli* using the MBP vector. *Ssb* was soon realized as inactive in the lymphocyte proliferation assay thus omitted from the list of *Brucella* immunodominant proteins. In contrast rUvrA, rGroEL and rGroES stimulated such a response of murine CD4⁺ primed T lymphocytes obtained from *B. abortus* inoculated mice. The cytokine profile of the proliferating cells was characteristic of a Th1 type detecting IL-2 and IFN- γ but not IL-4 cytokine in the supernatant. The recombinant proteins rGroEL and rUvrA also elicited DTH response in *B. melitensis* sensitized guinea pigs reflecting their involvement in the development of the host's immune cellular response (8).

Essential to understanding the protective value of the antigens is the evaluation of the reactive T cell subset population. Knock out mice lacking functional CD4⁺ or CD8⁺ subset were used to demonstrate the role of these cells in the immune response. CD8⁺ defective mice ($\beta 2-m^{-/-}$), CD4⁺ defective mice ($A\beta^{-/-}$) and normal cell population mice (C57BL/6) were infected with living *B. abortus* S19 cells. The course of brucella clearance was then tested by harvesting spleen cultures of the mice at different times after infection. The necessity of CD8⁺ T cells for optimal resistance to *B. abortus* infection was clearly demonstrated. CD4⁺ defective mice and normal mice cleared the organisms in their spleens within 4 weeks after infection. In contrast brucella actually replicated and reproduced in the first two weeks after infection of CD8⁺ deficient mice

being slightly eliminated in the next two weeks. The $A\beta^{-/-}$ and C57BL/6T cells exhibited a type 1 cytokine profile, marked by production of IFN- γ , a critical cytokine involved in host protection. Mice lacking CD8 $^{+}$ T cells ($\beta 2-m^{-/-}$) produced two fold higher levels of IL-10, an immunosuppressive cytokine, at the infection period (9). These results demonstrated the role CD8 $^{+}$ type 1 CD44 hi CD45 Rb lo T lymphocytes play in controlling intracellular *B. abortus* infection. The involvement of distinct molecules, previously suggested as immunodominant brucella proteins, in the development of cytotoxic immune activity remains to be elucidated.

4. Discussion

Our previous BARD project was aimed at identification of brucella immunodominant proteins. We used the terminology of immunodominant proteins to indicate those brucella proteins that elicit cellular immunity in the host. The lymphocyte proliferation assay and allergic skin test were used to identify such antigens. Using Western blot-T lymphocyte proliferation assay a 12kDa predominant immunodominant protein was identified in the brucella lysate. The N-terminus amino acid sequence was determined and the 5'-terminus gene sequence was deduced from it. A gene probe was synthesized and used in DNA hybridization to identify the gene in *B. abortus* and *B. melitensis*. Then, the gene was subcloned and expressed in *E. coli*. Our goal in the current BARD project was to characterize the protein and its function in brucella. The success achieved during the first project led us to believe that using similar approaches other immunodominant proteins can be identified and characterized.

DNA sequencing of the 12kDa encoding gene and comparison to known GenBank sequences revealed it is the *rplL* ribosomal gene. The sequence of the genes from *B. abortus* and *B. melitensis* were identical (1,2) demonstrating the high homology between the two species. The identification of an identical immunodominant protein in the two species provided the scientific rationale for development of a multi-species vaccine. Therefore, we pursued the subcloning and expression of the gene in *E. coli*. The two laboratories used different cloning vectors. In Israel the pQE30 plasmid was chosen. This plasmid includes a six histidine moiety tagged to the 5' gene's sequence. Therefore, the recombinant protein can be purified using this moiety as a binding site to a Ni-NTA column. In the USA the pMAL-c2 vector was used. This plasmid includes Maltose Binding Protein at the 5' terminus of the insertion site. Therefore, a fusion protein is obtained between MBP and the cloned protein. This protein can be purified from the *E. coli* lysate using anti MBP antibody affinity column. The native protein can then be separated from the fusion protein by Factor Xa proteinase digestion.

The L7/L12 recombinant protein was used to produce rabbit antibodies. These antibodies were bound to Sepharose column which was used as affinity column to purify the native protein from brucellergen - a protein enriched brucella cytosolic extract which elicits the allergic skin response in sensitized animals. This approach allowed us to compare the DTH antigenicity of rL7/L12 protein to that of the native protein. The data suggested a dual antigenicity profile of the protein. Whereas the native protein elicited a skin response in sensitized guinea pigs the rL7/L12 protein did not elicit such a response. In contrast the rL7/L12 protein showed CD4 $^{+}$ T-cell proliferation antigenicity when cattle PBM cells were tested (3). Similarly, using murine brucellosis as a model CD4 $^{+}$ cells were identified as important T-cells subset population that responded to the recombinant protein (4,8).

The discrepancy observed between the biological properties of the two proteins that have been shown to have identical amino acid sequence was puzzling. Therefore, we hypothesized that DTH antigenicity is a property endowed on the protein due to a post translation modification mechanism of brucella. This hypothesis was confirmed by demonstration of acylation of the recombinant protein with ^3H -palmitic acid after incubation of the protein in brucella lysate (6). The requirement that L7/L12 be modified to engender it activate in the DTH response may indicate that virulence factors of brucella can originate in plain structural proteins.

The success achieved during this research led us to further identify additional important immunodominant proteins. A new approach has been sought. We used T lymphocyte proliferation assay to identify recombinant clones in pBluescript II SK⁺ brucella S2308 genomic library that express immunodominant proteins. The UvaR protein was thus identified (7). In addition, GroEL and GroES were tested and found important immunodominant proteins. The three proteins were tested for their DTH antigenicity and rGroEL produced a significant response in *B. melitensis* Rev1 sensitized guinea pigs (8). The identification of additional immunodominant proteins posted us in the front line of identification of alternative vaccines and diagnostic antigens.

Analysis of the cytokine profile induced during in vitro incubation of PBM cells in the presence of immunodominant antigens was then applied. The results suggested that IFN- γ is the major cytokine produced by the primed CD4⁺ cells (4,7,8). IFN- γ is a major cytokine involved in the development of cytotoxic immune response against the invasive organism. Therefore, it can be suggested that L7/L12 is an important immunodominant protein candidate for use as a subunit vaccine. The fact that cytotoxic activity has been shown to be a major control mechanism of the disease in murine brucellosis supports this idea (9).

To conclude, the project has greatly improved our knowledge on the brucella immunodominant proteins and their role in the pathogenesis of the organism. The genes of three major immunodominant proteins were cloned and expressed in *E. coli* and the recombinant proteins were shown to participate in the cellular immune response. However, L7/L12 probably requires additional postranslation modification to endow it with the DTH antigenicity. Our results provided the logical frame to considering individual brucella proteins as either vaccines or diagnostic reagents. Future studies will be aimed at evaluating the use of these proteins for such purposes.

IV. Description of Cooperation

The research project was conceived after the two principal investigators from Israel and the USA mutually elaborated on the major ideas. It is a continuation project of a previous BARD project No. I-1434-89. Following identification of the L7/L12 immunodominant protein it was logical to clone, sequence and express the gene in *E. coli* to test its potential use in allergic skin test. Similarly, it was important to characterize its immunological properties and to use similar approaches to identify and characterize additional immunodominant proteins.

The *B. abortus* *rpIL* gene has been identified in the USA and subsequently it was identified in Israel using the *B. melitensis* genomic library as a DNA source. The two genes were subcloned and sequenced individually resulting in identification of identical nucleotide sequence in *B. abortus* and *B. melitensis*, respectively. Then, each laboratory used a different approach to subclone and express the gene in *E. coli*.

Biological activity of the two recombinant proteins had been studied individually. In Israel, rL7/L12 was compared with the native protein for DTH antigenicity. In the USA rL7/L12 was used in in vitro lymphocyte proliferation assays to determine its involvement in the development of cellular immunity.

The USA laboratory further pursued identification of additional immunodominant proteins. These studies resulted in identification, cloning and expression of rSsb, rUvrA, rGroEL and rGroES. The recombinant proteins were sent to Israel for evaluation of their DTH antigenicity proving UvrA and GroEL to have such a property (8). Therefore, these latest experiments demonstrated best the keen collaboration that existed between the two laboratories. We have corresponded by e-mail exchanging ideas and identifying mutual goals. We are currently active in coordination of our next proposal to BARD using the important data and conclusions achieved during this grant. Moreover, we agreed on expanding this collaboration by including also Dr. Tom Ficht, Texas A&M University, Texas, USA. Dr Ficht's input will be in identification of brucella virulence genes that will enable us to develop an attenuated living vaccine strain.

V. Evaluation of the Research Achievements

All four objectives were completed successfully. The L7/L12 ribosomal protein was identified a key antigen that elicits CD4⁺ cellular response against brucellosis. The CD4⁺ cells were from the Th1 type eliciting protective cytokines - i.e., IFN- γ and IL-2. The L7/L12 gene was cloned and expressed in *E. coli* by the two laboratories. It was demonstrated that *B. abortus* and *B. melitensis* share an identical gene suggesting a very close relatedness between the two species. Therefore, the scientific basis for using the recombinant protein as a subunit vaccine has been established. Moreover, the native protein was shown to be a major DTH inducing antigen in brucellergen.

Using lymphocyte proliferation to screen *B. abortus* expressive genomic library the immunodominant proteins Ssb and UvrA were identified. UvrA has also been demonstrated to possess DTH antigenicity proving the importance of this protein in the development of the immune response in the host. Similarly, *GroES* and *GroEL* were subcloned and expressed in *E. coli* allowing characterization of GroEL as an additional important immunodominant protein.

Besides identification of brucella immunodominant proteins use of CD4⁺ and CD8⁺ knock out mice demonstrated that CD8⁺ cells are important in the control of the disease. Therefore, our data provided the scientific rational to development of a subunit vaccine as well as use of several of these antigens for diagnostic purposes. In addition, the Israeli laboratory was central in planning and conducting a national eradication program. This field activity provided important epidemiological data on the disease. Specifically, the advantages and drawbacks of the Rev1 vaccine were studied. The data suggested that Rev1 vaccination caused adverse responses that seriously hampered control program of the disease. Therefore, it was concluded that development of an alternative vaccine (additional attenuation of Rev1 and development of subunit vaccine) is essential to achieving an effective local and regional control of the disease. This conclusion will be implemented in our next BARD proposal.

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VII. Appendix - Reprints