BARD

FINAL REPORT

PROJECT NO. US-2968-98

Attenuated *Brucella melitensis* Rough Rev1 Vaccine

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Cover Page**

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Investigators

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Signature
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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1	2	3
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Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted			1	1
Issued (allowed)				
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Final Scientific Report

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Abstract:

The original objectives of the proposal were: 1. Compare mutants 444 and 710 to Rev1 (parent strain), and 16M (field strain) in murine and human macrophage lines for phenotypic differences. 2. Determine *in vivo* virulence and survival of the mutants 444 and 710 in guinea pigs and mice. 3. Determine humoral and cell-mediated immune responses induced by mutants 444 and 710 in guinea pigs and mice. 4. Determine *in vivo* protection of mice and guinea pigs provided by mutants 444 and 710 compared to Rev1

Background: While human and animal brucellosis are rare in the U.S., brucellosis caused by *B. melitensis* remains relatively constant in Israel. Despite a national campaign to control brucellosis in Israel, the misuse of Rev1 Elberg vaccine strain among pregnant animals has produced abortion storms raising concern of human infection due to vaccine excretion in the milk. Further, some commercial Rev1 vaccine lots can: a) produce persistent infection, b) infect humans, c) be horizontally transmitted, d) cause abortion, and e) induce a persistent anti-O-polysaccharide antibody response confounding the distinction between infected and vaccinated animals. In Israel, vaccination practices have not optimally protected the milk supply from *Brucella* and Rev 1 vaccine can exacerbate the problem. In addition, cattle vaccinated against *B. abortus* are not protected against *B. melitensis* supporting the need for an improved vaccine.

A safe vaccine used in adult animals to produce herd resistance to infection and a vaccine that can be distinguished from virulent infection is needed. A rough Rev1 vaccine would be less virulent than the parental smooth strain and permit serologic distinction between vaccinated and infected animals. Advantages of the Rev1 vaccine foundation are: 1) Rev1 vaccination of sheep and goats against *B. melitensis* is approved; therefore, vaccines derived from the Rev1 foundation may be readily accepted by licensing agencies as well as commercial companies, and 2) considerable data exists on Rev1 vaccination and Rev1 proteins. Therefore, a post-genomic vaccine against *B. melitensis* based on the Rev1 foundation would provide a great advantage.

Major conclusions from our work are: 1) We have determined that mutant 710 is highly attenuated in macrophages compared to virulent field strain 16M and mutant 444. 2) We have confirmed that mutant 710 is highly attenuated in guinea pigs and mice. 3) We have determined immune responses induced by mutant 710 in animals. 4) We have determined *in vivo* protection of mice and guinea pigs provided by mutants 444 and 710 compared to Rev1, and importantly, mutant 710 provides a high level of protection against challenge with virulent *B. melitensis* 16M. Thus, our data support the goals of the grant and provide the foundation for a future vaccine useful against *B. melitensis* in Israel.

Because of patent considerations, many of our findings with 444 and 710 have not yet been published.

Scientific and Agricultural Implications: Our findings support the development of a vaccine against *B. melitensis* based on the mutant 710. Because strain 710 is a mutant of the Elberg Rev1 vaccine, commercialization is more likely than development of an entirely new, uncharacterized *Brucella* mutant or strain.

Achievements:

Significance of achievements: Our achievements are arranged under headings of the *Brucella* genome that describes our work with *Brucella* genes/genome or **The Host Response to Brucella and Vaccine Development** that describes selected work determining the host response to *Brucella* and forming a basis to evaluate vaccine candidates.

1. The *Brucella* Genome

(*Infection and Immunity*, 69:7736-7742, 2001) Although no vaccine exists for *Brucella*, vaccine development in other bacteria has been based on deletions of selective nutritional as well as regulatory systems. Our goal is to develop a vaccine for *Brucella*. To further this aim, we have used a green fluorescent protein (gfp) reporter system to identify constitutive and intracellular-induced *B. abortus* genes. Constitutively producing clones were identified but of greater interest, clones negative for constitutively produced GFP in agar were examined to detect promoter activity induced within macrophages 4 and 24 h following infection. Bacterial genes activated in macrophages 4 h post infection appear to be involved in adapting to intracellular environmental conditions. Genes activated 24 h following infection were biosynthesis and metabolism-associated genes. Identification of *Brucella* genes activated following macrophage invasion provides insight into the *Brucella* pathogenesis and thus is valuable in vaccine design utilizing selective targeted deletions of newly identified *Brucella* genes.

We have developed a microarray of *B. melitensis* 16M to compare the genomes of other *Brucella* species to potentially uncover the basis of host preference of different *Brucella* species and permit rapid evaluation of *Brucella* gene expression during infections. *B. abortus*, *B. melitensis*, and *B. suis* are pathogenic to humans; in contrast, human infections of *B. ovis* and *B. neotomae* have not been reported, while *B. canis* human infections are rare. Limited genome diversity exists among *Brucella* species. Comparison of *Brucella* species whole genomes is, therefore, likely to reveal factors responsible for differences in host preference and virulence restriction. To facilitate such studies, we used the complete genome sequence of *B. melitensis* 16M, the species highly pathogenic to humans, to construct a genomic microarray. Hybridization of labeled genomic DNA from *Brucella* species to this microarray allowed comparison of their gene

content. Results revealed a total of 217 ORFs altered in five *Brucella* species analyzed. These ORFs are often found in clusters (islands) in the 16M genome. Included in these islands are ORFs encoding hypothetical proteins, transporters, transposases, and transcriptional regulators that may aid successful establishment of *Brucella* in human infections. Examination of the genomic context of these islands suggests that many are horizontally acquired and may contribute to the variability in host preferences and virulence among *Brucella* species. (Comparative genome analysis of *Brucella* species reveals acquisition of genomic islands, Manuscript submitted)

2. The Host Response to *Brucella* and Vaccine Development

Identification of host responses at the gene transcription level provides a molecular profile of the events that occur following infection. *Brucella* is a facultative intracellular pathogen of macrophages that induces chronic infection in humans and domestic animals. Using microarray technology, the response of macrophages 4 h following *Brucella* infection was analyzed to identify early intracellular infection events that occur in macrophages (Infection and Immunity, 71:1125-33, 2003). Of the >6,000 genes, we identified over 140 genes that were reproducibly differentially transcribed. First, an increase in the transcription of a number of proinflammatory cytokines and chemokines, such as tumor necrosis factor alpha, interleukin-1 α (IL-1 α), IL-1 α , and members of the SCY family of proteins, that may constitute a general host recruitment of antibacterial defenses was evident. Alternatively, *Brucella* may subvert newly arriving macrophages for additional intracellular infection. Second, transcription of receptors and cytokines associated with antigen presentation, e.g., major histocompatibility complex class II and IL-12p40, were not evident at this 4-h period of infection. Third, *Brucella* inhibited transcription of various host genes involved in apoptosis, cell cycling, and intracellular vesicular trafficking. Identification of macrophage genes whose transcription was inhibited suggests that *Brucella* utilizes specific mechanisms to target certain cell pathways. In conclusion, these data suggest that *Brucella* can alter macrophage pathways to recruit additional macrophages for future infection while simultaneously inhibiting apoptosis and innate immune mechanisms within the macrophage, permitting

intracellular survival of the bacterium. These results provide insights into the pathogenic strategies used by *Brucella* for long-term survival within a hostile environment.

We have developed a useful animal model to evaluate *Brucella* virulence (**Infection and Immunity**, 70:7004-7012, 2002). Interferon regulatory factor 1-deficient (IRF-1^{-/-}) mice infected with virulent *Brucella abortus* 2308 at 5×10^5 CFU developed acute hepatitis similar to many natural hosts but, unlike natural hosts, IRF-1^{-/-} mice were unable to resolve infection and died. In contrast, IRF-1^{-/-} mice survived when infected at 5×10^5 CFU with several attenuated *Brucella* strains (S19, RB51, cbp, and cyd). The survival of infected IRF-1^{-/-} mice is likely a function of the level of virulence of each *Brucella* strain and the extent of retained immunity. Further, these findings suggest that adaptive immunity may be important to the survival of IRF-1^{-/-} mice since attenuated *Brucella* strains can protect IRF-1^{-/-} mice against lethal challenge with virulent *Brucella*. Using the IRF-1^{-/-} mouse model, the following set of criteria were identified to define *Brucella* virulence: (i) the day of death for 50% of mice infected with 5×10^5 CFU of *Brucella*, (ii) the extent of liver toxicity, and (iii) the minimum immunizing dose of *Brucella* to protect against challenge with virulent S2308. Thus, IRF-1^{-/-} mice are the key to determining the level of *Brucella* virulence, to evaluating *Brucella* mutants for attenuation, and to investigating adaptive immunity in brucellosis.

IFN- γ is a key cytokine controlling *Brucella* infection, and the diverse functions of this cytokine are mediated by IFN regulatory factors (IRFs) such as IRF-1, IRF-2, and IFN consensus sequence binding protein (ICSBP). However, the roles of these three IRFs in *Brucella* infection have not been investigated. The infection of each IRF-deficient mouse strain provides an opportunity to determine not only the significance of each IRF molecule but also the crucial immune components necessary for host defense during in vivo infection, because respective IRF-deficient mouse strains contain unique immunodeficient phenotypes (**Journal of Immunology**, 168:2433-2440, 2002). *Brucella abortus* S2308-infected IRF-1^{-/-} mice were dead within 2 wk postinfection, while IRF-2^{-/-} mice contained less splenic *Brucella* CFU than wild-type mice at the early stage of infection. Infected ICSBP^{-/-} mice maintained a plateau of splenic *Brucella* CFU

throughout the infection. Additional infection of IL-12p40-, NO synthase 2-, and gp91phox-deficient mice indicates that these immune components are crucial for *Brucella* immunity and may contribute to the susceptibility of IRF-1^{-/-} and ICSBP^{-/-} mice. Immunologic and histopathological analyses of infected IRF-1^{-/-} mice indicate that the absence of IL-12p40 induction and serious hepatic damage are involved in the death of IRF-1^{-/-} mice. These results indicate that 1) IRF-1 and ICSBP are essential transcriptional factors for IFN- γ -mediated protection against *Brucella*; 2) IL-12, reactive nitrogen intermediates, and reactive oxygen intermediates are crucial immune components against *Brucella*, and their absence may contribute to the susceptibility of IRF-1^{-/-} and ICSBP^{-/-} mice; and 3) hepatic damage caused by *Brucella* virulence contributes to the death of IRF-1^{-/-} mice.

Brucella establishes chronic infections in host macrophages while downregulating cytokines such as tumor necrosis factor alpha (TNF- α). When producing TNF- α , *Brucella abortus* rough lipopolysaccharide (LPS) activates the same mitogen-activated protein kinase signaling pathways (ERK and JNK) as *Escherichia coli* LPS, but *Brucella* LPS is a much less potent agonist. Nitric oxide (NO), produced by the iNOS protein, is known as a defense mechanism against various pathogens and an apoptotic inducer of cells. Apoptosis can also be a host protective mechanism against intracellular bacteria. The intracellular survival of *Brucella* in RAW264.7 macrophages was examined under conditions of the apoptotic inducer, NO. Since *Brucella* does not induce high output of NO, *Escherichia coli* LPS and IFN- γ , as potential therapeutic modalities, were added to increase the expression of iNOS, and thus NO. Using 10 ng/ml *E. coli* LPS and 25 U/ml IFN- γ , nitrite production was as high as 140 microM by 72 h. However, when macrophages were infected with *Brucella*, the nitrite concentration was 60 microM after 72 h post infection, greater than a two-fold decrease. The number of surviving bacteria decreased, from 6 to 24 h, in the presence of nitrite accumulation. In the absence of *Brucella*, there was an increase in apoptotic cells at 72 h with high nitrite accumulation. In contrast, the number of macrophage apoptotic bodies decreased in the presence of *Brucella*. The data suggest that: (i) NO accelerates the killing of intracellular *Brucella*, but not to completion during the first 24 h of infection; (ii) *Brucella* can prevent

apoptosis as an advantage for bacterial survival inside macrophages and (iii) surviving intracellular bacteria then replicate steadily after 24 h. *Brucella* probably expresses genes that counteract the effect of a high NO environment or activates genes to utilize NO as a nitrogen source, as the *Brucella* genome codes for nitric and nitrous oxide reductase genes.

Impacts of the research findings

Our studies have for the first time utilized microarrays of the host and pathogen to identify genes used in the host-pathogen response. These studies are particular novel because few scientists study changes in both the host and pathogen during infection. Further, our work with mutants 710 and 444 have certainly demonstrated the utility of 710 as a potential vaccine foundation of use in Israel against *B. melitensis*.

Details of cooperation

Brucella research is a major focus of our laboratories, and collaboration extends from three previously funded BARD proposals (Is-1434-89, Is-2138-92, and US-2968C). In 2002 Dr. Banai visited the U.S. lab and data was reviewed, while in 2003, Drs. Banai and Splitter met in Spain at the 56th annual Brucellosis Conference to review data and research progress.

GenBank Accession: GenBank AY518304 10kDa gene sequence of *B. melitensis* Rev.1

List of publications

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