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TITLE: Basic Research Effort Toward Development of a Vaccine Against Human Brucellosis

PRINCIPAL INVESTIGATOR: Gerhardt G. Schurig, Ph.D.

CONTRACTING ORGANIZATION: Virginia Polytechnic Institute Blacksburg, Virginia 24061-0249

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This project identified several potentially protective *Brucella* antigens: YajC, GroEL, GroES, SOD, SecD, 18kDa, 16kDa and TolB. Some potential virulence factors have also been identified. Antigens have been purified as fusion proteins and/or native antigens for further characterization. Selected antigens have also been cloned into Baculovirus for antigen characterization and immunization purposes. Several vaccinia/*Brucella* recombinants with selected *Brucella* antigens have been produced and have been tested in mice. Mice are able to respond to these antigens immunologically but protection has not yet been achieved. New recombinants using more effective early/late synthetic promoters and able to express IL-12 are being constructed to overcome this problem. Vaccinia/listeriolysin and Brucella L7/112 recombinants are being constructed to assess vector efficacy. To assess the true protective abilities of the selected *Brucella* antigens, *B. abortus* vaccine strain RB51 disruption mutants have been constructed and DNA vaccines have been produced. These are being tested in mice for their ability to induce specific immune responses and protection against challenge with virulent *Brucella* spp.
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(✓) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 32 CFR 219 and 45 CFR 46.

(✓) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Principal Investigator's Signature Date

Sept 30/98

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V.  INTRODUCTION.

a.  Nature of the problem, background and previous work.

Brucellosis affects millions of people worldwide. Humans contract the disease either by consuming infected foods or by coming in contact with animals shedding the organism. The disease is characterized by an undulant fever, cold sweats and general malaise; any exercise will produce pronounced fatigue. If untreated the disease can last from a few weeks to several years. Serious complications leading to death can occur. Tetracycline is the treatment of choice for infected humans. In severe cases tetracycline treatment is supplemented with streptomycin or rifampicin. Brucellosis affects a variety of animals including swine, cattle, sheep, goats, dogs, and camels. Important species of Brucella are suis, abortus, ovis, melitensis, canis, and neotomae each with certain predilection for a particular animal species.

Humans are susceptible to B. melitensis, B. suis, B. abortus and B. canis in decreasing order. The disease is endemic in many Middle East countries, Asia, Mexico, Central and parts of South America.

Antibiotic resistant mutants of Brucella, including tetracycline, rifampicin and streptomycin resistant ones, are not difficult to produce in the laboratory. Such mutants could be utilized in biological warfare resulting in high morbidity with no adequate treatment. Therefore, prevention of infection through vaccination is desirable.

Currently available vaccines can not be used in humans because of their side effects or lack of effectiveness. The current, most used vaccine for protecting cattle against B. abortus is strain 19 and for protecting goats against B. melitensis is strain Rev 1. Vaccination with these strains leads to seroconversion complicating diagnosis of the disease. Both of these vaccine strains are pathogenic for human beings. B. abortus strain RB51 was developed in our laboratory and has now replaced strain 19 (Federal Register 1996) as the vaccine of choice for bovine Brucellosis. This strain is a stable, rough natural mutant devoid of 0-side chain. The strain is able to induce protective immunity in mice (1) and cattle (2,3) without the induction of antibodies to the 0-side chain (1,2). Transfer of T cells from strain RB51 immunized mice will transfer protective immunity against a virulent challenge (4), similar to the immunity transferred to mice by T cells derived from strain 19 immunized mice (5). This confirms previous indications that immunity to B. abortus infection requires a strong CMI component (4,5,6,7,8). The specific antigens, which confer strong protective CMI, have yet to be defined.
Macrophages are the principle cells of residence for *Brucella* in infected animals. Resistance usually depends on the correct interaction between T lymphocytes, specific for particular bacterial antigens, and the macrophage (9,10,11,12,13,14). Activation of macrophages by interferon-gamma (INF-γ) will lead to the destruction of intracellular *Brucella* (4). This further suggests that T cells, particularly those responsible for the production of INF-γ (T helper1-CD4+ response) are of major importance in anti-*Brucella* immunity (4). These observations do not eliminate a concomitant protective role for cytotoxic T cells (Tc, CD8+), since protective immunity can be demonstrated by passive transfer of either Th or Tc cells (8). It is possible that in Brucellosis the major role of the Tc cells in protection is production of INF-γ and not direct cytotoxicity. Considering these observations, it is most likely that *Brucella* proteins involved in protective immunity will preferentially stimulate INF-γ producing T cells which in turn activate macrophages enhancing their brucellacidal capabilities. Therefore, if *Brucella* proteins with such characteristics could be identified, they would likely induce a strong and protective CMI response. They could be used in cloning and expression systems that are able to present these antigens appropriately to the immune system.

b. Purpose of current work.

This project intended to identify *Brucella* antigens that are likely to stimulate Th1 responses (with production of INF-γ) in lymphocytes from vaccinated mice and therefore, are likely to have a role in the induction of protective immunity against Brucellosis. Several such antigens have been identified, the genes encoding those antigens were isolated, sequenced and their products characterized to some extent. The genes were used to construct recombinant vaccinia viruses. Vaccinia recombinants were used to immunize mice to determine whether they can induce an immune response and protect mice against a challenge with virulent *B. abortus*. A recombinant vaccinia with protective characteristics against *Brucella* challenge may be a good vaccine candidate for humans.

c. Outline of approach.

The general outline of the approach is found in figure 1. Briefly, mice vaccinated with a protective rough, *Brucella* vaccine (mainly strain RB51) served as source of antibodies to specific *Brucella* antigens and as source of sensitized, reactive lymphocytes. These antibodies and lymphocytes were used to identify reactive *Brucella* antigens. The main approach was to screen *E. coli/Brucella* genomic libraries. In a few instances, *Brucella* antigenic fractions were screened for reactivity. Those clones or fractions reactive with antibodies of the IgG2a type
and/or inducers of lymphocyte proliferation, IL-2 and or Interferon gamma (INF-γ) production were selected as potentially expressing *Brucella* antigens of interest. Once the clones were identified the specific gene(s) were isolated and

Figure 1. Outline of project approach.
eventually expressed in vaccinia virus. Antigens identified within the fractions were purified, N-terminal amino acid sequenced and corresponding gene(s) located with the appropriately deduced oligonucleotide probe(s). Vaccinia/Brucella recombinants were tested in mice for their ability to protect mice against a *Brucella* challenge and their ability to induce an immune response to the recombinant *Brucella* antigen(s).

The approach which gave the best results was screening of the genomic libraries and based on this approach the main methodology used can be outlined as follows:

I. Express *Brucella* antigens in genomic expression libraries and detect potentially protective antigens by either or both:

1. Reactivity of the recombinant clones with specific antibodies of the IgG2a subisotype found in immunized animals (IgG2a is considered as a potential indicator of Th1 involvement) and

2. Ability of the recombinant clones to stimulate the lymphocytes from animals immunized with the protective *B. abortus* strain RB51 vaccine by testing for their ability to proliferate and produce INF-γ upon exposure to such antigens. At later stages of the project we added cytotoxicity assays to detect induction of T cytotoxic (Tc) cells and flowcytometric analysis.

II. Subclone genes identified by the above outlined procedures into vaccinia virus (Western Reserve and/or Wyeth strains). Test the recombinants for their ability to express the antigens *in vitro* and their ability to induce protection in Balb/C mice. Analyze to some extent the character of the immune response induced by the recombinant vaccine and if protective, analyze in detail the immunological parameters associated with protection.

III. In order to obtain unequivocal results for in vitro CMI analysis, antigens have to be purified. Three main approaches were used: a) overexpression of specific genes in *E.coli* as fusion proteins of either thioredoxin (pThio-His expression system, Invitrogen, Inc.) or maltose binding protein (pMal expression system, Novagen) and purification of the recombinant proteins on metal or amylose affinity chromatography. b) ion exchange purification of *Brucella* recombinant proteins from *E.coli* and c) production of the *Brucella* antigens of interest in bacculovirus.
VI. BODY.

A. Detection and characterization of potentially protective antigens.

This project has detected various Brucella proteins which could be involved in the protective immune response. Genes of most of these proteins have now been sequenced and characterized. Some of these genes are being or have been cloned into vaccinia for protection and immune response studies.

1. Preparation of antisera and sensitized lymphocytes.
In order to prepare antisera to be used in the detection of antigens goats and mice were immunized as follows:

1. Two Goats were immunized three times with killed (therefore preventing any potential infection), whole or sonicates of B. abortus strain RB51 cells emulsified in adjuvant (goats 47 and 48).
2. BALB/C mice were vaccinated with B. abortus strain RB51 or with B. melitensis strain VTRMI (produced in our laboratory). Groups of 5 to 10 mice were vaccinated once or twice intraperitoneally (ip) with 1 x 10^8 viable organisms and sera obtained 4 weeks after the last immunization were pooled. Preimmunization sera from nonimmunized animals are used as negative controls. Sera were absorbed with whole and sonicated E. coli before use in the screening assays to avoid non-specific background reactions.
3. Sensitized lymphocytes were produced as follows: Splenic lymphocytes obtained from mice 6 to 8 weeks post ip vaccination with strain RB51 were used as a T cell source. Three to 4 mice were immunized weekly when appropriate to keep a continuous supply of lymphocytes available. Preimmunization lymphocytes from nonimmunized animals are used as negative controls.
4. Monoclonal antibodies specific for INF-γ were obtained in the laboratory from hybridoma supernatants and purifying the monoclonal by affinity chromatography. The pure monoclonals were used as the capture antibody in the INF-γ ELISAS.

2. Genomic libraries prepared, screening procedures and antigens selected.

Lambda gt1 library.
The lambda gt1 library was initially obtained from Dr. R. Warren (WRAIR). Genomic DNA from Brucella melitensis l6M was partially digested with HaeIII giving fragments ranging from 15 to 1 Kb, the majority being 2.5 to 3.0 Kb. After precipitation, the DNA was treated with EcoRI methylase followed by attachment of EcoRI linkers. The linkers were then cut with EcoRI allowing subsequent ligation to EcoRI cut Lambda gt1 arms. The ligation mixture was packaged in vitro and used to infect E. coli Y109O. The lambda gt1 expression library was
screened by plaque lifts using goat anti-*B. abortus* RB51 serum (goats 47 & 48). Unfortunately the goat serum had a high background titer to *E. coli* which was very difficult to eliminate even after adsorption of the serum with intact, sonicated, and lambda gt11 lysed *E. coli*. To check that the library was expressing proteins detectable using the goat 47 & 48 serum, Western blot analysis and dot blot analysis of the whole library were done. There were several weakly detected proteins in the size range of 45 to 66 kDa that were not seen in either *E. coli* only or *E. coli* infected with native lambda gt11; no fusion proteins were detected. Further immunological screening of the library was done by plaque lift after adsorption of the serum, but most of the putative positive phage were negative on re-screening indicating that the frequency of serologically positive clones was extremely low, consistent with others (23). Lysates of the suspected positive phage were screened along with lysates from pools of different phage recombinants (pools of 10, 50, and 100) using the lymphocyte transformation assay (LTA), all lysates having been standardized for protein content. Once again the relatively low levels of expressed proteins compared to the high background stimulation by *E. coli* in this system proved problematic.

**E. coli** Cl1 bank and antigen selection.  
Due to the low frequency of positive clones being found in the lambda gt11 library we also constructed a *Brucella* gene bank in *E. coli*. Genomic DNA from *Brucella abortus* strain 2308 was partially digested with the restriction enzyme Clal. DNA fragments of approximately 5Kb were cloned into the Clal site within the multiple cloning site of plasmid pBBRIMCS. The resulting "Cl1 bank" was subsequently transformed into *E. coli* strain DH5a and transformants screened to determine the average insert size as well as to confirm a variety of different clones had been generated. Analyzing 2500 clones would screen a genomic equivalent.

Two approaches for screening this library were undertaken:  
a) Serological screening and,  
b) screening of sensitized lymphocytes using the LTA and INF-g, II-2 and II-4 detection systems based on a commercial ELISA kits.

Before doing extensive amounts of work with this library, dot-blot and western blot analysis were performed to check that there were clones present expressing *Brucella* proteins. Dot blot results indicated that the library contained clones that express *Brucella* proteins, however, the size range could not be determined as the reactivity on Western blot was weak. On secondary screening of all putative positive clones, there was a total of 15 IgG positive clones: 9 clones IgG positive with both goat and mouse sera, 4 more that were positive with mouse sera only and 1 more that was positive by goat serum only. All the confirmed mouse positive clones were found to react with mouse antibodies of the IgG2a subisotype indicating potential involvement of a Th1
lymphocyte response with INF-γ production. All the 15, IgG positive clones were tested for their ability to stimulate T cells from sensitized mice to produce INF-γ, an indicator of T cell stimulation but not B cell stimulation. Also, since INF-γ plays a major role in facilitating the elimination of Brucella from macrophages, antigens that induce production of INF-γ should have a high probability of being involved in protective immunity. Preliminary data indicated that 11 of the 15 clones stimulate interferon gamma production. All 15 of the clones stimulated lymphocytes to produce a small amount of interleukin-2 (IL-2) but no interleukin-4 (IL-4) indicating that the antigens being selected are most likely Th1 and not Th2 inducers.

The following Brucella antigens obtained from the E.coli clones were eventually selected for characterization and cloning into vaccinia: YajC, SecD, 18kDa, 16kDa, GroEl, GroES and Cu/Zn SOD. Additional antigens were identified but more work is needed to understand their potential importance. These are: TolB (38% similarity to colicin transport), L15 (17% similarity; ribosomal protein on 50S subunit) and Pal (26% similarity; peptidoglycan associated lipoprotein).

In order to detect additional antigens possibly associated with protection but which may not be detectable by the above outlined screening methods (appearance of specific antibodies or detection of reactive T cells using proliferation or INF-γ detection assays) we have also looked for potential virulence factors of Brucella. Immune responses to virulence factors do not have to be necessarily associated with protection but, often protective antigens are virulence factors. Therefore, isolation, identification and characterization of Brucella genes that may be responsible for the intracellular survival and or ability of the parasite to persist in animals appeared to be pertinent to this project.

We prepared a B. abortus chromosomal library in pBR322 (sal) and a smaller B. melitensis chromosomal library in pBlueScript (HindIII) in E.coli and tested them for their ability to survive in mice for 48 hr after intraperitoneal (ip) injection. Approximately 10 to 12 E. coli clones were pooled and injected simultaneously into mice in order to accommodate the large number of clones being tested for increased virulence. The E.coli strains with just the vectors (pBR322 and pBlueScript) served as controls. It was determined that 10^8 colony forming units (c.f.u) of these control E.coli were cleared within 24 hours, when given ip.

Twelve pools of E. coli clones from the B. abortus library and four pools from the B. melitensis library survived for over 48 hours in the spleens of mice after ip inoculations. The vast majority of the pools were cleared from the mice within 48 hours. At this stage we are concentrating our efforts on 4 clones able to
survive in mice for more than 48 hours and which were isolated from the _B. melitensis_ library (table 1). The clones were selected by isolating individual colonies on plates and testing for the presence and characterization of their plasmids.

Table 1. Preliminary Analysis of Sequencing data for the following four pBSMB clones

<table>
<thead>
<tr>
<th>Clone # the clone</th>
<th>Insert Size in kb (Fig.1)</th>
<th>Completed Sequence (bp)</th>
<th>Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBSMB31</td>
<td>~6-7</td>
<td>~1939</td>
<td>VACB Protein</td>
</tr>
<tr>
<td>pBSMB28F</td>
<td>~5-6</td>
<td>~712</td>
<td>ATP synthase</td>
</tr>
<tr>
<td>pBSMB16</td>
<td>~1.6</td>
<td>~1265</td>
<td>Transcriptional Regu</td>
</tr>
<tr>
<td>pBSMB12</td>
<td>~2.8</td>
<td>~800</td>
<td>unknown</td>
</tr>
</tbody>
</table>

From the above results, it can be concluded that three clones (pBSMB 31, pBSMB 28, and pBSMB 16) may be associated with intracellular survival or virulence in general. We will continue to study these clones and genes they contain to assess if they are involved in protection.

c. Immunological characteristics of selected antigens.

Some immunogenic characteristics of the selected antigens, effect of their deletion in vaccine strain RB51 and status regarding antigen purification and cloning into vaccinia are summarized in table 2.

As can be observed selected antigens 18kDa, 16kDa, GroEL, GroES, SOD and YajC are involved in the immune response to _Brucella_ and represent potential candidates for protective antigens. L7/L12 is a protective antigen described by others and is being included because of this fact. 18kDa deletion mutants of vaccine strain RB51 protect as well as the parent vaccine strain indicating that although the 18kDa antigen has the right in vitro immunological profile (induction of IgG2a antibodies and lymphocyte proliferation with INF-γ production) it may not be a protective antigen. On the other hand, deletion of SOD reduces the protective ability of the vaccine strain suggesting the potential importance of SOD in the induction of a protective immune response. Very little was known about protein YajC in Brucella and YajC in general. A special effort was made to characterize this protein and its gene.
Table 2
Proteins (Antigens) in use and stages of characterization.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>IR in Brucella exposed humoral</th>
<th>CMI INFγ LTA</th>
<th>ΔRB51</th>
<th>Δeffect on protection</th>
<th>purified</th>
<th>in vaccinia</th>
</tr>
</thead>
<tbody>
<tr>
<td>18kDa</td>
<td>yes</td>
<td>yes yes</td>
<td>yes</td>
<td>none</td>
<td>pmal</td>
<td>yes</td>
</tr>
<tr>
<td>16kDa</td>
<td>(yes)</td>
<td>NED yes</td>
<td>IP</td>
<td>ND</td>
<td>pmal</td>
<td>IP</td>
</tr>
<tr>
<td>GroEL</td>
<td>yes</td>
<td>NED yes**</td>
<td>Lethal</td>
<td>ND</td>
<td>pmal&amp;thio</td>
<td>yes</td>
</tr>
<tr>
<td>GroES</td>
<td>no</td>
<td>yes ND</td>
<td>Lethal</td>
<td>ND</td>
<td>thio</td>
<td>IP</td>
</tr>
<tr>
<td>SOD</td>
<td>no*</td>
<td>yes weak</td>
<td>yes</td>
<td>decrease</td>
<td>thio,native ionexch</td>
<td>yes</td>
</tr>
<tr>
<td>YajC</td>
<td>yes</td>
<td>yes yes</td>
<td>yes</td>
<td>ND</td>
<td>pmal</td>
<td>IP</td>
</tr>
<tr>
<td>SecD</td>
<td>no</td>
<td>NED NED</td>
<td>IP**</td>
<td>ND</td>
<td>pmal(NC)</td>
<td>ND</td>
</tr>
<tr>
<td>TolB</td>
<td>yes@</td>
<td>yes@ yes@</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L7/L12</td>
<td>yes</td>
<td>yes# yes#</td>
<td>Lethal</td>
<td>ND</td>
<td>pmal</td>
<td>IP</td>
</tr>
</tbody>
</table>

NED: Not enough data; IP: in progress; ND: Not done yet, NC: not completed
* = depends on animal species
** = A ΔYajc will also be A Δ SecD; *** = to be confirmed
@ = Observed with whole clone (may include additional gene products)
# = Splitter

YajC/SecD. One selected positive clone (clone E. coli MCB 68) contained an insert of 2.6 kb; nucleotide sequence analysis of this insert revealed two open reading frames (ORF). The deduced amino acid sequences of the first and second ORFs had significant similarities with the YajC and SecD proteins, respectively, of several bacterial species. Both the YajC and SecD proteins were expressed in E. coli as fusion proteins with maltose binding protein (MBP). In Western blots, sera from mice vaccinated with B. abortus strain RB51 recognized YajC but not SecD. Further Western blot analysis with purified recombinant YajC protein indicated that mice inoculated with B. abortus strains 19 or 2308, or B. melitensis strain RM1 also produced antibodies to YajC. In response to in vitro stimulation with recombinant MBP-YajC fusion protein, splenocytes from mice vaccinated with B. abortus strain RB51 were able to proliferate and, importantly, produce interferon-gamma (table 3), but not IL-4 indicating a type Th1 response. This demonstrates, for the first time, the involvement of YajC protein in an immune response to an infectious agent. Expression of B. abortus YajC and partial SecD proteins was achieved in E. coli as maltose binding protein (MBP) fusions.
Table 3. Production of Interferon-γ by splenocytes of RB51 vaccinated and naive mice after in vitro stimulation with specific antigens and mitogen.

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Interferon-γ (ng/ml) in splenocyte culture supernatants (Mean ± Standard deviation; n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vaccinated mice</td>
</tr>
<tr>
<td></td>
<td>3 day cultures</td>
</tr>
<tr>
<td>Media</td>
<td>0</td>
</tr>
<tr>
<td>ConA</td>
<td>30.55 ± 0.65</td>
</tr>
<tr>
<td>RB51</td>
<td>29.63 ± 1.00</td>
</tr>
<tr>
<td>MBP-YajC</td>
<td>0.42 ± 0.10</td>
</tr>
<tr>
<td>MBP</td>
<td>0</td>
</tr>
</tbody>
</table>

It is interesting to notice nevertheless that the whole, killed RB51 organisms induced highly significant higher levels of INF-γ when compared to the purified YajC::MBP.

16kDa protein. Originally, we received pRS44.8 (LSU); this is a recombinant plasmid of pRSET B containing the gene fragment (14 kDa fusion protein). It was thought that this was the gene fragment encoding the carboxyl terminus of a much larger, 60 kDa protein. Instead, our results independently confirmed that the entire gene encodes a much smaller 16 kDa protein. To obtain the amino-terminus of the 16 kDa gene, sets of primer pairs were designed by using Primer Select computer analysis software (DNASTAR, Inc., Madison, WI). The reverse primer, 14kDmid, was taken from known sequence of the carboxyl fragment, pRS44.8. Random primers, named P1-P5, were then used in concert (as forward primers) with 14kDmid. The primer pair P2-14kDmid produced a 518-bp fragment encoding for the amino-terminus; this was confirmed by Southern blot analysis. The PCR fragment was then cloned into pCR II (TA Cloning®, Invitrogen) and sequenced using the dideoxy-mediated chain termination method. This sequence data gave rise to the construction of the primer 16kDstart.

To obtain the full 16 kDa gene sequence, two primers, 16kDstart and 16rev were selected. Primer 16kDstart is a 36 basepair (bp) oligomer containing an internal BglII site. Primer 16rev is a 34 bp oligomer containing an internal KpnI site. PCR was performed and the 452 bp fragment was subcloned into pCR II. The resulting construct was designated pTA16start and was used to transform E. coli DH5α cells.

The entire 16 kDa protein gene is 441 bp (147 amino acids) in length. It contains a ribosomal binding site (RBS) upstream. Computer analysis of the deduced amino acid sequence indicated a signal sequence at the amino
terminus. Analysis of the nucleotide sequence indicated a potential prokaryotic promoter at 300 bp upstream to the start codon. Currently, we are testing the functional ability of this promoter region in *E. coli* and *B. abortus*.

**10.2kDa and 11.6kDa proteins.**

Two proteins (10.2 kDa and 11.6 kDa) present in an ammonium sulfate extract of strain RB51 (fraction S2) were identified which stimulated production of INF-γ by proliferating lymphocytes from RB51 vaccinated mice. The N-terminal amino acid sequences were determined. Amino acid sequences were back translated using the preferred codon usage of *Brucella* and the generated oligonucleotides probes were digoxigenin labeled. The oligonucleotide sequences used for the generation of probes were:

i.) for the 10.2kDa amino acid sequence:

```
gcctggccagtgtgggtacctgcatggcctggaagaagtt
```

ii.) for the 11.6 kDa amino acid sequence:

```
gccagccgatgtctactggctctgtgcagctgtgctgctgagtt
```

Unfortunately both the oligonucleotides probes failed to hybridize to the *Brucella* chromosomal DNA. As an alternative method we are planning to screen our *Brucella* genomic library for the presence of these proteins. We have prepared a specific antisera to these 2 proteins in goat (goat 52) since mice vaccinated with strain RB51 do not produce antibodies to these 2 proteins, although they do develop a good *in vitro* lymphocyte response to them. Goat (52) was immunized with the two specific proteins obtained by cutting the corresponding bands out of SDS-PAGE preparations of strain RB51 fraction S2. The bands were minced in physiological saline and emulsified in Freund's incomplete adjuvant. The goat was immunized subcutaneously 3 times with this preparation. The resulting antisera reacted very strongly with the 10.2 and 11.6 kDa proteins. We have now determined that the 10.2 kDa protein is GroES therefore, a specific sera for GroES is available.

**B. Purification of selected antigens.**

As mentioned before, selection of antigens as candidates of protective antigens is based on the ability of T cells from *Brucella* immunized mice to react with the antigens *in vitro* in proliferation assays and induction of INF-γ. In order to insure that the selected antigens are inducing these responses, it is important to have these antigens in pure form that can be achieved by using recombinant protein expression systems. In order to purify the expressed recombinant proteins, a practical route is to express them as fusion proteins. For this purpose we are overexpressing the selected *Brucella* genes in *E. coli* as fusion proteins of either thioredoxin (pThio-His expression system) or maltose binding protein (pMal expression system) and purification of the recombinant proteins on metal
or amylose affinity chromatography. This was done with commercial kits and modifications were introduced to optimize protein yield (into several mgs) and purity. Quantification of purified fusion proteins is carried out using BioRad microtiter protein assays and purity is assessed by Western blot analysis. We have now good quantities (in mgs range) of the following fusion proteins available for continuous testing: GroES::Thio-his, YajC::MBP, Cu/Zn SOD::Thio-his, Lysteriolysin::Thio-his and GroEL::MBP. The GroEL::MBP is missing approximately 30 aminoacids at the C terminal end of the GroEL. Because of this problem we are also producing a GroEL::Thio-his. Future work will use this GroEl fusion protein instead of the GroEL::MBP.

The complete genes of proteins L7/L12 and the 16 kDa antigens were obtained by PCR amplification. These antigens were also overexpressed as MBP fusion proteins in E. coli using the pMalC2 vector system. During the purification process it was observed that the MBP-L7/L12 fusion protein had low affinity to the amylose resin, leading to low quantities of purified protein. Hence, the MBP-L7/L12 protein has been purified from the E. coli extracts on an anion exchange resin (HiTrapQ) followed by size exclusion chromatography on Sephadex G-75.

The level of expression of the MBP-16 kDa fusion protein was much lower than the other fusion proteins. This could be because of the presence of a hydrophobic signal sequence at the amino terminus of the 16 kDa antigen. Presently, we are trying to increase the expression levels by removing the signal sequence.

The Cu/ZnSOD was also purified as a non-fusion native protein from E. coli. For this, the SOD gene along with its own promoter was cloned into a high copy number plasmid, pBluescript SK-. E. coli containing this plasmid expressed high levels of SOD. This SOD was purified by a Triton-X100 treatment of the E. coli cells followed by an anion exchange chromatography on a HiTrapQ column. The Triton-X100 treatment released mostly the periplasmic proteins of E. coli of which the SOD comprised a major proportion. On the anion exchange column all the proteins except SOD bound to the column. This method of purification resulted in high quantities of purified SOD in its native form.

Purification of recombinant fusion proteins from E. coli even by affinity chromatography can not always guarantee 100% purity. This is a problem if impurities have biological effects on our in vitro immune response testing system. Also, the fusion partner may influence the in vitro response. In order to have an additional antigen purification method that would not utilize fusion proteins, we selected the expression of antigens in Baculovirus (BV). Also, in vitro recombinant BV production systems utilize components (insect cells lines, insect virus) which are unlikely to stimulate non-specific reactions in our in vitro testing systems.
The BAC-TO-BAC Baculovirus expression system of GibcoBRL (Grand Island, NY) was used to yield Baculovirus recombinants. It is based on site-specific transposition of an expression cassette into a BV shuttle vector (bacmid) propagated in E. coli. Genes to be expressed are inserted into the multiple cloning site of pFASTBAC1 downstream from the polyhedrin promoter.

*B. abortus*-pFASTBAC1 donor plasmids were generated for the 60 kDa molecular chaperon GroEL, the 10 kDa GroES and the 54 kDa heatshock protein HtrA. The identity of the cloned *B. abortus* genes were demonstrated by hybridization of digoxigenin-labeled DNAs to Southern blotted restriction enzyme digests of the pFASTBAC1 donor plasmids and recombinant bacmids.

*Spodoptera frugiperda* (Sf9) cells were transfected with the recombinant bacmid DNA using CELLFECTIN reagent. Recombinant virus was harvested at 72 hrs. To analyze the protein expression, large volume of cultures was infected with recombinant viruses and the cells were harvested at 48 hours post-infection. Samples of lysed and infected cells were analyzed on 10 % or 15 % SD S-PAGE gels followed by Western blotting. Western membranes were incubated with polyclonal goat anti-*B. abortus*, polyclonal goat anti-GroES (Goat 52), monoclonal mouse anti-GroEL, or monoclonal mouse anti-HtrA antibodies. Bound antibodies were detected with goat anti-mouse IgG or rabbit anti-goat IgG, conjugated to horseradish peroxidase, using 4-chloro- 1 -naphthol. Recombinant viruses were titrated using Sf9 cells and the titers were calculated by the method of Reed and Muench and expressed in median infective dose per ml (TCID50/ml). We have used the Sf9 cells infected with *Brucella* antigen expressing BV as antigens for in vitro lymphocyte transformation assay using lymphocytes from non-immunized normal mice and have determined that these preparations will not cause non-specific stimulations of lymphocytes. This opens the possibility that the *Brucella* expressing BV can be used as specific antigens in our in vitro systems. Preliminary mouse immunization experiments with the recombinant BV indicate that these viruses can induce specific immune responses to the *Brucella* proteins.

**C. Cloning into Vaccinia virus.**

1. Characteristics of Early vaccinia/Brucella recombinants expressing *Brucella* HtrA, GroEL or Cu/Zn SOD.

Crucial to the success of this proposal was to demonstrate that vaccinia virus is able to express *Brucella* proteins and that animals vaccinated with these recombinants are able to recognize the expressed *Brucella* proteins. To test this point we cloned into vaccinia virus Western reserve (WR) strain the gene coding for the *Brucella* Cu/Zn SOD (recombinant WRpUBSOD) and the gene coding for the *Brucella* High temperature requirement A (HtrA) protein (recombinant WRpSBHtrA). We selected these 2 genes because of our previous experience
with these proteins and the availability the DNA sequences in our laboratory.

The results of this study clearly indicated that vaccinia virus can express Brucella proteins using our methodology since both proteins, Cu/Zn SOD and HtrA were expressed in vitro. We also demonstrated that this expression occurs in vivo since the mice responded immunologically to the HtrA protein. Mice did not respond immunologically (at least not with a humoral immune response) to the Cu/Zn SOD protein. This findings is not surprising since mice do not respond to this protein even when immunized with Brucella probably because of the similarity which exists between Brucella and the mouse Cu/Zn SOD (24). In addition to the production of vaccinia recombinants expressing Brucella proteins mentioned above we have constructed a GroEl/ vaccinia recombinant which expresses the whole GroEl protein using the Wyeth strain (WySBGroEL) and a protection study was completed. The following vaccinia/ Brucella recombinants were used in this protection study:

BALB/c mice were vaccinated once with either vaccinia recombinants WySBGroEL, WRPcSBHtrA and WRPcUBSOD and challenged with virulent B. abortus strain 2308 seven weeks later or vaccinated twice 3 weeks apart and challenged four weeks after the second vaccination. Mice were killed at 1 week post challenge and colony forming units (CFU) of Brucella per spleen enumerated and expressed as log10 values. Animals were bled before vaccination and at various intervals thereafter. The dose of vaccination was 10^7 median tissue culture infective doses (TCID50) recombinant viruses in 0.1 ml PBS intraperitoneally.

WySBGroEL recombinant did not replicate in mice based on lack of antibody production against both, vaccinia proteins and the Brucella GroEL. This observation corresponds with those of other workers concerning recombinants developed with the Wyeth strain. The other 2 recombinants did replicate in mice as reported previously (24) and produced antibodies to vaccinia proteins. The HtrA recombinants induced antibodies to Brucella HtrA but the SOD recombinant did not produce antibodies to the Brucella CuZn SOD. None of the recombinants protected against challenge with virulent Brucella while vaccination with B. abortus vaccine strain RB51 induced significant protection (Table 4). The lack of protection by the GroEL recombinant was not unexpected since it did not replicate in mice and therefore the Wyeth strain should not be used vaccinia. Two vaccinations with the recombinant expressing HtrA enhanced infection with the challenge strain suggesting that the HtrA antigen may have a deleterious effect on the protective immune response to Brucella. The data also indicate that two inoculations with vaccinia, 3 weeks apart will induce some level of non-specific immunity to Brucella (see statistically significant difference among groups 3, once vaccinated or twice vaccinated). Data also confirms previous non published observations that two immunizations with RB51 lead to higher protection levels than 1 vaccination.
Table 4. Vaccination with Vaccinia/Brucella recombinants. Results

<table>
<thead>
<tr>
<th>1 vaccination</th>
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<tr>
<td>Group</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

* No statistical differences

Psc11: Vaccinia recombinant not expressing *Brucella* antigens (negative control)

<table>
<thead>
<tr>
<th>2 vaccinations</th>
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<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

** statistically different from groups 3 and 4.

Group 3, 1 vaccination different from group 3, 2 vaccinations  p = 0.0477

Analysis of the immune response to *Brucella* antigens induced by the other 2 recombinant vaccines (WRpSBHtrA and WRpUBSOD) indicated a strong anti-
HtrA antibody response after one vaccination but did not reveal anti SOD antibodies. The anti HtrA antibody response is not expected to be protective since passive transfer of anti HtrA antibodies does not confer protection. Potentially, a CMI response to either HtrA or SOD could be protective since T cell transfer experiment with RB51 sensitized T cells is protective in mice. Therefore, we tested recombinant vaccinated mice for a CMI responses in vitro using lymphocyte transformation assays (LTA) and INF-γ production and in vivo using foot-pad DH reactions.

Mice vaccinated once with the HtrA and SOD vaccinia recombinants and the appropriate controls were tested at 7, 11 and 13 weeks post immunization using the corresponding purified antigens. We were unable to demonstrate a definitive in vivo or in vitro CMI response in any of the vaccinia vaccinated mice. These data suggest that the present constructs may not be inducing CMI against either HtrA or SOD that may explain the lack of protection. We believe that the failure to induce a strong and detectable CMI response to the Brucella antigens by the vaccinia recombinants may be due to the type of promoter we have used in our constructs i.e they do not lead to high expression of the recombinant proteins.

2. **WR vaccinia/Brucella GroEL recombinant.**

Since we discovered that the WySBGroEL recombinant would not replicate in mice new constructs were made with the WR vaccinia using an early/late vaccinia promoter p7.5 (fig 2).

![Diagram of recombinant plasmid pSBGroEL](image)

**Fig 2.** Diagram of recombinant plasmid pSBGroEL. A 1.7 kb fragment containing the *B. abortus* groEL gene (cross hatched line) was cloned into the
shuttle vector pSC11 (light gray regions). The early/late vaccinia virus promoter p7.5 (solid arrow) regulates expression of the groEL gene and a late vaccinia virus promoter p11 (dotted arrow) regulates the expression of the lacZ gene. The vaccinia virus thymidine kinase sequences (TKL, TKr) flank the expression cassette controlled by the p7.5 and p11 promoters.

This recombinant, denominated WRSBGroEL, was tested for its ability to induce specific antibodies and selected cell mediated immune responses in mice as well as being able to protect these animals against challenge.

Construction of WRSBGroEL (similar strategies have been followed with some modification for the production of additional vaccinia/Brucella recombinants described below).

In order to prepare the vaccinia virus expression vector coexpressing B. abortus GroEL heat shock protein, plasmid pBA2168 was digested with restriction enzymes EcoRI, SalI and DraI. Overhanging ends were filled in and the resulting 1.7 kb fragment containing the B. abortus groEL gene was purified from 1% agarose gel, the fragment was ligated into the SmaI site of shuttle plasmid pSC11 to produce plasmid pSBGroEL. Human thymidine kinase deficient 143B cells (HuTK- cells) were grown to 80% confluence in Eagle’s Minimum Essential Medium (EMEM) containing 5% fetal bovine serum (FBS) in 25 cm² flasks, infected with vaccinia virus strain Western Reserve (WR) (ATCC) at a multiplicity of infection (MOI) of 0.05 and incubated. One μg of pSBGroEL was dissolved together with lipofectin, incubated for 25 minutes at room temperature and the mixture (100μl) was added to 1 ml of EMEM and added to WR infected HuTK- cells at 80% confluence. After a 4+ cytopathic effect (CPE) had developed, the cells were ruptured by 3 cycles of freezing in liquid nitrogen and thawing at 37°C. The cell lysates, containing the putative recombinant virions, were serially diluted in 10-fold steps and subcultured onto a new monolayer of HuTK- cells in flat-bottom six-well plate with EMEM containing 25 μg of bromodeoxyuridine (BdUR) per ml for selection of recombinant virus. Following a 4+ CPE development, the medium was aspirated and the infected cells were overlaid with 1 ml of plaquing media (2x EMEM with 50μg BdUR) containing 0.6 mg/ml of Bluo-gal. Blue plaques, produced by replicating recombinant virions expressing the lacZ gene, were collected and used to enhance the virus content of the plaques by inoculating a confluent layer of HuTK- cells in either 25 cm² flasks or six well tissue culture plates. CPE and the presence of blue plaques in the cell monolayer assessed replication of the recombinant virus. Recombinant virus was harvested, plaque purified and enhanced by infecting larger volumes of cell monolayers two more times to develop the recombinant virus WRSBGroEL. Same experiments were also carried out for to generate the vaccinia virus recombinant WRSC11 (control) with shuttle vector pSC11.
Recombinant virus was tested for infectivity, purity and ability to induce GroEL antibodies in mice. 10⁴ plaque forming units (pfu) of recombinant virus were injected into each of three BALB/C mice. One of the mice was killed six days later and the recombinant virus was isolated from ovaries and tested for CPE and β-galactosidase activity in HuTK⁻ cells. Mouse was confirmed infected. The second mouse was killed on day thirteen post infection and the same procedures were followed in order to isolate and to test the infectivity of the recombinant virus in the cell culture. The third mouse was kept alive to obtain serum for serological tests. After isolating the virus from the second mouse, the recombinant virus was plaque purified again and enhanced three more times in order to reach 10⁷ pfu/ml, the desired infective dose for mice vaccination. Recombinant expression of Brucella GroEL was confirmed by western blotting using anti- B. abortus strain RB51 goat serum (fig. 3).

Fig. 3. Western blot analysis of vaccinia virus recombinant WRGroEL (2 different stocks) and plasmid control (WRSC11) and B. abortus strain RB51 antigens with goat anti-RB51 serum

Immunization for protection studies was carried out as follows: Five groups of 8 female BALB/C mice each were used. The first and second groups were injected with 10⁷ pfu of WRSBGroEL intradermally (id) and intraperitoneally (ip) respectively. The third group was inoculated ip with 10⁷ pfu/ml of vaccinia virus/shuttle plasmid recominant WRSC11. The fourth group received 2.6 x 10⁶ colony forming units (cfu) of B. abortus rough strain RB51 (ip) as a positive control for protection and the fifth group received saline ip. Five mice out of each group were challenged with virulent B. abortus 8 weeks post immunization and
killed 2 weeks later to assess the number of colony forming units (CFU) in the spleen. The remaining mice were used for serology and in vitro lymphocyte proliferation using various antigens including GroEL::MBP as the specific antigen. GroEL::MBP was missing the last 30 carboxyl end amino acids of GroEL.

Results can be summarized as follows: Mice vaccinated with the WRSBGroEL developed IgG class antibodies specific for *Brucella* GroEL, lymphocyte proliferation assays results were not conclusive and protection was not observed. Further studies are underway to understand why protection was not induced. The lack of *in vitro* lymphocyte stimulation may be related to the use of GroEL::MBP as the *in vitro* stimulating antigen, as mentioned above the GroEL was missing the last 30 amino acids and it is possible that the stimulatory epitopes are concentrated in that area. The detailed work with this recombinant is available in a recently MS thesis (Assessment of the expression of *Brucella abortus* heat shock protein, GroEL, in vaccinia virus to induce protection against a Brucella challenge in BALB/c mice. Simge Bologlu, Master of Sciences, Virginia Tech, June 1997).

3. **WR vaccinia/Brucella recombinants expressing the 18kDa antigen using synthetic early/late promoters.**

In general, we now believe that the nature of the promoter (p7.5) used in the previous constructions does not lead to the appropriate *in vivo* expression levels of GroEL needed to induce an appropriate CMI response and therefore, no protection is observed. This possibility had to be explored before more complex issues like appropriateness of GroEL presentation when delivered by vaccinia are investigated. For this reason, new vaccinia recombinant constructs were carried out using a synthetic early/late promoter.

As mentioned above, the lack of a strong specific CMI response in mice vaccinated with the recombinant vaccinia viruses could be due to the nature of the promoters present on the cloning vectors used in the preparation of the recombinant viruses. Recently, shuttle vectors (pMCO2 and pSC65) with a synthetic early/late promoter were developed. Glucoronidase is the marker enzyme in pMCO2 vector whereas in pSC65 it is β-galactosidase (fig. 4). Expression of the cloned genes by the recombinant vaccinia viruses prepared with these new vectors is 1000 times over natural early or late vaccinia promoters.

Higher levels of *Brucella* protein expression by the recombinant vaccinia viruses should enhance the specific and hopefully appropriate CMI responses in the vaccinated mice leading to a better chance of protection against a virulent *Brucella* strain challenge.
Fig. 4. Schematic representation of the vaccinia shuttle vector pSC65 where *Brucella* genes can be cloned under a synthetic early/late promoter. Note: pMCO2 contains glucoronidase as the marker enzyme, whereas pSC65 contains β-galactosidase.

Based on this rationale, we cloned the 18kDa gene in shuttle vector pMCO2 and recombinant vaccinia virus was constructed using the WR strain (vWRMC18). Mice were vaccinated ip 10⁷ pfu/mouse with vWRMC18 and bled at several time intervals post vaccination. Strong antibody responses were observed against the 18kDa antigen and the vaccinia virus antigens confirming replication of the virus as well as *Brucella* antigen recognition (fig. 5).

Fig. 5. Extracts from cells infected with the recombinant vaccinia virus containing the 18 kDa protein gene either in the wrong orientation (lane 1) or in the right orientation (lane 2), and *B. abortus* strain RB51 (lane 3) were reacted...
with sera from mice vaccinated with *B. abortus* strain RB51 (panel A), the recombinant vaccinia virus expressing the 18 kDa protein (panel B), and the recombinant vaccinia virus not expressing the 18 kDa protein (panel C).

An analysis of the immunoglobulin isotypes involved in the humoral immune response against the 18kDa protein revealed that both, vWRMC18 immunized mice and RB51 immunized mice produced IgG2a antibodies against the 18kDa protein (table 5).

### Table 5

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>ELISA reading against 18kDa antigen (mean +/-SD)</th>
<th>IgG1</th>
<th>IgG2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWR18kDa</td>
<td>1.16 +/- 0.44</td>
<td>0.67 +/- 0.02</td>
<td>0.74 +/- 0.25</td>
</tr>
<tr>
<td>VWR</td>
<td>0.08 +/- 0.06</td>
<td>0.01 +/- 0.004</td>
<td>0.01 +/- 0.01</td>
</tr>
<tr>
<td>RB51</td>
<td>1.91 +/- 0.26</td>
<td>0.002 +/- 0.001</td>
<td>1.35 +/- 0.21</td>
</tr>
</tbody>
</table>

Preliminary flowcytometric analysis of whole blood lymphocyte from vaccinated mice indicate that that the CD4+ cells are responding to the specific 18kDa protein but not the CD8+ cells as indicated by the number of CD69 expressing cells while the total number of CD4+ and CD8+ remains constant (tables 6 and 7).

Lymphocytes obtained from the spleens of these mice proliferated *in vitro* upon stimulation with purified MBP-18 kDa fusion protein and secreted IFN-γ into the culture supernatants. The *in vitro* CMI results combined with the strong antibody responses to 18 kDa, indicate that the synthetic promoter is achieving better results than the promoters used before and encouraged the use of the promoter in future constructions.

The lack of protection in spite of the development of strong humoral and cell mediated immune responses to the 18kDa protein could therefore be related to the following two reasons among others:

a) the 18 kDa antigen is not at all involved in the induction of protection against *Brucella* infection or

b) the recombinant vaccinia virus is unable to express *Brucella* antigens in a way leading to correct processing an induction of protective CMI.

To test the first possibility, we constructed a 18kDa-disruption mutant of vaccine strain RB51 by inserting a kanamycin cassette into the 5' end of the 18kDa gene. Mouse protection experiments performed with this mutant RB51 strain, showed similar level of protection as those obtained with the vaccine strain
strongly suggesting that the 18 kDa antigen is not involved in the elicitation of a protective immune response. This is probably the most likely explanation for the protective failure of vWRRMC18. The protection results obtained from this experiment emphasize the importance of using antigens of proven protective capabilities for the construction of the vaccinia recombinants. Therefore, we are now also constructing RB51 disruption mutants using those antigens selected for cloning into vaccinia to asses if they play a significant role in protection. Obviously, no disruption mutants can be obtained with genes that are essential to _Brucella_ survival (groEL, groES).

<table>
<thead>
<tr>
<th>Table 6. Percentages of CD69-expressing cells in CD4+ and CD8+ T cells, in peripheral blood, 4 weeks after vaccination. Cells were activated with 3.6 ug/mL 18 KDa protein for 8 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
</tr>
<tr>
<td>Vaccinia 18 KDa</td>
</tr>
<tr>
<td>Vaccinia 1.4</td>
</tr>
<tr>
<td>RB51</td>
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<tr>
<td>PMA+ionomycin</td>
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</table>

<table>
<thead>
<tr>
<th>Table 7. Percentages of Total CD4+ and CD8+ T cells, in peripheral blood, 4 weeks after vaccination.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CD4</td>
</tr>
<tr>
<td>Vaccinia 18 KDa</td>
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<tr>
<td>Vaccinia 20.9</td>
</tr>
<tr>
<td>RB51</td>
</tr>
<tr>
<td>PMA+ionomycin</td>
</tr>
</tbody>
</table>

To test the second possibility (the recombinant vaccinia virus is unable to express _Brucella_ antigens in a way leading to correct processing an induction of protective CMI), we will be testing the effectiveness of our vaccinia delivery system by constructing a recombinant virus expressing the listeriolysin protein of _L. monocytogenes_. Listeriolysin is a well demonstrated protective antigen in murine listerial infections (20). The gene for listeriolysin was PCR amplified from the genomic DNA of _L. monocytogenes_ strain EGD. Primers for the
amplification was designed based on the published sequence information (21). The part of the gene coding for the signal sequence was not included for the amplification. The amplified fragment was cloned in vaccinia shuttle vector pSC65 and recombinant virus was prepared using WR strain. Upon an initial screening, several recombinant viral plaques expressing the marker enzyme β-galactosidase were obtained. Clear expression of listeriolysin by these recombinant viruses is yet to be demonstrated. The use of recombinant vaccinia virus expressing listeriolysin in mice should demonstrate protection against challenge with L. monocytogenes and serve as a positive control for our vaccinia delivery system. Failure to protect mice against L. monocytogenes challenge by this recombinant would indicate that our vaccinia system is not an appropriate delivery system for protective Brucella antigens and that further research has to be carried out on the expression system itself before a successful vaccine can be engineered.


Production of INFγ is considered to be one of several crucial immunological events necessary to obtain protective immunity against Brucella. However, when challenged with the virulent B. abortus strain 2308, these mice did not show any level of protection. Although the immunization with vaccinia/18kDa protein did induce an in vitro INFγ response to stimulation with 18kDa::MBP, one can not assume that protection must be observed. Other immunological events may have to occur simultaneously, like the production of specific cytotoxic T cells, to obtain protective CMI. We have now standarized cytotoxicity assay in the laboratory and are testing for the presence of Brucella specific cytotoxic lymphocytes in recombinant vaccinia immunized mice. Establishment and standardization of this test using 51Cr and/or neutral red release from mouse macrophage cells lines (targets) took several months of work. A detailed description of the test follows and figure 6 compares de 51Cr release assay to the neutral red assay. Stepwise description of test:

1. Infect J774 with Brucella (RB51) usually 100 RB51: 1 Macrophage, incubate overnight (about 12 hrs) in a flask.
2. Wash with gentamycin containing media (add media with 50ug/ml to flask, leave mixing slowly for 5 minutes), remove cells and centrifuge. Resuspend in media with 50 ug gentamycin.
3. Do viable count.
4. Add to 96 well plate (round bottom) 50,000 macrophages per well. Ready to use. Also prepare plates with uninfected macrophages.
5. Get splenocytes from normal and immunized mice.
6. Separate on nylon wool columns (get “fall through” cells mostly T cells).
7. Do viable count
8. Add different proportion of T cells: 12:1 (T cell : Macrophages), 6:1, 3:1, 1.5:1.
9. Incubate overnight (12 hrs not less!).
10. Remove the media.
11. Add neutral red (0.036% in PBS) 200ul.
12. Incubate for 45 minutes
13. Remove the solution and wash cells with PBS very careful so you don’t detach or damage cells (3 times).
14. Lyse cells with acetic acid 0.5M + 10% SDS (200ul/well). Pipette up and down several times.
15. Add solution to flat bottom ELISA plate (for reading in machine, round bottom not very good).

Fig 6. Comparison of the 51Cr and neutral red cytotoxicity assays.

Comparison of colorimetric and 51Cr release assays

<table>
<thead>
<tr>
<th>Effector/Target Ratio</th>
<th>% specific lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:1</td>
<td>30</td>
</tr>
<tr>
<td>5:1</td>
<td>20</td>
</tr>
<tr>
<td>2:5:1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Colorimetric assay:
- ▲ Lymphocytes of RB51 vaccinated mouse
- ▼ Lymphocytes of normal mouse

51Cr release assay:
- ▲ Lymphocytes of RB51 vaccinated mouse
- ▼ Lymphocytes of normal mouse

Fifty thousand target cells were used in all the experiments. All experiments were performed in triplicates. Only the mean values were used in the graph. The standard deviations within the experiment were below 10% of the mean values.

5. **WR vaccinia/Brucella recombinants expressing Brucella Cu/ZN SOD using synthetic early/late promoters.**

Since the 18kDa protein role in protection is probably unimportant the question whether higher levels of *Brucella* protein expression by the recombinant vaccinia virus would enhance the specific and hopefully appropriate CMI responses in the vaccinated mice leading to a better chance of protection
against a virulent *Brucella* strain challenge could not be resolved. Therefore, Vaccinia/Brucella SOD recombinants using pMCO2 were made since there is at least circumstantial evidence that SOD is a protective antigen.

The Cu/Zn SOD gene of *Brucella* was amplified by PCR using a custom made primer-pair. Two different restriction enzyme sites were engineered into the primers to facilitate directional cloning of the PCR products. The amplified gene was cloned into the shuttle vector, pMCO2, under the synthetic early/late promoter. Using this vector, the recombinant vaccinia virus (wild type: WR strain) expressing the Cu/Zn SOD was constructed following the established protocols of our laboratory. The recombinant virus was enhanced to a titer of 10⁸ pfu/ml. The expression of the SOD protein was confirmed by a Western blot analysis using SOD-specific goat antiserum available in our laboratory.

Mice vaccinated, intraperitoneally, with 10⁷ pfu of recombinant virus did not produce any detectable antibody response to SOD and were not protected from the challenge infection with 2 X 10⁸ cfu of *B. abortus* strain 2308. Mice vaccinated with strain RB51 do not produce antibodies to Cu/Zn SOD, although they show a weak CMI response to this antigen. Our deletion experiments indicate that the absence of Cu/Zn SOD expression reduces the vaccine efficacy of strain RB51, thereby implying a potential protective role for this antigen. Accordingly, certain peptide sequences of this antigen along with an appropriate adjuvant had been shown to induce a protective response in mice. The lack of protective response in mice vaccinated with the recombinant vaccinia virus could be because of insufficient CMI responses to Cu/Zn SOD. Currently, we are analyzing the CMI responses of vaccinated mice to Cu/Zn SOD. Nevertheless, preliminary analysis of the results would indicate that level of *Brucella* antigen expression by the vaccinia recombinant although, probably important, is not the sole factor necessary to induce an appropriate protective immune response.

6. **WR vaccinia/Brucella recombinants expressing the 16 kDa antigen using synthetic early/late promoters.**

To further explore the question of expression level and induction of immune responses, the full length gene for the 16 kDa antigen was amplified by PCR and cloned into the shuttle vector, pSC65. Construction of the recombinant virus is currently under progress. Several recombinant viral plaques are being subjected to three rounds of plaque purification to select a stable recombinant virus.

D. **DNA vaccines.**

We are additionally testing the protective ability of the selected *Brucella* antigens by vaccinating mice with plasmid DNA (pCDNA, Invitrogen – fig. 7)
containing the specific *Brucella* genes under an eukaryotic promoter (cytomegaloviral promoter) and other essential transcriptional signal sequences. Currently we have immunized mice with DNA vaccines of GroEL using a variety of injection routes. Initial analysis indicated that all the immunized mice developed a strong antibody response against the specific proteins (fig. 7). CMI assays are being carried out. Mice in this pilot study were challenged with virulent *Brucella* to assess protection; no protection was obtained.

Figure 7.

We are initially targeting the GroEL, GroES, and SOD proteins since homologues of these proteins from other intracellular pathogens have been shown to be protective if a suitable antigen delivery system is used (15, 16, 17). It has been clearly demonstrated that delivery of appropriate antigens through DNA vaccination results in immune responses necessary for protection against several viral as well as bacterial pathogens (18,19).

E. Publications resulting from the contract support, personnel list and graduate degrees.


Thesis: Assessment of the expression of *Brucella abortus* heat shock protein, GroEL, in vaccinia virus to induce protection against a Brucella challenge in 30
BALB/c mice. Simge Bologlu, Master of Sciences, Virginia Tech, June 1997.**

Vemulapalli R, Duncan AJ, Boyle SM, Toth TE, Sriranganathan N, Schurig GG. Isolation and expression cloning of secD and yajC genes of Brucella abortus. Abstract# B-106, 97th General meeting, American Society for Microbiology, Miami Beach May 4-8, 1997.

Cravero S, Vemulapalli R, Toth TE, Calvert CL, Boyle SM, Sriranganathan N, Rosetti O, Schurig GG. Specific immune responses of BALB/c mice inoculated with recombinant vaccinia virus expressing B. abortus 18 kDa outer membrane protein. Abstract# E-73, 97th General meeting, American Society for Microbiology, Miami Beach May 4-8, 1997.


Eid, H.1,2, Y. Zhang1, S. M. Boyle1, G.G. Schurig1, M.E. Enany2, M.E.El-Sayed1,2 and N. Sriranganathan1. "Cloning and sequencing of a hypothetical transcriptional regulator gene from Brucella melitensis biotype 3." 50th Brucellosis Research Conference. Nov,8&9th, 1997 Chicago, IL.

The following individuals worked on this project and received pay from this contract as salary with benefits or hourly wages: J. Bard, D. Coker, L. Buccolo, R. Vemulapalli, C. Perdue, R. L. Speed, M. Knierien, G. Machin-Scarpaci, M. Fallon, P. Dillon-Long. R. Jack, R. Zeytun. L. Colby, J. Duncan, C. Calvert, R. Rice, E. Hughes, C. Tilton-Lewis
* A Ph.D. degree was obtained by Mrs. Darla Wise; this project contributed funds for materials and supply for one section of her work related to the sequencing and attempts to locate the genes for the 10.2 and 11.6 kDa Brucella proteins. Stipends and other cost were not covered by this project.
** A MS degree was obtained by Mrs. Simge Bologlu; this project contributed funds for material and supplies related to the construction of vaccinia/Brucella recombinants and testing in vivo and in vitro. Stipends and other cost were not covered by this project.

VII. Conclusions.

During this period we have been able to discover several additional Brucella antigens potentially involved in a protective immune response as indicated by in vitro correlates of CMI like the induction of IgG2a antibodies, stimulation of lymphocyte proliferation and induction of INF-γ production. Some of these "antigens" may be virulence factors. The antigens can be purified in mg quantities as fusion proteins allowing for studies leading to better characterization and understanding of the immune response to these putative protective antigens. An alternate method of producing specific Brucella antigens using recombinant Baculovirus, also appears useful for such studies. Studies with pure antigens are important to insure that we are dealing with antigens that will induce appropriate immune responses.

Studies with some of our vaccinia/Brucella recombinants expressing these putative protective antigens constructed in our laboratory clearly indicate that vaccinia/Brucella recombinants can express Brucella antigens and that mice immunized with the recombinants are able to respond with an immune response against these antigens. Nevertheless, protection has not yet been induced with any of our constructs indicating that additional research is needed to optimize the system in order to induce protective immunity.

One possible reason for the protective failure is that the promoters selected to achieve expression of the putatively protective Brucella antigens are not strong enough and not enough Brucella antigen is produced during the virus replication cycle. New constructs are being made using early/late synthetic promoters to overcome this problem but, results are starting to suggest that the use of stronger synthetic, early/late promoters does not resolve the problem by itself.

A second possible reason is that the vaccinia system as applied in this project, may not be appropriate to induce the correct CMI responses needed for protection. This area is being explored by constructing vaccinia recombinants expressing listeriolysin. These recombinants should protect against challenge with L. monocytogenes. Depending on the results obtained, we may have to
further alter the vaccinia expression system to achieve protection. For example the use of vaccinia vectors expressing IL-12 may be necessary to achieve a strong TH1, protective immune response.

A third possible reason is that the antigens selected to induce a protective immune response are not protective antigens. This area is being explored by constructing vaccinia/Brucella L7/L12 recombinants. The L7/L12 antigen has been demonstrated to induce a certain level of protection therefore, vaccinia/L7/L12 recombinants should induce a protective immune response. It is probably incorrect to assume that Brucella antigens that demonstrate in vitro reactivities that correlate with in vivo induction of CMI are good candidates as protective antigens. This is exemplified by the 18kDa Brucella antigen that induces INF-γ responses and IgG2a production but is not a protective antigen. Therefore, these assays are probably not enough to conclude that the selected Brucella antigens do play a role in protection. Additional assays should be added to the in vitro panel; we have initiated the use of cytotoxicity assay using sensitized lymphocytes. In addition, disruption mutants of putative protective Brucella antigens should be made from B. abortus strain RB51 and DNA vaccines should be prepared with the genes of the putatively protective antigens. These preparations can be tested for their ability to induce protection. After all these data are available a final selection of antigens of putative protective potential should be made for cloning into desired vectors. We were successful in producing both (disruption mutants and DNA vaccines) and they are being tested in mice for their ability to induce CMI and protection against challenge. Loss of protection using specific RB51 disruption mutants or protection afforded with DNA vaccines would strongly suggest that our selected antigens are of protective nature.

VIII. REFERENCES.


5. Bagchi, T. G. G. Schurig. 1990. Protection against Brucella abortus smooth strain by adoptive transfer of rough strain sensitized splenic lymphocytes. ADRWSS, Raleigh, NC abstract #3


