DEVELOPING A DNA VACCINE TO PROTECT AGAINST BRUCELLOSIS

A Senior Honors Thesis

by

DAVID MATTHEW OWEN

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2003

Group: Lifesciences

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Approved as to style and content by:

Tom Ficht
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ABSTRACT

Developing a DNA Vaccine to Protect Against Brucellosis. (April 2003)

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Brucella are Gram-negative intracellular pathogenic bacteria which represent a threat to human and animal health. Live vaccine strains are available to protect some animal species but no vaccines exist for human use. A DNA vaccine could potentially provide long lasting cell-mediated protection against human brucellosis while minimizing the virulence risks associated with live vaccines. Five DNA vaccine candidates, each containing a different stress response gene from *B. melitensis*, have been constructed to test the theory that stress response genes delivered as a DNA vaccine could provide protection against *Brucella* infection. A reporter vaccine expressing green fluorescent protein has also been constructed to facilitate vaccine trafficking studies. It is not yet clear whether these vaccines can provide protection against brucellosis.

This paper is dedicated to my parents, in appreciation of the love, support, guidance, instruction, and everything else they have provided over the years in getting me to this point when I'm about to graduate and enter the "real world"

"Train a child in the way he should go and when he is old he will not depart from it"
–Proverbs 22:6

Thanks Mom & Dad!

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I would like to thank several people for their contributions to this work. First of all I would like to thank Dr. Tom Ficht for having confidence in my idea and pretty much giving me free reign to design and carry out this project. It has been a tremendous learning experience and a great help in getting into med school/grad school. Thanks also go to the rest of the Ficht lab: To Josh Turse for sharing his bench, advice and willingness to answer all my questions, and for computer support. To Dr. Jianwu Pei for his expertise on tissue culture, providing BHK-21 cells and the pLEGFP-N1 plasmid, and for help taking pictures with the fluorescent microscope. To Melissa Kahl for providing the goat serum and Asp24 sequence, and for cleaning the BL3 all the times we were supposed to do it as a team but I wasn't there. To Carol Turse for help with orders and supplies. To my fellow student workers Sruti, Midhat, and Amanda for sharing office space and making solutions. Thanks also go out to Dr. Allison Ficht's lab for suggestions and critiques of the project.

At the Honors Office I would like to thank Betsy Pate for all her help with ordering supplies from the research stipend. I would also like to thank Heidi Bludau (now at the University of Maryland) for her help when I was putting my proposal together last spring. Thanks to Donna O'Connor and Dr. Finnie Coleman for their work in organizing the Fellows program and to Dr. Ed Funkhouser for running a great Honors Program.

Across the pond at Lancaster University I would like to thank Dr. P. Jane Owen-Lynch for teaching a great immunology class and assigning the paper that first got me interested in DNA vaccines. I would also like to thank Dr. Keith Jones for the chance to work in his lab and for the experience of making my first poster and writing a journal-style paper (even though it didn't get published).

Finally, I would like to thank my family and my roommates Ryan and Jordan for proofreading, critiquing, and challenging me to explain my research in a way that makes sense to a broader audience.

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I. INTRODUCTION

Brucella are Gram-negative intracellular pathogenic bacteria which cause disease in humans and livestock. In cattle the strain *B. abortus* causes pregnant cows to miscarry, resulting in economic loss. The strain *B. melitensis* causes disease in sheep and goats. Other species include *B. suis, B. ovis, B. canis,* and *B. maris* which infect pigs, sheep, dogs, and dolphins, respectively[1]. The National Brucellosis Eradication Program has virtually eliminated the occurrence of brucellosis in livestock populations in the United States, but the potential remains for contaminating infection from wild animals. Vaccines are an important tool to maintain *Brucella*-free livestock populations. The live vaccines S19 (from *B. abortus*) and Rev1 (from *B. melitensis*) were developed to protect animals against *Brucella* infection[2]. More recently another vaccine strain RB51 has replaced S19 as the standard animal vaccine strain. While animal brucellosis has been well controlled in most developed countries through vaccination and/or slaughter of infected animals, animal brucellosis remains endemic in many parts of the developing world and represents a threat to human health.

Humans can contract *Brucella* infection through ingestion of unpasteurized dairy products or through direct contact with an infected animal. Human infection results in symptoms including chronic fever, malaise, muscle pain, anorexia, and depression. *B. melitensis* is considered to be the most pathogenic strain in humans[3]. As human brucellosis is a zoonotic disease, the frequency of human infection is closely related to the incidence of infection in the animal population. A study conducted on a human population in Saudi Arabia where animal brucellosis was endemic showed that close to 20% of the population had been exposed to *Brucella* on the basis of serology[4]. In the United States, the occurrence of brucellosis in humans is about 100 cases per year, less than .5 cases per 100,000[5]. This low number is due to the success of the brucellosis eradication program. However, *Brucella* also represent a potential threat to human health

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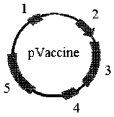
This thesis follows the style and formatting of *Nature*.

as a bioterrorist agent delivered in an aerosolized form. *Brucella* is listed by the CDC as a category B select agent. Interestingly, the first biological warfare agent developed by the US was a weaponized form of *B. suis* in 1954[6]. A human vaccine would be desirable both to protect at risk populations in the developing world and to protect military or civilian personnel from bioterrorism. Although a strain derived from S19 was widely used in the Soviet Union in the 1950s[7], there are currently no vaccines approved for human use. The animal vaccine strains S19 and Rev1 can cause brucellosis in humans[8].

An ideal vaccine would provide protection, minimize adverse effects, and be easy to deliver. In order to be protective the vaccine should stimulate a Th1 cell-mediated immune response[8]. Live vaccines have traditionally been the most effective at stimulating a cellular immune response, but they can also carry the risk of causing disease or other adverse effects. Efforts are underway to create live vaccine strains by making defined knockouts of virulence genes. This represents a safer approach than vaccines that are attenuated by unknown mutations. However, living systems by definition have the ability to change and possibly revert to virulence. Additionally, living systems are complex and it may be difficult to predict potential adverse reactions. This concern becomes especially important in developing vaccines for human use as there is a low tolerance for adverse reactions. Subunit vaccines, while generally safer than live vaccines, are expensive to prepare and are not as effective at stimulating cell-mediated immunity or long term protection[9].

DNA vaccines represent an alternative vaccination strategy that eliminates the virulence problem while at the same time maintaining the potential to stimulate protective cellular immunity. A DNA vaccine (Figure 1) is a plasmid that contains a gene or genes from the pathogen that is being vaccinated against. It contains a prokaryotic origin of replication and a eukaryotic promoter to drive expression.



1) Prokaryotic origin of replication
2) Eukaryotic promoter
3) Antigen gene
4) Poly-A signal
5) Selectable marker

Figure 1. Simple DNA vaccine.

It could also include genes that encode cytokines or immunostimulatory CpG sequences. A prokaryotic system can be used to produce the vaccine plasmid but it cannot express the antigen gene(s). In a cukaryotic host the antigen gene(s) can be expressed but the vaccine should not be able to spread out of control or get incorporated into the host genome. The host cells should pick up the vaccine plasmid, express the antigen gene(s), and then the immune system will recognize the foreign antigen. Since the antigen gene is expressed within the cell, endogenous antigen processing pathways can present the antigen on the MHC complex where it can be recognized by immune cells. This process is outlined in Figure 2.

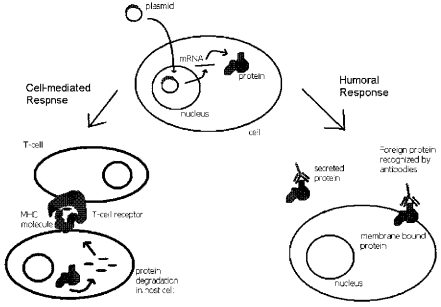


Figure 2. Hypothetical mechanism of DNA vaccine action.

The potential advantages of a DNA vaccine and the relative lack of work done in this area so far makes a DNA vaccine approach to brucellosis protection an attractive area for study.

II. SELECTION OF CANDIDATE GENES

After deciding to pursue a DNA vaccine approach, the next step is to choose which *Brucella* genes to test for protection. So far, seven genes have been tested for protection against brucellosis in an animal model[10,11,12,13,14,15]. These results are summarized in Table 1.

Table 1. *Brucella* genes already tested as DNA vaccines.

Gene	Immune response	Protection	Reference
L7/L12	Antibody and T-cell	Yes, against *B. abortus* 2308	Kurar & Splitter 1997
Bacterioferritin	Th1	No	Al-Mariri, et al. 2001
P39	Th1	Yes, against *B. abortus* 544	Al-Mariri, et al. 2001
GroEL	Th1	No	Leclerq, et al. 2002
RRF (CP24)	Ig2a antibody, no T-cell proliferation	No	Cassataro, et al. 2002
Lumazine Synthase	Th1	Yes, against *B. abortus* 544	Velikovsky, et al. 2002
GAPDH	Th1	Yes, against B. abortus 2308 (only when co-delivered with IL-12)	Rosinha, et al. 2002

The existing literature shows that a DNA vaccine approach could provide protection against *Brucella*, but the number of protective genes identified so far is quite limited. The *Brucella mellitensis* genome has been sequenced[16] which allows any of the 3,197 open reading frames to be tested at will. I did not have the time or resources to screen the entire genome for protective genes, although some have pursued this approach for other diseases in a process called expression library immunization[17]. My goal was to select 4-5 genes as I thought this would be a manageable number to work with and testing in an animal model would essentially double the number of *Brucella* genes thus far studied for protection.

Celio Silva's lab in Brazil has studied the ability of heat shock proteins to protect against *Mycobacteria* infection[18]. Heat shock proteins are proteins upregulated under cellular stress conditions. They generally function as chaperones which help to maintain other proteins in the correct conformation and protect them from denaturation. Silva's lab found that a DNA vaccine encoding HSP 65 gave protection against *M. tuberculosis* that was better than the standard Bacillus Calmette Guerin (BCG) live vaccine even eight months after vaccination[19]. Tuberculosis, like *Brucella*, is an intracellular pathogen so they may share some of the same types of antigenic proteins.

MHC II molecules become associated with antigen in endosomes[20]. *Brucella* infect macrophages by uptake in endosomes. Macrophages use low pH and reactive oxygen intermediates to break down the contents of endosomes and lysosomes. *Brucella*, however, resist this degradation by upregulating heat shock and other stress response genes. It is hypothesized that stress response genes in *Brucella* would make good vaccine candidates as they are upregulated in the same cellular compartment that selects antigens for presentation to the immune system, and they have been shown to be protective in another species of intracellular pathogenic bacteria.

I decided to pick five stress response genes to test as DNA vaccines against brucellosis. I initially searched GenBank for *Brucella* heat shock genes but later narrowed my search to select stress response genes based on two important criteria:

1) Genes which had been shown experimentally to be upregulated under heat, acid, or oxidative stress conditions

2) Genes which had not previously been tested for protection as DNA vaccines

I obtained the experimental regulation data from Teixeira-Gomes, Cloeckaert, and Zygmunt (2000) and Lin and Ficht (1995)[21,22]. The candidate genes selected are summarized in Table 2.

Table 2. DNA vaccine candidate genes.

Gene	Predicted size	Function	Upregulation	GenBank ID
AapJ	37.1 kDa	Amino acid binding	heat shock	AE009560 gi17983192
Asp24	20.4 kDa	Calcium binding	acid pH	AF014823 gi2353000
CuZn SOD	18.2 kDa	Superoxide dismutase	oxidative stress	AE009694 gi17984757
DnaK	68.7 kDa	Chaperone	heat shock, acid pH	AE009633 gi17984056
Mn SOD	22.5 kDa	Superoxide dismutase	heat shock	AE009560 gi17983362

AapJ, CuZn SOD, and DnaK have been shown to be immunogenic in sheep[21]. After making the decision to test these Brucella stress response genes I began the work of building the actual DNA vaccine constructs.

III. CONSTRUCTION OF DNA VACCINE VECTORS

Introduction

A rationally designed DNA-based vaccine approach allows a great variety of features to be included in the vaccine design. Such options include encoding multiple antigen genes, cytokines to modulate immune response, CpG or other immunostimulatory sequences, and tags to target proteins for trafficking. For the sake of simplicity and clarity in interpreting the results of future protection experiments I decided to construct basic vaccine vectors. I built 5 vectors each carrying a single gene under the control of the human cytomegalovirus (CMV) promoter. This promoter has been used successfully in many DNA vaccine experiments. I chose pVAX1 from Invitrogen as the vector backbone because it was designed to comply with FDA guidelines for developing DNA vaccines[23,24]. Genes were amplified by PCR from the *B. melitensis* genome and cloned into pVAX1. The resulting constructs were verified by digestion and sequencing.

Materials and methods

Bacteria. *E. coli* strains DH5α or Top10 were used for plasmid propagation. *E. coli* were grown at 37° in LB broth or on LB agar plates containing 100μg/ml kanamycin when appropriate. *B. melitensis* strain 16M was grown at 37° under BL-3 conditions. This culture was spotted onto FTA cards (Whatman, Clifton, NJ) to provide genomic DNA for PCR.

PCR primers. PCR primers to amplify each gene were designed by hand or with primer design tools in MacVector or Biology Workbench (workbench.sdsc.edu) using the GenBank sequence (Table 2). The forward primers were designed to contain a Kozak sequence for correct eukaryotic translation initiation[25]. A BamH1 restriction site was also added to the forward primers and an EcoRV site to the reverse primers to facilitate

directional cloning. These primers (Table 3) were ordered from Invitrogen (Carlsbad, CA).

Table 3. PCR primers.

Primer	Sequence
AapJ FWD (TAF 283)	5'-cgggatccatcatggcgggtgtattgggtgc-3'
AapJ REV (TAF 284)	5'-cggatatcgttcggtcttgctgtctgcc-3'
Asp24 FWD (TAF 285)	5'-cgggatccacaatggagtcaagaaccatgaaatcg-3'
Asp24 REV (TAF 286)	5'-cggatatcttatcgagaaggctgaaggc-3'
CuZnSOD FWD (TAF 287)	5'-cgggatccacgatggagtccttatttattgc-3'
CuZnSOD REV (TAF 288)	5'-cggatatccactagaattgggcatgg-3'
DnaK FWD (TAF 289)	5'-cgggatccagaatggagagaaatatggctaaag-3'
DnaK REV (TAF 290)	5'-cggatatcacttctcttttgcctgtccg-3'
MnSOD FWD (TAF 302)	5'-cgggatccaccatggctttcgaactgcc-3'
MnSOD REV (TAF 303)	5'-cggatatccttttcaaacaatcggcagg-3'

PCR. The iCycler (Bio-Rad, Hercules, CA) thermal cycler was used to PCR amplify each gene. A temperature gradient was used to obtain optimal PCR. FastStart Taq (Roche, Basel, Switzerland) and associated reagents were used to amplify each gene from a 1.2mm punch of an FTA card matrix (Whatman, Clifton, NJ) containing genomic B. melitensis template DNA.

Molecular cloning. Molecular cloning of PCR amplified genes into pVAX1 (Invitrogen, Carlsbad, CA) was carried out according to the protocols found in Molecular Cloning[26]. PCR amplified genes were double digested with BamH1 and EcoRV. pVAX was double digested with BamH1 and EcoRV and dephosphorylated with shrimp alkaline phosphatase (Roche, Basel, Switzerland). Vector and insert were ligated using T4 DNA ligase (Promega, Madison, WI) for 2 hours at room temperature. The ligation was transformed into chemically competent *E. coli* and plated on LB/kan. This process

resulted in the constructs pVAX-AapJ, pVAX-Asp24, pVAX-CuZnSOD, pVAX-DnaK, and pVAX-MnSOD.

Digestion verification. For each transformation, 5-10 colonies able to grow on LB/kan were picked to verify the presence of the hypothetical vaccine construct. Plasmid DNA was collected using a miniprep kit (Sigma, St. Louis, MO). Undigested plasmid DNA and plasmid DNA double digested with BamH1 and EcoRV was run on a 1% (w/v) agarose gel and visualized for the presence of the vector backbone and the expected size insert.

Sequencing. Sequencing primers based on the T7 and BGH sequences in pVAX1 were ordered from Invitrogen (Carlsbad, CA). The primer 5'-taatacgactcactatacgg-3' (TAF 304) allows forward (T7) sequencing and 5'-tagaaggcacagtcgagg-3'(TAF 305) allows reverse (BGH) sequencing of the insert region. A 15 µl sequencing reaction containing 6 µl Big Dye, 500ng plasmid template, and 5pmol of primer was carried out in the iCycler (Bio-Rad, Hercules, CA). The sequence was determined by the VTPB sequencing core (Texas A&M).

Results

Five vaccine vectors, pVAX-AapJ, pVAX-Asp24, pVAX-CuZnSOD, pVAX-DnaK, and pVAX-MnSOD, were successfully constructed. Figures 3 and 4 show that each construct contains the expected insert.

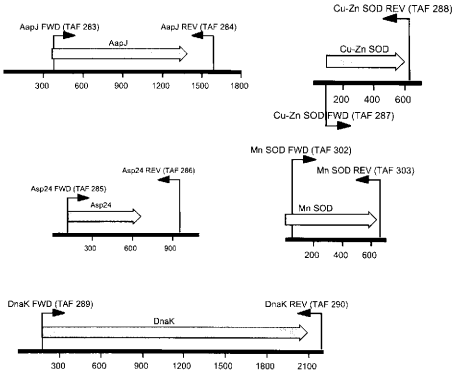
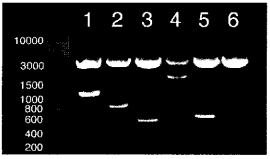


Figure 3. PCR amplified gene inserts.



Construct	Expected fragment
1) pVAX-AapJ	1227bp
2) pVAX-Asp24	858bp
3) pVAX-CuZnSOD	563bp
4) pVAX-DnaK	2034bp
5) pVAX-MnSOD	640bp
6) pVAX	

Figure 4. Digests of vaccine vectors.

The forward and reverse sequence for each construct was aligned to the GenBank sequence and found to match up as expected. In particular, the presence of the start codon and surrounding Kozak sequence was verified. The sequence analysis suggests that the vaccine vectors should be able to express the cloned gene.

IV. TESTING THE VACCINES FOR EXPRESSION

Introduction

The vaccines contain a eukaryotic promoter and a gene in the correct orientation with a eukaryotic translation initiation sequence, so eukaryotic cells should be able to express the antigen gene. However, in order to have confidence in the results of in vivo efficacy tests, it is necessary to demonstrate that the vaccines do in fact express the correct protein in eukaryotic cells. The strategy for testing for expression in eukaryotic cells is to transfect the vaccine plasmids into tissue culture and then to detect the protein in cell lysates by western blot.

Materials and methods

Tissue culture. Baby hamster kidney (BHK-21) cells were grown in DMEM supplemented with 10% FBS and non-essential amino acids. Tissue culture work was performed in a sterile hood. Cells were seeded at a density of $2x10^4$ cells/cm^2 in 75cm^2 tissue culture flasks and grown at 37° with 5% carbon dioxide.

Transfection. BHK-21 cells were seeded in 24 well plates at a density of $1x10^5$ cells/well. On the following day cells were transfected according to the Lipofectamine 2000 protocol with 100 µl of lipoplexes containing .8-1µg plasmid DNA and 2-3µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were transfected with the vaccine plasmids or pVAX-lacZ as a positive control. After 48 hours, cells were lysed and prepared for electrophoresis in SDS loading gel according to the Molecular Cloning protocol[26]. The control cells were stained with X-gal according to the Invitrogen protocol and observed under a microscope.

Protein electrophoresis. SDS-PAGE gels of the cell lysates were run using the reagents and protocols of the Mini-Protean II system (Bio-Rad, Hercules, CA). The proteins were run on 12% polyacrylamide gels prepared according to the Mini-Protean II protocols or on precast ReadyGels (Bio-Rad, Hercules, CA). Gels were stained with Coomassie to visualize proteins or were used for blotting.

Protein transfer. Proteins were transferred from the gels to Immobilon-P membrane (Millipore, Billerica, MA) by semi-dry transfer using the Immobilon-P protocol.

Western blots. Membranes were blocked with 3% gelatin in TBST. Blots were incubated with a 1:500 dilution of serum containing primary antibody and 1% gelatin in TBST overnight. Membranes were washed 4-5 times for 2 minutes with TBST. Blots were incubated 2 hours with a 1:1000 or 1:5000 dilution of secondary antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, MD) prepared in 1% gelatin in TBST. Blots were washed 4-5 times for 2 minutes with TBST and then TBS. Blots were visualized using TMB 1-component membrane peroxidase substrate (KPL, Gaithersburg, MD).

Results

Successful transfection was verified by the pVAX-LacZ positive control plasmid. 50-70% transfection efficiencies were observed (Figure 5). Serum from a *B. melitensis* infected goat was used as the source of primary antibody to detect protein expression in the tissue culture cells (Figure 6). A goat exposed to *Brucella* would presumably make antibodies against a large number of *Brucella* proteins. However, attempts to use this serum were not effective in identifying the expected proteins. There are at least two possible explanations for this result. Antibodies against the vaccine gene proteins may not be present in the infected goat or present only at low levels.

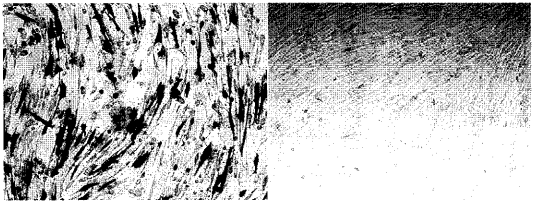
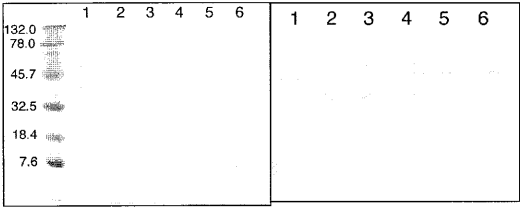


Figure 5. Transfected cells. BHK-21 cells transfected with pVAX-LacZ (left) or untransfected (right) stained with X-gal.



1) Nontransfected
2) Transfected with pVAX-AapJ
3) Transfected with pVAX-Asp24
4) Transfected with pVAX-CuZnSOD
5) Transfected with pVAX-DnaK
6) Transfected with pVAX-MnSOD

Figure 6. Protein gel and western blot. Protein gel stained with Coomassie (left) and western blot using *B. melitensis* infected goat sera (right).

A lack of antibodies against these proteins does not necessarily imply that the proteins are not good antigens. If the proteins are intended as T-cell antigens to stimulate a cell mediated response, the lack of a humoral response may be irrelevant. Alternatively,

antibodies against the proteins may be present in the sera, but the vaccines may not be expressing detectable amounts of protein. This particular experiment did not have sufficient controls, so the results were all or nothing. Unfortunately the results are inconclusive. Additionally, the blot showed a large amount of non-specific binding to proteins from the BHK cells. The background could potentially be reduced through some type of preabsorption step, but this was not attempted.

The western blot was repeated with sera from a rabbit exposed to Asp24 protein. This specific primary antibody should be able to determine whether or not BHK cells could express Asp-24 from the pVAX-Asp24 vaccine. The western blot was performed twice with a negative control (nontransfected BHK-21 lysate), a positive control (*B. melitensis* protein extract), and the unknown (BHK-21 transfected with pVAX-Asp24). Neither attempt showed binding to the unknown or to the positive control. This result combined with the non-specific binding observed in the other experiment suggests that there may be something wrong with my blotting technique. However, no specific actions were taken to induce Asp24 expression in the *B. melitensis* positive control. It is possible that the postitive control was not expressing detectable levels of Asp24. A new positive control should be made from a culture grown in low pH conditions to be sure of inducing Asp24 expression.

V. REPORTER FOR DNA VACCINE TRAFFICKING EXPERIMENTS

Introduction

The method whereby DNA vaccines provide protection is still not well understood. The ability to combine data on the efficacy of a vaccine with knowledge of how the vaccine is trafficked could provide a better understanding of the protective mechanism. A DNA vaccine encoding green fluorescent protein would allow one to identify the types of cells that pick up the vaccine plasmid under different delivery conditions. Tissue samples could be taken from the injection site, lymphatic system, or any other part of the body. Cells expressing GFP could be observed by fluorescence microscopy or flow cytometry. This approach could be combined with fluorescent antibodies to distinguish specific cell types that take up the vaccine.

Materials and Methods

Construction of pVAX-GFP. A 959 bp BamH1 EcoRV fragment containing the GFP gene was excised from pLEGFP-N1 (Bio-Rad, Hercules, CA). This fragment was cloned into the multiple cloning site of pVAX1 using methods similar to those described above. Success in building the desired construct was verified by digestion as described above.

Testing pVAX-GFP. pVAX GFP was transfected into BHK-21 cells as described above. 48 hours post transfection the cells were visualized by fluorescence microscopy and compared to a nontransfected control.

Results

The results of this experiment show that pVAX-GFP is a functional construct capable of expressing GFP in tissue culture (Figure 7).

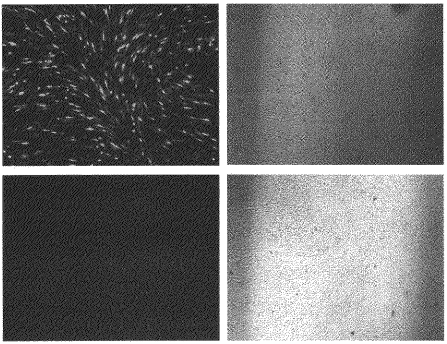


Figure 7. Expression of pVAX-GFP.
Top left: pVAX-GFP transfected cells (fluorescent view)
Top right: pVAX-GFP transfected cells (brightfield view)
Bottom left: Nontransfected cells (fluorescent view)
Bottom right: Nontransfected cells (brightfield view)

VI. CONCLUSIONS AND FUTURE WORK

Five DNA vaccines, each encoding a different stress response protein from B. melitensis were constructed to test the hypothesis that stress response genes delivered as DNA vaccines could protect against *Brucella* infection. The properties of these vaccine

constructs have been partially characterized, but it is not yet clear whether these vaccines can be expected to perform as designed when delivered in an animal study. Additionally, the primary goal of testing these vaccines for protection against brucellosis in an animal model has not yet been achieved.

The verification of protein expression from the vaccines has been hindered by the lack of specific antibodies against the proteins of interest. In order to carry out western blots to detect the proteins in the cell lysates it will be necessary to make specific antibodies. To accomplish this, the genes will need to be cloned into a bacterial expression vector, possibly using a poly histidine tag to aid in affinity chromatography purification. The purified protein can then be injected into animal and the sera containing antibody can be collected. This process may take a significant amount of time.

Alternatively, advances in proteomics technologies may offer another approach to detect the presence of these proteins in cell lysates. Dr. Russel's lab in the chemistry department at Texas A&M has explored the use of MALDI-TOF mass spectrometry to detect individual proteins present in a mixture of proteins[27]. This technology may be improved to detect individual proteins present in whole cell lysates. Proteins are digested into peptide fragments. These peptide fragments are ionized and the mass spectrum is taken for each peptide. These spectra are compared to a database of known spectra for defined peptides. Software converts this data to a list of the proteins present in the sample.

Once protein expression has been verified, these vaccines should be tested in an animal model for protection against brucellosis. Current research suggests that a massive dose of DNA (100 μg for a mouse) delivered by intramuscular injection can stimulate a Th1 immune response[28]. A Th1 immune response is believed to be important for protection against brucellosis, so delivery of the DNA vaccines by i.m. injection would be a good place to start. One could also test oral delivery, possibly in food grade bacteria, or targeted delivery using liposomes or other methods. If desired, the vaccines could be tested before expression in tissue culture is confirmed. Detection of expression

in tissue culture could be carried out later to determine why a particular vaccine did or did not provide protection.

Experiments using the pVAX-GFP plasmid in an animal model could also begin right away to understand how delivery method affects which cells pick up the vaccine plasmids. Experiments designed to target the vaccines to specific cell types could also be conducted. An understanding of how plasmids are moved through the body, taken up and expressed, or delivered to particular cell types would provide information that would assist in designing an optimal protection experiment.

It is unfortunate that time and unforeseen difficulties have not yet allowed the vaccines constructed in this work to be tested for protection in an animal model. However, the work presented here provides a foundation for the future development of DNA vaccines and delivery strategies to protect against brucellosis.

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