APPLICABILITY OF VACCINIA VIRUS AS CLONING AND EXPRESSION VECTOR FOR BACTERIAL GENES: MICE IMMUNE RESPONSES TO VACCINIA VIRUS EXPRESSING *BRUCELLA ABORTUS* AND *LISTERIA MONOCYTOGENES* ANTIGENS

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ABSTRACT

Previous studies by our group showed that vaccinia virus recombinants expressing *Brucella abortus* (*BA)* antigens heat shock protein GroEL, 18 kDa protein and Cu/Zn SOD, were unable to induce protective immune responses against *Brucella* challenge. This dissertation analyzes the possible reasons for this phenomenon, by using other genes/proteins from *BA* and *Listeria monocytogenes (LM)*, various shuttle plasmids (pSC65, pSC11) and immune response modulators (CpG, IL-12, B7-1).

As the first objective, a vaccinia virus recombinant (WRL7/L12), expressing the *BA L7/L12* gene was generated. L7/L12 ribosomal protein was used as a T-cell reactive antigen, with protective potential to *Brucella* challenge. The WRL7/L12 was able to express the gene of interest and induce IgG2A type antibody response, but not a protective immune response against *Brucella* challenge. As a control, an antigen from *LM* proven to induce CTL and protective immune responses, was used to test the efficacy of vaccinia virus to induce protection. A portion of *hly* gene, encoding partial listeriolysin (pLLO), was inserted into the same vaccinia virus stain. This recombinant (WRpLLO) was able to induce protection against a *Listeria* challenge.

Next another vaccinia virus recombinant expressing *Brucella abortus* Cu/Zn SOD was analyzed. Although a variety of approaches, including the enhancement of the protein expression by the pMCO2 synthetic promoter, booster immunization, addition of the oligomer CpG adjuvant (WRSODCpG) to enhance Th1 type response, were used, the SOD recombinant failed to protect mice against *Brucella* challenge.

Lastly, vaccinia virus produces a family of proteins that bind cytokines, chemokines and interferons to evade the host defensive systems. Therefore, a vaccinia

virus strain co-expressing murine IL-12, and cofactor B7-1, were used to generate the recombinant WRIL12L7/L12. In order to further boost the induction of Th 1 type response, the adjuvant CpG was used. A similar recombinant, WRIL12pLLO, was generated with partial *hly* gene to serve as a positive control for protection. Mice immune responses to these recombinants, with and without adjuvant CpG, were analyzed, and compared with the recombinants generated with vaccinia strain WR. Co-expression of IL12 and B7 abrogated the protective efficacy of the vaccinia/ pLLO recombinant.

DEDICATION

I would like to dedicate this work to my family in thanks for all of their love and support.

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1. LITERATURE REVIEW

1.1 VACCINIA VIRUS

1.1.1 History

Vaccinia virus is widely known for its role as the live vaccine used for the immunoprophylaxis of smallpox. This devastating disease killed indiscriminately affecting the young, not so young, the poor and the rich. Telltale pockmarks on the 3000 year old mummified head of Egyptian Pharaoh Rameses V (1157 BC), demonstrates the length of time that smallpox has been the scourge of humanity. Smallpox became prevalent in Europe during the 18th and 19th centuries and where it killed at least one tenth of the population. Since the disease attacked very young children in particular, it was accepted as a kind of natural control on family size, and in large number of households, a child was not considered as a true descendant until they survived an attack of smallpox. In India, during the year 1900, there were 88,585 smallpox deaths in a population of 227,621,486 inhabitants and 7,874,392 vaccinations or revaccinations. In the United States, during the first quarter of the 20th century, there were still deaths from smallpox including 791 in 1922 (21).

Two varieties of smallpox have been distinguished on the basis of the severity of the disease and mortality rate: variola major, the classic disease of earlier times and variola minor which was endemic along with variola major in the Americas and Africa. Although the rash was often quite extensive, variola minor produced much less toxemia; it had a case fatality rate in unvaccinated subjects of about 1% compared with 10-30 % for variola major. The incubation period of both varieties was between 10-14 days. The onset was acute, with fever, malaise, headache and backache. The initial toxemic phase lasted 4 or 5 days and in pale-skinned subjects sometimes accompanied by an erythematous, or rarely, a petechial rash. On about the 4th day after the onset of symptoms, the characteristic rash appeared first on the buccal and pharyngeal mucosa, the face and the forearms and hands and spread to the trunk and lower limbs within a day.

The lesions of rash began as macules that soon became opaque and pustular. They were usually raised from the skin, tense and firm to the touch. About 8 or 9 days after the onset of the rash, the pustules became umbilicated and dried up becoming crusted by 14-16 days. The distribution of the rash, as well as its evolution was highly characteristic being mostly profuse on the face and more abundant on the forearms than the upper arms and the lower legs than thighs and relatively sparse on the trunk especially the abdomen. The outcome of the disease was either death or recovery with elimination of the virus, but sometimes with sequelae. The most common sequelae were pockmarks that could occur all over the body but were usually most profuse on the face because of the large number of sebaceous glands. Blindness was a rare complication, usually occurring in cases where there was malnutrition and or secondary bacterial infection. Recovery was accompanied by prolonged immunity to reinfection with variola virus. Heterologous immunity to infection was less durable especially in cases of variola minor. Variola virus did not persist in the body after recovery (90).

The eradication of smallpox has been a human endeavor for a very long time, lasting for more than two centuries, from 1721-1979 in fact. The practice of variolation was brought to Europe early in the eighteenth century. A very beautiful English woman, named Lady Mary Montagu contracted smallpox that left her scarred. The next year her husband was appointed Ambassador to the Turkish (Ottoman Empire) court. While in Constantinople (Istanbul), Lady Mary learned of the Turkish practice of variolation, inoculation with variola virus, and had her children variolated. When she returned to London in 1718, she used her knowledge and position to campaign vigorously for variolation. Perhaps more than anyone else, Lady Mary was responsible for bringing the variolation to England and subsequently to Europe and the United States (96).Towards the end of the eighteenth century, Edward Jenner, a country physician in England, observed that women who worked with cows did not often contract smallpox. He conjectured that the reason was that they caught a mild case of cowpox, the bovine form of smallpox. Since the cowpox infections were so mild as to yield no symptoms other than smallpox immunity, Jenner developed an immunization procedure vastly superior to classic variolation. This method, later to be known as vaccination, involved transfer of

the vaccine virus from arm-to-arm (215). Some lymph was taken from a person who had a spot with lymph or pus containing the vaccine virus and was then transferred to another person with the aid of a lancet. This Jennerian method had the advantage of not using the material directly from an animal and of easily providing some lymph. In order to be properly protected, the patient had to contract the vaccine through the virus carried in the lymph. Afterwards the patient had to present himself to the presence or absence of a vaccine blister subsequently, in the course of this visit it was easy for the vaccinator to recoup a little of the variolous lymph to carry out new vaccinations (21). Vaccination rapidly replaced variolation during the early nineteenth century and was effective in reducing the incidence of smallpox throughout Europe and North America. However, the widespread use of the vaccine in less accessible regions of the world was hampered by the lack of an easily transportable stable vaccine. Because arm-to-arm transfer was the only effective way known, the Spanish, in attempting to transfer the vaccine to Mexico, resorted to transporting shiploads of orphan children and vaccinating groups of them at regular intervals. Despite the effectiveness of arm-to-arm transfer, this method had the disadvantages that the availability of the vaccine was sometimes limited and other human pathogens including measles and syphilis were spread. The development of calf lymph vaccine in 1860 produced a safer vaccine that was also available in much greater quantity (215).

In 1958, the World Health Organization (WHO) began its smallpox eradication program put forward by the Soviet Union. Subsequently mass vaccination campaigns, extensive surveillance programs and containment of fresh outbreaks resulted in smallpox disappearing form the West by 1971 and from Asia by 1975. On May 7, 1980 WHO officials held a news conference in Geneva in which they announced the eradication of smallpox (211). This also marked the year that vaccine virus, now known as vaccinia virus began to be used as a viral vector for the production of recombinant vaccines. As early as 1984, in the workshop on "Vaccinia Viruses as Vector for Vaccine Antigens", Enzo Paoletti reported the modification of vaccinia virus to express the foreign genetic material under vaccinia virus regulation. Immunization of animals with these

recombinants induced an immune response that is directed against the foreign antigen, which subsequently protected the vaccinated animals to infectious challenge (211).

1.1.2 Biology of Vaccinia Virus

The vaccinia virus is a member of the genus *Orthopoxvirus*, in the subfamily of Chordopoxvirinae, in the family of Poxviridae. Poxviruses are the largest and most complex of all animal viruses. The basic features of the family are large brick shaped or ovoid virions (Figure 1.1).

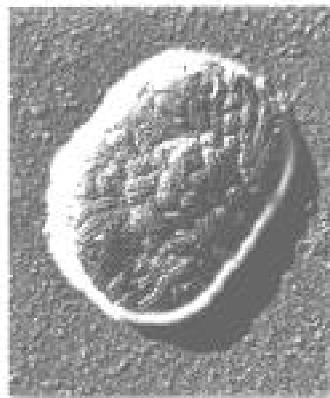


Figure 1.1 Electron micrograph of freeze-dried vaccinia virus.

(Courtesy of Dr. Milan V. Nermut (Institute for Biological Standards and Control, Hert, UK) with permission from http://www.tulane.edu/*dmsander/big…virology/special/Nermut/vaccinia.gif/)

They are 300-400 nm in diameter with lipoprotein membranes that surround the core structure containing a genome of a single linear molecule of a covalently closed double stranded DNA, between 130 and 220 kb in length (176). Members of the genus *Orthopoxvirus* are shown in Table 1.1, which indicates the separate transmission cycle of the main species. The origins of vaccinia virus are obscure but in smallpox vaccination times it has been produced in large quantities. Limited circulation of vaccinia virus has been detected in some outbreaks of "cowpox" in Holland and of "buffalo pox" in India, though these were minor variants of vaccinia virus (211). The majority of antigens of the *Orthopox* viruses are unrelated to those of other genera of poxviruses, but within the genus there is little antigenic diversity. The members are differentiated primarily by the characteristic pathological effects they produce in a variety of laboratory animals and cell culture systems. Although the members of the genus were given species status, the antigenic overlap is almost complete and there are no species-specific neutralizing antibodies. Profiles of the size distribution of intracellular virus polypeptides are characteristic for four of the main species, vaccinia, variola, cowpox and monkeypox (112).

Table 1.1 Species and natural hosts of the genus *Orthopoxvirus* (89).

Species	Host range in laboratory animals	Animals found naturally infected	Geographic range of natural infections
Camelpox virus	Narrow	Camels	Africa and Asia
Cowpox virus	Broad	Numerous: carnivores, cow, humans, rats, gerbils and other rodents	Europe and former USSR
Ectromelia virus	Narrow	Mice, possibly voles	Europe
Monkeypox virus	Broad	Apes, squirrels, humans and raccoons	Western and central Africa
Raccoonpox virus	Broad	Raccoon	USA
Tatera poxvirus	Narrow	Gerbil (*Tatera kempi*)	Western Africa
Uasin Gishu poxvirus	Medium	Horse (natural host unknown)	Kenya, Zambia
Vaccinia virus	Broad	Numerous buffalo, cow, human, pig, rabbit. Natural host unknown	
Variola virus	Narrow	Humans. Now eradicated	Formerly worldwide
Vole poxvirus	Broad?	Voles	USA

Two infectious forms of vaccinia virus exist. The intracellular one, which is predominant, contains a lipoprotein envelope, a biconcave core, and lateral bodies fitted into the concavities. The extracellular form, the amount of which may vary from less than 1 percent to more than 20% of the total infectious virus depending on the vaccinia virus strain and cultured cells used, has an additional lipoprotein envelope acquired from the Golgi membrane (202), (175). Although a minor component *in vitro*, the extracellular form is thought to be important for virus dissemination *in vivo* (203).

1.1.2.1 Molecular Biology of Vaccinia Virus

Vaccinia virus is the most thoroughly studied member of the genus. Like other species in the *Orthopox* genus, the virus is large, complex and has a double stranded DNA genome of 187 kb (240) located within the core structure. The DNA sequence at the two ends of the genome is identical for about 10,000 bps (101), (283). This very long inverted terminal repetition contains sets of tandem repeats that are 54, 70 and 125 bps long. Perhaps most unusual is the covalent linkage of the two DNA strands by incompletely base-paired hairpin loops at the end of each end of the genome (20). Its genome is noninfectious and contains approximately 200 closely packed genes. These genes are expressed in a strictly regulated cascade encoding numerous proteins including a multisubunit DNA dependent RNA polymerase, transcription factors, capping and methylating enzymes and a polyA polymerase. This enables vaccinia to synthesize translatable mRNAs with typical eukaryotic features after entry into a cell (176), (237) (Figure 1.2).

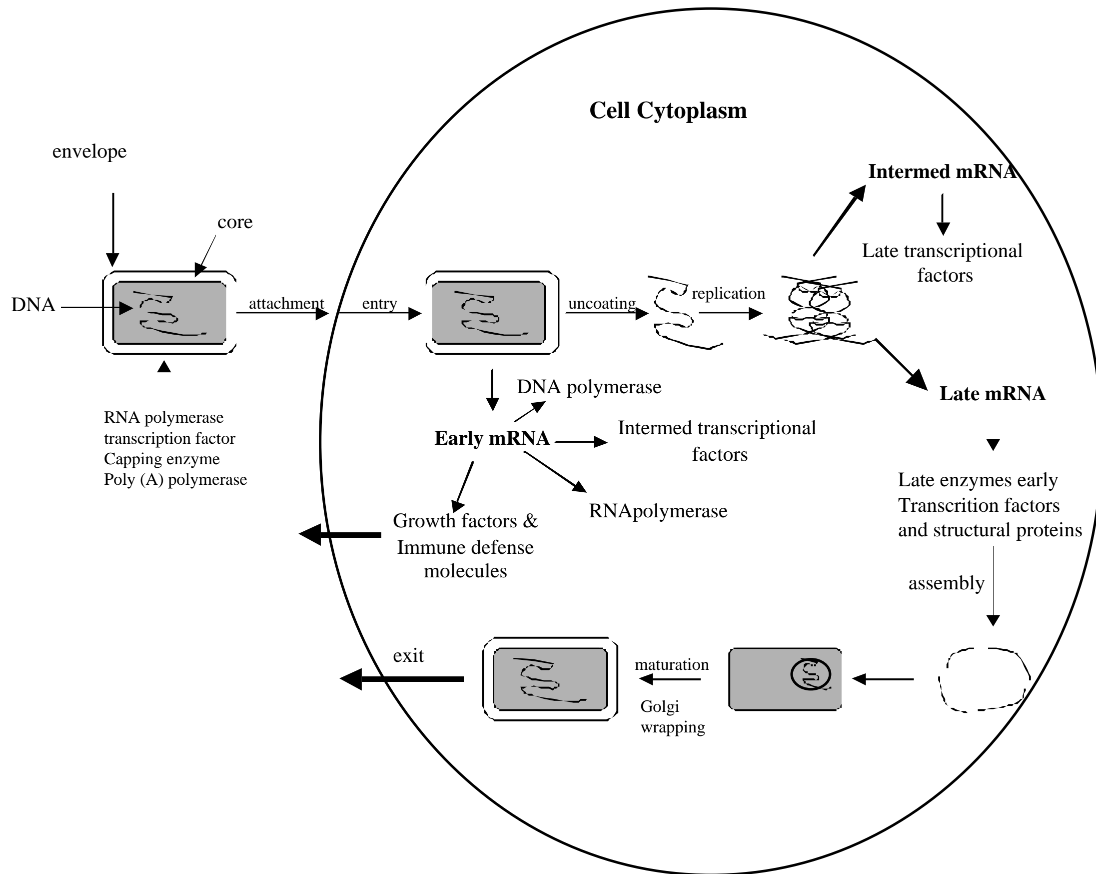


Figure 1.2 Infectious cycle of vaccinia virus.

[Adapted from reference (176).]

Following adsorption and penetration of host cells, the virus core is released into the cytoplasm where transcription occurs. DNA-RNA hybridization studies indicate that about half of the genome is expressed at this early or pre-replicative stage (200), (29). In this stage there are approximately 100 early genes distributed throughout the length of DNA (123). All of the genes have continuous coding segments; most, if not all, vaccinia virus mRNAs are polyadenylated (184).

Following the onset of DNA replication, the late class of genes are expressed and many of the early genes are no longer functionally active. Late genes, which encode major structural proteins, are distributed throughout the genome, however they appear to be more concentrated in the central region (123). Upon synthesis of large structural proteins, infectious virus particles are assembled. Vaccinia virus assembly is a complex process that occurs within the specialized areas of the cytoplasm. Mature particles are moved out of the assembly areas, become enveloped and modified in the Golgi membrane and then transported to the cell periphery where they are externalized (256).

The mechanisms controlling the temporal expression of vaccinia genes are under intensive investigation. Nucleotide sequence data for vaccinia genes are available, and these show that transcriptional control regions (promoters) of vaccinia genes are very A: T rich for 60 base pairs upstream from the transcriptional initiation site and do not share any extensive homology with either prokaryotic or eukaryotic transcriptional consensus sequences (238). This explains the noninfectious nature of vaccinia genomic DNA.

1.2 VACCINIA VIRUS AS AN EXPRESSION VECTOR

For over two decades vaccinia virus has been used to express many different genes. These vary from eukaryotic growth factors and protozoan structural proteins to prokaryotic enzymes and a variety of virus gene products. In addition to the ease of generation of recombinant viruses, foreign gene expression by recombinant vaccinia viruses offers several advantages:

1) Proteins are processed and modified correctly.
2) Proteins are properly transported and localized in the infected cell.
3)Uniform protein production is achieved within a target cell population using a high multiplicity of infection.

4)The extremely broad host range of vaccinia virus allows a wide array of primary and transformed tissue culture cell lines to be utilized.

5) Foreign gene expression can be achieved with high efficiency in cells that are refractory to nucleic acid transfection procedures such as primary macrophage cultures.

6) A variety of natural and synthetic vaccinia promoters as well as hybrid systems using bacteriophage T7 (99), (98), (81), T3 (218) and SP6 (271) promoters and repression via *Escherichia coli lac* repressor/operator permit varying levels and control of gene expression.

7) The problems and limitations associated with expression in permanently transformed cell lines are avoided due to the transient nature of the vaccinia virus expression system.

8) The cytoplasmic localization of transcription bypasses requirements for regulated export of unspliced mRNAs out of the nucleus. However, since the messenger RNAs are not spliced in the vaccinia virus system, open reading frames must be continuous (36).

The popularity and diverse uses of vaccinia virus vectors also derive from their ability to stably integrate and package large amounts of DNA up to 25 kb without loss of infectivity as well as from the development of simple and effective methods for isolating recombinant viruses (180).

Another advantage of recombinant vaccinia viruses over replication defective vectors is their ability to retain complete infectivity in animal models. This allows vaccination of experimental animals with live recombinant viruses expressing a single foreign antigen and analysis of the immunological response to that specific antigen. Recombinant vaccinia viruses induce both humoral and cellular immune responses (198), (25), (78). Since the proteins undergo normal postranslational modifications and intracellular trafficking, they are presented in their native configuration. This is an important feature as viral neutralizing antibodies are frequently directed to conformational epitopes of surface glycoproteins (178). In numerous examples

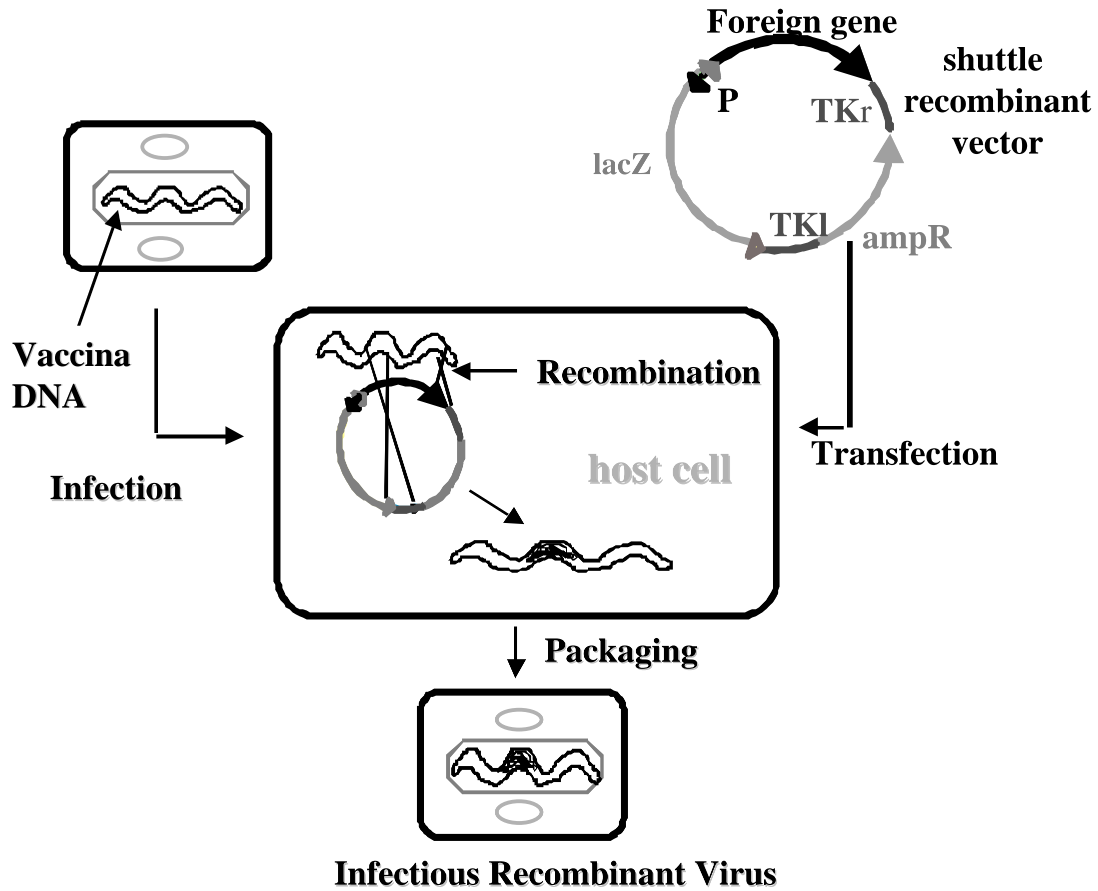
protection induced by recombinant viruses was correlated with neutralizing antibody against viral envelope proteins (180).

The induction of strong class I restricted cytotoxic T cell (CTL) response provides a major advantage of infectious recombinant viruses compared to inactivated or subunit vaccines. In some animal models CTL provided protection against a virulent strain challenge (126), (136), (172), (7). Antigen presentation by class I molecules may be decreased late in infection (72), (267). Consequently, gene regulation by early (or tandem early-late) promoters is recommended. Consistent with present concepts of presentation, expression of minigenes encoding short peptides is sufficient to induce CTL responses (106), (280), (7).

1.2.1 Genetic Engineering of Vaccinia Virus

1.2.1.1 Homologous Recombination

Most strategies for genetically engineering of vaccinia viruses have employed homologous DNA recombination in infected cells, a process that occurs naturally during the replication of poxviruses. In this strategy vaccinia virus recombinants can be generated by a two step procedure (Scheme 1.1). The first step involves assembling a shuttle plasmid vector, containing a foreign gene flanked by vaccinia virus DNA (158). The foreign gene is inserted after a vaccinia virus transcriptional start site and upstream regulatory sequences (promoter) between flanking vaccinia virus DNA. The second stage of the process is insertion of the foreign gene into vaccinia virus. Transfection of vaccinia virus infected cells with the constructed recombinant shuttle vector allows homologous recombination to occur between the sequences in virus genomic DNA, resulting insertion of foreign gene in the shuttle vector into the virus genome at low frequency.



Scheme 1.1 Homologous recombination.

A DNA fragment containing the gene encoding foreign protein is inserted in a shuttle vector downstream from a vaccinia virus promoter. The resulting recombinant shuttle vector is introduced by transfection into the cells infected with wild type vaccinia virus. Within the infected cells, homologous recombination results in site-specific insertion of the foreign gene into the viral genome.

To facilitate the rapid construction and insertion of foreign genes into vaccinia virus several applicable shuttle vectors have been generated (Table 1.2) (159), (51), (47).

Table 1.2 Selected vaccinia virus shuttle vectors

Selection/screening	Vector	Promoter	Flanking DNA	Reference
TK	pGS20	P7.5 (E/L)	TK	(159)
	pSC 59	Synthetic (E/L)	TK	(52)
TK and β−gal	pMJ601	Synthetic(L)	TK	(74)
	pSC65	Synthetic (E/L)	TK	(46)
	pSC11	(E/L)	TK	(51)
TK and β-glucuronidase (GUS)	pMCO2	Synthetic (E/L)	TK	(47)
β-gal	pCF11	P7.5 (E/L}	HindIII/C	(92)
Ecogpt and /orTK	PTKgptF1s	P11 (L)	*TK*	(86)
	pMC1107	P7.5 (E/L)	TK	(28)

These vectors have the following properties:

1) An isolated vaccinia virus promoter including the transcriptional initiation site and 200-300 base pairs of upstream DNA,
2) Several unique restriction nuclease cleavage sites positioned just downstream from the transcription start point that are suitable for in frame insertion of foreign DNA,

3) Nonessential vaccinia virus DNA that flanks both sides of the promoter and restriction sites which directs insertion of foreign gene into the homologous nonessential region of vaccinia virus DNA,
4) A bacterial origin of DNA replication, an antibiotic resistance marker and a reporter gene such as *lac* Z allows the easy generation of recombinant shuttle vector and selection of recombinant viral progeny (240).

The type of promoter used in the shuttle vector dictates both the level and time of expression. There are numerous combinations of promoter systems. Quantitative analysis of the expression of vaccinia virus genes has revealed that early promoters express genes from 0.5 hours (h) to a maximum of 1.5 h postinfection, intermediate promoters from approximately 1.5 h to a maximum of 2 h and late promoters from approximately 3 hours onward (179). Constitutive or compound promoters are those that contain both early and late transcriptional elements. Factors that influence the choice of promoter system come from assessing the desired use of recombinant vaccinia virus, or from the known properties of the gene product of interest (36). For large-scale protein production a strong vaccinia virus promoter such as the synthetic late (74), or early late promoter (52), or the hybrid vaccinia/T7 polymerase system (98), (279) is recommended. The conditions for optimal protein production, the fate of the gene product such as secretion may also need to be addressed (207). Finally, if a specialized cell type is used such as primary cell culture, it may be useful to characterize that cell type for its ability to support vaccinia virus infection, replication and gene expression by different classes of promoters (64), (35).

One of the most widely used types of shuttle vector utilizes recombination into the nonessential thymidine kinase (*tk*) gene of vaccinia virus. Not only is the *tk* gene nonessential, but disruption of this function provides a means of selecting recombinant viruses with a *tk* $^{-}$ phenotype by growth in the presence of thymidine kinase analog 5-bromodeoxyuridine (BrdU) (160). Using spontaneous *tk*$^{-}$ vaccinia viruses, the first foreign gene introduced and expressed in vaccinia virus was the herpes simplex virus *tk* gene (160), (199). Incorporation of a functional *tk* gene into the shuttle vector allowed

selection of new recombinants with tk^{+} phenotype over tk^{-} parent and complemented the *tk* gene deletion in the viral progeny (212). Another widely used selection mechanism employs the incorporation of the *Escherichia coli* xanthine-guanine phosphoribosyl transferase (XGPRT) gene *Ecogpt* into the shuttle vector (86), (32). Mycophenolic acid (MPA), an inhibitor of purine metabolism, blocks replication of vaccinia virus. Expression of *Ecogpt* by vaccinia virus and inclusion of xanthine and hypoxanthine in the growth medium rescues the virus from blockage. Thus, the shuttle vectors that include the *Ecogpt* gene controlled by vaccinia virus promoter in the recombination cassette will yield recombinant vaccinia viruses expressing both the *Ecogpt* gene and the gene of interest (36). Other mechanisms of selection include the use of neomycin (94) or hygromycin (297), changes in the plaque size (219), (28), red blood cell agglutination phenotypes (234) and alterations of host range (204), (242), (119).

Because homologous recombination occurs with a low frequency and most selection methods allow for growth of some parental virus, putative recombinants must be screened by plaquing to identify the ones containing recombinant virus. By far the easiest method involves the use of *E.coli lac*Z gene, which allows a positive colorimetric assay for the identification of recombinant viruses through the production of -galactosidase (-gal) (51). A more recent colorimetric assay is based on the *E.coli gus* A gene encoding -glucuronidase (GUS) which is significantly smaller in size making plasmid and cloning manipulations easier (47). In the absence of a colorimetric screening approach, plaques containing recombinant virus can be identified either by DNA hybridization in a dot blot procedure or polymerase chain reaction (PCR) or immunological assays including Western blotting, immunoprecipitation or immunostaining if an antibody is available.

1.2.2 Vaccinia Virus in the Development of Vaccine Candidates

The numerous examples, in which vaccination of experimental animals with recombinant vaccinia viruses that express one or more genes of viruses or parasites have

provided partial or complete protection against diseases caused by challenge are summarized in Table 1.3.

Table 1.3 Protective vaccinia virus recombinants.

[updated, and modified from Perkus et al., 1995 (173)]

Pathogen	Species protected	Antigen
1. Rabies	Mice, raccoons, foxes, dogs	Glycoprotein (gp)
2. Measles	Mice, rats, dogs (from CDV)	Fusion, hemagglutinin (HA), gp, nucleoprotein (N)
3. Hepatitis B virus (HB)	Chimpanzees	Surface antigen (HbsAg)
4. Herpes simplex virus (HSV)	Mice, guinea pigs	HSVgp B, HSVgpD
5. Mouse cytomegalovirus	Mice	Nonstructural protein pp89
6. Epstein-Barr virus (EBV)	Cottontop tamarins	EBVgp 340
7. Influenza	Hamsters, ferrets, mice	HA
8. Dengue	Mice	Capsid+envelope+nonstructural NS1+NS2+premebrane (PreM)
9. Yellow fever	Mice	NS1, NS2a, NS2b, PreM, E
10. Japanese encephalitis virus (JEV)	Mice, swine	PreM, E, NS1
11. Respiratory syncytial virus	Cotton rats, mice, chimpanzees	M2 ,gp,
12. Human papilloma virus	Mice, rats	E6, E7 oncoproteins
13. Human parainfluenza	Monkeys	Gp
14. Lassa fever	Guniea pigs	Internal nucleocapsid (nc), gp
15. Vesicular stomatitis virus	Mice, cattle	N, gp
16. *Leishmenia*	Mice	GP46/M2
17. Rinderpest	Rabbits, cattle	F, HA
18. Peste des petits ruminants	Goats	F, h
19. Equine herpes virus	Hamsters	EHV gp13, gp14
20. Pesudorabies virus	Mice, swine	Gp50, gpII, gpIII
21. Equine influenza (EIV)	Horses	HA
22. Bovine leukemia virus	Sheep	E, gp51
23. Bovine papilloma virus (BPV)	Rats	Early BPV proteins
24. Polyoma virus	Rats	Tumor specific antigens
25. Hog cholera virus	Swine	Structural proteins gp55,gp33
26. Venezuelan equine encephalomyelitis	Mice, monkey, horses	Capsid , gpE1, gpE2
27. Sendai virus	Mice	HN, F, NP, matrix (M)
28. Avian Influenza	Chicken	HA
29. Friend leukemia virus	Mice	Gag protein
30. Human Immuno-deficiency virus (HIV)	Small laboratory animals, macaques, chimpanzees, humans	HIV-1 Envelope gps, gag/pol, gag/protease HIV-2 Envelope gps, gag, pol,

Vaccinia viruses have several immunological applications, the most straightforward being the induction of immunoglobulin synthesis in the infected host against a known protein or the product of an open reading frame of unknown function. Although in most cases, protection was correlated with neutralizing antibody against viral envelope proteins expressed by recombinant vector (176), good immune responses were also detected against internal proteins such as the nucleocapsid proteins (157).

The second application was that recombinant vaccinia viruses prime and stimulate cell mediated immune responses (CMI) in vaccinated animals. Protection was due to induction of CTLs (126), (111), (172), (78). Recombinants, even those that expressed truncated genes, which were sufficient to be targets for CTLs induced protection to subsequent challenge (106), (280), (7). Cells infected with recombinant vaccinia viruses express the foreign antigen on the cell surface in conjunction with antigens of the major histocompatibility complex class I. These cells are recognized and lysed by autologous CTLs directed against the foreign antigen.

Vaccinia virus has also been extensively used for assessing the immunogenicity of tumor- associated antigens (TAAs) in various animal systems (201). Table 1.4 lists the examples demonstrating the effectiveness of vaccinia virus recombinants expressing TAAs in the immunoprophylaxis and immunotherapy of experimentally induced tumors (205). Probably the most studied TAA is the carcinoembryonic antigen. Carcinoembryonic antigen is expressed in a number of human tumors, including colorectal, gastric, pancreatic, breast and non-small cell lung carcinoma. Mice inoculated with vaccinia virus carcinoembryonic antigen recombinant developed antigen-specific antibodies, elicited CMI and had lower levels of tumor growth compared with control animals after challenge (134), (131), (130). Recombinant vaccinia virus, expressing antigens from viruses associated with cancer, have also prevented cancer formation in rodents. Human papilloma virus tumor antigens E6 or E7 protected rodents partially against tumor challenge. Protection was mediated by $CD8^+$ lymphocytes (169).

The vaccinia virus recombinants expressing the biological response modifiers such as co-stimulatory molecules like B7 and ever-expanding array of cytokines have been proven to enhance immune responses (205). The costimulatory molecule B7 is found on the surface of the professional antigen–presenting cells such as dendritic cells and interacts with its ligands CD28 on $CD4^+$ T cells. The simultaneous interactions of the complex containing the peptide and the major histocompatibility complex with a specific T-cell receptor and the B7 with CD28 are essential for the effective stimulation of antigen-specific cytotoxic T lymphocytes, mediated, in part by the up-regulation and stabilization of IL-2 messenger (153). Carroll et al. (1998) generated a panel of recombinant viruses that enabled them to analyze the activities and contributions of TAAs, B7 and IL-12 alone and in combination. Importantly, all recombinant vaccinia viruses stably expressed biologically active IL-12, B7 and or TAAs and produced antigen-specific treatment of a murine cancer (48). The direct antitumor effects were dependent on INF- release and its effect on CD8+ T lymphocytes.

Table 1.4 Protection against tumors by vaccinia virus recombinants

TAAs/Antigen (ag)	Origin	Protection
Carcinoembryonic ag	Colon	Mice
p97	Melanoma	Mice
p185	Rat neu oncogene	Mice
Epithelial tumor antigen	Breast	Rats
Viral		
E7	Human papilloma virus (HPV)	Mice
E6	HPV	Mice
E6, E7	HPV	Rats
E5, E6, E7	HPV	Rats
gp51, gp30	Bovine leukemia virus (BLV)	Sheep
gp340	Epstein- Barr virus	Cotton top tamarins

Vaccinia virus recombinants showed an impressive potential as vaccines expressing viral, tumor and parasite antigens but they had limited success with bacterial antigens. Similar to recombinants carrying viral or tumor antigens, foreign bacterial genes cloned into viral genome were expressed in high amounts. Immunizations with these recombinants induced high humoral and cellular immune responses. Vaccinia virus/bacterial antigen recombinants exhibiting protection to infectious strain challenge are summarized in Table 1.5.

Table 1.5 Protective vaccinia virus/bacterial antigen recombinants

Bacterium	Antigen	Species protected	Reference
Bacillus anthracis	Protective antigen (PA)	Mice, guinea pigs	(122)
Mycobacterium tuberculosis	19kDa, 38kDA	Mice	(298)
Streptococcus pyogenes	M	Mice	(121)
Listeria monocytogenes	91-99 CTL epitope	Mice	(7)

1.2.3 Attenuated Vaccinia Viruses

The significant adverse reaction accompanying smallpox vaccination prompted the development of highly attenuated poxviruses for recombinant vaccines. The four general approaches used were: 1) attenuation by serial passage in tissue culture for smallpox vaccination, 2) selective deletion of specific vaccinia virus genes, 3) use of poxviruses having a narrower host range than vaccinia virus and 4) insertion of lymphokine genes into vaccinia virus (178).

The modified vaccinia virus Ankara (MVA) is one of the most highly attenuated vaccine strains (163), (164). This strain was attenuated by more than 570 passages in chicken embryo fibroblasts, became host restricted and unable to replicate in human and other mammalian cells. Genetic analysis indicated that more than 30,000 base pairs of DNA, including at least two host range genes, had been deleted from MVA (171). Significantly, replication of MVA in non-permissive human cells is blocked at a step in virion assembly, rather than at an early stage as occurs with other host-range restricted

poxviruses (257). MVA is avirulent in normal and immunosuppressed animals and caused no significant side effect in 120,000 humans many of whom were at high risk for the conventional smallpox vaccines (178).

The LC16m8 strain of vaccinia virus, derived from the Lister vaccine, has decreased neurovirulence but is not as highly attenuated as MVA (114). This strain has also been used as a vaccine vector to express human T-cell leukemia virus type 1 envelope gene that provided protective immunity in the tested animals (233).

The phenotype of vaccinia virus can also be altered by specific gene deletions. Attenuation has been associated with inactivation of numerous genes including those encoding TK (42), growth factor (41), hemagglutinin (92), 13.8K secreted protein (139), envelope proteins (220), (84), (284) and host range genes (148). Paoletti and coworkers (1992) have developed and extensively tested host-range restricted mutant, called NYVAC with numerous gene deletions (261), (138).

The insertion of lymphokine genes into the genome of vaccinia virus offers another method of decreasing virulence without adversely affecting immunogenicity. Recombinant vaccinia viruses that express mouse (212), or human (92) IL-2 or interferon- (137) were much less pathogenic than wild type virus for immunodeficient athymic nude mice. Rapid clearance of IL-12 expressing virus occurred by natural killer and T-cells secreting INF- (132), (133). Attenuation was also noted upon infecting immunocompetent monkeys with an IL-2 expressing vaccinia virus (93), (223).

1.3 BRUCELLOSIS

Brucellosis is a disease caused by a gram-negative bacterial pathogen in the genus *Brucella*. The organism is named after Sir David Bruce who in 1886 first isolated the organism from the spleen of a soldier afflicted with what was then termed Malta fever (39). Aside from Western Europe, USA and Canada brucellosis remains a major zoonosis worldwide. Manifestations of the disease may range from abortion in livestock, to endocarditis, arthritis, meningitis and osteoarticular problems in humans. Characteristically, all *Brucella* species establish persistent infection in the reticuloendothelial system of the natural host species.

The four *Brucella* species considered zoonotic to humans are *B. melitensis*, *B. suis*, *B. abortus,* and rarely *B. canis* (66). Human brucellosis is characterized by biphasic or undulant fever, weakness and myalgia. The disease can lead to complications such as endocarditis, arthritis, meningitis and osteoarticular problems. In chronic cases neurological disorders may occur (66). Transmission of brucellosis to humans occurs principally through contact between infective animal secretions and human mucous membranes. Food borne brucellosis occurs when individuals consume infected milk or dairy products. The first reported case of human brucellosis in the US occurred in 1906. The incidence rate of human *Brucella* infections increased to a high level of 39.6 new cases per 100,000 individuals per year during 1945-1949 (63). Since then, the incidence markedly decreased due to a combination of public education and a national brucellosis eradication program. However, still a small number of food borne cases of brucellosis occur every year and many have been linked to consumption of imported soft cheeses or cheeses where raw milk has been used. Most of these cases have yielded *B. melintensis* and *B. abortus* as causative agents. Today in the U.S., the main source of transmission comes from occupational risk. Occupational risk of brucellosis occurs in the field and affects mainly livestock producers, veterinarians and laboratory workers. The risk comes from handling infected tissues such as aborted fetuses, placental membranes or fluids and

vaginal discharges. At present *B. melintensis* is the major cause of human brucellosis worldwide (277).

Animal brucellosis is a disease affecting various domestic and wild life species. Six species of *Brucella* exist, which are associated with several principal hosts: *B. abortus* (cattle), *B. canis* (dog), *B. melitensis* (goats), *B. neotomae* (wood rat), *B. ovis* (sheep) and *B. suis* (swine). Recently, a new species of *Brucella* has been recognized as infecting sea mammals (222). Infection of susceptible animals depends on the dose, exposure route, and virulence of the *Brucella* strain and the health of the host. Cattle are most frequently infected with *B. abortus* biovar one. Brucellosis in cattle may cause late term abortions, still births, retained placentas, sterility, lymphoplasmacytic mastitis and tissue granuloma (1). Typically, *B. abortus* is transmitted by ingestion of infected placentas, fetal tissues or fluids, which often contain high concentration of organisms (95), (5). Other transmission routes including respiratory, venereal and congenital transmissions have been documented in domestic cattle (214). Sexually mature cattle are much more susceptible to infection than immature ones. Susceptibility also increases with pregnancy and as gestation progresses. Although in many countries the causative agent of cattle brucellosis is *B. abortus,* in some areas *B. melitensis* infects this species as well as sheep and goats.

Swine brucellosis is a venereal disease. Females are readily infected when bred with an infected male. The habits of pigs favor the oral route of infection when susceptible pigs consume discharge from infected pigs. With brucellosis, considerable loss occurs from reduced reproduction. Fetal wastage as well as posterior paralysis and lameness occur as outward signs of the disease observed by the pig owner (http://www.ncopp.com/brucel.htm).

There are few known reservoirs of *B. suis* other than infected swine. Feral pigs have been named as reservoirs. Infected pigs can be a source of infection for other domestic animals, i.e. horses, cattle, dogs, and fowl. *B. suis* also infects cattle, thus extending its opportunities to infect humans (67).

1.3.1 **The Genus *Brucella***

1.3.1.1 General Characteristics

Brucellae are polymorphic gram-negative cells in the shape of cocci, coccobacilli, or short rods, 0.5-0.7μm in diameter and 0.6-1.5 μm in length (66). The members of the genus are closely related and can only be distinguished by rigorous metabolic, immunologic, and biochemical analyses. They are aerobic and do not grow under strict anaerobic conditions. Many strains, especially *B. abortus* and *B. ovis*, are carboxylic and require supplementary CO_2 for growth. Their metabolism is oxidative and energy is produced by utilization of various amino acids and carbohydrate substrates. For many strains, erythritol is a preferred energy source. Most strains require complex media containing multiple amino acids and vitamins including thiamin, biotin, nicotinamide and panthothenic acid for growth and especially on primary isolation (66). The optimal pH condition for growth ranges between 6.6-7.4. All strains lose viability at 56^0C; however, 1 hour incubation at temperatures over 70^0C may be required to insure complete killing of *Brucella*.

On serum-dextrose agar (SDA), smooth colonies appear transparent, raised, and convex and have a smooth shiny surface (66). Colony variants can be classified under four morphological categories: smooth, rough, smooth-rough intermediate and mucoid. This classification is made based on characteristics of the bacterium's lipopolysaccharide (LPS). Colony smoothness and roughness usually correlates with high and low virulence among *B. abortus*, *B. melitensis* and *B. suis*. Mutation from smoothness to roughness usually decreases the virulence of these species and decreases or eliminates the antibody production to the O-side chain in animal hosts. *B. abortus* strain RB51 illustrates this well; it is a highly attenuated, rough strain and does not induce O-side chain antibodies (115).

The *Brucella* genome consists of two chromosomes of 2.1 and $1.5x10^6$ base pairs (bp) (127). Sequencing efforts are underway for the *B. abortus* (http://www.iib.unsam.edu.ar/genomelab/brucella/gss.html), *B.suis*, (http://www.tigr.org) *B. melitensis* (http://www.integratedgenomics.com) genomes and data is available at http://www.tigr.org. Both chromosomes contain essential genes encoding for metabolic and replicative functions. Unlike some other pathogens, the pathogenicity of *Brucella* is not dependent on plasmids. Repeated attempts by many laboratories have failed to detect extrachromosomal DNA in *Brucella*. However, *Brucella* can maintain some plasmids introduced via electroporation or conjugative transfer (216).

Brucella species share greater than 90% DNA homology (243). This had led some researchers to describe the *Brucellae* as a monospecific genus (278). However, differences between species, based on restriction enzyme analysis and by analysis of restriction polymorphism within specific genes confirm current distinct taxonomic relationships. Molecular genetic studies have demonstrated phylogenetic relatedness to *Agrobacterium*, *Ochrobactrum*, *Phyllobacterium* and *Rhizobium*. (67)

The *Brucellae* are susceptible *in vitro* to gentamycin, tetracycline and rifampicin. Additionally, many strains are also susceptible to ampicillin, chloramphenicol, erythromycin, kanamycin, novobiocin, spectinomycin, streptomycin and sulfamethoxisole/trimethoprim. Susceptibility to antibiotics can differ among species, biovars and even strains therefore these differences can aid in identification of specific strains of *Brucella* (277).

1.3.1.2 Pathogenesis and Virulence Factors

The *Brucellae* are facultative intracellular parasites. They usually enter the body through cuts and abrasions in the oral mucosa, nasopharynx, conjunctiva or genitalia and even unbroken skin. After gaining entry to the body, the organism survives within the cells of the reticuloendothelial system particularly in the macrophages and/or monocytes. They can survive within cells derived from either ectodermal or mesodermal origin, but do not invade tissue of endodermal origin (243). Virulent *Brucella* can infect both

nonphagocytic and phagocytic cells. The mechanism of invasion of nonphagocytic cells is not clearly established. Cell components specifically promoting cell adhesion and invasion have not been characterized, and attempts to detect invasin genes homologous to those of enterobacteria have failed. Within nonphagocytic cells, *Brucella* tends to localize in the rough endoplasmic reticulum. In polymorphonuclear or mononuclear phagocytic cells, they use a number of mechanisms for avoiding or suppressing bactericidal responses. The smooth-lipopolysaccharide (S-LPS) plays a substantial role in intracellular survival as smooth organisms survive much more effectively than rough ones. Compared with enterobacterial LPS, S-LPS has many unusual properties: a relatively low toxicity for endotoxin-sensitive mice, low toxicity for macrophages, low pyrogenecity and hypoferremic activity. It is also a relatively poor inducer of interferon but paradoxically, is an effective inducer of interleukin-12 (IL-12) (46), (294).

Pathogenicity is related to production of lipopolysaccharides containing a poly N-formyl-perosamine O-chain, Cu-Zn superoxide dismutase, erythrulose phosphate dehydrogenase, stress-induced prot eins related to intracellular survival, and adenine and guanine monophosphate. An important survival factor in phagocytic cells is the production of adenine and guanine monophosphates, which inhibit phagolysosome fusion, degranulation and activation of myelo-peroxidase-halide system and production of tumor necrosis factor (45), (76).

An auxotrophic mutant defective in the 5'-phosphoribosyl-5-amino-4-imidazole carboxylase, an enzyme necessary for the *de novo* synthesis of purines, is essential for the intracellular survival of *B. melitensis*. Deletion of the *purE* gene encoding this enzyme drastically reduced the ability of *B. melitensis* to survive within macrophages and demonstrated attenuated behavior in mice and goats (54, 73, 75).

Brucella Cu-Zn superoxide dismutase is believed to play a significant role in early phase of intracellular infection (34). Survival within the macrophages is associated with the synthesis of 17, 24, 28, 60 and 62 kilodalton (kDa) proteins encoded by *Brucella*. The 62 kDa protein corresponds to the GroEL homologue heat shock protein HSP62 and

the 60 kDa protein is an acid induced variant of this. The 24 kDa protein is also acid induced and its production correlates with bacterial survival under acidic conditions. The 17 and 28 kDa proteins are specifically induced by macrophages and correlated with intracellular survival (152). Another stress induced protein, HtrA, is involved in the induction of an early granulomatous response to *B. abortus* in mice and is associated with a reduction in the levels of infection during the early phase (262). The role of iron-sequestering proteins or other siderophores in the pathogenesis of brucellosis is unknown. In general, the low availability of iron *in vivo* restricts the microbial growth. However, high iron concentrations promote the killing of *Brucella,* probably favoring production of hydroxylamine and hydroxyl radical (67).

Recently, a two component regulatory system has been discovered in *B. abortus*. The Bvr (*Brucella* virulence related proteins) system consists of regulatory (BvrR) and sensory (BvrS) proteins. This regulatory system may play a critical role in the ability of *B. abortus* to invade and multiply within cells (244). BvrR deficient mutants were obtained by transposon mutagenesis. Morphologically, these mutants produced smooth type LPS. They were increasingly sensitive to polycations and surfactants and showed decreased *in vivo* replication and persistence in mouse spleens without any obvious growth defects *in vivo*. Complementation with the *bvrR* gene restores resistance to polycations and partially restores the ability of these mutants to multiply intracellularly. The results further suggest that restoration of full virulence requires both components of the regulatory system to be intact. Since LPS, core and lipid A are known to be involved in polycationic resistance there is a good chance that these cell envelope components are under BvrR-BvrS regulatory system (277).

1.3.2 Immune Responses to Intracellular Bacterial Pathogens

Resistance to facultative intracellular bacterial pathogens depends on acquired cell mediated immunity, characterized by the activation of T-lymphocytes and subsequent activation of macrophages for increased killing of such intracellular pathogens. When

intracellular bacteria invade a host, they are phagocytosed by resident macrophages resulting in local inflammation. $CD4^+$ and $CD8^+$ T cells are then activated; $CD4^+$ T cells primarily produce IFN- that enhances macrophage functions including their bactericidal activity. They also activate pathogen specific $CD8^+$ T cells which subsequently lyse infected macrophages releasing the intracellular bacteria. These released bacteria are then phagocytosed again and killed by the INF- activated macrophages.

T helper cells of the $CD4^+$ $CD8^-$ phenotype include two distinct subsets based on the two different profiles of cytokine production. T helper type 1 (Th1) cells characteristically secrete Th1 type cytokines IFN- , IL-2 and TNF- . Th1 cells act primarily as helper cells for cell-mediated inflammatory reactions such as delayed hypersensitivity and macrophage activation. Although INF- promotes IgG2a secretion, the Th1 type cytokines do not stimulate specific antibody formation. T helper 2 (Th2) type cells typically produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. This mixture of cytokines stimulates antibody production particularly IgE, IgG1 and IgA but they have no effect on development of cell mediated responses. Th1 type cytokines inhibit the actions of Th2 cells, induce cellular type responses and promote resistance to intracellular pathogens (265).

Cytotoxic T cells ($CD8^+CD4^-$ phenotype) are also involved in protection against intracellular bacteria (135). The $CD8^+$ cytotoxic T lymphocytes (CTLs) kill target cells principally via two major pathways: 1) the Fas ligand on CTLs interacts with its Fas receptor on target cells and then activates a suicide pathway in the target cells; 2) the CTLs exocytose granules containing perforin and granzymes that form pores in the target cell membrane causing cell death.

1.3.2.1 Immune Responses to Brucella

1.3.2.1.1 Cell-Mediated Immune Response

Cell mediated immune response (CMI) is essential to induce protection against intracellular pathogens including *Listeria monocytogenes*, *Mycobacterium spp.*, *Brucella spp.*. Most of the pathogens are able to survive within the macrophages, enabling the bacteria evade the innate defense system such as complement mediated and phagocyte-mediated killing. *B. abortus* replicates within the endosomes of phagocytic and non-professional phagocytic cells by inhibiting the phago-lysosome fusion and escaping the action of degrading enzymes (67).

Although antibodies to O-side chain confer a certain level of protection against a virulent challenge in mice, CMI appears to play a major role in protection against brucellosis in naturally susceptible species of animals (9), (10), (67). The best example for this is the protection induced by *B. abortus* strain RB51. This strain does not induce O-side chain antibodies yet it provides good level of protection against *Brucella* infection. The protection induced by strain RB51 can be transferred by immune T cells but not by serum of immunized mice (125). Both $CD4^+$ and $CD8^+$ T cells are involved in immunity against brucellosis as indicated by cell depletion experiments using T-cell specific monoclonal antibodies (9), and further by using gene targeted knockout mice lacking either $CD4^+$ or $CD8^+$ T cell population (53), (189). *B. abortus* induces a Th1 type immune response and inhibits both the primary and secondary Th2 type immune responses (3), (289), (105).

Cytokines are essential molecules that modulate the development of protective immune responses by stimulation of $CD4^+$ T cells to produce IFN- *in vivo* and *in vitro*. IFN- is particularly important since it activates the macrophages and up-regulates their bactericidal activity. The important role of IFN- in mediating resistance to *Brucella* infection is demonstrated by depletion of endogenous IFN- with monoclonal antibody,

which causes increased *Brucella* replication *in vivo*. In addition, in an adoptive T-cell transfer experiment with IFN- , antibodies blocked the transfer of T cell mediated resistance in mice (292). Experiments *in vitro* demonstrated that macrophages activated with IFN- have enhanced brucellacidal and brucellastatic activities (124). This cytokine is produced mainly by natural killer (NK) cells, Th1 cells and its production is positively regulated by IL-12.

IL-12 is a heterodimeric protein consisting of P40 and P35 subunits. The biologic activities of IL-12 include stimulation of NK and cytotoxic T cells, induction of CD4+ T cells *in vivo* and *in vitro*. IL-12 is the pivotal factor for the differentiation of Th1 cells and induces proliferation of murine and human Th1 clones (289). Several studies in mice indicate that endogenous IL-12 produced during infection with *B. abortus* promotes the production of INF- and the clearance of bacteria in *vivo* (290, 293, 295). Depletion of endogenous IL-12 before infection of mice significantly exacerbated *Brucella* infection. IL-12 depleted mice also had reduced splenomegaly resulting from infection and showed a decrease in percentage and absolute number of macrophages compared with those in non IL-12 depleted control mice. Furthermore, spleen cells from IL-12 depleted mouse had reduced ability to produce nitrate, a product of activated macrophages (293).

Production of tumor necrosis factor-alpha (TNF-) also appears to be important for a protective immune response to *Brucella*. Only live *Brucella* elicited the production of TNF- from macrophage cultures (291). TNF- receptor knockout mice (TNF-$R^{-/-}$) were severely deficient in IL-12 production and *Brucella* infection in these knockout mice was exacerbated (53). In addition, the production of nitric oxide by macrophages was inhibited in the TNF-$R^{-/-}$ mice, suggesting that nitric oxide may also be an important mediator of brucellacidal activity by activated macrophages.

In summary, Th1 type cytokines such as IFN- and TNF- appear to be of high importance in protection against *Brucella* infections. The cytokines are induced by live attenuated *Brucella* vaccines and not by killed vaccines that tend to induce a Th2 type

immune response. A Th2 type immune response actually may interfere with protective immunity to brucellosis.

1.3.2.1.2 Humoral Immune Responses to Brucella

Convincing evidence as to the role of antibodies in protection against *Brucella* infection comes from the passive immunization of mice with either monoclonal antibodies (mAbs) or polyclonal immune serum. Passive immunization with immune serum from mice recovered from the infection or vaccinated with smooth strains of *Brucella* conferred protection against challenge with virulent *B. abortus* (10). A number of studies have investigated the protective effect of passive immunization with mAbs directed towards *Brucella* LPS and outer membrane proteins (OMPs) (281), (30), (31). The O antigen of *Brucella* LPS, although it is a carbohydrate, elicits a strong antibody response including IgM and IgG isotypes. The antibodies to the *Brucella* O antigen can confer passive as well as acquired partial protection against virulent *Brucella* infection in the mouse model (10), (173). Among these mAbs, IgM and IgG2a isotypes were reported to be better protectors (281). Protection afforded by an IgG3 mAb specific to rough LPS was found to confer significant protection, although it did not reach the level of protection afforded by S-LPS specific mAb (59). It was also found that *B. abortus* strain 19 and 2308 stimulated protracted polyclonal responses of both IgG2a and IgG3 isotypes specific to the O-side chain of smooth *Brucella* in BALB/c mice (83). The presence of high IgG2a and the absence or low IgG1 subisotype antibodies to the O-side chain indicated the induction of a Th1 type immune response (255).

Based on immunization experiments with *E. coli* extracts containing the *B. melitensis* 25 kDa major OMP, Bowden et al. (1995) proposed that antibodies against well exposed conformational epitopes of OMP25 can contribute to protection against *B. melitensis* infections in mice. Antibodies to OMPs and R-LPS have been demonstrated to be protective against infections with naturally rough strains of *Brucella* such as *B. ovis* (31).

Brucella infection induces the production of IgM, IgG1, IgG2a and IgA antibody isotypes detectable in both milk and sera of cattle (186). IgM is produced soon after infection but declines rapidly. IgM reacts nonspecifically in many serological tests and cause high false positive reactions (187). IgG1 is consistently produced at high levels in *Brucella*-exposed cattle and has high affinity and specificity for *Brucella* antigens particularly for the O-side chain.

Recent studies with strain RB51 indicated that it induces high levels of IgG2a and low level of IgG1 in response to *Brucella* Cu/Zn SOD antigen (276). Strain RB51 overexpressing *Brucella* O-side chain induces predominantly IgG2a and IgG3 responses and no detectable IgG1 response to SOD (275), (115).

1.3.3 Brucellosis Vaccines: Past, Present, Future

Since *B. melitensis* and *B. abortus* were isolated around the turn of 20th century, the magnitude of brucellosis problem in terms of economics to the domestic animal industry and human health has been widely recognized. Major efforts have been devoted to the prevention and treatment of this disease. In addition, prevention of the disease by vaccination has played an essential role in brucellosis eradication program. Both killed and live vaccines have been examined for their potential in the control and eradication of brucellosis in cattle, goats and swine. Live attenuated vaccines induce an effective, long lasting, protective, cell mediated immune response. Since they are administered live and the organism replicates in the host, they are inexpensive vaccines.

1.3.3.1 Past Brucella Vaccines

1.3.3.1.1 Brucella abortus Strain 19

Strain 19 (S19) is an attenuated smooth strain and was the one of the most commonly used vaccines to prevent bovine brucellosis. Although the basis of the

attenuation is unknown, there is a deletion in the erythritol catabolic genes (225). Vaccination with S19 protects cattle against subsequent *Brucella* infection. There is a degree of variation in the level of protection depending on a number of factors such as the age of the animal, dose, route and prevalence of brucellosis in vaccinated herds (185). Although S19 is of low virulence for cattle, vaccination of pregnant cattle can result in abortions even when a reduced dose (1/20-1/100) of the standard dose was used (70), (6). In addition, presence of an O-side chain in the LPS of S19 caused production of O-side chain antibodies. This often caused persistent antibody titers in vaccinated animals, which could not be distinguished from antibody in naturally infected animals using standard serological tests (116), (186), (251). For these two reasons, persistence of diagnostic titers and probability of causing abortion in the infected animals, Erasmus and Erasmus recommended that vaccination of adults with the reduced dose of S19 should be relegated to herds heavily infected with *B. abortus* (85). S19 was then recommended only for calves between 4 and 12 months of age and was designated as calfhood vaccine. However, this also caused some side effects as calves were found to be suffering from persistent orchitis in males and arthropathy in females and still resulted in persistent titers in some animals (44), (68).

1.3.3.1.2 B. abortus strain 45/20

The appearance of O-side chain antibodies due to S19 vaccination stimulated the search for other vaccines that would not cause this problem. *B. abortus* smooth strain 45 was isolated from a cow in 1922 and a rough derivative was obtained after 20 passages in guinea pigs (193). Unfortunately when used as a live vaccine this strain was not stable and tended to revert to the smooth virulent form thus defeating the purpose of attenuation (56). Therefore, strain 45/20 was used as a bacterin. The presence of severe local reactions observed with some animals at the site of vaccine inoculation and induction of anti-O antibodies in some animals which eventually caused discontinuation of strain 45/20 use.

Several other live vaccines have been used in the past but they have not been adopted widely or their use has been discontinued for a variety of reasons. Among these is the M vaccine a mucoid derivative of *B. suis*, the 104 M vaccine a derivative of *B. abortus* strain, *B. suis* strain 2 used as an oral vaccine in China for cattle, goat and sheep vaccination (285) and *B. melitensis* strain 5 is also used in China to prevent brucellosis in cattle, sheep, goats and deer (227).

1.3.3.1.3 B. melitensis Rev1

Rev1 vaccine is an attenuated, smooth live *B. melitensis* strain originating from a virulent *B. melitensis* isolate (79). In experimental challenge trials in goats, this strain induced significant protection against a virulent strain (2). The Rev1vaccine has some disadvantages as it can cause abortion if used in pregnant animals and can result in persisting agglutinins that interfere with various serological diagnostic tests (2). It is fairly pathogenic to humans via aerosol exposure (194); self-inoculation causes generalized brucellosis in affected individuals (277). It is still widely used in goats and no good substitute vaccine has been developed.

1.3.3.2 Current Brucella Vaccine

1.3.3.2.1 Brucella abortus RB51

In the search for a rough mutant that would be stable and sufficiently attenuated, strain RB51 was selected by serial passage of virulent strain 2308 on trypticase soy agar supplemented with varying concentrations of rifampin and penicillin. Strain RB51 is essentially devoid of the O-side chain and it remains stable after multiple passages *in vivo* and *in vitro* (228), (63). Most importantly, because of the lack of the O-side chain, it does not induce O-side chain antibodies regardless of age, dose, or frequency of injections, therefore, it does not interfere with serological tests used in the diagnosis of brucellosis.

Virulence studies on strain RB51 were first carried out in BALB/c mice and thereafter in guinea pigs, cattle and goats. *B. abortus* strain RB51 is cleared from mice much faster than even much lower doses of strain 19 (173), (253). Goat fetuses injected *in utero* with over $1x10^8$ strain RB51 became infected, but did not die or abort. Conventional serological tests remained negative for both does and kids (221). These observations *in vivo* confirmed the attenuated characteristics of the strain RB51. Abortion due to strain RB51 vaccination of pregnant animals is uncommon but can occur (226).

The advantages of using strain RB51 relative to other vaccines in order to induce protection against bovine brucellosis are numerous. It does not produce any clinical signs, nor does it produce a local reaction at the site of injection (55). It is cleared as early as 2 weeks post inoculation from the bloodstream. It is not shed in the nasal secretions, saliva, or urine. Therefore, the organism appears to be unable to spread from vaccinated to non-vaccinated animals through these routes. In immunocompromised animals, no recrudescence of infection has been documented. Vaccination of cattle with strain RB51 induces significant protection comparable or superior to the protection induced by strain 19 (154). *B. abortus* strain RB51 is able to induce protective immunity against *B. melitensis*, *B. abortus*, *B. suis* and *B.ovis* challenge in mice but appears to not to protect against *B. ovis* infection (228), (125), (252).

The use of strain RB51 has also helped clear up the issue of *Brucella*-positive herds. As of February 1996, the USDA Animal Plant Health Inspection Service (APHIS) approved the use of strain RB51 as the official calfhood vaccine for protection against brucellosis (http://www.aphis.usda.gov:80/vs/nahps/Brucellosis/rb51.html). Since this strain does not interfere with the serological tests, even adult cattle vaccinated with strain RB51 remained negative in all subsequent tests. This made it possible to distinguish between the infected versus vaccinated animals, which has greatly helped the brucellosis eradication program. The basic approach of the program has always been to test cattle for antibodies against LPS and slaughter the serologically positive animals. Identification

of market animals for tracing, surveillance to find infected animals, investigation of affected herds and vaccination of replacement calves in high risk areas are the important features of the program. Studies have shown that if the brucellosis eradication program efforts were stopped, the cost of producing beef and milk would increase by an estimated $80 million annually in less than 10 years. As of April 2001, it has been declared that there are no cattle in the United States quarantined for brucellosis (http://www.aphis.usda.gov:80/us/bruczero.pdf). States are designated brucellosis free when none of their cattle or bison are found to be infected for 12 consecutive months under an active surveillance. The states of Florida, Texas, Missouri and Oklahoma must complete 12 months without any brucellosis detection before they are declared class free.

1.3.3.3 Future Brucellosis Vaccines

1.3.3.3.1 Rough Mutants

Development of new attenuated live vaccines for humans or improvement of animal vaccines requires genetically defined mutants. An effective live vaccine must induce a strong immune response and cause no disease. In this respect, many groups are trying to generate deletion mutants. Our group at the Center for Molecular Medicine and Infectious Diseases (CMMID) identified the *wboA* gene in *Brucella,* which encodes a mannose glycosyltransferase that is essential for the biosynthesis of the *Brucella* O antigen (166). This finding was useful for two purposes: genetic characterization and complementation of O-side synthesis in strain RB51 (275) and construction of rough disruption mutants of *B. melitensis* 16M and *B. suis* (282). This strategy can be utilized to generate rough mutants from any smooth *Brucella* species. These mutants were tested in mice for their virulence and ability to protect mice against infection with heterologous and homologous strains of *Brucella*. These *wboA* deletion mutants showed lower virulence than their parent strain but the level of attenuation achieved was not as complete as with strain RB51 (282). This indicated that *wboA* gene is involved in virulence but also suggests involvement of other genes in the attenuation of strain RB51 (275). Protection induced by these *wboA* deletion mutants was assessed in mice and

found to be superior to the protection afforded by strain RB51. This is probably due to their more vigorous replication characteristics and the ability to stay longer in the host than strain RB51. Studies with the *B. melitensis wboA* deletion mutant indicated that it was able to colonize the lymph nodes of goats and did not induce abortion in pregnant goats. This *B. melitensis* deletion mutant did not induce O-side chain antibodies, however a single dose vaccination induced only partial protection against both infection and abortion following challenge (82).

A purine synthesis mutant of *B. melitensis* 16M was generated by Drazek et al., (75). This mutant was generated by electroporation of strain 16M strain with suicide plasmids containing a kanamycin resistance cassette that replaced 226bp of the carboxyl end of *purE*, the intergenic region and 18 bases of the *purK* open reading frame. These two genes encode two enzymes of the *de novo* purine synthesis pathway (195). Recombinant *B. melitensis* Δ*purE201* required exogenous purines for growth on minimal media and had a decreased ability to replicate in human monocyte derived macrophages (75). Infection of mice with strain Δ*purE201* induced a lesser infection than one caused by strain 16M with earlier clearance of bacteria from target organs and less profound alteration of spleen cell phenotype and proinflammatory cytokine response (73). This strain was further analyzed for its ability to elicit cellular and humoral immune responses and to protect mice against intranasal challenge with *B. melitensis* 16M. Mice inoculated intraperitoneally with Δ*purE201* made serum antibody to lipopolysaccharide and non-O-polysaccharide antigens. Splenocytes from immunized animals released interleukin-2 (IL-2), INF- and Il-10 when cultured with *Brucella* antigens. Immunization led to protection against disseminated infection but had only a slight effect on clearance of the challenge inoculum from the lungs. These studies suggested that Δ*purE201*should be further investigated as a vaccine to prevent human brucellosis (120).

Earlier it was indicated that brucellosis vaccines need improvement. An example of improved *Brucella* vaccine is strain RB51 used as a vector for homologous protein expression. Protection afforded by strain RB51 vaccination is through induction of specific CMI (10). Studies in our laboratories indicated that strain RB51 preferentially

induces the Th1 type of immune responses (273), (272). It was reasoned that strain RB51 as a vaccine could be improved by using it as a vector for the delivery of protective proteins (274). For this purpose *Brucella* Cu/Zn superoxide dismutase (Cu/Zn SOD) gene was overexpressed in strain RB51. This strain, RB51/SOD overexpressing a homogenous antigen, was tested in BALB/c mice for its ability to protect against challenge infection with virulent strain 2308. Mice vaccinated with strain RB51/SOD but not with RB51 strain developed antibodies and cell mediated immune responses to Cu/Zn SOD. Strain RB51/SOD vaccinated mice developed significantly higher resistance to challenge than those vaccinated with strain RB51 alone. The presence of the plasmid alone in strain RB51 did not alter its protective efficacy. In addition, overexpression of SOD did not alter the attenuation characteristic of strain RB51 (276). In a similar approach, the *wboA* gene mutation was complemented with plasmid carried *wboA* gene in strain RB51. Complemented mutant RB51/WboA expressed the O-side chain intracellularly, however it had a rough phenotype. Clearance studies in mice indicated no increase in the survivability of strain RB51/WboA *in vivo* compared to that of strain RB51. Vaccination of mice with live strain RB51/WboA induced antibodies to the O-side chain, which were predominantly of the immunoglobulin G2a (IgG2a) and IgG3 subisotypes. Mice vaccinated with strain RB51/WboA were better protected against a challenge infection with the virulent strain 2308 than those vaccinated with strain RB51 (275).

1.3.3.3.2 Recombinant Vaccines

The use of recombinant antigens as immunogens to induce protective immune response in animals is being investigated. Various *in vitro* assays such as production of cytokines, antigen specific lymphocyte proliferation, T-cell cytotoxicity toward *Brucella* infected target cells and *in vivo* assays can be used to select putative target antigens. Recombinant antigens can be delivered with an appropriate vector system or can be purified from recombinant bacteria or insect viruses such as baculovirus and injected in the presence of appropriate adjuvants or stimulatory cytokines. Our group at the CMMID has used vaccinia virus/ *Brucella abortus* recombinants (266), (16), (17), (273), or purified *B abortus* antigens expressed in the baculovirus system (13), (14) with and

without cytokines and adjuvants. These antigens included HtrA, GroEL, and GroES, 18 kDa antigen Cu/Zn SOD and L7/L12 ribosomal protein. These studies indicated that recombinant vaccinia viruses were able to express *Brucella* antigens and induce specific immune responses to these antigens *in vivo,* however, protection against challenge with virulent *Brucella* infection was not achieved. Other groups have also tried other vector systems including attenuated *Salmonella* as the carrier for the antigens. *S. typhimurium* recombinant expressing 31kDa *Brucella* protein has been used for oral immunization of mice that lead to induction of serum antibodies to the recombinant proteins although CMI responses were poor (247). Purified antigens such as Cu/Zn SOD (196) and L7/L12 (190) protected mice against virulent *Brucella* challenge. However, more research is still needed in order to optimize the vectors and levels of recombinant antigen production to induce a sufficient protective CMI response.

1.3.3.4 Brucella Antigens

Many of the proteins and carbohydrates of *B. abortus* have been investigated for their possible role in the immune response induced against brucellosis. The antigens can be classified in four groups: LPS, outer membrane proteins, periplasmic proteins and cytoplasmic proteins.

1.3.3.4.1 LPS

The outer membrane contains lipopolysaccharide and outer membrane proteins. The LPS of smooth strains consists of lipid A, a core region containing mannose and 2-amino-2,6 dideoxy-D-glucose (quinovosamine) and 3-deoxy-D-manno-2-octulosonate (KDO) and an O-side chain composed of a homopolymer of about 100 residues of *N*–formylated perosamine (43). Two distinct epitopes occur on the O-side chain depending upon the species of the strain. In *B. abortus* the A epitope is present predominantly and probably represents terminal *N*-formylated perosamine residues which are linked by the 1 and 2 carbon atoms throughout the chain. In *B. melitensis,* the M epitope is predominant. In this case, the O-side chain consists of repeating units of four

1,2-linked *N*-formylated perosamine residues and one 1, 3-linked residue (206). The M and A epitopes can be detected by cross-absorbed polyclonal antisera.

The toxic effects of *Brucella* LPS are attributable to the lipid portion. Increased survival of smooth organisms compared with rough avirulent organisms in macrophages suggests that the LPS plays a role in uptake and survival (217). In mice the passive transfer of monoclonal antibodies directed against the O-antigen produced effective immunization, while the antibodies directed against a number of outer membrane proteins induced no significant protection (173).

1.3.3.4.2 Outer Membrane Proteins

Outer membrane proteins (OMPs) were identified in the early 1980s by selective extraction techniques and classified according to their apparent molecular mass in either group 1(88 to 94 kDa), group 2 (35 to 39 kDa) and group 3 (25 to 31 kDa) proteins. These proteins have been further characterized by the use of monoclonal antibodies and include a) lipoproteins: OMP10, OMP16, and OMP19, b) two homologous group 3 proteins: OMP25 and OMP31, c) porin proteins: OMP2a/OMP2b and d) group 1 proteins: OMP1 or 89kDa protein (57). The OMPs were shown to be located exposed on the surface by monoclonal antibodies (mAbs) and enzyme-linked immunosorbent assay (ELISA), immunoelectron microscopy and flow cytometry. They are much less accessible on smooth than on rough *Brucella* strains. This may be the reason why mAbs to OMPs were poorly or not at all protective against a smooth *Brucella* challenge in a mouse model. In contrast, mAbs to OMPs particularly to OMP31 have shown high protective ability in mice against rough *B. ovis* (61). The genes encoding OMPs are particularly interesting for molecular typing purposes as they display diversity among *Brucella* species, biovars and strains allowing their differentiation (60).

Brucella YajC and SecD proteins are encoded by two open reading frames (ORFs) in one operon (273). Both proteins are suspected to be involved in the translocation of periplasmic and putative secretory proteins of *Brucella*. YajC induces

humoral immune response in mice. In response to a recombinant YajC-MBP fusion protein, splenocytes from mice vaccinated with B. *abortus* strain RB51 were able to proliferate and produce gamma interferon but not interleukin 4 (IL-4). Sec D did not induce antibody response detectable by Western blot analysis (273).

An 18-kDa lipoprotein is also present on the surface of *B. abortus* (140). This protein induces humoral immune response in infected mice, sheep, goats, dogs humans and CMI response in mice models. However, a vaccinia virus recombinant expressing this protein was not able to induce protection to a *Brucella* 2308 strain challenge in mice. In addition, disruption of the gene encoding this protein in strain RB51 was found not to affect this strain's vaccine characteristics or its *in vivo* attenuation characteristics (272). These data suggest that the 18kDa protein does not play a role in protective immunity.

1.3.3.4.3 Periplasmic Proteins

A number of immunogenic proteins have been identified in the periplasmic space including BCSP31, BP26 and Cu/Zn SOD. Among these, BCSP31 is a 31kDa salt-extractable protein and highly antigenic during natural infections and vaccinations (33), (162). The gene encoding this protein has been used to design PCR primers for rapid diagnosis of human brucellosis by PCR (210). Immunization of mice with recombinant BCSP31 did not provide protection (109), (247), (248). BP26 is a periplasmic protein and is highly antigenic in sheep cattle, goats and humans. Antibodies to the 26kDa protein can be detected in serological assays with sera from infected animals and could be used as a diagnostic antigen for detection of brucellosis in infected animals (58).

1.3.3.4.3.1 Cu-Zn SOD

Cu-Zn SOD activity is present in all oxygen metabolizing cells and protects cells from the toxic effects of reactive oxygen intermediates by converting superoxide radicals into hydrogen peroxide (H_2O_2) and oxygen (97). Superoxide radicals (O_2^-) are generated as intermediates during reduction of molecular oxygen. In addition, these oxygen radicals undergo further reduction to form hydrogen peroxide and hydroxyl radicals

(OH*). These active oxygen species can damage the DNA, RNA, protein and lipids leading to disruption of cellular architecture and activity (88). To protect themselves against these toxic species, cells that can grow in the presence of oxygen utilize antioxidant enzymes such as SOD, catalases and peroxidases. SODs also play an important role in bacteria permitting them to survive a phagocytic attack. Thus SOD is usually regarded as a virulence factor facilitating intracellular survival (88). Three forms of SOD have been characterized according to their metal prosthetic groups, manganese (Mn), iron (Fe) and copper-zinc (Cu/Zn) (19). Cu/Zn SOD is usually present in the cytosol of eukaryotes. Only few species of prokaryotes contain Cu/Zn SOD which are considered as the most recently evolved SOD (18), (149).

B. abortus possess two forms of SOD. One has been preliminarily characterized as a manganese [Mn SOD (245)], and the second one has been identified as Cu/Zn SOD (23). The specific activity of *Brucella* Cu/Zn SOD in crude extracts of *Brucella* strains is 10 to 100 fold higher than reported for other intracellular bacterial pathogens (13). However, the presence of larger or similar amounts of SOD, the presence or absence of SOD activities in the culture supernatants among the virulent and avirulent *B. abortus* strains studied suggests that SOD is not a major virulence factor. Deletion of Cu/Zn SOD from virulent *B. abortus* strain 2308 did not induce differences in virulence *in vivo*, bacterial survival or colony morphology (146). These data also suggest that Cu/Zn SOD is not a major virulence factor for *Brucella* smooth strains. It is possible that in the presence of a major virulence factor like the O-side chain, the virulence role of Cu/Zn SOD is overshadowed (146). It may well be that SOD contributes to the virulence of *B. abortus* but is only one of several factors (45). Others suggest that the presence of a novel Cu/Zn SOD in *Brucella* may be a critical virulence associated factor that allows the survival of the organism in the host (23). It has been suggested that Cu/Zn SOD contributes, but is not solely responsible for increased survival of *B. abortus* 2308 within murine macrophages during early stages of infection (263). Intracellular bacteria containing periplasmic Cu/Zn SOD, such as *B. abortus*, are able to survive the initial extracellular or extracytoplasmic oxidative stress associated with neutrophil or macrophage phagocytosis (249). A more recent study (196) demonstrated a significant

level of protection against a *B. abortus* virulent strain challenge in BALB/c mice injected with $1x10^6$ live *E. coli* cells expressing *B. abortus* SOD. Immunization of mice with purified *Brucella* Cu/Zn SOD or synthetic peptides induces significant protection against virulent strain challenge (13), (260). Moreover, vaccination of mice with *Brucella* strain RB51 overexpressing homologous Cu/Zn SOD also stimulated enhanced protection as compared to strain RB51 alone (276). Such protective and virulence roles of SOD have also been demonstrated in other facultative intracellular bacteria (22).

1.3.3.4.4 Cytoplasmic Proteins

The last category of immungenic proteins include the heat shock proteins (DnaK, HtrA GroEL, GroES), and ribosomal L7/L12 protein. The heat shock response is a widespread phenomenon found in all living cells. Proteins associated with this response are highly conserved. Bacterial heat shock proteins are immunodominant targets for humoral and cellular immune responses (151), (110). *B. abortus* GroEL and GroES are the members of the family of hsp60 and hsp10 homologs, respectively. These proteins are involved in chaperone functions in the cell (80). Due to the immunogenic characteristics of heat shock proteins, they attract considerable attention against unrelated bacterial infection (235). The immunodominance of *B. abortus* GroEL may be related to its high level of expression during infection of macrophages (152). Vaccination with GroEL proteins induces protective immunity against intracellular pathogens, such as *Mycobacterium tuberculosis* and *Legionella pneumophila* (235),(27). Members of the high-temperature requirement A (HtrA) class of stress response proteins are serine proteases which apparently function by degrading oxidatively or otherwise damaged proteins before they can accumulate to toxic levels in cells (13). Oxidative killing pathways are generally thought to be the primary mechanism by which host phagocytes kill intracellular bacteria (15). Thus, the HtrA protein of *Brucella* may contribute significantly to the successful survival in the host phagosomes. However recent studies indicated *Brucella* heat shock proteins expressed in baculovirus and vaccinia virus recombinants did not confer protection against virulent *Brucella* challenge in mice (13), (16), (17).

1.3.3.4.4.1 L7/L12 protein

The immunological stimulation by ribosomal protein preparations from 28 different pathogens including *B. abortus*, has been studied by several investigators (288), (65), (107). These ribosomal antigens confer protection when used as vaccines. Ribosomal protein L7/L12 (12,200 Da) is located in the large ribosomal unit (50S) of prokaryotes (129). This protein forms a well-defined domain in the ribosome involved in interactions with translation factors during protein biosynthesis. The proteins L7 and L12 have identical sequences except for the presence of an acetylated amino terminal serine residue in L7. Both proteins are acidic but they differ slightly in their isoelectric points (264). Because of the close similarity, they are often referred in the literature as the L7/L12 protein. Protein L7/L12 has been studied in detail because of the ease with which it can be removed selectively from the ribosomal submits. The N-terminal domain of L7/L12 is necessary for dimerizing with L10 ribosomal protein and the C-terminal is important in binding the elongation factor EF-Tu and EF-G and energy utilization from GTP hydrolysis. Therefore, L7/L12 is required for high efficiency and low error frequency in protein synthesis (144).

The *B. abortus* L7/L12 ribosomal protein was identified as the most immunodominant antigen (38) among 38 studied *B. abortus* proteins using a T-lymphocyte Western blot technique. An oligonucleotide corresponding to the N-terminal of this protein was used as a probe to screen a *B. melitensis* genomic library. The gene encoding the 12 kDa *B. melitensis* protein was identified and sequenced. This protein is similar to *E. coli* ribosomal protein L7/L12 and identical to the *B. abortus* protein (192).

Studies with recombinant *B. abortus* L7/L12 ribosomal protein indicated, this protein induces a Th1 subset response by murine $CD4^+$ T cells producing significant levels of INF- production (193). Antigens that preferentially induce IFN- producing Th1 subset response are desirable subunits of any vaccine preparation against brucellosis. Furthermore, peripheral blood mononuclear (PBM) cells from *B. abortus* primed cattle

are able to respond to the recombinant L7/L12 *in vitro*, demonstrating that T-cell recognition of this particular antigen is not species specific (191). Other studies also indicated that the, L7/L12 protein is a major component in the Brucellin used for delayed-type hypersensitivity (DTH) testing in *Brucella* sensitized guinea pigs (12). In addition, the immune response to the *in vivo*-expressed *B. abortus* ribosomal L7/L12 gene in muscle cells was examined (144). This naked DNA application demonstrated specific antibody and T-cell responses compared with negative controls and *B. abortus* strain 19 injected controls. After being characterized as a T-cell reactive ribosomal protein, L7/L12 was tested for its ability to confer protection to mice. Recombinant *Brucella abortus* L7/L12 ribosomal protein fused to maltose binding protein (MBP) was used to immunize BALB/c mice (190). *B. abortus* L7/L12 ribosomal protein conferred a significant degree of protection when compared to mice vaccinated with adjuvant alone, adjuvant plus MBP or *B. abortus*. These data indicated that a recombinant protein previously identified as T-cell reactive, engendered protective immunity to mice against brucellosis.

1.4 LISTERIOSIS

Listeriosis is a food-borne disease caused by *Listeria monocytogenes.* This bacterium is ubiquitous and found throughout the environment including soil, water and decaying vegetation. Substantial proportion of the sporadic cases of listeriosis are caused by consumption of the organism in foods. Disease usually occurs in well-defined high-risk groups, including pregnant women, neonates and immunocompromised adults, but may occasionally occur in persons who have no predisposing underlying condition. Susceptible groups and the clinical presentations of listeriosis are summarized in Table 1.6 (224).

Table 1.6 Clinical Syndromes in humans associated with infection with *L. monocytogenes*.

Population	Clinical Presentation	Diagnosis
Pregnant women	Fever, myalgia, diarrhea, preterm delivery, abortion, stillbirth	Blood culture, amniotic fluid culture
Newborns		
< 7 days old	Sepsis, pneumonia	Blood culture
$\geq$7 days old	Meningitis, sepsis	Cerebrospinal fluid culture
Immunosupressed adults	Sepsis, meningitis, focal infections	Blood culture, cerebrospinal fluid
Adults	Diarrhea and fever	Stool culture

In addition to sepsis, meningitis and meningoencephalitis, a variety of other clinical manifestations of infection with *L. monocytogenes* include endocarditis, endopthalmitis, septic arthritis, osteomyelitis, pleural infection and peritonitis. Focal infections are rare and usually result from seeding during a preceding bacteremic phase (224).

Numerous animal species are susceptible to listerial infection, with large proportion of healthy asymptomatic animals shedding *L. monocytogenes* in their feces. Although most infections are subclinical, listeriosis in animals occurs either sporadically

or as epidemics and often leads to fatal forms of encephalitis. Virtually all domestic animals are susceptible to listeriosis (155). Animal listeriosis is frequently associated with late term abortions, placentitis, gastrointestinal septicemia with hepatitis, splenitis, pneumonitis and encephalitis (165).

Listeria species are small gram positive, non-spore forming, facultatively anaerobic rods with rounded ends. They possess peritrichous flagella and are motile when cultured at 20- 25^{0}C. *Listeria* usually grows well on most commonly used bacteriological media. The normal temperature limits for growth are -2 ^{0}C to 45 ^{0}C (128). They are catalase positive and oxidase negative and express a -hemolysin that produces zones of clearing on blood agar (87).

Since *L. monocytogenes* is commonly found in the environment, avoiding exposure presents a difficult challenge. Dietary and food preparation measures have been recommended to the general public. These measures include thorough cooking of raw food from animal sources, washing raw vegetables before eating, keeping uncooked meats separate from vegetables, cooked foods and ready-to-eat foods and washing hands after handling uncooked foods (50).

For immunocompromised people, there are specific dietary measures that can be taken to decrease risk. Such persons should avoid foods epidemiologically linked with listeriosis such as soft cheeses and ready to eat foods. In addition to individual advice for consumers, control of listeriosis requires action from public health agencies and the food industry.

1.4.1 Pathogenesis of *Listeria monocytogenes* and Listeriolysin

Factors affecting the pathogenicity of *L. monocytogenes* are multifactorial. These include:

1) Capacity for intracellular growth
2) Surface components, hemolysin

3) Catalase and superoxide dismutase
4) Iron compounds

Most of the known virulence gene products are involved in the intracellular life cycle of *L. monocytogenes.* These genes are clustered on the chromosome in the so-called PrfA dependent virulence gene cluster. The cluster comprises six well-characterized genes, *prfA*, *plcA*, *hly*, *mpl*, *actA* and *plcB.* Products of these genes are: listeriolysin (*hly*), a phosphatidylinositol-specific phospholipase C (*plcA*), a phophatidylcholine-specific phospholipase C (*plcB*), a metalloprotease (*mpl*), ActA protein involved in actin polymerization (*actA*) and the positive regulatory factor PrfA (*prfA*). The internalin genes *inlA*, *inlB*, and *inlC* coding for internalin proteins A, B, C respectively also play a major role in virulence and are located outside of the virulence gene cluster (143).

Hemolytic activity detected around colonies of *L. monocytogenes* growing on blood-agar plates results from a cytolysin called listeriolysin (LLO). LLO is a sulfhydryl (SH)-activated cytotoxin secreted by virulent *L. monocytogenes* that permits entry of the organism into the cytoplasm of eukaryotic cells from the endocytic pathway. The role of LLO in virulence was determined by injection of wild type and non-hemolytic mutants of *L. monocytogenes* into mice intraperitoneally (ip) and intravenously (iv) and the fate of *Listeria* in liver and spleen were followed. In contrast to wild type strains, non-hemolytic mutants were eliminated from these organs within few hours (71), (100), (209). The role of LLO in intracellular survival was determined using different mouse and human cell lines. Non-hemolytic mutants were incapable of intracellular growth and survival within these host cells and also cultured cells like mouse bone marrow derived macrophages and mouse macrophage like cell line J774 (209). Electron microscopy of infected macrophages and epithelial cells revealed that non-hemolytic *L. monocytogenes* mutants, which were found inside host cells, were unable to open the phagosome to escape into the cytoplasm (100). These data suggests hemolytic activity is indispensable for lysis of the phagosomal membrane.

LLO secretion is necessary for *L. monocytogenes* to induce protective immunity *in vivo* (26) and to elicit IFN- production from specific CD8+ T cells *in vitro* (40). Heat killed organisms and organisms mutant in LLO production are incapable of inducing these responses. These observations suggest a model in which the requirement for LLO secretion for the induction of protective immunity is based on access of the bacterium to the cytoplasm of the infected cell (40). Cytoplasmic localization is sufficient to introduce bacterial proteins to the class I MHC antigen processing pathway (174) resulting in the potential for CD8+ T cell recognition (113). A vaccinia virus recombinant expressing nanomeric CTL epitope (91-99) was protective against *L monocytogenes* challenge (6).

2. RATIONALE OF DISSERTATION

In an attempt to develop a genetically engineered vaccine for human brucellosis, vaccinia virus recombinants expressing *B. abortus* antigens were generated and immune responses induced in BALB/c mice were analyzed. The *Brucella* antigens included GroEL (16), (17), HtrA (266), (13), SOD (266) and 18kDa outer membrane proteins (272). Although these vaccinia virus recombinants did express *Brucella* antigens and induced humoral immune responses in BALB/c mice, no protective immune responses to *Brucella* challenge were induced. To explain these results, several hypotheses were formulated:

1) The previous *B. abortus* antigens chosen were not protective.

2) When expressed by vaccinia virus the *Brucella* antigens were not protective.

3) Vaccinia virus did not present the *Brucella* antigen in the right major histocompatability complex (MHC) context.

4) Vaccinia virus has intrinsic properties inhibiting induction of protective immune responses against virulent bacterial strain challenge.

Therefore the following rationales were used:

1) Cloning and expressing other proteins from *B. abortus* and other bacteria with more protective potential.

2) Using different shuttle vectors to enhance the protein expression

3) Using compounds favoring a Th1 type immune response.

4)Using vaccinia virus strains expressing murine IL-12 subunits and co-stimulatory molecule B7 to enhance T-cell mediated immunity.

5) Exploring combinations of these parameters to test for a synergistic effect.

Recombinant vaccinia viruses induce humoral and cellular immune responses (198), (25), (78). The viral and eukaryotic proteins expressed in vaccinia virus undergo

normal postranslational modifications and intracellular trafficking and they are presented in their native configuration. This is an important advantage as viral neutralizing antibodies are frequently directed to conformational epitopes of surface glycoproteins (178). In numerous examples, protection induced by recombinant vaccinia viruses was correlated with neutralizing antibody against viral envelope proteins (180).

The induction of strong class I restricted cytotoxic T cell (CTL) response is another major advantage that recombinant vaccinia viruses offer. In many studies protection against virulent pathogens was correlated with the induction of CTL responses (126), (136), (172), (7). Antigen was presented by class I molecules (72), (267) and even expression of minigenes encoding short peptides was sufficient to induce protective CTL responses (106), (280), (7).

Protective immune response against *B. abortus* requires induction of strong cell mediated immunity (9), (10), (67). Both $CD4^+$ and $CD8^+$ T cells are involved in immunity against brucellosis as indicated by cell depletion experiments using T-cell specific monoclonal antibodies (9), and further by using gene targeted knockout mice lacking either $CD4^+$ or $CD8^+$ T cell populations (53), (189). *B. abortus* infection induces a Th1 type immune response and inhibits primary and secondary Th2 type immune responses (3), (289), (105). $CD4^+$T cells secrete INF- , which enhances the antimicrobial activity of macrophages allowing bacterial killing (124). INF- production is positively regulated by IL-12. The biologic activities of IL-12 include stimulation of natural killer (NK) and cytotoxic T cells as well as induction of CD4+ T cells *in vivo* and *in vitro*. IL-12 is the pivotal factor for the differentiation of Th1 cells and induces proliferation of murine and human Th1 clones (289). Several studies indicate that endogenous IL-12 produced during infection with *B. abortus* promotes the production of INF- and clearance of the pathogen in *vivo* (290, 293, 295). Depletion of endogenous IL-12 before infection of mice significantly exacerbated *Brucella* infection.

Production of TNF- also appears to be important for a protective immune response to *Brucella*. Only live *Brucella* elicited the production of TNF- from

macrophage cultures (291). TNF- receptor knockout mice (TNF-$R^{-/-}$) were severely deficient in IL-12 production and *Brucella* infection was exacerbated (53). In summary, Th1 type cytokines such as IFN- and TNF- appear to be critical in developing resistance against *Brucella* infections. A Th2 type immune response actually may interfere with protective immunity to brucellosis.

Several hypotheses were tested in this dissertation:

1) A vaccinia virus recombinant expressing a *Brucella* antigen, which specifically induces IFN- producing Th1 subset, could induce a protective immune response against *Brucella* challenge in BALB/c mice. Recombinant *B. abortus* L7/L12 ribosomal protein induces a Th1 subset response in mice in which $CD4^+$ T cells produced significant levels of INF- (193). In addition, when fused to maltose binding protein (MBP), the *B. abortus* L7/L12 ribosomal protein conferred a significant degree of protection compared to mice vaccinated with adjuvant alone or adjuvant plus MBP (190). In chapter 3 the question of whether a vaccinia virus / *B. abortus* L7/L12 recombinant (WRL7/L12) would induce a protective immune response against a *Brucella* challenge in BALB/c mice was answered.

2) Vaccinia virus as an expression vector is not the factor for the failure of recombinants to induce a protective immune response. If a protective bacterial antigen is expressed, a recombinant vaccinia virus would induce protection against challenge. In order to test this hypothesis, a vaccinia virus recombinant (WRpLLO) expressing protective part of listeriolysin (pLLO) was generated. Listeriolysin is a cytotoxin secreted by virulent *L. monocytogenes* and permits entry of the organism into the cytoplasm of eukaryotic cells. In an earlier study, a vaccinia virus recombinant carrying a nanomeric CTL epitope of listeriolysin was protective against a *Listeria* challenge (7).

In addition, in chapter 3 mice immune responses to these two recombinants WRL7/L12 and WRpLLO were assessed and compared to explore the differences in the immune responses induced by these recombinants.

3) An adjuvant that induces a strong Th1 type response could be sufficient to induce protective immune responses by vaccinia/*Brucella* recombinants. It was hypothesized that the immune responses induced by vaccinia virus/*Brucella* recombinants are not sufficiently skewed to a Th 1 type response. The capacity of CpG to enhance Th1 type immune responses and thus protective efficacy against a virulent *B. abortus* challenge was explored in chapter 4. In a previous work, a vaccinia virus/ *B. abortus* SOD recombinant (WRSOD) did not protect BALB/c mice against a *Brucella* challenge. This recombinant was also tested in a diversified immunization experiment in which vaccinia virus recombinant was followed by naked DNA vaccination. In chapter 4 BALB/c mice immune responses induced to several SOD recombinants were compared: *Brucella abortus* strain RB51 (RB51SOD), *Ochrobactrum anthropi* (OASOD) and WRSOD. In addition the capacity of CpG adjuvant to boost the immune responses induced by WRSOD was assessed.

4) A vaccinia virus strain expressing a functional murine IL-12 and co-stimulatory factor B7.1 could further enhance the cellular immune responses induced by vaccinia virus recombinants in mice. IL-12 is the pivotal factor for the differentiation of Th1 cells and induces proliferation of murine and human Th1 clones (289). Several studies indicate that endogenous IL-12 produced during infection with *B. abortus* promotes the production of INF- and clearance of the pathogen in *vivo* (290, 293, 295). Co-stimulatory signals are also important in the activation of T cells to proliferate and secrete cytokines. These signals are delivered by interaction between B7.1 and B7.2 molecules expressed on antigens presenting cells (APC) with CD28 or CTLA-4 on T cells. It has further been suggested that B7.1 and B7.2 molecules preferentially costimulate production of cytokines: B7.1 elicits Th1 and B7.2 evokes release of Th2 cytokines (3).

In chapter 5, the ability of vaccinia virus expressing the IL-12 and co- stimulatory factor B7-1 along with the foreign bacterial antigen was examined for induction of the cell mediated immunity and whether this response could be boosted with CpG adjuvant. The cellular and protective immune responses induced by this vaccinia virus strain in

BALB/c mice were compared to ones induced by wild type WR strain, as a function of adjuvant CpG.

3. MICE IMMUNE RESPONSES TO TWO RECOMBINANTS: VACCINIA VIRUS EXPRESSING *LISTERIA MONOCYTOGENES* PARTIAL LISTERIOLYSIN AND *BRUCELLA ABORTUS* RIBOSOMAL L7/L12 PROTEIN

3.1 ABSTRACT

The *Brucella abortus L7/L12* gene encoding ribosomal protein L7/L12 and *Listeria monocytogenes* partial *hly* gene encoding the protective region of the hemolysin (partial listeriolysin, pLLO) were cloned into vaccinia virus by homologous recombination. Initially plasmid pSC65 carrying a synthetic early/late promoter was used as a shuttle vector to enhance the expression of the cloned gene. However recombinants generated with this plasmid were unstable and were lost during subsequent recombinant generation steps. A tertiary enhancement of the unstable recombinant WRpSC65L7/L12 was used to inject BALB/c mice and was unable to induce protection against challenge with virulent *B. abortus*. Although purified MBP-L7/L12 fusion protein induced some proliferation of BALB/c mice splenocytes it failed to induce IFN- secretion *in vitro*. In order to generate stable recombinants, plasmid pSC11, a natural early/late vaccinia promoter was used to produce WRL7/L12 and WRpLLO recombinants. Although mice inoculated with WRL7/L12 recombinant produced antibodies specific to vaccinia virus and L7/L12 antigens, the mice were not protected against virulent *B. abortus* 2308 strain challenge. In contrast, WRpLLO inoculation of mice induced resistance to virulent *L. monocytogenes* challenge. Splenocytes from WRpLLO inoculated mice when stimulated with maltose binding protein (MBP)-LLO fusion protein (MBP-LLO), but not with MBP, secreted significantly higher amounts of INF- than saline inoculated mice. Mice inoculated with either WRpLLO or WRL7/L12 recombinants produced predominantly IgG2a isotype antibody responses. Mice inoculated with either WRpLLO or WRL7/L12

recombinants produced predominantly IgG2a isotype antibody responses, indicative of a Th1 type response. The protective potential of WRpLLO recombinant was correlated with the ability to induce the production of INF- in mice.

This study demonstrated that a single immunization with a recombinant virus expressing partial listeriolysin was able to confer protection to *Listeria* challenge. However, vaccinia virus expressing the *L7/L12* gene was unable to induce protective immune response to *Brucella* challenge.

3.2 INTRODUCTION

The ability of vaccinia virus to express foreign genes make it a very effective tool for the development of a recombinant vaccine. The DNA genome of the virus can accept up to 25 Kb foreign DNA without much change in the infectivity and functional characteristics of the virus (239). Certain genes within the genome, such as *thymidine kinase (TK)* (160), *vaccinia growth factor* (296) and *hemagglutinin (HA)* allow subcloning of foreign genes by homologous recombination. Cytoplasmic replication of the virus eliminates special requirements for nuclear processing and transport of RNA (176). Cloned genes are efficiently processed after translation (118). Vaccinia DNA contains strong promoters that are not recognized by eukaryotic transcription machinery. This enables high expression of the desired protein encoded by a foreign gene without any interruption from the eukaryotic host. Recombinant vaccinia viruses induce both humoral and cellular immune responses (25), (78), (198). Induction of a strong MHC class I cytotoxic T- cell response provides a major advantage to infectious recombinant vaccinia virus compared to inactivated or subunit virus vaccines (178), (287).

Vaccinia virus recombinants showed an impressive potential as vaccines expressing viral, tumor and parasite antigens, but they have had limited success with bacterial antigens. Similar to vaccinia recombinants carrying viral or tumor antigens, foreign bacterial genes cloned into viral genome were expressed in high amounts. A number of studies demonstrated vaccinia virus expressing a single antigen from a variety of bacterial pathogens, produced significant humoral and cell mediated immune responses in animals (8), (16), (17), (7), (91), (121), (122), (161), (272), however

resistance to virulent strain challenge was achieved only in limited number of studies (7), (91), (121), (122).

In an attempt to develop a genetically engineered vaccine for human brucellosis, vaccinia virus recombinants expressing *B. abortus* antigens were generated and the immune responses induced in BALB/c mice were analyzed. The *Brucella* antigens included GroEL (16), (17), HtrA (266), (13), SOD (266) and an 18kDa outer membrane protein (272). Although these vaccinia virus recombinants expressed *Brucella* antigens and induced humoral immune responses in BALB/c mice, no protective immune responses to *Brucella* challenge were induced. To explain the lack of protective immune response, it was rationalized that these *B. abortus* antigens were not protective. It was hypothesized that cloning and expression of other *B. abortus* genes and genes from other bacteria with more protective potential should yield protective vaccinia virus recombinants. In order to test this hypothesis two genes were cloned into vaccinia virus genome of the *B. abortus: L7/L12* gene encoding *B. abortus* L7/L12 ribosomal protein and a partial *L. monocytogenes hly* gene encoding protective portion of listeriolysin (pLLO).

Using a T-lymphocyte Western Blot technique, the *B. abortus* L7/L12 protein has been identified as the most immunodominant antigen (38) among 38 *B. abortus* proteins studied. An oligonucleotide corresponding to the N-terminal of this protein was used as a probe to screen a *B. melitensis* genomic library. The gene encoding the 12kDa *B. melitensis* protein was identified and sequenced. This protein is similar to *E. coli* ribosomal protein L7/L12 and is identical to the *B. abortus* L7/L12 protein (192).

Studies with recombinant *B. abortus* L7/L12 ribosomal protein indicated, that it induces a Th1 subset response by murine $CD4^+$ T cells with significant levels of INF- production (193). Antigens that preferentially induce IFN- producing Th1 subset response are desirable subunits of any vaccine preparation against brucellosis. Furthermore, peripheral blood mononuclear (PBM) cells from *B. abortus* primed cattle are able to respond to the recombinant L7/L12 *in vitro*, demonstrating that T-cell

recognition of this particular antigen is not animal species specific (191). Other studies also indicated that the L7/L12 protein is a major component in the Brucellin used for delayed-type hypersensitivity (DTH) testing in *Brucella* sensitized guinea pigs (12). The immune response to the *in vivo*-expressed *B. abortus* ribosomal *L7/L12* gene in muscle cells was examined (144). This naked DNA application induced higher level of specific antibody and T-cell immune responses when compared with negative controls and *B. abortus* strain 19 injected controls. After being characterized as a T-cell reactive protein, L7/L12 was tested for its ability to confer protection in mice against *Brucella* challenge. Recombinant *B. abortus* L7/L12 ribosomal protein fused to maltose binding protein (MBP) was used to inoculate BALB/c mice (190). The *B. abortus* L7/L12 ribosomal protein conferred a significantly higher degree of protection, when compared to mice inoculated with adjuvant alone or adjuvant plus MBP. These data indicated that a recombinant protein, previously identified as T-cell reactive, engendered protective immunity to mice against brucellosis.

LLO is a sulfhydryl (SH)-activated cytotoxin secreted by virulent *L. monocytogenes* that permits entry of the organism into the cytoplasm of eukaryotic cells from the endocytic pathway. The role of LLO in virulence was determined by injecting mice with wild type and non-hemolytic mutants of *L. monocytogenes* intraperitoneally (ip) and intravenously (iv). The fate of *Listeria* in liver and spleen was followed. In contrast to wild type strains, non-hemolytic mutants were eliminated from these organs within few hours (71), (100), (209).

LLO secretion is necessary for *L. monocytogenes* to induce protective immunity *in vivo* (26) and to elicit IFN- production from specific CD8+ T cells *in vitro* (40). Heat killed organisms and organisms mutant in LLO production are incapable of inducing these responses. These observations suggest a model in which the requirement for LLO secretion for the induction of protective immunity is based on access of the bacterium to the cytoplasm of the infected cell (40). Cytoplasmic localization is sufficient to introduce bacterial proteins to the class I MHC antigen processing pathway (174) resulting in the potential for CD8+ T cell recognition (113). More importantly, a recombinant vaccinia

virus carrying a minigene that expressed nanomeric CTL epitope of listeriolysin protected the mice against *Listeria* challenge (7).

In this study the generation of two vaccinia virus recombinants WRL7/L12 and WRpLLO and mice immune responses to these recombinants is described.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial and Viral Strains and Cell lines

E. coli DH5 cells used for construction of recombinant shuttle vectors were purchased from Invitrogen (Invitrogen, Carlsbad, CA). *B. abortus* strains RB51 and 2308 were supplied by Dr. G. G. Schurig (Virginia Polytechnic Institute and State University, Blacksburg, VA). *Listeria monocytogenes* strain was obtained from Dr. P. Elzer (Louisiana State University, Baton Rouge, LA). *Brucella* and *E. coli* cultures were grown in tryptic–soy broth (TSB) or on tryptic soy agar (TSA) plates. All *Brucella* strains were manipulated under Biosafety Level 3 (BL-3) conditions. *L. monocytogenes* was grown on blood agar plates and in brain heart infusion broth (BHI).

Vaccinia virus Western Reserve (WR) strain and human thymidine kinase deficient 143B cells ($HuTK^-$ cells) were purchased from the American Type Culture Collection, Rockville, MD.

3.3.2 Construction of Recombinant Shuttle Vectors

For generation of PSC65L7/L12, a 387 base pairs (bps) long *l7/l12* gene was digested from pCR2.1L7/Ll12 (Dr. R. Vemulapalli, Virginia Polytechnic Institute and State University, Blacksburg, VA) with *Kpn* I and *EcoR* V (Promega, Madison, WI) restriction endonucleases. The digested 387 bp DNA fragment was purified from 1% agarose gel using Qiagen gel extraction kit (Qiagen, Valencia, CA), and was directionally ligated into a *Kpn* I and *Sma* I double digested vector pSC65. The recombinant plasmid

pSC65L7/L12 was confirmed by restriction enzyme digestion followed by gel electrophoresis to size the cloned insert.

Plasmid pSC11L7/L12 was also subcloned from PCR2.1L7/L12 by restricting the plasmid using *Bgl* I and *Sca* I endonucleases. The overhanging *Bgl* I restricted end was filled in using Klenow enzyme (Promega, Madison, WI). The shuttle vector pSC11 was linearized using *Sma* I endonuclease. The restricted ends then were dephosphorylated using shrimp alkaline phosphatase (U.S. Biochemical Corporation (USB), Cleveland, Ohio) to prevent religation of the vector. The *L7/L12* gene then was inserted to the *Sma* I site of pSC11 using T4 DNA ligase (Promega, Madison, WI). Restriction enzyme digestion followed by gel electrophoresis and PCR analysis confirmed the correct orientation of the gene within the plasmid pSC11L7/L12.

Plasmid pSC11pllo was subcloned from PC2.1LLO (Dr. R. Vemulapalli, Virginia Polytechnic Institute and State University, Blacksburg, VA) by digesting the *hly* gene with *Bgl* II and *Sca* I at sites 1440 and 2424 respectively. This partial *hly* gene then was extracted from the 1% agarose gel using Qiagen gel extraction kit (Qiagen, Valencia, CA) and then the overhanging ends were filled in with Klenow enzyme. The shuttle plasmid pSC11 was digested at the *Sma* I sites and dephosphorylated. The 984 bps long partial *hly* gene was ligated into the *Sma* I site of the plasmid using T4 DNA ligase (Promega, Madison, WI). Restriction enzyme digestion followed by gel electrophoresis and PCR analysis confirmed the correct orientation of the gene within the plasmid.

To confirm the recombinant pSC11 vectors, two different sets of primers were used. The first set of primers was designed using upstream and downstream sites off the *Sma* I site in the plasmid (forward: CTAATTTATTGCACGGTAAGG, reverse: GAAATGTCCCATCGAGTGCGG). The second set of primers were specific for the genes, *L7/L12* (forward: GTTTAAACATGGCTGATCTCGCAAAGATCGTTGAATG, reverse: TCCAAACTTACTTGAGTTCAACCTTGGCGC) and for partial *hly* (LM forward: CCTAAGACGCCAATCGAA, LM reverse: AAGCGCTTGCAACTGCTC).

Primers used for *hly* gene amplification were designed by Broder et al. (1990), amplifying 702bps fragment within the cloned partial hly gene (37).

3.3.3 Generation of Vaccinia Virus Recombinants

Human thymidine kinase deficient 143B cells (HuTK$^-$ cells) were grown to 80% confluency in Eagle's Minimum Essential Medium (EMEM) (ICN, Costa Mesa, CA) containing 5% fetal bovine serum (FBS) (Intergen, Purchase, NY) in 25 cm^2 flasks. They were infected with vaccinia virus strain WR at a multiplicity of infection (MOI) of 0.05 and incubated for 2 hours at 37^{0}C, in a 5% CO_2 incubator. One microgram (μg) of recombinant shuttle vectors (pSC65L7/L12, pSC11L7/L12, pSC11pllo) was dissolved in 50 microliter (μl) of sterile distilled water and mixed with 50 μl of undiluted lipofectin reagent (Gibco-BRL, Grand Island, NY) and incubated for 25 minutes at room temperature. The DNA/lipofectin mixture (100μl) was mixed with 1 milliliter (ml) of EMEM which was then added to WR-infected HuTK$^-$ cells at 80% confluency. The infected cells were supplemented with 3.0 ml of EMEM. After a 4+ cytopathic effect (CPE) had developed (usually in 48-72 hours), the cells were ruptured by 3 cycles of freezing in liquid nitrogen and thawing at 42^0 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 [(about 9.6 centimeter square (cm^2) surface area) (Falcon™, Franklin Lakes, NJ)] with EMEM containing 25 μg of bromodeoxyuridine (BdUR) per milliliter (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 50 μg BdUR) containing 0.6 mg/ml of Bluo-gal (Gibco-BRL, Grand Island, NY). Blue plaques, produced by replicating recombinant virions expressing the *lac*Z gene, were collected and used to enhance the virus content of the plaques by inoculating a confluent layer of HuTK$^-$ cells in either 25 cm^2 flasks or six well tissue culture plates. Replication of the recombinant virus was assessed by CPE and the presence of blue plaques in the cell monolayer. Recombinant viruses were harvested, plaque purified and enhanced by infecting larger

volumes of cell monolayers (in a 25 cm^2 flask) two more times to develop the recombinant virus. For viral stock preparations 150 cm^2 flasks were inoculated with recombinant viruses at MOI of 0.1 and after a 4+ CPE was observed, the content of the flask was aspirated and centrifuged at 1000 *g* for 5 min. The pellet was saved and resuspended in 1ml of minimal cell culture medium Optimem (Gibco-BRL, Grand Island, NY). The virus was released from the cells by three consecutive freeze- thaw cycles.

3.3.4 Analysis of Vaccinia Recombinants

DNA was extracted from vaccinia infected and uninfected $HuTK^-$ cells using QIAamp DNA extraction kit (Qiagen, Valencia, CA). DNA samples were used in a PCR to amplify the gene of the interest using the same primers described in section 3.3.1.

Recombinants generated with pSC11vector were also analyzed by sequencing using the pSC11 primers. Automated DNA sequencing was performed in the Core Laboratory Facility at the Virginia Bioinformatics Institute at Virginia Tech using standard methods on an ABI 377 or 3100 automated DNA Sequencer and using PE Biosystems BigDye Terminator chemistry. Cycle sequencing reactions were performed using 10ng/100bp of PCR product (or if plasmid template, 500ng total) and PE Biosystems (Foster City, CA) Big Dye Terminator (version 2) ready reaction kit. Primer amounts in the reaction were 3.2pmol, and total reaction volume was 15ul. Cycling parameters were: 25 cycles of 30s@95C, 15s@50C, 4m@60C and refrigerated until used. Reactions were purified using the Millipore Multiscreen plates, dried and resuspended as per manufacturer's protocols for loading the automated sequencer. Sequencher software (Ann Arbor, MI) was used for editing and aligning chromatograms and sequences.

3.3.5 SDS-Page and Western Blotting

SDS-PAGE and Western blotting was used to determine foreign gene expression in vaccinia virus recombinants.

3.3.5.1 SDS-Page

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 15% gel as per standard protocol (11) using the Mini-Protean®II gel apparatus (Bio-Rad, Rockville, NY). Gels were run approximately for 60-90minutes at 25mA/gel in SDS-PAGE electrophoresis buffer (25mM Tris, 0.19M Glycine, 0.1%SDS pH:8.3).

3.3.5.2 Western Blotting

All protein samples were separated on 15% SDS-PAGE and transferred to a nitrocellulose membrane (0.45 micron Nitropure membrane) (MSI Filters, Westboro, MA). The membranes were blocked with TBS containing 2% bovine serum albumin (BSA) and 1.5% dry milk and reacted with tested sera as primary antibody and appropriate HRP-conjugated IgG as secondary antibody. The serological reactions were visualized by incubation in a developing solution consisting of 0.060g 4-chloro-1-napthol in 20ml methanol added to 100 ml of Tris buffered saline (TBS)(0.15M NaCl, 20mM Tris, pH 7.5) with 36µl of 30 % hydrogen peroxide. The development of the color reaction was stopped after 5 min by placing the membranes in distilled water.

3.3.6 Mice Experiments

3.3.6.1 Protection Studies

3.3.6.1.1 WRpSC65L7/12 Recombinant

Four groups of 8 mice each (BALB/c mice, Charles River laboratories, Wilmington MA) were used. Groups were injected with WRpSC65L7/L12 ($1x10^5$TCID$_{50}$/mice), vaccinia control strain WR ($1x10^5$ TCID$_{50}$/mice), *B. abortus* RB51 strain (positive vaccine control) ($5x10^8$ CFU/mice), or saline solution intraperitoneally (ip). Vaccinia virus- injected mice were boosted at the 5th week intrarectally (ir) with the same dose and the control groups received 50µl of saline solution. Five mice from each group were challenged ip at 8th week with *B. abortus* strain 2308 ($2.4x10^4$). *Brucella* challenged mice were euthanized by CO_2 asphyxiation 2 weeks post challenge for bacterial clearance analysis. The remaining 3 mice in each group were sacrificed for CMI analysis at 10th week post injection.

3.3.6.1.2 WRL7/L12 Recombinant (pSC11)

Three groups, of 8 BALB/c mice each, were used. Groups were inoculated with WRL7/L12 ($1x10^{6.5}$ TCID$_{50}$/mice), RB51 ($5x10^8$CFU/mice), 300 µl saline solution (negative control) ip. Five mice from each group were challenged at 8th week with *B. abortus* strain 2308 ($2.4x10^4$). *Brucella*-challenged mice were euthanized by CO_2 asphyxiation 2 weeks post challenge for bacterial clearance analysis. The remaining 3 mice in each group were sacrificed for CMI analysis at 10th week post injection.

3.3.6.1.3 WRpLLO Recombinant

Two groups of 8 BALB/c mice each were injected ip with $1X10^{6.5}$ TCID$_{50}$/mice of WRpLLO and 300 µl saline. Five mice were challenged with $2.5x10^4$CFU/ml of *L monocytogenes* (0.1 LD$_{50}$) 8 weeks pi. *Listeria*-challenged mice were sacrificed 60 hours

after challenge for bacterial clearance analysis. The remaining 3 mice in each group were sacrificed for CMI analysis at 10th week post injection.

3.3.6.1.4 Bacterial clearance analysis

Mice were euthanized by CO_2 asphyxiation then the spleens were removed aseptically and placed in individual plastic tubes containing sterile sand (0.75g/tube) and 1 ml TSB. Spleens were homogenized using a sterile pestle. Ten-fold serial dilutions of the homogenates were prepared in TSB. Five, 10 µl drops, from each dilution were plated on TSA plates and incubated at 37^0C for 2 days for *Listeria*, 5 days for *Brucella* incubation. To minimize statistical error, number of colonies in the lowest dilution that could be counted, was used to quantify the bacteria per spleen in colony forming units (CFUs/spleen). To determine the number of CFU/spleen, the following formula was used (Detection limit:$2x10^2$ CFUs).

CFU/spleen =Total CFU/dilution **x** 20 **x** dilution factor

The results were analyzed by t-Test (Sigma Plot ™, Scientific Graphic Software version 5.0, Jandel Scientific, San Rafael, CA) by comparing each group to the negative control group.

3.3.6.2 Serological analysis

Three mice per group were bled by puncturing the retroorbital plexus at the third, fifth, seventh, ninth and twelfth weeks post immunization. The serum was diluted 1:50 and analyzed for antibodies by a Western Blot analysis

3.3.6.2.1 Indirect ELISA

The presence of antigen specific serum IgG, IgG1 and IgG2a isotypes were determined by indirect ELISAs. The antigens at 20µg/ml concentration in carbonate

buffer (pH 9.6) was used to coat the wells (50 µl/well) of MaxiSorp™ immunoplates (Nalge-Nunc International, Rochester, NY). After coating at 4^0 C overnight, plates were blocked using blocking buffer (2% bovine serum albumin (BSA) in phosphate buffered saline (PBS). After two hours of blocking at room temperature, the blocking buffer was discarded and serum samples (1:50 dilution in blocking buffer) were added to the wells (100µl/well). Each serum sample was tested in duplicate wells. The plates were incubated for three hours at room temperature (RT) and washed four times with PBS with 0.05% Tween® 20 (Fisher Scientific, Springfield, NJ). Isotype specific (IgG, IgG1, IgG2a) goat anti-mouse horseradish peroxide conjugated secondary antibodies (Caltag Laboratories, San Fransisco, CA) were added to each well at 1:7400 dilution and incubated at RT for 1 hour (h). Following the secondary antibody incubation the plates were washed four times and 100 µl of TMB Microwell substrate (Kirkegaard &Perry laboratories, Gaithersburg, MD) was added to each well. After 30 minutes (min) incubation at room temperature, the enzyme reaction was stopped by adding 100 µls of stop solution (0.185M sulfuric acid). The plates were read and analyzed at 450nm absorbance using a microplate reader (Molecular Devices, Sunnyvale, CA).

3.3.6.3 Cell-Mediated Immune Response Analysis

3.3.6.3.1 Culturing Splenocytes from Mice Spleens

Mice were sacrificed by CO_2 asphyxiation and their spleens were removed under aseptic conditions. Single spleen cell suspensions were prepared from the spleens. Briefly, red blood cells were lysed using ACK solution (0.1 M NH_4Cl, 1mM $KHCO_3$, 0.1mM EDTA [pH7.3]). The splenocytes were cultured in 96-well U bottom plates (Corning, Acton, MD) at a concentration of $5x10^5$ viable cells/well. RPMI 1640 medium (Gibco, BRL, Grand Island, NY) supplemented with 2mM L-glutamine (Gibco, BRL, Grand Island, NY), 10% heat-inactivated fetal bovine serum (FBS) (Intergen, Purchase, NY) and 1ml of penicillin/streptomycin (Penicillin 100U/ml; Streptomycin 100µg/ml) was used for culturing splenocytes. Splenocytes were stimulated with the following antigens: 1µg of concanavalin A (ConA); heat inactivated $1x10^6$ (low dose), $1x10^7$ (high

dose) of *B. abortus* RB51 or *L. monocytogenes* as positive controls, purified antigens at concentrations of 0.125, 0.25, 0.5, 1, 2 μg/well; and no additives/well as negative control. The splenocytes were incubated in 5% CO_2, at 37^0C for 5 days for cytokine ELISAs and for 2 days for lymphocyte proliferation assays.

3.3.6.3.2 Lymphocyte Proliferation Assays

Splenocytes were cultured from mice as described above. After 48h incubation at 37^0C in a 5% CO_2 incubator, the cells were pulsed for 18 hours with 0.5mCi (20μl of 1:20 dilution with CRPMI) of tritiated thymidine (ICN, Costa Mesa, CA) with specific activity of 6.7 Ci/mmole. Cells were harvested and insoluble tritiated thymidine counts were determined by liquid scintillation spectrometry (TopCount NXT, Packard Instruments, Meriden, CT). The results are expressed as stimulation index (SI) calculated by the following formula:

$$\frac{\text{Insoluble } ^3\text{H thymidine cpm of spleen cells exposed to antigen}}{\text{Insoluble } ^3\text{H thymidine cpm of spleen cells unexposed to antigen}}$$

3.3.6.3.3 Cytokine ELISAs

Splenocytes were cultured from mice as described above and incubated in U bottom 96-well plates (Corning, Acton, MD) for 5 days at 5%CO_2 incubator at 37^0C. The culture supernatants were transferred to a new 96-well plate and stored at -70^0C until a sandwich ELISA was performed to quantify cytokine levels in the supernatants. Briefly, 96- well MaxiSorp™ immunoplates (Nalge-Nunc International, Rochester, NY) were coated overnight with 0.1μg/well of purified rat- antimouse cytokine coating antibody (Pharmingen, San Diego, CA) diluted in (PBS) at pH: 7.4. Following overnight coating the plates were blocked for 1 h with assay buffer (2% BSA in PBS pH: 7.2-7.4). Plates were washed 5 times using wash buffer (50mM Tris, 0.2% Tween 20 ® [Fisher Scientific, Springfield, NJ] pH: 7.0-7.4). During the blocking, cytokine standards (Pharmingen, San

Diego, CA) were serially diluted (1:2) in the range of 50-0.39 ng/ml with RPMI 1640 medium (Gibco, BRL, Grand Island, NY) supplemented with 2mM L-glutamine (Gibco, BRL, Grand Island, NY), 10% heat-inactivated fetal bovine serum (FBS) (Intergen, Purchase, NY) and 50μM of penicillin/streptomycin. Samples from the previously frozen plates and standards were added to each well, mixed 1:1 with assay buffer. Plates were incubated overnight at room temperature. Plates were then washed 5 times with wash buffer and incubated for 1 h with 0.25μg/ml diluted biotinylated detecting antibody (100μl/well) (Pharmingen, San Diego, CA). Following the biotinylated detecting antibody, plates were washed five times with wash buffer and then incubated 30 min with 1:5000 diluted (assay buffer with 0.05%Tween 20®) horseradish peroxidase (HRP) conjugated streptavidin antibody (Vector, Burlingams, CA). Following the incubation with streptavidin antibody, the plates were washed five times and 100 μl of TMB Microwell substrate (Kirkregaard &Perry Laboratories, Gaithersburg, MD) was added to each well. The enzyme reaction was stopped by adding 100 μls of stop solution (0.185M sulfuric acid) after 30 minutes (min) incubation at room temperature. The plate was read and analyzed at 450nm absorbance by a microplate reader (Molecular Devices, Sunnyvale, CA). The quantity of cytokines was determined according to the linear regression equation of the standard curve.

3.3.7 Stimulant Antigen Preparation

The wLLO and L7/L12 antigens used in the immune response analysis were prepared using HiTrap Q (Pharmacia,/LKB Biotechnology, Inc., Piscataway, NJ) anion-exchange column chromotography. Briefly complete *L monocytogenes hly* gene and *B. abortus* L7/L12 gene were subcloned from pCR2.1wLLO and pCR2.1L7/L12 to pMALp2 expression vector generating pMalwLLO and pMalL7/L12 respectively. Both proteins were expressed in *E. coli* as fusion proteins to the maltose binding protein (MBP). *E. coli* cultures were grown to 2 X 10^8 cells/ml and induced with isopropyl -D-thiogalactopyranoside (IPTG) (Sigma/Aldrich, St. Louis, MO) for 2 hours. Following induction, bacterial cells were harvested by centrifugation and resuspended in start buffer

(20 mM Tris-HCl pH 8.0). Cells were sonicated 2min with 15 second intervals and centrifuged 30 min 4000*xg*. Crude extract was diluted 1g/2ml and antigens were purified using Hi-Trap Q column according to manifacturer's recommendation. The wLLO antigen was mainly found in flow through and L7/L12 antigen was eluted from the column using 0.3 M NaCl containing start buffer. After column purification antigens were dialyzed in Spectra/Por® molecularporous membrane (12-14 kDa MWCO) (Spectrum, Medical Industries, Inc, Los Angeles, CA) in PBS buffer and LPS was removed using Affi-prep® Polymyxin Support (BIO-RAD, Hercules, CA). Purified antigens were analyzed with SDS-PAGE and Western Blotting. Protein concentrations were determined using BIO-RAD protein microtiter plate assay.

3.4 RESULTS

3.4.1 Unstable WRpSC65L7/L12 Recombinant

The *Brucella abortus L7/L12* gene was subcloned to shuttle vector pSC65 generating pSC65L7/L12. This recombinant shuttle vector was used to generate vaccinia virus recombinant using homologous recombination (refer to Scheme 1.1).

Homologous recombination occurs with a low frequency therefore, putative recombinants must be screened by 3 consecutive plaquing and enhancement to identify plaques containing recombinant virus. This screening involves the use a colorimetric assay, expression of *E.coli lac*Z gene, positive (blue color development) for the identification of recombinant viruses through the production of -galactosidase (-gal). During these screening and enhancement steps WRpSC65L7/L12 recombinant appeared to be unstable. Although the initial plaquing experiment produced good size plaques (about 2-3mm in diameter), in the subsequent steps either the size of the plaques was reduced (<0.5mm in diameter), or there was no color development in the enhancement step following the plaquing. Although some color development was observed in some

enhancement experiments due to -galactosidase expression, the color was not stable and would be lost before a recombinant viral stock could be made. DNA was extracted from these non-colored or slightly blue-colored enhancement experiments and analyzed by PCR using L7/L12 primers (Figure 3.1). *L7/L12* gene was amplified in all of the tested samples except HuTK^{-} cell control. Brightness of the amplified *L7/L12* band was correlated with the -galactosidase expression in the cultures.

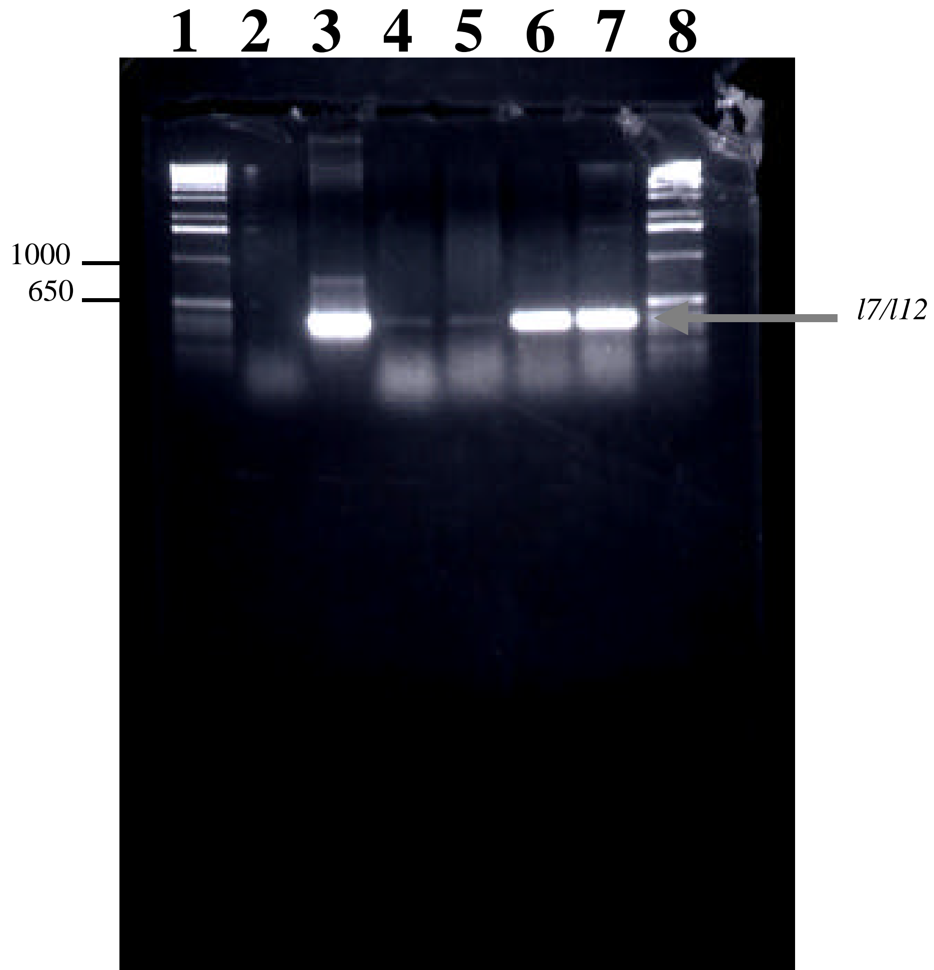


Figure 3.1 PCR analysis of unstable WRpSC65L7/L12 recombinant.

Lanes are 1) and 8) 1 kb ladder , PCR amplification of 2)DNA extracted from HuTK$^-$ cell control, 3) pSC65L7/L12 shuttle vector 4), 5), 6), 7), virus DNA extracted from cultures with varying degree of -galactosidase expression: colorless (4) to some blue color (7).

3.4.1.1 Mice Immune Responses to unstable WRpSC65L7/L12

3.4.1.1.1 Humoral Immune Response

Unstable vaccinia virus WRpSC65L7/L12 recombinant was able to induce antibodies to vaccinia virus and L7/L12 in BALB/c mice. However the antibody response to L7/L12 antigen was very weak and only recognized the MBP-L7/L12 fusion protein at 60 kDa. The L7/L12 protein was not recognized in the WRpSC65L7/L12 antigen (Figure 3.2).

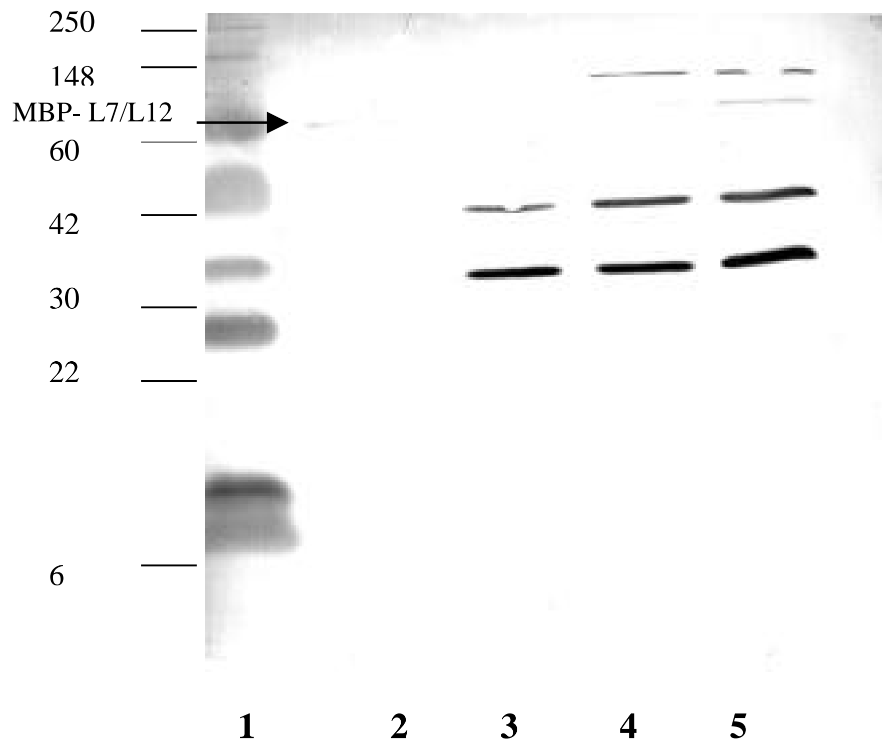


Figure 3.2. Western Blot developed using WRpSC65L7/L12-injected mice sera.

Antigens in lanes 1)MW markers; 2)Purified fusion MBP-L7/L12 protein (60kDa); 3)WRpSC65L7/L12 (non-colored); 4) WR; 5)WRpSC65L7/L12 (blue).

3.4.1.1.2 Resistance to ***Brucella*** *Challenge*

Based on the average bacterial clearance from spleens, the WRpSC65L7/L12 recombinant did not induce any protection against *Brucella* 2308 challenge when compared to negative control. There was significant difference in the splenic bacterial counts of mice vaccinated with of the *B. abortus* strain RB51 and the control group. Strain RB51 vaccination protected mice against *B. abortus* 2308 challenge ($p<0.05$) (Figure 3.3).

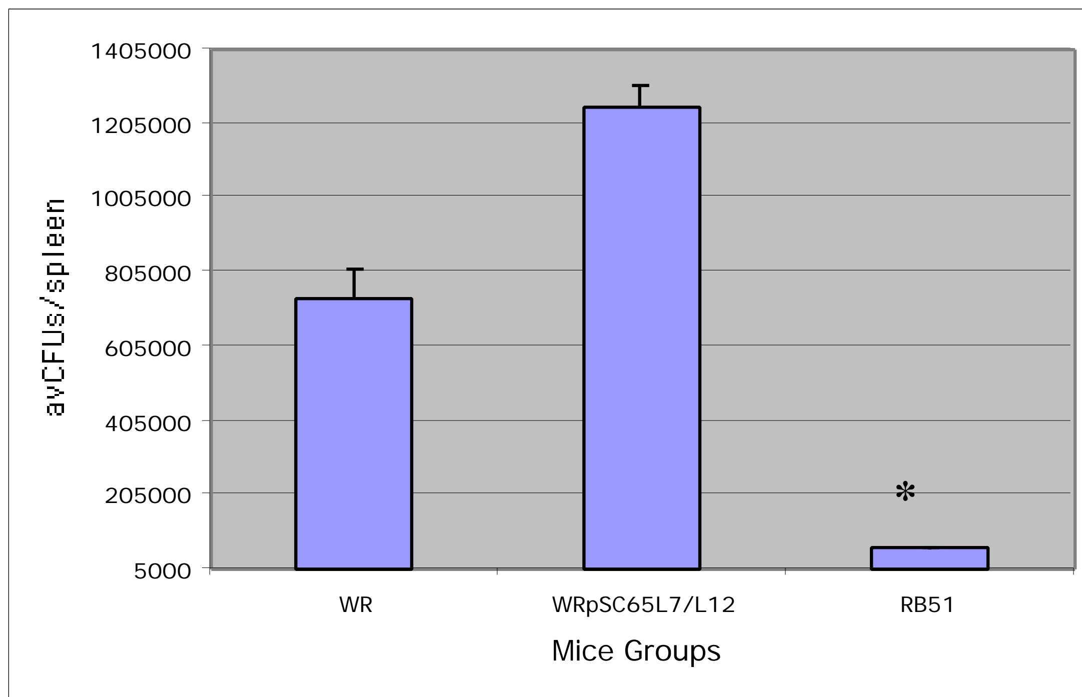


Figure 3.3 Protective immune response by WRpSC65L7/L12 recombinant inoculated mice.

WRpSC65L7/L12 recombinant was unable to induce protective immunity against *Brucella* challenge. RB51 immunized mice had significantly lower bacterial counts in spleens (* *P*-value: <0.05, student T-test).

3.4.1.1.3 Cell mediated Immune Responses

3.4.1.1.3.1 Analysis of INF- Production

Splenocytes of *B. abortus* strain RB51 vaccinated mice released INF- *in vitro* after stimulation with purified MBP-L7/L12 fusion protein. INF- release by splenocytes of WR or WRpSC65L7/L12-injected mice was below the detection limit (Table 3.1).

Table 3.1 Production of INF- (ng/ml) by splenocytes of inoculated mice

	IFN-γ ng/ml		
Mice groups	**RB51**	**WRpSC65L7/L12**	**WR**
Antigens			
RB51(20µg/well)	5.76	-	-
RB51(10 µg/well)	5.03	-	-
L7/L12(2µg/well)	3.19	-	-
L7/l12(1µg/well)	1.38	-	-
L7/L12(0.5µg/well)	2.10	-	-
L7/L12(0.25 µg/well)	4.46	-	-
L7/L12(0.125µg/well)	2.48	-	-
Media	-	-	-

3.4.1.1.3.2 MBP-L7/L12 fusion antigen was able to induce *in vitro* proliferation of splenocytes from WRpSC65L7/L12 and RB51 injected mice

In vitro stimulation indices of splenocytes from RB51-, WRpSC65L7/L12- and WR-injected mice stimulated with RB51,MBP-L7/L12 fusion protein (L7/L12) are given in Table 3.2.

Table 3.2 SI values

	Mice groups*		
	RB51	**WRpSC65L7/L12**	**WR**
Antigens			
RB51(20μg/well)	7.5	3.5	<1
RB51(10 μg/well)	5.6	4.9	<1
L7/L12(2μg/well)	6.4	2.5	<1
L7/l12(1μg/well)	3.3	1.74	<1
L7/L12(0.5μg/well)	2.3	4.5	<1
L7/L12(0.25 μg/well)	1.4	<1	1.19
L7/L12(0.125μg/well)	1.6	<1	<1
Media	1	1	1

*Averages from 3 mice per group

3.4.2 Stable Vaccinia Virus Recombinants Generated with Shuttle Vector pSC11: WRL7/L12 and WRpLLO

3.4.2.1 Generation of Recombinants WRL7/L12 and WRpLLO

Due to the instability of the recombinants generated with the pSC65 shuttle vector, shuttle vector pSC11with early/late natural vaccinia virus promoter was used to clone B. *abortus l7/l12* and *L monocytogenes* partial *hly* gene into the vaccinia virus genome. Recombinant pSC11shuttle vectors pSC11L7/L12 and pSC11pLLO are diagrammed in Figure 3.4 and 3.5 respectively. The generated vaccinia virus recombinants were plaqued-purified and enhanced to high titers without difficulty. Recombinants were stable and recombinant plaques have been recognized without difficulty (blue color, -galactosidase expression) throughout the recombinant virus generation steps. After high titers were reached DNA was extracted from recombinant virus-infected HuTK$^-$ cells. This DNA was used as a template in PCR to determine the presence of foreign gene in the recombinants. Figure 3.6 shows amplification of partial *hly* and *l7/l12* genes. Primers used were designed for upstream and downstream of the *Sma* I insertion site in the vector, therefore, along with genes of interest a small fragment of pSC11 (about 200 base pairs) was also amplified. In this experiment DNA of pSC11 and HuTK$^-$ cells served as negative control and pSC11L7/L12 and pSC11pLLO served as positive control. Most importantly, Figure 3.6 shows the amplification of *l7/l12* gene in WRL7/L12 recombinant. Figure 3.7 shows a PCR amplification of partial *hly* gene in WRpLLO recombinant. Diagnostic primers amplifiying a 702bps fragment of partial *hly* gene were used (37). The expression vector carrying whole listeriolysin pMalwLLO and pSC11pLLO DNA, were used as positive control. DNA extracted from HuTK$^-$ cells and pSC11 vector were the templates for negative controls. The amplification of 702bps DNA fragment was clearly demonstrated in WRpLLO DNA.

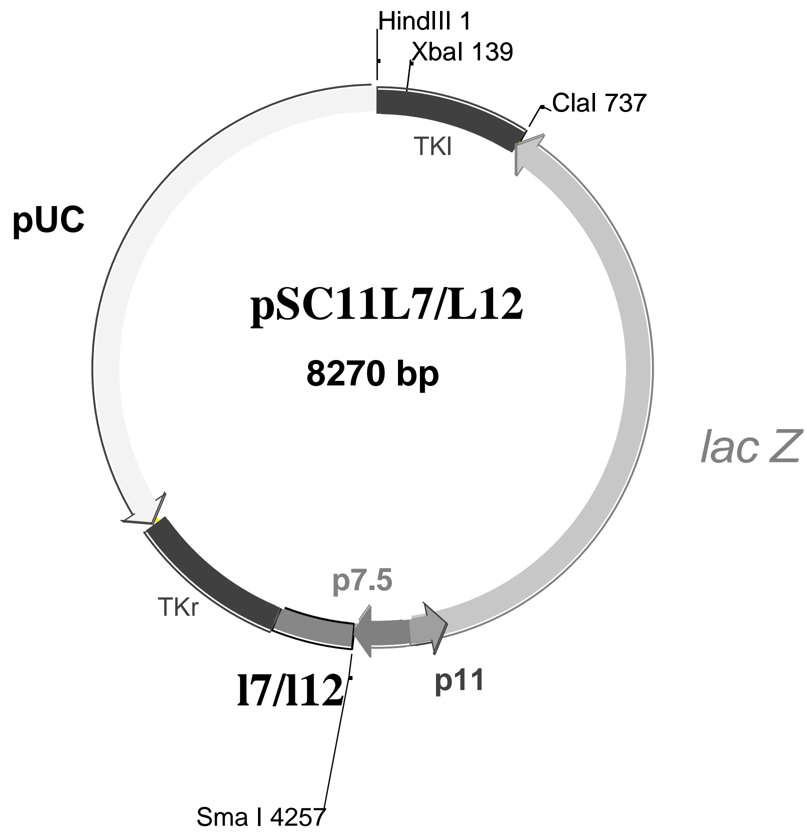


Figure 3.4 Recombinant shuttle vector pSC11L7/L12.

The *B. abortus l7/l12* gene (387bps) was ligated into the *Sma* I site.

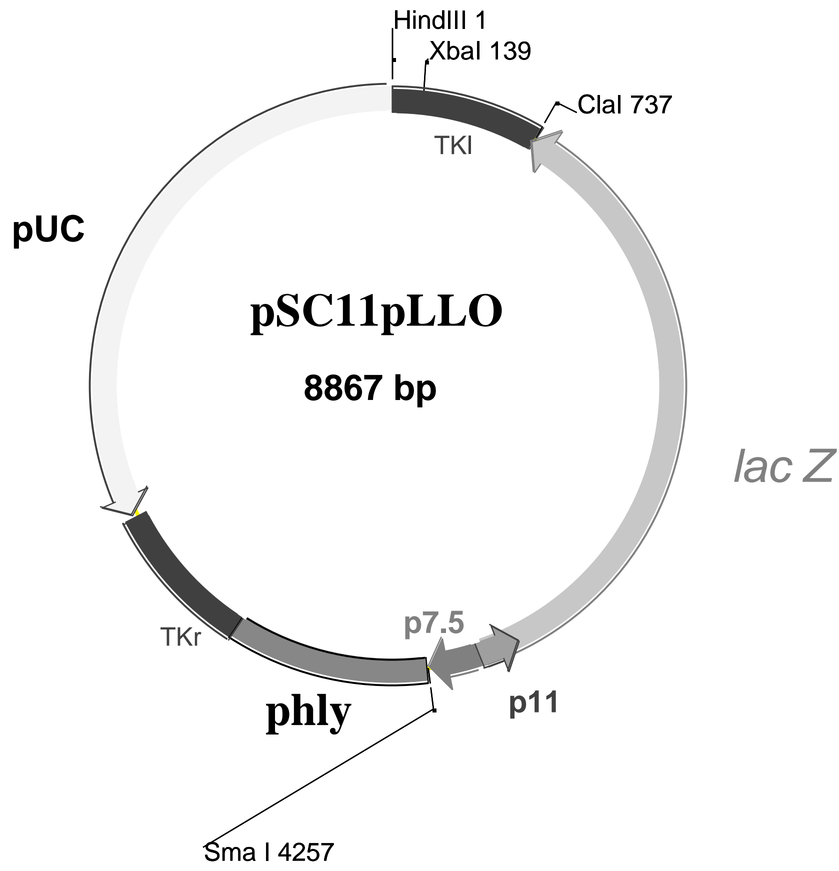


Figure 3.5 Recombinant shuttle vector pSC11pllo.

A partial *L monocytogenes hly* gene encoding for partial listeriolysin was cloned into the *Sma* I polylinker site.

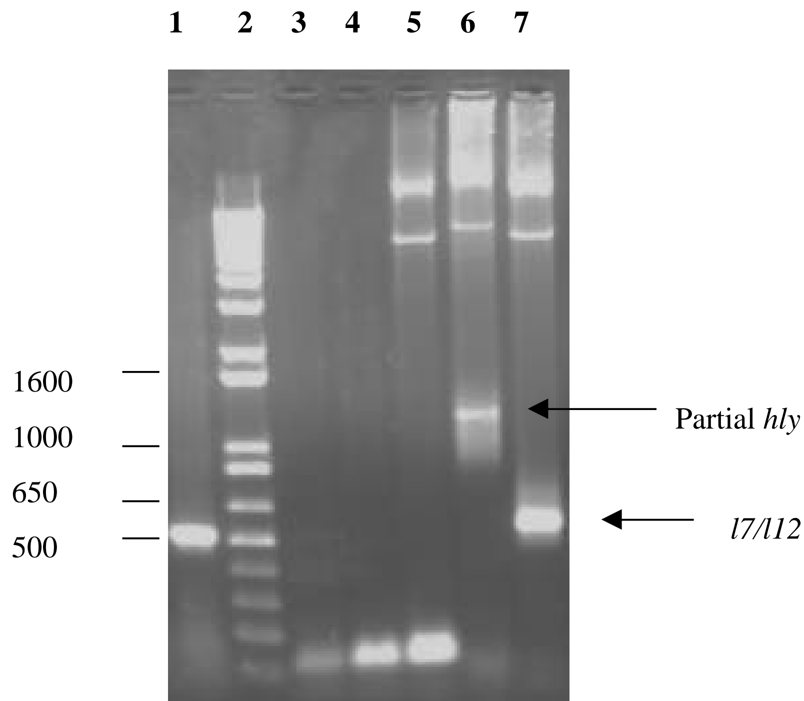


Figure 3.6 PCR amplification of WRL7/L12 recombinant generated using pSC11L7/12.

Lanes include template DNA of 1) HuTK$^-$ cells infected with WRL7/L12; 2)1 kb ladder; 3)HuTK$^-$ cells infected with WRpSC11(vaccinia virus/plasmid recombinant); 4)HuTK$^-$ cells; 5) pSC11 shuttle vector, 6) pSC11pLLO recombinant vector; and 7) pSC11L7/12. PSC11primers were used to amplify L7/L12 and pLLO.

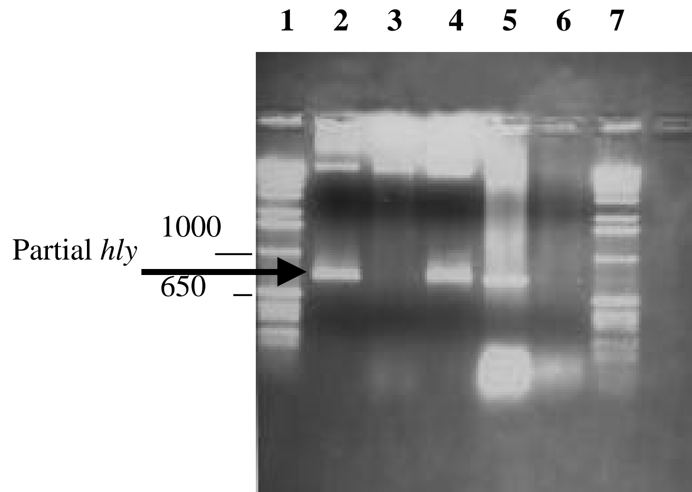
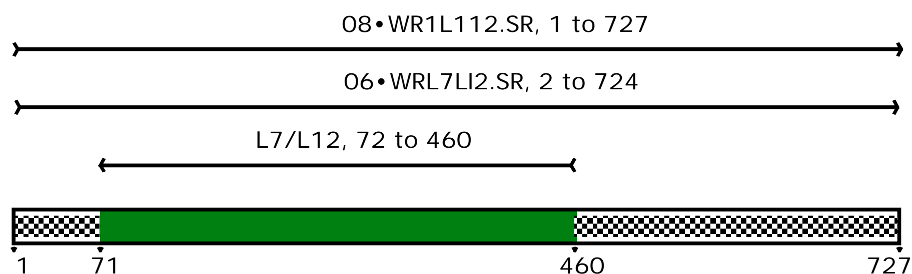


Figure 3.7 PCR amplification of vaccinia recombinant WRpLLO generated with pSC11pLLO.

Lanes include DNA of: 1) 1kb ladder; 2) pMalWLLO; 3) pSC11 (-control); 4) pSC11pLLO; 5) HuTK$^-$ cells infected with WRpLLO; 6)HuTK$^-$ cell culture. Primers used for *hly* gene amplification were designed for diagnostic detection of *Listeria monocytogenes* using PCR, amplifying a 702bps fragment within the partial *hly* gene cloned.

3.4.2.1.1 Sequencing Analysis of Recombinant Viruses

Recombinant viruses were sequenced using pSC11 primers; the sequences were analyzed by comparing the sequenced data with gene bank data.



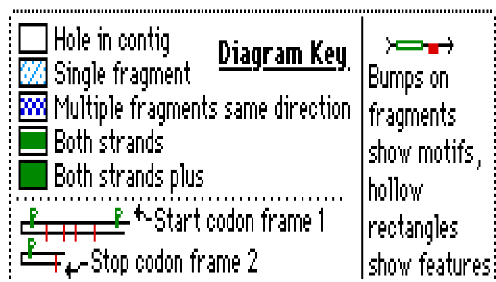
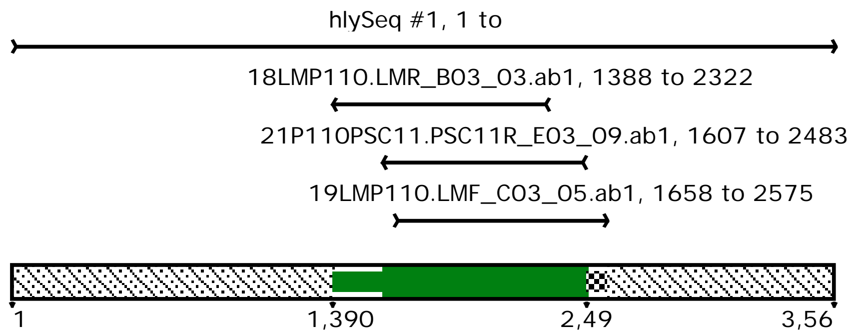


Figure 3.8 Sequence alignment of *L7/L12* gene

Sequence alignment of *L7/L12* amplified from WRL7/L12 (08.WR1L112.SR 1 to724 and 06.WRL7/L12.SR. 2 to 724 shown in the diagram) using pSC11 primers with *B. abortus l7/l12* sequence retrieved from EMBL+GenBank Release 102 accession number: L19101 (L7/L12, 72 to 462 in the diagram). There were no bumps on fragments and list of start and stop codon frames were removed from the figure to reduce the confusion.



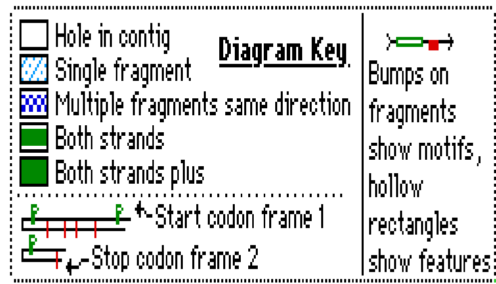


Figure 3.9 Sequence alignment of partial *hly* gene

Sequence alignment of partial *hly* gene amplified from WRpLLO using pSC11 and LM primers with *L. monocytogenes hly* gene sequence retrieved from EMBL+GenBank Release 102 accession number: M29171 (hlySeq #1 in the diagram).

3.4.2.1.2 Expression of L7/L12 by vaccinia virus WRL7/L12 recombinant

A polyclonal anti-L7/L12 serum was able to recognize the 12 kDa L7/L12 antigen expressed in vaccinia virus recombinant WRL7/L12. The L7/L12 protein recognized was at the same molecular weight as that in strain RB51. Recognition of L7/L12 (tested at the same conditions) was stronger in extracts of WRL7/L12 antigen than of WRpSC65L7/L12 antigen.

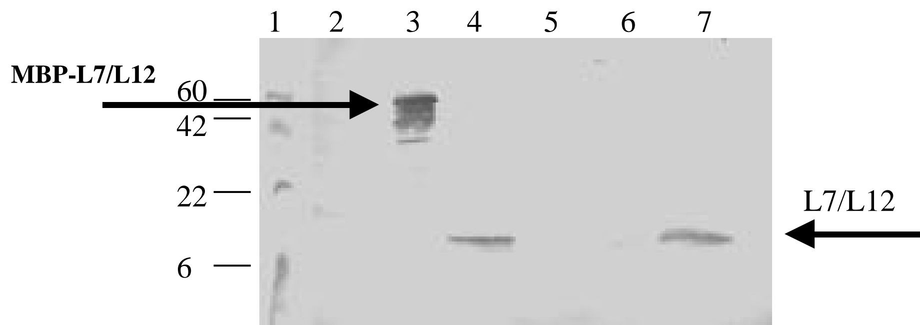


Figure 3.10 Western Blot analysis of recombinant WRL7/L12.

Antigens include: 1) MW markers; 2) WRpSC11; 3) MBP-L7/L2 fusion protein; 4) WRL7/L12 recombinant; 5) WRpSC65L7/L12; 6) WRL7/L12 (1^0 enhancement stage); 7) RB51 antigen. Western blot was probed with polyclonal anti-L7/L12 mouse sera (1:50 dilution).

3.4.2.1.3 Expression of pLLO by WRpLLO recombinant

A monoclonal antibody to whole LLO antigen (SE1 monoclonals, Dr. J.A Ainsworth, College of Veterinary Sciences, Mississippi State University) weakly recognized the pLLO expression at 32 kDa. (Data not shown due to weak expression).

3.4.2.2 Mice Immune Responses to WRL7/L12 and WRpLLO Recombinants

3.4.2.2.1 Resistance to Bacterial Challenge

3.4.2.2.1.1 Resistance against *Brucella* Challenge

The clearance of *B. abortus* 2308 from mice was determined by assessing viable *Brucella* recovered from spleens. The cfus of each dilution for each mouse spleen was determined and averaged. These values were graphed to compare the efficacy of the vaccinia virus recombinant as a vaccine candidate relative to the vaccine *B. abortus* strain RB51 and the negative control saline group. The bar graph (Figure 3.11) shows the average cfus values of each group of mice spleens. A student t-test (Sigma Plot™) indicated that the difference in cfus between strain RB51 and saline group treatments was the only statistically significant difference (*P* value:<0.01) i.e., protection. In contrast, there was no significant difference between the WRL7/L12 and the saline injected groups, WRL7/L12 inoculation did not protect the mice against challenge under the conditions tested.

3.4.2.2.1.2 Resistance against *Listeria* Challenge

To analyze the protective efficacy of WRpLLO the same protocol *in vivo* in BALB/c mice as for *Brucella* challenge (bacterial clearance from spleens) was used. Compared to saline inoculated control, inoculation of mice with WRpLLO recombinant 8 weeks prior to challenge significantly reduced the number of *Listeria* in mice spleens (Figure 3.12). The protection afforded by WRpLLO compared to saline group by student t-test (sigma plot) is demonstrated as a bar graph with standard errors (*P* value: <0.05).

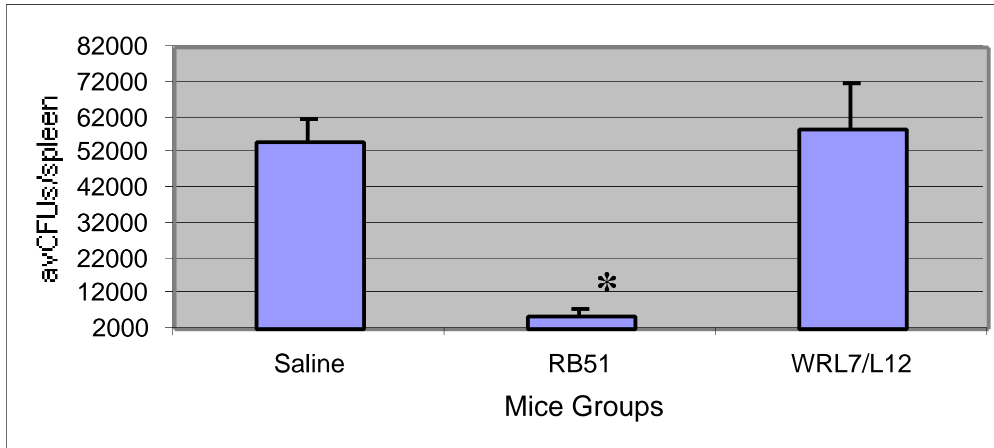


Figure 3.11 Protection studies with WRL7/L12 recombinant

Bar graph of mean *B*. *abortus* cfus in mice spleens 2 weeks following challenge. Groups of mice were injected ip with 1) saline, 2)*B. abortus* strain RB51 3)WRL7/L12 . The bars on each group represents standard error. * indicates statistical significance (*P* value: <0.01).

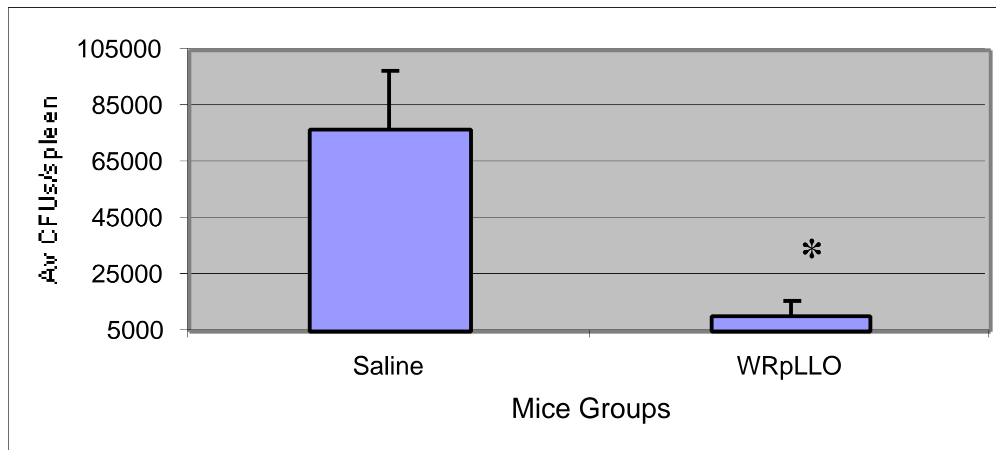
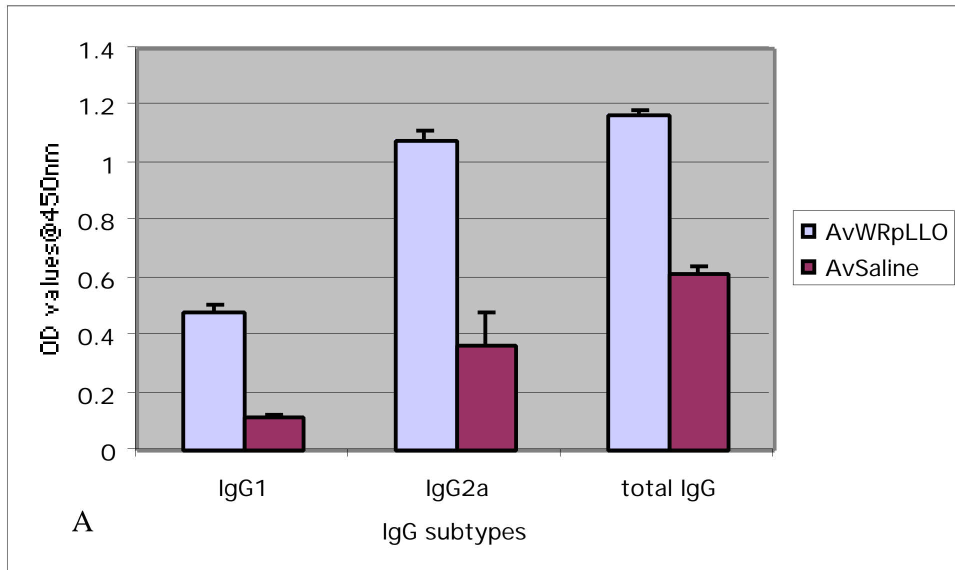


Figure 3.12 WRpLLO protects BALB/c mice against *Listeria* challenge.

Mice inoculated with WRpLLO had significantly lower cfu/spleen of *Listeria* recovered than mice inoculated with saline injected group. (**P* value :<0.05)

3.4.2.2.2 Serologic Analysis of Mice immune responses

Recognition of antibody isotypes in sera of mice inoculated with WRpLLO and WRL7/L12 vaccinia virus recombinants were analyzed with indirect ELISA by using purified proteins MBP-wLLO, and WRL7/L12. Mice responded predominantly with IgG2a response to recombinant vaccinia virus injections (Figures 3.13-3.14).



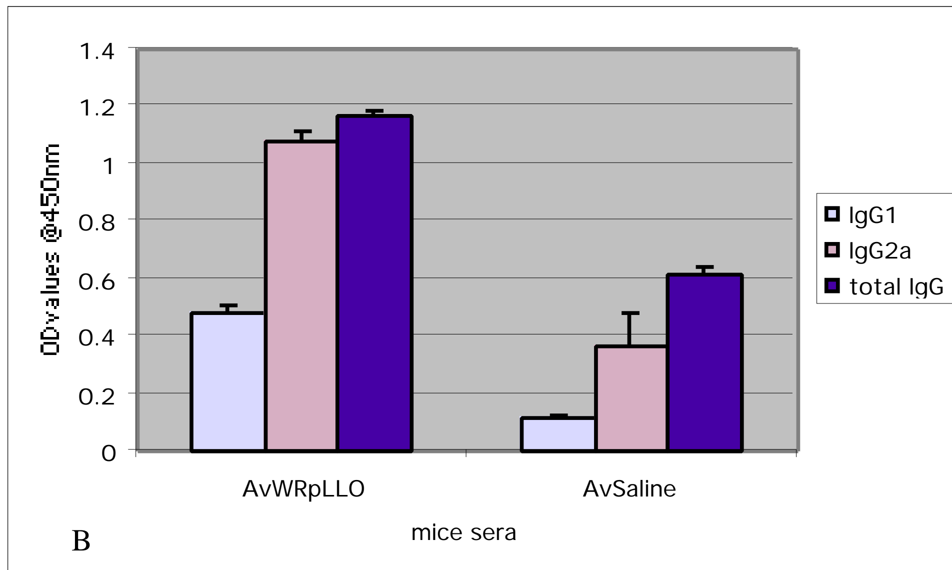
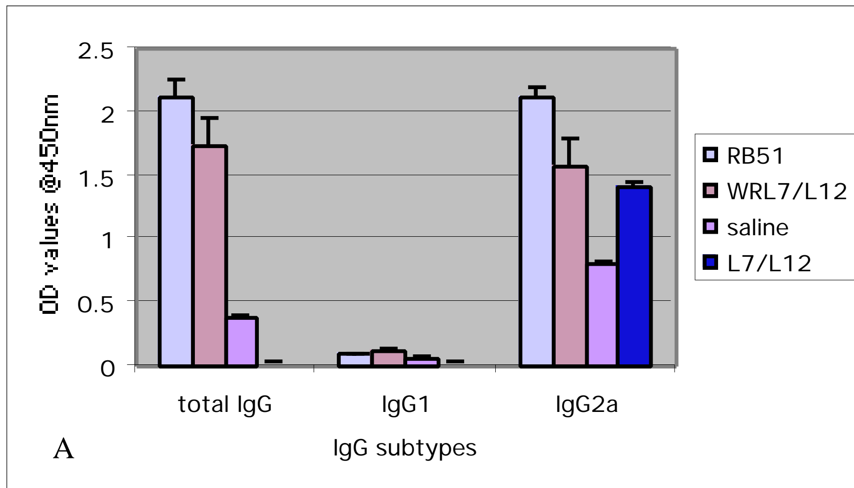


Figure 3.13 Antibody isotype responses of saline and WRpLLO injected mice 7th week postinjection (pi).

Same experiment represented in two charts. Figure 3.13A. Data represented by IgG subtypes, Figure 3.13B Data represented by mice groups. Sera from 3 mice/ group were tested in duplicates. Standard error between the 6 six readings for each group are indicated on the bar graphs.



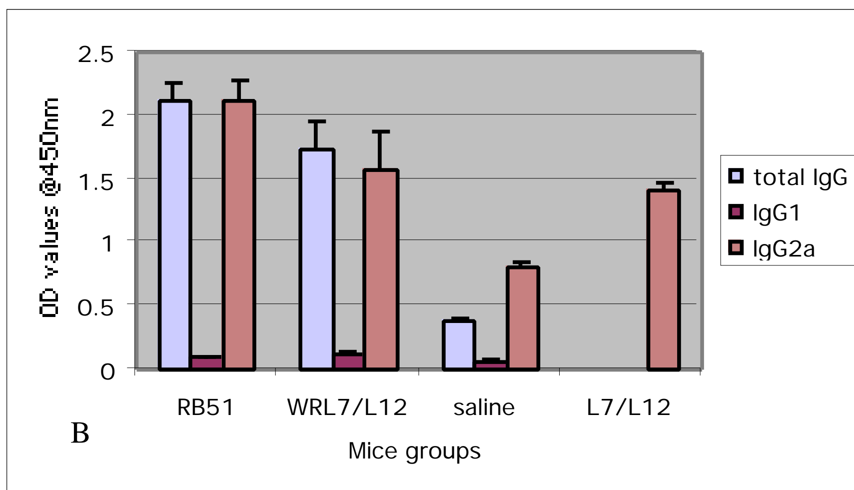


Figure 3.14 Antibody isotype responses of RB51, WRL7/L12, Wrpsc11, L7/L12 and saline injected mice 7th week pi.

Same experiment represented in two charts. Figure 3.14A. Data represent IgG subtypes. Figure 3.14B. Data represent mice groups. Sera from 3 mice/ group were tested in duplicates. Standard error between the 6 six readings for each group are indicated on the bar graphs.

3.4.2.2.3 Analysis of cytokine production by splenocytes in vitro

INF- production *in vitro* by splenocytes from WRpLLO and saline- injected mice upon stimulation with MPB-wLLO was analyzed by sandwich ELISA. The INF- produced by WRpLLO injected mice were significantly higher than saline injected mice (Figure 3.15). IL-4 production analyzed under same conditions was below detectable levels for both treatments.

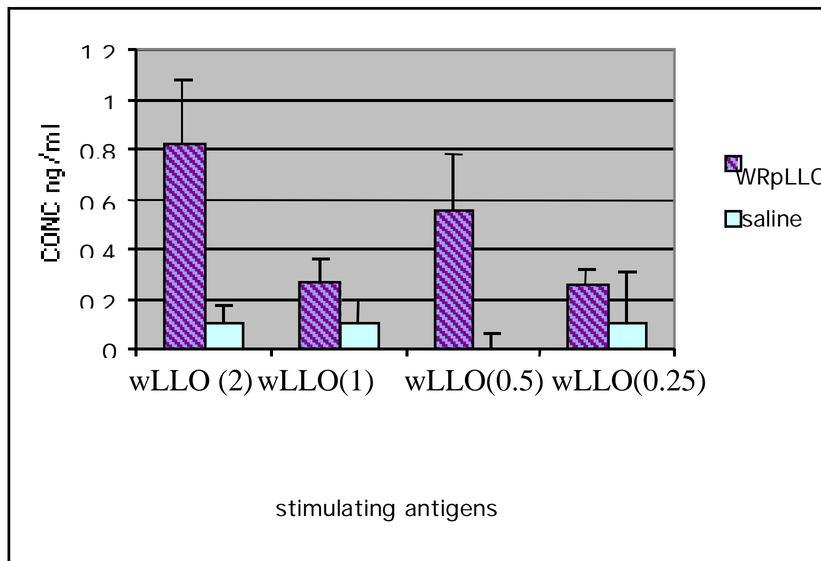


Figure 3.15 Average concentration of INF- (ng/ml) released after *in vitro* stimulation of splenocytes from WRpLLO and saline injected mice.

Stimulation of splenocytes with MBP-wLLO fusion protein induces significantly different INF- release (*P* value:<0.05).

3.5 DISCUSSION

A significant outcome of this study is that a single inoculation of mice with the WRpLLO recombinant was able to induce protective immune response against a virulent *Listeria* challenge. Mice immune responses were characterized as predominantly Th1 type as indicated by predominant IgG2a antibody responses and production of INF- by splenocytes upon stimulation with purified MBP-wLLO antigen; no IL-4 levels were detectable (data not shown). In contrast, mice inoculated with vaccinia/*Brucella* L7/L12 recombinants were unable to demonstrate protection against *Brucella* challenge.

Both *Listeria* and *Brucella* are intracellular bacterial pathogens and it is likely that the immune responses necessary to prevent these diseases are very similar. The immune cells involved in clearing infection with *Listeria* are phagocytic cells, including macrophages, probably neutrophils and T- cells especially $CD8^+$ subset. Antibody production by B cells seems not to play an important role in resistance to *Listeria* infection (170). *Listeria* survives by growing intracellularly in permissive cells but once $CD8^+$ *Listeria* specific T cells appear, permissive cells are lysed and bacteria are again exposed to extracellular milieu. *Listeria* then are phagocytosed and killed by nonpermissive macrophages. Compelling *in vivo* studies document an important role for INF- in resistance to *L monocytogenes*, and show that it is required early not late during infection (183). Adoptive transfer of resistance to *Listeria*, which can be achieved by transfer of $CD8^+$ T cells alone and is not inhibited by antibodies to IFN- showing that IFN- is not required for events mediated by $CD8^+$ T cells (113). In rodent models, the expression of protective immunity against *L monocytogenes* can be mediated by the immune $CD8^+$ T-subset alone. One major target antigen of *Listeria* specific $CD8^+$ T cells is the secreted bacterial Listeriolysin O (LLO), a hemolysin that also functions as an essential virulence factor for this pathogen. Experimental studies with BALB/c mice revealed that an H2-K^d-restricted LLO derivative is the target of immune $CD8^+$ T cells that are induced following sublethal infection with *L monocytogenes*. These $CD8^+$ T

cells exhibit *in vitro* cytotoxicity against LLO pulsed target cells and *Listeria* infected phagocytic cell monolayers and also provide *in vivo* protection following systemic challenge with this pathogen (69).

Both *Listeria* and *Brucella* are able to survive within the macrophages, enabling the bacteria evade the innate defense system such as complement mediated and phagocyte-mediated killing. *B. abortus* replicates within the endosomes of phagocytic and non professional phagocytic cells by inhibiting the phago-lysosome fusion and escaping the action of degrading enzymes (67).

Although antibodies to O-side chain confer a certain level of protection against a virulent challenge in mice, CMI appears to play a major role in protection against brucellosis (9), (10), (67). The best example of this is the protection induced by *B. abortus* strain RB51. This strain does not induce O-side chain antibodies yet it provides good protection against *Brucella* infection. The protection induced by strain RB51 can be transferred by immune T cells (125). Both $CD4^+$ and $CD8^+$ T cells are involved in immunity against brucellosis as indicated by cell depletion experiments using T-cell specific monoclonal antibodies (9), and by using gene targeted knockout mice lacking either $CD4^+$ or $CD8^+$ T cell population (53), (189). *B. abortus* induces a Th1 type immune response and inhibits both the primary and secondary Th2 type immune responses (3), (289), (105). Current knowledge about the role of CTLs in the acquired resistance to brucellosis is limited. The development of *Brucella*-specific CTLs in vaccinated animals and the phenotypic and functional characterization of such CTLs have not been studied in detail. Recently, our group developed a highly sensitive, non-radioactive assay for analysis of *Brucella* specific CTLs in mice immunized with various strains of *Brucella* [He, 2001 #8839]. According to these studies, antigen specific cytotoxic acitivity was exerted by T lymphocytes but not by NK cells. CD3+ CD8+ T cells secreted low levels of IFN- but demonstrated high levels of specific lysis of *Brucella*- infected macrophages.

Cytokines are molecules that modulate the development of protective immune responses by stimulation of $CD4^+$ T cells to produce IFN- *in vivo* and *in vitro*. IFN- is particularly important since it activates the macrophages and up-regulates their bactericidal activity. The important role of IFN- in mediating resistance to *Brucella* infection is demonstrated by depletion of endogenous IFN- with monoclonal antibody, which causes increased *Brucella* replication *in vivo*. In addition, in an adoptive T-cell transfer experiment with IFN- , antibodies blocked the transfer of T cell mediated resistance in mice (292). *In vitro* experiments demonstrated that macrophages activated with IFN- have enhanced brucellacidal and brucellastatic activities (124). This cytokine is produced mainly by natural killer (NK) cells, Th1 cells and its production is positively regulated by IL-12. The biologic activities of IL-12 include stimulation of NK and cytotoxic T cells, induction of CD4+ T cells *in vivo* and *in vitro*. Several studies indicate that endogenous IL-12 produced during infection with *B. abortus* promotes the production of INF- and the clearance of bacteria in *vivo* (290, 293, 295). Depletion of endogenous IL-12 before infection of mice significantly exacerbated *Brucella* infection. IL-12 depleted mice also had reduced splenomegaly resulting from infection and showed a decrease in percentage and absolute number of macrophages compared with those in non IL-12 depleted control mice. Furthermore, spleen cells from IL-12 depleted mouse had reduced ability to produce nitrate, a product of activated macrophages (293).

Production of tumor necrosis factor-alpha (TNF-) also appears to be important for a protective immune response to *Brucella*. Only live *Brucella* elicited the production of TNF- from macrophage cultures (291). TNF- receptor knockout mice ($TNF\text{-}R^{-/-}$) were severely deficient in IL-12 production and *Brucella* infection in these knockout mice was exacerbated (53). In addition, the production of nitric oxide by macrophages was inhibited in the $TNF\text{-}R^{-/-}$ mice, suggesting that nitric oxide may also be an important mediator of brucellacidal activity by activated macrophages.

In summary, Th1 type cytokines, such as IFN- and TNF- appear to be of high importance in resistance against *Brucella* infections. They can be induced by live attenuated *Brucella* vaccines but not by killed vaccines that tend to induce a Th2 type

immune response. A Th2 type immune response actually may interfere with protective immunity to brucellosis.

Vaccinia virus exhibit several unique characteristics including, those capable of overcoming the specific and non-specific fighting mechanisms of the infected hosts. Vaccinia induces the release a family of proteins that bind cytokines, chemokines and interferons from infected cells and block their activities (237). In order to successfully replicate within a host cell, poxviruses have acquired genes encoding proteins that modulate the host response to infection (236), (4). Some of these encode homologs of the extracellular binding domain of cytokine receptors that are secreted from infected cells and intercept the normal activities of particular cytokines. One of these proteins is a soluble IFN- receptor homolog that counteracts the pleiotropic effects of IFN- . Cells infected with poxviruses secrete a 37-43 kDa protein which possesses homology to the ligand binding domain of the known mammalian IFN- receptors and effectively functions as a soluble IFN- receptor homolog (270), (181). Mossman et al., (1995) demonstrated the ability of radiolabeled rabbit IFN- to bind to secreted proteins from the supernatants of cells, infected with various poxviruses. Later, Alcami and Smith (1995) reported that IFN- binding activity is encoded by the B8R ORF of vaccinia virus Western Reserve (WR) strain and also demonstrated the expression of these soluble IFN- receptor homologs by 17 different orthopoxviruses (4).

Moreover, the WR strain of vaccinia virus encodes a protein, B18R, with specific binding and neutralizing capacities against type I IFNs of human, mouse, rat, bovine and rabbit hosts. The high affinity of the B18R protein for type 1 IFNs and its abundance both on the cell surface and in the extracellular milieu indicated that this protein could be an extremely powerful blocker of type I IFN autocrine and paracrine functions (62).

In addition to the previously mentioned mechanisms, vaccinia viruses encode many other proteins that antagonize host responses to virus infections. These are complement binding proteins C21L, C3L which modulate the development of the

inflammatory processes (232); inhibitors of specific protein kinases E3 and K3 which act within the infected cell to override the inhibition of protein synthesis (117).

Cytotoxic T lymphocytes represent one of the most potent ways the host recognizes and destroys the virus infected cells. CTLs recognize virus peptides associated with class I major histocompatibility complex (MHC) antigens on the virus infected cell surface and kill the target cell. Although most viruses (such as adenoviruses and herpesviruses) are efficient in inhibiting the cell-surface expression of class I associated with virus peptides so that the infected cell is not recognized by CTL, pox viruses are less effective at doing this. In fact vaccinia virus has been widely used as a vector to express foreign antigens to determine which antigens and peptides are recognized by CTL (24).

In this study as well as other studies, mice injected with vaccinia virus recombinants exhibited higher splenic *Brucella* counts than saline inoculated mice following challenge. This is significant since it shows that vaccinia virus interferes with the immune response required for *Brucella.* The most critical property of vaccinia virus contributing to its virulence is the ability to counteract pleiotropic effects of IFN- . Even when the L7/L12 protein, which induces a Th1 subset response by murine $CD4^+$ T cells with significant levels of INF- production (193), was used to generate vaccinia recombinants, a protective immune response against *Brucella* challenge was not observed. In addition, upon stimulation with fusion MBP-L7/L12 protein, INF- levels produced by splenocytes from WRL7/L12 inoculated mice splenocytes, was below detectable levels. In order to be protective, the vaccinia/*Brucella* recombinants generated not only have to express the foreign *Brucella* antigens at a high level, but also overcome properties of vaccinia virus that inhibit effects of secreted IFN- and complement mediated killing responses of host. Indirect evidence indicating that this was not achieved by WRL7/L12 recombinants is that the level of lymphocyte proliferation induced with MBP-L7/L12 fusion protein in WRpSC65L7/L12 recombinant injected mice spleens was as high as reported by Oliveira and et al. (1994). In this study, investigators reported that CD4+ T cells isolated from spleens of mice infected with *B. abortus* strain 19 responded

to MBP-L7/L12 fusion antigen with a lymphocyte proliferation 5 times higher than medium-pulsed splenocytes of same animals (SI:5). In this current study a SI of 4.5 was observed in splenocytes of WRpSC65L7/L12-injected mice pulsed with purified MBP-L7/L12 relative to media pulsed splenocytes. However, when cytokine responses were analyzed, Oliveira et al. (1994) detected 4000 pg/ml INF release whereas INF release in vaccinia virus recombinant injected animals was below detectable levels (<390pg/ml). These results show vaccinia virus recombinants injected mice splenocytes proliferated upon stimulation with MBP-L7/L12 fusion antigen but INF- secretion was not detected upon same stimulation. These *in vitro* result could likely be due to persistence of vaccinia virus in the spleens of mice at the time of testing and suppressing the INF- production .

Another difference between two vaccinia virus recombinants is that the WRpLLO recombinant expresses partial listeriolysin that contains a very strong CTL epitope (7). In a previous study, a vaccinia virus recombinant expressing only this epitope was used to inoculate mice. Memory CTLs cultured from these mice specifically lysed target cells pulsed with a synthetic nonamer peptide of LLO (7). It is very likely that the WRpLLO recombinant induced similar cytotoxic activity, however, CTL activities induced by recombinants was not analyzed in this study. On the other hand, studies with *Brucella* L7/L12, identify this protein as an inducer of $CD4^+$ T cells to secrete a pattern Th1 type cytokines. As of yet no *Brucella* antigens have been identified that contain a CTL epitope.

It has been reported that inoculation of mice with recombinant vaccinia viruses induces elevation of natural killer (NK) cell responses (108). NK cells are cabable of lysing microbe-infected cells mostly due to the production of IFN- . Early, but not late, production of IFN- by NK cells was essential for resistance to listeriosis (77). Studies with *B. abortus* using the YAC-1 cell cytotoxicity assay indicated no role of NK cells in the early control of *B. abortus* 2308 infections. Depletion of NK cells from BALB/c mice had no effect on cytotoxic T cell responses (286). Moreover, unlike *B. abortus, L monocytogenes* can cause acute infections, thus early IFN- production by NK cells and

NK cell elevation maybe a determining factor in protective immune response induced by WRpLLO recombinant. However, since *B. abortus* causes only chronic infection and IFN- produced by NK cells is at a level not sufficient enough to induce resistance to *Brucella* challenge, high amounts of IFN- have to be produced by antigen-specific $CD4^+$ cells.

In conclusion, data represented in this study indicate that vaccinia virus can be a suitable expression vector for bacterial antigens. When the correct combination of shuttle vector and protective antigen is used the immune responses induced by vaccinia virus recombinants correlate well with the required immune response to prevent the bacterial disease.

4. VACCINIA VIRUS RECOMBINANT EXPRESSING *BRUCELLA ABORTUS* CUPPER/ ZINC SUPEROXIDE DISMUTASE PROTEIN DOES NOT INDUCE A PROTECTIVE IMMUNE RESPONSE AGAINST *BRUCELLA* CHALLENGE

4.1 ABSTRACT

Brucella is a small gram-negative, facultative intracellular bacterial pathogen. Although several immuno-reactive proteins of *B. abortus* have been characterized, little is known about the specific proteins necessary for inducing the protective immune responses. Peptides containing certain epitopes of copper/zinc superoxide dismutase (Cu/Zn SOD), but not the complete recombinant protein of Cu/Zn SOD of *B. abortus*, have been shown to induce partial protection against *Brucella* challenge. Recent studies, involving inoculation of mice with *E. coli* over-expressing Cu/Zn SOD, indicated a protective role for this protein. Also, over-expression of this SOD in the *B. abortus* vaccine strain RB51 induced a better protective immune response than the strain RB51 alone. Although these studies indicated a protective potential of Cu/Zn SOD, a vaccinia virus recombinant expressing *B. abortus* Cu/Zn SOD (WRSOD) failed to induce protective immune response against *Brucella* challenge. In order to improve the protective efficacy of vaccinia recombinant WRSOD, three different approaches were used: 1) increasing the protein expression by using a shuttle vector (pMCO2) with synthetic vaccinia virus promoter; 2) boosting with a naked SOD DNA vaccine following WRSOD inoculation and 3) using an oligomer CpG as an adjuvant to enhance Th1 type of immune response induced. In spite of successfully implementing these approaches, the WRSOD recombinant, failed to induce a protective immune response as measured by bacterial clearance from the spleens of BALB/c mice. Although there was a significantly lower CFU in the spleens of WRSODCpG treated group than in the spleens of WRSOD-inoculated group, the CFUs were not significantly lower than in the saline control group. The protective ability of other recombinants, such as *B. abortus* RB51SOD and *Ochrobactrum anthropi* SOD and purified SOD were also tested. Only strain RB51SOD

immunization protected mice against a *Brucella* challenge. Only splenocytes from strain RB51SOD inoculated group was able to secrete INF- upon stimulation with RB51 antigens. Stimulation of splenocytes from WRSOD- and WRSODCpG-inoculated mice by purified SOD did not induce any INF- release *in vitro*. This observation provides a partial explanation as to why protection against a *Brucella* challenge was not achieved with the vaccinia recombinants.

4.2 INTRODUCTION

SOD activity is present in all oxygen metabolizing cells, protecting cells from the toxic effects of reactive oxygen intermediates by converting superoxide radicals into hydrogen peroxide (H_2O_2) and oxygen (97). Superoxide radicals (O_2^-) are generated as intermediates during reduction of molecular oxygen. In addition, these oxygen radicals undergo further reduction to form hydrogen peroxide and hydroxyl radicals (OH*). These active oxygen species can damage the DNA, RNA, protein and lipids leading to disruption of cellular architecture and activity (88). To protect themselves against these toxic species, cells that can grow in the presence of oxygen utilize antioxidant enzymes such as SOD, catalases and peroxidases. SODs also play an important role in intracellular pathogenic bacteria permitting them to survive in phagocytes. Thus SOD is usually regarded as a virulence factor facilitating intracellular survival (88). Three forms of SOD have been characterized according to their metal prosthetic groups, manganese (Mn), iron (Fe) and copper-zinc (Cu/Zn) (19). Cu/Zn SOD is usually present in the cytosol of eukaryotes. Only few species of prokaryotes contain Cu/Zn SOD, which is considered as the most recently evolved SOD (18), (149).

B. abortus possesses two forms of SOD. One has been preliminarily characterized as a Mn SOD (245), and the second one as a Cu/Zn SOD (23). The specific activity of *Brucella* Cu/Zn SOD in crude extracts of *Brucella* strains is 10 to 100 fold higher than reported for other intracellular bacterial pathogens (13). However, the presence or absence of SOD activities in the culture supernatants or cytosolic extracts among the virulent and avirulent *B. abortus* strains suggests that SOD is not a major virulence factor. Deletion of Cu/Zn SOD from virulent *B. abortus* strain 2308 did not alter itsvirulence, survival in the infected animals or colony morphology (146). These data also suggest that Cu/Zn SOD is not a major virulence factor for *Brucella* smooth strains. It is possible that in the presence of a major virulence factor like O-side chain, the virulence role of Cu/Zn SOD is overshadowed (146). It may well be that SOD contributes to the virulence of *B. abortus* but is only one of several factors (45). Others

suggest that the presence of a novel Cu/Zn SOD in *Brucella* may be a critical virulence associated factor that allows the survival of the organism in the host (23). It has been suggested that Cu/Zn SOD contributes, but is not solely responsible, for increased survival of *B. abortus* 2308 within murine macrophages during early stages of infection (263). Intracellular bacteria containing periplasmic Cu/Zn SOD, such as *B. abortus*, are able to survive the initial extracellular or extracytoplasmic oxidative stress associated with neutrophil or macrophage phagocytosis (249). A more recent study (196) demonstrated a significant level of protection in BALB/c mice injected with $1x10^6$ live *E. coli* expressing *B. abortus* SOD against a *B. abortus* 2308 virulent strain challenge. Immunization of mice with purified *Brucella* Cu/Zn SOD or synthetic peptides representing Cu/Zn SOD epitopes induces significant protection against virulent strain challenge (13), (260). Moreover, vaccination of mice with *Brucella* strain RB51 overexpressing homologous Cu/Zn SOD also induced an enhanced protection as compared to strain RB51 alone (276). Such protective and virulence roles of SOD have also been demonstrated in other facultative intracellular bacteria (22).

In spite of these results indicating the protective potential of *Brucella* Cu/Zn SOD, a previous study by our group demonstrated that a vaccinia virus/*Brucella* Cu/Zn SOD recombinant, generated by using the plasmid pUV-1 that contains a natural late promoter, even failed to induce humoral immune response in mice (266). In order to improve the efficacy of the vaccinia/ *Brucella* SOD recombinant three different approaches were used.

First, a new vaccinia virus recombinant WRSOD was generated using a shuttle vector $pMCO_2$ containing a synthetic stronger early/late promoter instead of the natural late promoter in pUV-1.

Second, the enhancement of the protective ability of the vaccinia virus/SOD recombinant was attempted with a diversified booster immunization protocol. Since, the intense immune response of mice against vaccinia proteins limits the efficacy of repeated inoculations with recombinant viruses. Therefore diversified prime and booster

inoculation protocols have been used. Following recombinant vaccinia virus injection, a booster with either another non-replicating poxvirus carrier (MVA strain, Avian poxviruses) or nonhomologous carrier has been recommended (118), (230). In this work, to enhance the primary immune response, after the initial inoculation of mice with WRSOD, a naked DNA/SOD construct (pcDNA$_3$SOD) was injected to BALB/c mice as a booster.

Third, a strong immune modulator, the oligodeoxynucleotide adjuvant CpG was used. Synthetic oligodeoxynucleotides, containing CpG dinucleotides (CpG), mimic the immunostimulatory qualities of bacterial DNA. Unmethylated CpG dinucleotides are found more frequently in genomes of bacteria and viruses than in vertebrate DNA. *In vitro,* these CpG motives in a given base context activated antigen-presenting cells (APCs) to upregulate certain surface molecules such as CD69 and major histocompatibility complex class II as well as costimulatory molecules such as B7-1 and B7-2 (141), (208), (250). In addition, CpG containing oligonucleotides induced cytokine secretion by activated APCs including IL-6, TNF- , IL-12 and IFN- (250). In-vivo administration of CpG induced TNF- production by macrophages or INF- production by NK cells. Co-administration of CpG with soluble proteins in complete Freund's adjuvant promoted Th1 response whereas without CpG Th2 responses were obtained (49).

A recent unpublished study with *Ochrobacterum anthropi* which is a gram negative nonpathogenic bacteria with close genetic relatedness to *Brucella*, was used to express *B. abortus* Cu/Zn SOD (115). In this study, it was demonstrated that recombinant *O. anthropi* expressing SOD (OASOD) did not protect mice against *Brucella* challenge. However, when OASOD recombinant was used with CpG, significant protection against *Brucella* infection was achieved.

Our previous work with vaccinia/*Brucella* recombinants suggested that although vaccinia virus appeared to induce a Th1 type response as demonstrated by predominant IgG2a antibodies in mice, cytokines associated with this Th1 type response were not

detected. It was reasoned that this phenomenon was due to soluble IFN- and TNF-binding proteins secreted by vaccinia virus-infected cells interfering with cytokine production. It was hypothesized that inoculation of the strong immune modulator CpG along with vaccinia virus recombinant administration would enhance the production of cytokines associated with Th1 type in injected animals. In this study, mice immune responses to WRSOD inoculation as a function of CpG were analyzed and compared to immune responses induced by other bacterial recombinants RB51SOD and OASOD.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial and Viral Strains and Cell lines:

B. abortus strain RB51SOD and vaccinia virus recombinant WRSOD was acquired from Dr. Ramesh Vemulapalli, the OASOD strain was acquired from Dr. Yongqun He and the challenge strain *B. abortus* 2308 was supplied by Dr. Gerhardt G. Schurig, Virginia Polytechnic Institute and State University, Blacksburg, VA. *Brucella* and OASOD cultures were grown in tryptic–soy broth (TSB) or on tryptic soy agar plates (TSA). All *Brucella* strains were manipulated under BL-3 conditions.

WRSOD stocks were generated using human thymidine kinase deficient 143B cells ($HuTK^-$ cells) were purchased from American Type Culture Collection (ATCC, Rockville, MD). Briefly, 150 cm^2 flasks containing $HuTK^-$ cells at 80% confluency were inoculated with recombinant viruses at MOI of 0.1. After a 4+ CPE was observed, the content of the flask was aspirated and centrifuged at 1000*g* for 5 min. The pellet was saved and resuspended in 1ml of minimal cell culture medium Optimem (Gibco-BRL, Grand Island, NY). The virus was released from the cells by three consecutive freeze-thaw cycles.

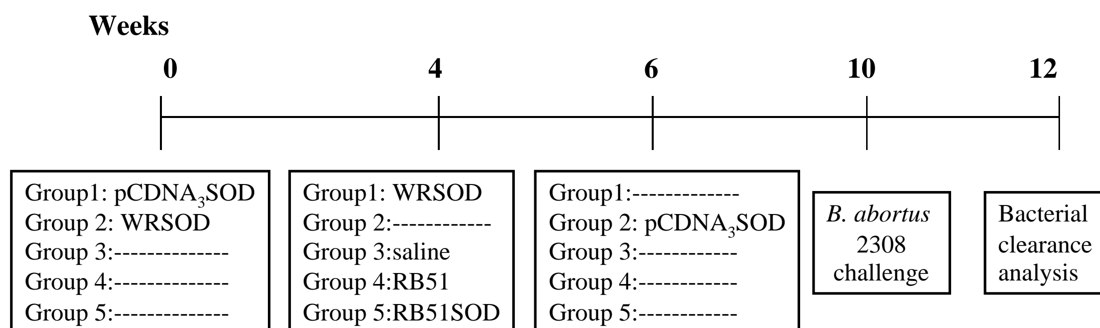
4.3.2 Purified SOD Antigen Preparation

The SOD protein used for mice immunizations and for immune response analysis was prepared using HiTrap Q (Pharmacia,/LKB Biotechnology, Inc., Piscataway, NJ) anion-exchange column chromatography. Briefly, plasmid pBBSOD (acquired from Dr. Ramesh Vemulapalli) was used to transform *E. coli* (Top10 F'®cells, Invitrogen, Carlsbad, CA) cells according to manufacturers recommendations. Recombinant *E. coli* cultures, grown to $2x10^8$ cells/ml, were harvested by centrifugation and resuspended in 200 mM Tris-HCl pH 8.0 containing 0.1% Triton X-100. The suspension was incubated at 37^0C overnight with moderate shaking (100 rpm). After overnight incubation the suspension was centrifuged and the cell supernatant was collected. The crude extract was diluted 1g/2ml and antigens were purified from Hi-Trap Q column according to

manufacturers recommendation. The SOD antigen was mainly found in flow through volume. After column purification, antigen was dialyzed in Spectra/Por® molecularporous membrane bags (12-14 kDa MWCO) (Spectrum, Medical Industries, Inc, Los Angeles, CA) in PBS buffer and LPS was removed using Affi-prep® Polymyxin Support (BIO-RAD, Hercules, CA). Purified antigen was analyzed with SDS-PAGE and Western Blotting. Protein concentration was determined using BIO-RAD protein microtiter plate assay.

4.3.3 Mice Experiments

4.3.3.1 Primary Injection and PCDNA$_3$SOD Booster (Diversified immunization)



Scheme 4.1 Primary inoculation scheme of BALB/c mice for priming and boosting with WRSOD.

Five groups of BALB/c mice (Charles River laboratories, Wilmington MA), each containing five mice, were used. In this experiment, a diversified priming and boosting treatment schedule was tried, as shown in scheme 4.1. At week 0, Group 1 mice received 2 injections of 50μg PCDNA$_3$SOD in 100 μl of PBS each. One injection was given intradermally (id) and the second one intramuscularly (im). Group 2 mice received WRSOD at a dose of $1x10^7$ $TCID_{50}$/mice at week 0. At week 4, Group 1 mice received a booster injection with WRSOD ($1x10^7$ $TCID_{50}$/mice). At week 4, Group 3, 4 and 5 mice were injected as follows: Group 3 mice were served as a negative control group and was injected only with 300 μl of saline. Groups 4 and 5 were the positive control groups Group 4 mice were vaccinated with RB51 ($4x10^8$ CFU/mice); Group 5 mice received RB51SOD ($4x10^8$ CFU/mice) ip, respectively. At week 6, Group 2 mice, which were

previously treated with WRSOD recombinant, received 2 injections of 50μg of $PCDNA_3SOD$ in 100 ml PBS, one given id and the other one im. All mice groups were challenged with *B. abortus* virulent strain 2308 ip ($2.5x10^4$) at week 10. Mice were sacrificed by CO_2 asphyxiation at week 12 for bacterial clearance analysis. The results were analyzed by t-Test (Sigma Plot ™, Scientific Graphic Software version 5.0, Jandel Scientific, San Rafael, CA) by comparing each group to the negative control group.

4.3.3.2 Protection Studies

4.3.3.2.1 Mice Treatments

Six groups of eight mice each, were used. Three groups of mice were injected initially with 5 nM/mouse of phoshothioate modified CpG (Sigma-Genosys, Woodlands, TX) intraperitoneally (ip) with the sequence TCCATGACGTTCCTGATGCT. Four hours post inoculation (pi), the 3 mice groups previously injected with CpG received another 5 nM of CpG and one of the following treatments Group 1 received WRSOD ($1x10^7 TCID_{50}$/mice); Group 2 received 30μg of purified SOD and Group 3 $5x10^8$ CFU/mice OASOD. The other 3 mice groups were injected as follows: Group 4, *B. abortus* RB51 strain overexpressing SOD (RB51SOD, positive vaccine control) ($3.5x10^8$ CFU/mice); Group 5 saline solution (300μl); and Group 6 WRSOD ($1x10^7$ $TCID_{50}$/mice) ip. Mice inoculated with purified SOD were boosted 4 weeks pi with 30μg SOD with 10 nM CpG (5nM CpG 4 hours before, and 5nM simultaneously with the SOD inoculation). Five mice from each group were challenged at 8th week pi with *B. abortus* strain 2308 ($2.4x10^4$ CFU/mouse). *Brucella* challenged mice were euthanized by CO_2 asphyxiation 2 weeks post challenge for bacterial clearance analysis. Remaining three mice in each group was sacrificed for CMI analysis at 10th week pi.

4.3.3.2.2 Bacterial clearance analysis

Mice were euthanized by CO_2 asphyxiation, the spleens were removed aseptically and placed in individual plastic tubes containing sterile sand (0.75g/tube) and 1 ml TSB. Spleens were homogenized using a sterile pestle. Ten-fold serial dilutions of homogenates were prepared in TSB. Five, 10 µl drops, from each dilution were plated on TSA plates and incubated at 37^0C for 5 days. To minimize statistical error, number of colonies in the lowest dilution that could be counted, was used to quantify the bacteria per spleen in colony forming units (CFU/spleen). To determine the number of CFU/spleen, the following formula was used (Detection limit:$2x10^2$)

CFU/spleen =Total CFU/dilution **X** 20 **X** dilution factor

The data were analyzed by t-Test (Sigma Plot ™, Scientific Graphic Software version 5.0, Jandel Scientific, San Rafael, CA) by comparing each group to the saline-inoculated group.

4.3.3.3 Serological analysis

Three mice per group were bled by puncturing the retroorbital plexus on the third, fifth, seventh, tenth and twelfth weeks post immunization. On the fifth and tenth weeks pi sera was used in indirect ELISA for IgG isotype analysis. The serum acquired at week 7 was diluted 1:50 and analyzed for SOD antibodies by a Western Blot analysis.

4.3.3.3.1 Indirect ELISA

The presence of antigen-specific serum -IgG, -IgG1 and -IgG2a isotypes were determined by indirect ELISAs. The SOD antigen at 20µg/ml concentration in carbonate buffer (pH9.6) was used to coat the wells (50µl/well) of MaxiSorp™ immunoplates (Nalge-Nunc International, Rochester, NY). After coating at 4^0 C overnight, plates were

blocked using blocking buffer (2% bovine serum albumin (BSA) in phosphate buffered saline (PBS). After two hours of blocking at room temperature, the blocking buffer was discarded and serum samples (1:50 dilution in blocking buffer) were added to duplicate wells (100µl/well). The plates were incubated for three hours at room temperature (RT) and washed four times with PBS with 0.05% Tween® 20 (Fisher Scientific, Springfield, NJ). Isotype specific (IgG, IgG1, IgG2a) goat anti-mouse horseradish peroxidase conjugated secondary antibodies (Caltag Laboratories, San Fransisco, CA) were added to each well at 1:7400 dilution and incubated at RT for one hour (h). Following the secondary antibody incubation the plates were washed four times and 100 µl of TMB Microwell substrate (Kirkegaard &Perry laboratories, Gaithersburg, MD) was added to each well. After 30min incubation at room temperature, the enzyme reaction was stopped, by adding 100 µls of stop solution (0.185M sulfuric acid). The plates were read and analyzed at 450nm absorbance by a microplate reader (Molecular Devices, Sunnyvale, CA).

4.3.3.4 Cell-Mediated Immune Response Analysis

Cell mediated immune responses induced in mice were analyzed by lymphocyte proliferation assays and determination of cytokines produced by mice splenocytes upon stimulation with antigens *in vitro* (cytokine ELISAs).

4.3.3.4.1 Culturing Splenocytes from Mice Spleens

Mice were sacrificed by CO_2 asphyxiation and their spleens were removed under aseptic conditions. Single spleen cell suspensions were prepared from the spleens. Briefly red blood cells were lysed using ACK solution (0.1 M NH_4Cl, 1mM $KHCO_3$, 0.1mM EDTA [pH7.3]). The splenocytes were cultured in 96-well U bottom plates (Corning, Acton, MD) at a concentration of $5x10^5$ viable cells/well. RPMI 1640 medium (Gibco, BRL, Grand Island, NY) supplemented with 2mM L-glutamine (Gibco, BRL, Grand Island, NY), 10% heat-inactivated fetal bovine serum (FBS) (Intergen, Purchase, NY) and 50µM of penicillin/streptomycin was used for culturing splenocytes. Splenocytes were stimulated with following antigens: 1µg of concanavalin A (ConA);

heat inactivated $1x10^6$ (low dose), $1x10^7$ (high dose) of *B. abortus* RB51 as positive controls, purified SOD at concentrations at 0.25, 0.5, 1 and 2µg/well; and no additives/well as negative control. The splenocytes were incubated in a 5% CO_2, at 37^0C for 5 days for cytokine ELISAs and for 2 days for lymphocyte proliferation assays.

4.3.3.4.2 Lymphocyte Proliferation Assays

Splenocytes were cultured from mice as described above. After 48h incubation at 37^0C in a 5% CO_2 incubator, the cells were pulsed for 18 hours with 0.5mCi (20µl of 1:20 dilution with CRPMI) of tritiated thymidine (^{3}H)(ICN, Costa Mesa, CA) with specific activity of 6.7 Ci/mmole. Cells were harvested and insoluble tritiated thymidine counts were determined by liquid scintillation spectrometry (TopCount NXT, Packard Instruments, Meriden, CT). The results are expressed as stimulation index (SI) calculated by the following formula.

The SI is calculated by:

$$\frac{\text{Insoluble } ^3\text{H thymidine cpm of spleen cells exposed to antigen}}{\text{Insoluble } ^3\text{Hthymidine cpm of spleen cells unexposed to antigen}}$$

4.3.3.4.3 Cytokine ELISAs

Splenocytes were cultured from mice as described above and incubated in U bottom 96-well plates (Corning, Acton, MD) for 5 days at 5%CO_2 incubator at 37^0C. The culture supernatants were transferred to a new 96-well plate and stored at -70^0C until a sandwich ELISA was performed to quantify cytokine levels in the supernatants. Briefly, 96- well MaxiSorp™ immunoplates (Nalge-Nunc International, Rochester, NY) were coated overnight with 0.1µg/well of purified rat- antimouse cytokine coating antibody (Pharmingen, San Diego, CA) diluted in (PBS) at pH: 7.4. Following overnight coating, the plates were blocked for 1 h with assay buffer (2% BSA in PBS pH: 7.2-7.4). Plates were washed 5 times using wash buffer (50mM Tris, 0.2% Tween 20 ® [Fisher Scientific, Springfield, NJ] pH: 7.0-7.4). During the blocking, cytokine standards (Pharmingen, San Diego, CA) were serially diluted (1:2) in the range of 50-0.39 ng/ml with RPMI 1640 medium (Gibco, BRL, Grand Island, NY) supplemented with 2mM L-glutamine (Gibco,

BRL, Grand Island, NY), 10% heat-inactivated fetal bovine serum (FBS) (Intergen, Purchase, NY) and 50µM of penicillin/streptomycin. Samples from the previously frozen plates and standards were added to each well, mixed 1:1 mixed with assay buffer. Plates were incubated overnight at room temperature. Plates were then washed five times with wash buffer and incubated for one h with 0.25µg/ml diluted biotinylated detecting antibody (100µl/well) (Pharmingen, San Diego, CA). Then, plates were washed five times with wash buffer and then incubated 30 min with 1:5000 diluted (assay buffer with 0.05%Tween 20®) horseradish peroxidase (HRP) conjugated streptavidin antibody (Vector, Burlingams, CA). Following the incubation with streptavidin antibody, the plates were washed five times and 100 µl of TMB Microwell substrate was added to each well. The enzyme reaction was stopped by, adding 100 µls of stop solution (0.185M sulfuric acid) and incubating for 30 min at room temperature. The plate was read and analyzed at 450nm absorbance by a microplate reader (Molecular Devices, Sunnyvale, CA). The quantity of cytokines was determined according to the linear regression equation of the standard curve.

4.4 RESULTS

4.4.1 Bacterial Clearance Study after Diversified Treatment Schedule

Due to the highly immunogenic nature of vaccinia virus, a previous treatment with vaccinia virus will likely interfere with subsequent booster injections. Therefore, after WRSOD inoculation, one group of mice received a booster consisting of naked DNA construct expressing SOD gene (pCDNA$_3$SOD). As a control, one group of mice received the treatment sequence in reverse: injection of pCDNA$_3$SOD was followed by WRSOD recombinant. Compared to the negative control saline-injected group, bacterial clearance studies indicated that the diversified priming and booster treatments (WRSOD followed by pCDNA$_3$SOD and vice versa) had no effect on the level of resistance induced against *Brucella* challenge (Figure 4.1.). Strain RB51SOD- and RB51-inoculated mice cleared the bacteria faster than the mice treated by either diversified scheme and the *Brucella* CFUs in the spleen of RB51 and RB51SOD mice were significantly lower than in the saline treated group.

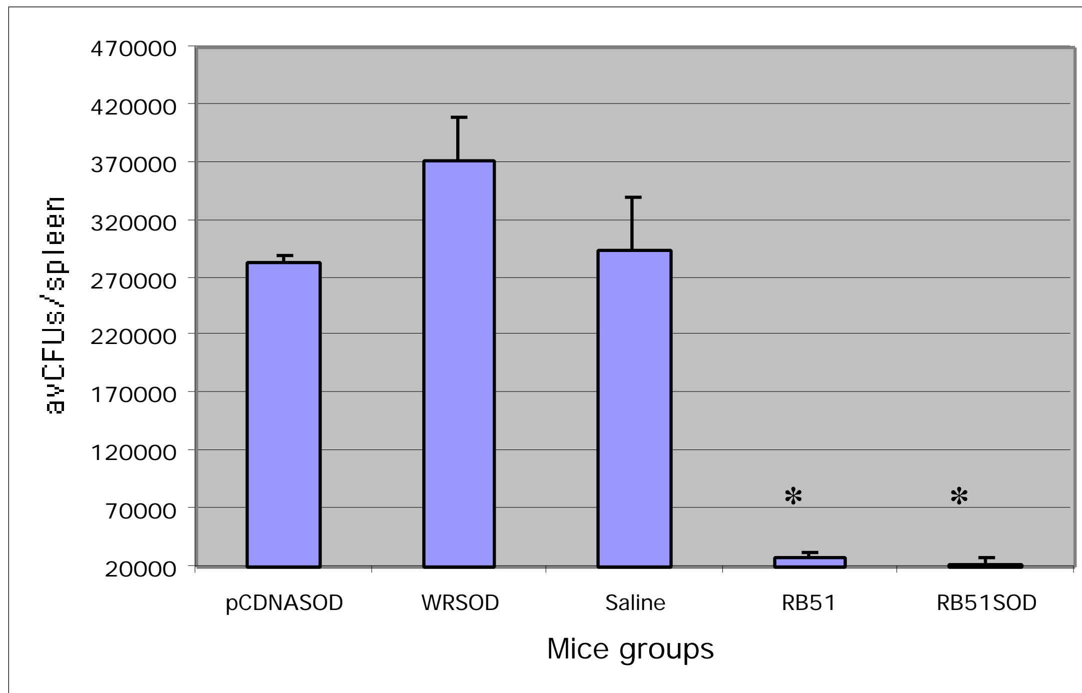


Figure 4.1 Splenic clearance of *B. abortus* 2308 challenge after diversified prime and boosting treatments.

Data is represented as bar graphs showing log values of mean *B. abortus* cfus in mice spleens 2 weeks after the challenge. Groups of mice treatments were 1) pCDNA$_3$SOD, initial, WRSOD booster; 2) WRSOD initial, pCDNA$_3$SOD booster; 3) saline; 4) RB51; 5) RB51SOD. The bars on each group represents standard error. * indicates statistical significance (*P value* :$<$0.01) relative to saline control.

4.4.2 Humoral Immune Response Analysis

4.4.2.1 Western Blotting

Mice sera from each group obtained at the seventh week pi were used in a Western blotting to reveal antibodies against the various antigens. The results indicated, that RB51SOD-injected mouse sera weakly recognized expressed SOD antigen in the WRSOD recombinant (lane 3), but SOD was not recognized in the WR antigen (lane 2) (Figure 4.2). The same sera recognized strongly various proteins of OASOD antigen (lane 4) and of RB51SOD antigen (lane 5). Sera of RB51SOD-inoculated mice only recognized SOD at 20kDa position in purified SOD antigen (lane 6).

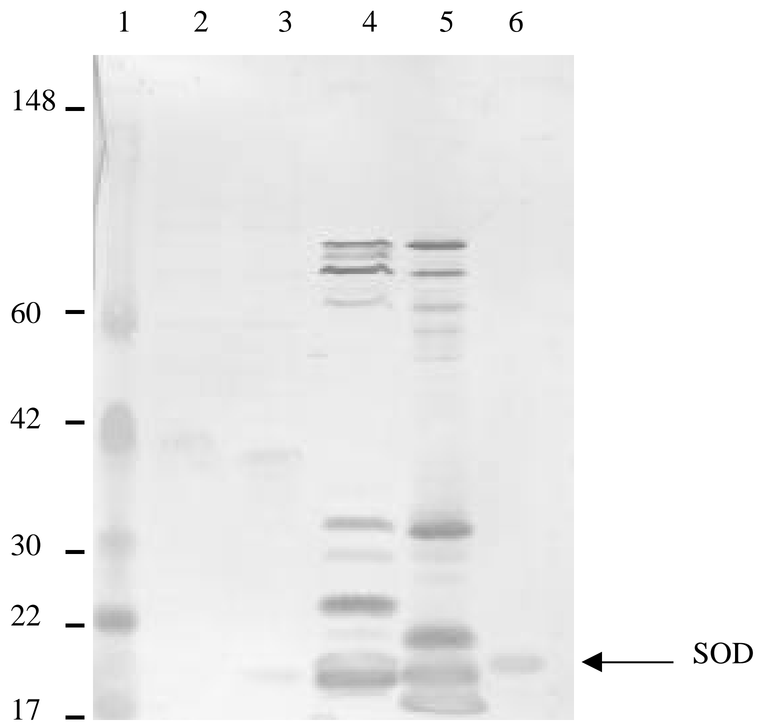


Figure 4.2 Western Blot analysis of 7 week pi sera of RB51SOD-inoculated mice.

Lanes: 1)MW markers; antigens 2)WR, 3)WRSOD, 4)OASOD, 5)RB51SOD; and 6) purified *B. abortus* SOD.

The antigen recognition pattern by sera of OASOD-inoculated mice was similar to that by sera of strain RB51-inoculated mice. Sera of OASOD-inoculated mice recognized strongly various proteins of strain OASOD antigen (lane 4) and of strain RB51SOD (lane 5). SOD is recognized by sera of OASOD-inoculated mice in all the antigen preparations except the WR negative control (lane 2) Figure 4.3.

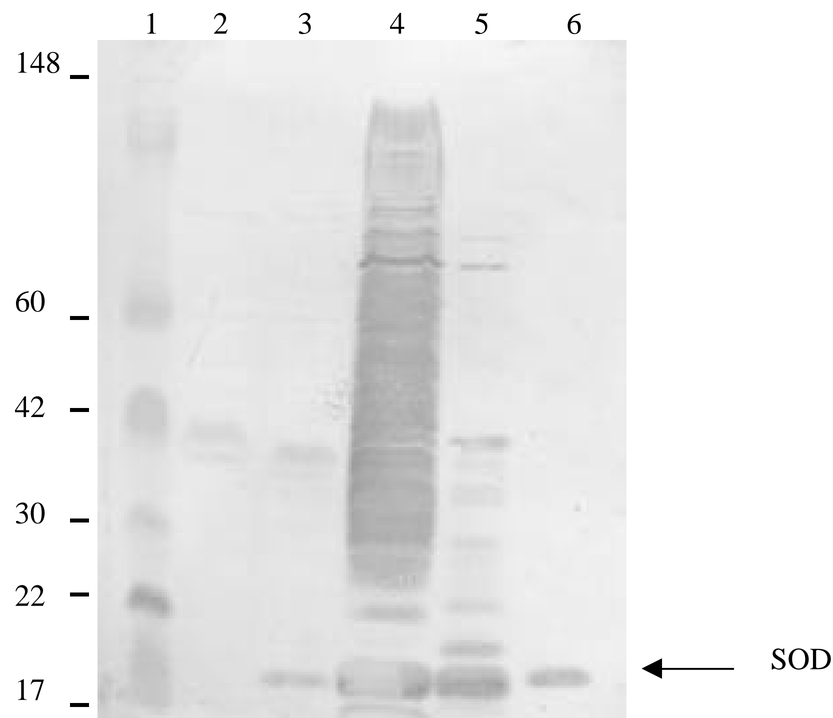


Figure 4.3 Western Blot analysis of serum 7 week pi sera of OASOD-inoculated mice.

Lanes: 1)MW markers; antigens 2)WR, 3)WRSOD, 4)OASOD,5)RB51SOD; and 6) purified *B. abortus* SOD.

Serum of SOD-injected mice recognized SOD in all of lanes containing antigens WRSOD, OASOD, RB51SOD and purified SOD except negative control WR antigen (lane 2) (Figure 4.4).

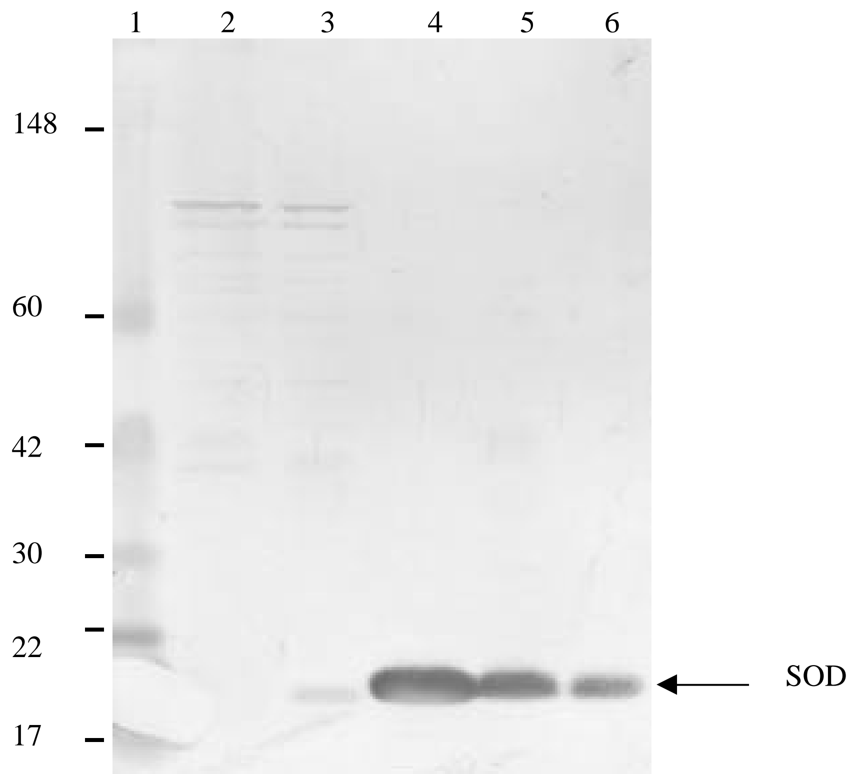


Figure 4.4 Western Blot analysis of 7 weeks pi sera of SOD-inoculated mice.

Lanes: 1)MW markers; antigens 2)WR, 3)WRSOD, 4)OASOD,5)RB51SOD; and 6) purified *B. abortus* SOD.

Serum of saline-injected mice did not recognize any of the tested proteins (data not shown). Sera of WRSOD-inoculated mice recognized only vaccinia virus specific antigens but did not recognize SOD in any of the antigen preparations OASOD, RB51SOD or SOD Figure 4.5.

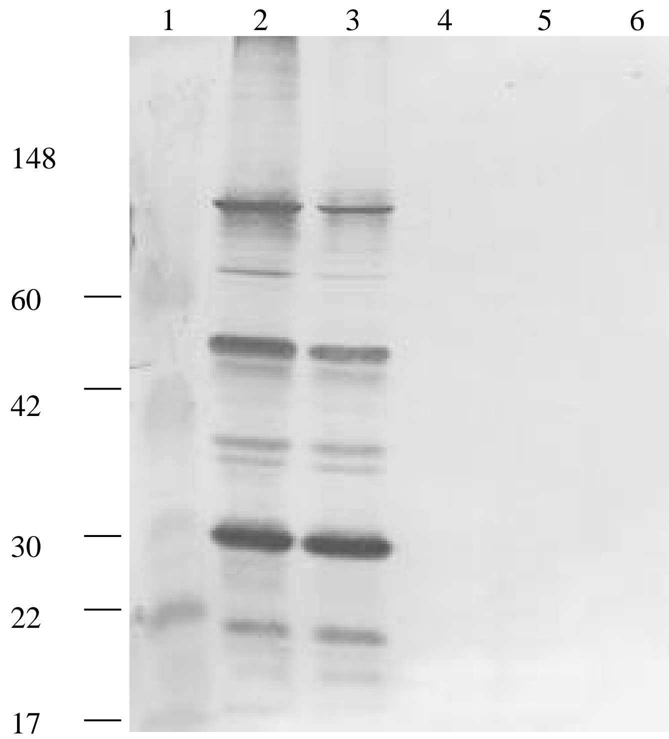


Figure 4.5 Western Blot analysis of 7 weeks pi sera of WRSOD-inoculated mice.

Lanes: 1)MW markers; antigens 2)WR, 3)WRSOD, 4)OASOD,5)RB51SOD; and 6) purified *B. abortus* SOD.

4.4.2.2 Indirect ELISA

The presence of antigen specific IgG, IgG1 and IgG2a isotypes in mice sera were determined by indirect ELISA. Three mice from each group were bled at the fifth and tenth week pi. Sera were diluted 1:50 and each dilution was tested in duplicates and averaged. Data are represented in Figure 4.6. All mice groups, except saline-inoculated mice responded predominantly with IgG2a isotype. Antibody responses of SODCpG-inoculated mice seemed greater than any of the other groups. This is likely due to the booster inoculation with another ip injection of SODCpG at the 4th week. Therefore, the SOD-specific antibody titers of sera collected ten week pi, were tested by indirect ELISA. Data are represented in Figure 4.7.

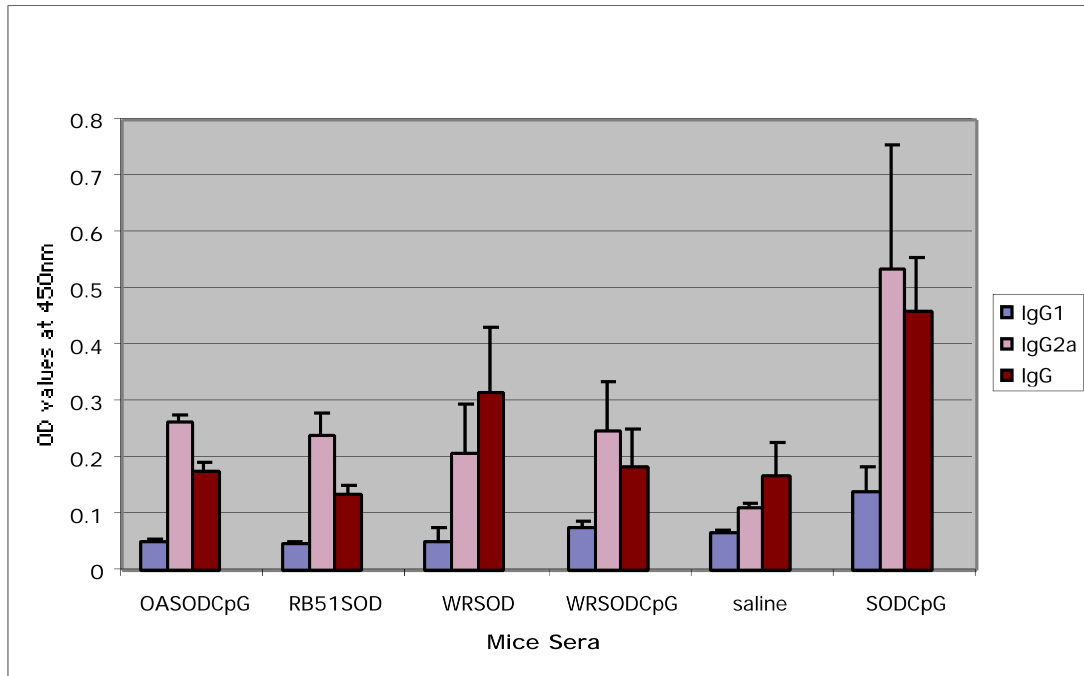


Figure 4.6 Antibody isotype responses with mice sera acquired at week 5

Antibody isotype responses of OASODCpG, RB51SOD, WRSOD, WRSODCpG, saline and SODCpG injected mice 5th week pi. Standard errors of the mean OD values of three mice representing each inoculation are indicated on the bar graphs.

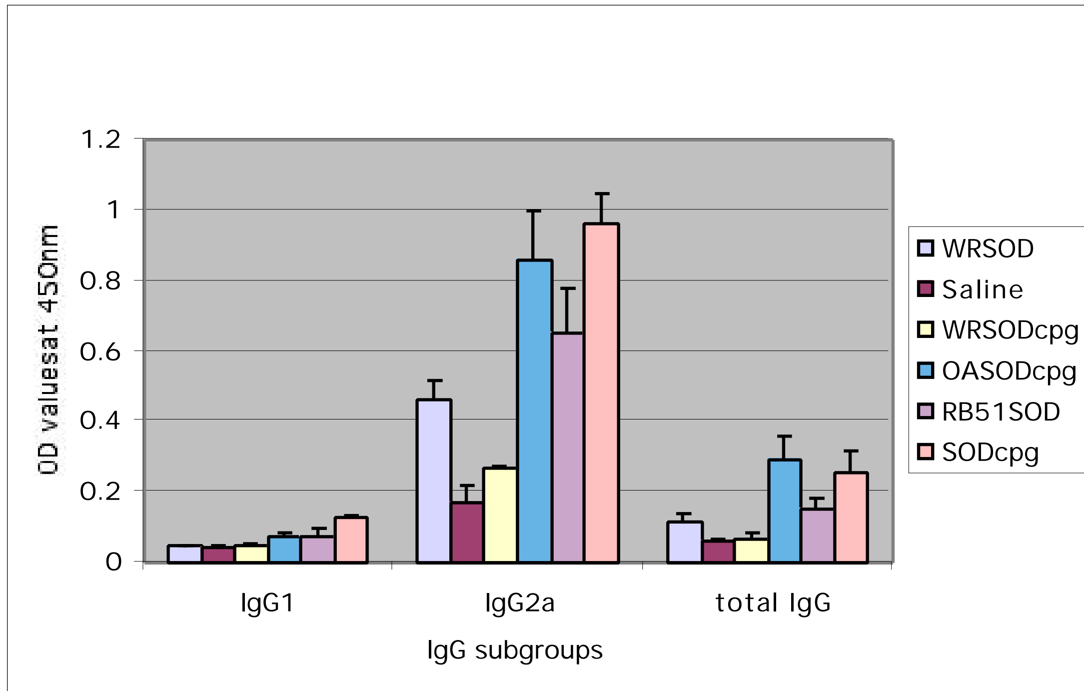


Figure 4.7 Antibody isotype responses with mice sera acquired at week 10

Antibody isotype responses of OASODCpG-, RB51SOD-, WRSOD-, WRSODCpG-, saline- and SODCpG- injected mice. Data represented by IgG isotypes. Standard errors of the mean OD values of three mice representing each inoculation are indicated on the bar graphs.

4.4.3 Protective Immune Response Analysis

The clearance of *B. abortus* 2308 from mice was determined by assessing viable *Brucella* recovered from spleens. These CFU/spleen values were averaged and graphed to compare the protective efficacy of the SOD recombinants relative to saline inoculated mice. Figure 4.8 shows the average cfus of each group of mice spleens. The analysis by student t-test (Sigma Plot™) indicated that the difference between strain RB51SOD and saline treatments was the only statistically significant change (*P* value:<0.01) i.e., protection. In contrast, the cfus/spleen of mice inoculated with any other treatment did not differ significantly from cfus/spleen of mice inoculated with saline. Although there was no difference between the WRSODCpG and saline treated group, when the WRSODCpG treated group compared to WRSOD group cfus of WRSODCpG treated mice was significantly lower than WRSOD treated group (*P* value :<0.023).

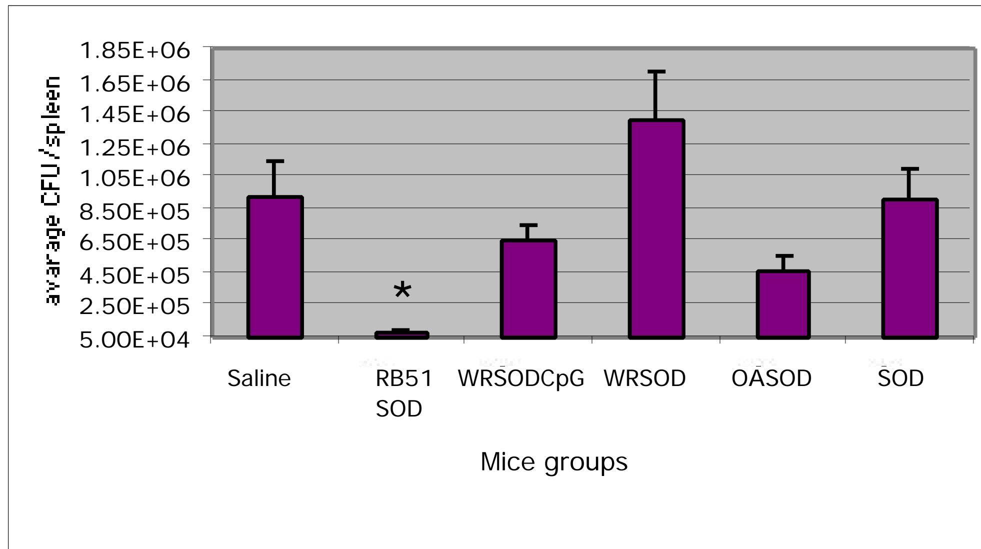


Figure 4.8 Protection studies

Bar graph of mean B. *abortus* cfu values per spleens 2 weeks following challenge. Groups of mice were injected ip with 1) saline; 2) RB51SOD; 3) WRSODCpG; 4)WRSOD; 5) OASODCpG; and 6) SODCpG respectively. When the group means were compared, only RB51SOD inoculated group mean was statistically lower than saline control group (*P* value: <0.01).

4.4.4 Cell Mediated Immune Response Analysis

Lymphocyte proliferation and cytokine ELISAs were performed to determine the cellular immune responses.

4.4.4.1 Lymphocyte Proliferation Assays

Mice splenocytes were stimulated with the following preparations: $1x10^6$, $1x10^7$ heat killed RB51 cell antigen (L, H respectively), and 2, 1, 0.5, 0.25 μg/ml of purified SOD antigen, 0.5 μg conA and no additives (media, non-stimulated control). After

stimulation splenocytes were pulsed with [^{3}H] thymidine and the SI values for each mice group were calculated (Table 4.1)

Table 4.1 SI indices of splenocytes from inoculated mice.

	Mice Groups					
Antigens	RB51SOD	Saline	OASODCpG	SODCpG	WRSOD	WRSODCpG
ConA	54.7	52.4	119.4	115.43	304.5	150.8
RB51 (H)	6.3	<1	1.98	1.27	9.34	1.82
RB51 (L)	1.23	<1	1.19	1.13	3.08	<1
SOD (2)	1.03	<1	<1	1.73	4.35	<1
SOD (1)	<1	<1	<1	1.38	1.66	1.49
SOD (0.5)	2.25	<1	<1	1.92	1.58	2.08
SOD (0.25)	<1	<1	<1	<1	<1	<1
Media	1	1	1	1	1	1

4.4.4.2 Cytokine ELISAs

Mice splenocytes were analyzed *in vitro* for INF- and IL-4 secretion upon stimulation with tested antigens: conA, RB51 (H, L), SOD (2, 1, 0.5, 0.25 µg/well). INF- levels secreted from saline-, OASODCpG-, SODCpG-, WRSOD- and WRSODCpG-treated mice spleens were below detectable limits (0.14ng/ml) upon stimulation with either RB51 (H, L) or SOD antigens. Only splenocytes of RB51SOD treated mice secreted INF- upon stimulation with RB51 antigen. Splenocytes of RB51SOD-inoculated mice upon stimulation with high and low doses of RB51 secreted 0.33 ±0.11µg/ml and 0.35±0.11 µg/ml INF- levels respectively. Splenocytes of RB51SOD-injected mice secreted INF- below detectable level when stimulated with SOD.

IL-4 secretion by splenocytes of each mouse group upon stimulation with RB51 or SOD antigens were below detectable levels.

4.5 DISCUSSION

Numerous vaccinia virus recombinants carrying *Brucella abortus* genes have been generated. These include recombinants expressing *Brucella* L7/L12 (chapter 3), GroEL (16), (17), HtrA (13), GroES, 18 kDa (272) and SOD (266). None of these recombinants were able to induce protective immune response against a *Brucella* challenge. Most of these vaccinia virus recombinants induced antigen specific humoral immune responses in inoculated animals, only a vaccinia virus/SOD recombinant (VVSOD) was not able to induce humoral immune responses in BALB/c mice (266).

Instead of the previously generated recombinant VVSOD, using pUV-1 shuttle vector, vaccinia virus/*B. abortus* SOD recombinant (WRSOD) using shuttle vector $pMCO_2$ was generated. These two plasmids not only use a different gene for colorimetric secreening of the recombinants, but most importantly, they differ in the vaccinia promoter they use for the expression of the foreign gene. The shuttle vector pUV-1 contains a natural late vaccinia promoter and the *lac* Z gene of *E. coli* for colorimetric secreening. In contrast the shuttle vector $pMCO_2$ contains a synthetic early/late promoter and the *gus* A gene of *E. coli* expressing -glucuronidase for colorimetric screening. The literature indicates that the type of the promoter employed in vaccinia virus recombinants dictate both the level and time of antigen expression (36). Factors that influence the choice of promoter system include the desired use of the recombinant vaccinia virus. Chakrabarti et al. (1997) indicated that for large-scale protein production induced by a vaccinia virus promoter, a strong synthetic early/late should be used. In addition to high levels of protein production, the early/late promoter use was recommended for the induction of cytotoxic immune responses (177). In our work, in spite of using of the $pMCO_2$ shuttle vector with synthetic early/late promoter the WRSOD recombinant failed to induce a strong humoral immune response in BALB/c mice. In Western blots, the sera of WRSOD-inoculated mice acquired at seventh week pi failed to recognize SOD antigen in purified SOD, WRSOD, RB51SOD, OASOD antigens (Figure 4.5). The indirect ELISAs revealed that sera acquired from WRSOD inoculated mice were able to recognize SOD antigen. Although the IgG levels were not too high, they were as high as

the ones induced with OASOD or RB51SOD inoculations (Figure 4.6). The antibody responses of inoculated mice were also tested using sera obtained at week 10 pi. Results demonstrated even lower IgG and IgG2a levels, almost as low as in sera of the saline-inoculated group (Figure 4.7). The fact that Cu/Zn SOD might be not be a good inducer of antibody response has been demonstrated in several studies (266), (259), (260). Mice inoculated, with recombinant purified SOD or synthetic peptides of SOD were not able to produce antibody responses in mice and cattle, and mice developed SOD specific antibodies only after challenge with *Brucella* (260), (254). Moreover, vaccination of cattle and mice with *Brucella* strains RB51 and strain 19 did not induce antibodies specific to SOD (55), (196), (254). It is very likely the amount of SOD produced by WRSOD recombinant, like the amount produced by the *Brucella* injection was insufficient to induce SOD specific antibodies. The weak antibody response induced could also be due to 27% homology of *Brucella* Cu/Zn SOD to eukaryotic SOD. Thus SOD is not recognized as foreign and SOD specific antibody responses only appear when this protein is over-expressed in B. *abortus* strain RB51 or *O. anthropi* (115), (276). A passive transfer experiment with mice sera in this study shown that, sera from mice which had been inoculated with purified SOD, OASOD, RB51SOD and which had SOD specific antibody titers (Figures 4.2, 4.3, 4.3) were unable to convey resistance against *Brucella* challenge in BALB/c mice (data not shown). Therefore, the evidence suggests that antibodies to *Brucella* Cu/Zn SOD do not protect against *Brucella*.

In contrast to the above results, several publications point to the protective role of Cu/Zn SOD (115), (276), (196), (254). In all of these studies SOD antigen was able to induce lymphocyte proliferation of the treated animals. Stevens et al., (1994) inoculated several synthetic SOD peptides and was able to show at least one of these synthetic peptides was responsible for the cellular immune response. Our results correlate with these results, since the WRSOD recombinant was able to induce splenocyte proliferation upon stimulation with purified SOD antigen (Table 4.1). However, this cellular immune response was not sufficient to protect mice against a *Brucella* challenge.

As indicated earlier, there are two problems associated with the use of vaccinia viruses as carriers for antigens. One of these problems is that vaccinia virus proteins are highly immunogenic and therefore a secondary inoculation of the same recombinant will be neutralized due to preexisting vaccinia virus specific immunity and a secondary immune response for the foreign antigen won't be achieved or will only materialize at a low level. The second problem is the ability of vaccinia virus to induce vaccinia virus-infected cells to secrete soluble IFN- and TNF- binding proteins interfering with Th1 type immune response induction.

In this study, both of the problems were addressed in attempts to overcome them and increase the protective efficacy of the WRSOD recombinant. In order to avoid the neutralization of vaccinia virus upon secondary inoculation, a booster immunization with a naked DNA construct pCDNA$_3$SOD was used. Conversely, in one group of mice priming was done using pCDNA$_3$SOD followed by the WRSOD booster. Although literature indicates that this type of diversified immunization increases the immunogenicity and protective efficacy of recombinants expressing the foreign antigen (229), this was not achieved for the WRSOD recombinant with either inoculation scheme (Figure 4.1).

In order to overcome INF- and TNF- inhibiting characteristics of vaccinia virus a strong immune modulator, the oligodeoxynucleotide adjuvant CpG was used. As indicated in the literature, CpG treatments induced TNF- production by antigen presenting cells (APCs), and INF- secretion by NK cells (250). In addition, injection of CpG together with antigen skewed antigen (Ag) specific IgG isotypes toward IgG2a and promoted the induction of Ag-specific CD8+ cytolytic T cells. Our results suggested, however, that, WRSODCpG treatment induced much lower SI values than the WRSOD inoculation as determined by lymphocyte proliferation assays (Table 4.1). Both, IgG2a and total IgG responses, induced in WRSODCpG treated mice, were lower than in mice inoculated with WRSOD. A recent study indicates that CpG treatment alone was able to induce protection against vaccinia virus infection and this response was not antigen specific or mediated by B cells (197). This could very likely be the possible explanation

for the detection of lower antibody titers and cellular immune responses induced with CpG treatment. Vaccinia virus, when it is simultaneously inoculated with CpG, may be cleared from the mice faster than the WRSOD treatment alone. Rapid clearance of WRSOD from these mice, due to CpG treatment, might have not allowed sufficient SOD expression to induce SOD specific antibody and cellular immune responses.

Although CpG adjuvant was not able to induce higher IgG2a titers or lymphocyte proliferation than the treatment without the CpG, WRSODCpG inoculated mice had significantly lower CFU/spleens compared to WRSOD inoculated mice (student T-test, *P* value: 0.023). However, the difference for the CFUs/spleen of WRSODCpG-inoculated treated mice was not significantly lower than CFUs/spleen of saline treated mice (student T-test, *P* value: >0.05). Neither inoculation with WRSOD alone or with simultaneous treatment with CpG protected mice against *Brucella* challenge. However those that have been simultaneously treated with CpG did better. What could be the explanation?

Although, vaccinia virus is an efficient cloning, expression and immunizing vector for numerous antigens, use of recombinants involve certain risks. We showed previously (chapter 3, (17)), that mice inoculated with vaccinia virus controls that were not carrying any *Brucella* genes, had significantly higher CFU/spleens following challenge with *Brucella*. This suggests that replication efficient vaccinia virus interferes with the immune responses needed against *Brucella* and we speculated this phenomenon could be due to the soluble IFN- and TNF- binding proteins secreted by vaccinia virus-infected cells interfering with cytokine production. The assumption was supported by the observation that, in spite of the positive lymphocyte proliferation upon stimulation with SOD, INF- was not released. It is suspected that the lower CFU/spleen induced by WRSODCpG inoculation was not because this treatment enhanced the production of cytokines associated with Th1 type or skewed the induced immune response to Th1. Rather it is believed that the simultaneous treatment with CpG diminished the undesirable characteristics of vaccinia virus interfering with induction of protective *Brucella* immunity.

In conclusion all the attempts to improve the protective efficacy of WRSOD recombinant these included; a) increasing the protein expression by using a shuttle vector (pMCO2) with synthetic vaccinia virus promoter, b) boosting with a naked SOD DNA construct and c) using an oligomer CpG adjuvant, failed. Only strain RB51SOD immunization (positive control group) induced a protective immune response against *Brucella* challenge. This protection was correlated with high IFN- production. However, stimulation of splenocytes from WRSOD- and WRSODCpG-inoculated mice by purified SOD did not induce any INF- release *in vitro,* providing a partial explanation why protection against challenge was not achieved.

5. THE EFFECTS OF IMMUNOMODULATORS IL-12, B7-1 AND CPG ON THE EFFICACY OF VACCINIA VIRUS RECOMBINANTS EXPRESSING PROTEINS FROM TWO INTRACELLULAR PATHOGENS: *BRUCELLA ABORTUS* AND *LISTERIA MONOCYTOGENES*

5.1 ABSTRACT

A number of cytokines and costimulatory molecules such as cytokine IL-12 and B7-1 a costimulatory molecule are involved in the activation of T lymphocytes. The biologic activities of IL-12 include stimulation of NK and cytotoxic T cells, induction of CD4+ T cells *in vivo* and *in vitro*. Several studies indicate that endogenous IL-12 produced during infection with *B. abortus* promotes the production of INF- and the clearance of bacteria *in vivo.* Also, depletion of endogenous IL-12 in mice significantly exacerbated *Brucella* and *Listeria* infection. Two recombinant vaccinia viruses, co-expressing IL-12 and B7-1, were explored as expression vectors. One expressed the *Brucella* L7/L12 (WRIL2L7/12) and *L. monocytogenes* partial listeriolysin proteins (pLLO) (WRIL12pLLO). Immune responses of BALB/c mice to these vaccinia recombinants were analyzed. In order to further drive the immune responses induced towards Th1 type response, a strong immunomodulator CpG was also used. The effects of IL-12 and B7-1 in vaccinia virus recombinants were compared with the recombinants generated earlier with WR strain alone. The recombinants WRIL12L7L12 and WRIL12pLLO induced weaker immune responses in BALB/c mice than the WRL7/L12 and WRpLLO. The IL-12 and B7-1 coexpression in vaccinia virus diminished the protective ability of WRpLLO recombinant against a *Listeria* challenge but simultaneous CpG treatment restored this ability. IL-12 and B7-1 coexpression did not help to induce protective immune response against a *Brucella* challenge. WRIL12L7/L12-inoculated mice had significantly higher *Brucella* counts in their spleens than WRL7/L12-inoculated mice. Although CpG treatment seemed to help to clear the bacteria from the spleens of mice, none of the treatments WRIL12L7/L12, WRIL12L7/L12CpG, WRL7/L12 or

WRL7/L12CpG induced protective immune responses against *Brucella* challenge. The weaker immune responses induced by WRIL12 recombinants were confirmed by western blotting, immunoglobulin serotype- specific and cytokine ELISAs. In conclusion, instead of improving the protective efficacy of vaccinia virus recombinants, co-expression of IL12 of B7-1decreased the antigen specific immune responses in BALB/c mice.

5.2 INTRODUCTION

Cytokines are signaling molecules involved in communication between cells, mainly those of the immune system. The cytokine Il-12 is a heterodimer composed of two glycoproteins, p40 and p35, and is expressed primarily by activated B cells, monocytes and macrophages. This immunostimulatory cytokine has a variety functions, including the induction of non-specific natural killer cells and the maturation of CD8+ T cells into antigen-specific cytotoxic T-lymphocytes (268). In addition, IL-12 can stimulate Type 1 CD4+ helper T cells that can lead to the production of interferon gamma (INF-) and the induction of a cell-mediated response (48). IL-12 was used as a vaccine adjuvant and contributed to the clearance of a *Leishmania major* infection from BALB/c mice, which was otherwise lethal (258). Several studies indicate that endogenous IL-12 secreted during infection with *B. abortus* promotes the production of INF- and the clearance of bacteria in *vivo* (290, 293, 295). Depletion of endogenous IL-12 before infection of mice significantly exacerbated *Brucella* infection. IL-12 depleted mice also had reduced splenomegaly resulting from infection and showed a decrease in percentage and absolute number of macrophages compared with those in non IL-12 depleted control mice. Furthermore, spleen cells from IL-12 depleted mouse had reduced ability to produce nitrate, a product of activated macrophages (293). Similar effects of IL-12 were also observed for *Listeria monocytogenes* infections. The neutralization of IL-12 was found to exacerbate *L monocytogenes* infection (269).

The costimulatory molecule B7-1 is found on the surface of professional antigen presenting cells, such as macrophages and dendritic cells and interacts with its ligand CD28, expressed on most T-cells. Both the engagement of the T cell receptor (TCR) with major histocompatibility complex/antigen (MHC/Ag) and a second signal, are needed for the complete activation of the T cell. The CD28/ B7 receptor ligand system is one of the dominant costimulatory pathways (150). Interaction of B7-1 with CD28 is essential for the effective stimulation of antigen-specific cytotoxic T-lymphocytes, mediated in part by the up-regulation and stabilization of IL-2 messenger RNA. Stimulation via T-cell receptor without co-stimulation can result in T-cell anergy or

apoptosis. Several reports indicate that the effects of IL-12 are greatly enhanced when B7-1 and IL-12 are added together (142), (182), (213). Murphy et al. (1994) showed that B7 and IL-12 cooperate for proliferation and IFN- production by mouse T helper clones that are unresponsive to B7-1 co-stimulation.

Previous work demonstrated that vaccinia virus recombinants expressing *B. abortus* antigens failed to induce resistance against a virulent *B. abortus* challenge (16), (17), (266), (13), (266) (272). The inability to induce protective immune response by these recombinants was correlated with low or lack of INF- production of inoculated mice (chapter 3, chapter 4, (272)). It is very clear from the literature that vaccinia viruses can induce infected cells to secrete soluble INF- and TNF- binding proteins (241). It was reasoned that these host immune system inhibiting properties of the vaccinia virus were interfering with the immune responses needed to clear a *Brucella* infection. Therefore, in order to overcome cytokine inhibiting properties, a vaccinia virus recombinant expressing IL-12 and B7-1 costimulatory molecules was used to generate a vaccinia/ *B. abortus* L7/L12 recombinant. It was hypothesized that by using this strain of vaccinia virus, that a cytokine microenvironment favorable to the antigen specific activation of cell mediated immune response could be induced. In this work the generation of two vaccinia virus recombinants using the WRIL12 strain is described. These are: WRIL12L7/L12 expressing T-cell reactive *B. abortus* L7/L12 protein and WRIL12pLLO expressing partial listeriolysin (pLLO) of *L monocytogenes* is described and corresponding immune responses. These recombinants were used to inoculate BALB/c mice with and without a strong immuno-modulator CpG and their mice immune responses to these treatments were analyzed. The recombinants generated earlier in another study (chapter 3) along with wild type strain WR, WRL7/L12 and WRpLLO, were also used to compare the mice immune responses induced as a function of IL-12 and costimulatory molecule B7-1. The immune responses induced by these 4 recombinants (WRL7/L12, WRIL12L7/L12, WRpLLO, WRIL12pLLO), simultaneously administered with and without CpG, were compared to immune responses induced to saline-injection (negative control) and *B. abortus* strain RB51 and *L monocytogenes* injection (positive control groups). These analysis included protection studies,

immunoglobulin serotype-specific ELISAs, lymphocyte proliferation assays and cytokine ELISAs.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial and Viral Strains and Cell lines:

E. coli DH5 cells used for construction of recombinant shuttle vectors were purchased from Invitrogen (Invitrogen, Carlsbad, CA). *B. abortus* strains RB51 and 2308 were from Dr. Gerhardt G. (Virginia Polytechnic Institute and State University, Blacksburg, VA). *Listeria monocytogenes* strain was obtained from Dr. P Elzer (Louisiana State University, Baton Rouge, LA). *Brucella* and *E. coli* cultures were grown in tryptic–soy broth (TSB) or on tryptic soy agar (TSA) plates. All *Brucella* strains were manipulated under Biosafety Level 3 (BL-3) conditions. *L. monocytogenes* strain was grown on blood agar plates and in brain heart infusion broth (BHI).

Vaccinia virus strain co-expressing murine IL-12 and B7-1 (WRIL12) was obtained from Dr. Bernard Moss (National Institutes of Health, Bethesda, MA). Human thymidine kinase deficient 143B cells (HuTK$^-$ cells) were purchased from the American Type Culture Collection, Rockville, MD.

5.3.2 Generation of Vaccinia Virus Recombinants

Recombinant shuttle vectors pSC11L7/L12 and pSC11pLLO, which were previously described in section 3.3.2, were used to generate vaccinia virus recombinants, WRIL12L7/L12 and WRIL12pLLO respectively. Briefly, human thymidine kinase deficient 143B cells (HuTK$^-$ cells) (were grown to 80% confluency in Eagle's Minimum Essential Medium (EMEM) (ICN, Costa Mesa, CA) containing 5% fetal bovine serum (FBS) (Intergen, Purchase, NY) in 25 cm^2 flasks and infected with vaccinia virus strain WRIL12 at a multiplicity of infection (MOI) of 0.05 and incubated for 2 hours at 37^{0}C, in a 5% CO_2 incubator. One microgram (μg) of recombinant shuttle vectors (pSC11L7/L12, pSC11pLLO) was dissolved in 50 microliter (μl) of sterile distilled water and mixed with 50 μl of undiluted lipofectin reagent (Gibco-BRL, Grand Island, NY) and

incubated for 25 minutes at room temperature. The DNA/lipofectin mixture (100µl) was mixed with 1 milliliter (ml) of EMEM and added to WRIL12-infected HuTK$^-$ cells at 80% confluency. The infected cells were supplemented with 3.0 ml of EMEM. After a 4+ cytopathic effect (CPE) had developed (usually in 48-72 hours), the cells were ruptured by 3 cycles of freezing in liquid nitrogen and thawing at 42^0 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 plates (about 9.6 centimeter square [cm^2] surface area) (Falcon™, Franklin Lakes, NJ) with EMEM containing 25 µg of bromodeoxyuridine (BdUR)/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 50 µg BdUR) containing 0.6 mg/ml of Bluo-gal (Gibco-BRL, Grand Island, NY). Blue plaques, produced by replicating recombinant virions expressing the *lac*Z gene, were collected and used to enhance the virus content of the plaques by inoculating a confluent layer of HuTK$^-$ cells in either 25 cm^2 flasks or six well tissue culture plates. Replication of the recombinant virus was assessed by CPE and the presence of blue plaques in the cell monolayer. Recombinant viruses were harvested, plaque purified and enhanced by infecting larger volumes of cell monolayers (in a 25 cm^2 flask) two more times to develop the recombinant virus. For viral stock preparations 150 cm^2 flasks were inoculated with recombinant viruses at MOI of 0.1 and after a 4+ CPE was observed, the content of the flask was aspirated and centrifuged at 1000*g* for 5 min. The pellet was saved and resuspended in 1ml of minimal cell culture medium Optimem (Gibco-BRL, Grand Island, NY). The virus was released from the cells by three consecutive freeze- thaw cycles.

5.3.3 Analysis of Vaccinia Recombinants

Recombinants, WRIL12L7/L12 and WRIL12pLLO were used to infect a 80% confluent HuTK$^-$ cells at MOI 1. After a 4+ CPE has developed DNA was extracted from vaccinia infected HuTK$^-$ cells using QIAamp DNA extraction kit (Qiagen,.Valencia, CA). Uninfected HuTK$^-$ cell DNA was also extracted to serve as a

control. DNA samples were used in PCR to amplify the gene of the interest using the same primers described previously in section 3.3.1.

5.3.4 SDS-Page and Western Blotting

SDS-PAGE gel and Western blotting was used to determine foreign gene expression in vaccinia virus recombinants as previously described in section 3.3.5.

5.3.5 Mice Experiments

5.3.5.1 Protection Studies

Twelve groups, each consisting of 8 BALB/c mice were inoculated at week 0 as summarized in Table 5.1. Groups 1-6 were designated as *Listeria* study groups and groups 7-12 designated as *Brucella* study groups.

Table 5.1 Group names of BALB/c mice and the treatments

	TREATMENT	
Group Names	0 hour	4 hour
1. WRpLLO	-	WRpLLO
2. WRpLLOCpG	5 nM CpG	5nMCpG +WRpLLO
3. WRIL12pLLO	-	WRIL12pLLO
4. WRIL12pLLOCpG	5 nM CpG	5nMCpG+ WRIL12pLLO
5. *L monocytogenes*	-	*L. monocytogenes*
6. Saline	-	PBS
7. WRL7/L12	-	WRL7/L12
8. WRL7/L12CpG	5 nM CpG	5nM CpG+WRL7/L12
9. WRIL12L7/L12	-	WRIL12L7/L12
10. WRIL12L7/L12CpG	5 nM CpG	5nM CpG + WRIL12L7/L12
11 *B. abortus* RB51	-	*B. abortus*
12. Saline	-	PBS

All injections were given ip with the following doses: vaccinia virus recombinants $1x10^{6.5}$ $TCID_{50}$/mouse; *L monocytogenes* $2.5x10^{4}$ CFU/mouse; *B. abortus* strain RB51 $5x10^{8}$; saline 300µl.

5.3.5.1.1 Listeria Study

Listeria study groups were inoculated as summarized in Table 5.1. Five out of eight mice were challenged ip at week 8 with 2.5×10^4 *L. monocytogenes*. Mice were sacrificed 60h later and cfu/spleen for each group was determined as previously described in section 3.3.6.1.4. The remaining three mice were sacrificed at week 10 for cell mediated immune response analysis.

5.3.5.1.2 Brucella study

Brucella study groups were inoculated as summarized in Table 5.1. Five out of eight mice were challenged ip at week 8 with 2.5×10^4 *B. abortus* 2308. Mice were sacrificed at week 10 and cfu/spleen for each group was determined as previously described in section 3.3.6.1.4. The remaining three mice were also sacrificed at week 10 for cell mediated immune response analysis.

5.3.5.2 Serological analysis

Three mice per group were bled by puncturing the retroorbital plexus at the third, fifth, seventh, ninth and tenth weeks post immunization. The serum was diluted 1:50 and analyzed for antibodies by Western Blot analysis.

5.3.5.2.1 Indirect ELISA

The presence of antigen specific serum IgG, IgG1 and IgG2a isotypes were determined by indirect ELISAs with sera collected at 7 week pi, as described previously in section 3.3.6.2.1.

5.3.5.3 Cell-Mediated Immune Response Analysis

Cell mediated immune responses induced were analyzed by lymphocyte proliferation assays and by determination of levels of cytokines secreted by mice splenocytes upon stimulation with antigens *in vitro* (cytokine ELISAs) as described in section 3.3.6.3. The INF- secretion data was subjected to the analysis of variance and

the group means for each antigen stimulation was compared by using Tukey's honest significant difference procedure.

5.3.6 Stimulant Antigen Preparation

Antigens wLLO and L7/L12 used immune response analysis were prepared using HiTrap Q anion-exchange column chromatography as described in section 3.3.7 in detail.

5.4 RESULTS

5.4.1 PCR analysis of WRIL12L7/L12, WRIL12pLLO recombinants

Using primers specific for pSC11 shuttle vector DNA from WRIL12pLLO or WRIL12L7/L12 infected HuTK$^-$ cells were used as templates in a PCR to amplify the *hly* and *l7/l12* genes. Shuttle vectors used to generate recombinants, pSC11pLLO and pSC11L7/L12 and DNA of previously generated vaccinia virus recombinants WRpLLO and WRL7/L12 served as positive controls in this reaction. DNA from HuTK$^-$ cells, WRpSC11-infected HuTK$^-$ cells (vaccinia/pSC11 control recombinant) and shuttle plasmid pSC11, were used as the negative controls. Figure 5.1 shows amplification of partial *hly* and *l7/l12* genes at positions approximately 1200 and 600 bps, respectively. Primers used were designed to be complementary to sequences upstream and downstream of *Sma* I insertion site of the pSC11 vector, therefore, along with the genes of interest a small amount of pSC11 (about 200 base pairs) was also amplified.

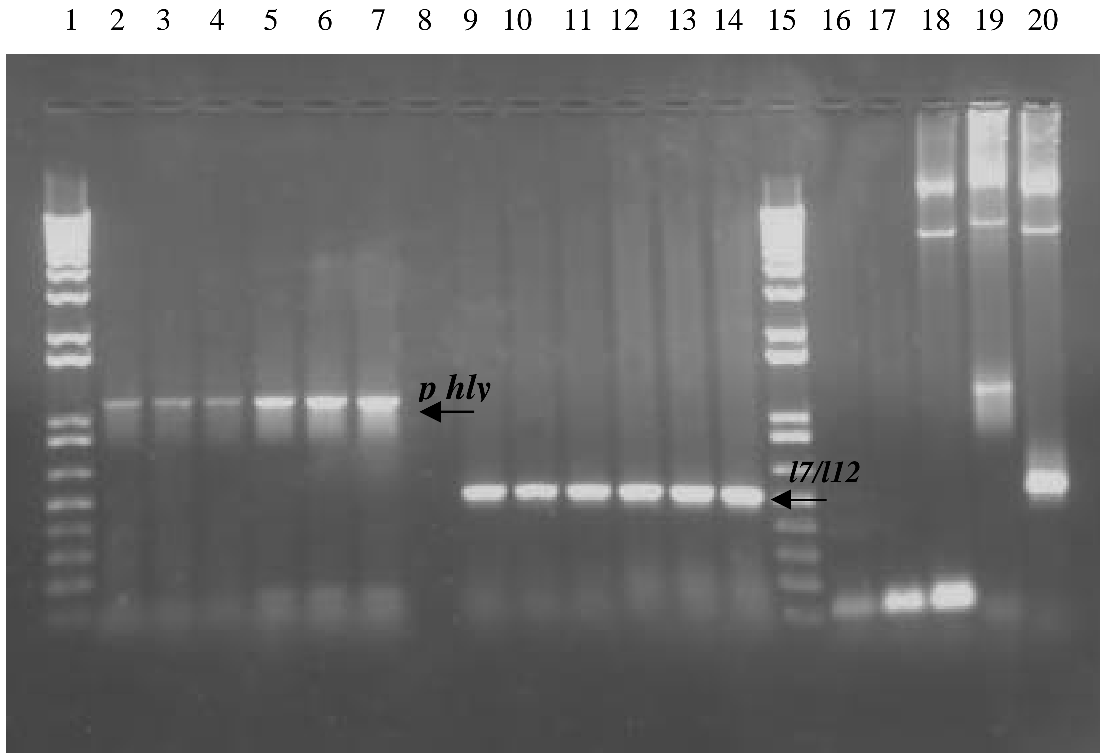


Figure 5.1 PCR amplification of partial *hly* and *l7/l12* genes in WRIL12pLLO, WRpLLO, WRIL12L7/L12, WRL7/L12 recombinants.

The fragments amplified from recombinant virus DNA were loaded in triplicate lanes. Lanes: 1) 1kb ladder; 2-4) WRIL12pLLO; 5-7) WRpLLO; 9-11) WRIL12L7/L12; 12-14) WRL7/L12; 15) 1kb ladder; 16) HuTK$^-$ cells, 17) WRpSC11; 18) pSC11 shuttle vector; 19) recombinant shuttle vector, pSC11pLLO; 20) recombinant shuttle vector pSC11L7/L12.

5.4.1.1 Expression of L7/L12 by WRIL12L7/L12

A polyclonal anti-L7/L12 mouse serum obtained from Dr. Y. Wu (Virginia Tech, Blacksburg, VA) was able to recognize the L7/L12 antigen in WRIL12L7/L12 recombinant at 12 kDa in Figure 5.2. L7/L12 protein was recognized at the same location in RB51 antigen.

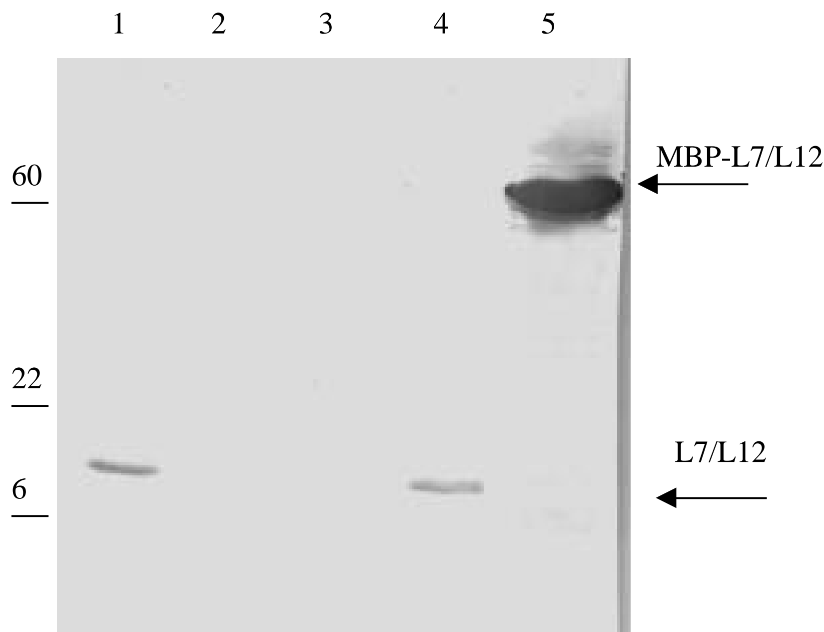


Figure 5.2 Western Blot analysis of recombinant WRIL12L7/L12.

Antigens: 1) RB51; 2) WRpSC11, 3) $HuTK^{-}$ cells, 4) WRIL12L7/L12; 5) MBP-L7/L12 fusion protein. Western Blot was probed with polyclonal anti-L7/L12 mouse sera.

5.4.1.2 Expression of pLLO by WRIL12pLLO Recombinant

A monoclonal antibody to whole LLO antigen (SE1 monoclonals, Dr. J.A Ainsworth, College of Veterinary Sciences, Mississippi State University) weakly recognized the partial listeriolysin approximately at 35kDa Figure 5.3.

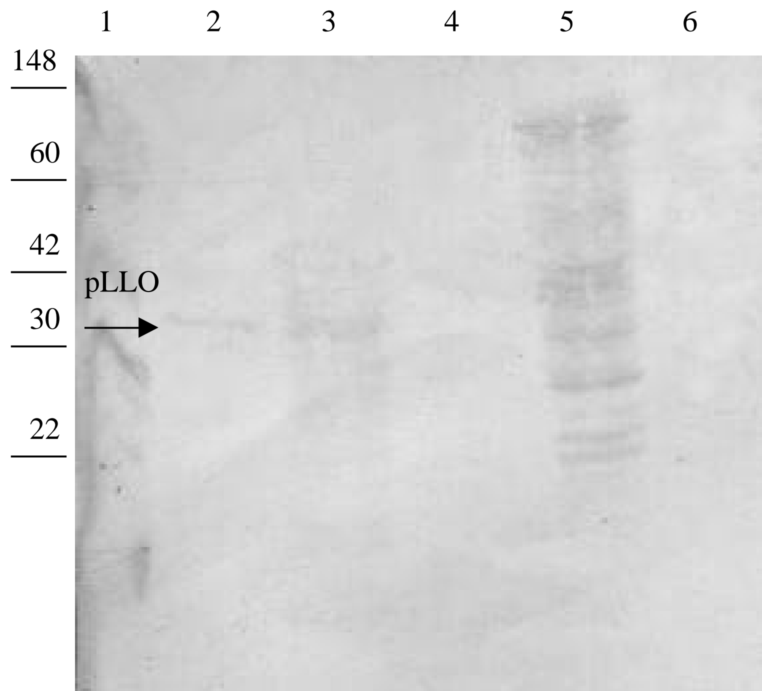


Figure 5.3 Western Blot analysis of recombinant WRIL12pLLO.

Antigens: 1)MW markers; 2)WRIL12pLLO; 3) WRpLLO; 4) WRpSC11; 5) crude *E. coli* expressing MBP-wLLO fusion protein; 6) HuTK$^-$ cells

5.4.2 Mice Immune Responses

5.4.2.1 Listeria study

5.4.2.1.1 Protective Immune Response

The clearance of *L. monocytogenes* from mice was determined by assessing viable *Listeria* recovered from spleens following *Listeria* challenge. The cfus of each dilution for each mouse spleen was determined and averaged. These values were graphed to compare the efficacy of the vaccinia virus recombinants as a vaccine candidates relative to a *Listeria* immunization and the negative control saline group. Figure 5.4 shows the average CFUs/spleen from each group of mice as analyzed by a student t-test (Sigma Plot™). The *Listeria* immunized mice group (Group 5) had no bacterial count indicating sterile immunity. All the groups that had CFUs/spleens were compared to the negative control group. Both of the WRpLLO-inoculated groups (Group1 and 2) with or without CpG treatment had significantly lower bacterial counts in theirs spleens than saline inoculated mice (Group 6), indicating protection against *Listeria* challenge. The protective effect was enhanced by simultaneous treatment with CpG as reflected in the significantly lower CFUs in the WRpLLOCpG treated group than in mice inoculated solely with WRpLLO (Group 1). In contrast to these results, WRIL12pLLO- inoculated mice (Group 3) had the highest CFU/spleen, even higher than the CFUs of mice inoculated with saline suggesting not only lack of protection but a suppression of the immune response by mice to this inoculation. When CpG was given simultaneously with WRIL12pLLO, the negative effect was reversed, the high CFU/spleen in Group 3 was lowered and mice in Group 4 had significantly lower CFU/spleen values than in saline inoculated mice (Group 6).

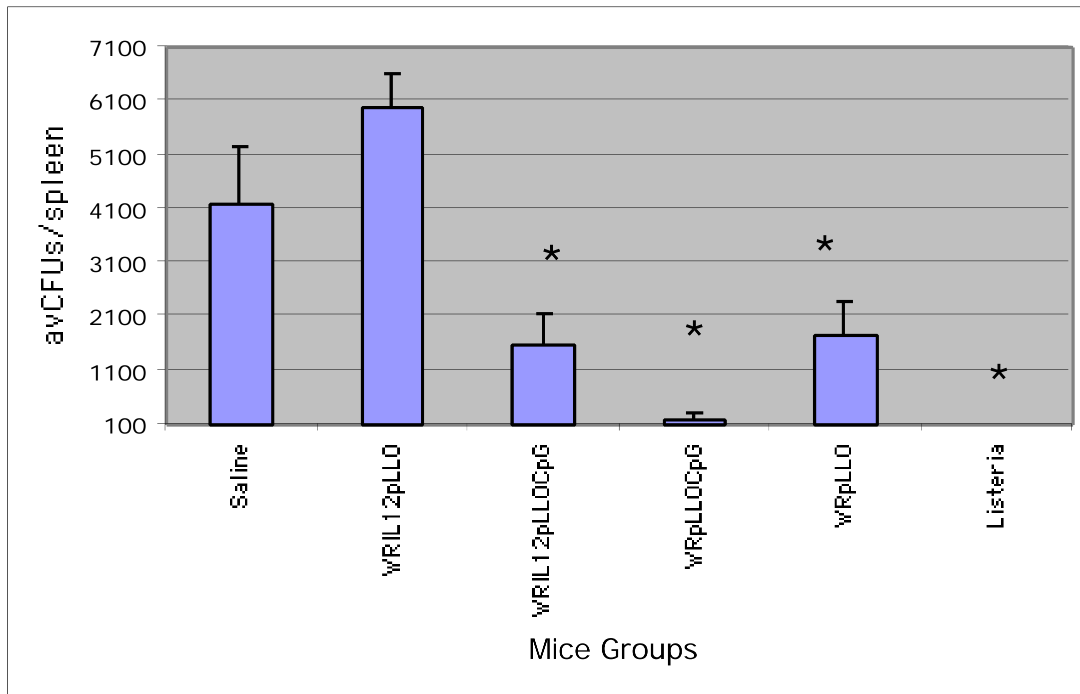


Figure 5.4 Protective immune response by WRpLLO and WRIL12pLLO-inoculated mice.

Bar graph of mean *L. monocytogenes* cfus in mice spleens. Mice groups 1)Saline; 2)WRIL12pLLO; 3)WRIL12pLLOCpG; 4)WRpLLOCpG; 5) WRpLLO; 6)*L monocytogenes*. The bar on each represents the standard error. * Indicates groups with CFUs/spleen significantly lower than saline inoculated groups. (*P* value:<0.05)

5.4.2.1.2 Humoral Immune Response

Three mice per group were bled, by puncturing the retroorbital plexus. Sera collected at the third week pi were analyzed for antibodies by Western blots and sera collected at seventh week pi were used to determine the presence of antigen specific serum IgG, IgG1 and IgG2a isotypes by indirect ELISAs.

5.4.2.1.2.1 Indirect ELISA

The presence of wLLO-specific IgG, IgG1 and IgG2a isotypes in sera of mice inoculated with WRpLLO, WRpLLOCpG, WRIL12pLLO, WRIL12pLLOCpG, *Listeria* and saline were determined by indirect ELISA. In all groups IgG2a levels were higher than IgG1 levels. WRpLLO-inoculated mice with or without CpG had higher antibody titers than WRI12pLLO-inoculated mice (Figure 5.5).

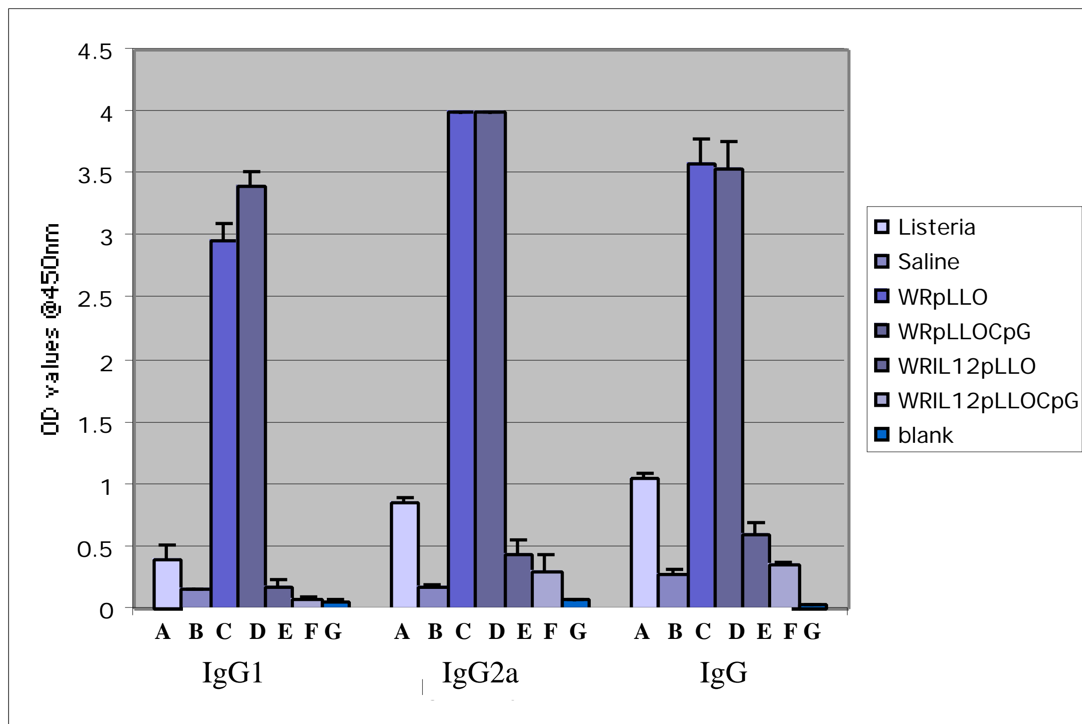


Figure 5.5 Antibody isotype responses of mice in the *Listeria* study.

Antibody isotype responses of: A) *Listeria*-, B) saline-, C)WRpLLO-, D)WRpLLOCpG-, E)WRIL12pLLO-, F)WRIL12pLLOCpG- inoculated mice at 7th week pi G is media control levels. Standard errors of the mean OD values of three mice for each group are indicated on the bar graphs.

5.4.2.1.2.2 Western Blotting

Indirect ELISA results showed antibody responses of WRpLLO- inoculated mice that were highly elevated compared to WRIL12pLLO-inoculated mice. In order to confirm these results, western blotting was performed with sera acquired at the third week pi from WRpLLO- and WRIL12pLLO-inoculated mice. Figure 5.6 contains two strips of western blots. The difference in the recognition level correlates well with the indirect ELISA results. Blot A was probed with sera of WRpLLO-inoculated mice and Blot B was probed with sera of WRIL12pLLO-inoculated mice. Western blotting was performed using exactly the same conditions (antigen and antibody dose, incubation periods etc) for both of the blots. The sera of WRpLLO inoculated mice recognizes MBP-wLLO and WRIL12pLLO antigens stronger than sera of WRIL12pLLO.

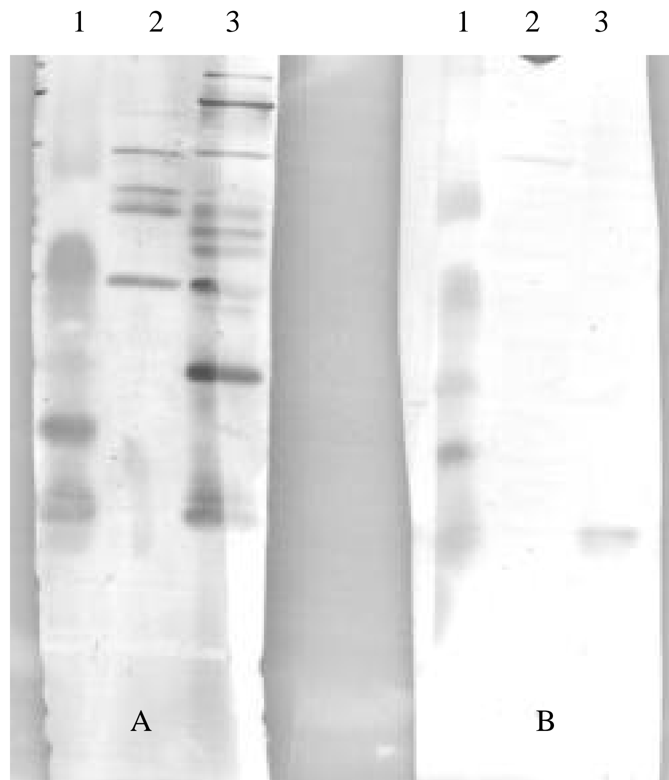


Figure 5.6 Comparative Western Blotting with sera of WRpLLO- and WRIL12pLLO-inoculated mice.

Lanes: 1) MW markers; 2)MBP-wLLO fusion protein; 3)WRIL12pLLO antigen. Blot A was developed with pooled sera of WRpLLO-inoculated mice and Blot B developed with pooled sera of WRIL12pLLO- inoculated mice.

5.4.2.1.3 Cell Mediated Immune Response

Cell mediated immune responses in mice induced by WRpLLO-, WRpLLOCpG-, WRIL12pLLO-, WRIL12pLLOCpG-, and *Listeria*- inoculations respectively were analyzed by lymphocyte proliferation assays. Table 5.2 shows the SI values. Lymphocytes of WRpLLOCpG- and *Listeria*- inoculated mice proliferated upon stimulation with MBP-wLLO fusion. Heat killed *Listeria* antigen stimulated the lymphocytes of *Listeria* -inoculated mice but not the lymphocytes of mice inoculated with vaccinia virus recombinants.

Table 5.2 Stimulation Indices in the *Listeria* study

	MICE TREATMENTS					
	Saline	*Listeria*	WRpLLO	WRpLLOCpG	WRIL12pLLO	WRIL12pLLOCpG
ANTIGENS						
Media	1	1	1	1	1	1
LM (H)	1.7	31.3	**<1**	3.3	1.3	2.9
LM (L)	<1	12.7	3.0	1.7	<1	1.3
wLLO (2)	9.0	17.0	4.7	20.7	7.9	8.1
wLLO (1)	9.0	17.1	5.6	21.5	10.5	9.5
wLLO (0.5)	5.6	11.9	3.1	12.9	8.1	6.7
MBP	1.1	<1	2.9	1	<1	<1

5.4.2.2 Brucella Study

5.4.2.2.1 Protective Immune Response

The clearance of *B. abortus* from mice was determined by assessing viable *Brucella* recovered from spleens. The cfus of each dilution for each mouse spleen was determined and averaged. These values were graphed to compare the efficacy of the vaccinia virus recombinants as vaccine candidates relative to *B. abortus* strain RB51 immunization and the negative control saline group. Figure 5.7 shows the average cfus values of each group of mice spleens. These values were analyzed in student t-test (Sigma Plot™).

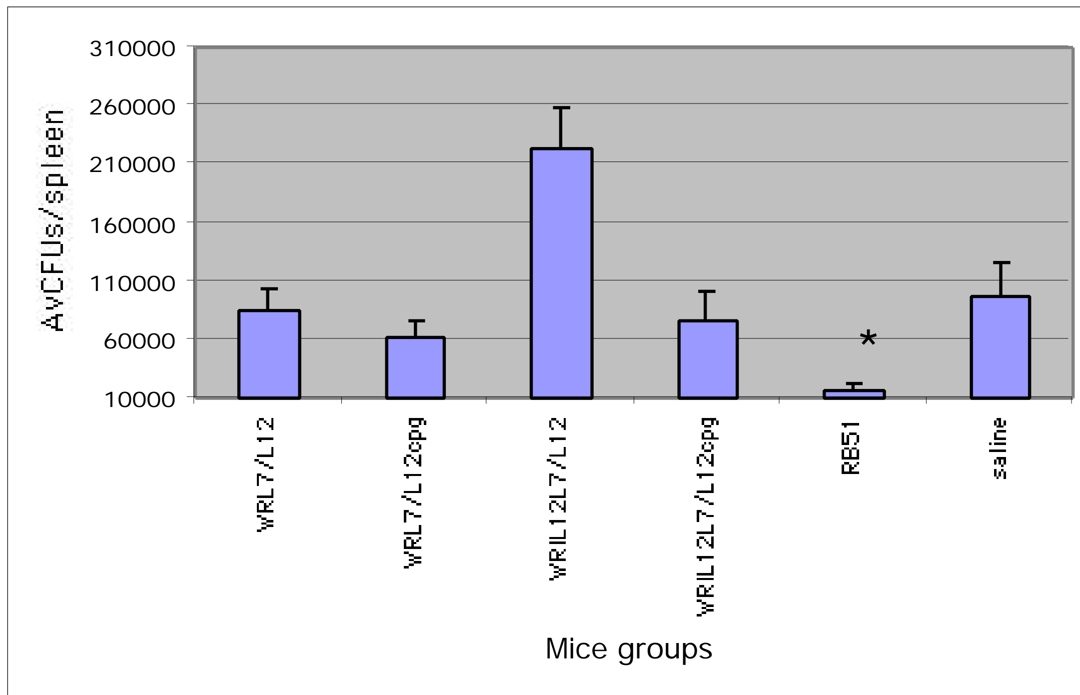


Figure 5.7 Protective immune response by WRL7/L12 and WRIL12L7/L12 recombinants.

Bar graph of mean *B. abortus* cfus in mice spleens. Mice groups: 7)WRL7/L12; 8)WRL7/L12CpG; 9)WRIL12L7/L12; 10)WRIL12L7/L12CpG; 11)RB51, 12) Saline. The bar on each represents the standard error. * Indicates groups with CFUs/spleen significantly lower than saline inoculated groups. (*P* value:<0.05)

5.4.2.2.2 Humoral Immune Response

The presence of L7/L12 specific IgG, IgG1 and IgG2a isotypes in sera of mice inoculated with WRL7/L12, WRL7/L12CpG, WRIL12L7/L12, WRIL12L7/L12CpG, RB51 and saline were determined by indirect ELISA (Figure 5.8). In all groups IgG2a levels were higher than IgG1 levels except in the WRIL12L7/L12CpG- inoculated mice group. IgG1, IgG2a and IgG levels in this serum were similar. WRL7/L12CpG treatment induced highest the IgG2a serotype titers.

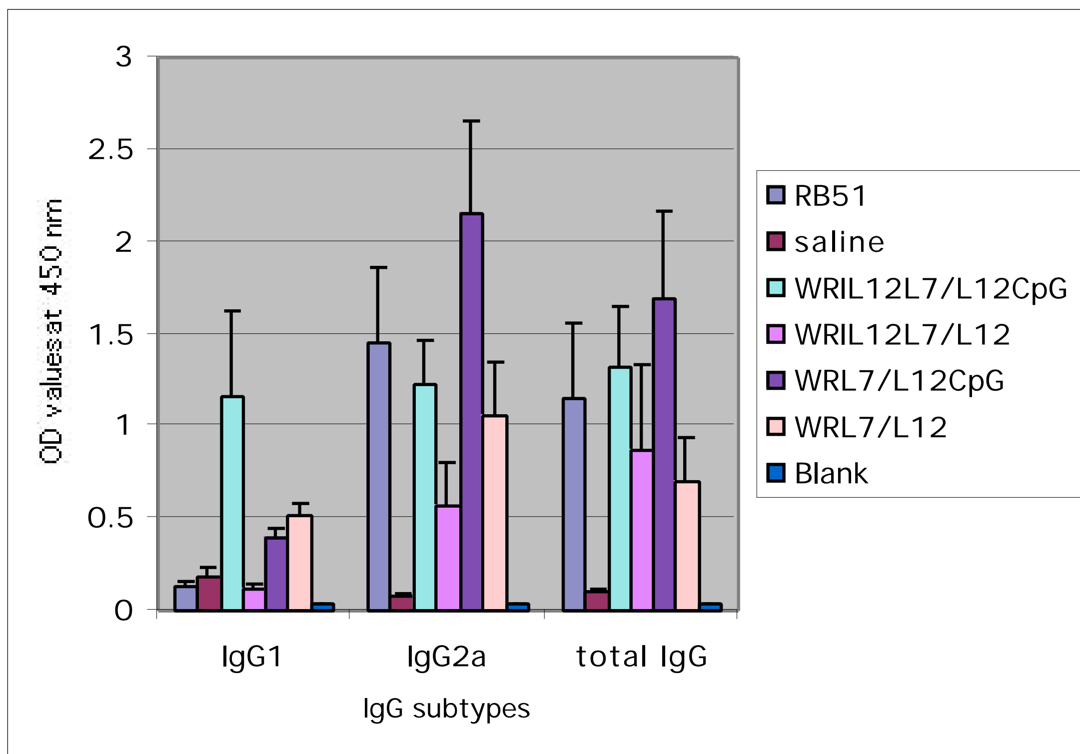


Figure 5.7 Antibody isotype responses of mice of the *Brucella* study.

Antibody isotype responses of WRL7/L12-, WRL7/L12CpG-, WRIL12L7/L12-, WRIL12L7/L12CpG-, *RB51*- and saline-inoculated mice 7th week pi. Standard errors of the mean OD values of three mice for each group are indicated on the bar graphs.

5.4.2.2.3 Cell Mediated Immune Response

Cell mediated immune responses in mice induced by WRL7/L12-, WRL7/L12CpG-, WRIL12L7/L12, WRIL12L7/L12CpG, and RB51 inoculations were analyzed by lymphocyte proliferation assays and cytokine ELISAs. Table 5.3 shows the SI values. Splenocytes of WRL7/L12CpG and RB51- inoculated mice proliferated upon stimulation with MBP-L7/L12 fusion and heat killed RB51 antigen.

Table 5.3 Stimulation Indices in the *Brucella* study

	MICE TREATMENTS					
	Saline	WRL7/L12	WRL7/L12 CpG	WRIL12L7/L12	WRIL12L7/L12 CpG	RB51
ANTIGENS						
RB51 (H)	7.39	6.37	14.99	10.37	3.90	35.84
RB51 (L)	2	2.47	4.54	2.84	1.46	18.72
MBP-L7/L12 (2)	2.14	1.64	3.08	1.26	<1	2.47
MBP-L7/L12 (1)	1.76	1.20	2.27	1.62	<1	3.52
MBP-L7L12 (0.5)	1.31	1.3	2.51	1.34	1.23	3.87
MBP	1.26	1.0	1.16	<1	<1	<1
media	1	1	1	1	1	1

5.4.2.2.3.1 Cytokine ELISAs

Mice splenocytes were analyzed *in vitro* for INF- and IL-4 secretion upon stimulation with tested antigens: conA, RB51 ($1x10^6$[L], $1x10^7$[H]), MBP-L7/L12 (2, 1, 0.5 μg/well). Mean INF- levels secreted from saline-, RB51-, WRL7/L12-, WRL7/L12CpG-, WRIL12L7/L12- and WRIL12L7/L12CpG- inoculated mice groups were subjected to the analysis of variance and the group means for each antigen stimulation were compared by using Tukey's honest significant difference procedure. Results indicated RB51-inoculated mice upon stimulation with RB51 antigens, secreted

significantly higher INF- titers (19±0.7ng/ml, high RB51 antigen dose; 14±2.24, low RB51 antigen dose) than any other groups of mice (*P* value :< 0.023). However, the amount of INF- secreted by splenocytes of RB51-inoculated mice upon stimulation with MBP-L7/L12 were not different than secreted by splenocytes of mice receiving the other treatments. Only splenocytes of WRL7/L12CpG-inoculated mice splenocytes secreted upon stimulation with (1mg/ml) MBP-L7/L12 protein significantly higher amount of INF- , 7.67±3.4ng/ml titers than splenocytes any of the other mice groups (*P* value: 0.01).

IL-4 secretion by splenocytes of each mouse group upon stimulation with RB51 or MBP-L7/L12 antigens were below detectable levels.

5.5 DISCUSSION

Vaccinia virus encodes proteins that counteracts different branches of the host defense system and interferes with the effects of cytokines, inflammation and complement mediated killing (246). One of the most important virulence mechanisms of vaccinia virus is its ability to inhibit the effects of INF- . Vaccinia virus achieves this by producing soluble interferon- receptors that bind to secreted INF- and prevents the cytokine binding to cellular receptors (237). This binding ability was suspected to be the reason why vaccinia virus recombinants expressing *Brucella* antigens did not induce protective immune responses against *Brucella* challenge and was addressed in detail in the discussion section of Chapter 3. In this study in order to improve the immune responses induced by vaccinia virus/ *B. abortus* recombinants and to overcome INF- inhibiting properties, a vaccinia virus recombinant co-expressing murine IL-12 and costimulatory B7-1 molecule (WRIL12) was used.

The results reported here demonstrate that coexpression of IL-12 and B7-1 did not increase the efficacy of the vaccinia virus recombinants used. In contrast, co-expression of these molecules significantly reduced the protective ability of vaccinia virus recombinant expressing partial listeriolysin (WRIL12pLLO). This outcome was unexpected since the literature indicates the importance of IL-12 for the elimination of *Listeria* (102). This study (102) demonstrated that neutralization of IL-12 during the primary response resulted in increased susceptibility to *Listeria* infection and IL-12 deficient mice were killed by normally sublethal doses of *Listeria*. IL-12 has also been shown to play a central role in the innate and acquired immune responses; this includes activities such as enhancement of natural killer and cytotoxic T lymphocyte activity and promotion of CD4 Th1 T cell development. Thus IL-12 coexpression should induce a better protective immune response against *Listeria* since cell mediated immune responses required to resolve *Listeria* infection involves NK and CD8+ T cells (188).

However this current study showed that the WRpLLO recombinant induced significantly better protective immune response than a vaccinia virus recombinant,

WRIL12pLLO, co-expressing IL-12 and B7. There could be several reasons for this. The IL-12 co-expression may have enhanced the mice immune responses against vaccinia virus to a degree that replication of the virus became self-limiting or attenuated. There are several studies pointing to this possibility. Co-expression of cytokines influences vaccinia virus virulence and can attenuate or render the vaccinia virus more virulent depending on the cytokine it expresses. A report demonstrated that a vaccinia virus expressing IL-4 persisted in the mice longer and had enhanced virulence (231) compared to the unmodified virus. In contrast, when cytokines associated with Th1 type responses were expressed by vaccinia viruses, the recombinant virus became more attenuated and was cleared from the mice faster. Another study demonstrated that vaccinia virus expressing IFN- persists only for 3 weeks compared to 12 weeks for unmodified virus (137). Similar observations were reported for vaccinia virus expressing IL-2 and tumor necrosis factor. These recombinants were so highly attenuated that even nude mice, normally killed within 12 days due to a vaccinia virus infection, were readily able to control the growth of the virus. Similar reports were also available for vaccinia virus expressing IL-12 (104), (167), (213). However, vaccinia virus recombinants encoding IL-12 stimulates Type 1 CD4+ helper T cells that can lead to the production of interferon gamma (INF-) and the induction of a cell-mediated response (48). All of these studies were perfomed in immunocompromised, tumor bearing mice or immunodeficient mice (213), (168), (48). When immunocompetent mice were used, the replication of the vaccinia virus expressing IL-12 was attenuated and rapidly cleared from mice (104). This could very likely be the possible explanation for the inefficiency of WRIL12 strain as an immunizing vector. Since immunocompetent BALB/c mice were used in this current study, the WRIL12pLLO recombinant may have been cleared faster than WRpLLO recombinant. This failed to induce sufficiently high level of specific immune response against the cloned antigen. A comparative humoral immune response analysis by Western blotting and indirect ELISA results supports this interpretation. Sera of WRpLLO inoculated mice had highly elevated antibody titers and recognized both vaccinia and MBP-wLLO antigens than sera of WRIL12pLLO inoculated mice (Figure 5.5, 5.6).

Another reason for reduced protective ability of WRIL12pLLO recombinant could be that the levels of IL-12 were not optimal. There are several reports indicating that the dose and the timing of IL-12 administration should be precisely established for adjuvant effect. Gherardi et al., (103) demonstrated that enhancement of cellular immune response due to IL-12 co-expression by vaccinia virus is dependent on time and dose. When a different schedule is used, IL-12 can have immunosuppressive effects mediated by nitric oxide. Another group also demonstrated that the dose and schedule of IL-12 administration significantly affect adjuvant activity, leading to either enhancement or suppression of antigen specific responses (147). In this dissertation, IL-12 was expressed by vaccinia virus recombinant and due to limitations neither the dose nor the schedule of IL-12 administration could be explored. Thus, it is possible that the exposure or the timing of IL-12 expression was not appropriate and therefore, instead of an adjuvant effect, a suppressive effect was observed.

In the current research, it was also demonstrated that CpG treatment, along with WRL7/L12 and WRIL12L7/L12 inoculations lowered the CFU/spleen after challenge. Although even with simultaneous CpG treatment, neither WRIL12L7/L12 nor WRL7/L12 induced a protective immune response against *Brucella* challenge. The IgG2a responses induced with simultaneous CpG treatment were higher than the responses induced by vaccinia virus recombinant inoculations. In addition, mice inoculated with WRL7/L12CpG were the only group that produced significantly higher levels of IFN- levels upon MBP-L7/L12 fusion protein stimulation. It is evident that although CpG skewed the response towards type Th1 CMI, the level of response was not sufficient to protect against a *Brucella* challenge.

Simultaneous CpG treatments with WRpLLO and WRIL12pLLO also lowered the CFU/spleen *Listeria* values following challenge. Mice inoculated with WRIL12pLLOCpG were protected against a *Listeria* challenge. In contrast, mice inoculated only with WRIL12pLLO had higher splenic *Listeria* counts than saline-inoculated mice. WRpLLOCpG-treated mice were also better protected than WRpLLO inoculated mice. When cellular immune responses were analyzed, splenocytes from

WRpLLO mice, which had the lowest CFU/spleen in protection studies, proliferated upon MBP-wLLO stimulation better than any other groups. Although there was a significant difference in the level of protective immune response induced using CpG treatment, this difference was not reflected in results of indirect ELISAs. The levels of IgG2a induced were similar to the ones induced with the recombinant virus alone. Mice inoculated with vaccinia virus/pLLO recombinants exhibited predominantly IgG2a type responses and this was more evident in WRpLLO- and WRpLLOCpG- inoculated mice than any other mice groups.

In summary, these experiments demonstrate that a vaccinia strain co-expressing murine IL-12 and costimulatory factor B7-1 was not efficient in inducing protective immune response against intracellular pathogens. It is very likely that IL-12 coexpression attenuates the vaccinia virus and accelerates its clearance form the immunocompetent host. Thus rapid clearance of recombinants generated with WRIL12 might have not allowed sufficient antigen expression to induce antigen specific antibody and cellular immune responses.

The current work demonstrates the complexity of developing protective vaccinia virus recombinants expressing antigens from intracellular bacteria. It also demonstrates that various factors need to be very carefully evaluated for the specific circumstances. These include: the vaccinia virus strain, the effect of replicating vaccinia virus on aspects of the host immune response, the nature of the protein cloned, the nature of bacteria against which protective immune response is to be induced, the nature, dose scheme of administration of the various immunomodulators used to drive the immune response towards the desired type. Although literature data about these factors may help reasoning and planning experimental protocols, the expected protective outcome may be quite different from those expected.

6. CONCLUSIONS AND SUMMARY

The research described in this dissertation was designed to better understand vaccinia virus as a cloning, expression and immunizing vector for proteins of intracellular pathogens. The two intracellular pathogens chosen in this study were *Listeria monocytogenes* and *Brucella abortus*.

For over two decades now, vaccinia virus has been used as an expression and immunizing vector by numerous researchers. Vaccinia virus has shown an impressive potential as a component of recombinant vaccines expressing viral, tumor and parasite antigens. The situation is different with bacterial antigens. To date there are only 5 vaccinia virus/bacterial antigen recombinants capable of inducing protective immune response against infectious strain challenge. These include vaccinia virus expressing *Bacillus anthracis* protective antigen; *Streptococcus pyogenes* M protein; a CTL epitope of *Listeria monocytogenes*; and 2 secreted proteins of *Mycobacterium tuberculosis*. In contrast to bacterial antigens, the list of vaccinia virus recombinants expressing viral and tumor antigens with protective potential is quite impressive (for a summary, refer to Table 1.3 and Table 1.4).

Although *Brucella* antigens have been expressed by vaccinia virus, the immune responses induced by these recombinants were either not the appropriate type or not at a level sufficient to protect. In this dissertation, two putatively protective *B. abortus* proteins, Cu/Zn SOD and L7/L12 ribosomal protein, were used to generate vaccinia virus recombinants. The initial testing of WRL7/L12 and WRSOD recombinants failed to demonstrate protective immune response in mice against a *Brucella* challenge. These recombinants induced antigen specific cellular and humoral immune responses with high IgG2a titers, indicative of a Th1 type cell mediated response. However, cytokine ELISAs indicated either very low or no detectable levels of INF- production contradicting the expected cytokine profile of a Th1 type response characterized by high INF- and low IL-4 levels. The low levels of INF- provided a partial explanation why vaccinia virus/*Brucella* recombinants were not protective. Another significant observation was

that when mice were injected with vaccinia/plasmid controls, i.e. with recombinants not carrying *Brucella abortus* proteins, *Brucella*/CFU spleen after challenge were significantly higher than in those mice inoculated with saline alone. This indicated that vaccinia virus as an immunizing vector may actually interfere with the immune responses required to protect against a *Brucella* challenge.

Replicating vaccinia virus has several mechanisms that counteract or interfere with the host immune responses. Most important of these mechanisms is the ability to counteract the effects of INF- . It is well known that INF- is a key cytokine involved in the protective immune response against *Brucella*. To enhance the levels of INF- produced and to minimize the undesired characteristics of vaccinia virus, various immunomodulators were used in the current work. CpG is an oligonucleotide that favors the development of a Th1 type immune response. Co-administration of CpG with vaccinia virus recombinants significantly lowered the *Brucella* CFUs in the spleens of mice compared to mice inoculated with the recombinants alone. However, the CpG effect was not sufficient to induce significantly lower counts than mice inoculated with saline only. Thus the effect of CpG under these circumstances cannot be considered protective. When the antigen-specific humoral and cell mediated immune responses were analyzed with ELISAs and lymphocyte proliferation assays, the results indicated that there was no significant improvement attributable to CpG treatment. Data in the literature indicate that CpG treatment alone was able to induce protection against vaccinia virus infection and this response was not antigen specific or mediated by B cells (197). In the current work there was no improvement in the antigen-specific immune responses but there was improvement in protective immune responses induced by CpG co-administration. Therefore, it is assumed that the improvement was due to the faster clearance of the vaccinia virus thereby suppressing undesirable effects of the replicating vaccinia virus.

As a next step, the immunostimulatory effects of IL-12 and B7-1 costimulatory molecules were explored. A vaccinia virus strain co-expressing IL-12 and B7-1 (WRIL12) was used to generate a vaccinia virus recombinant expressing *Brucella*

abortus L7/L12 protein. Although IL-12 and B7-1 expression by vaccinia virus improved cellular immune responses and induced anti-tumor CTL responses in immunocompromised mice, this strain was rapidly cleared from immunocompetent BALB/c mice (104). When this vaccinia virus strain was used to express *Brucella* genes, mice inoculated with this recombinant (WRIL12L7/L12) did not develop strong antigen-specific immune responses. Both the cellular and humoral immune responses induced by WRIL12 recombinants were lower than the immune responses induced by recombinant generated with WR strain WRL7/L12. CpG administered with WRIL12 recombinant helped to lower *Brucella* CFU/spleens. However, as previously seen with the WR strain, CFU/spleen were not significantly lower than CFU/spleen of saline–inoculated mice and therefore not considered protective. It is possible that IL-12 co-expression enhanced the immune responses against vaccinia virus and thus caused a faster clearance of the recombinant virus. This in turn would prevent sufficient expression of the cloned protein and the induction of an antigen specific immune response.

In order to improve the efficacy of vaccinia virus/ *Brucella* recombinants, other methods such as a diversified schedule of booster immunizations, different shuttle vectors and different routes of administration were tried. All these methods failed to improve the immunizing efficacy of these recombinants.

In contrast to the lack of protection seen with *Brucella*/vaccinia virus recombinants, a vaccinia virus recombinant expressing partial listeriolysin (WRpLLO) induced protective immune response and CpG treatment improved the protective efficacy against a *Listeria* challenge. Again, coexpression of IL-12 and B7-1 significantly lowered the protective efficacy of vaccinia/pLLO recombinants. Antigen specific immune responses induced by WRIL12pLLO were significantly lower than the one induced with WRpLLO. These results confirmed those observed with the WRIL12/*Brucella* recombinant, i.e. that the vaccinia virus co-expressing IL12 is a less effective immunizing vector whether *Brucella* or *Listeria* proteins are expressed.

The findings here are valuable in analyzing vaccinia virus as an expression vector for intracellular pathogens by contrasting the success of one recombinant, WRpLLO, with failure of the other, WRL7/L12. Development of a strong cell mediated immune response is essential to prevent both diseases. Both pathogens are able to survive within the macrophages, enabling the bacteria to evade the innate defense system, such as complement mediated and phagocyte-mediated killing. It has been reported that inoculation of mice with recombinant vaccinia viruses induces elevation of natural killer (NK) cell responses (28). NK cells are capable of lysing microbe-infected cells mostly due to the production of IFN- . Early, but not late, production of IFN- by NK cells was essential for resistance to listeriosis (23). Studies with *B. abortus* using the YAC-1 cell cytotoxicity assay indicated no role of NK cells in the early control of *B. abortus* 2308 infections. Depletion of NK cells from BALB/c mice had no effect on cytotoxic T cell responses (59). Moreover, unlike *B. abortus, L monocytogenes* can cause acute infections, thus early IFN- production by NK cells and NK cell elevation maybe a determining factor in protective immune response induced by WRpLLO recombinant. However, since *B. abortus* causes only chronic infection and IFN- produced by NK cells is at a level not sufficient enough to induce resistance to *Brucella* challenge, high amounts of IFN- have to be produced by antigen-specific $CD4^+$ cells.

Vaccinia virus as an immunizing agent is particularly suitable for inducing humoral immune responses against the glycoproteins or secreted antigens as well as inducing high cytotoxic T-lymphocyte responses. However, there are numerous reports demonstrating interference by vaccinia virus with Th1 type $CD4^+$ responses. Unfortunately, there are very limited number of *Brucella* antigens that have been identified as having protective potential; such as L7/L12 ribosomal protein and Cu/Zn SOD. Both of these proteins have been shown to induce proliferation of lymphocytes of *in vivo* and L7/L12 was shown as to enhance $CD4^+$ T-cell responses. So far, no *Brucella* proteins have been identified with specific CTL epitopes. Listeriolysin, on the other hand, contains a strong CTL epitope and has been shown to induce strong protective CTL responses. It is very likely that vaccinia virus expressing partial listeriolysin induced a

strong cytotoxic T lymphocyte response providing protective immunity. However, it appears that vaccinia virus recombinants expressing $CD4^+$ T-cell reactive proteins have been inhibited by vaccinia virus virulence characteristics that interfered with the induction of Th1 type response and prevented induction of a protective immune response. These results indicate that the immunogenicity of vaccinia virus recombinants is very much dependent on the nature of the protective antigen expressed, the type of the immune responses induced and whether the type of the immune responses induced are needed for the protection against pathogenic bacteria.

Analysis of published studies obtained with other protective vaccinia-bacterial antigen recombinants reveals some interesting data. Two of these recombinants were vaccinia virus expressing *Streptococcus pyogenes* M protein (vv-M) and vaccinia virus expressing *Bacillus anthracis* PA (vv-PA) protein. Both of these pathogens are gram positive, toxin producing bacteria. Similar to these recombinants, a protective vaccinia virus recombinant generated in this study, WRpLLO, expressed a protein associated with a cytotoxin of a gram-positive pathogenic bacteria, *Listeria*. In contrast, vaccinia virus recombinants expressing proteins of gram negative bacteria seem to fail to induce protective immune responses, (13) (17), (272). This dissertation and our previous work showed that recombinants, generated with 6 different *Brucella* proteins, i.e. GroEL, GroES, HtrA, Cu/ZnSOD, 18kDa, L7/L12 failed to induce protective immune responses. Two of these proteins, L7/L12 and Cu/Zn SOD,were shown by our group and others to have protective potential (144), (196), (276). These proteins however, were unable to induce protective immune response when expressed by vaccinia virus. Vaccinia virus was used as a cloning and expression vector for many of the *Mycobacterium* proteins such as 71, 65, 38, 35, 19, 18 and 12 kDa proteins (156), (298). Only two recombinants, either expressing *M. tuberculosis* 19 kDa or a 38 kDa glyco-protein that are normally excreted, induced a protective immune response. Our results corroborated these findings and were not surprising since recombinant vaccinia virus is particularly good for inducing production antisera against the glycoproteins or secreted antigens. It is possible that recombinant vaccinia virus may be only useful as an immunizing agent when expressing antigens that are normally excreted from the bacterial pathogens.

When amino acids are encoded by more than one codon each organism carries its own bias in the usage of the 61 available amino-acid codons. Correspondingly when the mRNA of heterologous target genes is overexpressed in vaccinia virus, differences in the codon usage can impede translation which can lead to translational stalling, premature translational termination, translational frameshifting and amino acid misincorporation which causes errors in the expressed antigens (145). Examination of the preferred codon usage by vaccinia virus genes and comparing it with the preferred codon usage of *Brucella*, *Listeria*, and *Bacillus anthracis* reveals that vaccinia prefers similar codon usage to that by *Listeria* and *Bacillus* but quite different for *Brucella* (Table 6.1) (http://www.kazusa.or.jp/codon). To be exact, the preferred codon usages of *B. anthracis* and *Listeria* are 93%, 87% synonymous respectively with preferred codon usage of vaccinia virus. However, *Brucella* uses only 30% of preferred codons of vaccinia virus. Although vaccinia virus was able to express all analyzed *Brucella* antigens, that in turn, induced cellular and humoral responses in mice, the level of expression may have been suppressed by codon bias of vaccinia virus, or more importantly, *Brucella* antigens may have been expressed with errors. Such errors in the accuracy of translation or in the level of expression of *Brucella* proteins may have rendered vaccinia virus-*Brucella abortus* protein recombinants unable to induce protective immune responses. The level of expression of the *Brucella* antigens in vaccinia virus may also contribute to the lack of protection seen in this dissertation.

Table 6.1 Codon usage analysis

Aminoacids	Codons	*Listeria*	*Brucella*	Vaccinia	*Bacillus*
Ala	GCA	GCA	GCC	GCU	GCA
	GCC	GCU	GCG	GCA	GCU
	GCG				
	GCU				
Arg	AGA	CGU	CGC	AGA	AGA
	AGG	AGA	CGU	CGU	CGU
	CGA				
	CGC				
	CGG				
	CGU				
Asn	AAC	AAU	AAC	AAU	AAU
	AAU	AAC	AAU	AAC	AAC
Asp	GAC	GAU	GAC	GAU	GAU
	GAU	GAC	GAU	GAC	GAC
Cys	UGC	UGU	UGC	UGU	UGU
	UGU	UGC	UGU	UGC	UGC
Gln	CAA	CAA	CAG	CAA	CAA
	CAG	CAG	CAA	CAG	CAG
Glu	GAA	GAA	GAA	GAA	GAA
	GAG	GAG	GAG	GAG	GAG
Gly	GGA	GGU	GGC	GGA	GGA
	GGC	GGA	GGU	GGU	GGU
	GGG				
	GGU				
His	CAC	CAU	CAU	CAU	CAU
	CAU	CAC	CAC	CAC	CAC
Ile	AUA	AUU	AUC	AUU	AUU
	AUC	AUC	AUU	AUA	AUA
	AUU				
Leu	CUA	UUA	CUG	UUA	UUA
	CUC	CUU	CUU	UUG	CUU
	CUG				
	CUU				
	UUA				
	UUG				
Lys	AAA	AAA	AAG	AAA	AAA
	AAG	AAG	AAA	AAG	AAG
Met	AUG	AUG	AUG	AUG	AUG
Phe	UUC	UUU	UUC	UUU	UUU
	UUU	UUC	UUU	UUC	UUC
Pro	CCA	CCA	CCG	CCA	CCA
	CCC	CCU	CCC	CCU	CCU
	CCG				
	CCU				
Ser	AGC	AGU	UCG	UCU	UCU
	AGU	UCU	UCC	UCA	AGU
	UCA				
	UCC				
	UCG				
	UCU				
Ter (stop)	UAA	UAA	UGA	UAA	UAA
	UGA	UGA	UAA	UGA	UAG
	UAG				
Thr	ACA	ACU	ACC	ACU	ACA
	ACC	ACG	ACG	ACA	ACU
	ACG				
	ACU				
Trp	UGG	UGG	UGG	UGG	UGG
Try	UAC	UAU	UAC	UAU	UAU
	UAU	UAC	UAU	UAC	UAC
Val	GUA	GUU	GUC	GUA	GUA
	GUC	GUA	GUG	GUU	GUU
	GUG				
	GUU				

In summary, the results of this work do not contradict the large body of data in the literature demonstrating that vaccinia virus in general is a good cloning, expression and immunizing vector. Although the literature may guide the overall reasoning of a project and the detailed planing of the experimental protocols, the outcome may be quite different from those expected.

Whether vaccinia virus, as an expression vector, is suitable for generation of recombinants with bacterial proteins that are capable of inducing protective immune response, is a complex issue. This study demonstrates that various factors can influence the outcome: the nature of the strain, the type of shuttle vector used, the effect of replicating virus on various aspects of the host immune response; the nature and the integrity of protein cloned; the nature of the bacteria against which protective immune response is to be induced; the nature, dose and the scheme of administration of the various immuno-modulators used to drive the immune responses towards the desired type. The appropriate synergism among all of these various factors is very important. If even one factor is out of synchrony the entire mechanism may unravel, resulting in the problems with quality and quantity of the expressed antigen and in the quality, type and magnitude of the immune response by the host.

Vaccinia virus seems to be not a suitable vector to express *Brucella* antigens and produce a protective immune response. This may be because the integrity or the level of antigen expression by vaccinia virus or it may be because of INF- inhibiting properties of vaccinia virus. In order to overcome the INF- inhibiting properties of vaccinia, and an attenuated strain devoid of these interferon inhibiting properties can be used. The modified vaccinia strain Ankara, which lacks these properties, would probably be a suitable candidate as an expression vector for *Brucella* genes.

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8. **VITA**

Simge Baloglu was born on July 16, 1971 in Erzurum, Turkey. After finishing high school at Ozel Dost Lisesi, Istanbul, Turkey in May 1989, she attended Bogazici University, Istanbul, Turkey and majored in Biology in Department of Molecular Biology and Genetics, where she was graduated with a Bachelor of Science degree in June of 1994.

In August of 1994, she enrolled in a Master of Science program at the Virginia-Maryland Regional College of Veterinary Medicine, Department of Biomedical Sciences and Pathobiology and received her Masters in Veterinary Medical Sciences degree in 1997. She continued her academic pursuit as a doctoral student under Dr. Toth's and Dr. Boyle's supervision. Upon graduation she will relocate to Boston MA where she will start her post-doctoral appointment at the Harvard Institutes of Medicine.