INDUCTION OF PROTECTION, ANTIBODIES AND CELL MEDIATED IMMUNE RESPONSES BY *BRUCELLA ABORTUS* STRAIN RB51, *OCHROBACTRUM ANTHROPI* AND RECOMBINANTS THEREOF

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Induction of Protection, Antibodies and Cell Mediated Immune Responses by *Brucella abortus* Strain RB51, *Ochrobactrum anthropi* and Recombinants Thereof

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(ABSTRACT)

Although it is known that cell-mediated immunity (CMI) plays a key role in protection against brucellosis, the exact immune mechanisms leading to protection are still not fully understood. Better understanding of the mechanisms would help in the development of a human *Brucella* vaccine and help in improving animal vaccines. In this research, *B. abortus* strain RB51 and a closely-related, nonpathogenic *Ochrobactrum anthropi* (strain 49237) bacterium were used to study the immune response against brucellosis in mice. Both *O. anthropi* strain 49237 and recombinant strain 49237 expressing *Brucella* protective antigen copper-zinc superoxide dismutase (Cu/Zn SOD) induced a mix of Th1 and Th2 type immune responses but failed to provide protection against virulent *Brucella* challenge. After changing the immune response to a predominantly Th1 type of response using CpG oligonucleotides as an adjuvant, both strains provided protection with the recombinant strain inducing significantly higher protection. It was also demonstrated that vaccination with strain RB51 induced Th1 immune responses characterized by high interferon-gamma (IFN-γ) production with no interleukin-4 (IL-4) secretion as well as high IgG2a and minimal IgG1 production. A colorimetric cytotoxic T lymphocytes (CTL) assay was developed to demonstrate that strain RB51 induced an antigen-specific CTL reaction that probably plays an important role in protection. The results suggest that optimal protection against brucellosis requires IFN-γ-secreting T cells and antigen-specific CTLs. Recombinant strain RB51 overexpressing *Brucella* Cu/Zn SOD and simultaneously expressing mycobacterial 85A antigen induced higher IFN-γ production and CTL activity than the parent RB51 strain. The combined results suggest that the recombinant *O. anthropi* strain could be used as a human vaccine against brucellosis and that the recombinant RB51 strain could be used as an effective vaccine against both brucellosis and tuberculosis in animals.

DEDICATION

This Dissertation Is Dedicated To My Parents And Wife,

Longgao He, Deyu Chen, And Yuying Tian,

With My Deepest Love And Gratitude

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
ATCC	Amerian Type Culture Collection
BCG	Bacillus Calmette-Guerin
BL-3	biosafety level 3 laboratory
CFU	Colony Forming Units
CMI	Cell-mediated Immunity
ConA	concanavalin A
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco minimal essential medium
DMSO	dimethyl sulfoxide
ELISA	Enzyme-linked Immunosorbant Assay
FITC	fluorescein isothiocyanate
Ig	Immunoglobulin
IL	interleukin
IFN	interferon
kb	kilobases
kDa	kilodalton
LPS	lipopolysschoride
mAb	monoclonal antibody
MBP	maltose-binding protein
MHC	major histocompatibility complex
MW	molecular weight
NCBI	National Center for Biotechnology Information
NK cells	natural killer cells
PE	phycoerythrin
PVDF	polyvinylidene difluoride
PBS	phosphate buffered saline
RPMI	Rosewell Park Memorial Institute, Buffalo, NY
SDS-PAGE	sodium dodecyl sulfate polyacrylamyde gel electrophoresis
SOD	superoxide dismutase
%T	percent transmittance
TBS	Tris buffered saline
TEM	transmission electron microscopy
TNF	tumor necrosis factor
TSA	trypticase soy agar
TSB	trypticase soy broth
Tween 20	Polyoxyethylene 20 - sorbitan monolaurate

CHAPTER ONE

LITERATURE REVIEW

OCHROBACTRUM ANTHROPI

General Characteristics of the Genus *Ochrobactrum*

O. anthropi is a gram-negative, rod-shaped, strictly aerobic, nonpigmented, oxidase-producing, non-lactose-fermenting bacillus (108). *O. anthropi* produce acid from several carbohydrates and reduce both nitrate and nitrite. *O. anthropi* is motile by means of peritrichous flagella (108). There are at least 56 strains and they are rarely pathogenic (108). *O. anthropi* were previously known as *"Achromobacter"* Centers for Disease Control (CDC) group Vd. Parameters of DNA-rRNA hybridization indicate that *Ochrobactrum* belongs to the *Brucella* rRNA branch within rRNA superfamily IV (61). Similar to *Brucella*, *O. anthropi* also belongs to the alpha-2 subdivision of *Proteobacteria* and has two chromosomes (122). The type strain is ATCC strain 49188 (= LMG 3331 = CIP82.115 = NCTC 12168).

O. anthropi strain LMG 3301 has been shown to be closer to *Brucella spp*. than *O. anthropi* type strain LMG 3331 based on genetic and antigenic determinants (251). Therefore, a new species name, *Ochrobactrum intermedium sp. nov*, was proposed to be in the genus together with *O. anthropi* (251).

The Close Relationship of *O. anthropi* with *Brucella*

Extensive research has been done on the genetic and antigenic characteristics of *O. anthropi*. Studies have clearly shown the close relationship between *O. anthropi* and *Brucella*.

According to the DNA-rRNA hybridization data, the nearest neighbors of *O. anthropi are Brucella, Phyllobacterium, Agrobacterium, Mycoplana,* and *Rhizobium*. All of these organisms belong to rRNA superfamily IV. *Brucella* is the nearest rRNA neighbor to *O. anthropi* and *Phyllobacterium* is the next closest. Phenotypically, *Phyllobacterium* is the closest to *O. anthropi* and *Brucella* shows the lowest phenotypic level of similarity within the superfamily IV group (61, 108).

Besides DNA-rRNA hybridization, the close relationship between *O. anthropi* and *Brucella* is evidenced through several other characteristics. For example, a PCR assay developed by Romero et al (211) with a pair of primers derived from the 16S rRNA sequence of *B. abortus* amplified a 905bp fragment from *O. anthropi* biotype D, suggesting a close relationship between *Brucella* spp. and *O. anthropi* biotype D. No amplification was detected using the same primers with the DNAs from 10 strains phylogenetically related to *Brucella spp*., 5 gram-negative bacteria showing serological

cross-reactions with *Brucella spp*., and 36 different clinical isolates of non-*Brucella* species. Another PCR study showed that 4 pairs of PCR primers specific for the genes encoding *Brucella* 16S RNA and heat shock proteins DnaK, DnaJ, and GroEL could not differentiate *Brucella* from *O. anthropi* (58).

Immunological cross reactivities have been demonstrated. For example, *O. anthropi* cytosolic proteins frequently evoked a delayed-type hypersensitivity (DTH) reaction of an intensity similar to that observed with *B. melitensis* brucellin in *Brucella* infected animals (61). *O. anthropi* cytosolic proteins reacted in double gel immunodiffusion and immunoelectrophoretic tests with antibodies obtained from *B. melitensis* infected animals (250). Protein profiling and Western blot analysis showed many antigenic cross-reactivities between *Brucella* and *O. anthropi* (250, 251, 259). A recent report indicated that most monoclonal antibodies to the *Brucella* outer membrane lipoproteins Omp10, Omp16, and Omp19 cross-reacted with *O. anthropi* and *Phyllobacterium rubiacearum*, but not with *Yersinia enterocolitica* O:9, *Escherichia coli* O:157, or *Salmonella urbana* (43). Unpublished results from our laboratory also indicated that mouse or goat sera obtained after infection or vaccination with *B. abortus* or *B. melitensis* recognized many *O. anthropi* antigens.

***O. anthropi* as a Potential brucellosis Vaccine & Vaccine Vector**

The cross-protection strategy has been widely used in the development of vaccines against bacterial pathogens. Certain serotypes of bacteria can be used as live vaccines to cross-protect against different serotypes of the same bacterial pathogens, e.g., *Shigella* (177), *Pasteurella* (109, 202), and *Salmonella* (57). Also, active immunization with either the Smith diffuse strain of *Staphylococcus aureus* or a type Ia strain of group G *Streptococci* protected against challenge by either the homologous or heterologous bacterial strains (115). Attenuated bacteria, e.g., *Brucella* vaccine strain RB51 (120, 148), *Mycobacterium* vaccine strain Bacille Calmette-Guerin (BCG) (140), and *Salmonella typhimurium* (57) have been used to protect against infection with heterologous strains or species. These same organisms including *E. coli* (81) have been successfully used at least experimentally as vaccine vectors to express foreign antigens specified by cloned genes from other pathogens.

Since *O. anthropi* is so closely related to *Brucella*, we hypothesized that *O. anthropi* might be directly used as a vaccine against *Brucella* infection, or that *O. anthropi* could be used as a vaccine vector to express protective *Brucella* antigens in order to induce protection against virulent *Brucella* challenge. Although wild type *O. anthropi* has been used to degrade or detoxify mycotoxins present in grains (patent #6,001,638), *O. anthropi* has not been used as a vaccine or vaccine vector before.

If protection can be achieved, *O. anthropi* could have many advantages over the direct use of *Brucella* as vaccines. For example, *O. anthropi* does not infect people with an uncompromised immune system (108). It also grows rapidly at temperature below 37^0C, one day being sufficient for *O. anthropi* to replicate to visible colonies while

Brucella usually take at least three days to achieve comparable number. Some *O. anthropi* strains are sensitive to common antibiotics. Most *O. anthropi* strains appear to contain plasmids, but some do not have any native plasmids. These could be used as vaccine vectors by transforming them with a broad-host-range plasmid that is commonly used for the generation of recombinant experimental *Brucella* vaccines.

BRUCELLA ABORTUS

General Characteristics of *Brucella abortus*

The *Brucella*e are taxonomically placed in the alpha-2 subdivision of the class *Proteobacteria* (167). There are six species of *Brucella* based on the preferential host specificity: *B. melitensis* (goats), *B. abortus* (cattle), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (sheep) and *B. neotomae* (desert mice). The first four species are pathogenic to humans in decreasing order of severity making brucellosis a zoonotic disease. *B. melitensis* is found in goats and sheep. In addition to cattle, *B. abortus* can also infect swine, goats, and many wildlife animals such as elk and bison (45). *B. suis* infects swine and reindeer, *B. canis* infects dogs, and *B. neotomae* infects desert mice. It appears that a seventh species exists and was isolated from sea mammals (86, 214, 215).

The *Brucella*e are small, nonmotile, nonsporulating, gram-negative coccobacilli or short rods (0.5 to 0.7 μm by 0.5 to 1.5 μm) (143). Growth occurs aerobically and often requires or is enhanced by 5-10% CO_2. The *Brucella*e are facultative intracellular bacteria that replicate within host macrophages (12). Molecular genetic studies have demonstrated phylogenetic relatedness to *Agrobacterium*, *Ochrobactrum*, *Phyllobacterium*, and *Rhizobium* (51). *B. abortus* can be differentiated from other species within the *Brucella* genus and be classified into seven biovars according to a combination of characteristics such as bacteriophage susceptibility (50, 53, 54), dye sensitivity to basic fuchsin and thionin (77), H_2S production, CO_2 requirement, and the presence of A or M surface antigens (92).

Genetics of the *Brucellae*

Classic genetic studies of *Brucella*e began with spontaneous mutants early this century. The most widely studied spontaneous mutants are vaccine strains, such as *B. melitensis* Rev 1, *B. abortus* strain 19 and recently *B. abortus* strain RB51. The classic genetic studies focused on phenotypic appearance, stability, metabolism and virulence of mutant colonies (99). Colony smoothness and roughness usually correlates with high and low virulence among *B. abortus*, *B. suis*, and *B. melitensis*. Mutation from smoothness to roughness usually decreases the virulence of these species and decreases or eliminates the stimulation of antibodies to the O antigen in animal hosts. *B. abortus* strain RB51 illustrates this well, it is a rough strain which is highly attenuated and does not induce anti-O antibodies (223).

The *Brucella* genome has a GC content of approximately 58%. *B. melitensis, B. abortus, B. ovis, B. neotomae, and B. suis* biovar 1 each have two chromosomes of 2,100 kb and 1,150 kb (121, 160). However, *B. suis* biovar 2 and 4 have two chromosomes of 1.85 Mb and 1.35 Mb, and *B. suis* biovar 3 has only one chromosome with a size of 3.1 Mb (121). These difference in chromosome size and number can be explained by rearrangements resulting from homologous recombination at chromosome regions containing the three *rrn* genes (121). The DNA sequences among different *Brucella spp.*

share greater than 90% homology (111). According to the present taxonomy and phylogeny based on 16S RNA, the classic 6 species belong to a single species. This fact has been used to propose that the genus *Brucella* contains only a single species *B. melitensis*, and that the remaining classic species be considered biovars (99).

Insertion sequences (IS) are discrete segments of DNA that can transpose from one genomic site to another and promote genetic rearrangements (44). Insertion sequences are found on both chromosomes of *Brucella*e. All *Brucella* spp. contain approximately 8-35 copies of an insertion sequence denominated IS711 (also known as IS6501). The position and copy number of this insertion sequence seems to vary in different species, a characteristic which can be used to differentiate them (191). For example, the *wboA* gene in *B. abortus* RB51 is disrupted by an IS711-like element (159, 258). Based on this, a PCR assay has been developed to distinguish strain RB51 from other *Brucella* spp. and strains including its parent strain 2308 (258). Many PCR assays based on gene differences have been developed to detect or differentiate various *Brucella* strains (11, 23, 24, 90, 142, 191, 204, 211, 226, 243).

There are more than 50 *Brucella*e genes with a variety of functions listed in GenBank. For example, GenBank includes genes that encode the chaperones such as *dnaK*, *groEL*, and *groES*. Both 16S RNA and 23S RNA DNA sequences of *Brucella* are found in GenBank. No resident plasmids have been found in *Brucella*. However, several plasmids have been shown to be able to replicate in the *Brucella*e (99).

Brucella Infections and Control in Humans and Domestic Cattle

Brucellosis in humans is also known as undulant fever or Malta fever. Bruce and coworkers first isolated *B. melitensis* from military personnel in Malta in 1897. This disease was derived from infected goats. *B. melitensis* does not exist in the USA. *B. abortus* was isolated from aborting cattle by Bang later in 1897 (14). Therefore, brucellosis caused by *B. abortus* is also known as Bang's disease. *B. suis*, *B. abortus*, and rarely *B. canis* can also infect humans. As of April, 2000 there are still 6 *B. abortus* infected cattle herds and 1 or 2 bison herds in the USA. *B. suis* infected herds do exist in the USA and the organism can also be found in ferral swine. *B. canis* is rarely found in the USA.

The first reported human brucellosis infection within the U.S.A. occurred in 1906. The incidence rate of human *Brucella* infections increased to a high level of 36.9 new cases per 100,000 individuals per year from 1945-1949 (45). Since then, the incidence markedly decreased largely due to a combination of milk pasteurization, public education programs, and the national brucellosis eradication program.

Bovine brucellosis is mainly caused by *B. abortus* that causes abortion in pregnant cows and sterility in bulls and undulant fever in humans (51). It currently infects millions of cattle and many humans throughout the world and causes large economic losses as well as suffering in both animals and humans (51, 158, 171, 265).

The national brucellosis eradication program in the U.S.A was initiated in 1954 and aimed at the eradication of brucellosis in cattle caused by *B. abortus*. The eradication program was designed as a cooperative effort among the federal government, the states, and livestock producers. As a result, the domestic cattle population is now virtually free of brucellosis. However, a re-introduction of the disease within the U.S. is still possible as many countries still have a high prevalence of brucellosis in cattle, which could infect US herds through importation of infected animals. brucellosis also exists in the U.S. in wildlife, such as elk, bison, deer, moose, coyotes, and raccoons and reintroduction of the disease from these animals into cattle is possible (45). *Brucella* is also one of several pathogens which could be used for biological terrorism and bio-warfare.

Transmission and Pathogenesis of *B. abortus* Infections in Domestic cattle

A *Brucella* infection in a susceptible animal depends on the dose, exposure route, virulence of the strain and the health of the animal host. Cattle are most frequently infected with *B. abortus* biovar one. Typically, *B. abortus* is transmitted by oral ingestion after exposure to infected placentas, fetal tissues or fluids which often contain high concentrations of organisms (3, 87). Other transmission routes including respiratory, venereal and congenital transmissions have also been documented in domestic cattle (55, 206). Sexually mature cattle are much more susceptible to infection than immature cattle. Susceptibility also increases with pregnancy and as gestation progresses (174). After ingestion of *Brucella*, the organisms survive and replicate in the reticuloendothelial system of the local lymph nodes resulting in regional lymphadenopathy. Eventually bacteremia occurs and the organism migrates to the spleen, lymph nodes, the mammary gland, the reproductive tract particularly the pregnant uterus (173). The virulent strains of *B. abortus* localize preferentially in areas with high concentrations of erythritol such as the pregnant uterus, fetal tissues, and the male genital tract (227). The *Brucellae* can also be cultured from bones, joints, eyes and brain in adult cattle and from the stomach, lung and spleen of bovine feti (45).

B. abortus infection in cattle may cause late term abortions, still births, retained placentas, sterility, lymphoplasmacytic mastitis and tissue granuloma (1, 52). However, the exact mechanism of abortion in infected cows is still not clear. The principal virulence factors of *Brucella*e include endotoxin (cell wall lipopolysaccharide) (102, 166), and their ability to survive within reticuloendothelial cells probably by inhibiting phagolysosomal fusion (28, 88, 200) and inhibition of primary granule release (19). On the other hand, hosts can have innate immunity (78) and can develop acquired immunity (52) against *Brucella* infections. The successful co-existence of *Brucella* and their preferred host is thought to be the outcome of ancient co-evolutionary relationship and selection pressures (1).

Vaccination against *B. abortus* infection

Since *Brucella melitensis* and *Brucella abortus* were first isolated in 1897, 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. Although *Brucella*e are sensitive to many antibiotics, treatment is not practical and too expensive in most animal species. On the other hand, prevention of the disease by vaccination is and has played a key role in brucellosis eradication programs. Several vaccines have been used around the world to control brucellosis in cattle and goats. *Brucella abortus* strain 19 has been used to control *Brucella abortus* infections in cattle and *Brucella melitensis* strain Rev 1 to control brucellosis in goats and sheep. Strain 19 had been used for calfhood vaccination in the USA since 1941. The newest *Brucella abortus* vaccine is strain RB51, which has recently replaced *B. abortus* strain 19 in the USA.

Characteristics of *B. abortus* vaccine strains 19 and RB51

B. abortus strain 19 was originally isolated as a virulent strain from the milk of a Jersey cow in 1921 and left at room temperature for over a year in the laboratory (27). This "aged" strain was attenuated and able to induce protection against bovine brucellosis caused by *B. abortus*.

A distinct disadvantage of strain 19 is that it stimulates the production of O-chain specific antibodies which can be detected in serum and milk, interfering with diagnosis of the disease and in the eradication program.

Endotoxin or lipopolysaccharide (LPS) is the major antigen as well as the main toxic component of gram-negative bacteria. LPS stimulates B lymphocytes to proliferate, differentiate, and secrete antibodies. LPS also induces transduction signaling cascade and cause endotoxic shock. The LPS includes lipid A, oligosaccharide core and O polysaccharide chain (O chain, or O antigen). The lipid A is the biologically active part of the endotoxin. Although the structure of lipid A is highly conserved among enterobacteria such as *Escherichia coli*, *Shigella* spp., or *Salmonella* spp., its structure in *Brucella* spp. is quite different. Consequently, *Brucella* LPS displays very low endotoxic activity but remains immunogenic (165, 205). The O antigen of *Brucella* is a homopolymer of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues joined by an α-1,2 linkage in A-epitope-dominant strains, but is joined by an α-1,3 linkage at every fifth residues in M-epitope-dominant strains (32). Smooth bacterial strains have intact O-chain while rough strains lack this O-chain on their LPS molecule partially or completely. Smooth strains *of B. abortus*, *B. suis* and *B. melitensis* are usually virulent while rough *Brucella* strains are usually less virulent except *B. ovis* and *B. canis* which are pathogenic for sheep and dogs, respectively. The O-chain plays a key role in the serological diagnosis of brucellosis because it is an immunodominant antigen able to induce antibody responses in most animals exposed to smooth *Brucella* organism and the detection of antibodies to the O-chain is used in most brucellosis diagnostic serological tests (65, 221-223).

B. abortus strain RB51 was originally generated through successive passages of virulent strain 2308 on TSA plates containing rifampicin (223). Strain RB51 has been shown to induce protection against challenge with smooth virulent *Brucella* spp. in cattle (38), swine (148) and mice (120, 261). RB51 is a rough mutant devoid of the O-chain and its roughness is very stable as demonstrated after multiple passages *in vitro* and *in vivo* through various species of animals (45, 223, 236, 237). One reason for RB51 to be rough is that the *wboA* gene in RB51 is disrupted by an IS711 element so that RB51 cannot encode a glycosyltranferase, an essential enzyme required for the synthesis of O antigen (256, 258).

Strain RB51 is a live, attenuated vaccine and one injection provides similar or better protection than one injection of strain 19 (36, 37, 149, 185). One clear advantage of strain RB51 over strain 19 is that RB51 cannot induce any detectable antibody response against the O-chain of lipopolysacchride (LPS) since strain RB51 is rough and does not contain O-chain (223). Antibodies to the O chain are the antibodies detected by a variety of serum tests commonly used to diagnose brucellosis (65, 221-223). Many strain 19-vaccinated animals “seroconvert” after vaccination making them indistinguishable or at least difficult to distinguish from animals infected with *Brucella* field strains. The use of strain RB51 will greatly benefit brucellosis eradication programs due to the fact that strain RB51 can not induce false positive results even if injected multiple times for “boosting” purposes. It is known, at least in mice, that the protective mechanisms induced by strain RB51 are cell-mediated and not antibody-mediated (6). The exact cell-mediated immunological mechanisms operative in protection are unclear. Existing vaccines including strain RB51 do not induce "sterile" immunity in the mouse model ("sterile" means that the challenged virulent *B. abortus* strain 2308 cannot be completely cleared from mouse spleens although some logs of decrease can be found). strain RB51 induces good protection in cattle and swine but it does not protect sheep against *B. ovis* (119) and the level of protection achieved in goats against *B. melitensis* is still not clear (184, 212). Therefore, some very important immunological questions are still pending: which are and how many protective antigens exist in the *Brucella*e? What specific immune responses can these protective antigens stimulate? What immunological *in vitro* and *in vivo* tests correlate with strong protection against *Brucellae* infection? The answers to these questions are very important in order to rationally develop more efficient vaccines against brucellosis.

Use of the mouse model in *Brucella* research

The mouse model has been extensively used in brucellosis research (15, 201, 240). Mice are relatively easy to handle and cheap to house. Their immune systems have been extensively characterized (201). Moreover, a variety of inbred mice are available which allow all researchers throughout the world work on the same genetically identical mice and share information without worrying about individual animal variation.

BALB/c and C57BL/10 mice differ in their abilities to induce immune responses and protection against virulent *Brucella* infections (80, 144, 273). BALB/c mice are more

sensitive than C57BL mice to virulent infections of *Brucella* such as *B. melitensis*, *B. abortus*, and *B. suis* (80, 273). It was also reported that *B. melitensis*, *B. abortus*, and *B. suis* all caused chronic pathological lesions in lymph nodes, spleen and liver, and that the lesions developed in BALB/c mice were more remarkable than those in C57BL/10 mice (144). C57BL/10 mouse splenocytes produced more IFN-γ than those in BALB/c mice upon stimulation with *Brucella* antigens (80). This information suggests that C57BL/10 mice should be better bovine brucellosis model than BALB/c mice since the ability to develop high INF-γ levels appears to be important in protection. BALB/c mice are nevertheless more commonly used as a model for *Brucella* infections. There was no solid scientific reason for this choice. However, later studies indicated that the BALB/c model was better than the C57BL/10 model because the BALB/c mouse mimicked the infection kinetics observed in cattle with virulent *B. abortus* strains or strain 19 (6, 162). For example, virulent *B. abortus* strain 2308 causes chronic infections without overt disease in both cattle and BALB/c mice (162). In contrast, the level of infection caused by strain 2308 in C57BL/10 mice is consistently low and a chronic infection is not established (6). The relatively faster clearance of strain 19 than of strain 2308 in BALB/c mice also matches the relative virulence of these two strains in cattle (162).

Antigens of *Brucella*

The O-chain in the *Brucella* LPS is the most exposed antigenic structure on the surface of smooth *Brucella* and is the immunodominant antigen in serological responses to smooth *Brucella* infections. The O-chain is also known to be a protective antigen as shown in the mouse model by passive transfer immunization experiments with anti-O-chain monoclonal antibodies or active immunization experiments with purified O-chain or smooth *Brucella* LPS conjugated to carrier proteins (6, 163). The protective effect is antibody mediated.

Outer membrane proteins (OMPs) were initially identified in the early 1980s by selective extraction techniques and classified according to their molecular mass as group 1 (88 - 94 kDa), group 2 (35 - 39 kDa), and group 3 (25 - 31 kDa) OMPs (260). These proteins have since been characterized by monoclonal antibodies and some of the genes encoding them have been cloned. For example, well-characterized and cloned OMPs are: the Omp1 or 89 kDa within the group 1 proteins; Omp2a and Omp2b, two homologous porin proteins within the group 2 OMPs; Omp25 and Omp31, two homologous group 3 proteins. In addition, several low molecular weight proteins including Omp10, Omp16, Omp19 have been identified as minor OMPs and as lipoproteins. OMPs appear to be poor immunogens in animals, however, some of them such as Omp31, may stimulate protective antibody response in mice against rough *Brucella* such as rough *B. ovis* (168). It is possible that some may induce protective CMI.

Brucella YajC and SecD proteins are two membrane proteins encoded by two open reading frames (ORFs) in one operon (254). Both proteins are probably involved in the translocation of *Brucella* periplasmic or putative secretory proteins. YajC stimulates

both antibodies and IFN-γ responses in mice, but appears to be non-protective. SecD cannot stimulate any detectable antibody response by immunoblotting analysis (254).

An 18-kDa lipoprotein is present on the surface of *Brucella* (135). This protein can induce antibody production in infected mice, sheep, goats, dogs, and humans (135, 244). This protein can also induce CMI in the mouse model. However, a recombinant vaccinia virus expressing this protein does not protect against virulent *Brucella* infection and disruption of the gene in strain RB51 does not affect either the vaccine strain's protective capabilities or its *in vivo* attenuation characteristics (252). These data suggest that the 18-kDa protein plays no role in protective immunity.

Brucella Cu/Zn SOD is a protective periplasmic *Brucella* antigen (9, 241). *E.coli* expressing *Brucella* Cu/Zn SOD induces significant protection in mice against virulent *Brucella* strain 2308 infection (186). Immunization of mice with purified *Brucella* Cu/Zn SOD (9) or SOD synthetic peptides (241) induced significant protection against virulent strain 2308 infection in mice. Vaccination of mice with *Brucella* strain RB51 overexpressing homologous Cu/Zn SOD also stimulated enhanced protection as compared to strain RB51 alone (257). The data indicate that Cu/Zn SOD is a protective antigen.

Other periplasmic proteins that have been described are BCSP31 and BP26. BCSP31 is a 31-kDa salt-extractable protein and highly antigenic during natural infections and after vaccination (25, 156). Its gene has been used to design PCR primers for rapid diagnosis of human brucellosis using PCR assay (204). Immunization of mice with recombinant BCSP31 did not provide protection (41) and it appears that BCSP31 is not a protective protein (100, 231, 232). BP26 is a 26-kDa periplasmic protein (216). Antibodies to the 26-kDa protein can be detected in serological assays with sera from infected animals and could be useful for the purpose of diagnosing brucellosis in different mammals (21, 42, 63). Its protective characteristic is still unclear.

The last category of immunogenic *Brucella* proteins is cytoplasmic and ribosomal proteins. One example is the L7/L12 ribosomal protein that has been shown to be another protective antigen (8, 182). Immunization of mice with recombinant L7/L12 (181) or a DNA vaccine of L7/L12 genes (138) induced protection against virulent *Brucella* infection. It was further found that L7/L12 stimulated a delayed-type hypersensitivity (7) and Th1 type immune responses with the production of IL-2 and IFN-g (183).

Heat shock proteins (Hsp's) are also cytoplasmic proteins. They are chaperones or stress proteins that are important in protein biogenesis. For instance, Hsp GroEL is involved in the folding, assembly, and transport of factors necessary for growth under stress conditions (146). Hsp's are expressed at higher levels under stress stimuli such as increased temperature, exposure to toxic oxygen radicals, nutritional deficiencies, and intracellular growth. In addition, hsp's were identified as highly immunogenic antigens in many microbial pathogens (128). *B. abortus groES* and *groEL* genes have been cloned (95, 145, 213). It was found that cattle vaccinated with *B. abortus* strain 19 (145) and

cattle and mice infected with virulent strain 2308 (213) developed an antibody responses to recombinant GroEL. Hsp GroES and GroEL also induce proliferation of and IFN-γ secretion by $CD4^+$ T cells from *Brucella*-vaccinated mice and elicit delayed-type hypersensitivity reactions in *Brucella*-sensitized guinea pigs (179). However, it is still unclear if they are protective antigens.

The *Brucella* HtrA (high-temperature-requirement A) protein is a cytoplasmic stress response serine protease that is important in cellular defense against oxidative killing (73). The deletion of *htrA* gene in *B. abortus* strain 2308, *B. abortus* strain 19, or *B. melitensis* strain 16M results in the transient attenuation of its parent strain in the BALB/c mouse model, strongly suggesting it contributes to virulence (72, 198, 208). These HtrA mutants are also more sensitive to oxidative killing *in vitro*. Their *in vitro* attenuation characteristics are also demonstrated in goats (70, 197).

Other cytoplasmic proteins include the Ssb and UvrA proteins, bacterioferritin, and proteins with unknown functions called p15, p17 and p39. Not much is known about their immunological role in brucellosis (41).

Antibody Response to *Brucella*

Upon infection with gram-negative bacteria, host cells are exposed to bacterial lipopolysaccharide (LPS) and other types of antigens. Antibodies to these structures may be induced. Carbohydrate and glycolipid molecules, such as bacterial LPS, can induce antibodies but are traditionally considered as T-independent antigens based on the facts that they can activate B lymphocytes and induce antibody production without apparent direct activation of T cells (17, 56). It is believed that this is due to the fact that pure polysaccharide and glycolipids fail to bind to the MHC class II groove due to their chemical structure (101, 116). However, many studies indicated that bacterial LPS is capable of binding MHC class II molecules in B cells (203, 264). *B. abortus* smooth LPS and purified O antigen can bind to MHC class II molecules in murine B lymphocytes (74). It was further found that structurally different LPSs from *Brucella* and *Shigella* strains are delivered to lysosomal compartments of B cells which are enriched in MHC class II molecules (85). However, only *B. abortus* LPS associates with MHC class II molecules in a haplotype- and species-independent manner. The *B. abortus* LPS-MHC class II complex wase transferred to the cell surface and presented to T cells (85).

The O antigen of the *Brucella* LPS, although 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 at least in the mouse model (6, 163). It was recently found that vaccination of mice with live strain RB51 that overexpressed the O antigen in the cytoplasm induced antibodies to the O antigen which were predominantly of the IgG2a and IgG3 isotypes (256). It was also found that *B. abortus* strain 19 and strain 2308 stimulated protracted polyclonal responses of both IgG2a and IgG3 isotypes specific to the O antigen of smooth *Brucella* in BALB/c mice (71). The presence of high IgG2a and absence or low IgG1

subisotype antibodies to the O antigen indicated the induction of a Th1 type of immune response at some stage of the immune response (239).

B. abortus infection induces the production of IgM, IgG1, IgG2a, and IgA antibody isotypes detectable in both milk and sera of cattle (175). IgM is produced soon after infection but declines quickly when production of IgG increases. IgM reacts nonspecifically in many serological tests and can cause high rates of false positive reactions (172). IgG1 is consistently produced at high levels in Brucella-exposed cattle sera and has a high affinity and specificity for *Brucella* antigens particularly the O-chain (176).

Recent studies in our laboratory indicate that *B. abortus* strain RB51 stimulates high level of IgG2a and low level of IgG1 to *Brucella* antigens other than the O antigen, such as *Brucella* Cu/Zn SOD (257). Strain RB51 overexpressing *Brucella* O antigen in the cytoplasm induces predominantly IgG2a and IgG3 responses to the O antigen and no detectable IgG1 response (256).

General CMI to facultative intracellular parasites

Although it is not possible to consider cell-mediated and antibody-mediated responses entirely separately, the term "cell-mediated immunity (CMI)" is currently used to describe any immune reactions against organisms or tumors that are mediated by cells rather than by antibody or other humoral factors.

Mackaness first demonstrated in the history of immunology the importance of CMI for protection against intracellular pathogens by injecting *B. abortus* strain 19 intravenously into mice tails in 1964 (151, 152). He found a non-specific immune response by showing that macrophages activated by *Brucella* infection provided cross-protection against *Listeria monocytogenes*. He also demonstrated a specific immune response that once waned, could be recalled later by a second dose of *Brucella* injection (151). The term "cell mediated immunity" was first coined to describe this phenomenon (35, 246). The importance of CMI to intracellular pathogens was later confirmed by extensive studies for various intracellular bacterial pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Salmonella typhimurium* (10, 35)]. Since the discovery of T helper (Th) cells and cytotoxic T lymphocytes (CTLs) by Cantor and Boyse (29-31), considerable work has been done to demonstrate their specific immune functions within the CMI response.

Models for explaining how CMI works against intracellular bacteria have been formulated for years (35). When intracellular bacteria invade host animals, they are phagocytosed by resident macrophages resulting in a local inflammation. $CD4^+$ and $CD8^+$ T cell are then activated. $CD4^+$ T cells primarily produce IFN-γ that enhances macrophage functions including bactericidal activity and activated, pathogen-specific $CD8^+$ T cells lyse any infected cells including macrophages releasing the intracellular bacteria. These released bacteria are then phagocytosed and killed by the IFN-γ-activated

macrophages. Antibodies and complement may also play a role during this "extracellular" phase. This CMI model has been extensively supported for different intracellular bacteria in many animal models (35).

T helper cells are of the $CD4^+CD8^-$ phenotype ($CD4^+$ cells) and they include two distinct subsets based on the two different profiles of cytokine production. T helper type 1 (Th1) cells characteristically secrete IFN-γ, IL-2, and TNF-β. Th1 cells act primarily as helper cells for cell-mediated inflammatory reactions such as delayed hypersensitivity and macrophage activation. Although IFN-γ promotes IgG2a secretion, the Th1 type cytokines do not stimulate specific antibody formation (245). T helper type 2 (Th2) cells typically can 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 the development of delayed hypersensitivity and other cell-mediated reactions. Th1 type cytokines (e.g., IFN-γ) inhibits the actions of Th2 cells and Th2 type cytokines (e.g., IL-10) inhibits the actions of Th1 cells. Thus, an immune response tends to bias into a Th1 type response or a Th2 type response (210). Evidence indicates that Th1 response promotes resistance to intracellular pathogens (245).

T cytotoxic lymphocytes (CTL's) are of the $CD4^-CD8^+$ phenotype and are also involved in protection against intracellular bacteria (123). The $CD8^+$ CTLs kill target cells principally via two major pathways: (1) The Fas ligand (FasL) 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 target cell membrane and eventual cell death. The first killing mechanism also frequently occurs during apoptosis (programmed cell death). The second one is unique to CTLs (123). It was described a decade ago that CTLs could lyse mouse target cells infected with intracellular bacteria such as *M. tuberculosis* (124).

$CD4^+$ T helper cells and $CD8^+$ cytotoxic cells recognize cell-bound antigens in form of peptides in association with MHC class II and class I molecules, respectively (209). MHC class I binding antigenic peptides are generated from cytosolic proteins. MHC class I molecules are loaded with peptides actively transported from the cytosole into the endoplasmic reticulum (ER) by an ATP-dependent transporter associated with an antigen processing (TAP) heterodimer. The class I MHC-peptide complex then travels through the Golgi apparatus onto the cell surface, where it presents the peptides to CD8+ T cells. This process is known as the classic MHC class I pathway. On the other hand, MHC class II binding peptides are derived from more exogenous antigens. The exogenous antigen is endocytosed and degraded in the endosomal/lysosomal compartments. Degraded peptides are transferred to class II molecules synthesized and transported from rough endoplasmic reticulum into the endosomal compartment. The newly formed class II complexes then move to the cell surface where they are recognized by the CD4+ T cells. This is the classic MHC class II pathway (209). Therefore, the subcellular localization of intracellular bacteria has important implication for the predominant type of protective immunity the host will develop, namely, CD4+ and/or CD8+ mediated. For example, some intracellular bacteria, e.g., *L. monocytogenes*, and

Shigella, enter the host cell's cytosol. They readily induce MHC class I pathway and CD8+ T cell activation (225). *M. bovis BCG* and *S. enterica*, on the other hand, remain in vacuolar compartments. They primarily stimulate $CD4^+$ T cells via antigen presentation through MHC class II pathway (126). Nevertheless, although *M. bovis* induces preferentially $CD4^+$ T cells, it also induces $CD8^+$ cells indicating that some of the exogenous antigens find their way onto MHC class I molecules. This indicates that, facultative intracelllular parasites are able to induce both, specific $CD4^+$ and $CD8^+$ cells regardless of their main intracellular location.

A typical example of facultative intracellular bacteria is *M. tuberculosis*, which causes tuberculosis. Tuberculosis accounts for 3 million human deaths per year (20). *M. tuberculosis* replicates primarily within host macrophages and thereby escape the threat of complement- and antibody-mediated defense systems somwhat comparable to *Brucella*. *Mycobacteria* reside within vacuolar phagosomes of infected cells and elicit $CD4^+$ T cells following infection (219). The activation of macrophages induced by IFN-γ (83) and TNF-α (84) is therefore very important for the control of tuberculosis and activated $CD4^+$ T cells are essential for protective immunity in part due to their production of lymphokines such as IFN-γ (33, 82). Despite the *Mycobacterial* noncytosolic location, *M. tuberculosis*-derived antigens are also presented to $CD8^+$ T cells. It suggests that *M. tuberculosis* antigens or peptides somehow escape the phagosomal membrane and thus stimulate both $CD4^+$ and $CD8^+$ T cells (126). It is also possible that the process of $CD8^+$ induction occurs via an alternative MHC class I antigen-processing pathway (196). It has been found that a new $CD8^+$ T cell subset also recognizes *Mycobacterial* glycolipids presented by CD1 molecules instead of the classic MHC class I pathway (157, 235). Adoptive transfer of $CD8^+$ cells and *in vivo* T cell depletion studies suggest that $CD8^+$ T cells are involved in the immune response to *M. tuberculosis* (113, 169, 188, 190). This same observation is probably true for *M. bovis* infections.

Cell-mediated Immune Response to *Brucella*

The above illustrated example with *M. tuberculosis* probably also applies to *Brucella spp*. In infected host, virulent *B. abortus* replicate within the endosomes of phagocytic and non-professional phagocytic cells by inhibiting the phago-lysosome fusion and escaping the action of degrading enzymes (51). *In vitro* studies show that *Brucella* can invade phagocytic and non-professional phagocytic cells within 10 min post-inoculation (199). The virulent *B. abortus* strain 2308 prevents the phago-lysosome fusion and distributes within autophagosome-like compartments (200). However, the attenuated *B. abortus* strain 19 cannot prevent the later phago-lysosome fusion and is easily degraded. Its bacterial degradation products are found scattered throughout the host cell cytoplasm (199). Strain 2308 can also transit through autophagic pathway and replicate in the endoplasmic reticulum probably for the purpose of obtaining metabolites (199). Peptides could reach both MHC class I and MHC class II molecules and therefore, induce both $CD4^+$ and $CD8^+$ *Brucella* specific T cells.

Although antibodies to *Brucella* O antigen confer a certain level of protection against a challenge infection in mice (17, 56), CMI appears to play the major role in protection against brucellosis like most other intracellular bacterial infections (5, 6, 51). This can be demonstrated using rough *B. abortus* vaccine strain RB51. This vaccine strain does not induce anti-O antigen antibodies (223) but provides good protection against *Brucella* infection and the protection conferred by strain RB51 vaccination that can only be transferred by immune T cells and not by antibodies (120).

Both $CD4^+$ and $CD8^+$ T cells are involved in immunity against brucellosis as indicated by cell depletion experiments using T cell specific monoclonal antibodies (5, 195) and further, by using gene targeted knockout mice lacking either $CD4^+$ or $CD8^+$ T cell populations (35, 180). *B. abortus* induces a Th1 type immune response and inhibits both the primary and secondary Th2 type immune responses (2, 224).

As products of CMI, cytokines are key molecules that modulate or mediate the development of protective immune responses (210). Among them, IFN-γ is an important cytokine that activates macrophages and up-regulates their bactericidal activity. IFN-γ is produced mainly by Th1 cells and NK cells and its production is positively regulated by IL-12, which is released by macrophages and dendritic cells. The importance of IL-12 in brucellosis was confirmed *in vivo* by depleting endogenous IL-12 before infection of mice with virulent *Brucella*. This leads to exacerbated *Brucella* infection and low IFN-γ production (270). The important role of IFN-γ in mediating resistance to *Brucella* infection can be shown by the facts that depletion of endogenous IFN-γ with monoclonal antibody causes increased *Brucella* replication *in vivo* and that treatment of recipient mice in T cell adoptive transfer experiments with anti-IFN-γ antibody blocks the adoptive transfer of the T cell-mediated resistance (269). *In vitro* experiments also demonstrate that macrophages activated with IFN-γ have enhanced Brucellacidal and Brucellastatic activities (117).

Live bacterial vaccines are essential to effectively induce appropriate protective CMI responses (130). Live bacterial vaccines favor IFN-γ and IgG2a production while killed bacteria preferentially stimulate IL-4 and IgG1 production (271).

Production of TNF-α also appears to be important in a protective immune response to *Brucella*. Only live *Brucella* elicited the production of TNF-α from macrophage cultures (268). It was found that TNF-α-receptor knockout mice (TNF-R-/-) were severely deficient in IL-12 production and *Brucella* infection in the TNF-R-/- mice was exacerbated (35). 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 the *Brucellacidal* activity by activated macrophages.

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

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

Many *Brucella* antigens stimulate CMI in animal models. However, the detection of such a response does not necessarily implicate that the antigen is protective. They may, nevertheless, be involved in enhancing the immune response to truly protective antigens. For example, bacterial LPS is able to activate murine or human T cells in an antigen presenting cell (APC) dependent manner (34, 155). Splenocytes derived from recombinant RB51WboA-immunized mice stimulated higher levels of IFN-γ production than those from RB51-immunized mice upon *in vitro* stimulation with killed RB51 bacteria (256), suggesting that the overexpressed O antigen somehow enhances Th1 immune responses. It is possible that the O antigen binds to MHC class II of APCs and specifically activates T cells (85) which may have a role in protection. Another possibility is that the O antigen binds to *Brucella* peptides and serves to focus or concentrate these peptides onto the MHC class II molecules and enhances efficient, specific sensitization of T cells to the peptides.

Our study also found that mice vaccinated with recombinant RB51 overexpressing *Brucella* Cu/Zn SOD, but not RB51, developed specific Th1 type CMI response to Cu/Zn SOD as indicated by antigen-specific IFN-γ but not IL-4 stimulation (257). We now know that SOD is a protective antigen (9, 186).

Several *Brucella* proteins including L7/L12, UvrA, GroEL, and GroES have been shown to stimulate Th1 immune responses with the production of IL-12 and IFN-γ but not necessarily protection (41). Thus, one should be very careful in using induction of IFN-γ by an antigen as an indicator of protection candidates. *Brucella* Cu/Zn SOD and L7/L12 have been shown to be protective antigens as described above. Probably both afford protection against *B. abortus* infection through the induction of protective Th1 immune responses of which IFN-g production is an important component.

GENERAL RATIONALE OF THE PRESENT RESEARCH

Although the cattle population in the US is now virtually free of brucellosis, the disease is still widespread in many other countries and can potentially infect US cattle in different ways. brucellosis still exists in many wildlife species in the USA. An ideal brucellosis vaccine for wildlife is still not available. *Brucella* is also a zoonotic disease and can infect humans. Although *Brucella* could be used for biological terrorism and bio-warfare, we still do not have a human vaccine against brucellosis. Therefore, further research on *Brucella* and brucellosis is still necessary. We have to develop useful vaccines against brucellosis in humans and wildlife. The vaccines currently used in domestic animals can also be improved. In addition, *Brucella* infection is a good model for studying the CMI responses induced by intracellular pathogens in general. The forthcoming chapters address some of these points.

The general hypothesis for this dissertation is that an effective *Brucella* vaccine would require a predominantly Th1 type immune response and stimulate antigen-specific cytotoxic T lymphocyte (CTL) activity.

Chapter 2 in the dissertation is focused on developing a non-pathogenic vaccine vector to express a *Brucella* protective antigen with the final goal of making a genetically-engineered vaccine that can be potentially used for humans and does not use *Brucella* as a vector. This cross-reactive bacterium is a good model for studying the immune mechanism against virulent *Brucella* infections and suggests that Th1 type of immune responses are necessary for protection.

Chapter 3 in the dissertation is focused on standardizing a colorimetric cytotoxic T lymphocytes (CTL) assay to replace the classic ^{51}Cr-release assay and in using it to study the role of CTLs in brucellosis. It analyzes various aspects of the role of CTLs in the immune response to *Brucella*.

Chapter 4 studies the possibility of improving the performance of current *B. abortus* vaccine RB51 and analyzes the immune response of a recombinant *B. abortus* RB51 vaccine developed to better protect against *Brucella* and simultaneously against *Mycobacterial* infections.

CHAPTER TWO

THE USE OF *O. ANTHROPI* AS A VECTOR FOR *BRUCELLA* VACCINES

INTRODUCTION:

Brucella abortus is a gram-negative, facultative intracellular bacterial pathogen that causes brucellosis in humans and many animal species. Protection against *Brucella* infection requires cell-mediated immune responses that can be stimulated by live attenuated vaccines. Here I report that a highly related bacterium, *Ochrobactrum anthropi*, can be used as a vector for *Brucella* antigens to protect against *brucellosis*. *O. anthropi* is a gram-negative, rod-shaped, strictly aerobic, motile by means of peritrichous flagella, nonpigmented, oxidase-producing, non-lactose-fermenting bacillus (108). There are at least 56 strains and they are rarely pathogenic. The close relationship between *O. anthropi* and *Brucella* has been clearly demonstrated through DNA-rRNA hybridization (61), polymerase chain reaction (211), delayed-type hypersensitivity (DTH) reactivity of *Brucella* infected animals (61), double gel immunodiffusion (250) and Western blot analysis (250). For instance, the DNA-rRNA hybridization showed that *Brucella* was the nearest rRNA neighbor to *O. anthropi* (61). Unpublished results from our laboratory indicated that mouse sera obtained after infection or vaccination with *B. abortus* strains RB51 and 2308 recognized many *O. anthropi* antigens.

O. anthropi strain 47237 was originally isolated from soil and it is sensitive to almost all the common antibiotics. *O. anthropi* strain 47237 does not carry a plasmid but can be easily transformed with the broad-host-range plasmid pBBR1MCS allowing the expression of a variety of *Brucella* antigens (this study). *Brucella* Cu/Zn SOD is a protective *Brucella* antigen (9, 186, 241). *E.coli* expressing *Brucella* Cu/Zn SOD induced significant protection in mice against virulent *B. abortus* strain 2308 infection (186). Mice immunized with purified *Brucella* Cu/Zn SOD (9) or SOD synthetic peptides (241) developed significant protection against virulent strain 2308 infection in mice. Mice vaccinated with *Brucella* RB51 overexpressing homologous Cu/Zn SOD also stimulated enhanced protection (257). Since *O. anthropi* has many antigens that cross-react with *Brucella*, we hypothesized that intact *O. anthropi* strain 49237 alone or with expressing *Brucella* Cu/Zn SOD would provide protection against virulent *Brucella* infection. In this study it was demonstrated that *O. anthropi* or recombinant *O. anthropi* expressing SOD does not protect mice against *Brucella* challenge. Nevertheless, if the immune response is switched to a Th1 type of response by co-administrating a CpG adjuvant, significant protection against *Brucella* infection is achieved.

RATIONALE AND HYPOTHESIS

Due to the high genetic relatedness between *O. anthropi* and *Brucella*, it is reasonable to hypothesize that both bacteria induce similar immune responses in animal hosts. *O. anthropi* may induce strong immune responses to some of the common epitopes,

this in turn might stimulate resistance against virulent *Brucella* infection. This cross-protection strategy has been used in the development of many vaccines against bacterial or viral pathogens (57, 81, 109, 115, 120, 140, 177, 202).

Since *O. anthropi* strain 49237 has no native plasmid but can be electroporated with a broad-host range plasmid pBBR1MCS, it is possible to express *Brucella* protective antigens in *O. anthropi* strain 49237. If *O. anthropi* strain 49237 alone could not induce protection against virulent *B. abortus* challenge infection, it was hypothesized that a recombinant *O. anthropi* strain 49237 expressing *Brucella* protective antigens would induce protection (this study). If *O. anthropi* strain 49237 alone induces the protection, the recombinant strain may induce better protection. It was further hypothesized that if the recombinant *O. anthropi* strain expressing protective *Brucella* antigens induces protection against *Brucella* infection, the immune response induced against these protective antigens may be very similar, if not identical, to the immune response induced by these antigens in *Brucella*. On the other hand, if protection was not achieved, the immune response to these antigens must be different. *O. anthropi* would be an excellent model for the study of the precise immune mechanisms induced by selected *Brucella* antigen(s).

Beside the protective *Brucella* antigen SOD, two *Brucella* heat shock proteins GroEL and GroES were used in this study. *Brucella* GroES and GroEL stimulate good immune responses at least in the mouse model (145, 179, 213). However, it is still unknown if they could induce protective immunity and therefore be protective antigens. Large amounts of *Brucella* GroES and GroEL were expressed in *O. anthropi* strain 49237 with using the pBBR1MCS plasmid. They would be considered protective antigens if their expression in recombinant *O. anthropi* increased the protection against virulent *Brucella* challenge.

MATERIALS AND METHODS:

Bacterial strains and growth conditions. *O. anthropi* type strain 49188 and strain 49237 were purchased from ATCC (Betheda, MD); strain 3301 was kindly provided by Dr. Moriyon (Pamplona, Spain). *Brucella abortus* strains 2308 and RB51 were from Dr. Gerhardt Schurig's culture collection. All bacteria were grown in Tryptic soy broth (TSB) or on Tryptic soy agar (TSA) plates. Chloramphenicol at the concentration of 30 μg/ml was added to the broth or agar while culturing bacteria containing the broad-host-range plasmid pBBR1MCS (136).

Detection of antibiotic susceptibility and plasmids existence To assess *O. anthropi* strain susceptibility, thirteen antibiotics were chosen to run the standard diffusion test (233) as well as dilution test (234). Tests were carried out in the Clinical Bacteriology Laboratory, Veterinary Teaching Hospital, Virginia-Maryland Regional College of Veterinary Medicine. To detect the plasmid existence in the three *O. anthropi* strains, plasmid isolation was carried out using QIAprep Spin Miniprep kit (Qiagen, Valencia,

CA). Plasmids were resuspended in distilled water, and electrophoresed in a 0.8% agarose gel containing 0.5 μg/ml of ethidium bromide.

Reagents, antigens and antisera. Phosphothioate-modified CpG motif was custom synthesized by Genosys Biotechnologies, Inc. (The Woodlands, Texas). The sequence was 5'-TCC AT**G ACG TT**C CTG ATG CT-3' (the bold letters depict the active motif). *Brucella* Cu/Zn SOD was purified by ion exchange chromatography as reported previously (26, 257). Goat anti-*B. abortus* RB51 and goat anti-Cu/Zn SOD serum were available in our laboratory. *E. coli* strain DH5α (GIBCOBRL, Gaithersburg, MD) was used for cloning the necessary plasmid constructs. Heat-inactivation of live *B. abortus* or *O. anthropi* strains was carried out by incubating sealed tubes with bacteria in 65 ^{0}C water-bath for 30 min.

Electron Microscopy J774.A1 cells were cultured to full confluency in one 25 cm^2 tissue culture flasks and infected with live *O. anthropi* strain 49237 (1 x 10^8 CFUs/ml, 5 ml/flask) for 1 h at 37^{0}C in 5% CO_2 in air. Complete DMEM medium containing 50 μg/ml gentamicin was then used to rinse off the extracellular bacteria three times and used for further culturing. The flask of cells was fixed at 5 h post-washing in general fixative (5% glutaraldehyde, 4.4% formaldehyde, 2.75% picric acid in 0.05% sodium cacodylate buffer). A transmission electron microscope (JEOL 100 CX II) was used to visualize *O. anthropi* within J774.A1 as previously described (117).

Construction of plasmids containing genes for *Brucella* SOD, and/or GroEL and GroESThe genes for *B. abortus* Cu/Zn SOD (*sodC*), GroES (*groES*) and GroEL (*groEL*) were initially obtained from a pUC19 genomic library of *B. abortus* strain 2308. As shown in Fig. 2.xA, a 1.1 kb DNA fragment containing the *sodC* gene and its promoter sequence was excised with *Cla* I restriction enzyme from recombinant plasmid pBAII-3 (Latimer E, et al, 1992) and subcloned into pBBR1MCS (136). The resulting plasmid was designated pBBSOD (257) (Fig. 2.1.A). Similarly, a 4.5 kb *Sma* I-*Hpa* I DNA insert containing the *groES* and *groEL* genes and their common promoter was excised from pBA2131 and cloned into the *Sma* I site of pBBR1MCS. This plasmid was designated pBBGroESL (Fig. 2.1.B). The 1.1 kb *Cla* I insert from pBBSOD was excised and then cloned into *Cla* I site of pBBGroESL and the resulting plasmid was denominated pBBSOD/GroESL.

Construction of recombinant *O. anthropi* 49237 strains expressing *Brucella* SOD, GroEL and GroES *E. coli* strain DH5α was initially transformed with pBBSOD, pBBGroESL or pBBSOD/GroESL. A colony of *E. coli* containing pBBSOD, pBBGroESL or pBBSOD/GroESL was selected on a TSA plate containing chloramphenicol at a concentration of 30 μg/ml. The expression of *Brucella* Cu/Zn SOD, GroES or GroEL was confirmed by Western blot analysis (257).

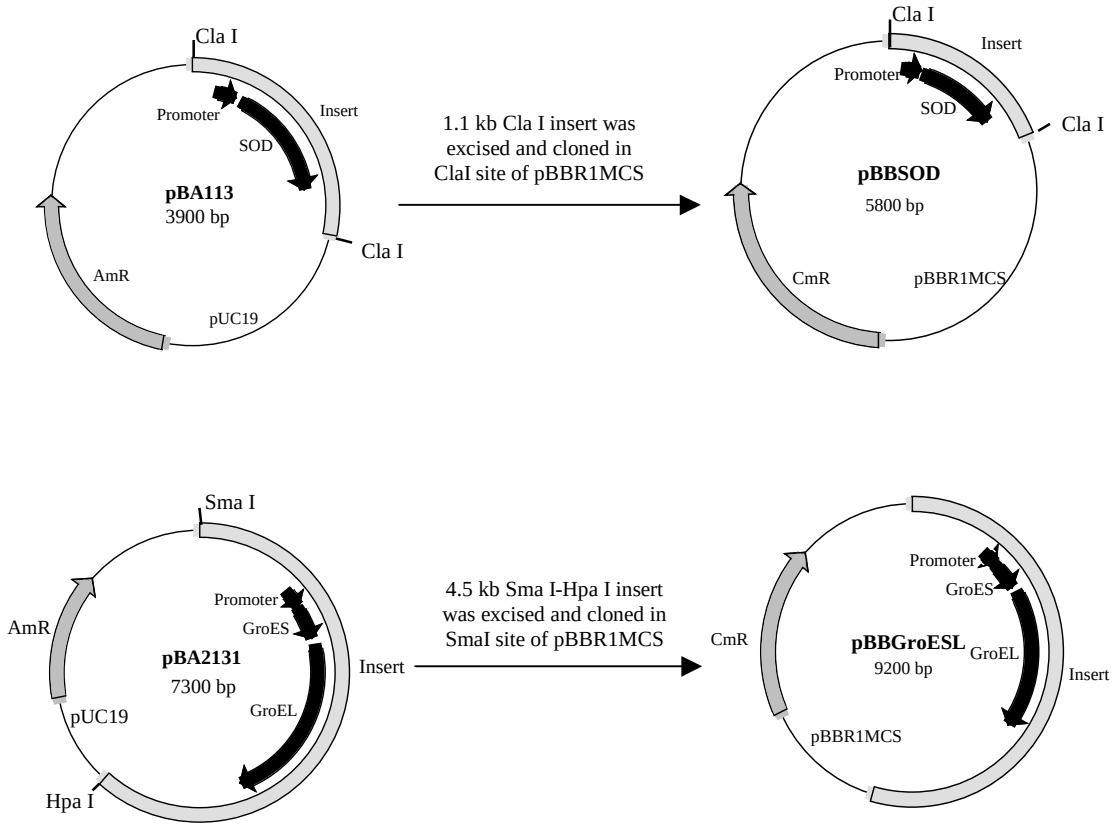


Fig. 2.1. Construction maps of plasmids of pBBSOD and pBBGroESL for overexpression of Cu/Zn SOD (A) and GroES and GroEL (B) in *O. anthropi* strain 49237. The inserts containing these genes were excised from the pBA113 (SOD) and pBA2131 (GroES and GroEL) regions and subcloned into the broad-host range plasmid pBBR1MCS. The resulting recombinant plasmids were denominated pBBSOD and pBBGroESL. The 1.1 kb Cla I insert was also excised and cloned in Cla I site of pBBGroESL. The resuting plasmid was denominated pBBSOD/GroESL.

The pBBSOD, pBBGroESL and pBBSOD/GroESL were then isolated from *E. coli* and electroporated into electro-competent *O. anthropi* strain 49237 according to a protocol described previously for *Brucella* (26). Colonies of the *O. anthropi* strain 49237 containing pBBSOD, pBBGroESL or pBBSOD/GroESL (denominated *O. anthropi* strains 49237SOD, 49237GroESL, and 49237SOD/GroESL, respectively) were selected from TSA plates containing chloramphenicol at a concentration of 30 μg/ml. The expression of *Brucella* Cu/Zn SOD, GroES or GroEL by these recombinant *O. anthropi* strains was confirmed by Coomassie blue staining of SDS-PAGE gels of whole recombinant *O. anthropi* 49237 bacterial extracts and by Western blot analysis with goat anti-*B. abortus* RB51 and goat anti-Cu/Zn SOD sera.

Survival of *O. anthropi* strain 49237 and *O. anthropi* strain 49237SOD in mice. 6-weeks-old female BALB/c mice (Charles River Laboratories, MA) were used. Groups of 15 mice each were inoculated intraperitoneally (i.p.) with approximately 4×10^8 CFU of *O. anthropi* strain 49237, *O. anthropi* strain 49237SOD, or strain 49237SOD plus CpG adjuvant. The exact CFUs of injected bacteria were determined retrospectively by plating serial dilutions of the bacterial suspensions used for infection on TSA plates or chloramphenicol containing TSA plates (257). The CpG adjuvant was inoculated at a dose of 10 nmol at 4 hours before and again at the time of injection with strain 49237SOD. At 1, 5, and 16 days postinoculation, 5 mice from each group were killed by CO_2 asphyxiation. Their spleens were collected and the bacterial CFUs per individual spleens were determined by plating the serial dilutions of the spleen homogenates on TSA plates as well as on chloramphenicol containing TSA plates (223).

Protection experiment in mice. To determine whether *O. anthropi* 49237, *O. anthropi* 49237pBB, *O. anthropi* 49237SOD, *O. anthropi* 49237GroESL, or *O. anthropi* 49237SOD/GroESL could stimulate protection against virulent *Brucella* infection in mice, 4 different protection experiments were performed. In experiment one, groups of 8 mice were inoculated i.p. with saline (negative control), *B. abortus* strain RB51 (2×10^8 CFU/mouse, positive control), recombinant *B. abortus* strain RB51SOD (2×10^8 CFU/mouse, positive control), *O. anthropi* 49237 (5×10^8 CFU/mouse) and recombinant strains *O. anthropi* 49237pBBR1MCR (5×10^8 CFU/mouse), 49237SOD (5×10^8 CFU/mouse), 49237GroESL (5×10^8 CFU/mouse), and 49237SOD/GroESL (5×10^8 CFU/mouse). At 2, 4, and 6 weeks after inoculation 5 mice from each group were bled retroorbitally, sera were obtained and stored at -40°C until use in ELISA or Western blot analysis. Six weeks after inoculation, 5 mice from each group were challenged i.p. with 2×10^4 CFU/mouse of virulent *B. abortus* 2308. The mice were killed 2 weeks later, spleens were collected and homogenized, and dilutions were plated to determine the numbers of *Brucella* CFUs per spleens (223). The remaining 3 unchallenged mice from each group were killed 6-8 weeks post-inoculation and spleen cells collected for *in vitro* cell culture work.

Experiment two was carried out to determine whether multiple injections of *O. anthropi* 49237SOD were required for protection. Groups of 7 mice were immunized i.p. with saline, *B. abortus* strain RB51 or *O. anthropi* 49237SOD using the same doses

described in Experiment 1. Two weeks later, mice inoculated with *O. anthropi* 49237SOD were re-immunized with *O. anthropi* 49237SOD (5 x 10^8 CFU/mouse). All groups of mice were challenged at 6 weeks after the first immunization and killed 2 weeks later.

A third experiment was carried out to determine whether different doses of *O. anthropi* 49237SOD influenced the results of protection. Four groups of 5 mice each were inoculated with *O. anthropi* 49237 in 4 different doses: 5 x 10^8 CFU/mouse, 5 x 10^7 CFU/mouse, 5 x 10^6 CFU/mouse, and 5 x 10^5 CFU/mouse. An additional group of five mice was inoculated i.p. with saline as the negative control. All mice in Experiment 2 & 3 were challenged and killed following the protocol of experiment 1.

In experiment 4, synthetic CpG-containing oligonucleotides (CpG-ODN) was administered as an immunostimulatory adjuvant. Groups of 8 mice were inoculated i.p. with *O. anthropi* strains 49237 (5×10^8 CFU/mouse) alone, 49237 (5×10^8 CFU/mouse) with CpG adjuvant, 49237SOD (5×10^8 CFU/mouse) alone and 49237SOD (5×10^8 CFU/mouse) with CpG adjuvant. The CpG adjuvant was administered as described previously under *O. anthropi* survival experiments. As controls, three groups of mice were inoculated with saline alone, CpG-ODN alone or *E. coli* DH5α (1 x 10^6 CFU/mouse) with CpG-ODN. Mice were bled at 2, 4, and 6 weeks after inoculation. At six weeks after inoculation, 5 mice from each group were challenged with strain 2308 and two weeks later killed for protection study as described above. The remaining 3 unchallenged mice were used for cell-mediated immune response detection. This experiment was repeated three times.

Serological tests. Colony immunoblot assay and Western blotting were performed according to the previously published procedures (257). An indirect ELISA was performed to measure the isotypes of specific anti-*Brucella* Cu/Zn SOD antibodies in the sera of mice. *Brucella* Cu/Zn SOD purified by ion exchange chromatography (257) was adsorbed to wells of polystyrene plates (Nunc Maxisorp) at a concentration of 1 μg SOD/well in 50 μl of bicarbonate buffer (pH 9.6). After overnight incubation at 4^oC, plates were blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (pH 7.4) for 2 h at room temperature. Mice serum samples at 1:100 dilution were added to wells in duplicate and incubated for 3 h at room temperature. The plates were washed three times with PBS containing 0.05% Tween-20. Isotype specific goat anti-mouse horseradish peroxidase conjugated (ICN Pharmaceuticals, Inc.) antisera were added for 30 min at room temperature, plates were washed for three times, 100 μl of TMB substrate solution (KPL, Gaithersburg, MD) was added and incubated in dark for 20 min. The reaction was stopped by adding 100 μl/well of 0.18M sulfuric acid, and the absorbance of the developed color was measured at 450 nm.

Splenocyte cultures and quantitation of interferon-γ (IFN-γ) and IL-4. Mice were killed by CO_2 asphyxiation and their spleens removed under aseptic conditions. Single spleen cell suspensions were prepared from the spleens according to the standard procedures (223). Red blood cells were lysed with ACK solution (150 mM NH_4Cl, 1mM

$KHCO_3$, 0.1 mM Na_2EDTA [pH 7.3]). The splenocytes were cultured in 96-well flat bottom plates at a concentration of 5×10^5 viable cells/well in the presence of heat-inactivated RB51, *O. anthropi* strain 49237 or 49237SOD bacteria equivalent to 10^6 CFUs, 1 μg of purified SOD, 1 μg of concanavalin A (ConA) or no additives (unstimulated control). RPMI 1640 medium (GIBCO BRL) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, and 50 μM of penicillin/streptomycin was used for culturing the splenocytes. The cells were cultured for 5 days and the plates were centrifuged at $250 \times g$ for 10 min. The clear culture supernatants were transferred to a new 96-well plate and stored at -70°C until ELISA was performed to determine the IFN-γ concentrations (254). For detection of IL-4 in culture supernatants, cells were cultured with the different stimulants for 4 days at which time 50 ng of phorbol myristate acetate (PMA) per ml and 1 μM ionophore were added (229). Supernatants were collected 16 hours after stimulation with PMA-ionopnore. The IFN-γ and IL-4 levels in culture supernatants were detected by sandwich ELISA as previously described using purified recombinant mouse IFN-γ or IL-4 as standard (254). All assays were performed in triplicate. The concentration of IFN-γ or IL-4 in the culture supernatants was calculated by using a linear-regression equation obtained from the absorbance values of the standards.

Statistical analysis. The CFUs data in the protection study were analyzed by performing analysis of variance with unequal variance model. The mixed procedure of SAS was used (147). Single degree of freedom contrasts were used to test specific hypotheses of interest. The student's t-test was performed in bacterial clearance study and the Ig isotype study using *O. anthropi* strain 49237SOD with or without CpG-ODN.

RESULTS

Close relationship between RB51 and three *O. anthropi* strains The cross-reaction between *B. abortus* RB51 and *O. anthropi* strains 3301, 49188 and 49237 was clearly demonstrated by Western blot analysis using the goat hyperimmune serum against strain RB51 (Fig. 2.2). This serum not only detected many strain RB51 antigens but also detected many *O. anthropi* antigens at similar sizes, strongly illustrating their antigenic similarity.

Antibiotic susceptibility of *O. anthropi* strains As shown by Table 2.1, *O. anthropi* strains 49188 and 3301 are resistant to many antibiotics while *O. anthropi* strain 49237 is susceptible to almost all antibiotics. Because of this, only *O. anthropi* strain 49237 was considered a suitable vaccine candidate since a live bacterium that is resistant to a wide spectrum of antibiotics would be a poor choice.

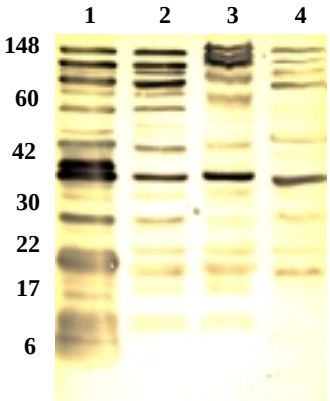


Fig. 2.2 Close relationship between *B. abortus* RB51 and three *O. anthropi* strains shown by Western blot analysis. The primary antibody was a goat hyperimmune serum against *B. abortus* RB51. Antigens: Lane 1 – RB51, lane 2 – *O.anthropi* strain 3301, lane 3 – *O. anthropi* strain 49188, lane 4 – *O. anthropi* strain 49237.

Table 2.1. Antibiotic Susceptibility of different *O. anthropi* strains.

	O. anthropi 49188	*O. anthropi* 49237	*O. anthropi* 3301
Amikacin	S	S	S
Ampicillin	R	I	R
Carbenicillin	R	S	R
Ceftiofur	R	S	R
Cephalothin	R	S	R
Chloramphenicol	R	S	R
Clavamox	R	S	R
Enrofloxacin	S	S	S
Gentamicin	S	S	S
Neomycin	S	S	I
Sulfamethoxazole with Trimethoprim	S	S	S
Tetracyclin	S	S	S
Ticarcillin	R	I	R

* The results of antimicrobial suscptibility were obtained through the standard diffusion test as well as dilution test. R -- resistant, S -- susceptible, I -- intermediate (or moderately susceptible).
* Strain 49237 contains no plasmids.

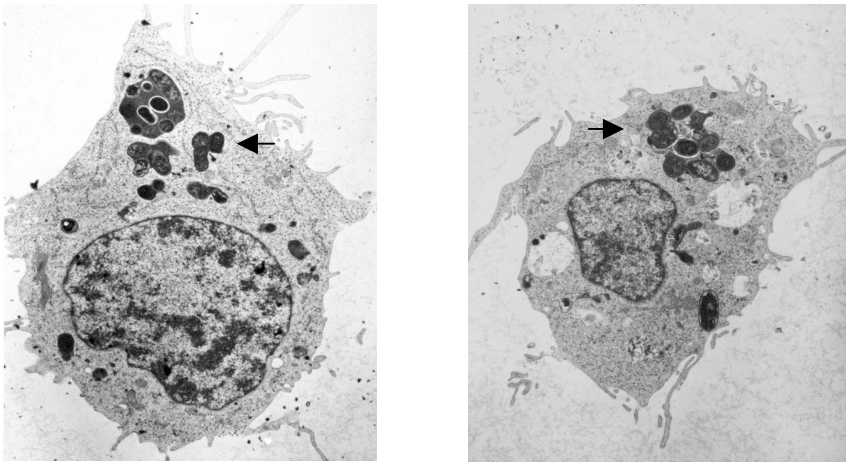
***O. anthropi* strain 49237 invades macrophages** Our electron microscopy showed that *O. anthropi* strain 49237 invaded macrophage J774.A1 cells for at least 6 h (Fig. 2.3). J774.A1 cells were originally derived from BALB/c mouse and had been used previously in *Brucella* studies employing electron microscopy (117, 262). *O. anthropi* strain 49237 bacteria were found inside macrophages individually or in a package.

Plasmid existence in *O. anthropi* strains Our studies showed that *O. anthropi* strain 49237 does not contain any native plasmid but both *O. anthropi* strain 49188 and 3301 have one or several plasmid(s) (data not shown). Since plasmids usually contain antibiotics-resistant gene(s), the results are consistent with the antibiotic susceptibility results. No further work was pursued along this line.

No cross-reactive O antigen expression in *O. anthropi* strains Since the O antigen of *Brucella* LPS induces protective antibodies in the mouse model (69, 159, 223), we tested for possible cross-reactivity between *O. anthropi* O antigen and *Brucella* O antigen using monoclonal antibody Bru38 which is specific for *Brucella* O antigen (223). *O. anthropi* strains 49237, 49237pBB and 49237pBBSOD and *Brucella* strain RB51 did not react with the monoclonal antibody against *Brucella* O antigen, while *Brucella* smooth strain 2308 reacted strongly with the monoclonal antibody in Western blots as expected (Fig.2.4) (223). Western blot analysis using polyclonal antibodies against *Brucella* strain 2308 and containing anti-O chain antibodies also indicated that *O. anthropi* 49237 or the recombinant *O. anthropi* 49237SOD did not have cross reactive O antigens (data not shown). Antisera to *O. anthropi* did not react with *Brucella* O-chain (data not shown).

Construction of the recombinant *O. anthropi* 49237 strains and their expressions of *Brucella* Cu/Zn SOD and/or GroES and GroEL The *Brucella sodC* and/or *gro*ESL genes were successfully subcloned into pBBR1MCS. The recombinant plasmids pBBSOD, pBBGroESL and pBBSOD/GroESL were successfully electroporated into *O. anthropi* 49237 to form recombinant *O. anthropi* strains 49237pBBSOD, 49237pBBGroESL and 49237pBBSOD/GroESL. The expressed *Brucella* Cu/Zn SOD, GroES and GroEL were visualized by Coomassie blue staining (Fig. 2.5.A) and Western blot analyses (Fig. 2.5.B and C). It was shown that the *Brucella* Cu/Zn SOD, GroES and GroEL were proteins expressed by recombinant *O. anthropi* 49237 strains while *O. anthropi* strain 49237 alone did not express any detectable Cu/Zn SOD or *Brucella* GroES or GroEL.

***O. anthropi* strain 49237 and recombinant *O. anthropi* 49237SOD failed to stimulate protection against virulent *Brucella* infection.** In Experiment 1, mice immunized with *O. anthropi* strains developed strong antibody responses to *O. anthropi* and *Brucella* antigen extracts at 4 and 6 weeks postimmunization as detected by Western blot analysis and indirect ELISA (data not shown). *O. anthropi* 49237SOD also stimulated a strong antibody response to purified *Brucella* SOD while other strains did not (Fig. 2.6).



(A) (B)

Fig. 2.3. Electron microscopy of J774.A1 macrophages exposed to viable *O. anthropi* strain 49237 bacteria. After J774.A1 cells grew to full confluency in one 25 cm^2 tissue culture flask, *O. anthropi* strain 49237 was used to infect them. Extracellular bacteria were rinsed off after 1 h and new medium with 50 μg/ml gentamicin was added and the flask was re-incubated for 5 h. The flask of cells was then fixed and transmission electron microscopy was carried out. Two individual cells (A and B) are shown; arrows indicate the presence of *O. anthropi* inside the macrophages.

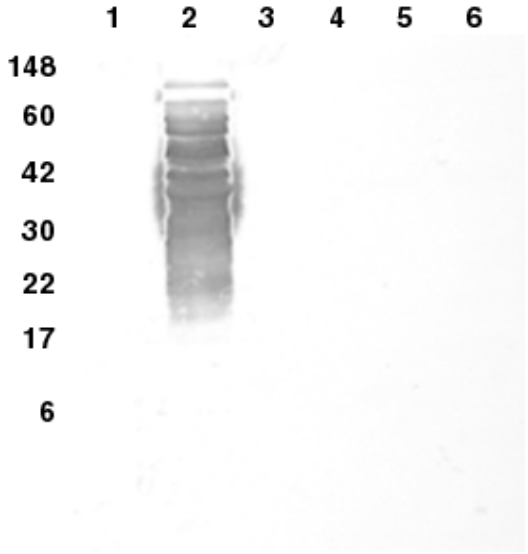
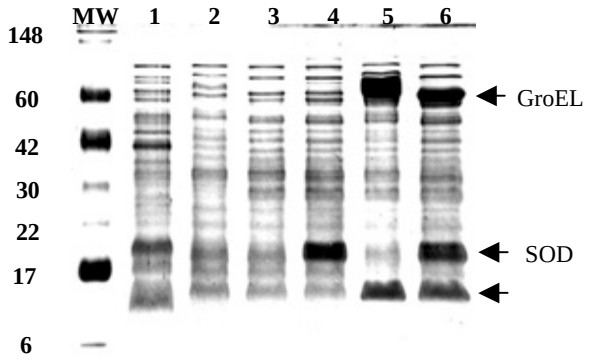
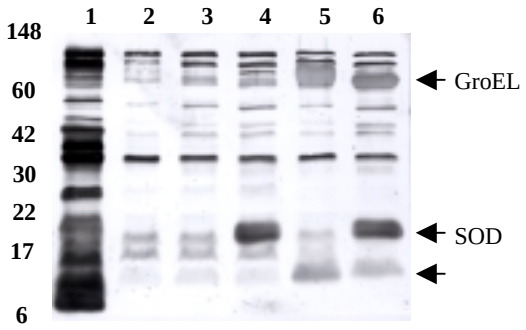


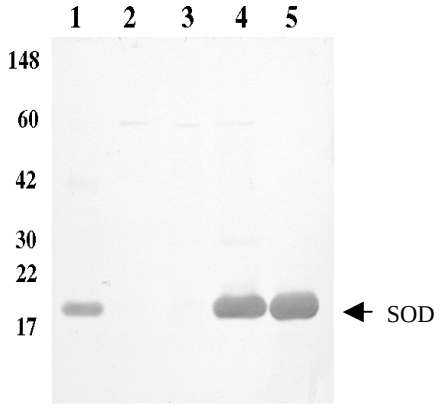
Fig. 2.4. Western blot analysis of O chain cross-reaction between *B. abortus* and *O. anthropi* strains. The extracted bacterial antigens were: lane 1 -- *B. abortus* strain RB51, lane 2 -- *B. abortus* strain 2308, lane 3 -- *O. anthropi* strain 49237, lane 4 – *O. anthropi* strain 49237SOD, lane 5 – *O. anthropi* strain 49188, Lane 6 – *O. anthropi* strain 3301. Bru 38, monoclonal antibody against *B. abortus* O chain, was used as the primary antibody.



(A)



(B)



(C)

Fig. 2.5. Coomassie blue staining of SDS-PAGE and Western blot analysis. (A). Coomassie blue staining of molecular weight marker (WM), *B. abortus* strain RB51 (1), *O. anthropi* strain 49237 (2), *O. anthropi* strain 49237pBB (3), and *O. anthropi* strain 49237pBBSOD (4), *O. anthropi* strain 49237pBBGroESL, and *O. anthropi* strain 49237pBBGroESL/SOD. (B). Western blot analyses of expression of *B. abortus* Cu/Zn SOD and GroESL in *O. anthropi* strain 49237 using goat anti-RB51 serum containing anti-SOD, anti-GroES and anti-GroEL antibodies. (B) and mouse anti-SOD (C) as the primary antibody. The antigens used in (B) were the same as those in (A). The first 4 lanes of antigens used in (C) were the same as the first 4 lanes in (A). The last lane (5) of (C) was purified *B. abortus* Cu/Zn SOD.

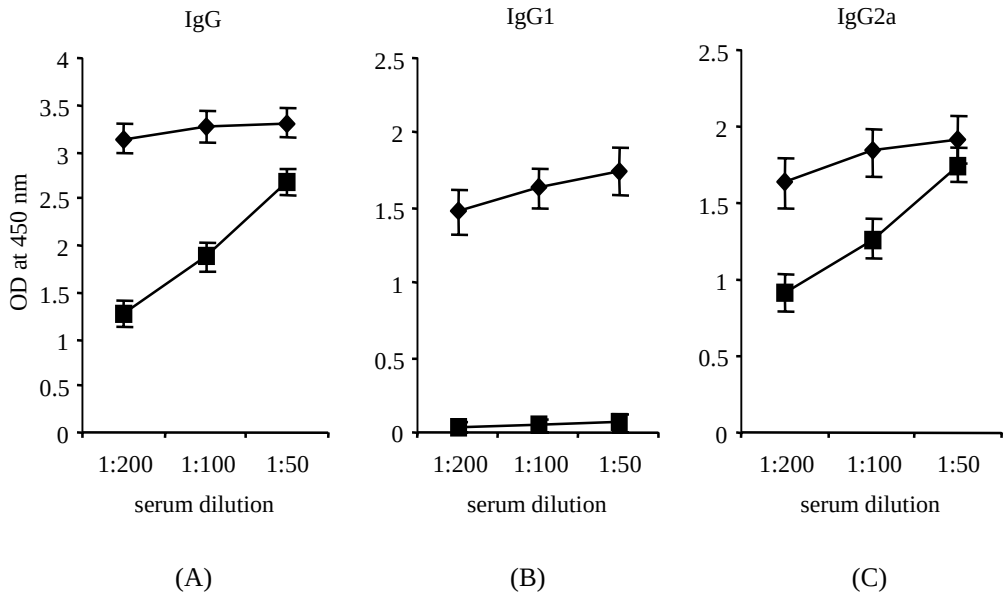


Fig 2.6. Comparison of IgG, IgG1 and IgG2a responses to purified *Brucella* Cu/Zn SOD by *O. anthropi* strain 49237SOD (—◆—) and *Brucella* RB51SOD (—■—) immunizations. Groups of 5 mice were immunized i.p. with saline, *O. anthropi* strain 49237 or 49237SOD, *Brucella* RB51 or RB51SOD as indicated previously. Mice were bled individually at 6 weeks after immunization. 96 - well plates were coated with purified *Brucella* Cu/Zn SOD and three dilutions (1:200, 1:100 and 1:50) of serum samples were used for detection of IgG, IgG1 and IgG2a responses to purified *Brucella* Cu/Zn SOD by indirect ELISA as shown. Each serum sample was tested in duplicates. Saline, *O. anthropi* strain 49237 or *Brucella* RB51 did not induce detectable antibody responses to purified *Brucella* Cu/Zn SOD (Data not shown)

Mice immunized with *B. abortus* strain RB51 demonstrated 1.13 Log_{10} of protection (when compared to mice immunized with saline alone, $P < 0.01$), and mice immunized with *B. abortus* strain RB51SOD induced 3.0 Log_{10} of protection ($P < 0.01$) (Table 2.2). However, none of *O. anthropi* strains provided protection against *B. abortus* strain 2308 challenge (Table 2.2). After the first mice experiment, *O. anthropi* strain 49237SOD was further analyzed because *Brucella* Cu/Zn SOD is a known protective antigen. In Experiment #2, mice were immunized twice with *O. anthropi* 49237SOD (5×10^8 CFU/mouse), the booster being two weeks after the first immunization; no protection was obtained (Table 2.2). Since dose might play an important role in directing the development of a predominantly Th1 or Th2 immune responses (22, 49, 94), Experiment 3 was carried out to study dose effect on protection; none of the doses used induced protection (Table 2.2). Indirect ELISA demonstrated that *O. anthropi* 49237SOD stimulated a mix of IgG1 and IgG2a responses to SOD while strain RB51SOD stimulated exclusively an IgG2a response (Fig. 2.6). Western blot analyses confirmed that *O. anthropi* 49237SOD induced both IgG1 and IgG2a immune responses to *Brucella* Cu/Zn SOD and many other *Brucella* and *O. anthropi* antigens (Fig. 2.7). In addition, *O. anthropi* 49237 or *O. anthropi* 49237SOD stimulated both *in vitro* IFN-γ and IL-4 production while strains RB51 and RB51SOD stimulated only IFN-γ production *in vitro* (data not shown). Later experiments showed these same results (see Fig. 2.9 and Fig. 2.10). The data suggest that a mix of Th1 and Th2 responses were induced by immunization with *O. anthropi* and that the Th2 responses might be interfering with protection. It was hypothesized that switching the observed mixed Th1 and Th2 responses to a predominantly Th1 response could make *O. anthropi* 49237SOD stimulate protection against virulent *Brucella* infection. Our strategy for switching the immune response to a Th1 response was to use the CpG DNA adjuvant.

***O. anthropi* strain 49237 and *O. anthropi* strain 49237SOD plus CpG-ODN stimulated significant protection against virulent *Brucella* infection.** In Experiment 4, vaccination of mice with CpG-ODN alone, *E. coli* plus CpG-ODN, *O. anthropi* 49237 alone, or *O. anthropi* 49237SOD alone could not stimulate significant protection as compared to the saline inoculated controls. However, co-administration of the immunostimulatory CpG-ODN with *O. anthropi* 49237 or 49237SOD stimulated significant protection ($P < 0.01$) (Table 2.3). *O. anthropi* strain 49237SOD with CpG-ODN stimulated significantly better protection than strain 49237 with CpG-ODN ($P < 0.05$), further confirming that SOD was a protective *Brucella* antigen under the right immune response direction.

Addition of CpG-ODN helped develop an IgG2a predominant antibody response against *Brucella* Cu/Zn SOD. Mice vaccinated with *O. anthropi* strain 49237SOD developed specific antibodies to the *B. abortus* Cu/Zn SOD antigen which were detectable as early as 2 weeks post-vaccination and the antibody levels increased at 4 and 6 weeks (data not shown). *O. anthropi* strain 49237 immunization did not develop any antibody response to the SOD antigen as expected (data not shown).

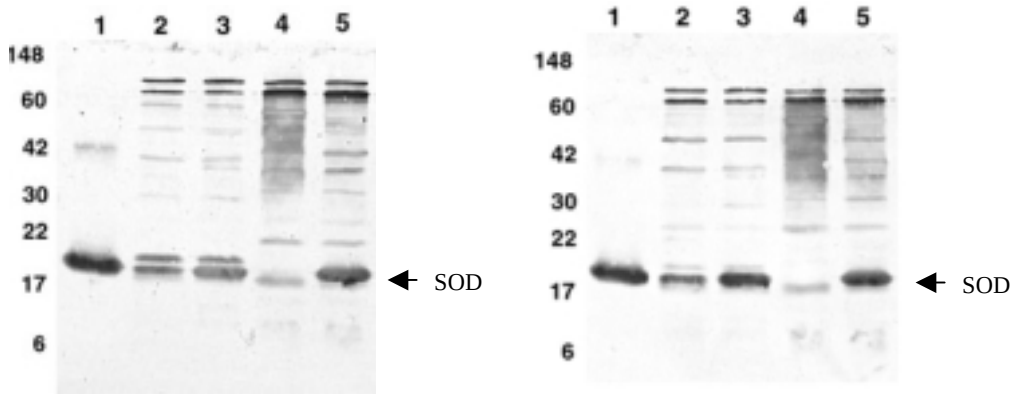
Table 2.2. Protection studies in experiment 1,2, and 3.

Exp. No.	Treatment group	mice No.	Vaccines and dose (CFUs)	Log10 strain 2308 in spleen (x ± SD)	P value
1[a]	1	5	Saline	5.37 ± 0.19	>0.5
	2	5	RB51, 5 x 10^8	4.24 ± 0.29	<0.5
	3	5	RB51SOD, 5 x 10^8	3.01 ± 0.34	<0.01
	4	5	O.a 49237, 5 x 10^8	5.27 ± 0.33	>0.5
	5	5	O.a 49237pBB, 5 x 10^8	5.40 ± 0.21	>0.5
	6	5	O.a 49237SOD, 5 x 10^8	5.29 ± 0.19	>0.5
	7	5	O.a 49237GroESL, 5 x 10^8	5.29 ± 0.13	>0.5
	8	5	O.a 49237SOD/GroESL, 5 x 10^8	5.39 ± 0.42	>0.5
2[b]	1	7	Saline	5.50 ± 0.19	>0.5
	2	7	RB51, 5 x 10^8	4.54 ± 0.32	<0.5
	3	7	O.a 49237SOD, 5 x 10^8 (2 immunization**)	5.28 ± 0.44	>0.5
3[c]	1	5	Saline	5.43 ± 0.24	>0.5
	2	5	RB51, 5 x 10^8	4.31 ± 0.27	<0.5
	3	5	O.a 49237SOD, 5 x 10^8	5.37 ± 0.26	>0.5
	4	5	O.a 49237SOD, 5 x 10^7	5.51 ± 0.41	>0.5
	5	5	O.a 49237SOD, 5 x 10^6	5.28 ± 0.32	>0.5
	6	5	O.a 49237SOD, 5 x 10^5	5.42 ± 0.23	>0.5

[a] In experiment 1, 8 groups of 5 mice were injected i.p. with saline, RB51, RB51SOD, *O. anthropi* 49237, *O. anthropi* 49237pBB, *O. anthropi* 49237SOD, *O. anthropi* 49237GroESL, or *O. anthropi* 49237SOD/GroESL. At 6 weeks after injection, mice were challenge-infected i.p. with 3 x 10^4 CFU/mouse of virulent *B. abortus* 2308. Two weeks after challenge infection, the mice were sacrificed and the CFUs in their spleens were determined.

[b] In experiment 2, 3 groups of 7 mice were injected i.p. with saline, RB51, RB51SOD, or *O. anthropi* 49237SOD. Two weeks later, mice inoculated with *O. anthropi* 49237SOD were re-immunized with the same dose of *O. anthropi* 49237SOD. All mice were challenged at 6 wks after the first immunization.

[c] In experiment 3, 6 groups of 5 mice were injected i.p. with saline, RB51, or three different doses of *O. anthropi* 49237SOD. Mice were challenged at 6 weeks after injection.



(A) (B)

Fig. 2.7. Western blot analyses of IgG1 as well as IgG2a antibodies in the antisera to *O. anthropi* strain 49237SOD. Lane 1: Purified SOD antigen; lane 2: RB51; lane 3: RB51SOD; lane 4: *O. anthropi* 49237; lane 5: *O. anthropi* 49237SOD. The primary antibody was mice antisera to *O. anthropi* 49237SOD. The secondary antibodies were goat anti-mouse IgG2a (A) and goat anti-mouse IgG1 (B). *O. anthropi* 49237SOD induced both IgG1 and IgG2a immune responses to *Brucella* SOD and many other *Brucella* and *O. anthropi* antigens.

Table 2.3. Protection study; experiment 4.

Groups	Treatment	(CFU ± SE) per spleen
1	Saline	130000 ± 21000
2	*E. coli* + CpG-ODN	130000 ± 23000
3	CpG-ODN	130000 ± 20000
4	*O.a.* 49237	136000 ± 26000
5	*O.a.* 49237 + CpG-ODN	34000 ± 14000
6	*O.a.* 49237SOD	137000 ± 23000
7	*O.a.* 49237SOD + CpG-ODN	810 ± 480

* 6 groups of 5 mice were injected i.p. with (1) Saline, (2) *E.coli* DH5α (10^6 CFU/mouse) + CpG-ODN, (3) CpG-ODN alone, (4) *O. anthropi* (*O.a.*) 49237 (5 x 10^8 CFU/mouse), (5) *O. anthropi* 49237 (5 x 10^8 CFU/mouse) + CpG-ODN, (6) *O. anthropi* 49237SOD (5 x 108 CFU/mouse), (7) *O. anthropi* 49237 (5 x 10^8 CFU/mouse) + CpG-ODN. At 6 weeks after injection, mice were challenge-infected i.p. with 3 x 10^4 CFU/mouse of virulent *B. abortus* 2308. Two weeks after challenge infection, the mice were sacrificed and the CFUs in their spleens were determined. To calculate different contrasts, the P value of group 1 verse groups 2, 3, 4 or 6: $P > 0.05$; Group 1 verse group 5 or 7: $P < 0.01$; Group 4 verse group 5, group 6 verse group 7: $P < 0.01$; Group 5 verse group 7: $P < 0.05$. This experiment was repeated three times with similar results.

It was found that addition of CpG-ODN to the vaccination protocol with strain 49237SOD significantly suppressed the SOD-specific IgG1 response ($P < 0.01$) (Fig. 2.8.B); the SOD-specific IgG and IgG2a levels were not significantly changed (Fig. 2.8A and C).

Addition of CpG-ODN to the vaccination protocol helped increase IFN-γ secretion and suppressed IL-4 secretion. Splenocytes from *O. anthropi* strain 49237 or 49237SOD immunized mice produced IFN-γ upon stimulation with heat-inactivated *O. anthropi* strain 49237 or 49237SOD bacteria; however, splenocytes from mice immunized with *O. anthropi* strain 49237 or 49237SOD in combination with the CpG-ODN produced significantly higher levels of IFN-γ upon stimulation with the same antigens ($P < 0.05$) (Fig. 2.9). When stimulated *in vitro* with heat-inactivated strain 49237SOD, splenocytes from strain 49237SOD plus CpG-ODN immunized mice induced significantly higher IFN-γ production than splenocytes from strain 49237 plus CpG-ODN immunized mice ($P < 0.05$) (Fig. 2.9). Heat-inactivated strain RB51 antigen stimulated minimum amount of IFN-γ in splenocytes from mice immunized with bacteria only but stimulated high amount of IFN-γ in splenocytes from mice immunized with bacteria in combination with CpG (Fig. 2.9). More specifically, *in vitro* stimulation of splenocytes with purified SOD produced significantly higher IFN-γ levels when derived from mice immunized with *O. anthropi* strain 49237SOD plus CpG-ODN than when obtained derived from mice immunized with strain 49237SOD only (Fig. 2.9).

Since IL-4 cytokine levels in supernatants were too low to detect, PMA and ionophore were used to trigger IL-4 production in the last 16 hours of culturing as described previously (229). *O. anthropi* 49237 or 49237SOD could stimulate high level of IL-4 production by splenocytes *in vitro* if derived from *O. anthropi* 49237 or 49237SOD vaccinated mice. Addition of CpG-ODN to the vaccination protocol significantly decreased the production of IL-4 ($P < 0.01$). Interestingly, *in vitro* stimulation of splenocytes with heat inactivated strain RB51 did not lead to the production of IL-4 when derived from *O. anthropi* 49237 or 49237SOD vaccinated mice with or without CpG adjuvant. Addition of *Brucella* Cu/Zn SOD to splenocytes *in vitro* could induce high amount of IL-4 production if splenocytes were derived from mice immunized with strain 49237SOD, but none if the splenocytes were derived from mice immunized with strain 49237SOD plus CpG-ODN (Fig. 2.10).

Bacterial persistence. To investigate whether there were any differences in the virulence characteristics of *O. anthropi* 49237 and 49237SOD with or without CpG adjuvant, the bacterial clearance of the immunized mice was assessed. As shown in Table 2.4, the CpG adjuvant did not influence the survival status of *O. anthropi* strain 49237SOD ($P > 0.05$). Although the spleens of mice in different groups were free of any bacteria by 16 days post-inoculation, it demonstrated that *O. anthropi* strain 49237 was eliminated faster than strain 49237SOD ($P < 0.01$), suggesting that SOD facilitated the survival of *O. anthropi* strain 49237 and might be a virulence factor in *O. anthropi* strains.

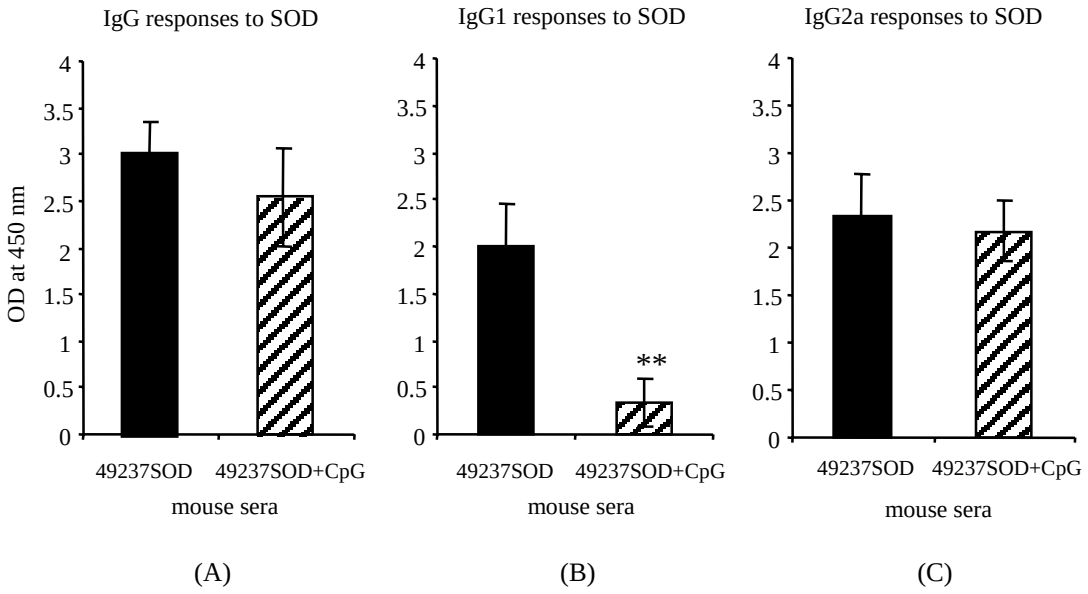


Fig. 2.8. Antibody isotype responses to purified *Brucella* Cu/Zn SOD protein stimulated by *O. anthropi* strain 49237SOD with (■) or without (□) CpG DNA adjuvant. Groups of 5 mice were immunized i.p. with saline, or with *O. anthropi* strain 49237SOD with or without CpG DNA adjuvant. Mice were bled individually at 5 weeks after immunization. 96 - well plates were coated with purified *Brucella* Cu/Zn SOD and serum samples of 1:100 dilution were used for detection of IgG (A), IgG1 (B) and IgG2a (C) responses to purified *Brucella* Cu/Zn SOD by indirect ELISA as shown previously. Each serum sample was tested in duplicates. Significant difference was shown only in the production level of IgG1 between vaccination with *O. anthropi* strain 49237SOD and strain 49237SOD plus CpG-ODN (B) ($P < 0.01$).

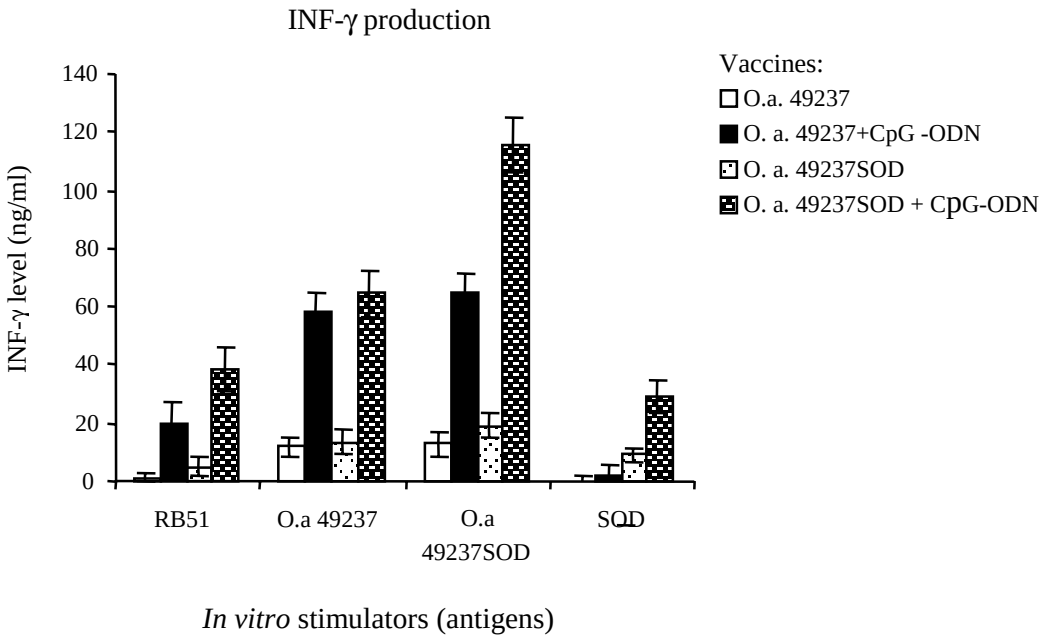


Fig. 2.9. INF-γ production *in vitro* by splenocytes from immunized mice upon stimulation with different antigens. BALB/c mice were inoculated i.p. with *O. anthropi* strain 49237 (5 x 10^8 CFU/mouse) and recombinant strain 49237SOD 49237 (5 x 10^8 CFU/mouse) with or without CpG adjuvant as described. Mice were killed after 6 weeks. 5 x 10^5 of splenocytes were isolated and cultured in 96-well plates in triplicate with stimulation of heat-inactivated *B. abortus* strain RB51 (10^6 CFU/well), *O. anthropi* strains 49237 (10^6 CFU/well) or 49237SOD (10^6 CFU/well). After 5 days supernatants were collected and tested for INF-gamma production by sandwich ELISA.

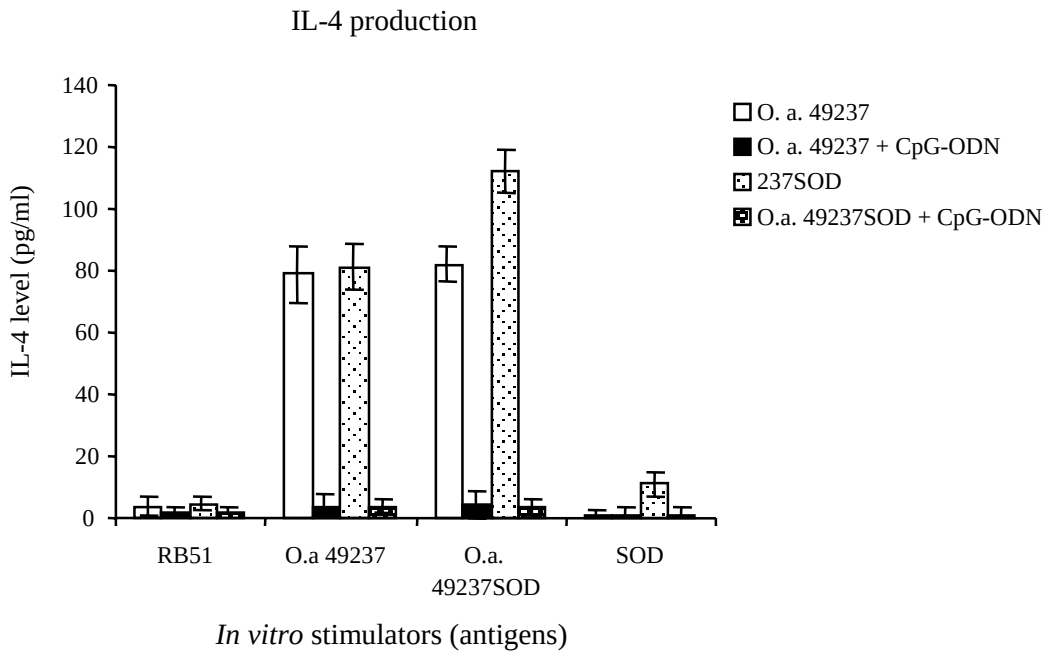


Fig. 2.10. IL-4 production *in vitro* by splenocytes from mice upon stimulation with different antigens. BALB/c mice were inoculated i.p. with *O. anthropi* strain 49237 (5 x 10^8 CFU/mouse) and recombinant strain 49237SOD (5 x 10^8 CFU/mouse) with or without CpG adjuvant as described. Mice were killed after 6 weeks. 5 x 10^5 of splenocytes were isolated and cultured in 96-well plates in triplicate with stimulation of heat-inactivated *B. abortus* strain RB51 (10^6 CFU/well), *O. anthropi* strains 49237 (10^6 CFU/well) and 49237SOD (10^6 CFU/well). After 5 days supernatants were collected and tested for IL-4 production by sandwich ELISA.

Table 2.4: Clearance of *O. anthropi* 49237containing pBBR1MCS plasmid, *O. anthropi* strain 49237 and recombinant strain 49237SOD with or without CpG adjuvant from mice spleen[a].

No. of Days Postinfection	Mean $\log_{10}$CFU/spleen ± SD in				
	49237pBB[a]	49237	40237+CpG	49237SOD	49237SOD+CpG
5	4.08 ± 0.36	3.27 ± 0.36	3.59 ± 0.26	4.91 ± 0.33	4.74 ± 0.51
8	2.06 ± 0.49	2.01 ± 0.24	2.11 ± 0.22	2.89 ± 0.26	2.70 ± 0.35
14	-[b]	-	-	-	-

[a] Infection doses, 3.6 x 10^8 (log=8.56) for *O. anthropi* 49237, 2.2 x 10^8 (log=8.34) for *O. anthropi* 49237SOD, given intraperitoneally to each of 5 mice per group.

[b] -, the infection had cleared to undetectable level (CFU/spleen < 20, or $\log_{10}$CFU/spleen < 1.3).

*** N**o significant difference was found between *O. anthropi* strain 49237 and strain 49237 plus CpG-ODN ($P > 0.05$) or between *O. anthropi* strain 49237SOD and strain 49237SOD plus CpG-ODN ($P > 0.05$) at 5 or 8 days post-infection. However, strain 49237 (or 49237 plus CpG-ODN) had significant lower CFU's than strain 49237SOD (or 49237SOD plus CpG-ODN) at 5 or 8 days post-infection ($P < 0.05$). Clearance of strain 49237pBB was significantly different from all the other groups at 5 days post-infection ($P < 0.05$) but only different from strain 49237SOD and strain 49237SOD plus CpG-ODN ($P < 0.05$).

DISCUSSION

In summary, the co-administration of CpG-ODN with *O. anthropi* strains 49237 and 49237SOD stimulated a Th1 immune response characterized by a high IgG2a/IgG1 ratio, increased IFN-γ production and decreased IL-4 production. The results indicated that CpG-ODN switched the somewhat mixed Th1 and Th2 immune responses to a predominantly Th1 immune response when added to the *O. anthropi* vaccination protocol. This was particularly true with the SOD antigen. Addition of CpG-ODN to strain 49237 or 49237SOD leads to protective immunity.

The most important observation of this study is the protection induced by *O. anthropi* expressing *Brucella* SOD against a *Brucella* challenge when CpG-ODN is added to the vaccination protocol. A T helper-1 response is usually needed for protection against intracellular pathogens such as *Leishmania major* or others (274). Th2 immune responses may interfere with Th1 responses and block the protection against virulent infection (274). It has been known that the Th-1 response and CMI in general are critical in protection against *Brucella* infection (253, 255, 257).

O. anthropi strain 49237 shows many cross-reactive antigens with *Brucella*. However, neither strain 49237 nor recombinant 49237SOD expressing protective *Brucella* antigen SOD stimulated protection against virulent *Brucella* infection. It was found that strain 49237 stimulated both Th1 and Th2 immune responses to the SOD antigen, characterized by mixed IgG1 and IgG2a antibody isotype responses and both IFN-γ and IL-4 responses. This Th2 immune response might interfere with the protective response offered by an exclusive Th1 immune response. It was therefore hypothesized that the recombinant *O. anthropi* strain 49237SOD would stimulate protection against virulent *Brucella* infection if the immune response could be switched to an exclusive Th1 response. This was achieved by the use of CpG-ODN that is known to favor Th1 responses. The *in vivo* injection of the DNA adjuvant with antigen can skew an antigen-specific response to a Th1 immune response (39, 40, 274). In this study, switching to a Th1 response was achieved by adding CpG-ODN to the immunizing procedures. After switching to a predominantly Th1 response, both *O. anthropi* strains 49237 and 492237SOD provided protection against *Brucella* challenge. Recombinant strain 49237SOD gave better protection than strain 49237. These results suggested that the cross-reactive antigens in *O. anthropi* can stimulate protection against *Brucella* infection if they induce the right immune reactions but that expression of *Brucella* SOD increases the protection. It is also clear from our studies that the broad-host-range plasmid pBBR1MCS replicates in the *O. anthropi* system and that the *O. anthropi* strain 49237 readily expresses *Brucella* proteins (e.g., *Brucella* Cu/Zn SOD, GroES, GroEL) using *Brucella* promoters.

The CpG motifs have been widely studied as vaccine adjuvant (60, 104, 137). Synthetic DNA motifs containing an unmethylated CpG dinucleotide flanked by two 5' purines (optimally GpA) and two 3' pyrimidines (optimally TpC or TpT) mimic the

immunostimulatory effects of bacterial DNA. Due to CpG methylation and CpG suppression in eukaryotic genomes, these sequence motifs are 20 times more common in prokaryotic than eukaryotic DNA (68, 131). Bacterial DNA or synthetic CpG-containing oligonucleotides (CpG-ODN) stimulate an innate immune response characterized by activation of B cells, T cells, and macrophages that proliferate, secrete antibodies, and produce Th1 cytokines such as IFN-γ and TNF-α. Together with protein-based antigens, the *in vivo* injection of CpG-containing oligonucleotides (CpG-ODN) also induces a Th1-dominant antigen-specific immune response. CpG-ODN can even switch antigen-specific Th2-dominant immune response to Th1-dominant immune response through increased secretion of IL-12 and IFN-γ (40). It was found that CpG-ODN triggered protective Th1 responses in lethal Th2-driven *Leishmania major* infection (274). CpG-ODN were even curative when injected as late as 20 days after lethal *L. major* infection, indicating that CpG-ODN reverted an established Th2 response (274). Previous studies suggested that CpG motifs in the bacterial plasmid backbone of DNA vaccines were required for vaccine effectiveness (132, 218). It is therefore not surprising that CpG-ODN changed the immune response to *O. anthropi* to a Th1 response and consequently immunization led to protection since Th1 responses are required for anti-*Brucella* immunity.

It appears that co-administration of CpG-ODN with different antigens can increase IgG2a/IgG1 ratio by different ways. Co-administration of live *Leishmania major* with CpG-ODN induced decreased *L. major*-specific IgG1 protection and increased IgG2a protection (274). Immunization with IFA-HEL (incomplete Freud's adjuvant - hen egg lysozyme) plus CpG-ODN enhanced anti-HEL IgG2a but did not decrease high level of anti-HEL IgG1 production (40). Our study showed that co-administration of *O. anthropi* strain 49237SOD with CpG-ODN significantly decreased the anti-SOD IgG1 level indicating a Th2 to Th1 switch although it did not increase the IgG2a levels significantly.

O. anthropi strain 49237SOD induced an average of 2.1 $\log_{10}$ protection against *Brucella* infection when co-administrated with CpG-ODN. In 3 separate experiments, *B. abortus* strain RB51 only induced an average of 1.07 log10 protection against *Brucella* infection. Although in separate experiments, control animals in protection experiments with *O. anthropi* SOD + CpG and strain RB51 presented similar infection levels. This suggests that vaccination with *O. anthropi* SOD + CpG is more effective than immunization with strain RB51. The experiments gave additional evidence that *Brucella* Cu/Zn SOD is a protective antigen since good protection levels were reached when the antigen-specific immune response was of the correct Th1 type. Although SOD is a protective antigen, vaccination with strain RB51 does not stimulate an antibody response nor a cell-mediated immune response to SOD (257) probably due to its low expression level in strain RB51. However, overexpression of SOD by strain RB51 results in higher protection and induction of both antibody and CMI responses to SOD.

Cu/Zn SOD is an enzyme involved in protecting cells from exogenous sources of superoxide, such as the oxidative burst of phagocytes (96). It is therefore often regarded as a virulence factor in many bacterial pathogens. Although overexpression of SOD in

attenuated *B. abortus* strain RB51 does not alter the virulence characteristics of strain RB51 (257), this study found that expression of SOD in *O. anthropi* extends the *in vivo* survival of the organism although, both *O. anthropi* strain 49237 and its recombinant strain expressing *Brucella* Cu/Zn SOD were cleared within 2 wks post-inoculation. This suggests that *Brucella* SOD can be considered a mild virulence factor under the right conditions. Even so, the fast clearance of the recombinant *O. anthropi* makes this organism a potentially good, highly attenuated *Brucella* vaccine.

Production of IFN-γ during a Th1 response is very important for protection against virulent *Brucella* infection (80, 117, 266, 267, 271). IFN-γ activates macrophages and enhances their bactericidal effects through production of reactive oxygen intermediates (76, 118) and nitrogen intermediates (76, 97, 118). The nitric oxide (NO)-mediated killing possibly plays a role in *Brucella* killing but, this role appears to be more important in *B. suis* than in *B. melitensis* or *B. abortus* (76, 97, 118). IFN-γ also stimulates development of Th1 cells. It was shown that addition of IFN-γ *in vitro* inhibits *Brucella* replication in macrophages (117) and injection of recombinant murine IFN-γ *in vivo* increased resistance of mice to infection with *B. abortus* (238). When endogenous IFN-γ was depleted with anti-IFN-γ monoclonal antibody, increased numbers of *B. abortus* were found in the spleens and livers of infected mice (269). IL-4 is typically produced by Th2 lymphocytes and can down-regulate the production of a Th1 response. It was shown that vaccination with live *Brucella* induced IFN-γ-producing $CD4^+$ Th1 cells while vaccination with soluble *Brucella* proteins induced IL-4-producing $CD4^+$ Th2 cells (271). Furthermore, IFN-γ-producing $CD4^+$ Th1 cells from immunized donor mice were able to mediate resistance against virulent *Brucella* challenge, but IL-4-producing $CD4^+$ Th2 cells from immunized mice failed to provide resistance (271). *O. anthropi* strain 49237 or 49237SOD immunization was unable to induce protection and stimulated both antigen-specific IFN-γ and IL-4 production. The data suggest that the IL-4-producing Th2 response to *Brucella* antigens can interfere with the IFN-γ-producing Th1 response and prevent protection. On the other hand, as demonstrated in our study by the addition of CpG-ODN, once the response is devoid of IL-4 and has an enhanced IFN-γ component, protection is induced.

The failure of protection stimulated by either strain 49237 or recombinant 49237SOD might be due to the incorrect vaccination doses. For instance, 10^6 CFU/mouse of recombinant *E. coli* expressing the same *Brucella* SOD used in this study stimulated protection in BALB/c mice but not higher or lower doses (186). However, four different doses of strain 49237SOD were used in this study and no protection was observed with any of them. In addition, two immunizations with strain 49237SOD did not provide any protection against *Brucella* infection. Since *O. anthropi* strain 49237 and recombinant *O. anthropi* strain 49237SOD stimulated a strong antibody response as early as 2 weeks postimmunization, booster immunization were not expected to change protection results significantly.

The cross-protection strategy has been widely used in the development of vaccines against bacterial pathogens. Many attenuated bacteria, e.g., *Brucella* vaccine strain RB51

(255), *Mycobacterial* vaccine strain Bacille Calmette-Guerin (BCG) (140), *Salmonella typhimurium* (57), and *E. coli* (81), have been successfully manipulated as vaccine vectors to express foreign antigens specified by cloned genes from other pathogens. Many bacteria can be used directly as vaccines against different bacterial pathogens using the cross protection strategy. For instance, active immunization of either the Smith diffuse strain of *Staphylococcus aureus* or a type Ia strain of group G *streptococci* protected against challenge by either the homologous or heterologous bacterial strains (115). However, our study is the first report to our knowledge in which a cross-reactive bacterium was used as a vaccine vector to express a protective antigen of a pathogenic bacterium and induced protection against the virulent pathogen only after switching to an antigen specific Th1 response.

CHAPTER THREE

THE ROLE OF CYTOTOXIC LYMPHOCYTES INDUCED BY *BRUCELLA ABORTUS* STRAIN RB51 VACCINATION

INTRODUCTION

Brucella abortus is a gram-negative, facultative intracellular bacterial pathogen that causes brucellosis in humans and cattle (51). In the infected host, *B. abortus* multiplies within the phagosomes of phagocytic cells (e.g., macrophages) by inhibiting the phagosome-lysosome fusion (Corbel MJ, 1997). Similar to most of the intracellular bacterial infections, cell-mediated immunity (CMI) seems to play a critical role in protection against virulent *Brucella* infection. Protective CMI is usually stimulated by live attenuated vaccines (257), such as *B. abortus* strain RB51, an attenuated stable rough vaccine currently used in the USA and many other countries as the vaccine of choice against bovine brucellosis. Strain RB51 induces immune protection against challenge of virulent *B. abortus* solely by CMI, as indicated by the fact that strain RB51 induced immunity can only be passively transferred by immune T cells and not by antibodies in the mouse model (120).

The classic CTL assay uses the release of ^{51}Cr from lysed target cells as an indicator. Unfortunately, the assay involves the use of radioisotope, is expensive to run and is not a very sensitive assay (62, 194). For health and environmental reasons there is a trend to replace radioactive assays with non-radioactive assays. Basically, in the ^{51}Cr-release assay, target cells are labelled with ^{51}Cr before the lysis event and lysis of target cells is determined by measuring released ^{51}Cr. Among different non-radioactive CTL assays, the neutral red uptake assay has been used effectively to analyze cytolytic activity induced by many viruses and bacteria including *Listeria monocytogenes*, a facultative intracellular parasite like *Brucella* (62, 127, 129). The main fact which permits the use of neutral red in CTL assays is that the neutral red dye can only be taken up by live macrophages but not by lymphocytes. In contrast to the ^{51}Cr assay, the unlysed target cells are labeled after the activity of the effector cells has been carried out; cells are then artificially lysed with a detergent and relative light absorbance is used to determine the numbers of unlysed cells left (194). The neutral red uptake assay for *B. abortus* strain RB51-induced CTL activity was standardized and found that more sensitive, faster, cheaper, and safer than the classic ^{51}Cr-release assay.

In this study a macrophage cell line J774.A1 as the antigen-presenting cell was used. The J774.A1 macrophage cell line originated from a tumor of a BALB/c female mouse. It has been widely used in brucellosis research (117, 118, 262). When compared to the progress of *in vivo* infections in mice, the J774.A1 cells closely predicted the kinetics of infection (262). J774.A1 cells have been widely used as antigen presenting cells for CTL assays in other systems involving bacteria, viruses, and tumors (89, 161,

193, 249). In this study, we used live strain RB51 and strain 2308-pulsed J774.A1 macrophages as target cells.

It is suspected that cytotoxic T cells play a crucial role in the protective CMI response to facultative intracellular parasites. In this study it was demonstrated that RB51 can induce strong *in vivo* cytotoxic T lymphocytes (CTL) activity in the mouse brucellosis model. The specific anti-*Brucella* cytolytic activity was mainly exerted by $CD3^+CD8^+$ T cells derived from strain RB51-immunized mice but, $CD3^+CD4^+$ T cells secreted high amount of IFN-γ and were able to induce certain levels of specific and non-specific lysis of *Brucella* infected target cells. NK cells appeared not to play a major role.

RATIONALE AND HYPOTHESIS

B. ABORTUS strain RB51 vaccination in mice is a good model for studying and understanding the induction of CMI against virulent *Brucella* and potentially other intracellular bacterial infections. This is because strain RB51 vaccination induces protective immunity in mice and the antibody response does not play a role in the acquired, specific resistance (10). Recent studies carried out in our laboratory indicated that protection induced by strain RB51 vaccination is preceded by a Th1 type immune response characterized by high IFN-γ but no IL-4 production and high specific IgG2a but no or low IgG1 responses (255-257). Since a Th1 immune response usually invokes CTL, it is reasonable to hypothesize that strain RB51 induces antigen-specific CTL activity in the mouse model, which should be able to lyse *Brucella*-infected macrophages. Both *B. abortus* strains RB51 and 2308 infected macrophages should be lysed, the CTL activity would be exerted mainly by $CD8^+$ cells and a neutral red assay can be a useful assay for detection of CTL activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Brucella abortus* strains 2308 and RB51 were supplied by Dr. Gerhardt Schurig (Virginia Tech). *Listeria monocytogenes* strain was obtained from Dr. P. Elzer (Department of Veterinary Sciences, Lousiana State University, Baton Rouge, LA). All bacteria were grown in either Tryptic soy broth (TSB) or Tryptic soy agar (TSA) plates as described in Chapter 2.

Vaccination of mice Six-week-old BALB/c female mice were inoculated intraperitoneally (i.p.) with 4×10^8 CFU of *B. abortus* strain RB51 or the same volume of saline (negative control) as described in Chapter 2. The mice were killed at 6 wks post-immunization by CO_2 inhalation. These mice served as splenocyte sources for the CTL assays using the *in vitro* stimulation step. For "residential CTL assays", the *in vitro* stimulation step was omitted. Instead, the strain RB51-vaccinated mice were boost-injected i.p. with 2×10^8 CFUs of strain RB51 at 6 wks post-first immunization. As control, saline-injected mice were re-injected with saline at the same time. Two weeks post-booster the mice were killed.

Cell lines J774.Al macrophage cell line was purchased from the American Type Culture Collection (ATCC). The cells were cultured in complete tissue culture medium (c-DMEM) consisting of DMEM (ATCC, Manassas, VA) with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA). The A/Sn mouse lymphoma YAC-1 cell line was also purchased from ATCC (ATCC TIB-160). This cell line is sensitive to the cytotoxic activity of NK cells of mice (79, 263). Therefore, YAC-1 cells were used as target cells in the NK cytotoxicity assay. YAC-1 cells were cultured in complete RPMI medium (c-RPMI) consisting of RPMI 1640 (GIBCO BRL) with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2mM of L-glutamine (MediaTech, VA) and 10 mM HEPES buffer (Sigma, St. Louis, MO).

Relationship of macrophage cell number and uptake of neutral red dye. One 75 cm^2 tissue culture flask with confluent J774.A1 macrophage growth was infected with live *B. abortus* strain RB51 at a ratio of 1:100 (cells : RB51) for 4-5 hours. The medium was then discarded and extracellular bacteria were rinsed away with c-DMEM containing 50 μg/ml of gentamicin. Another flask of normal macrophages without *Brucella* was prepared as control. The macrophages were scraped off with a sterile rubber policeman and centrifuged at 200 x g for 5 min. Trypan blue exclusion was used to count macrophage cell numbers and determinate viability (47). The infected and normal macrophages were distributated in triplicate to wells of a 96-well round-bottom cell culture plate (Corning, NY) at numbers ranging from 10,000 to 70,000 and were allowed to adhere to the bottom at 37^0C for 4 h. A working solution of neutral red was prepared by diluting a 1% (w/v) stock solution to a 0.036% working solution in warm phosphate-buffered saline solution (PBS, pH7.1-7.2) just prior to use. A 200 μl aliquot of the diluted neutral red was added to each well containing macrophages. After incubation at 37^0C, 5% CO_2 for 30 min the dye was discarded and the macrophage monolayers were washed for three times with warm PBS and then lysed with 0.22 ml of 0.05 M acetic acid-0.05% SDS solution (lysis buffer). OD was measured with an ELISA microplate reader (Molecular Devices, Sunnyvale, CA) at the wave length of 570 nm. The linear relationship between macrophage cell number and uptake of neutral red dye was determined using Microsoft Excel Software.

Cytotoxicity assay An overview for the colorimetric CTL assay procedure is depicted by the flow chart in Fig. 3.1. Splenocytes from normal or strain RB51-vaccinated mice were passed through a nylon wool column to enrich for T cells. The eluted, T cell enriched splenocytes were co-cultured with strain RB51-pulsed, mitomycin C-treated J774.A1 stimulator cells for 5 days. The cocultured cells were collected and centrifuged on an histopaque (Sigma-Aldrich, St Louis, MO) column to separate the live effector T cells. The live effector cells were obtained from the interface and washed, counted and viability established by trpan blue exclusion. The target cells were strain RB51-pulsed J774.A1 macophages.

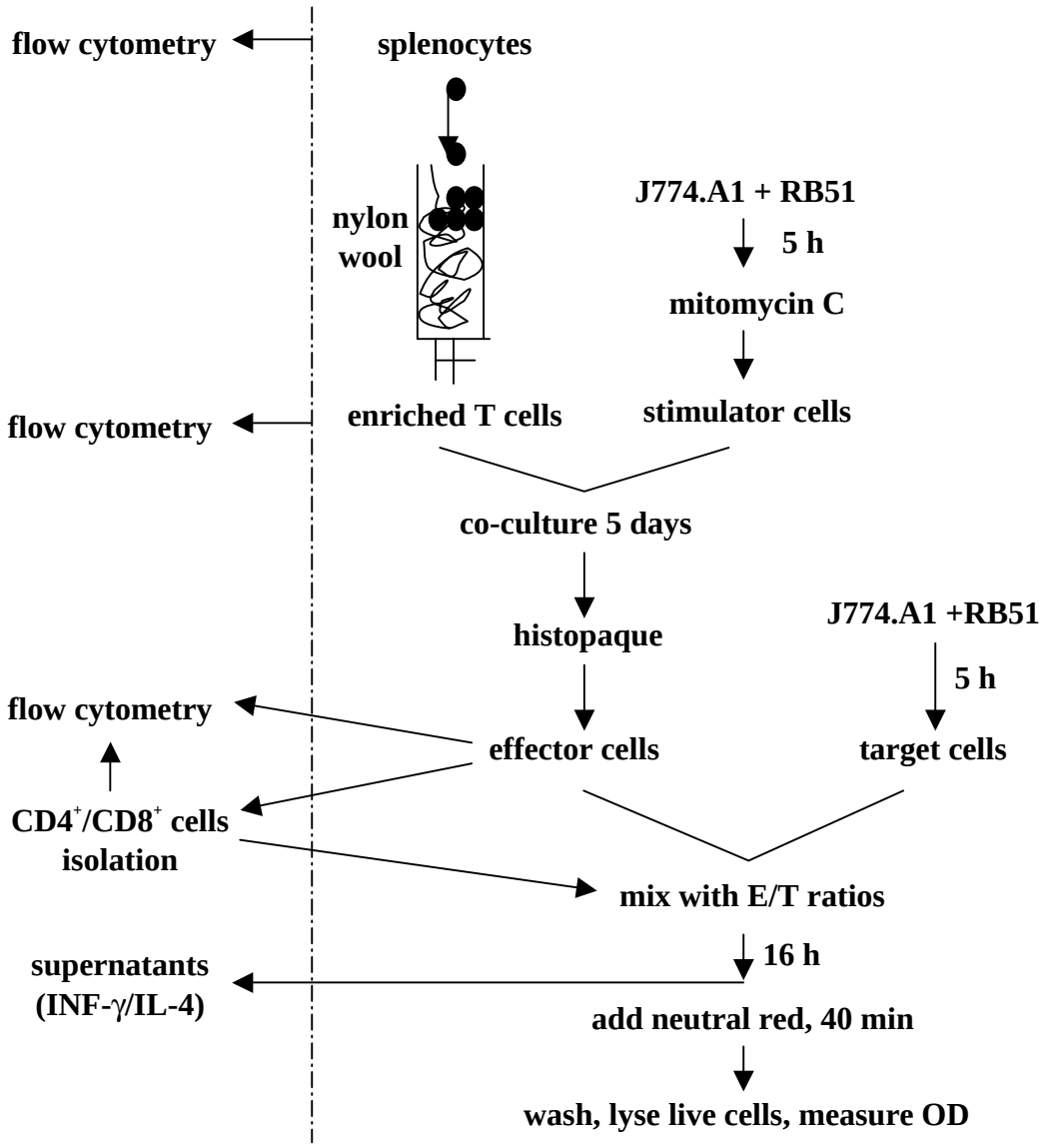


Fig. 3.**1.** Flow Chart of the neutral red uptake CTL assay. At 6-8 wks after vaccination, splenocytes from normal and vaccinated mice were pooled and passed through nylon wool columns to enrich T cells. The enriched T cells were co-cultured with RB51-pulsed, mitomycin C-treated J774.A1 macrophages (stimulator cells) for 5 days. Cells were then collected and live effector CTL were collected using histopaque column. The effector cells were mixed with RB51—pulsed J774.A1 target cells and incubated for 16 h. Neutral red was finally used to stain live target cells and OD readings were calculated for lysis levels. Flow cytometry was used to analyze different cell populations at different stages. Sandwich ELISA was used to detect INF-g and IL-4 secretion from the co-culture of effector and target cells. The different roles of CD4+ and CD8+ in the lysis reaction was also analyzed by first isolating the two populations by a magnetic kit.

The effector cells and target cells were mixed in different ratios (E/T ratios) and co-cultured for 16 h in 24-well cell culture plates. During the co-culturing period, target cells could be lysed by the effector T cells. The supernatants were discarded eliminating non-attached cells, the neutral red dye was added as described before and the cells were further incubated for 40 min with the dye. The neutral red is taken up only by live, infected or non-infected target macrophages but not by the remaining live effector cells or dead cells. Three-time gentle washes with warm PBS (pH7.2) were then performed to wash out extracellular neutral red dye. After wash, live cells were lysed by the lysis buffer and the uptake of neutral red dye was measured by ELISA microplate reader. The ^{51}Cr-release assay was also performed using the same E/T ratios to compare it to the neutral red uptake assay. The diffference among the two assays is that ^{51}Cr is added to the target cells before they are mixed with the effector cells. A gamma counter was used to measure the release of ^{51}Cr by lysed target cells.

Effector cells were also used to count and/or isolate $CD4^+$ and $CD8^+$ T cells to analyze their different roles in the cytolytic reaction. Flow cytometry was used to analyze different cell populations at different stages and the sandwich ELISA was used to analyze cytokine levels in supernatants resulting from the co-culture of the effector cells and the target cells. CTL assays were carried out with a) lymphocytes obtained from mice immunized once with strain RB51 and stimulated *in vitro* with mitomycin C treated macrophages, and b) with lymphocytes obtained from mice immunized twice with strain RB51; this assay is referred to as the "residential CTL assay". In residential CTL assays, the effector cells were the enriched T cells without the *in vitro* stimulations.

Preparation of target cells and stimulator cells Macrophage J774.A1 cells were infected with viable strain RB51 at a ratio of 1:100 and incubated for 4-5 hours at 37^0C, 5% CO_2. The strain RB51-pulsed J774.A1 cells were washed with c-DMEM containing 50 μg/ml of gentamicin. The cells were scraped off with a sterile rubber policeman and washed twice by centrifugation at 200 x g for 5 min. The cell pellet was resuspended in c-RPMI and the cells were used as target cells after counting the cells with Trypan blue. For stimulator cells, the resuspended strain RB51-infected J774.A1 were treated with mitomycin C at a concentration of 35 μg/ml and incubated in a 37^0C water-bath for 45 min. The mitomycin C was then washed out by centrifugations for 4 times. The cells were counted, resuspended in c-RPMI containg 50 μg/ml gentamicin, and used as stimulator cells.

Preparation of effector cells. Mice were killed by CO_2 asphyxiation at 6 weeks postimmunization. The spleens were removed under aseptic conditions. Single spleen cell suspensions were prepared from the spleens according to the standard procedures (223). Red blood cells were lysed with ACK solution (150 mM NH_4Cl, 1mM $KHCO_3$, 0.1 mM Na_2EDTA [pH 7.3]). Splenocytes from strain RB51-infected or saline-inoculated mice were resuspended in 2 ml c-RPMI and passed through nylon wool columns to enrich for T lymphocytes as described previously (46) with modifications. Briefly, 1.2 g of fluffy nylon wool (DuPont) was inserted into a 10 ml syringe tube with a 3-way stopcock and the whole package was autoclaved. The sterilized nylon wool column with a 23-G needle

was clamped to a ring stand, and the nylon wool was soaked with 12 ml c-RPMI, and equilibated by standing at room temperature for at least 1 h. The splenocytes were suspended in 2ml media and laid on the top of the nylon wool with the stopcock open. After the cell suspension entered into the column, the stopcock was closed and 2 ml more media was added on the top of nylon wool. This step was repeated for 4 more times and 20 min were allowerd to let cells bind to nylon wool between steps. Finally a 18-G needle was used to replace the 23-G one and 40 ml of media were used to elute T cells into a graduated 50-ml conical tube. The cell number and viability were determined by the trypan blue exclusion test. The enriched T cells were resuspended in c-RPMI containing 50 μg/ml gentamicin and distributed to wells of 24-well cell culture plates (Corning, NY) at a concentration of 4 million viable cells/well. The stimulator cells (prepared before) were also added to the wells at a concentration of 0.4 million cells/well. The mix of enriched T cells and stimulator cells was then incubated for 5 days at 37^0C, 5% CO2. After the 5-day incubation, cells were collected and the live effector cells were obtained by removing dead cells by Histopaque (1083) centrifugation as described previously (75).

Colorimetric CTL Assay A previously described procedure (194) was followed with some modifications. Effector and target cells were mixed at various ratios and incubated for 16 hours at 37^0C. After one wash with warm phosphate-buffed saline (PBS, pH7.2-7.4), 200 μl of 0.036% neutral red solution in PBS was added to stain unlysed target cells. After 30 min, the cells were thoroughly washed and then lysed with 0.22 ml of 0.05M acetic acid-0.05% SDS solution. The amount of dye released was measured by taking OD readings at 570nm. As control for non-lysis and maximal uptake of neutral red stain, target cells were cultured alone without effector cells. The percentage of specific lysis was established applying the following formula:

$$\% \text{ specific lysis} = (\text{OD of control} - \text{OD of experimental}) / \text{OD of control} \times 100$$

^{51}Cr-release CTL Assay. Standard procedures were followed (48) with slight modifications. Briefly, target cells were mixed with 0.1 mCi ^{51}Cr (specific activity: 571 mCi/mg^{Cr}; Catalog number: 62015, ICN Pharmaceuticals, CA) in 100 μl fetal bovine serum and incubated in 37^0C water bath for 1 h. The labeled target cells were washed thereafter. Then effector and target cells were mixed at various ratios and incubated for 16 hours at 37^0C, 5% CO_2 incubator and the ^{51}Cr released into supernatants was measured in a gamma counter and specific lysis was established through the formula:

$$\% \text{ specific lysis} = (cpm_{experiment} - cpm_{spontaneous}) / (cpm_{total} - cpm_{spontaneous}) \times 100$$

Flow Cytometric analysis. Lymphocytes were incubated with both fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and phycoerythrin (PE)-conjugated anti-CD4 (or anti-CD8 or anti-Nkpan labeled) monoclonal antibodies on ice in the dark for 30 min. Control experiments consisted of staining with conjugated, isotype-matched irrelevant mAb at the same concentrations. All the mAbs were purchased from PharMingen (San Diego, CA). Cells were then washed with phosphate-buffered saline (PBS) and fixed with PBS containing 2% paraformaldehyde. Dual-color immunophenotyping of cell samples

was processed on a Coulter Epics XL/MXL flow cytometer (Hialeah, FL) (Fig. 3.1). A gated lymphocyte population was derived from a bivariate histogram display of forward and side scatter, and immunofluorescence data were analyzed with the immuno-4 software program (66, 228).

IFN-γ and IL-4 Detection. Sandwich ELISA assays were used to determine IFN-γ and IL-4 levels in the supernatants of the effector cells and the target cells co-cultures, which were collected just before adding neutral red solution (Fig. 3.1). For the ELISA detection, rat monoclonal antibodies to IFN-γ or IL-4 (ATCC, Rockville, MD) were first absorbed to wells of polystyrene ELISA plates (Nunc Maxisorp) at a concentration of 0.1 μg/well in 50 ul of coating buffer (1 x PBS, pH7.4). After overnight incubation at room temperature (RT), the plates were blocked with 2% bovine serum albumin (BSA) in 1 x PBS (pH7.4) for one hour at RT. The plates were washed three times with 50 mM Tris-HCl containing 0.2% Tween 20 (pH7.0-7.4). The culture supernatants and standard recombinant mouse IFN-γ or IL-4 (PharMingen, San Diego, CA) were added and incubated for 3 h at RT. After the plates were washed for three times, 100 μl of biotinylated rat anti-mouse IFN-γ or IL-4 (PharMingen, San Diego, CA) was added at the concentraton of 0.25 ug/ml. After the one-hour incubation at RT, the plates were washed and 100 μl of 1:4000 horseradish peroxidase (HRP) conjugated with streptavidin (Vector Laboratories, Burlingame, CA) was added to the wells and the plates were incubated at RT for half an hour. After washing, 100 ul of the TMB Microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaitherburg, MD) was added and plates were incubated in the dark for 30 min. The reaction was stopped by adding 100 μl/well of 0.18 M of sulfuric acid, and the plates were read at the absorbance at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). The assays were performed in triplicate. The concentration of IFN-γ or IL-4 in the culture supernatants was calculated by using a linear-regression equation obtained from the absorbance values of the standards as indicated by the manufacturer.

Electron Microscopy J774.A1 cells were cultured to full confluency in two 25 cm^2 tissue culture flasks and infected with live *Brucella abortus* strain RB51 (1 x 10^8 CFUs/ml, 5 ml/flask) for 4.5 h at 37^0C in 5% CO_2 in air. The complete DMEM medium containing 50 μg/ml gentamicin was used to rinse off the extracellular bacteria three times and used for further culturing. One flask of cells was fixed at 0.5 h and one at 11.5 h post-washing in general fixative (5% glutaraldehyde, 4.4% formaldehyde, 2.75% picric acid in 0.05% sodium cacodylate buffer). A transmission electron microscope (JEOL 100 CX II) was used to visualize strain RB51 within J774.A1 as previously described (117).

Magnetic cell sorting. $CD4^+$ and $CD8^+$ T cells were purified by immunomagnetic methods (153). Briefly, live T cells isolated by Histopaque column purification were incubated with MACS magnetic MicroBeads to which mAb against CD4 or CD8 molecule had been coupled (Miltenyi Biotec, Auburn, CA) at the concentration of 10 μl of MicroBeads per 10^7 total cells for 15 min in the refrigerator of 4 ^{0}C (Fig. 3.1). The cells were then washed with PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA). Following passing the cells through a steel wool column in a

magnetic field, the positive selected $CD4^+$ or $CD8^+$ T cells were eluted out. The purity of the selected $CD4^+$ or $CD8^+$ T cell population was above 92% as determined by flow cytometry using the appropriate specific monoclonal antibodies as described before.

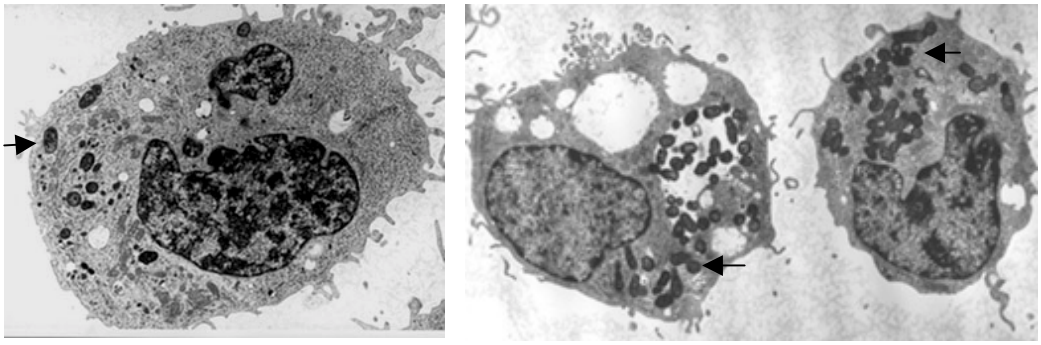
Statistical analysis. The data for the CTL lysis and cytokine secretion were subjected to the analysis of variance, and the means were compared by using Turkey's honest significant difference procedure (147).

RESULTS

***B. abortus* strain RB51 infected macrophages (EM).** In our CTL experiments, J774.A1 macrophage-like cells were used as antigen presenting cells. J774.A1 cells were originally derived from BALB/c mouse and had been used previously in *Brucella* work using electron microscopy (117, 262). We found that approximately 50% of J774.A1 cells were infected with *B. abortus* strain RB51 within 4-5 hours post-incubation (Fig. 3.2A). Large numbers of bacteria were observed if the cells were analyzed at a later stage (within 16 h) (Fig. 3.2B). Since macrophages were infected at 4-5 h post bacterial pulsing and in order to save time for our CTL assays, a 4-5 h infection period of macrophages with strain RB51 was used throughout our study. Experiments using 16 h infection periods also showed positive results in cytotoxic assays.

Linear relationship between macrophage cell number and their uptake of neutral red dye In this study, the dye neutral red was used in a colorimetric CTL assay instead of the ^{51}Cr-release assay. Although the literature clearly indicates the validity of the neutral red CTL assay (62, 127, 129, 194)], a few experiments were carried out to compare it to the ^{51}Cr-release assay using *Brucella*. The rationale of this new assay is that neutral red can be taken up by live macrophages but not by T lymphocytes (194) and there is a linear relationship between neutral red uptake and the number of macrophages present in a well (Fig. 3.3) (194). The higher the cell number, the higher the OD reading. The linear relationship was indicated by the R^2 value of near 1 for both normal and strain RB51-infected macrophages. The infection with *B. abortus* did not change the linear pattern as demonstrated by the two parallel lines (Fig. 3.3).

The cell patterns of $CD3^+CD4^+$, $CD3^+CD8^+$ T lymphocytes and NK cells at different stages of CTL assays Flow cytometry data showed that the nylon wool treatment greatly enriched $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells (Table 3.1). Both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell percentages increased and the combined cell population increased to approximately 88%. The ratio of $CD4^+$: $CD8^+$ T cells ranged from 2.3 to 2.7 (Table 3.1) and did not change much before or after nylon wool separation.



(A) (B)

Fig. 3.2. Electron microscopy (EM) of J774.A1 macrophages exposed to viable RB51 bacteria. After J774.A1 cells grew fully in two 25 cm^2 tissue culture flasks, RB51 was used to infect J774.A1 cells. Extracellular bacteria were rinsed off after 4.5 h, new medium with 50 μg/ml gentamicin was added and the flasks were re-incubated for 0.5 h or 11 h. After the 0.5 h and 11 h of culturing, one flask of cells respectively was fixed and transmission electron microscopy was carried out. Within 5 h of infection, most J774.A1 cells were infected with RB51 but with lower bacterial number per cell (A) than macrophages left in culture for an other 11 h (B). Arrows point out *Brucella*.

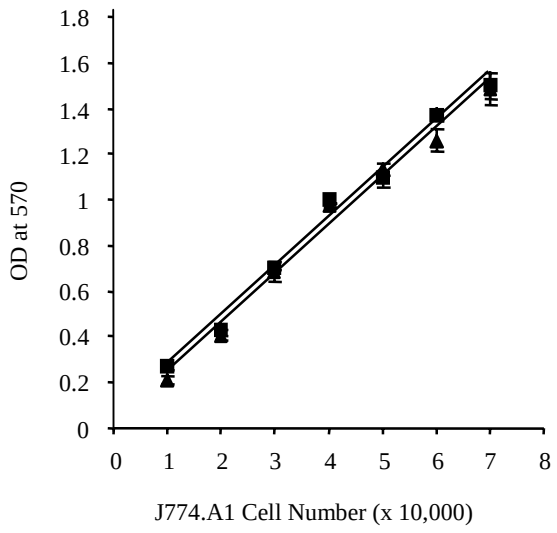


Fig. 3.3. Linear relationship between macrophage cell number added to each well of a 96-well round-bottomed microplate and uptake of neutral red dye by macrophages. The normal J774.A1 macrophages (—▲—) and the strain RB51-pulsed J774.A1 macrophages (—■—) were allowed to adhere for 4 h before addition of neutral red dye and were stained for 45 min. Each point represents the mean ± standard deviation (SD) of 4 determinations. Both were linear relationships shown by their R2 values of 0.9881 for RB51-pulsed J774 and 0.9867 for normal J774.A1 macrophages.

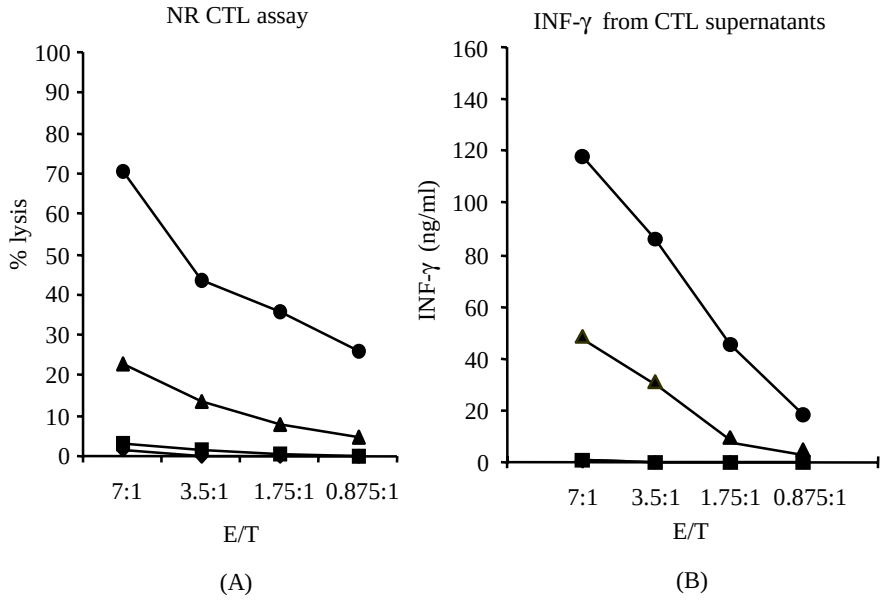
Table 3.1. Cell analysis by flow cytometry of one representative experiment. Normal and RB51-immunized mice were killed after 6 weeks of immunization. The spleens were collected and pooled in each group. The pooled splenocytes were processed through nylon wool column to enrich T lymphocytes, which were further cocultured with RB51-pulsed mitomycin C-treated J774 macrophages for 5 days. Viable live cells were separated by Hystopaque centrifugation. The different cell populations were analyzed by flow cytometry. The experiment was repeated three times with similar results.

Cell label / Cell population analyzed	CD3+ CD4+ (%)	CD3+ CD8+ (%)	CD3+CD4+ CD3+CD8+ (%)	CD3+CD4+ / CD3+CD8+	CD3+ NKpan+ (%)
Splenocytes from normal mice before nylon wool enrichment	31.2	13.0	44.2	2.4	4.6
Splenocytes from normal mice after nylon wool enrichment	64.7	23.6	88.3	2.7	4.4
Splenocytes from RB51 mice before nylon wool enrichment	28.7	12.7	41.4	2.3	3.4
Splenocytes from RB51 mice after nylon wool enrichment	62.9	25.4	88.3	2.5	2.4
Live cells from coculture of T cells from normal mice with RB51-pulsed macrophages	63.5	25.1	88.6	2.5	2.6
Live cells from coculture of T cells from RB51 mice with RB51-pulsed macrophages	62.3	25.9	88.2	2.4	2.8

The $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell percentages and their ratio did not change dramatically after 5-day co-culturing of the enriched T cells and stimulator cells (Table 3.1). Nylon wool treatment did not enrich NK cells and the NK cell proportion did not increase after the 5-day co-culture (Table 3.1). T lymphocytes used for mixing with the target cells therefore comprised 62% of $CD3^+CD4^+$ and 26%of $CD3^+CD8^+$ cells.

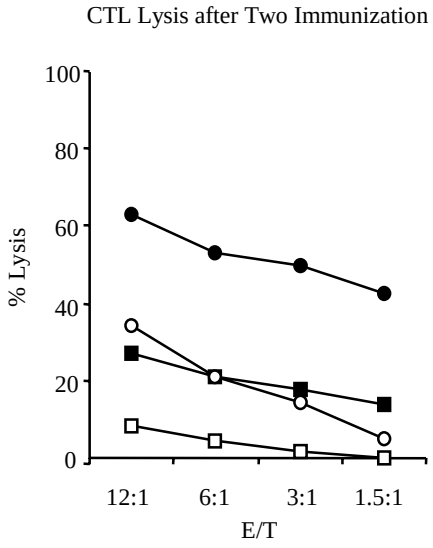
T cells from *B. abortus* RB51-vaccinated mice specifically lysed RB51-infected macrophages and secreted IFN-γ The results described are from CTL assays using the *in vitro* stimulatory step. As depicted in Fig. 3.4, cytotoxic T cells derived from strain RB51-immunized mice specifically lysed strain RB51-infected J774.A1 macrophages (Fig. 3.4A). The lysis level reached approximately 70% when the effector cells / target cells ratio (E/T) was 7:1. The same CTL population also demonstrated some low level of non-specific lysis of normal J774. A1 macrophages reaching about 20% at E/T ratios of 7:1. The effector T cells derived from normal saline-injected mice did not exert any obvious cytotoxic lysis against either normal or strain RB51-infected macrophages (Fig. 3.4A), suggesting that the cytolytic T cell activity was specific and induced by strain RB51-vaccination of the mice. Before neutral red was added, the supernatants from the coculture of effector cells and stimulatory cells were analyzed for secretion of IFN-γ and IL-4 (Fig. 3.4B). The effector cells derived from strain RB51-immunized mice secreted high amount of IFN-γ when cocultured with strain RB51-pulsed macrophages (Fig. 3.4B). The same population secreted over 50% less of IFN-γ when cultured with normal macrophages. The T effector cells derived from normal mice did not secrete any IFN-γ regardless of the target cell population (Fig. 3.4B). The secretion levels of IFN-γ paralleled the lysis level of target cells by CTL effector cells, i.e., the more lysis, the higher IFN-γ production. However, IL-4 was never detectable. The results suggested that an exclusive Th1 response was induced.

No significant, specific lysis against strain RB51-pulsed J774A.1 target cells was detected by the enriched T cells derived from mice immunized once with strain RB51 without the *in vitro* stimulation step (data not shown). I further investigated if this would change if mice received a strain RB51 booster. At 8 wks after the first immunization, three mice were re-immunized i.p. with strain RB51 using a decreased dose. Two weeks later, these mice and three control mice were killed and the prepared splenocytes were passed though a nylon wool column to enrich T cells. The enriched T cells were directly incubated with normal or strain RB51-pulsed target macrophages and the CTL lysis ability was detected by the colorimetric neutral red uptake assay in a 16 h lysis assay and a 4 h lysis assay (Fig. 3.5). It was found that the enriched T cells derived from strain RB51 double immunized mice specifically lysed strain RB51-pulsed J774.A1 macrophages in both 4 h and 16 h lysis assays; more lysis was observed in the 16 h lysis assay. Some non-specific lysis against normal J774.A1 macrophages was also found (Fig. 3.5).



—◆— : T cells derived from normal mice against non-infected target macrophages
—■— : T cells derived from normal mice against RB51-pulsed target macrophages
—▲— : T cells derived from RB51-immunized mice against non-infected target cells
—●— : T cells derived from RB51-immunized mice against RB51-pulsed target cells

Fig. 3.4. Demonstration of CTL activity and INF-γ secretion. The neutral red uptake CTL assay (A) showed strong, RB51-specific lysis by cytotoxic T cells derived from RB51-immunized mice against RB51-pulsed target macrophages. The same effector cells also showed some non-specific lysis against non-infected J774.A1 macrophages. T cells derived from normal mice did not lyse either of the target cells. The INF-γ secretion levels (B) from co-culture supernatants correlated positively with the CTL lysis levels; i.e., the more lysis, the more INF-γ. T cells used had an in vitro stimulatory step. The data are means for triplicate estimations, and standard deviations did not exceed 20% of the means.



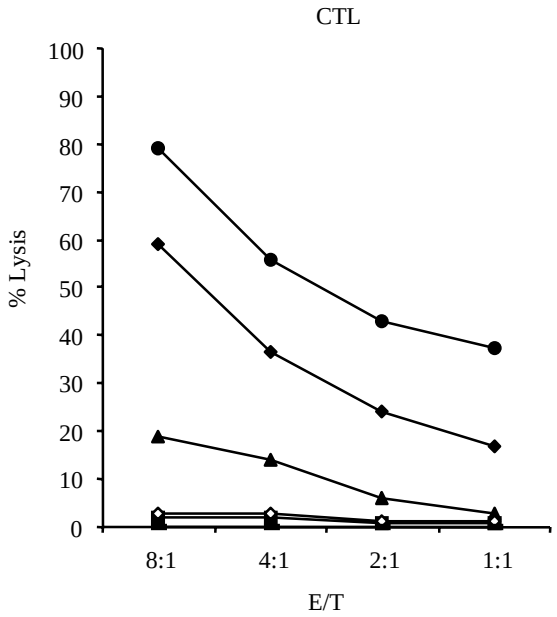
—□—: T cells derived from RB51-immunized mice against non-infected target cells in a 4 h CTL assay
—■—: T cells derived from RB51-immunized mice against RB51-pulsed target cells in a 4 h CTL assay
—○—: T cells derived from RB51-immunized mice against non-infected target cells in a 16 h CTL assay
—●—: T cells derived from RB51-immunized mice against RB51-pulsed target cells in a 16 h CTL assay

Fig. 3.5. Residential CTL reaction without *in vitro* stimulation. At 8 weeks after RB51 vaccination, 3 mice were boost-injected i.p. with 2 x 10^8 CFUs of RB51. Mice were killed after 2 wks and splenocytes were collected and pooled as described at M & M. After the nylon wool process, enriched T cells were mixed with normal or RB51-pulsed J774.A1 target cells and cocultured for 4 h or 16 h. Neutral red was used to detect the CTL activity. The data are means for triplicate estimations, and standard deviations did not exceed 20% of the means.

T cells from *B. abortus* RB51-vaccinated mice lysed virulent *B. abortus*-infected macrophages. Virulent *B. abortus* strain 2308 is used for challenge protection experiments. The ability of CTLs derived from strain RB51-vaccinated mice to lyse strain 2308-infected J774.A1 macrophages was detected and compared with the ability to lyse strain RB51-pulsed J774.A1 macrophages (Fig. 3.6). The CTLs derived from strain RB51-vaccinated mice specifically lysed strain 2308-infected J774 macrophages but the lysis levels were approximately 20% lower than the lysis levels achieved against strain RB51-pulsed target cells. The results indicate that it is reasonable to use strain RB51-pulsed macrophages as a model target cell population instead to using the virulent strain 2308 to pulse macrophages.

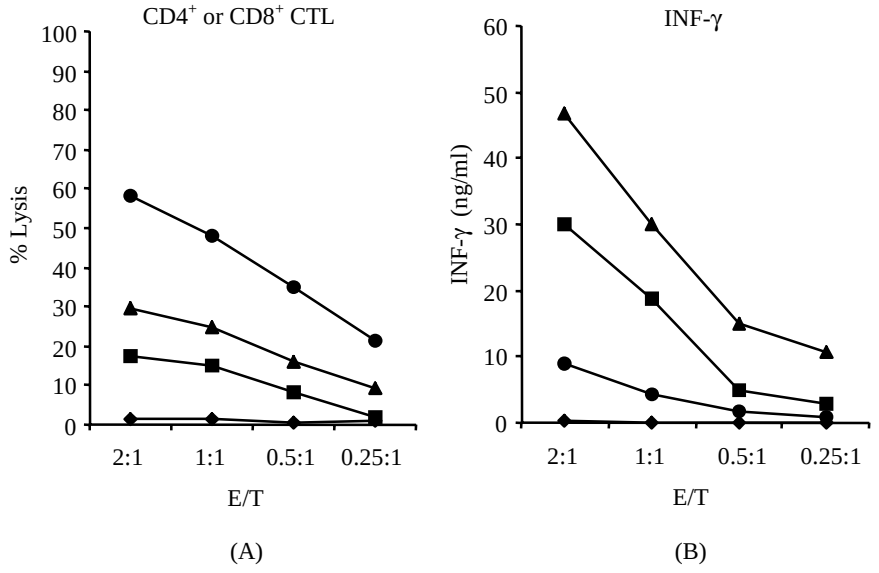
To analyze the specificity of the CTL assays, *Listeria monocytogenes* - infected J774.A1 macrophages were used as target cells. The effector cells derived from strain RB51-vaccinated mice did not lyse any *L. monocytegenes* – infected macrophages (date not shown), indicating that it was an antigen-specific assay without cross-reaction.

Different roles of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in CTL lysis reaction. It was demonstrated that the CTLs derived from strain RB51-vaccinated mice were able to non-specifically lyse uninfected J774 macrophages although this lysis level was significantly lower than the specific lysis levels achieved against strain RB51-pulsed J774 macrophages (Fig. 3.4A - 3.6). The CTL population consisted of approximately 62% of $CD3^+CD4^+$ T cells and approximately 26% of $CD3^+CD8^+$ T cells (Table 3.1). These two cell populations appeared to perform different functions. After the 5-day coculturing of stimulator cells and enriched T cells derived from strain RB51-immunized mice, the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations were separated by a magnetic cell sorting method and tested for their individual abilities to lyse target cells and secrete IFN-γ and IL-4 during the lysis process. The purity of isloated $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells was typically more than 92%. The isolated $CD3^+CD8^+$ cells never had more than 0.5% of $CD3^+CD4^+$ contaminating T cells or more than 0.5% NK cells. The same was true for isolated $CD3^+CD8^+$ T cells. The $CD3^+CD8^+$ T cells achieved high levels of lytic ability against strain RB51-pulsed macrophages but did not lyse any normal macrophages (Fig. 3.7A). These $CD3^+CD8^+$ T cells also secreted low levels of IFN-γ when cocultured with strain RB51-pulsed target cells and did not secrete any IFN-γ when incubated with normal macrophages (Fig. 3.7B). Contrary to the $CD3^+CD8^+$ T cells, the $CD3^+CD4^+$ T cells lysed normal macrophages and lysed significantly less strain RB51-pulsed target cells than the $CD3^+CD8^+$ T cells (Fig. 3.7A). The same $CD3^+CD4^+$ T cells secreted high amounts of IFN-γ whenever the target cells were normal or strain RB51-pulsed macrophages although IFN-γ levels tended to be lower with the non-infected target cells (Fig. 7B). IL-4 was never detected (data not shown).



—▬— : T cells derived from normal mice against non-infected target macrophages
—■— : T cells derived from normal mice against RB51-pulsed target macrophages
—◇— : T cells derived from normal mice against strain 2308-infected target cells
—▲— : T cells derived from RB51-immunized mice against non-infected target cells
—●— : T cells derived from RB51-immunized mice against RB51-pulsed target cells
—◆— : T cells derived from RB51-immunized mice against strain 2308-infected target cells

Fig. 3.6. CTL activity against strain 2308 and RB51–infected target cells *in vitro*. The preparation of *B. abortus* strain 2308-infected target cells followed the same protocol used for RB51-pulsed target cells. The T cells isolated at 6 wks postinoculation (i.p.) with strain RB51 or strain 2308 were stimulated *in vitro* as described in Materials and Methods. The data are means for triplicate estimations, and standard deviations did not exceed 20% of the means.

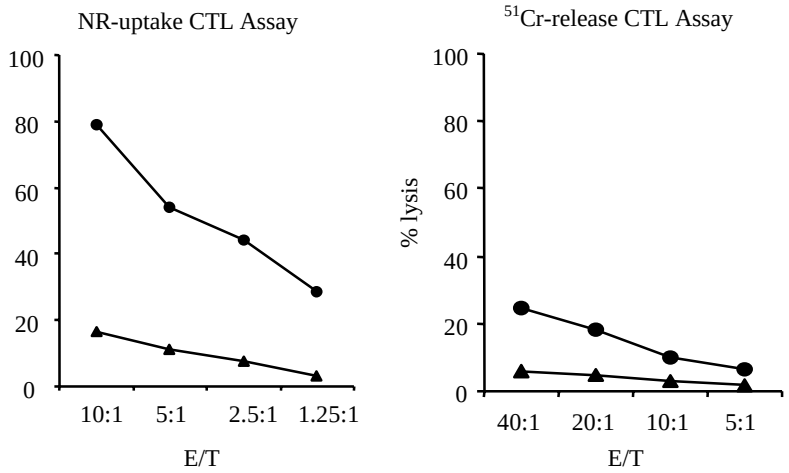


: CD4+ T cells derived from RB51-immunized mice against non-infected target macrophages
: CD4+ T cells derived from RB51-immunized mice against RB51-pulsed target macrophages
: CD8+ T cells derived from RB51-immunized mice against non-infected target cells
: CD8+ T cells derived from RB51-immunized mice against RB51-pulsed target cells

Fig. 3.7. Different roles of CD4+ and CD8+ in the cytotoxic lysis and release of INF-γ. The two T cell populations were isolated by the MACS magnetic kit and showed more than 92% purity by flow cytometry. The data indicated that CD8+ T cells specifically lysed RB51-pulsed target cells and showed no non-specific lysis against normal macrophages. The CD4+ T cells showed both specific and non-specific lyses (A). The same CD8+ T cells could secrete some level of INF-γ in response to RB51-pulsed target cells. However, the CD4+ T cells showed high levels of INF-γ secretion in response to normal and RB51-pulsed target cells (B). The data are means for triplicate estimations, and standard deviations did not exceed 20% of the means.

NK cells did not play important role in the cytotoxicity activity. As shown by flow cytometry (Table 3.1), there were less than 5% of NK cells within the splenocyte population and this percentage decreased after nylon wool separation. Although the number of NK cells did not increase after the incubation of the enriched T cells with the stimulator cells, the NK cells might be able to undergo activation during this process and lyse target cells. The possibility of whether these NK cells became highly active in the cytotoxicity reaction was investigated by using a YAC-1 cells as target cells. YAC-1 cells are very sensitive to the lysis by active NK cells and therefore are frequently used to detect NK-cell activity (79). Our studies indicated that the effector cells derived from strain RB51-immunized mice did not lyse any YAC-1 target cells (data not shown). This indicated that a low amount of NK cells did not get activated during the CTL activation process and suggested the NK cells did not play an important part in the cytotoxicity reaction.

Neutral red uptake assay versus ^{51}Cr-release assay. For comparison, the ability of *B. abortus* strain strain RB51-specific CTL to lyse strain RB51-infected J774.A1 macrophages was assayed by the classical ^{51}Cr-release assay and the neutral red uptake assay. T cells derived from normal saline-injected mice did not show any ability to lyse *Brucella*-infected macrophages or normal macophages in both assays (data not shown). However, the two assays demonstrated specific lysis of strain RB51-infected target cells by the effector cells derived from strain RB51-immunized mice (Fig. 3.8). The lysis patterns were similar: the higher the E/T ratio, the more lysis. Nevertheless, the detected lysis level was much higher with the neutral red assay, i.e., with 10:1 E/T cell ratio neutral red gave 80% lysis (Fig. 3.8A) while the ^{51}Cr-release assay gave less than 20% of lysis (Fig. 3.8B). The 20% lysis level was detected in the ^{51}Cr-release assay with a 40:1 E/T ratio (Fig. 3.5B). These results strongly suggest that the neutral red assay is more sensitive than the classic ^{51}Cr-release assay. Therefore, the neutral red assay is being routinely used through this study.



—▲— : T cells derived from RB51-immunized mice against non-infected target cells
—●— : T cells derived from RB51-immunized mice against RB51-infected target cells

Fig. 3.8. Comparison of neutral red uptake CTL and ^{51}Cr-release assay. T cells were isolated at 6 wks from mice immunized with RB51, enriched by nylon wool process and stimulated *in vitro* as described in Materials and Methods. The target cells were non-infected or RB51-infected J774.A1 macrophages. For the ^{51}Cr-release assay, ^{51}Cr was incubated with the target cells, extracellular ^{51}Cr was removed by washing, and the ^{51}Cr-labeled target cells were incubated with the effector cells. For neutral red uptake assay, the effector cells were incubated with target cells before neutral red was added. The data are means for triplicate estimations, and standard deviations did not exceed 20% of the means.

DISCUSSION

Specific activation of $CD8^+$ cytotoxic T lymphocytes (CTL) is crucial to elicit immunity against intracellular pathogens including viruses, bacteria and protozoa (123). The data presented in this study demonstrate that *B. abortus* strain RB51 induced strong and specific cytolytic T cell activity in a mouse model using a newly standardized colorimetric CTL assay. Data also suggested that the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells play different but probably collaborative roles in the lysis process.

B. abortus strain RB51 was found in macrophages at 4-5 and 16 hours post feeding. The ability of these cells to ingest *Brucella* as demonstrated here and before by Wise et al (262) makes them credible antigen presenting cells. This result was consistent with previous work by Jiang et al (117) using strain 19. It was found by Jiang et al that according to Giemsa stain and light-microscopic evaluation, approximately 30 to 40% of J774.A1 macrophages were infected with *B. abortus* strain 19 and there was an approximately 3 bacteria/macrophage at 3 h following infection (117). Wise et al (262) also demonstrated that J774.A1 cells also get infected with *B. abortus* strain 2308. In this study, it was found that macrophage viability at 5 or 16 h after infection was more than 80% in various experiments according to trypan blue exclusion assay which allowed us to proceed with the CTL assay. It was consistent with previous result that infection of *B. abortus* strain 19 did not kill J774A.1 even at 48 h post-infection (117).

A non-radioactive assay for T cell cytotoxicity against *Brucella*-infected target cells has been adopted in this study to replace the traditional ^{51}Cr release assay. The use of ^{51}Cr is expensive and creates radioisotope waste. ^{51}Cr has a relatively short half-life that reduces flexibility in experimental designs. Furthermore, since ^{51}Cr is slowly released from unlysed target cells, sensitivity of the assay is somewhat impaired due to relatively high background readings. The non-radioactive assay to detect *Brucella*-specific CTL is based on the ability of surviving target macrophages to incorporate the dye neutral red. Only live phagocytes have the ability to pinocytize neutral red. After addition of neutral red to cultures the dye can enter macrophages whereas lymphocytes cannot take up neutral red. The number of surviving target cells is determined by lysing the stained cells with a lysing solution consisting of SDS and acetic acid and measuring the amount of dye released with a spectrophotometer. Afterwards, the percentage of lysed cells can be calculated. The neutral red assay was more sensitive than the ^{51}Cr release assay. The increased sensitivity of the colorimetric neutral red uptake assay compared to the traditional ^{51}Cr-release assay found in this study was reported in many other systems (62, 194). In many virus systems, the sensitivity of neutral red assay is 6- to over 25-fold higher than ^{51}Cr release assay (194). By comparing the cytolytic activity of T cells from *L. monocytogenes*-infected mice against specific target cells using ^{51}Cr release assay and neutral red assay, it was found that the effector/target (E/T) ratio of 1, 0.5, 0.25, and 0.12 in neutral red assay had similar sensitivity compared to the E/T ratios of 100, 50, 25, and 12 in ^{51}Cr release assay (62). These numbers compare well to our findings.

Nylon wool columns were successfully used to enrich T lymphocytes from the general splenocyte populations. This process approximately doubled the concentration of the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells to 88% and reduced other types of cells to 12% of which, approximately 4% were NK cells. Enrichment of T cells was important in order to assure some level of certainty that observed effects were due to T cells. Nylon wool enrichment did not change the ratio of the two populations significantly, a potential useful characteristic for those experiments where cell ratio is important. The CTL effector cells were obtained by culturing the enriched T lymphocytes from mice immunized once with strain RB51 (or non-immunized control mice) with mitomycin C (MMC)-treated, RB51-pulsed J774.A1 macrophages for 5 days. The viable effector cells were purified by Histopaque (Sigma-Aldrich, St Louis, MO) centrifugation and confronted with strain RB51-infected J774.A1 target cells. The specific CTL lysis was analyzed by the neutral red uptake assay after the mix of effector and target cells at different ratios. Using this assay it was demonstrated that T lymphocytes isolated at 6 weeks after one strain RB51 immunization needed the *in vitro* stimulatory step to lyse target cells (Fig. 3.4, and 3.6-3.8) while T lymphocytes obtained at 2 weeks post second RB51 immunization did not require this step (Fig. 3.5). It therefore appears that one immunization seems not to be enough to obtain optimally stimulated CTL populations at least not if obtained at 6 weeks postimmunization.

T lymphocytes from strain RB51 immunized mice were also able to kill strain 2308 infected target cells (Fig. 3.6). Considering the fact that the practical reason to immunize animals with strain RB51 is to achieve protection against virulent strains like strain 2308, this observation is highly important. If strain RB51 immunization would not have induced CTL against strain 2308 infected target cells, the effectiveness of such immunizations could be questioned. On the other hand, lack of CTL activity against strain 2308 infected target cells would eliminate the CTL assay as a potential *in vitro* correlate of protective immunity. Comparable lytic results were obtained using strain 2308 or strain RB51 infected cells (Fig. 3.6). This was expected since strain RB51 is very closely related to strain 2308 since it is a mutant derived from strain 2308. Since it is safer to work with the attenuated strain RB51 than with the virulent strain 2308, we decided to routinely use strain RB51-infected macrophages as target cells instead of strain 2308-infected macrophages.

One additional advantage of using neutral red CTL assay is that the supernatants from the co-culture of effector cells and target cells can be easily collected and analyzed for cytokine secretion analysis without worrying about any contamination of radioacive material. The supernatants from the co-culture of effector cells and target cells were analyzed for the secretion of IFN-γ and IL-4. No detectable level of IL-4 secretion at any time was observed. It was consistent with relevant studies indicating that strain RB51 induces Th1 type of immune response (180, 254, 255, 257). Live *B. abortus* vaccines induce IFN-γ but not IL-4 production *in vitro* and *in vivo* in mouse models (271). It was previously reported by our group that splenocytes from strain RB51-immunized mice could not secrete any detectable IL-4 upon *in vitro* stimulation with heat-inactivated strain RB51, strain RB51 antigen extracts or any specific recombinant *Brucella* antigens (254,

255, 257). On the contrary, IFN-γ production could be readily detected. The high IFN-γ and no IL-4 secretion kinetics indicated again the induction of exclusive Th1-type immune response; this mediates the cell-mediated immunity against intracellular pathogens (210) by strain RB51 immunizations. In this CTL study, the effector T cells derived from strain RB51-immunized mice secreted high amount of IFN-γ when co-cultured with strain RB51-pulsed target cells and these IFN-γ levels paralleled the lysis levels of strain RB51-pulsed target cells (Fig. 3.4B). This strongly suggests that probably both, IFN-γ production and development of CTL activity is necessary for optimal protection levels. When the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were separated and analyzed individually for their secretion of IFN-γ, it was found that the $CD3^+CD4^+$ T cells secreted the majority of IFN-γ while the $CD3^+CD8^+$ T cells secreted low amounts of IFN-γ when co-cultured with strain RB51-pulsed target cells (Fig. 3.7B). Consistent with these results, it was previously reported that $CD8^+$ T cells derived from *B. abortus* strain 19 immunized mice could produce IFN-γ (80, 272) and that the $CD4^+$ T helper 1 cells were the main sources of IFN-γ production (80). It appears therefore that after strain RB51 immunization the $CD3^+CD4^+$ T cells are the main source of IFN-γ.

This study clearly found that certain level of cytotoxic activity by antigen-specific $CD3^+CD4^+$ T cells is induced after strain RB51 immunization although their cytolytic ability was much less than $CD3^+CD8^+$ T cells (Fig. 3.7). Similar observations have been made in other systems. For example, it was reported that MHC class II-restricted $CD4^+$ T cells specifically lysed *L. monocytogenes*-infected macrophages (127) and that these "killer" cells belong to the Th1 subset and are capable of expressing Fas-ligand (FasL) to induces apoptosis in Fas-positive target cells (98). The major target cells for these $CD4^+$ CTLs are cells from the immune system such as T, B and macrophages that express Fas upon activation.

It is possible for T cells to exert antigen specific and nonspecific lysis of target cells. In this study, when examing specific and non-specific lysis by the $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells, only $CD3^+CD8^+$ T cells demonstrated antigen-specific responses with no non-specific lysis (Fig. 3.7).

Some non-antigen-specific lysis of normal macrophages by the effector T cells derived from strain RB51-immunized mice was detected (Fig. 3.4A, 3.5, 3.6, 3.8) and more precisely by the $CD3^+CD4^+$ T cells derived from strain RB51-immunized mice (Fig. 3.7A). As discussed, $CD3^+CD4^+$ T cells secreted much higher IFN-g levels than $CD3^+CD8^+$ T cells. It is possible that the high amount of IFN-g induced the lysis of normal macrophages probably by apoptosis (64, 170, 242). Lysis did not occur when T cells derived from normal mice were used because these T cells did not secrete any IFN-g. Another possibility is that minor MHC differences between J774.A1 and native macrophages in BALB/c mice exist which trigger the non-antigen-specific lysis. Since the colorimetric assay was a very sensitive assay, it made this effect appear obvious but not necessarily unique. The difference found between $CD4^+$ and $CD8^+$ T cells is also shown in other systems. For example, the $CD4^+$ T cells from HIV-infected patients exerted nonspecific cytolytic activity against target cells expressing HIV envelop glycoprotein

(105). It was described as a non-MHC-restricted, calcium-independent cytotoxic effect probably based on cell-to-cell fusion. This non-specific lysis did not occur with another HIV antigen (gag/p55 protein) expressing target cells. However, $CD8^+$ T cells did not show any of the $CD4^+$ T cell mediated nonspecific lysis and only presented classical antigen-specific lysis. This non-specific lysis was also shown in ^{51}Cr-release assay using strain 19-induced CTL (180) and was also shown in *L. monocytogenes* - related CTL studies with ^{51}Cr-release or neutral red uptake assays (127). The findings were ignored due to the significant difference between non-specific and specific CTL lyses.

NK cells are capable of lysing microbe-infected cells mostly due to the production of IFN-γ (91). It was reported that early production of IFN-γ by NK cells and not by T cells was essential in resistance to listeriosis (67) and that the early production of IFN-γ by NK cells is also important to induce Th1 immune responses (141, 220). It was also shown that an aqueous-ether extract residue of *B. abortus* strain 456 stimulated NK cell cytolytic activity against YAC-1 cells in the mouse model (263). These studies suggest a probable important role of NK cells in our assay system. However, we could not detect any lytic NK cells activity in our cytolytic system. This lack of activity may be explained by the low number of NK cells in the system (Table 1) which did not secrete detectable IFN-γ levels. Another explanation is that the NK cells did not get activated as indicated by their inability to lyse YAC-1 cells. Previous studies also indicated no role of NK cells in the early control of *B. abortus 2308* infections by using the YAC-1 cell cytotoxicity assay and the lack of effect after depletion of NK cells in BLAB/c and C57BL/10 mouse models (79). Unlike *L. monocytogenes*, *B. abortus* only causes chronic infections instead of acute infections. Thus the early non-specific production of IFN-γ is probably minor compared to the IFN-γ produced by antigen-specific $CD4^+$ T cells (79).

Many studies have been undertaken regarding the roles of macrophages and cytokines secreted mainly by $CD4^+$ T cells against *Brucella* infection. However, research on CTL is very limited partly due to the complexity of the assays involved and the use of radioactive ^{51}Cr. There is only one ^{51}Cr-release assay report in *Brucella* research; it demonstrated that *B. abortus* strain 19 stimulated MHC class I-restricted T cells might play an important role in controlling virulent *B. abortus* infection in C57BL/6 mice (180). In that system a high amount of IFN-g was used *in vitro* to pre-activate macrophages before adding *Brucella*, which might have impaired its validity when compared to *in vivo* situations. The assay reported here did not use IFN-g *in vitro* as a pre-activation step. In addition, both *B. abortus* vaccine strain RB51 and virulent strain 2308-infected macrophages were used as target cells making the *in vitro* observations more related to the *in vivo* situation. The resulting high cytotoxic lysis validates the existing CTL activity in *Brucella* system and the neutral red assay developed somewhat simplifies the assay. The standardization of the colorimetric neutral red uptake assay should facilitate and strengthen future efforts in elucidating the role of CTL in *Brucella* immunity. These studies clearly indicate that strain RB51 immunization leads to the development of $CD3^+CD4^+$ T helper cell population which produce high levels of IFN-γ and can exert some antigen-specific cytotoxicty as well as non-antigen-specific cytotoxicity and a $CD3^+CD8^+$ T cell population which has very strong and highly antigen-specific

cytotoxicity activity. NK cells, on the other hand, seems not to play an important role in the cytotoxic phenomenon. Considering that both $CD4^+$ and $CD8^+$ T cells play important roles in protective immunity as demonstrated by Araya et al (6) in active T lymphocyte transfer experiments, it is suggest that optimal protection against brucellosis requires IFN-g secreting T cells and antigen-specific CTL.

Since the CMI is critical to the protection against virulent *Brucella* infection, it has been hypothesized that the CTL activity may correlate well with protection levels. If that is proven to be correct, the assay may allow the prediction of the level of immunity inducible by vaccine candidates. Some vaccine candidates developed at Virginia Tech are being used to test their ability to stimulate CTL activity. For instance, it has been showed that recombinant *B. abortus* strain RB51SOD (strain RB51 overexpressing protective antigen SOD) improved the vaccine efficacy of strain RB51 in the mouse model (257). Thus it is logical to hypothesize that the strain RB51SOD immunization stimulates higher CTL cytotoxic activity against *B. abortus* infected target cells and even SOD-pulsed target cells. This issue will be discussed in the next chapter.

Chapter Four

The Antibody and CMI Response to RB51 Overexpressing Homologous Antigen SOD and Expressing the *Mycobacterial* 85a Antigen

INTRODUCTION

Mycobacterium and *Brucella* are both facultative intracellular bacteria and the etiological agents of tuberculosis (130) and brucellosis (51), respectively. It is estimated that *M. tuberculosis* infects one third of the world's human population and causes the highest mortality of adults among infectious pathogens throughout the world (133, 134). *B. abortus* causes abortion and/or infertility in cattle and undulant fever in humans (51). brucellosis due to *B. abortus* in humans is under better control than tuberculosis partly due to the availability of vaccines against brucellosis in cattle which reduces the zoonoses. The attenuated *M. bovis* strain Bacillus Calmette-Guerin (BCG) has been used as a vaccine against human tuberculosis for decades but its protective efficacy is very variable ranging from 0 to 80% (13). This vaccine variability makes research to develop a more effective vaccine using advanced genetic engineering techniques very attractive and pertinent. At present, the new *B. abortus* vaccine strain RB51 has replaced previous vaccines in the U.S., Central and South America and is being widely used to control cattle brucellosis since early 1996. Recently it was reported that strain RB51 could be used to overexpress its own protective antigen for improvement of its vaccine efficacy (257) or express heterologous antigens to induce antigen-specific Th1-type immune responses (255). This work suggests that strain RB51 could be used as an excellent "bi-valent" vaccine against both brucellosis and tuberculosis by overexpressing its own protective antigen(s) and expressing *Mycobacterium* protective antigen(s).

Several *Mycobacterium* protective antigens have been described. Among them are the antigen 85 complex proteins (Ag85A, Ag85B, and Ag85C), which are mycolyltransferases (16). These proteins are major secretory *Mycobacterial* products and represent about 45% of the total amount of extracellular proteins produced by *M. tuberculosis* (103, 178). The Ag85 complex proteins share high sequence homology at the nucleotide and protein level with each other and among different *Mycobacterial* species. For instance, the amino acid sequence of 85A is identical between *M. bovis* BCG and *M. tuberculosis* (217).

Multiple evidence suggest that secreted *Mycobacterial* antigens, rather than constitutive or stress proteins, are critical protective *Mycobacterial* antigens (187, 189). Studies from four separate laboratories all indicated that *Mycobacterial* culture filtrate proteins stimulated protection against experimental tuberculosis in mouse or guinea pig models (4, 112, 192, 207). As a major component of the *Mycobacterial* culture filtrate proteins, Ag85A has been shown to stimulate protection against experimental infection and induce humoral and cellular immune responses through DNA vaccination of guinea

pigs and mice (13, 114, 150, 164, 247, 248) (13). It is therefore conceivable that production of 85A by a suitable vector could serve as a protective vaccine.

As described in Chapter 2, *B. abortus* Cu/Zn SOD is an important *Brucella* protective antigen. It also stimulates specific Th1 type immune responses in the mouse model if administrated overexpressed in strain RB51. However, it is unclear if administration of overexpressed SOD in strain RB51 leads to stronger cytotoxic T lymphocytes (CTL) activity against virulent *Brucella*-infected target cells as compared to administration of strain RB51 alone. Administration of strain RB51 overexpressing Cu/Zn SOD stimulates antibody and cellular immune responses (as measured by IFN-g production) to SOD while administration of strain RB51 alone does not lead to these SOD responses. In this study SOD was found in the growth media of strain RB51 overexpressing Cu/Zn SOD and this "leaking" of SOD out of strain RB51 probably induces the SOD-specific humoral and cellular immune responses. The strain RB51 strain overexpressing Cu/Zn SOD induced stronger CTL cytolytic activity against virulent *Brucella*-infected macrophage target cells. In this study it was demonstrate that recombinant strain RB51 both overexpressing Cu/Zn SOD and expressing *Mycobacterial* Ag85A induces Ag85A-specific IFN-γ production.

RATIONALE AND HYPOTHESIS

It was previously hypothesized that overexpression of a bacterial protective antigen(s) in its vaccine strain would greatly enhance the vaccine's efficacy (257). This hypothesis was validated by overexpressing *Brucella* protective antigen SOD in strain RB51 (257). It was also found that strain RB51 can be used as a vector for heterologous protein expression (e.g., *Mycobacterial* heat shock protein HSP65) and induction of antigen-specific Th1 type immune responses (255). The heterologous protein expression did not alter either the attenuation characteristics of strain RB51 or its vaccine efficacy against virulent *Brucella* challenge.

It was hypothesized that strain RB51 could be used as a vaccine vector to overexpress its homologous antigen(s) and express *Mycobacterial* heterologous protein(s) at the same time. This recombinant RB51 strain would have an increased vaccine efficacy against virulent *Brucella* infection and have the ability to protect against *Mycobacterial* infection. Chapter 3 indicated that strain RB51 vaccination induces cytotoxic T lymphocytes (CTL) able to specifically lyse *Brucella*-infected macrophages. Since it is believed that CTLs play an important role in protection and that a CMI response would develop against the 85A antigen, it was hypothesized that increased vaccine efficacy by RB51 overexpressing SOD and expressing heterologous antigen 85A would be accompanied by increased CTL activity against virulent *Brucella* infected target macrophages.

MATERIALS AND METHODS

Bacterial strains *B. abortus* strains 2308, RB51 and recombinant strain RB51SOD (257) were from the culture collection of Dr. Gerhardt G. Schurig (Virginia Tech). *E. coli* strain DH5a (Gibco-BRL, Gaithersburg, MD) was used for producing the necessary plasmid constructs. All bacteria were grown either in trypticase soy broth (TSB) or on trypticase soy agar (TSA) plates with or without addition of chloramphenicol as described in Chapter II. All experiments with live *Brucella* were carried out in a biosafety level 3 facility.

Construction of recombinant RB51 overexpressing *Brucella* Cu/Zn SOD and expressing mycobacterial 85A Strain RB51SOD/85A was provided by Dr. Ramesh Vemulappali (Virginia Tech). This strain was generated by transforming strain RB51 with plasmid pBBSOD/groE85A. To construct pBBSOD/groE85A, initially, the gene for the 85A protein was amplified via PCR using the genomic DNA of *M. bovis* BCG as template. The amplified product was subsequently cloned under the groE promoter of *Brucella* in pBBgroE (255) to generate pBBgroE85A. From pBBgroE85A, the 85A protein's gene along with the groE promoter was excised and subcloned in pBBSOD to generate pBBSOD/groE85A. In this plasmid, the expression of SOD is driven by its own promoter whereas the expression of the 85A protein is under the control of groE promoter of *Brucella*. The expression of the two proteins were detected by Coomassie blue staining and Western blot analysis as described below.

Preparation of antigens Recombinant *Brucella* Cu/Zn SOD was prepared by expressing SOD in *E. coli* DH5a and purifying it using an anion-exchange column (HiTrapQ; Pharmacia Biotech) as described previously (257). *Mycobacterial* protein 85A was expressed in *E. coli* as a fusion protein and purified according to protocol described previoiusly (254). Briefly, mycobacterial *85A* gene was PCR amplified from the genomic DNA of *M. bovis* strain BCG and cloned into expression vector pMalP2 (New England Biolabs Inc.). In this vector, the mycobacterial *85A* gene was expressed as a fusion protein with maltose-binding protein (MBP) at the amino terminus; this facilitated purification of the recombinant fusion protein using affinity chromatography with amylose resin. The strain RB51 crude extract prepared previously (254) was available in the laboratory.

SDS-PAGE. Denaturing gel electropheresis was performed using 12.5% acrylamide gels according to standard procedures (139). The *B. abortus* samples for SDS-PAGE were prepared as follows. *B. abortus* grown on TSA plates was harvested and killed by incubating for 30 min in a 65^0C water bath. The killed bacterial cells were washed twice with 10 mM Tris-HCL buffer (pH8.0), and their cell concentration was adjusted to 10% transmittance at 525 nm. Aliquots of 1ml bacterial suspensions were centrifuged, resuspended in 100 ul of 10 mM Tris-HCL buffer, and stored at -20^0C. Before use, aliquots were mixed with 100 ul of 2 x Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO), boiled for min, and 20 ul of each sample was used to run 12.5% acrylamide gel for SDS-PAGE. Gels containing the separated proteins were either stained with Coomassie brilliant blue R (Sigma-Aldrich, St. Louis, MO) or used for Western blot analysis.

Western blot analysis. Immunoblotting analysis was performed as previously described (257). Briefly, proteins separated by SDS-PAGE were transferred to a nitrocellular membrane by using a Trans-blot semidry system (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 2% bovine serum albumin solution and used for reaction with polyclonal antibodies to SOD or protein 85A. The membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) (ICN Biochemicals, Inc., Aurora, OH), and visualized by the HRP reaction with 0.015% hydrogen peroxide and 4-chloro-1-naphthol.

Analysis of "secreted" proteins. Strains RB51, RB51SOD, and RB51SOD/85A were grown in TSB medium, respectively, to a cell density of ~5 x 10^8 cells per ml. Culture supernatants were precipitated with cold trichloroacetic acid (TCA, final concentration: 20%) and incubation at 4 ^{0}C overnight. Following centrifugation, precipitated proteins were washed with acetone, dissolved in 2 x Laemmli sample buffer, and boiled for 5 min. Samples were used for SDS-PAGE in 12.5% acrylamide gels as described above. Gels were stained with Coomassie brilliant blue R-250 if no western blot was intended. Proteins from unstained gels were transferred to nitrocellulose membranes for Western blot analyses. The “secreted” protein was used for amino acid sequencing. For this purpose, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane in CAPS transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0) in accordance with the method of Matsudaira (154). The proteins were visulized by staining with Coomassie brilliant blue R-250, destained with 50% methanol and then distilled water. The protein bands could still be weakly seen. The whole membranes were then mailed to Midwest Analytical, Inc. (St. Louis, MO) for amino acid sequencing. Once sequences were received, a National Center for Biotechnology Information (NCBI) BLAST search was conducted to identify the "secreted" protein.

Mice protection and clearance experiment. Four- to five-week-old female BALB/c mice were purchased from Charles River laboratories (Wilmington, MA). Mice were given one week of rest before the experiments were started. Twelve mice in each group were each vaccinated intraperitoneally (i.p.) with ~4 x 10^8 of strains RB51, RB51SOD, at RB51SOD/85. As a negative control, 12 mice in another group were injected i.p. with saline alone. Five mice from each group were bled at 3 and 6 weeks postinoculation to obtain sera for ELISA and western blot analyses. Also at 6 weeks postinoculation, five mice from each group were challenged i.p. with 2 x 10^4 of virulent *B. abortus* strain 2308. Two weeks after the challenge infection, the mice were killed, *Brucella* from their spleens were recovered, and the numbers of CFU’s were determined. In addition, at 6-7 weeks postvaccination, three mice from each group were killed and their splenocytes were used for *in vitro* culturing to determine cytokine production. The other four mice each group were killed at 6-7 weeks post-vaccination to conduct neutral red uptake CTL assays. Fig. 4.1 depicts the experimental design.

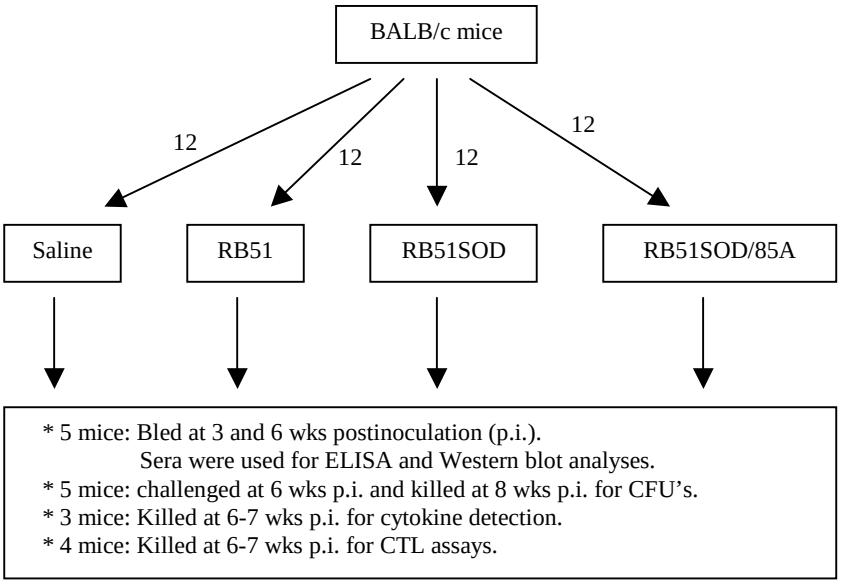


Fig. 4.1 Flow chart of experiments with mice. 12 mice in each group were inoculated i.p. with saline, RB51, RB51SOD or RB51SOD/85A as described in Materials and Methods. 5 mice per group were bled at 3 and 6 wks postinoculation (p.i.). The sera were used for ELISA and Western blot analyses. Protection study was performed by infecting 5 mice per group with strain 2308 at 6 wks p.i. and killing them at 8 wks p.i. to detect the bacterial CFU's in spleens. 3 mice per group were used for cytokine study and the last 4 mice per group were used for CTL assays. This diagram does not include the bacterial clearance study. The mice bled at 3 and 6 weeks post-vaccination were randomly assigned to the challenge, cytokine detection or CTL group mice.

To determine *in vivo* clearance, groups of 15 mice were inoculated i.p. with ~4 x 10^8 CFU of strains RB51 and RB51SOD/85A in a separate experiment. Five mice from each group were sacrificed at 3, 4 and 5.5 wks postinoculation, and the bacterial numbers in their spleens were determined.

Indirect ELISA The presence of serum IgG, IgG1, and IgG2a isotypes with specificity to the strain RB51 extract, Cu/Zn SOD or MBP-85A was determined by indirect ELISAs. The strain RB51 extract, purified recombinant Cu/Zn SOD, or MBP-85A was diluted to 10 μg/ml in carbonate buffer (pH9.6) and the dose of 50 μl/well was used to coat the wells of polystyrene plates (Nunc-Immuno plate with MaxiSorp surface). After overnight culture at 4^0C, the plates were washed four times in wash buffer (Tris-buffered saline [pH7.4] with 0.05% Tween 20) and blocked with 2% bovine serum albumin in Tris-buffered saline. After 1 h at room termperature, the blocking solution was discarded, and the srum samples (1:100 dilution in blocking solution) was added to the wells (100 ul/well). Each serum sample was tested in triplicate wells. The plates were incubated for three hours at room temperature and washed for 4 times, and isotype-specific goat anti-mouse horseradish peroxidase conjugates (Caltag Laboratories, San Francisco, CA) were added (100 ul/ml) at an appropriate dilution. After 1 h of incubation at room temperature, the plates were washed four times, and 100 ul of TMB Microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. After 30 min of incubation at room temperature, the enzyme reaction was stopped by adding 100 ul of stop solution (0.185 M sulfuric acid), and absorbance at 450 nm was recorded with a microplate reader (Molecular Devices, Sunnyvale, CA).

Cytokine quantitation. Splenocytes from inoculated mice were obtained as previously described in Chapter 2 and cultured in the presence of 0.5 to 2.0 μg (protein) of Cu/Zn SOD, 1 μg/well of MBP-85A, 10^7 CFU's of heat-inactivated *B. abortus* strain RB51, 0.5 μg of concanavalin A, or no additives (unstimulated control). After 5-day culture, the cell supernatants were collected and tested for the secretion of IFN-g and IL-4 by previously described sandwich ELISA method using recombinant mouse IFN-g and IL-4 (PharMingen, San Diego, CA) as standards. In these assays, the lower detection limits were 100 pg and 10 pg for the IFN-g and IL-4 assays, respectively. The assays were performed in triplicate.

Neutral red uptake CTL assay. The cytolytic ability to lyse virulent *Brucella* infected target macrophages by effector T cells from immunized mice was detected by the neutral red uptake assay described in Chapter 3. Briefly, each time the assay was performed, two mice in each group vaccinated with strain RB51, strain RB51SOD or strain RB51SOD/85A were killed at 6-7 weeks post-vaccination. The spleens from each group were pooled and splenocytes were isolated and passed though nylon wool columns to enrich for T cells. At the same time, viable strain RB51 were used to infect J774.A1 macrophages at a ratio of 100:1 for 4-5 h. The infected macrophages were then scraped off and treated with mitomycin C at the concentration of 35 μg/ml for 45 min at 37^0C water bath. After washing out the mitomycin C by four centrifugations, the macrophages

were mixed with the enriched T cells at a ratio of 1:10 at 24-well cell culture plates and incubated at 37^0C, 5% CO_2 for 5 days. The cells were then collected and the live effector T cells were purified by Histopaque column centrifugation. The infected target cells were prepared by infecting J774.A1 macrophages with virulent *B. abortus* strain 2308 at a ratio of 100:1 for 4-5 h at 37^0C, 5% CO_2. Normal J774.A1 cells were used as control target cells. A serial dilutions of the effector cells were first added to wells of 96-well round-bottom cell culture plates in triplicate and then the target cells were added at the concentration of 50,000 cells/well. The mix of effector T cells and target cells were incubated for 16 h at 37^0C, 5% CO_2. Wells of target cells without any effector cells were also included as controls. The culture media were carefully discarded and 200 µl of warm neutral red (0.036%) was added to all wells. After the following incubation for 40 min, the monolayers of cells were carefully washed three times with warm (37^0C) phosphate-buffered saline (pH7.2-7.4) and the cells were then lysed with 220 µl per well of 0.05 M acetic acid-0.05% SDS solution. A 200 µl aliquot from each well was transferred to a 96-well ELISA microplate and the OD reading at the wavelength of 570 nm was recorded using an ELISA microplate reader. Complete DMEM (10% fetal bovine serum, DMEM medium purchased from ATCC) medium was used for the macrophage cultures. Complete RPMI medium (RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2mM of L-glutamine (MediaTech, VA) and 10 mM HEPES buffer (Sigma-Aldrich, St. Louis, MO)) was used whenever T cells were cultured. The viable cell numbers were always determined by trypan blue exclusion.

As control for non-lysis and maximal uptake of neutral red stain, target cells were cultured alone without effector cells. The percentage of specific lysis was established applying the following formula:

% specific lysis = (OD of control – OD of experimental) / OD of control x 100

Statistical analyses. The counts of bacterial CFU in the spleens of mice were analyzed by Student's t test. IFN-g production data were subjected to analysis of variance, and the means were compared using Turkey's honestly signifinant difference procedure (147).

RESULTS

Expression of mycobacterial 85A protein by strain RB51SOD/85A The expression of mycobacterial 85A in strain RB51SOD/85A was analyzed by SDS-PAGE and Western blot analysis. Using mouse antisera against strain RB5185A (Dr. Gerhardt Schurig, Virginia Tech), the mycobacterial 85A protein (approximately 32 kDa) expressed by strain RB51SOD/85A could be detected by Western blot analysis (Data not shown due to the low expression level and weak protein band in the membrane). However, its expression level was too low to be detectable using Coomassie blue staining of SDS-PAGE gels.

SOD overexpression and "secretion" by recombinant strains *Brucella* Cu/Zn SOD was overexpressed by strain RB51SOD as shown by SDS-PAGE and Western blot analysis of washed strain RB51 bacteria (257). The same was true for recombinant *Brucella* strain RB51SOD/85A. Based on Coomassie blue staining of SDS-PAGE gels, the TCA precipitated culture supernatants of recombinant strains RB51SOD and RB51SOD/85A contained a 19 Kda protein (Fig. 4.2) which reacted with anti-SOD antibodies in Western blots (data not shown). This protein was the only protein found in the TCA precipitated culture supernatants of strain RB51SOD and RB51SOD/85A and no proteins could be found in the TCA precipitated culture supernatant of strain RB51. In order to confirm that the culture supernatant of strain RB51SOD contained SOD, the TCA precipitate was used to run a SDS-PAGE gel and the protein was electro-transferred to PVDF membrane for sequencing (Fig. 4.2.B). According to sequencing results, the first 10 amino acids from N-terminal end of the 19Kda protein were determined to be ESTTVKMYEA. This amino acid sequence matches the *B. abortus* Cu/Zn superoxide dismutase sequence in the NCBI protein database (Accession number P15453, PID number g134603) and also indicates that the signal sequence is missing.

Induction of Th1 type immune responses in mice Specific antibody and CMI responses of the vaccinated mice were determined by indirect ELISA and cytokine quantitaion, respectively. Mice vaccinated with strains RB51SOD and RB51SOD/85A, but not those vaccinated with strain RB51 or inoculated with saline, developed *B. abortus* Cu/Zn SOD-specific IgG (Fig. 4.3.A). Subisotype analysis indicated that the developed antibodies were predominantly IgG2a (Fig. 4.3.B). Minimal levels of IgG1 antibody isotypes to Cu/Zn SOD were detected (Fig. 4.3.C). Both strain RB51SOD and strain RB51SOD/85A developed similar levels of *B. abortus* Cu/Zn SOD-specific antibodies. Antibodies to MBP (previous study) and MBP-85A were not detected.

In response to stimulation with heat-killed strain RB51 equivalent to 10^6 CFU/well, splenocytes of normal mice did not secrete detectable IFN-γ; however, splenocytes of strain RB51-, RB51SOD-, RB51SOD/85A-vaccinated mice secreted high amounts of IFN-γ and no significant difference was found between the three groups ($P > 0.05$) (Table 4.1). Upon *in vitro* stimulation with recombinant Cu/Zn SOD, splenocytes of normal mice did not secrete detectable IFN-γ and splenocytes of strain RB51-vaccinated mice secreted a minimal amount of IFN-γ; in contrast, splenocytes from mice vaccinated with strains RB51SOD and RB51SOD/85A produced significantly higher levels of IFN-γ than control RB51 mice but did not significantly differed among each other ($P > 0.05$) (Table 4.1). MBP-85A did not stimulate any detectable IFN-γ production by splenocytes from normal mice or strain RB51-vaccinated mice but did stimulate minimal amounts of IFN-γ by splenocytes of strain RB51SOD-vaccinated mice. The IFN-γ level secreted by the lymphocytes obtained from the strain RB51SOD/85A immunized mice was signicantly higher than the level of the other groups upon stimulation with antigen 85A (Table 4.1). IL-4 was never detected under any conditions (data not shown).

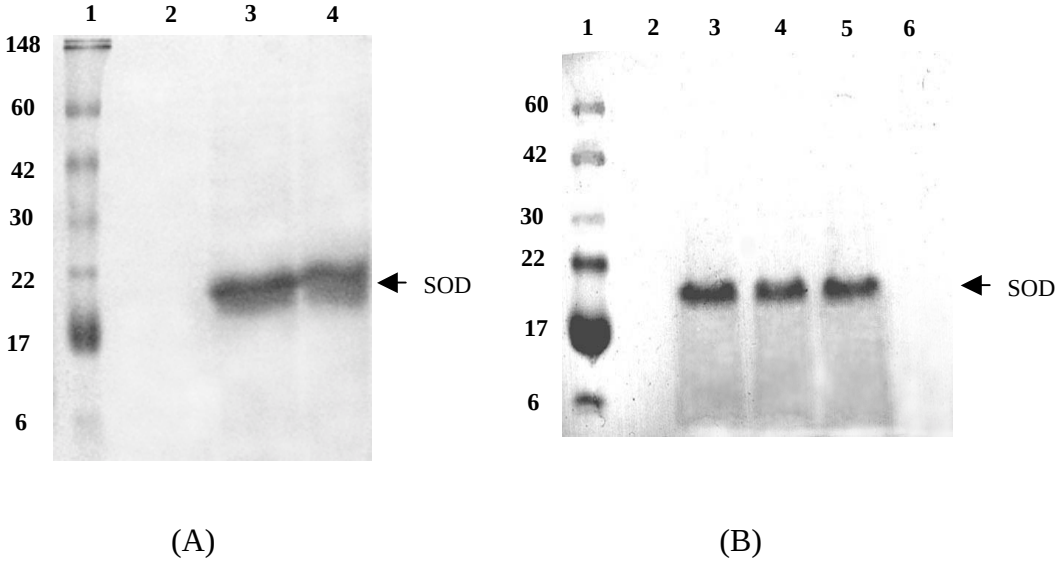


Fig. 4.2. Secretion of overexpressed SOD out of recombinant strain RB51SOD and RB51SOD/85A. Bacterial culture supernatants precipitated by TCA were processed by SDS-PAGE, and strained with Coomassie brilliant blue (A) or transferred to PVDF membrane and then stained with Coomassie blue R250 (B). The only protein band observed was approximately 19 kDa. The protein band in (B) membrane was used for amino acid sequencing. The amino acid sequence matched *B. abortus* Cu/Zn SOD in the NCBI protein database. The protein antigens used were: (A) lane1 -- molecular weight markers; lane -- RB51 culture supernatants; lane 3 -- RB51SOD culture supernatants; lane 4 -- RB51SOD/85A culture supernatants. (B) lane1 -- molecular weight markers; lane 2 and 6 --blank; lane 3, 4, 5 -- RB51SOD culture supernatants. All supernatants are TCA precipitated.

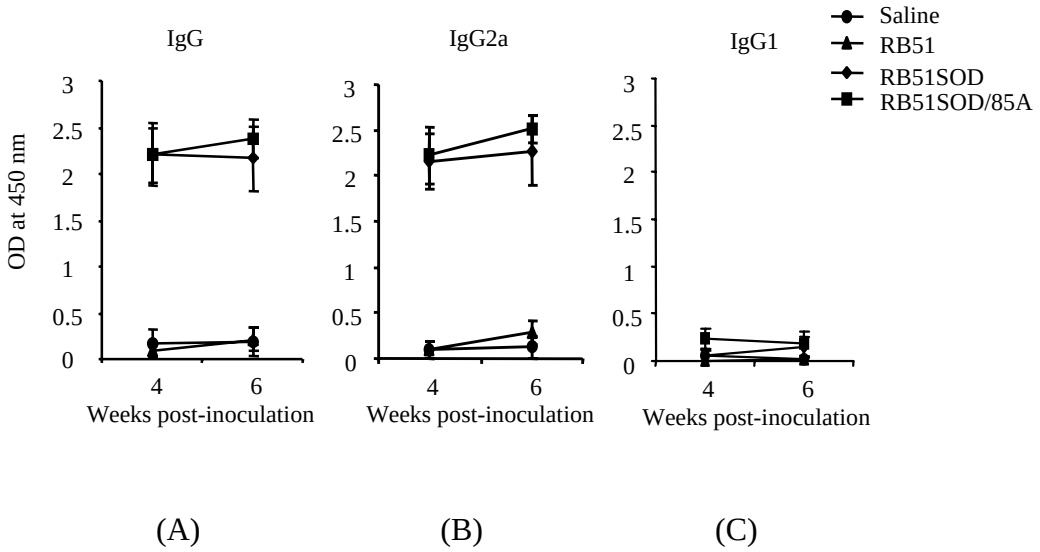


Fig. 4.3. ELISA detection of *Brucella* Cu/Zn SOD-specific IgG, IgG1 and IgG2a antibodies in serum of mice vaccinated with strain RB51 (▲), RB51SOD (◆), RB51SOD/85A (■) or inoculated with saline (●) alone. Sera collected from 5 mice of each group at 3 and 6 weeks post-vaccination were diluted 1:100 and assayed for the presence of specific antibodies. Results were shown as the means ± standard deviation of OD_{450} of the color developed.

Table 4.1. Production of INF-γ by splenocytes of naïve and vaccinated mice after *in vitro* stimulation with specific antigens: Cu/Zn SOD (1 μg protein/well), heat-killed RB51 (10^6 CFUs/well), MBP-85A (1 μg/well).

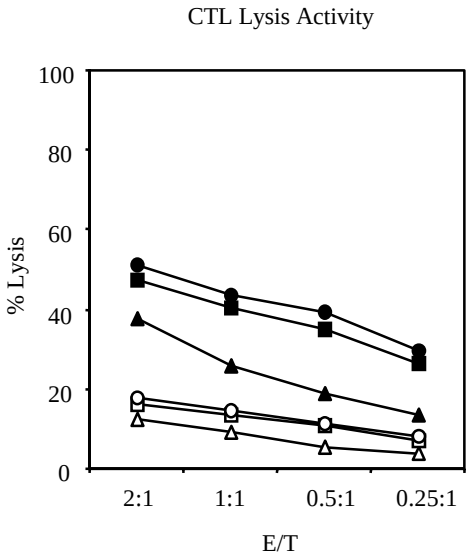
Stimulant	Concentration of INF-γ (ng/ml) in mice groups			
	Naïve	RB51-vaccinated	RB51SOD-vaccinated	RB51SOD85A-vaccinated
Media	—[a]	—	—	—
Heat-killed RB51	—	35.47 ± 6.41*	39.67 ± 3.62*	42.15 ± 3.34*
Cu/Zn SOD	—	0.36 ± 0.08[Δ]	13.11 ± 3.24**	14.37 ± 2.25**
MBP-85A	—	—	0.54 ± 0.23[O]	5.69 ± 1.16

[a] —, below detection limit (less than 0.1 ng/ml)
* No significant difference between the three groups.
** No significant difference between the two groups.
[Δ] and [O] are considered background levels of activity.

Cytotoxic activity of strains RB51, RB51SOD and RB51SOD/85A induced T effector cells The ability of *B. abortus*-specific CTLs from strain RB51, RB51SOD or RB51SOD/85A immunized mice to lyse virulent *B. abortus* strain 2308-infected or non-infected J774.A1 macrophage cells was assayed (Fig. 4.4). Specific lysis of strain 2308-infected target cells was detected by the T effector cells derived from the three different *B. abortus* strains with all four different E/T ratios used. However, strains RB51SOD and RB51SOD/85A induced significantly more lysis against strain 2308-infected macrophages than strain RB51 ($P < 0.05$). No significant differences were found between strains RB51SOD and RB51SOD/85A. Minimal lysis was observed when the T effector cells were incubated with non-infected J774.A1 macrophages as observed in the previous Chapter (Fig. 4.4).

Bacterial persistence and clearance Strain RB51SOD had attenuation levels similar to strain RB51 in mice as revealed by spleen clearance kinetics, indicating overexpression of *B. abortus* Cu/Zn SOD in strain RB51 does not increase its virulence (257). To verify whether there were any differences in the attenuation characteristics of strains RB51SOD/85A and RB51, the bacterial clearance from the spleens of mice inoculated i.p. with strains RB51SOD/85A and RB51 were compared. The spleens of mice inoculated with either strain RB51 or RB51SOD/85A were free of detectable *Brucella*e by 5.5 weeks (39 days) post-vaccination, and no significant differences were observed at 3 or 4 weeks post-vaccination ($P > 0.05$) (Fig. 4. 5).

To analyse the vaccine efficacy of strains RB51, RB51SOD and RB51SOD/85A against virulent *B. abortus* strain 2308 challenge infection, an *in vivo* protection study in BALB/c mice was performed. Compared to the saline-inoculated control, vaccination of mice with strains RB51, RB51SOD, and RB51SOD/85A at 6 weeks prior to the challenge infection significantly reduced the number of strain 2308 bacteria in spleens at 2 weeks post-challenge infection in all three vaccinated groups ($P < 0.05$). Protection afforded through strain RB51SOD or RB51SOD/85A was significantly higher than protection afforded by strain RB51 ($P < 0.05$). The protection afforded by strains RB51SOD and RB51SOD/85A was similar ($P > 0.05$) (Fig. 4.6).



: T cells derived from RB51-immunized mice against non-infected target cells.

: T cells derived from RB51-immunized mice against strain 2308-infected target cells.

: T cells derived from RB51SOD-immunized mice against non-infected target cells.

: T cells derived from RB51SOD-immunized mice against 2308J-infected target cells.

: T cells derived from RB51SOD/85A-immunized mice against non-infected target cells.

: T cells derived from RB51SOD/85A-immunized mice against strain 2308-infected target cells.

Fig. 4.4. Lysis of *B. abortus* strain 2308-infected or non-infected J774.A1 target cells by the T effector cells derived from strain RB51, RB51SOD or RB51SOD/85A immunized mice. The T effector cells derived from saline-injected mice showed no lysis against any target cells (data not shown). This CTL cytolytic reaction was performed with the neutral red uptake assay as described in Materials and Methods. The data are means for triplicate estimations, and standard deviations did not exceed 20% of the means.

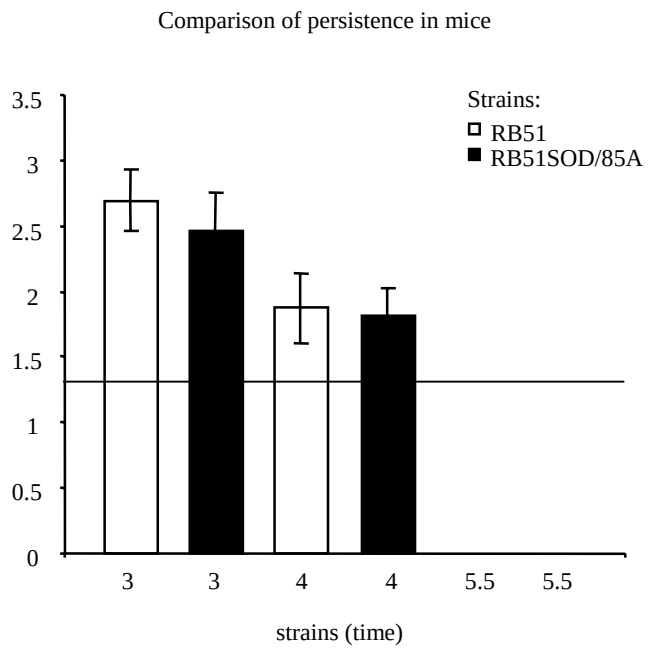


Fig. 4.5. Persistence of strain RB51 and RB51SOD/85A in spleens of vaccinated mice. Fifteen mice per group were inoculated i.p. with RB51 or RB51SOD/85A at a concentration of approximately 4 x 10^8 cfu/mouse. At 3, 4 and 5.5 wks post-vaccination, five mice from each group were killed and the number of CFU in their spleens was determined as described in Materials and Methods. The numbers of CFU at 5.5 wks were below detectable limits (less than $1.3\log_{10}$). There was no significant difference between the two groups at 3 wks or 4 wks concerning the CFU numbers ($P > 0.05$).

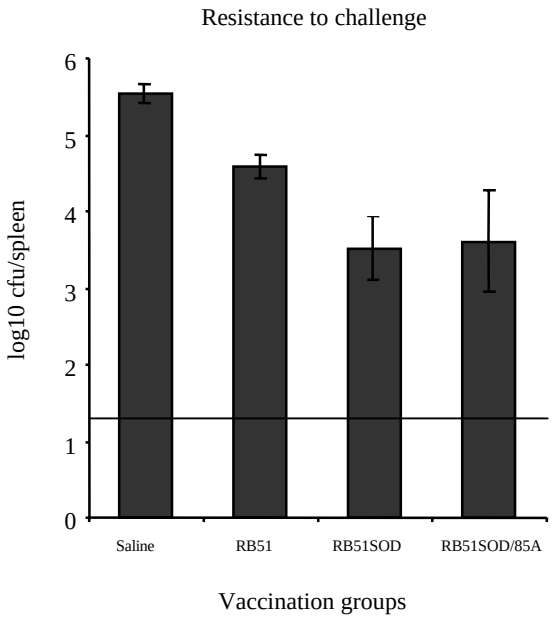


Fig. 4.6. Resistance to *B. abortus* strain 2308 challenge infection in mice vaccinated with RB51 and its recombinants. Mice were vaccinated 6 weeks prior to the challenge infection. Two weeks post-challenge infection, the number of strain 2308 CFU in their spleens was determined. The horizontal line above the χ axis indicates the lower detection limit. The saline group was significantly different from the other three groups ($P < 0.05$), the RB51 group (*) was significantly different from the RB51SOD (**) and RB51SOD/85A (**) groups ($P < 0.05$). No significant different was found between the RB51SOD (**) and RB51SOD/85A (**) groups ($P > 0.05$).

DISCUSSION

Previous studies indicated that *B. abortus* strain RB51 could be used to overexpress *Brucella* Cu/Zn SOD to increase its vaccine efficacy and keep its attenuation characteristics (257). It was also demonstrated that strain RB51 could also express heterologous proteins, for example, the 65-kDa heat shock protein (HSP65) of *Mycobacterium bovis*, and that immunization with such an RB51 induced antigen-specific Th1 type immune responses (255). In this study, strain RB51 overexpressing *Brucella* Cu/Zn SOD and simultaneously expressing the mycobacterial 85A protein was used to immunized mice to study the induction of antigen specific Th1 immune responses, CTL cytolytic reactions and protection against *Brucella* challenge. To the best of our knowledge, it is the first report to use such a strategy for development of a bi-valent, attenuated live vaccine.

Intracellular bacteria including *Listeria monocytogenes*, *M. tuberculosis*, *B. abortus* are controlled by T-cell-mediated immune mechanisms. Satisfactory control of these pathogens would be best achieved by vaccines that efficiently stimulate protective T cells. More and more evidences are showing that secreted proteins are preferred antigen candidates for vaccines against intracellular bacteria (4, 59, Hess, Immunol Med Microbiol 2000 Apr #544, 106, 107, 110). For example, it has been shown that mice can be protected against challenge with *M. tuberculosis* by vaccination with *Mycobacterial* culture filtrate proteins and that protection is equal to immunization with live *M. bovis* strain BCG (4). When splenic T cells from C57BL/6 and BALB/c mice vaccinated with live BCG organisms were used to screen the stimulatory potential of fractionated somatic and secreted *Mycobacterial* proteins by production of IFN-γ, it was found that maximum responses were obtained with fractionated secreted proteins of *M. tuberculosis* (59). The two protective antigens in *L. monocytogenes*, listeriolysin O and protein p60, are natural secreted proteins. However, these two proteins if expressed by *Salmonella typhimurium* strains induced protection against listeriosis only in secreted but not in somatic forms (107). *L. monocytogenes* Cu/Zn SOD is a non-secreted protein just like *Brucella* Cu/Zn SOD. When a recombinant *S. typhimurium* aroA vaccine strain was constructed to secrete the SOD protein, it provided protection against lethal *L. monocytogenes* challenge (106). However, when the SOD was kept inside the bacterium, it did not induce protection (106).

A possible mechanism aimed at explaining why secreted proteins are better protective antigen candidates is that proteins secreted by intracellular microorganisms are available for T cell recognition from the early stages of infection (125). Secreted bacterial proteins are accessible to the class I antigen processing pathway and provide the best targets for protective $CD8^+$ T cells.

Since the recombinant RB51 strain overexpressing *Brucella* Cu/Zn SOD induces enhanced vaccine efficacy (257), the question whether the recombinant SOD may be secreted by strain RB51 must be raised. Our study indicated that *Brucella* Cu/Zn SOD

was the only “secreted” protein in the bacterial culture (Fig. 4.2). It was demonstrated that the strain RB51 overexpressing SOD but not normal strain RB51 stimulated SOD-specific IgG2a antibody (Fig. 4.3) and IFN-γ production (Table 4.1). The recombinant strains RB51SOD and RB51SOD/85A also stimulated higher CTL activity against virulent *Brucella* 2308-infected macrophages (Fig. 4.4). It is possible that these increased activities were due to the “secretion” of SOD since the phenomenon was consistent with the one observed with *L. monocytogenes* SOD (106). However, at this point we cannot eliminate the possibility that increased immunological activities, including protection, were due solely to the higher level of SOD expression achieved by these strains and were not related to the "secretion" phenomenon discussed (257). Unlike *L. monocytogenes* and *M. tuberculosis*, *Brucella spp.* have not been found to secrete proteins. Nevertheless, attenuated *Brucella* vaccines like strain RB51 induce protection and Th1 and CTL activities. It remains an important question whether the extracellular presence of SOD is crucial for the enhanced protection. To answer this question it may be necessary to construct a strain RB51 which overexpresses SOD but retains it in the cytoplasm by removing the SOD signal sequence and assess its protective potential. It will also be useful to construct RB51 strains that overexpress the SOD signal sequence without the SOD since the signal sequence by itself may have a role in protection.

At this point it is unknown how the overexpressed SOD becomes extracellular. It is possible that some specific secretion mechanism is activated to truly "secrete" the overexpressed SOD when it reaches the periplasmic area. It is also possible that the periplasmic area may not be able to hold all the overexpressed SOD and "leaking" through the outer membrane occurs.

Although we could not detect any IgG antibody response against recombinant *Mycobacterial* 85A antigen possibly due to the low expression level of Ag85A, the recombinant strain RB51SOD/85A induced an Ag85A-specific IFN-γ response (Table 4.1). The dose of Ag85A might be important. It was reported that microgram doses of Ag85 elicited delayed hypersensitivity reactions in sensitized guinea pigs while nanogram doses of Ag85 inhibited local delayed hypersensitivity (18, 93). This study suggests that the low expression of Ag85A observed *in vitro* was enough to stimulate a Th1 type immune response. It is possible that this low-dose Th1 immune response inhibits the humoral antibody response (210). Since the CMI response plays a critical role in protection against tuberculosis, it is hypothesized that the recombinant strain RB51SOD85A may be able to induce a certain level of protection against tuberculosis. This hypothesis will be tested in an animal model.

Splenocytes from mice immunized with strains RB51SOD and RB51SOD/85A produced similar levels of IFN-g upon stimulation *in vitro* with purified SOD (Table 4.1), suggesting the expression of mycobacterial 85A antigen in strain RB51SOD/85A did not significantly increase or decrease the effect of SOD on stimulating a specific Th1 type of immune response. The expression of mycobacterial 85A in strain RB51SOD/85A is under control of its own promoter probably without interference from SOD expression

and as expected both strains RB51SOD and RB51SOD/85A have similar expression levels of SOD.

Since the Cu/Zn SOD benefits bacterial survival by neutralizing products of the host's respiratory burst, it can be regarded as a virulence factor (230). On the other hand, Ag85A is a mycolyltransferase and plays an essential role in *Mycobacterial* cell wall synthesis (18) but has no apparent role in virulence. Ag85A readily induces cellular immune responses in humans and animal models. By overexpressing a virulence factor with or without expressing a heterologous antigen in an attenuated pathogen its virulence characteristic may change. It was previously demonstrated that overexpression of SOD did not alter the virulence characteristics of strain RB51 (257) and our studies indicate that adding the expression of a heterologous antigen does not affect this characteristic. strain RB51 appears to be an excellent carrier to express homologous and heterologous antigens with the ability of inducing enhanced Th1 immune responses to the specific antigens and potentially protection without sacrificing its attenuation characteristic.

Chapter 5 Overall Summary and Discussion

The research described in this dissertation was designed to better understand the role of CMI in protection against virulent *B. abortus* infection in order to develop better vaccines against brucellosis and other diseases in the future. *B. abortus* vaccine strain RB51 and a new approach, based on vaccination with *O. anthropi* strain 49237, were used as two parallel models for the CMI analyses.

Live *O. anthropi* was used as a new vaccine for brucellosis because of its many potential advantages over attenuated, live *B. abortus* strains. *O. anthropi* is nonpathogenic for humans with an uncompromised immune system, and thus can be considered as a safe vaccine in the laboratory and in the field. *O. anthropi* strain 49237 does not contain any native plasmid, is susceptible to all the commonly-used antibiotics and grows much faster than *B. abortus* making it a potentially safer and cheaper vaccine. Also, due to this close genetic relationship to *Brucella*, *O. anthropi* shares many antigens with *Brucella*. In Chapter 2, it was demonstrated that *O. anthropi* strain 49237 can be easily transformed with the broad-range-host plasmid pBBR1MCS that is routinely used in *Brucella* genetic engineering. Furthermore, the promoters from *Brucella* genes (e.g., *Brucella sodC* and *groES/groEL*) can be used in *O. anthropi* strain 49237 to achieve high expression levels of *Brucella* proteins.

It was demonstrated that *O. anthropi* strain 49237 and its recombinant strains expressing *Brucella* Cu/Zn SOD and/or GroEL/GroES induced a mix of antigen-specific Th1 and Th2 immune responses in BALB/c mice. *Brucella* Cu/Zn SOD is a protective antigen in *Brucella* although the protective characteristics of GroEL and GroES remain unclear. The mixed immune responses were characterized by both IgG1 and IgG2a responses as well as both IFN-g and IL-4 responses to purified *Brucella* Cu/Zn SOD and whole *O. anthropi* strain 49237 sonicated antigens. All the strains failed to provide protection against virulent *B. abortus* strain 2308 challenge infection. Since *Brucella* protection requires a Th1 type immune response, it was hypothesized that at least *O. anthropi* recombinant strain 49237SOD would induce protection against virulent *Brucella* infection if the induced immune response could be switched to a predominantly Th1 type of response. The strategy used in this study was to use the CpG motifs as an adjuvant. CpG motifs are immunostimulatory oligonucleotides which favor the development of a Th1 type immune response. The co-administration of strain 49237 or strain 49237SOD and CpG-ODN stimulated antigen-specific Th1 type immune responses, characterized by high IgG2a and low IgG1as well as high IFN-g and very low IL-4 immune responses. Under these circumstances both strain 49237 and strain 49237SOD induced protection against virulent *Brucella* infection. The latter induced significantly higher protection, indicating that the expressed *Brucella* SOD stimulated protection *in vivo*. Strain 49237 most probably induces protection because it contains many *Brucella* cross-reactive antigens. Our data indicate that induction of the correct immune response is crucial for induction of protection. This appears to be the first report which indicates that non-

protective, live recombinant bacteria which are expressing protective antigens can be made protective by changing the immune response to the protective antigen(s).

Brucella survives and replicates within macrophages. Murine macrophages only have limited capability to control *Brucella* infection even when activated by IFN-g (76). This suggests that additional immune responses such as the development of CTL activity may play an important role in the control of murine brucellosis (76).

In Chapter 3, a safe, more sensitive, non-radioactive neutral red-uptake CTL assay was adopted to replace the traditional ^{51}Cr-release assay for detection of *Brucella*-specific CTL cytolytic activity. It was shown that *B. abortus* strain RB51 induced specific CTL against both strains RB51 and 2308-infected macrophages, but not to *L. monocytogenes*-infected macrophages. The antigen-specific cytotoxic activity was carried out by T lymphocytes but not by NK cells. The level of CTL activity was paralleled by the capability of the lymphocytes to secrete IFN-g. $CD3^+CD4^+$ T cells secreted the highest amount of IFN-g and exerted low levels of specific lysis of *Brucella*-infected macrophages as well as low levels of non-specific lysis of normal macrophages. In contrast, $CD3^+CD8^+$ T cells secreted low levels of IFN-g and demonstrated high levels of specific lysis of *Brucella*-infected macrophages but no non-specific lysis. It suggests that $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells play synergistic roles in the anti-*Brucella* activity. The IFN-g produced mainly by the $CD3^+CD4^+$ Th1 cells activates macrophages and enhances their bactericidal function as well as stimulates the production of CTL activity. On the other hand, the CTL lyse *Brucella*-infected cells with release of *Brucella* into the extracellular environment where antibody- and complement- mediated immune responses may kill the bacteria or where the released bacteria are ingested by IFN-g activated macrophages.

In Chapter 4, *B. abortus* vaccine strain RB51 was used as a vaccine carrier to overexpress *Brucella* Cu/Zn SOD and simultaneously express mycobacterial 85A antigen (strain RB51SOD/85A). Its purpose was to improve the vaccine performance of strain RB51 and simultaneously use it to protect against virulent mycobacterial infection. A protective immune response to *Mycobacterium* species requires the development of a Th1 type of immune response. Since strain RB51 induces strong Th1 responses, the expression of protective mycobacterial antigen by strain RB51 should lead to the development of Th1 responses to these antigens and potentially, to protection. On the other hand, overexpression of a homologous antigen like Cu/Zn SOD by strain RB51 (RB51SOD) will lead to better protection against brucellosis. The recombinant strain showed enhanced protection against strain 2308 infection in mice when compared to RB51 and the protection level was similar to that achieved by recombinant strain RB51SOD described previously (257). Strain RB51SOD/85A also demonstrated attenuation characteristics similar to strain RB51 or strain RB51SOD. As expected, strain RB51SOD/85A stimulated antigen-specific Th1 immune responses including high IFN-g and no IL-4 production upon stimulation with purified SOD or 85A proteins, and high IgG2a and low IgG1 response against purified SOD antigen. Also, as compared to strain RB51, higher CTL activity against strain 2308-infected macrophages was induced by

strains RB51SOD and RB51SOD/85A. The immunological results indicate that strain RB51 does serve as a Th1 inducing vector for both heterologous and homologous antigens and should induce enhanced protective immunity against mycobacterial disease.

The recombinant *O. anthropi* strain 49237SOD may be used as a human and animal vaccine against brucellosis if combined with CpG-ODN and the recombinant *B. abortus* strain RB51SOD/85A might be used as a vaccine simultaneously protecting against both brucellosis and tuberculosis. Following this line of thought, it may be possible to develop RB51 based vaccines able to protect simultaneously against a variety of diseases which require a strong Th1 response for protection.

APPENDIX
RECIPES OF SOLUTIONS
10 x Stock Phosphate-buffered Saline (PBS) (pH7.4)
80 g NaCl
2 g KCl
14.4 g $Na_2HPO_4 \cdot 7H_2O$ (or 7.63 g Na_2HPO_4)
2.4 g KH_2PO_4
800 ml dH_2O
Titrate to pH7.4
Add more dH_2O to 1 L

50 x TAE Buffer

SOLUTIONS USED IN SDS-PAGE:
Resolving gel buffer: 3 M Tris-Hcl (pH 8.8)
36.6 g Tris base
48 ml 1M HCL
Add dH_2O to final 100 ml
Titrate to pH 8.8
Filter through a Whatman No. 1 fliter
Store at 4 ^{0}C

Stacking gel buffer: 0.5 M Tris-HCl (pH 6.8)
6.0 g Tris base
40 ml dH_2O
Titrate to pH 6.8 with 6 M Hcl
Add more dH_2O to final 100 ml
Filter through a Whatman No. 1 fliter
Store at 4 ^{0}C

Reservoir buffer stock (10 x): 0.25 M Tris, 1.92 M glycine, 1% SDS (pH 8,3)
30.3 g Tris base
144.0 g glycine
10.0 g SDS
Dissolve in dH_2O to 1 L final volume
Store at 4 ^{0}C

1.5% ammonium persulfate
0.09 g ammonium persulfate
Dissolve in 6.25 dH_2O
Prepare before use, enough for 4 gels

Resolving gel (12.5%)
4.68 ml 40% acrylamide stock

1.9 ml resolving gel buffer
0.15 ml 10% SDS
7.57 ml HPLC-grade water
0.75 ml 1.5% ammonium persulfate
7.5 µl TEMED
(Enough for 2 gels)

Resolving gel (15.0%)
5.62 ml 40% acrylamide stock
1.9 ml resolving gel buffer
0.15 ml 10% SDS
6.6 ml HPLC-grade water
0.75 ml 1.5% ammonium persulfate
7.5 µl TEMED
(Enough for 2 gels)

Stacking gel
0.94 ml 40% acrylamide stock
2.5 ml stacking gel buffer
0.1 ml 10% SDS
5.96 ml HPLC-grade water
0.5 ml 1.5% ammonium persulfate
10 µl TEMED
(Enough for 4 gels)

2 x Laemmli Sample Buffer:
4% SDS
20% glycine
10% 2-mercaptoethanol
0.004% bromphenol blue
0.125 M Tris-HCL, pH 6.8

SOLUTIONS USED IN WESTERN BLOT ANALYSIS:
Trans Blot Buffer:
14.41 g Glycine
3.02 g Tris
200 ml Methanol
5 ml 10% SDS
Add dH_2O up to 1 L

Ponceau S Stain Solution
0.5 g ponceau S
1 ml glacial acetic acid
98.5 ml dH_2O

Tris-buffered Saline (TBS)
100 mM Tris-HCL, pH7.5
0.9% NaCl

Tris-buffered Saline / Tween 20 (TBST)
0.05% Tween 20 in TBS

SOLUTIONS USED IN ELISA:
Bicarbonate phosphate solution (coating buffer)
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
Add dH_2O to final 1 L
Titrate to pH9.6

0.18 M H_2SO_4 (Stop Solution)
4.5 ml 8 N H2SO4
95.5 ml dH_2O

SOLUTIONS USED IN CELL CULTURE:
ACK Lysis Buffer
8.29 g NaCl (0.15 M)
1 g $KHCO_3$ (1.0 mM)
37.2 mg Na_2EDTA (0.1 mM)
800 ml dH_2O
Adjust pH to 7.2 - 7.4 with 1 N HCl
Add dH_2O to 1 L
Filter sterilize through a 0.2 µm filter
Store at room temperature

Complete RPMI Medium
10% fetal bovine serum
2 mM L-glutamine
5 mM HEPES (?)
Option: 1 x penicillin and streptomycin
Commercial RPMI medium

Incomplete RPMI Medium
5% fetal bovine serum
2 mM L-glutamine
5 mM HEPES (?)
Option: 1 x penicillin and streptomycin
Commercial RPMI medium

SOC Medium

2% Bacto tryptone (Difco)
0.5% Bacto yeast extract (Difco)
10 mM NaCl
2.5 mM KCl
10 mM MgCl2
20 mM glucose

YENB Medium

0.75% Bacto yeast extract (Difco)
0.8% Bacto nutrient broth (Difco)
(* from: P42 BioTechniques, Vol 20, No. 1, 1996)

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