INTRACELLULAR GROWTH OF *BRUCELLA ABORTUS* AND *B. MELITENSIS* IN MURINE MACROPHAGE-LIKE CELL LINES AND PARTIAL CHARACTERIZATION OF A BIOLOGICALLY ACTIVE EXTRACT FROM *B. ABORTUS* STRAIN RB51

by

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(ABSTRACT)

Brucella abortus is a gram negative, facultative intracellular bacterial pathogen, capable of growth and replication within macrophages, and is the causative agent of bovine brucellosis. The progression of brucellosis within the host is determined by the interaction of *Brucella* with its host. Therefore, it was of interest to specifically examine several features of the *Brucella*-host interaction. The *Brucella*-macrophage interaction is central in the progression of brucellosis and, therefore, it was possible to study this interaction *in vitro* in the form of the abilities of *Brucella* strains to grow and replicate within macrophages. Furthermore, it was of interest to see if the *in vitro* model was capable of assessing the degree of attenuation of the *Brucella*. Various strains of *B. abortus* and *B. melitensis* were used to infect two murine macrophage-like cell lines to study their intracellular growth kinetics and to compare these kinetics with the growth characteristics observed in mice. It was determined that *Brucella* growth in one murine macrophage-like cell line (J774.A1) clearance pattern reflected the *in vivo* growth kinetics of the various *Brucella* tested. All strains tested in the macrophage

model had a significant 1-4 log decrease in intracellular bacteria at 24 hours post infection. The decrease in intracellular numbers at 24 hours postinfection was due to the bactericidal activities of the macrophages as opposed to changes in the *Brucella*. This model was determined not a satisfactory means *in vitro* for assessing the degree of attenuation of *Brucella* mutants, as the model was not capable of predicting the reduced virulence of the *B. melitensis* RM1 rifampin resistant mutant.

Vaccination with live *B. abortus* strain RB51 protects both mice and cattle against challenge with virulent strain 2308. As *B. abortus* is a facultative intracellular pathogen, the host's cell-mediated immunity is assumed to be important in the clearance of the bacteria. Therefore, selected antigens from strain RB51 could be used and tested *in vitro* for their ability to induce a cellular immune, specifically a T helper type 1 (T_{H1}) response, by splenocytes from mice vaccinated with strain RB51. An extract from strain RB51, designated S2, was found to stimulate the proliferation of splenic lymphocytes from strain RB51 vaccinated mice as well as the production of interleukin (IL)-2 and interferon (INF)-γ, but not IL-4. This cytokine profile is consistent with a cell-mediated T_H1 immune response. As the T_{H1} response is assumed to be important in the clearance of *Brucella* by the host, mice were immunized with S2 extract in adjuvant. Upon virulent challenge with strain 2308, no protection was observed as compared with challenged control mice.

The S2 extract contained nine proteins ranging in size from 10 kDa to 80 kDa. In order to determine which of the individual S2 proteins was responsible for the observed T_H1 activity, a means of separating the proteins into individual bands for

testing was needed. Sufficient resolution of the nine proteins could not be accomplished by isoelectric focusing (Rotofor) or by fast performance liquid chromatography (FPLC). Therefore, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate S2 into individual proteins. Two proteins, 10.2 kDa and 11.5 kDa in size, were observed to stimulate the production of INF-γ by sensitized splenocytes. The N-terminal amino acid sequences of these two proteins were obtained and their putative DNA sequences deduced.

Another antigen known to be a component of the S2 extract is the lipopolysaccharide (LPS). Polymyxin B is thought not to bind *B. abortus* LPS as it does not contain 3-myristic acid. However, it was important to evaluate the role, if any, of LPS contamination of the S2 extract in the *in vitro* responses observed with splenocytes from mice vaccinated with strain RB51. The S2 extract was treated with polymyxin B linked to agarose beads. The treated S2 extract contained less 2-keto-3-deoxyoctonate (KDO) which indirectly suggests a decrease in LPS. Furthermore, the proliferation of sensitized splenocytes induced by polymyxin B treated S2 extract was observed to decrease slightly, while the INF-γ levels were observed to increase following the treatment. Reactivity of a monoclonal antibody (Bru 48) specific for *B. abortus* rough LPS was observed by immunoblot analysis only with samples not treated with polymyxin B beads. This suggests that the amount of rough *B. abortus* LPS contained in the preparation is less than is detectable by the monoclonal antibody when the S2 extract was treated with polymyxin B beads. In contrast to published results, these data suggest that polymyxin B interacts with *B. abortus* LPS.

The use of a live vector delivery system as a means of vaccination needs to be examined further in the case of brucellosis. In this manner, the expression of the S2 proteins may more accurately be assessed as to their role in eliciting a protective immune response.

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In conclusion, I wish to say that no major work is ever the endeavor of an individual. There are countless others who helped in many ways: technical, spiritual, and emotional. It is to all of the individuals, both mentioned and unmentioned, that this work is dedicated.

DECLARATION OF WORK PERFORMED

I declare that, with the exception of the items listed below, all work reported in this dissertation was performed by myself.

J774.A1 and PU5-1.8 cells infected with *Brucella abortus* strains 2308 and RB51 were prepared by myself and taken to the Electron Microscopy (EM) Facility of the Veterinary College where the cells were processed, sectioned and placed on nickel grids. The immunogold labeling of the grids was performed by myself, with cell staining performed by Ms. Kathy Hayman at the EM Facility of the Veterinary College.

The individual protein bands for N-terminal amino acid sequencing were submitted to the Amino Acid Sequencing Facility, Department of Biology, Virginia Tech and the sequencing performed by Ms. Laura Sporakowski.

The mouse *Brucella* clearance data was obtained with the technical assistance of Ms. Betty Mitchell.

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ACK	ammonium chloride, potassium carbonate lysis buffer
APC	antigen presenting cell
BSA	bovine serum albumin
CMI	cell mediated immunity
CFU	colony forming units
CPM	counts per minute
DDA	dimethyldioctadecylammonium chloride
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
Ig	immunoglobulin
INF	interferon
IL	interleukin
kb	kilobases
kDa	kilodalton
KDO	2-keto-3-deoxyoctonate
x log(s)	10^x
LPS	lipopolysaccharide
LTA	lymphocyte transformation assay
MHC	major histocompatibility complex

mwco	molecular weight cut off
NCBI	National Center for Biotechnology Information
PBS	phosphate buffered saline
proK	proteinase K
RPMI	Roswell Park Memorial Institute, Buffalo, NY
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI	stimulation index
SOD	superoxide dismutase
T	transmittance
TBS	Tris buffered saline
TBST	Tris buffered saline-Tween 20
TEM	transmission electron microscopy
TSA	trypticase soy agar

CHAPTER ONE

LITERATURE REVIEW OF BRUCELLOSIS

Characterization and Historical Perspectives of *Brucella*

The genus *Brucella* consists of five bacterial species: *melitensis, abortus, suis, canis,* and *neotomae*. This genus represents a group of gram negative rods which are facultative intracellular pathogens, capable of growth and replication within macrophages (39). Phenotypically, smooth morphology is typically associated with virulence within the genus with the exceptions of *B. ovis* and *B. canis* (21, 72). The individual species of the group differ somewhat as to their biochemical properties and host specificity. At the DNA level, the genus has a G+C content of 56-58 mole percent and share >94% homology between species (39, 48).

Historically, *B. abortus* was originally described by Bang in 1897 and hence brucellosis in cattle often being referred to as Bang's disease (29). In 1906, Bang reported that cattle infected with virulent *B. abortus* were afforded protection from abortion (9). From this observation, he suggested that heifers should be infected with *B. abortus* before breeding age in order to decrease the numbers of abortions resulting from infection during pregnancy. Unfortunately, it was observed that infection was not a good means of prevention in this case. Thus began the search for less virulent mutants of *B. abortus* which could protect the animal against abortion without causing disease.

Brucella abortus

B. abortus is a facultative intracellular pathogen which causes abortion in pregnant cows and sterility in bulls and undulant fever in humans (29). In addition to swine and goats, *B. abortus* infections have been reported in the elk and bison populations (77). The smooth strains of *B. abortus* are more virulent than the rough strains. Rough strains can be differentiated from smooth strains, as rough strains by staining of colonies on agar plates with crystal violet and clumping in acriflavine (3). Biochemically, *B. abortus* preferentially utilizes the polyalcohol erythritol, is urease and oxidase positive, and most biotypes produce hydrogen sulfide and are capable of growth on basic fuchsin (3). Additionally, *B. abortus* can be identified by its ability to be lysed by the Tb phage (26).

Pathogenesis

Typically, *B. abortus* is transmitted to an uninfected host by ingestion, although other routes such as inhalation and the conjunctivae have been suggested (28, 38). Bovine brucellosis is not considered a venereal disease. Following ingestion, the bacteria localize in the draining lymph nodes where the *Brucella* replicate and disseminate to other sites, i.e. the spleen. Further dissemination leads to *Brucella* localization in the pregnant uterus and supramammary lymph nodes. Some evidence suggests that the virulent strains of *B. abortus* localize preferentially in the pregnant uterus and fetal tissues as the result of erythritol production at these sites (89). The

bacteria are capable of crossing the placenta and infecting the fetus (38). The localization of the bacteria in the placenta and fetal tissues results in the fetal death and/or fetal abortion. Although some animals clear the infection, others become chronically infected. The chronically infected animals are capable of transmitting the disease to other individuals. Since the bacteria are often shed in the milk and uterine secretions, particularly during and after abortions, entire herds are slaughtered when *B. abortus* infections are detected within a herd as part of the U.S. Brucellosis Eradication Program (47).

B. abortus Lipopolysaccharide

The *Brucella* lipopolysaccharide (LPS) is structurally similar, but chemically different than that observed in the Enterobacteriaceae family. The structure is similar to that in other gram negative bacteria in that there is a lipid A moiety joined by a core oligosaccharide to the O-chain polysaccharide (19). The lipid A moiety is unique in *Brucella* LPS in that it is composed of acyloxyacyl residues with amide linkages. Additionally the backbone sugar of the lipid A moiety is 2,3-diamino-2,3 dideoxy-D-glucose and its structure is similar to that observed in some species of photosynthetic bacteria (75). The O-chain polysaccharide is composed of repeating 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units, known as perosamine (19).

The LPS of *B. abortus* plays a central role in the humoral response of an infected host to the pathogen. The O-chain polysaccharide appears to be an

immunodominant antigen since the vast majority of the host's humoral immune response is to this antigen (80). Due to the immunodominant nature of this serological response, antibody reactivity with the O-chain polysaccharide is used as a diagnostic serologic test for the detection of infected animals (110). The LPS has been suggested for use as a carrier in vaccine development, as it stimulates B cells *in vivo* without the toxicity observed with the use of *Escherichia coli* LPS (43). The reason for the decreased toxicity of the *B. abortus* LPS was not determined. Classically, *B. abortus* LPS has been used as a T cell independent type 1 antigen (42). Furthermore, *B. abortus* LPS stimulates the production of the IgG1 antibody isotype (12). The LPS interaction with the host in a T-cell -independent manner was also able to induce a humoral response to other antigens due to the LPS stimulation of B cells *in vivo* in the mouse model (42). The authors of this study suggest that *B. abortus* LPS could potentially be used as a vaccine carrier.

The Murine Model of Bovine Brucellosis

Several investigators have used the murine model to study brucellosis, since experimental work with cattle is expensive and no inbred lines of cattle are available (14, 63, 80). The use of mice has the benefit of the vast information available on the immune system and the availability of inbred strains (80). The mouse model of brucellosis is similar to that observed in cattle in that mice are infected chronically by virulent *Brucella* and studies have shown that infection with virulent strains result in

the colonization of the placenta and fetal tissues, and ultimately fetal death in the pregnant mouse model (14, 106). Differences include antibody isotypes elicited and bovine macrophages do not have myloperoxidase involvement in the oxidative burst (2, 14).

Passive transfer studies have shown that cell-mediated immunity (CMI) and humoral immune responses are both important in the clearance of *Brucella* (6, 14, 63, 109). The humoral immune response in mice vaccinated with strain 19 (the current vaccine strain) and strain 2308 (the wildtype, virulent strain) has been characterized by a predominance of the IgG2a and IgG3 isotypes at 4 and 6 weeks postinfection (11, 34, 110). Winter *et. al.* observed that mice passively transferred with a monoclonal antibody (IgG2a isotype) specific for the O-chain polysaccharide of *B. abortus* strain 2308 conferred protection from virulent challenge to 82% of the treated mice, while 95% of the control mice were infected (109). Another monoclonal antibody specific for the O-chain but of the IgG1 isotype or convalescent serum decreased the numbers of viable *Brucella* in the spleen. However, at higher challenge doses these serums were not able to provide protection. The authors suggest that the ideal vaccine, at least in mice, should be able to induce both the appropriate humoral immune response as well as an appropriate cell mediated immune response. Using the pregnant mouse model, Bosseray observed that passive transfer of serum (from mice infected with *B. abortus* strain 19) prior to infection or at the time of infection significantly decreased *B. abortus* colonization of the placenta (14). He also observed that immunity

transferred in this manner lasted approximately 3 days. Plommet, *et. al.* injected immune serum into mice, subsequently footpad challenged them with *B. abortus* strain 544, and they observed an approximately 2 log decrease in numbers of *Brucella* from the spleens (79). If the immune serum and challenge bacteria were given to mice simultaneously, decreased numbers of viable strain 544 were obtained from the liver and the spleen at different time points following infection. Additionally, the mice were able to clear the infection within 49 days instead of remaining chronically infected.

Mackaness observed that a delayed-type hypersensitivity reaction noted with mice infected with intracellular pathogens could not be transferred passively with serum (63). Moreover, he found that the macrophages of the infected animals had increased bacteriocidal activity. Araya *et. al.* observed that serum and T-cells passively transferred together provided more protection in mice against virulent challenge with *Brucella* than serum or T-cells passively transferred individually (6). They also observed that passive transfer of equal numbers of $CD4^+$ and $CD8^+$ T cells were both protective in mice, suggesting that both T cell subsets have a role in protection. In another study, Araya and Winter observed that passively transferred T cells conferred better protection when the mice were challenged with the same strain of *B. abortus* as the T cell donor (5). Jiménez de Bagüés, *et. al.* passively transferred T cells from strain RB51 vaccinated donor mice and observed protection against virulent challenge with strain 2308 or *B. melitensis* 16M, but not *B. ovis* PA (54). However, the highest levels of protection were observed when the mice were challenged with the same

strain as the T cell donor (homologous challenge).

The murine model has elucidated some very important information regarding the role of cell-mediated immunity versus the role of the humoral immune response in the clearance of the bacteria from the host (109). The progression of brucellosis *in vitro* in intracellular models and the role of a plethora of cytokines has gained popularity in the literature (20, 52, 53, 56, 78, 92, 98, 113, 115- 117). Many of these models have been developed in either murine macrophage-like cell lines or murine peritoneal macrophages.

Rationale for the Development of New Vaccines for Brucellosis

Brucellosis has been almost completely eradicated in the United States as a result of Brucellosis Eradication Program initiated in the 1940's (47, 77). This program was devised as essentially a rigorous evaluation of all cattle within the United States. Those cattle that tested positive for brucellosis were subsequently slaughtered. However, isolated outbreaks do occur as the result of transport or movement of infected animals from inside or outside the United States or transmission to herds by the natural reservoirs, for example, elk and bison (77). Due to the impracticality of test and slaughter programs for elk and bison, other means of immunizing wildlife against brucellosis are currently being sought.

The current, approved vaccine strain for brucellosis in cattle is *B. abortus* strain 19. Strain 19 is a naturally attenuated strain, originally isolated from milk of an

infected cow named Matilda and incubated at room temperature for approximately one year (18, 44). Strain 19 possesses a semi-rough phenotype and genetically has a 702 base pair (bp) deletion in D-erythrulose-1-phosphate dehydrogenase gene necessary for the utilization of erythritol (17, 85). It was found that strain 19 typically provided 65-75% protection in vaccinated bovines (2). However, the strain 19 vaccine has some limitations: (i) causes chronic brucellosis in some animals; (ii) as strain 19 vaccination can sometimes cause abortion, only female calves prior to breeding age can be vaccinated; (iii) in some cases vaccination has been observed to cause orchitis and consequent sterility in male calves; and (iv) strain 19 possess the immunodominant O-chain polysaccharide antigen which stimulates the production of anti-O-chain polysaccharide antibodies in vaccinated animals. Vaccination of female calves before the age of 11 months was recommended, as persistent serological responses to the O-chain polysaccharide occur with adult strain 19 vaccination (2).

Since eradication of brucellosis is based on serologically identifying infected cattle, two approaches in developing an ideal vaccine could be investigated as a means of serologically differentiating between infected and vaccinated cattle. First, a mutant which lacks the O-chain polysaccharide antigen would not induce the production of anti-O-chain polysaccharide antibodies and thus could allow differentiation between vaccinated and infected animals. Second, a mutant which elicits a humoral immune response to a novel antigen could also be used to serologically differentiate between vaccinated and infected animals.

Due to the many disadvantages associated with vaccination of cattle with strain 19, alternative vaccines have been proposed and sought. The ideal *B. abortus* vaccine would be one which would (a) easily distinguish vaccinated from infected animals serologically, (b) not cause disease in humans or vaccinated animals, (c) elicit appropriate, protective humoral and cell-mediated immune responses by the host, (d) allow vaccination regardless of age, (e) not induce side-effects (such as abortion) in vaccinates, (f) not revert to a virulent type, and (g) require only one vaccination to provide life long protection.

One school of thought has been to develop an attenuated, rough and stable *B. abortus* mutant which does not express the O-chain (88). Others have constructed mutants with deletions in a variety of genes not related to O-chain polysaccharide biosynthesis as potential vaccine candidates (35, 45, 103, 104). Alternatively, *Brucella* antigens including LPS, are being investigated for their ability to stimulate specific, protective immune responses which may ultimately lead to development of a subunit or recombinant vaccine (14, 15, 33, 102, 115). A continuance of this line of thought lead to the design of a vector which expresses protective antigens and able to immunize an animal against several diseases simultaneously (33a).

One candidate which fits the order of a live, attenuated, rough *B. abortus* vaccine is *B. abortus* strain RB51 (88). Strain RB51 is a rough, attenuated mutant of *B. abortus* strain 2308, which was derived by serial passage on media containing rifampin. The specific mutations leading to the rough phenotype and rifampin

resistance of RB51 have not yet been characterized. Studies in mice have demonstrated that strain RB51 is cleared from the spleen within 4 weeks post inoculation (88). Serologically, RB51 does not induce anti-O-chain polysaccharide antibody production in mice, goats, rabbits, and cattle (88, 94, 97). Moreover, vaccination of mice with RB51 confers protection against virulent challenge with strain *B. abortus* 2308 and *B. melitensis,* but not against *B. ovis* (54, 88). Cattle are also protected from virulent challenge by prior vaccination with strain RB51 (24).

In a series of studies, Stevens, *et. al.* have examined the humoral and cell-mediated immune responses of cattle and mice to infection with strains RB51, 2308, and 19 (94-97). Proliferation of sensitized bovine lymphocytes were observed to respond at similar levels to a *B. abortus* protein fraction containing proteins ranging in size from 18kDa to 27kDa extracted from either strain RB51 or strain 2308 (96). The authors suggested that the responses are to the proteins and not to contaminating O-chain polysaccharide of the antigens isolated from strain 2308, as the responses to the same proteins extracted from strain RB51 were similar. In another study, it was observed that sensitized murine lymphocytes responded similarly to 22 protein fractions obtained from either strain 2308 or strain RB51 (97). In particular, the greatest lymphoproliferative response was observed to proteins which were 18kDa or smaller, from either strain 2308 or strain RB51. Furthermore, it was observed that protein fractions which induced proliferation by sensitized murine lymphocytes were the same for fractions from both strains 2308 and strain RB51. This is consistent with

the possibility that strain RB51 possesses the antigens necessary to elicit a protective immune response in vaccinated hosts.

In comparing splenic clearance of strains RB51 and 19 from mice, it was observed that infections with strain RB51 were of shorter duration (8 weeks) than with strain 19 (12 weeks) as measured by the numbers of viable brucellae (95). No anti-O-chain polysaccharide antibodies could be detected in the sera of the mice vaccinated with RB51. However, Stevens, *et. al.* observed that RB51 vaccinated mice had a lower "resistance to infection" than mice vaccinated with strain 19. This may be due to the short duration of strain RB51 persistence in the mice or, probably more importantly, the presence of the anti-O-chain polysaccharide antibodies due to strain 19 vaccination.

Stevens, *et. al.* also examined the immune responses of cattle vaccinated with strain 19 compared with strain RB51 (94). No anti-O-chain polysaccharide antibodies were detected in any of the cattle vaccinated with strain RB51. Lymphocytes from the lymph nodes of both strain 19 and strain RB51 vaccinated cattle were observed to proliferate in response to the same 12 of 22 protein fractions of strain 2308, suggesting the response is to the protein components of the fractions. Two fractions which contained a 27kDa protein were found to give the highest levels of stimulation in both groups of vaccinated cattle. No detectable differences in immune responses could be detected between the cattle vaccinated with strain 19 and those vaccinated with strain RB51 other than the lack of anti-O-chain polysaccharide antibodies in the strain RB51 vaccinated cattle.

Jensen, *et. al.* used pulse-field gel electrophoresis as a possible means of differentiating strain RB51 from *B. abortus* field isolates obtained from cattle, bison, and elk (51). A unique characteristic DNA pattern was observed with strain RB51 DNA digested with the restriction endonuclease *Xba* I. The RB51 digested DNA contained a band at 104 kilobases (kb), while the digested DNA of the field isolates possessed a band at 109 kb.

Rationale for Dissertation

As the interaction of *B. abortus* with the macrophage is central in brucellosis (63), it was of relevance to examine this interaction *in vitro*. Using two macrophage-like cell lines, J774.A1 and PU5-1.8, the growth kinetics of various species of *Brucella* was examined. Furthermore, based upon the growth kinetics of the *Brucella* within the macrophages, it was of interest to determine whether or not this assay was able to assess the degree of attenuation of various *Brucella* mutants. For comparison, although at different time intervals, the number of viable *Brucella* obtained from the spleens of vaccinated mice was used to assess the ability of the *in vitro* test to determine the degree of attenuation of the various mutants. An attenuated mutant was one which was cleared more quickly than the wildtype in mice and was hypothesized to replicate poorly in the *in vitro* macrophage model as determine by enumeration of viable intracellular CFU of bacteria.

Once a macrophage has phagocytized, processed, and presented antigen on its

surface in context of MHC class I or class II molecules, it is available for interaction with specific T cell clones. Examining the intracellular location of the *Brucella* within the macrophage-like cell lines by electron microscopy may help determine whether the macrophage-like cells are able to present *Brucella* antigens in the context of MHC class II, if the *Brucella* are observed only in vesicles. Alternatively, the antigens may be presented in context of MHC class I, if the *Brucella* are observed intracytoplasmically.

Vaccination of both mice and cattle with *B. abortus* strain RB51 confers protection upon virulent challenge (24, 88). Furthermore, passive transfer of T cells from strain RB51 vaccinated mice confers protection from challenge with virulent strain 2308 in recipient mice (8). The T cells responding including T_{H1}, T_{H2}, and T_{H0} (62).The T_H1 response is important in brucellosis, as the cell-mediated immune response is thought to be of importance in the clearance of facultative intracellular pathogens by an infected host (63). The T_H1 response is characterized by the stimulation of lymphoproliferation and the production of interleukin (IL)-2 and interferon (INF)-γ in response to macrophage-T cell interactions (62). While T_{H2} is characterized by the production of IL-4, IL-5, IL-6, and IL-10. T_{H0} cells are capable of producing both T_{H1} and T_{H2} cytokines, such as INF-γ and IL-4. Therefore, strain RB51 was used to prepare an antigen extract (S2) for investigation into antigens which may stimulate a T_H1 immune response by splenocytes from strain RB51 vaccinated mice.

Although the S2 extract was found to induce a T_{H1} type response, it was

unclear as to which individual antigens in the extract were responsible for generating this response. In order to determine which of the S2 antigens were eliciting the T_{H1} type response, the individual proteins of the strain RB51 antigen extract were separated to determine which antigens were responsible for the immune responses observed *in vitro*. The antigens observed to be stimulatory were further characterized by N-terminal amino acid sequencing.

It has been reported that live bacteria are better able to induce protective immune responses than killed vaccines (113). However, as S2 antigens were capable of eliciting an *in vitro* T_{H1} immune response, it was of interest to determine whether immunization of mice with S2 antigens was able to confer protection from virulent challenge. If protection was not observed with the S2 antigens, it would not eliminate the possibility that any of the S2 proteins has a role in protection. Active expression, and active infection may be required for a more accurate assessment of the role of protection for these antigens. For example, once the genes encoding these proteins are identified, their expression in a *S. typhimurium aroA* mutant (77A) may provide more information about the role of protection for any of the individual S2 proteins.

CHAPTER TWO

THE ROLE OF THE MACROPHAGE IN BRUCELLOSIS: GROWTH KINETICS OF VARIOUS *BRUCELLA* SPP. IN J774.A1 and PU5-1.8 MACROPHAGE-LIKE CELL LINES

INTRODUCTION

The ability of an infected host to successfully eliminate a *Brucella* infection lies in the combined effects of humoral and cell-mediated immunity (6, 109). An ideal vaccine candidate would elicit the appropriate humoral and cell-mediated immune responses of the host and therefore confer protection against virulent challenge. In the case of bovine brucellosis, the vaccine candidate should provide a means of distinguishing vaccinated from infected animals as the present vaccine does not (2).

The Role of the Macrophage

As a facultative intracellular pathogen, *Brucella* spp. are capable of survival, growth, and replication in macrophages (39). The interaction of the *Brucella* with the macrophage plays a central role in brucellosis. The *Brucella* is phagocytized by the macrophage and the bacteria is then subjected to the acidic pH of the phagosome (58). Fusion of the phagosome with a lysosome (resulting in the formation of a phagolysosome) results in exposure of bacteria to an oxidative burst, nitrogen

intermediates, lysozyme, and an iron-limiting environment. Those bacteria incapable of surviving such an antimicrobial attack are killed. However, facultative intracellular pathogens have developed a variety of ways of evading or withstanding these harsh conditions within the macrophage. For example, it has been observed that *B. abortus* can inhibit phagosome-lysosome fusion (40). *Listeria monocytogenes* is also capable of escaping from the phagosome into the cytoplasm (81). However, if the macrophages are activated by interferon (INF)-γ, the phagocytized *L. monocytogenes* is destroyed, a situation similar to the one occurring with *Brucella* (56).

Historical Perspectives of *In Vitro* Cell Cultures Examining Growth Characteristics of Facultative Intracellular Pathogens

Examination of the growth of facultative intracellular pathogens in macrophage models *in vitro* is by no means a new approach for modeling infections. Early studies by Mackaness examined growth rates of *Mycobacterium tuberculosis* in tissue culture with macrophages from vaccinated and normal rabbits (64). Furthermore, Mackaness, Smith, and Wells reported that there was a relationship between the growth rate of several *M. tuberculosis* strains in cultured macrophages and its virulence (65). This finding was earlier described by Suter using mononuclear leukocyte cultures (99, 100). Measurement of the growth rates of facultative intracellular pathogens in tissue explants and the similarity of the model *in vitro* as compared with observations *in vivo* has been available since 1924 (39,63, 68).

The early tissue culture models of infection provide insight into present day *in vitro* models for studying intracellular pathogenesis (39). Without using a chemotactic agent, peritoneal macrophages were obtained from guinea pigs. The monocytes were washed, counted, and resuspended in small amount of heat-inactivated horse serum in siliconized tubes to prevent attachment of the macrophages to the tubes and rough or smooth *B. abortus* were added. Once phagocytosis was complete (incubation at 37°C for 3 hours), the monocytes were mixed, subdivided into nonsiliconized tubes, and brought up to a volume of 2 ml in Hanks Balanced Salt Solution with a final serum concentration of 25%. The growth rate of the smooth and rough *B. abortus* strains were evaluated at T_0 and subsequent 18-24 hour periods. The number of both rough and smooth *Brucella* increased over the time course and no differences in the growth rates of smooth versus rough *B. abortus* strains were observed. This lack of difference may have been an artifact since extracellular bacteria were not removed from the assay following phagocytosis.

In a later study, Fitzgeorge, *et.al.* examined the growth curves of *B. abortus* smooth strain 544 in macrophages from immunized and nonimmunized bovines (37). They observed that the numbers of viable bacteria increased slowly in macrophages from immune cattle compared to nonimmunized cattle.

More recent studies have utilized macrophage-like cell lines, both mouse and human, for investigating the intracellular growth characteristics of facultative intracellular pathogens. These include the murine macrophage-like cell lines J774.A1

(J774), PU5-1.8 (formerly PU5-1R), and P388D1. J774 is a cell line cultured from a tumor of a BALB/c female mouse (7). This cell line produces lysozyme, possesses F_c receptors and complement receptors, capable of phagocytosis primarily via antibody-mediated opsonization, and constitutively produces IL-1. Additionally, the growth of J774 cells is inhibited by *E. coli* LPS. PU5-1.8 cells were derived from a spontaneous lymphoid tumor of a BALB/c mouse. Like J774 cells, PU5-1.8 cells produce lysozyme, possesses both F_c and complement receptors, and their growth is inhibited by *E. coli* LPS. The P388D1 cell line originated from a lymphoid neoplasm of a DBA/2 mouse. Also like the J774 cells, this cell line produces lysozyme, possesses F_c and complement receptors, and produces high levels of IL-1. Examination of growth kinetics of some *Brucella* spp. in J774 and P388D1 monocytic cell lines has been reported in the literature (8, 52). These macrophage-like cell lines do exhibit many of the traits observed with splenic or peritoneal macrophages. However, they are unlike splenic or peritoneal macrophages in that they are capable of replication *in vitro* and constitutively produce particular cytokines, such as IL-1.

Macrophage *In Vitro* Models in the Study of Brucellosis

Brucella spp. are gram negative, facultative intracellular pathogens capable of growth and replication within macrophages (39). Many individuals have used macrophage models in order to study pathogenesis of many facultative intracellular pathogens, including *Brucella abortus*, *Salmonella typhimurium*, *Listeria*

monocytogenes, and *Mycobacterium* spp. (10, 52, 69, 107). In a recent study, Caron *et. al.* infected the human monocytic cell line U937 with virulent and avirulent *Listeria* and *Brucella* spp. (22). They observed optimum uptake of bacteria when U937 cells were incubated with lipopolysaccharide (from *S. typhimurium*) for 30 minutes prior to infection with the *Listeria* and *Brucella* spp. and when opsonized bacteria were used. They observed that virulent *L. monocytogenes* and *B. suis* S1 were capable of replicating within the macrophages, in contrast *L. ivanovii* was eliminated from the cell line over a 24 hour period. *B. canis,* a rough strain and only considered pathogenic for dogs, was not capable of replication, but did survive. Due to the distinct differences in the growth kinetics of the virulent and avirulent *Listeria* and *Brucella* spp., the authors suggested that this model may provide a means of differentiating virulent from avirulent facultative intracellular pathogens.

Jiang and Baldwin used the murine macrophage-like cell line J774 to study the effects of the addition of different cytokines to the macrophages on the intracellular growth of antibody opsonized *B. abortus* strains 2308, 19, and RB51 (52). Additionally, they compared the responses of the J774 cells with those observed with murine splenic macrophages and thioglycolate-induced macrophages. They observed that approximately 30-40% of the J774 cells were initially infected with brucellae and at 48 hours postinfection 80-98% of the macrophages were still viable. Moreover, a decrease in intracellular strain 19 occurred at 12-24 hours postinfection, followed by an increase at 48 and 72 hours and a second decrease at 96 hours postinfection.

Although the initial decrease at 24 hours was not addressed in this paper, the decline at 96 hours was attributed to the death of the macrophages. Furthermore, J774 cells activated with interleukin 2 (IL-2) and interferon (INF)-γ prior to infection, killed both *B. abortus* strains 2308 and RB51 efficiently. Differences in intracellular brucellae numbers were apparent in the different populations of macrophages tested. However, a larger decrease in the number of intracellular brucellae was observed when the macrophage cultures were activated with INF-γ.

In contrast with this study, Jones and Winter observed that opsonized strain 2308 was phagocytosed more readily by murine peritoneal macrophages than opsonized strain 19 (56). Moreover, they observed that the addition of exogenous INF-γ to the system decreased the numbers of viable strain 19 to a larger degree than strain 2308. The strain 2308 was able to sustain an intracellular infection within the macrophages treated with exogenous INF-γ. During the course of the strain 2308 and 19 infections within the peritoneal macrophage, a decrease of viable bacteria was observed at 24 hours with a subsequent increase in intracellular numbers at 48 hours postinfection.

RATIONALE AND HYPOTHESIS

This portion of study had three major goals:

1) Ascertain if macrophage-like cell lines can be used to assess attenuation of

Brucella vaccine candidates *in vivo*. To do this, J774 and PU5-1.8 cells were infected with various *B. abortus* and *B. melitensis* strains which had been characterized regarding their degree of attenuation in mice. It was postulated that *Brucella* with attenuated characteristics *in vivo* would be cleared faster *in vitro*.

2) Ascertain if *Brucella* replicating within macrophages are a selected population. A conspicuous drop in intracellular numbers of *Brucella* at 24 hours post infection of macrophages was observed by us and others (52, 56). It was postulated that the intracellular bacteria present at 48 hours postinfection were a selected population refractive to killing during the early macrophage infection period. To investigate this phenomenon, *Brucella* obtained from macrophages past 24 hours postinfection were used to infect macrophages. Moreover, determine if the phenomenon was related to the action of the macrophages as opposed to nonphagocytic cells, such as Vero cells.

3) Ascertain the intracellular location of strains RB51 and 2308 within the J774 and PU5-1.8 macrophage-like cell lines by using electron microscopy. It was postulated that both cell lines would allow the growth of these strains within the cytoplasm.

MATERIALS AND METHODS

Cell Culture

J774, PU5-1.8 and P338D1 macrophage-like cell lines were obtained from the

American Type Tissue Collection (Rockville, MD). The cells were cultured at 37° C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (Gibco-BRL) supplemented with 2mM L-glutamine (ICN Immunochemicals) and 10% heat inactivated fetal bovine serum (Hyclone).

Cells were passaged twice weekly by scraping the bottom of the flask (Nunc) and diluting the cells 1:10 for 25 cm^2 flasks or 1:3 for 80 cm^2 flasks.

For the *Brucella* intracellular growth kinetics assays, exponentially growing macrophages were diluted to a cell density of 3 X 10^5 cells/mL and 1 mL of the cell suspension seeded into wells of 24-well plates (Nunc). The macrophages were incubated at 37° C in 5% CO_2 for 1 hour and then infected with the appropriate *Brucella* strains under P3 conditions.

Bacterial Strains

Brucella abortus strains 2308, RB51, RA1, rifampin-resistant RA1, and *Brucella melitensis* strains 16M, RM1, and rifampin-resistant RM1 were obtained from the *Brucella* collection of the Virginia-Maryland Regional College of Veterinary Medicine (VPI & SU, Blacksburg, Va). All *Brucella* strains were grown on trypticase soy agar (TSA) plates at 37° C in 5% CO_2 for 72 hours.

For the infection assays, the *Brucella* were harvested by washing the plates with 0.1M phosphate-buffered saline pH 7.2 (PBS) and adjusted to a density of 10% transmittance (T) at 525nm. An aliquot of the suspension was removed and ten-fold

serial dilutions prepared. Five 10μL aliquots of each dilution were plated on TSA plates and incubated at 37°C in 5% CO_2 for 72 hours. The resulting colony forming units (CFU's) were used to determine the numbers of viable bacteria.

Opsonization of *B. abortus* Strain 2308

B. abortus strain 2308 was grown on TSA, harvested from the plates, and cell density adjusted to 10%T at 525 nm in sterile 0.1M PBS. In separate 1.5 mL microfuge tubes (Eppendorf), 100uL of the bacterial suspension/well to be infected was centrifuged at 8060 X g for 5 minutes (TOMY Microcentrifuge). The supernatant was removed, and the bacteria were resuspended in the appropriate volume of DMEM complete medium (2mM L-glutamine and 10% heat inactivated fetal bovine serum) or DMEM complete medium with a 1:100 dilution of stock monoclonal antibody Bru38 (87). Bru 38 (provided by Dr. G. Schurig) is a monoclonal antibody specific for the O-chain polysaccharide of *B. abortus, B. melitensis, and B. suis* lipopolysaccharide (LPS). The bacterial suspensions were vortexed and incubated at room temperature for 30 minutes to allow for opsonization. Aliquots of the opsonized or unopsonized bacteria was used to determine the number of viable bacteria or to infect the macrophages.

Infection of Macrophages and Growth Kinetics of Intracellular *Brucella* spp.

J774 or PU5-1.8 cells were seeded at a cell density of $3x10^5$ cells/well and

incubated at 37°C in 5% CO_2 for 1 hour. A 100 µL of a 10%T suspension of the appropriate *Brucella* spp. was added to each well and incubated for 30 minutes at room temperature. The actual numbers used in infection per well were as follows: *B. abortus* strain 2308-8.4 X 10^8 , strain 19- 6.4 X 10^8, strain RB51- 9.6 X 10^8, strain RA1- 6.3 X 10^8, strain RA1 rifampin resistant- 5.4 X 10^8; *B. melitensis* strain 16M- 6.6 X 10^8, strain RM1- 2.4 X 10^8, and RM1 rifampin resistant- 4.4 X 10^8. The cells were washed three times with 1 mL aliquots of sterile 0.1M PBS. One milliliter of DMEM complete medium + 50 µg/mL gentamicin (Sigma Chemical Co., MO) was added to the wells and incubated an additional 30 minutes at room temperature to kill extracellular *Brucella*. The medium was removed and replaced with 1mL of DMEM complete medium + 12.5 µg/mL gentamicin. The cultures were then incubated at 37°C in 5% CO_2 .

At intervals of 0, 24, 48, and 72 hours postinfection, a sample of the supernatant was plated on trypticase soy agar to assess growth of extracellular brucellae. The macrophages were washed three times with sterile 0.1M PBS, incubated at room temperature for 15 minutes in 1 mL 0.1% deoxycholate (Sigma Chemical Co., MO) to lyse the cells and release intracellular brucellae. The suspension was washed from the well by pipeting and placed into a sterile 1.5 mL microfuge tube and centrifuged at 8060 X g for 5 minutes. The supernatant was removed and the intracellular brucellae were resuspended by vortexing in 100 µL 0.1M PBS. Ten-fold serial dilutions of the entire aliquot of each dilution was plated on TSA to determine

the number of viable bacteria. All results were expressed as the mean±standard deviation of six individual wells per time interval.

Splenic Clearance of *Brucella* spp. by BALB/c Mice

Groups of five BALB/c female mice per *B. abortus* and *B. melitensis* strain to be tested (Charles River Breeding Laboratories, MA) were vaccinated intraperitoneally with the following doses of *Brucella* known to consistently infect mice: $5x10^4$ strain 2308 (6), $1x10^8$ strain RB51 (88), $5x10^4$ strain 19 (6), $1x10^7$ strain RA1, $1x10^4$ strain 16M, $1x10^6$ strain RM1, and $1x10^6$ rifampin resistant strain RM1 (Schurig, personal communication). Mice were killed by CO_2 inhalation at weekly time intervals from 1 to 6 weeks, depending upon the strain tested. The spleens were aspectically removed, ground on sterile sand (fine granular, Fisher Scientific, GA) in sterile 0.85% saline, ten-fold serial dilutions of the spleen suspension plated on TSA, and the CFU on bacteria per spleen enumerated. Results are given as the mean and standard deviation of the 5 mice per group at the given time points.

Comparison of Macrophage-like Cell Lines Infected with Strain 2308 Cultured in DMEM Complete Media or TSA

Strain 2308 was either cultured on TSA and resuspended at 10%T as described. Fifty mL of DMEM complete media was inoculated with strain 2308 and incubated at 37°C in a shaking bath set at 120 rpm for 72 hours. The DMEM grown strain 2308

were then centrifuged (8060 X g for 5 min.), the pellet was resuspended in sterile 0.1M PBS and the cell density adjusted to 10% T as described. A 100μL aliquot of either the TSA or DMEM cultured strain 2308 was then added to wells containing $3X10^5$ PU5-1.8 cells and incubated at room temperature for 30 minutes. The media was removed, the cells were washed 3X with 1mL aliquots of sterile 0.1M PBS, replaced with DMEM complete media + 50μg/mL gentamicin, and incubated at room temperature for 30 minutes. This media was removed and replaced with DMEM complete media + 12.5 μg/mL gentamicin. Intracellular 2308 were harvested and enumerated as described for the growth kinetics study.

Comparison of Strain 2308 Intracellular Growth in Vero Cells vs J774 and PU5-1.8 Cells

Strain 2308 was cultured on TSA and adjusted to a cell density of 10% T at 525 nm as described. Vero cells, African green monkey kidney cells, were enumerated and 3 X 10^5 cells/well seeded into 24-well plates and incubated at 37°C in 5% CO_2 for 4 hours to allow for cell attachment. The J774 and PU5-1.8 cells were plated as described. Vero cell and macrophage infections were carried out as described for macrophages. All cell types were evaluated in triplicate per time interval.

Intracellular Growth Kinetics of Strain 2308 Derived from J774 Infected Cells in J774 and P388D1 Macrophage-like Cell Lines

J774 cells were plated at a cell density of 3 X 10^5 cells/well in all 24 wells of a 24-well plate. J774 cells were then infected with strain 2308 as described. At 48 hours post infection the intracellular strain 2308 were collected as described above and resuspended in 2.5 ml DMEM complete media. The media from freshly adhered J774 cells (3X 10^5 cells/well) was completely removed and 200ul aliquots of the resuspended strain 2308 was added to the cells directly and incubated at room temperature for 30 minutes. The extracellular organisms were killed with gentamicin as described previously. The cells were maintained in media and macrophages were lysed at 0, 24, 48, and 72 hours post-infection. Similarly, intracellularly grown strain 2308, 48 hours post-infection from J774 macrophages, were used to infect J774 or P388D1 macrophages. These experiments were carried out with strain 2308 grown originally on TSA or DMEM media.

Electron Microscopy

Exponentially growing J774 or PU5-1.8 cells were enumerated and cell density adjusted to 3X10^5 cells/mL in DMEM complete media and 10mL seeded in a 25cm^2 tissue culture flask. The flasks were then incubated at 37°C in 5% CO_2 for 1 hour. The macrophages were then infected with 100μL of a 5% T (525nm) suspension of either *B. abortus* strain RB51 or strain 2308 in 0.1 M PBS and incubated at room temperature for 30 minutes. The media was discarded and the cells were washed 3X with 10mL aliquots of sterile 0.1M PBS. After the final rinse, 10mL DMEM complete

media + 50μg/mL gentamicin was added to the cultures for 30 minutes at room temperature. This media was removed and replaced with DMEM complete media + 12.5μg/mL gentamicin and incubated at 37°C in 5% CO_2 for 48 hours.

At 24 or 48 hours postinfection, the cells were fixed in general fixative (5% glutaraldehyde and 3% formalin in 0.05M sodium cacodylate buffer with 2.5% picric acid) by carefully tilting the culture media away from the cells and adding 1mL general fixative to the media, gently mixing without splashing the media onto the cells. Once mixed the flask was inverted to allow the general fixative solution onto the cells for 15 minutes at room temperature. This solution was then discarded and replaced with 5 mL general fixative.

The transmission electron microscopy (TEM) processing was carried out by the Electron Microscopy Facility of the Veterinary College at VPI & SU (Blacksburg, VA) in the following manner. The samples were washed twice in 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.05M sodium cacodylate buffer, and washed twice for 10 minutes each in 0.1 cacodylate buffer. The samples were dehydrated in graded alcohols (15%, 30%, 50%, 70%, 95%, 100%), for 15 minutes each. The dehydrated samples were incubated in propylene oxide for 15 minutes and placed in 50:50 propylene oxide resin for 12 hours. Thereafter, the samples were infiltrated in pure resin for an additional 12 hours, the resin was changed and the samples cured at 60°C for 48 hours. Cured samples were then thin sectioned for immunogold labeling of the intracellular brucellae. Using a Reichert-Jung Ultracut E

microtome, 60-90 nm sections were cut and placed on uncoated nickel grids.

Immunogold Labeling

The thin sectioned nickel grids were processed and immunogold labeled as described by Merighi and Polak for post-embedded immunogold staining (71). The grids were floated, sample down, on a 50μL drop of saturated aqueous sodium metaperiodate for 10 minutes at room temperature. The grids were rinsed in 0.5M Tris buffered saline (TBS) pH 7.2 + 1% Triton X-100. The rinsed grids were blocked on 50μL drops of 0.5M TBS + 1% bovine serum albumin, fraction V (TBS-BSA) + 10% normal goat serum for 1 hour at room temperature in a 96-well plate. The blocked grids were placed on 50μL drops of 1:20 dilution of rabbit 3187 (anti-2308) or 3189 (anti-RB51) sera in TBS-BSA in a 96-well plate and incubated at 4°C for 24 hours. These anti-sera were characterized previously by Tobias *et. al.* (105). Grids were washed in TBS-BSA and incubated in a 1:15 dilution of goat anti-rabbit IgG conjugated with 10nm gold particles (Sigma Immunochemicals, MO) at 37°C for 1 hour. The grids were washed in TBS-BSA, postfixed for 10 minutes in 2.5% glutaraldehyde in 0.05M cacodylate buffer followed by several rinses in double distilled water and air dried.

The air dried grids were stained with 2% uranyl acetate for 12 minutes, rinsed in distilled water, filter dried, and placed in Reynold's lead citrate (83) for 5 minutes, rinsed in distilled water, and air dried overnight.

Statistical Analysis

Comparisons in colony forming units (CFU's) between groups were made using the Student's t-test and the probability values reported as calculated by the Microsoft Excel statistical software package (Microsoft Corporation, USA).

RESULTS

Opsonized vs Nonopsonized Strain 2308 Growth in PU5-1.8 Cells

The results of strain 2308 infection in PU5-1.8 cells, with or without the use of specific antibody as an opsonin, are depicted in Figure 1. Both growth curves are very similar in that comparable numbers of bacteria are taken up by the macrophages, both have a distinct drop in the number of viable organisms at 24 hours, and there was an increase in the number of viable organisms at 48 hours. However, at 72 hours postinfection the number of intracellular bacteria is higher in the macrophages which were fed specific antibody opsonized bacteria ($p=0.015$). At all other time points there is no statistically significant difference between the groups ($p > 0.05$). As there was essentially no difference in the uptake and survivability through 48 hours postinfection of strain 2308 in the macrophage cell lines, no specific antibody opsonins were used in the remaining assays unless otherwise noted. Similar results were obtained with J774 cells infected with strain 2308 with or without specific opsonin.

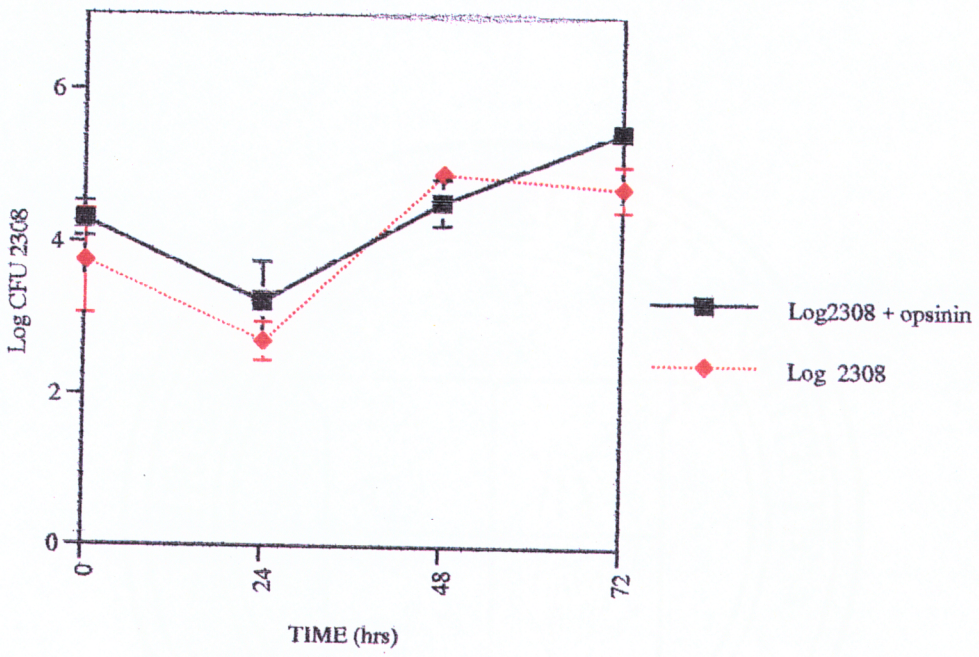


Fig. 1. Growth curve of strain 2308 with or without opsonin (Bru 38) in PU5-1.8 macrophages. Time 0 represents the number of intracellular bacteria per 3 x 10^5 macrophages after 30 minutes of incubation with strain 2308. Each point represents 6 replicates per time period.

Intracellular Growth Kinetics of *Brucella* spp. in J774 and PU5-1.8 Macrophages: General Observations

Intracellular growth characteristics of each *Brucella* spp. tested was unique to the cell line infected as can be observed in Figures 2-7. However, there were several characteristics of the model which are of note. There is a drop in intracellular *Brucella* numbers at 24 hours postinfection ranging from a 1 to 3 log decrease. After the initial drop the intracellular numbers of several *Brucella* strains increased over the next 48 to 72 hours. These kinetics demonstrate that the macrophages are capable of killing a significant number of ingested brucellae but, depending on the strain, the brucellae surviving the early reduction period are capable of survival and replication within the macrophages.

In comparing the smooth (2308, 16M), semi-rough (S19), and rough strains (RB51, RA1, RM1) of *Brucella*, the ingestion of rough bacteria without the addition of specific opsonins is approximately 1 log higher than that of smooth bacteria as determined by the mean T_0 values in both macrophage cell lines. Also, strains RA1, RM1, and RB51 had different numbers of intracellular bacteria at 24- 72 hours ($p<0.05$) in the two cell lines, indicating the macrophages interact differently with the intracellular *Brucella* (Fig. 3A, 5A, 7A).

Comparison of Intracellular Growth Kinetics of *Brucella* sp. *In Vitro* and *In Vivo*

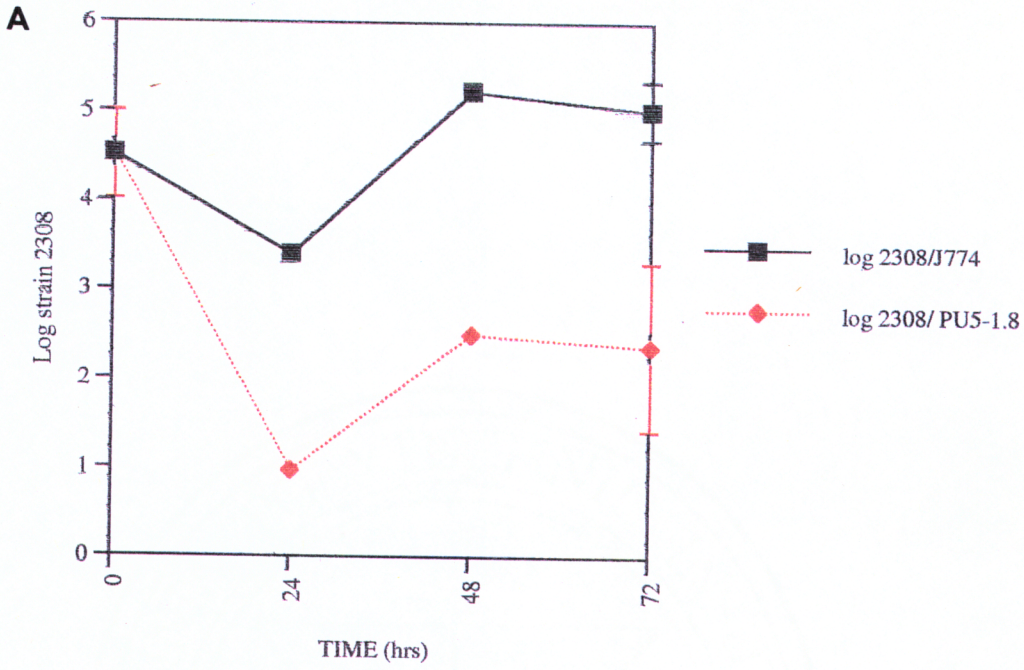
Overall the growth curves of the *Brucella* species examined in both the *in vitro*

macrophage assay and the clearance patterns observed in mice were similar, albeit at different time intervals (Fig. 2-7). Strain 2308 replicated in the macrophages following the initial 24 postinfection drop to achieve a plateau of intracellular numbers at 48 and 72 hours postinfection (Fig. 2A). In mice, strain 2308 produces a chronic infection (Fig. 2B). The clearance of strain 2308 is significantly different at 24, 48, and 72 h, the decrease in intracellular numbers at 24 hours is consistent in both cell lines.

Strain RB51 was readily cleared by the J774 macrophages, as intracellular numbers decreased consistently throughout 72 hours postinfection (Fig.3A). In the PU5-1.8 cell line, RB51 decreased to numbers significantly below those in J774 at 24 hours, but then numbers increased to plateau at 48 and 72 hours. The numbers reached were less than the initial uptake of strain RB51. In mice, RB51 is readily cleared by 4 weeks postinfection (Fig. 3B). The data *in vivo* closely resemble the intracellular strain RB51 growth kinetics observed with the J774 macrophage cell line in that RB51 is readily cleared from mice and the J774 cell line, albeit at different time intervals.

In the case of strain 19, the current vaccine strain, intracellular numbers decreased at 24 with a tendency of intracellular bacteria to increase in number at 48 and 72 hours postinfection in the J774 cell line (Fig. 4A). In the PU5-1.8 cell line, there is a decrease in intracellular numbers at 24 hours, followed by a plateau in intracellular numbers at 48 and 72 hours postinfection. There was no statistical difference ($p>0.05$) between intracellular numbers at the 24 to 48 hour and 48 to 72 hour time periods due to the large amount of variation in intracellular numbers

Fig. 2 A. Growth curve of strain 2308 in J774 and PU5-1.8 macrophage-like cell lines. Time 0 represents the number of intracellular bacteria per 3 x 10^5 macrophages after 30 minutes of incubation with strain 2308. Each point represents 6 replicates per time period per macrophage-like cell line. **B.** Clearance of *B. abortus* strain 2308 from the spleens of mice infected with 5 x 10^4 CFU. Each point represents the mean CFU ± standard deviation of 5 spleens.


A
Log strain 2308
0
1
2
3
4
5
6
0
24
48
72
TIME (hrs)
log 2308/J774
log 2308/ PU5-1.8

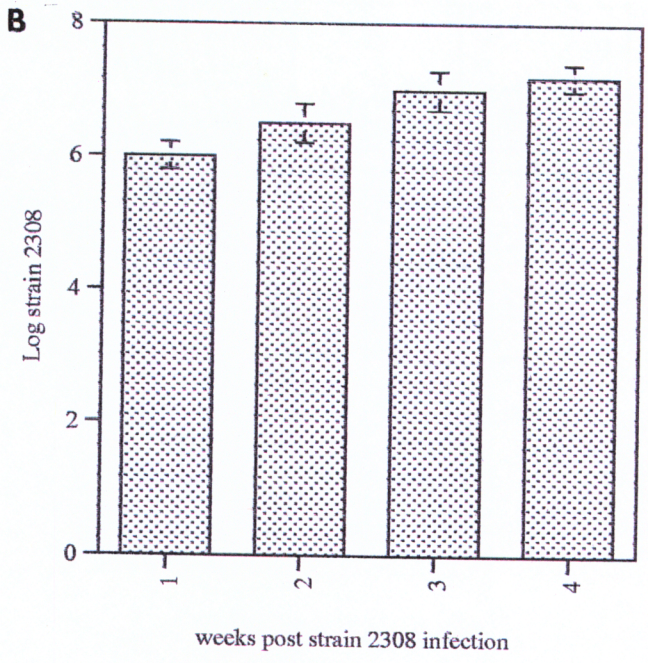
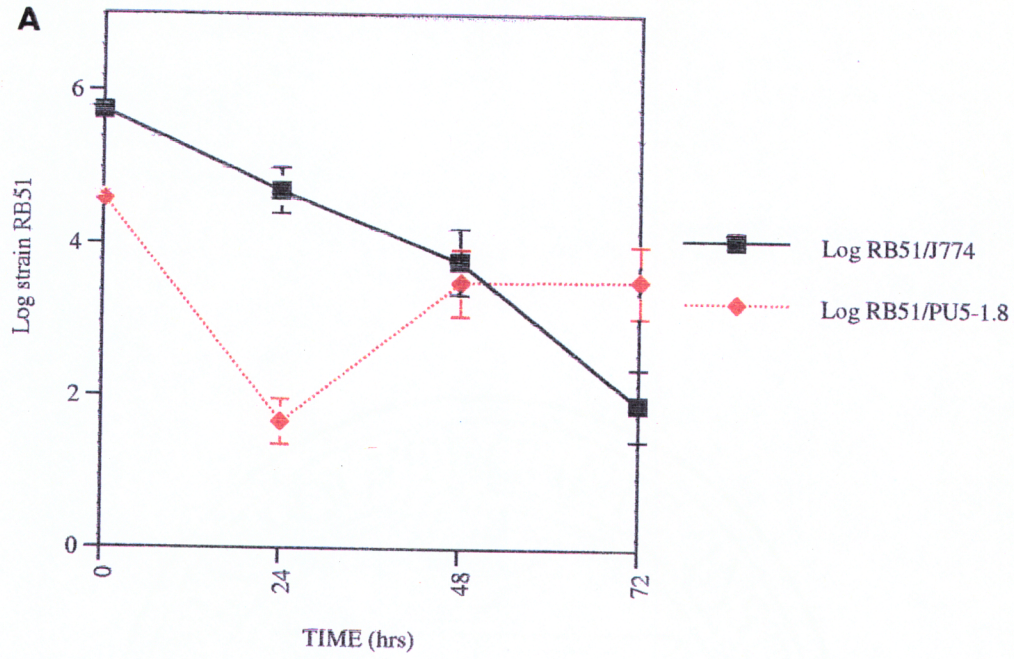

B
Log strain 2308
0
2
4
6
8
1
2
3
4
weeks post strain 2308 infection

Fig. 3. A. Growth curve of strain RB51 in J774 and PU5-1.8 macrophage-like cell lines. Time 0 represents the number of intracellular bacteria per 3 x 10^5 macrophages after 30 minutes of incubation with strain RB51. Each point represents 6 replicates per time period per macrophage-like cell line. **B.** Clearance of *B. abortus* strain RB51 from the spleens of mice infected with 1 x 10^8 CFU. Each point represents the mean CFU ± standard deviation of 5 spleens.


A
Log strain RB51
6
4
2
0
0
24
48
72
TIME (hrs)
Log RB51/J774
Log RB51/PU5-1.8

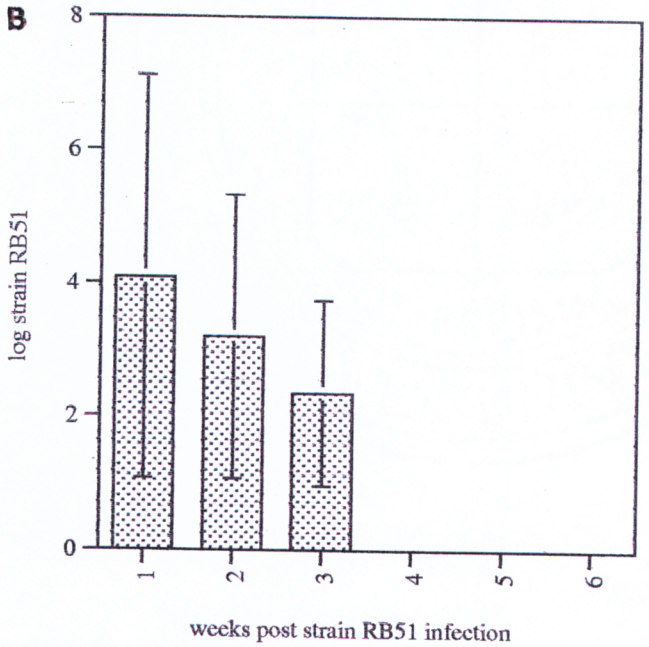
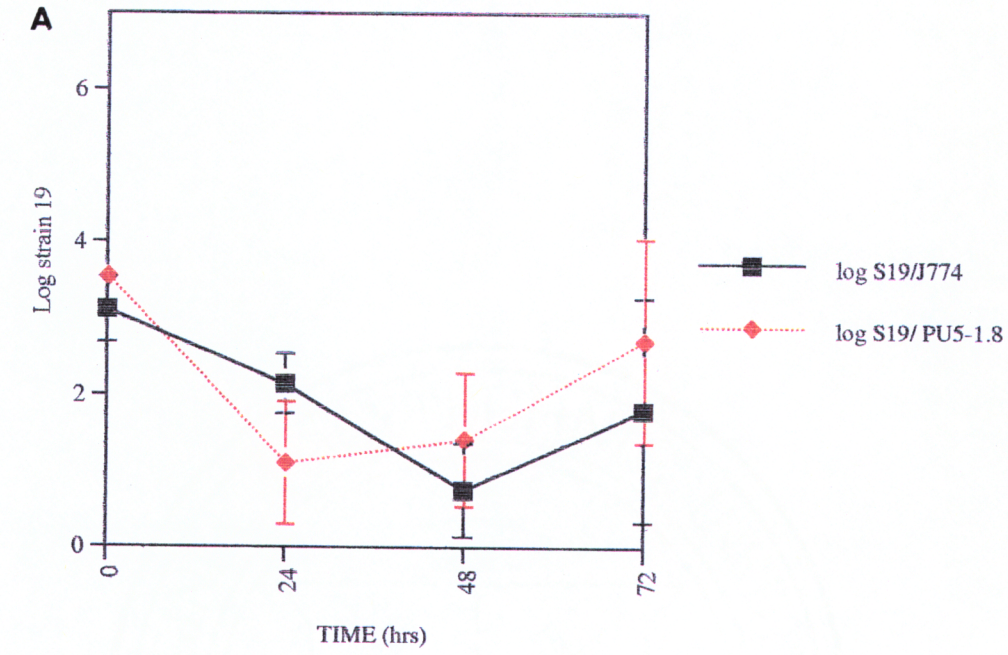
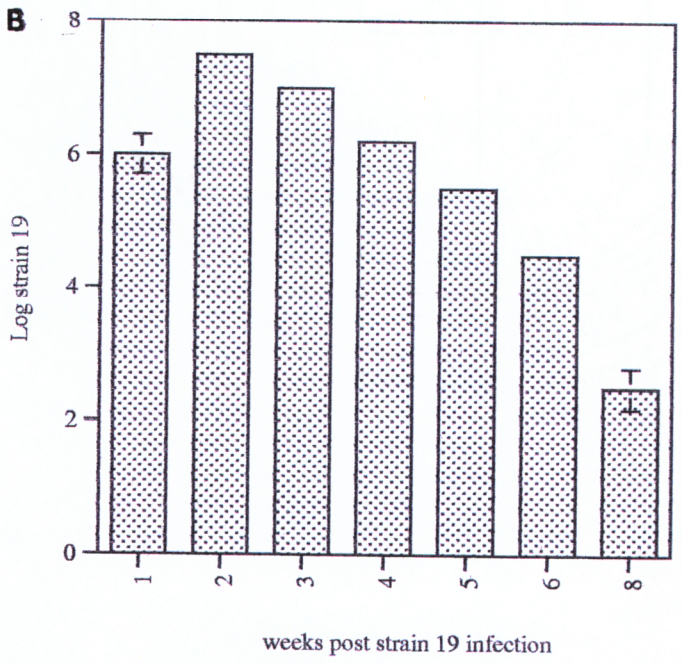

B
log strain RB51
8
6
4
2
0
1
2
3
4
5
6
weeks post strain RB51 infection

Fig. 4. A. Growth curve of strain 19 in J774 and PU5-1.8 macrophage-like cell lines. Time 0 represents the number of intracellular bacteria per 3 x 10^5 macrophages after 30 minutes of incubation with strain 19. Each point represents 6 replicates per time period per macrophage-like cell line. **B.** Clearance of *B. abortus* strain 19 from the spleens of mice infected with 5 x 10^4 CFU. Each point represents the mean CFU ± standard deviation of 5 spleens.


A
Log strain 19
0
2
4
6
0
24
48
72
TIME (hrs)
log S19/J774
log S19/ PU5-1.8


B
Log strain 19
0
2
4
6
8
1
2
3
4
5
6
8
weeks post strain 19 infection

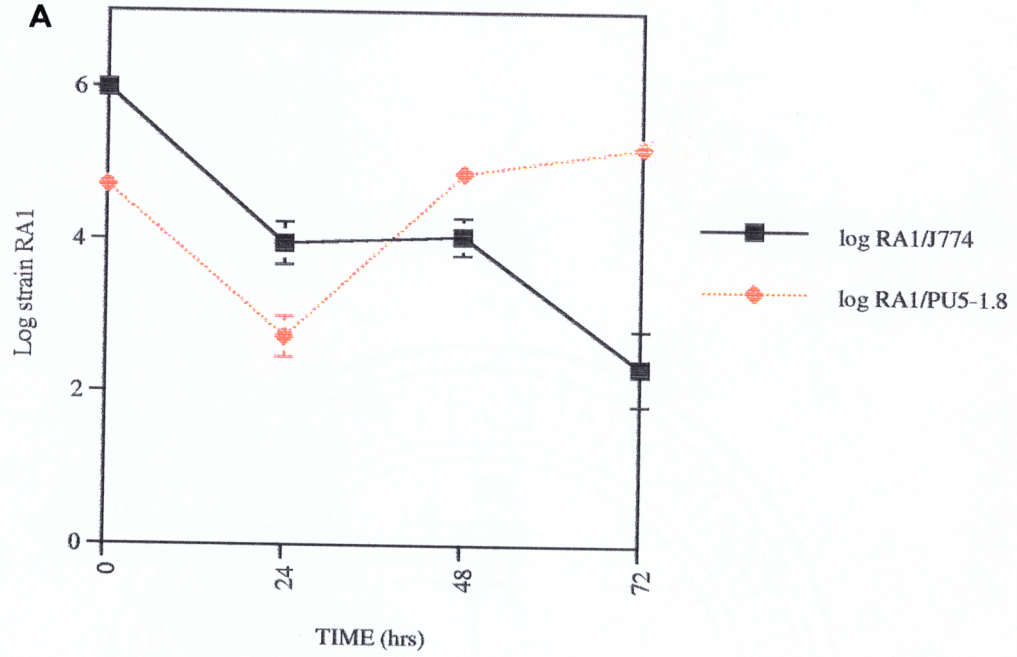
between replicates in both cell lines. However, such is not the case with the decrease in intracellular numbers between 0 and 24 hours ($p < 0.05$). In mice, strain 19 numbers increase, followed by a steady decrease in viable organisms isolated from the spleen over an 8 week course of infection (Fig. 4B).

Intracellular strain RA1 numbers decreased over the 72 hour time course, with a temporary plateau between 24 and 48 hours post infection in J774 macrophages (Fig 5A). In contrast, the growth curve in PU5-1.8 cells reflects a significant amount of intracellular growth and replication of strain RA1. In mice, strain RA1 tended to increase in numbers obtained from the spleen at 14 days postinfection and then steadily declined over 28 days post infection (Fig. 5B). In this manner, the intracellular growth kinetics of strain RA1 in J774 macrophages most closely reflects the *in vivo* mouse data, although at different time intervals.

After an initial fall, the number of *B. melitensis* strain 16M increased to levels greater than those initially taken up by J774 macrophages at T_0 (Fig. 6A). In contrast, in PU5-1.8 macrophages, the intracellular numbers of strain 16M do not surpass T_0 decrease after 48 hours post infection. In mice, strain 16M growth produced a chronic infection (Fig. 6B). The growth kinetics of strain 16M in J774 macrophages most closely resembles that observed *in vivo,* although at different time intervals, in that it attains high intracellular numbers following the initial decline in intracellular numbers at 24 hours post infection.

B. melitensis strain RM1, decreased somewhat in intracellular numbers in the

Fig. 5. A. Growth curve of strain RA1 in J774 and PU5-1.8 macrophage-like cell lines. Time 0 represents the number of intracellular bacteria per 3 x 10^5 macrophages after 30 minutes of incubation with strain RA1. Each point represents 6 replicates per time period per macrophage-like cell line. **B.** Clearance of *B. abortus* strain RA1 from the spleens of mice infected with 1 x 10^7 CFU. Each point represents mean CFU ± standard deviation of 5 spleens.


A
Log strain RA1
6
4
2
0
0
24
48
72
TIME (hrs)
log RA1/J774
log RA1/PU5-1.8

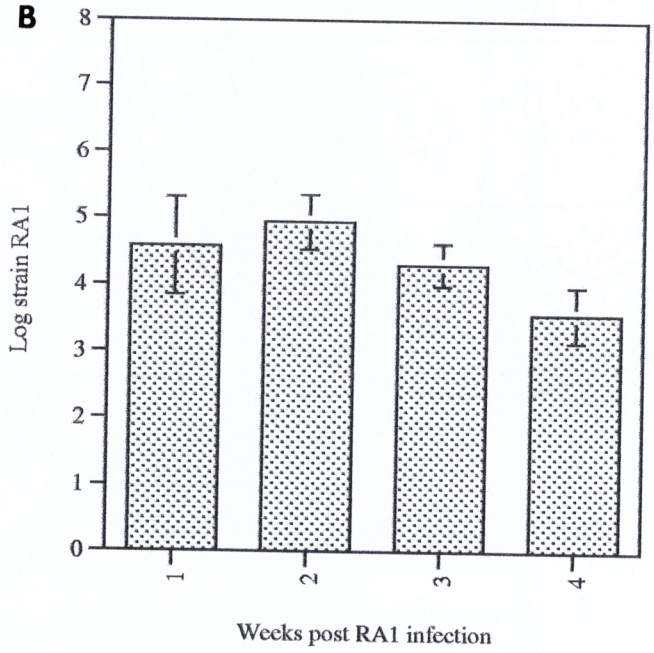
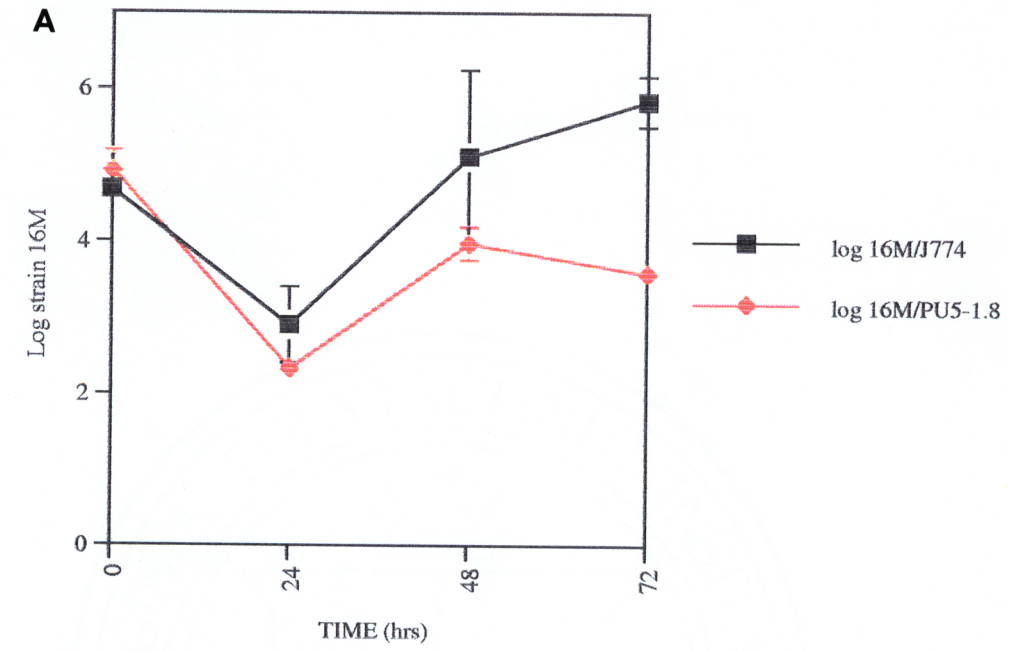
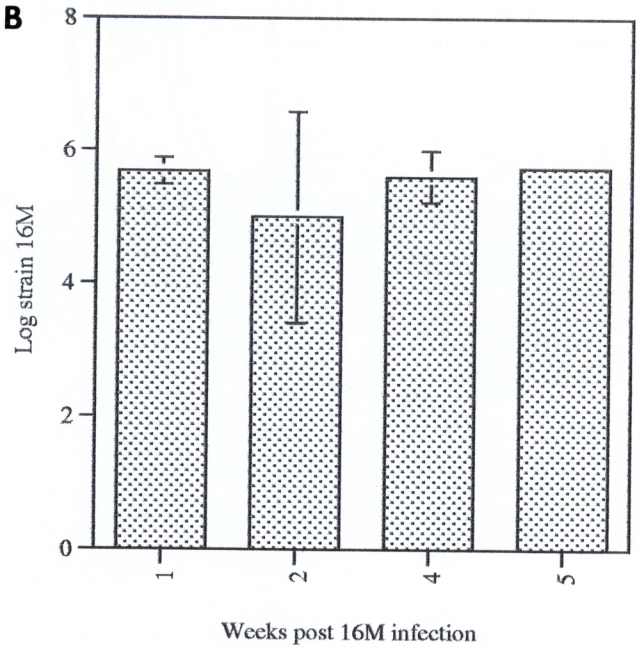

B
Log strain RA1
8
7
6
5
4
3
2
1
0
1
2
3
4
Weeks post RA1 infection

Fig. 6. A. Growth curve of *B. melitensis* strain 16M in J774 and PU5-1.8 macrophage-like cell lines. Time 0 represents the number of intracellular bacteria per 3 x 10^5 macrophages after 30 minutes of incubation with strain 16M. Each point represents 6 replicates per time period per macrophage-like cell line. **B.** Clearance of *B. melitensis* strain 16M from the spleens of mice infected with 1 x 10^4 CFU. Each point represents the mean CFU ± standard deviation of 5 spleens.


A
6
4
2
0
Log strain 16M
0
24
48
72
TIME (hrs)
log 16M/J774
log 16M/PU5-1.8

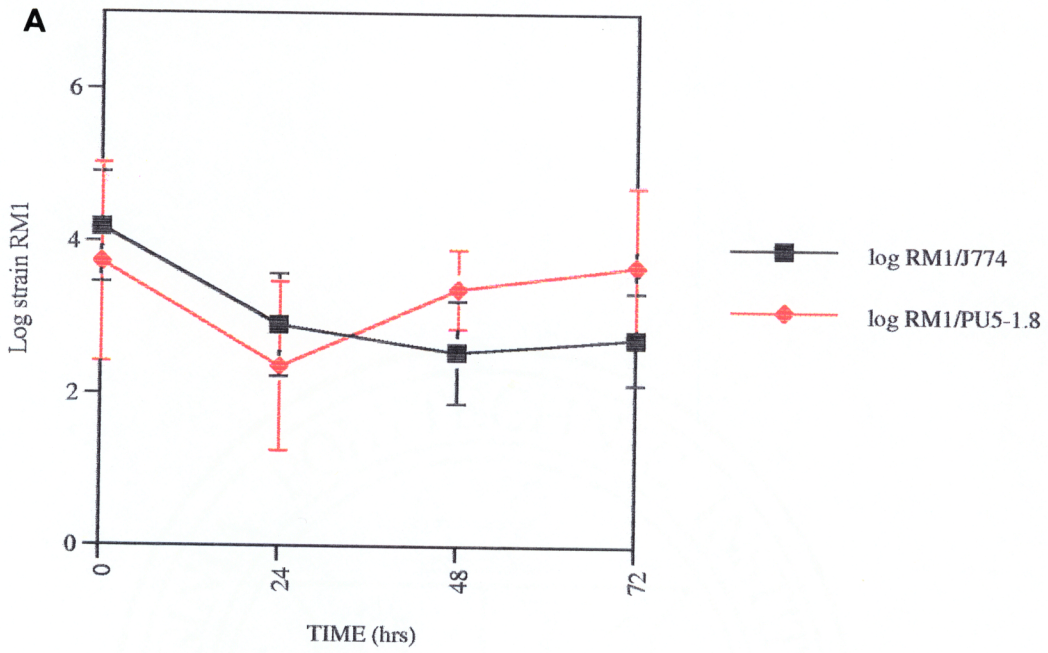

B
8
6
4
2
0
Log strain 16M
1
2
4
5
Weeks post 16M infection

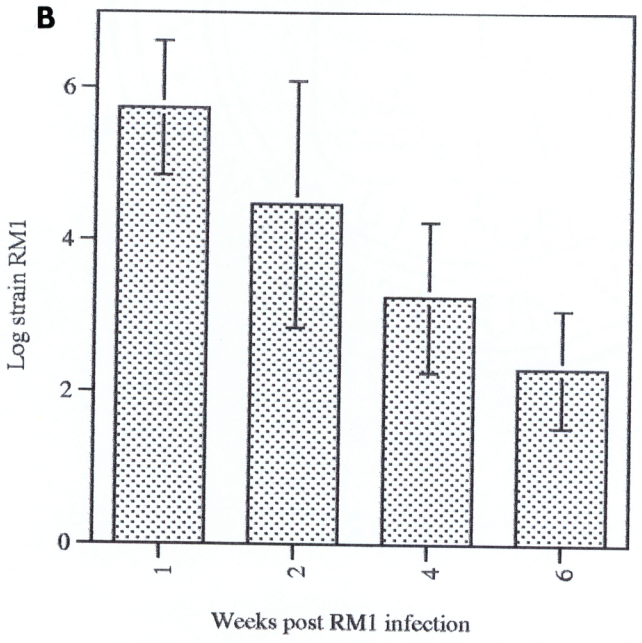
J774 macrophage cell line (Fig. 7A). However, the number of intracellular strain RM1 in the PU5-1.8 cell line appeared to increase over the 72 hour infection time. In mice, strain RM1 are cleared steadily over the 42 day course of infection (Fig. 7B). The growth kinetic curve of intracellular strain RM1 in J774 macrophages most accurately resembled that of the *in vivo* course of infection, although at different time intervals, in that intracellular numbers decline at 24 hours and then plateau at 48 and 72 hours postinfection.

In order to evaluate the efficacy of this assay in determining the attenuation of *Brucella* mutants, the growth curves of two strains, RA1 rifampicin resistant (Fig. 8) and RM1 rifampicin resistant (Fig. 9A) mutants were tested in the assay. Mouse clearance data is currently only available for the RM1 rifampicin resistant strain. The kinetics of strain RA1 rifampicin resistant showed the significant decline in intracellular numbers at 24 hours and growth at 48 hours reached a plateau in J774, while intracellular numbers declined at 72 hours in PU5-1.8 macrophages (Fig. 8).

Rifampicin-resistant strain RM1 growth kinetics appeared to show some degree of attenuation in that recovery of intracellular numbers is well below the number taken up at T_0 (Fig. 9A). However, these numbers were approximately 2 logs of bacteria higher than observed with strain RM1 in the macrophage-like cell lines (Fig 6A, 9A). In mice, this mutant decreased steadily over the 42 day time course of infection. The number of viable RM1 was approximately 1.5 logs bacteria higher than rifampin resistant RM1 in mice, even though the vaccination dose of both strains was $1x10^6$

Fig. 7. A. Growth curve of *B. melitensis* strain RM1 in J774 and PU5-1.8 macrophage-like cell lines. Time 0 represents the number of intracellular bacteria per 3 x 10^5 macrophages after 30 minutes of incubation with strain RM1. Each point represents 6 replicates per time period per macrophage-like cell line. **B.** Clearance of *B. melitensis* strain RM1 from the spleens of mice infected with 1 x 10^6 CFU. Each point represents the mean CFU ± standard deviation of 5 spleens.


A
Log strain RM1
0
2
4
6
0
24
48
72
TIME (hrs)
log RM1/J774
log RM1/PU5-1.8


B
Log strain RM1
0
2
4
6
1
2
4
6
Weeks post RM1 infection

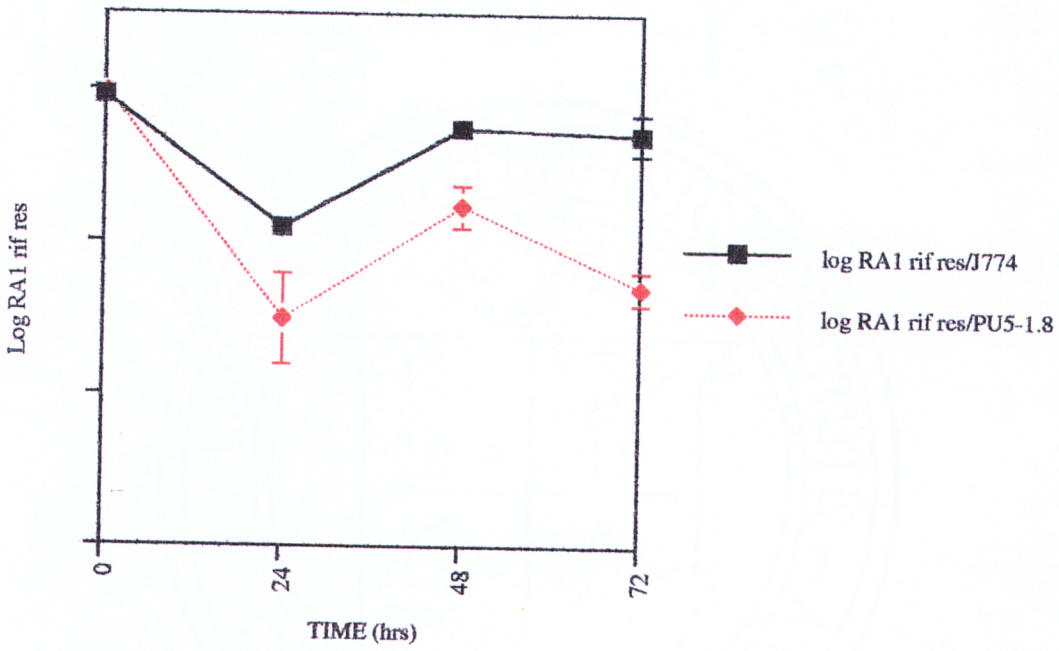
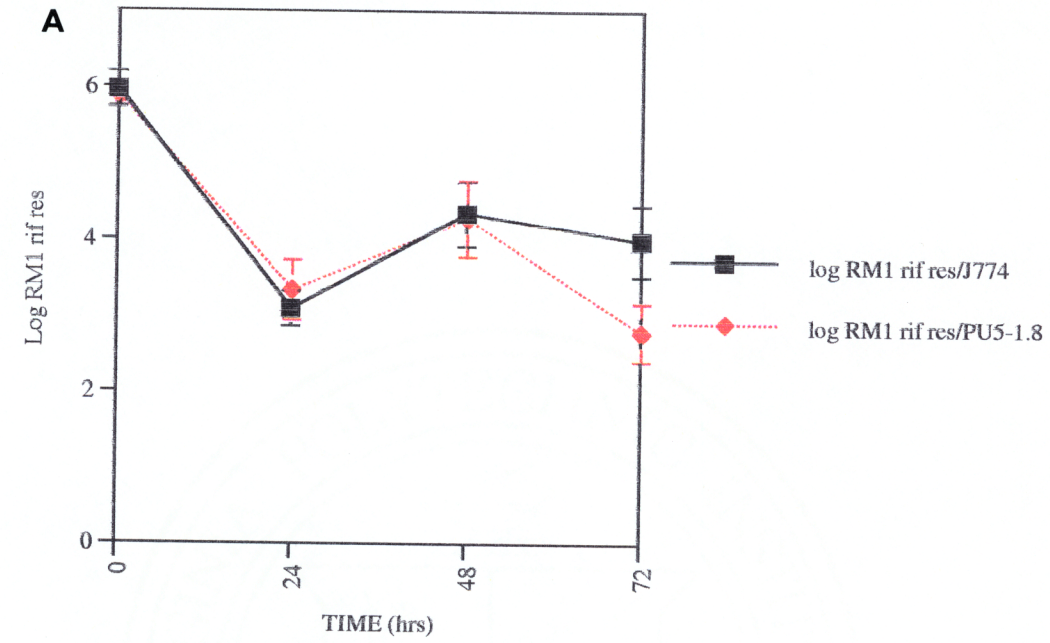
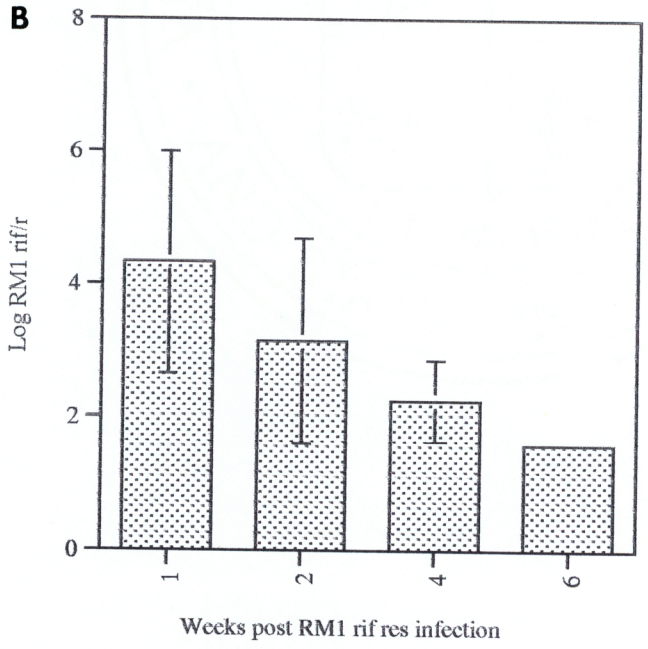


Fig. 8. Growth curve of rifampin resistant *B. abortus* strain RA1 in J774 and PU5-1.8 macrophage-like cell lines. Time 0 represents the number of intracellular bacteria per 3 x 105 macrophages after 30 minutes of incubation with rifampin resistant *B. abortus* strain RA1. Each point represents 6 replicates per time period per macrophage-like cell line.

Fig. 9. A. Growth curve of rifampin resistant *B. melitensis* strain RM1 in the J774 macrophage-like cell line. Time 0 represents the number of intracellular bacteria per 3 x 10^5 macrophages after 30 minutes of incubation with rifampin resistant *B. melitensis* strain RM1. Each point represents 6 replicates per time period per macrophage-like cell line. **B.** Clearance of rifampin resistant *B. melitensis* strain RM1 from the spleens of mice infected with 1 x 10^6 CFU. Each point represents the mean CFU ± standard deviation of 5 spleens.


A
Log RM1 rif res
6
4
2
0
0
24
48
72
TIME (hrs)
log RM1 rif res/J774
log RM1 rif res/PU5-1.8


B
Log RM1 rif/r
8
6
4
2
0
1
2
4
6
Weeks post RM1 rif res infection

bacteria (Fig. 6B, 9B).

Intracellular Growth Kinetics of 2308 Cultured in DMEM Complete Media vs TSA Cultures

The objective of this experiment was to assess whether or not the nutritional shift from TSA to DMEM complete media was causing the observed decrease in intracellular viability of *Brucella* at 24 hours postinfection. The intracellular growth curves of both 2308 cultures are very similar (Fig. 10) and there is no difference between the curves at 24 hours post infection ($p=0.06$). However, statistically significant differences exist in intracellular numbers at 48 and 72 hours post infection ($p< 0.001$) with strain 2308 grown on TSA achieving higher intracellular numbers.

Comparison of 2308 Intracellular Growth in Vero Cells vs. J774 and PU5-1.8 Cells

This study was designed to investigate the role of the macrophage in the decrease in viable intracellular bacteria at 24 hours postinfection. Vero cells, J774 cells, and PU5-1.8 cells were infected with the same number of viable strain 2308. The intracellular growth kinetics are illustrated in Figure 11 and show that at 24 hours postinfection the Vero cell growth curve is significantly different from both the J774 and PU5-1.8 curves ($p <0.005$) when comparing Vero cells at T_0 with Vero cells at T_{24}, there is no statistical difference ($p=0.06$). The data suggest that the decreased numbers of viable intracellular bacteria at 24 hours in the macrophages is due to the

bactericidal action of these cells. However, at T_{48} the Vero growth curve is not statistically different from J774 (T_0; p=0.6).

Intracellular Growth Kinetics of J774 Cultured 2308 in J774 and P388D1 Macrophages

The rationale for this experiment was to observe whether the *Brucella* which survived the early bactericidal attack by macrophages were better adapted to survival within macrophages. Intracellular strain 2308 were cultured within macrophages for 48 hours, at which time the macrophages were lysed, the intracellular 2308 harvested, resuspended, and used to reinfect J774 or P388D1 macrophages. As illustrated in Figures 12 and 13 respectively, reinfection of J774 macrophages, there is a decrease in viable intracellular organisms at 24 hours postinfection. This decrease is not statistically different than the decrease observed with non-macrophage passaged, TSA grown strain 2308 (p=0.37). Only the TSA grown *Brucella* show an increase at T_{48} ($p< 0.05$) resembling the kinetics observed previously. The macrophage passaged *Brucella* did not recover from the initial T_{24} drop in P388D1 cells.

Electron Microscopy of Intracellular 2308 and RB51 in J774 or PU5-1.8 Cells

Immunogold labeling of intracellularly growing 2308 or RB51 was performed to determine their intracellular location within the macrophage-like cell lines. Within these cell lines the bacteria are observed within vesicles and in the cytoplasm of the macrophages (Fig. 14-17). This observation suggests that the *Brucella* are capable of

escaping from the phagolysosome into the cytoplasm in these macrophage cell lines. Even though *Brucella* were evident in the cytoplasm of these cells, none were observed associated with the rough endoplasmic reticulum (30, 105).

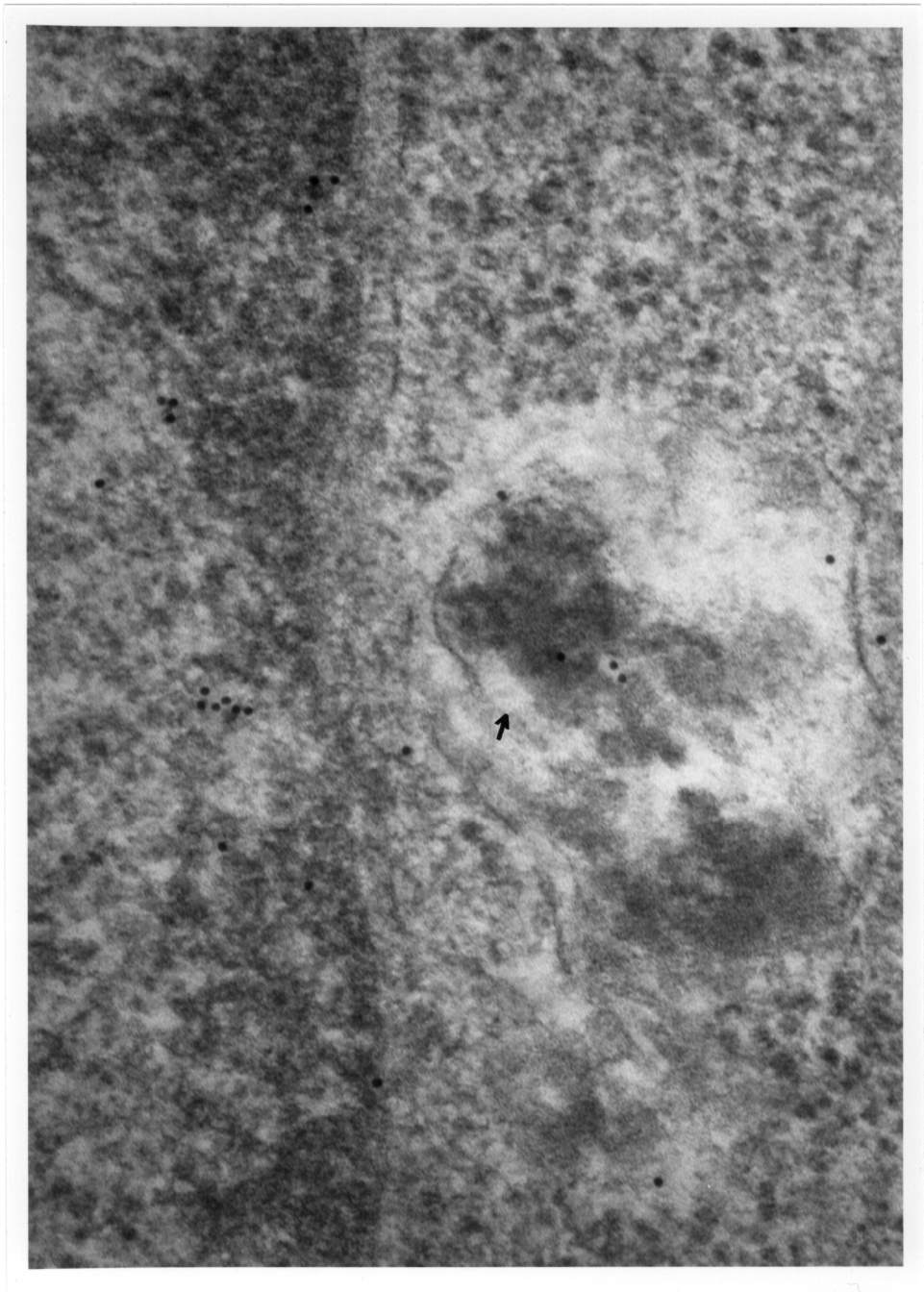


Fig. 14. Electron microscopy of strain 2308 contained within a vesicle in J774 macrophages. The *Brucella* are immunogold labeled with 10nm gold particles. (Magnification: 144,000X)

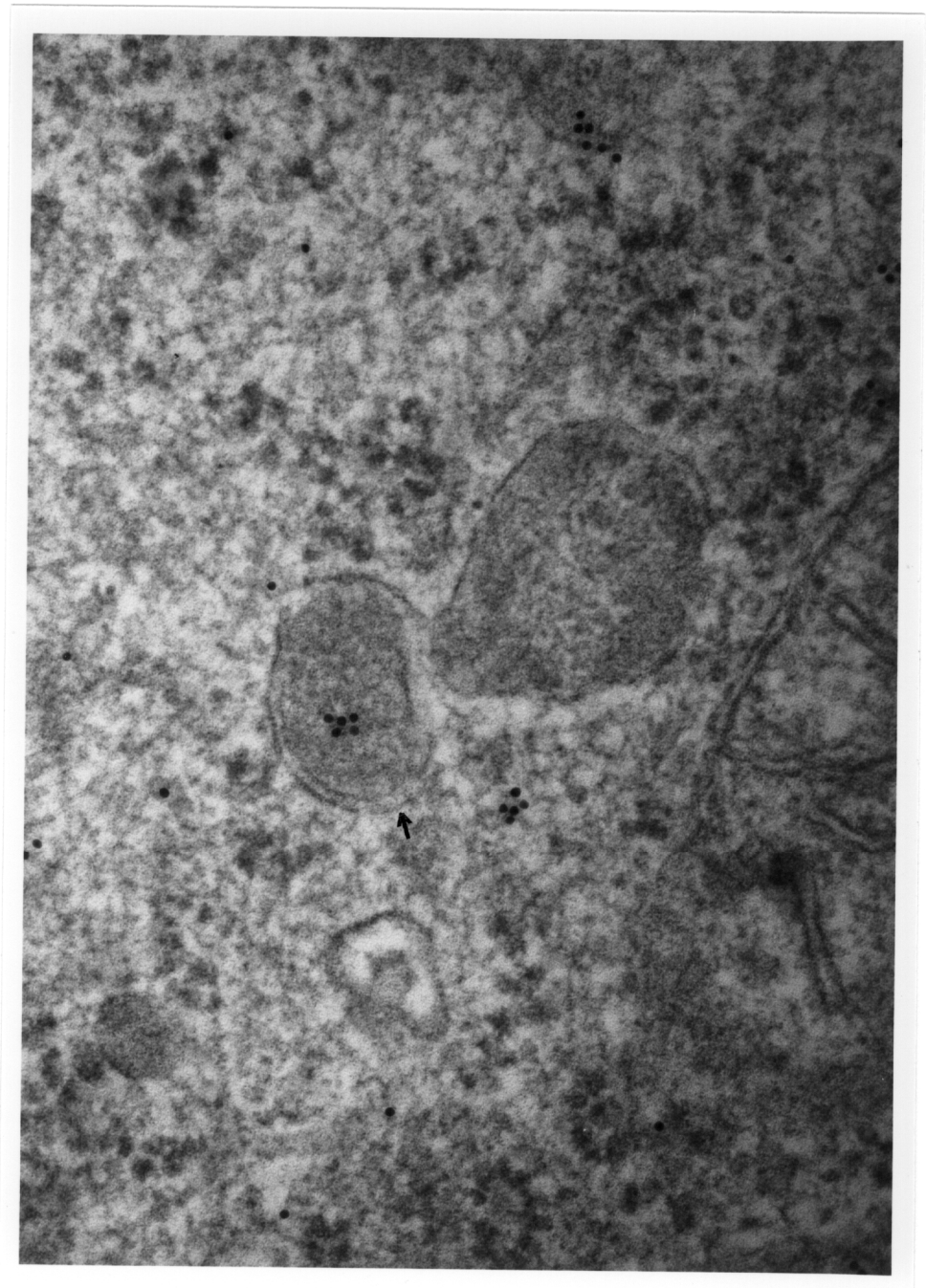


Fig. 15. Electron microscopy of strain 2308 contained in the cytoplasm of J774 macrophages. The *Brucella* are immunogold labeled with 10nm gold particles. (Magnification: 91,800 X)

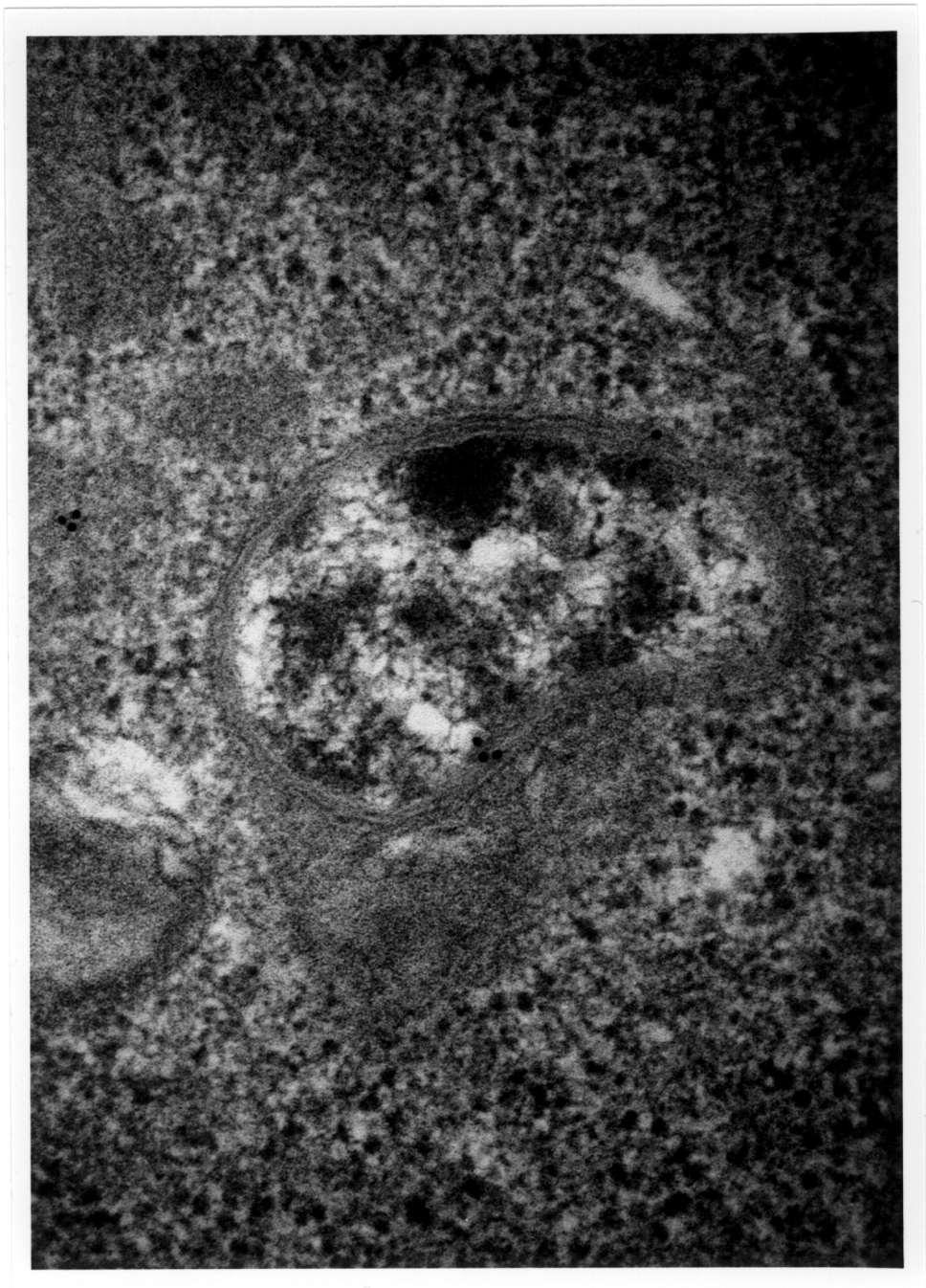


Fig. 16. Electron microscopy of strain RB51 contained within a vesicle in PU5-1.8 macrophages. The *Brucella* are immunogold labeled with 10nm gold particles. (Magnification: 91,800 X)

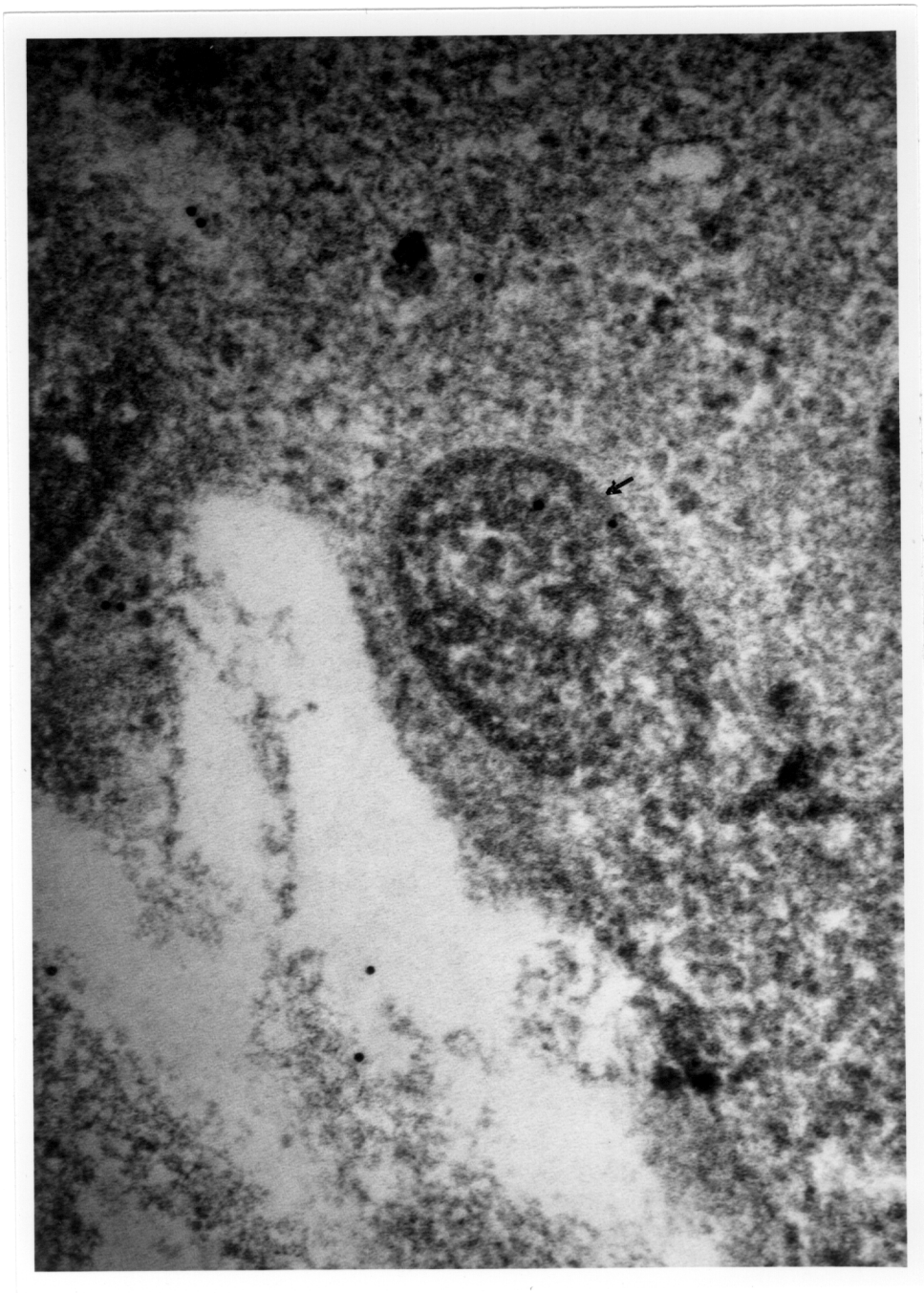


Fig. 17. Electron microscopy of strain RB51 contained in the cytoplasm of PU5-1.8 macrophages. The *Brucella* are immunogold labeled with 10nm gold particles. (Magnification: 144,000 X)

DISCUSSION

The macrophage model for examining intracellular bacterial growth has been used for at least 3 decades. In this study, the intracellular growth characteristics of several species of *B. abortus* and *B. melitensis* have been examined. The PU5-1.8 cell line does support the intracellular growth of *Brucella* spp. Although measured at different time intervals, when compared with *in vivo* infections in mice, the J774 cells more accurately reflect the kinetics of infection. However, the degree of *in vivo* attenuation or true attenuation could not be assessed using the macrophage-like cell lines, as exemplified by the inability to assess the reduced virulence of rifampin resistant RM1 in mice. Therefore, the *in vitro* model is not an accurate tool in screening for attenuation.

The growth kinetics yielded some valuable information regarding the *Brucella*-macrophage relationship *in vitro*. First of all, the decrease in viable organisms at 24 hours postinfection is consistent with the findings of others and occurs with both cell lines (55, 56). Furthermore, the Vero cell experiment supports the decrease in intracellular viability largely being the result of macrophage function as there is no decrease at 24 hours observed with bacterial intracellular growth within Vero cells.

The opsonization of strain 2308 did not affect the intracellular growth kinetics until 72 hours of macrophage infection. However, this particular point was not extensively examined in this study. Others have noted the benefit of increased uptake of strain 19 by macrophages with opsonization of the organisms in macrophage

infection studies *in vitro* (46, 52, 56, 109, 111). Consequently, the differences between the growth curve of strain 19 presented here and that in the literature may be due to the use of specific opsonins. For example, using specific opsinins Jiang and Baldwin observed 10^6 bacteria at 48 and 72 hours postinfection (52). Further evidence supporting the use of specific opsonins is that macrophages obtained from *Brucella* vaccinated mice have more F_c receptors, as well as enhanced mobility of the macrophages, and C3 complement receptor-mediated phagocytosis than the macrophages from nonvaccinated mice (13).

The culture of strain 2308 in DMEM vs TSA was an attempt to determine whether the observed decrease in viability at 24 hours post-phagocytosis was due to macrophage action or due to an effect on *Brucella* by the nutritional shift from TSA to DMEM media. No statistical difference was observed in the intracellular growth curves at T_0 and T_{24} suggesting that the decrease in viability at 24 hours post infection is due to the action of the macrophages.

No studies have addressed the reasons for the early decrease of intracellular *Brucella* after macrophage infection, although such a drop has been consistently reported (52, 56). This question was addressed by examining the ability of intracellularly passaged 2308 to reinfect macrophages. The objective of the study was to determine whether survivor-*Brucella* were select population. Passage of strain 2308 in macrophages did not appear to make it more able to survive within macrophages during the first 24 hours in J774 cells. However, at 48 and 72 hours postinfection in

J774 cells, the macrophage-cultured, intracellular strain 2308 was better able to survive and replicate than the TSA grown strain 2308 ($p < 0.05$). Infection of the macrophage-cultured strain 2308 actually made them less able to survive within P388D1 macrophages, which are known to be highly brucellicidal (8). The differences observed at T_{24} were statistically significant between TSA cultured and macrophage-cultured strain 2308 in P388D1 cells ($p < 0.5$), the TSA cultured 2308 having more intracellular bacteria. These data suggest that intracellular survivors are not the result of a selection process.

Various mechanisms could account for the lack of a significant difference between macrophage-cultured and TSA grown intracellular strain 2308 numbers at 24 hours postinfection. For example, genes induced at particular time points of the internalization of *Brucella* by the macrophage in direct response to macrophage activities may be crucial for the growth and replication of *Brucella* intracellularly (61). Alternatively, not all phagosomes within a macrophage fuse with lysosomes, and thus *Brucella* in these unfused phagosomes would not be killed, while other *Brucella* are destroyed (40). Destruction of *Brucella* within phagolysosomes may be reflected in the decrease in intracellular *Brucella* viability at 24 hours. However, the increase in intracellular numbers at 48 hours may reflect the replication of *Brucella* residing in unfused phagosomes. In nonphagocytic Vero cells, the decrease in intracellular strain 2308 viability at 24 hours postinfection was not observed ($p > 0.05$), as it was observed with both J774 and PU5-1.8 cell lines ($p < 0.05$). This suggests that the decrease of

intracellular organisms at 24 hours postinfection is due to macrophage bactericidal activity .

The electron microscopy of immunogold labeled *Brucella* revealed the intracellular location of the smooth (strain 2308) or rough (strain RB51) bacteria. Both strains were observed within vesicles as well as in the cytoplasm. Current studies indicate that the intracellular location of *Brucella* in peritoneal and other macrophages is only within vesicles, like the phagolysosome (70). It is unknown whether the cytoplasmic location of the bacteria observed in macrophage cell lines is what occurs in chronic brucellosis or if it is an artifact resulting from the nature of the macrophage-like cell lines. Cytoplasmic localization has been reported for other cell lines, including Vero cells *in vitro* and murine trophoblast cells *in vivo* (30, 105). However, the intracellular location of the bacteria may be an important factor in the pathogenesis of brucellosis.

The role of T-cytotoxic ($CD8^+$) cells in brucellosis is, at best, poorly understood. Passive transfer studies in mice suggest that $CD8^+$ cells are as important as $CD4^+$ cells in the clearance of viable *Brucella* from a host (6). In general, antigens in or being produced in the cytoplasm can be coupled to the major histocompatibility complex (MHC) class I molecules. This complex is transported to the surface of antigen presenting cell (APC) where it is available to interact with $CD8^+$ T cells (74). The interaction of the $CD8^+$ T-cell and APC may results in destruction of the APC. Conversely, traditionally it has been observed that antigens which are phagocytosed

and do not become intracytoplasmic are processed and complexed with MHC class II molecules and transported to the surface of the APC (41). These antigens are available to interact with specific $CD4^+$ T-helper cells. A more detailed explanation of this interaction is found in the next chapter. The result of such interaction ultimately leads to clonal proliferation of specific T-cells and potentially to the activation of macrophages through the production of INF-γ. This response is believed to be very important in the host response to a *Brucella* infection (32, 56). Recent work indicates that antigens from the phagosome can be transferred to the cytoplasm and complexed with MHC class I molecules, avoiding the need for intracytoplasmic location of the microorganisms as a requirement for MHC class I presentation (59). The authors suggest that antigens transferred into the cytoplasm from the phagosome share a "common pathway" which allows these antigens to be complexed with MHC class I molecules. The cytoplasmic localization of *Brucella* may be important for complexing *Brucella* antigens with MHC class I molecules in nonphagocytic cells, but may not be necessary in macrophages. This may explain why intracellular *Brucella* have not been observed in the cytoplasm of macrophages in infected animals, despite the apparent presence of specific $CD8^+$ cells (6, 70). Therefore, the ability of antigens to be transported in the cytoplasm by macrophages to be complexed with MHC class I molecules may be important in the host response to a *Brucella* infection. The cell lines described here may be particularly efficient in presenting *Brucella* antigens or MHC class I molecules due to the intracellular location of *Brucella* and may serve as a

useful tool to detect *Brucella* antigens important in the induction of protective $CD8^+$ cells (6).

CHAPTER THREE

I. PREPARATION, IMMUNOLOGICAL EVALUATION, AND PROTECTION OF A *BRUCELLA ABORTUS* RB51 ANTIGEN EXTRACT

INTRODUCTION

The Cellular Immune Responses

It has been reported that both humoral and cell-mediated immune responses are important for the ability of an infected host to clear *B. abortus* (6). Moreover, the cell-mediated immune response is hypothesized to be of crucial importance due to the intracellular nature of *Brucella* within an infected host (32). Passive transfer of both antibodies specific for the O-chain polysaccharide and T-cells from mice vaccinated with strain 19 afforded the greatest amount of protection against virulent challenge (8, 109). Vaccination with strain RB51 confers protection against virulent challenge (88). When antibodies and T-cells from RB51 vaccinated mice were passively transferred, only the T-cells afforded protection against virulent challenge with strain 2308 (8, 54). Therefore, a basic description of the cell-mediated immune response and main cytokines associated with these responses will be reviewed. The role of T-independent antigens will not be reviewed here.

In order for the immune cells (T and B cells) to respond to the *B. abortus* antigens, the antigens must first be presented to the reactive T cell clones in

association with major histocompatibility complex (MHC) molecules (84). (For schematic representation of this process, see Fig. 18). Initially the antigens are processed and presented to the T cells complexed to either MHC class I, class II, or both types of molecules (41, 59, 74). Antigens in context with class I molecules predominately stimulates $CD8^+$ or cytotoxic T cells (74). The exact role of the $CD8^+$ population of T cells in brucellosis is currently unclear. Passive transfer experiments with $CD4^+$ and $CD8^+$ subsets of T cells demonstrated that both subsets were capable of conferring protection from virulent challenge (6). These data suggest that $CD8^+$ T cells, as well as $CD4^+$ T cells, play an important role in the clearance of *B. abortus* by the infected host.

The role of the $CD4^+$ cells is somewhat more clear as to the roles played in the progression of either a humoral or CMI mediated response. The cytokines elicited during this process are additionally important in that the outcome of T_{H1} vs T_{H2} response is decided by their interplay in an active infection (62). A T_{H1} response is the observed responses which lead to a CMI response. The T_{H1} response is characterized by $CD4^+$ T cell interaction with the antigen complexed with MHC class II. The T cell is stimulated to produce interleukin (IL)-2, and interferon (INF)-γ in response to this interaction (23). This results in the clonal expansion of reactive T-cell clones (lymphoproliferation) and the activation of macrophages by INF-γ. In addition to the activation of macrophages, INF-γ also suppresses the T_{H2} response.

Recently a role for IL-12 in the stimulation of a T_{H1} response has been reported

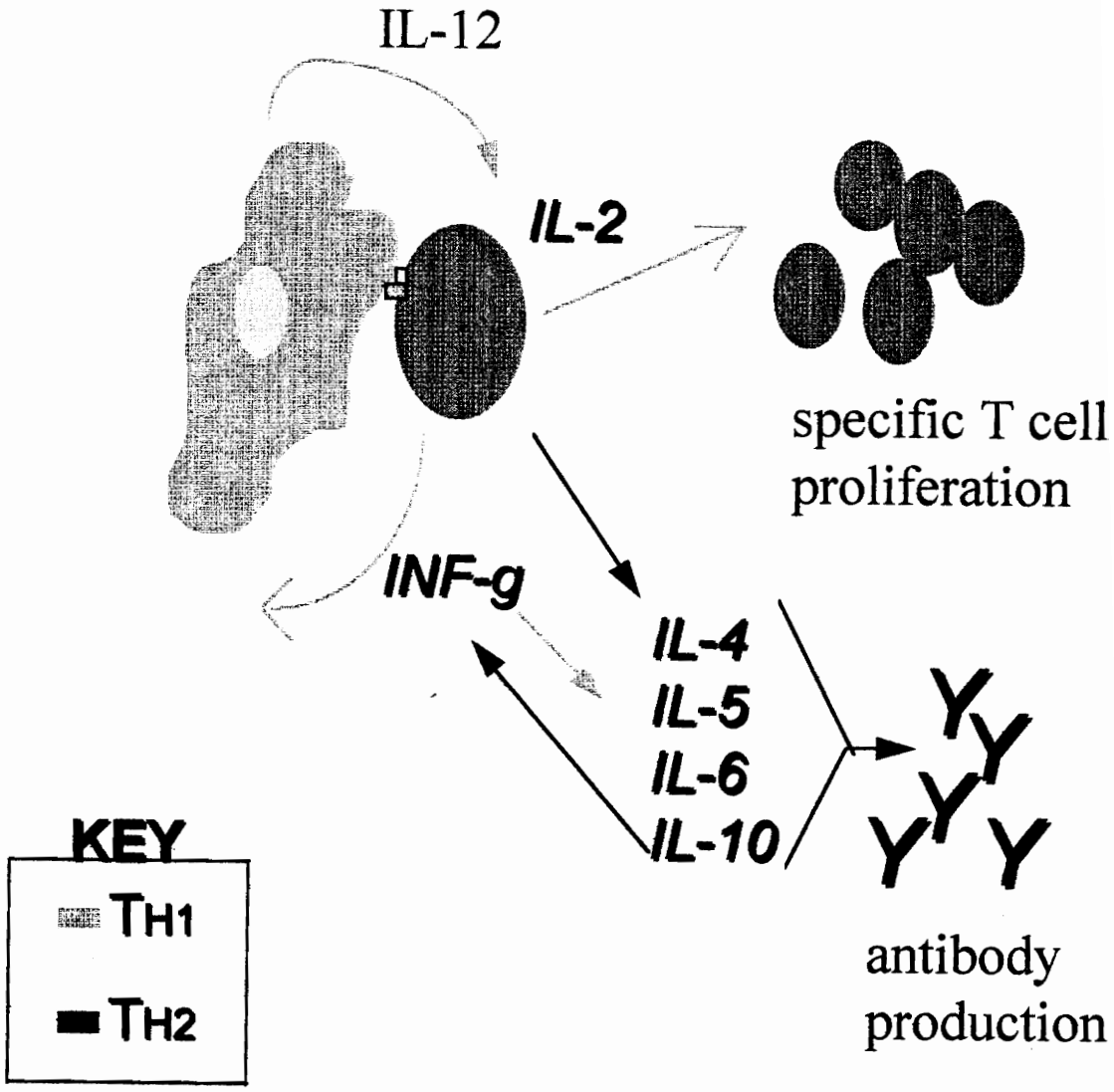


Fig. 18. Schematic representation of the T_{H1} and T_{H2} immune responses in the murine model.

(49). In this study, IL-12 production by the macrophages in response to interaction with T cells was observed to promote a T_{H1} response to *Listeria*. The authors suggest that IL-10 may inhibit the T_{H1} response by prohibiting the production of IL-12 by the macrophages.

The T_{H2} response leads to a humoral immune response (23). The $CD4^+$ T cell interacts with the antigen presented in context of MHC class II. In contrast of the T_{H1} response, this interaction stimulates the production of IL-4, IL-5, IL-6, and IL-10. This combination of cytokines results in activation of specific B cell clones and the production of antibodies. An additional role for IL-4 and IL-10 is to inhibit the T_{H1} response (50).

The Role of Cytokines in the Pathogenesis of Brucellosis

A number of studies have examined the production of cytokines as well as the addition of exogenous cytokines and the pathogenesis of brucellosis in the bovine or murine systems. Additionally, some have used macrophage-like cell lines to look at the influence of cytokines on bacterial growth or stimulation of cytokines in response to particular antigens (52, 53).

INF-γ plays a pivotal role in the production of a T_{H1} response. Stevens, *et.al.* observed that mice given two injections of recombinant INF-γ, one prior and one during infection with *B. abortus* 2308 were observed to have fewer live strain 2308 in the spleen than control mice or those given an injection of INF-γ only prior to

infection (98). From another perspective, Zhan and Cheers injected mice with an anti-INF-γ- monoclonal antibody and observed more strain 2308 in the spleens and livers of the vaccinated mice (116). These data confirm that INF-γ production is a necessary component in eliminating *B. abortus* by an infected host. Jones and Winter observed that exogenously added INF-γ to murine peritoneal macrophages limited the intracellular growth and replication of strains 19 and 2308, although strain 2308 was able to maintain viability in the INF-γ treated macrophages (56).

IL-1 injected in mice prior to vaccination with *B. abortus* strain 19 decreased the number of S19 observed in the spleen as compared with the controls (117). However, injection of IL-1 into chronically infected mice had no affect upon the numbers of bacteria isolated from the spleen. These data suggest that the timing of an IL-1 response is a critical parameter involved with disease progression.

IL-12 is produced by macrophages, B lymphoblastoid cells, and is involved with the development of a T_{H1} response (49). The role of IL-12, in the pathogenesis of brucellosis has also been investigated (114). Mice injected with an anti-IL-12-antibody prior to infection with S19 were observed to have an increased number of viable bacteria in the spleens and reduced splenomegaly. These mice were observed to have fewer macrophages in the spleen than the infected control mice, and these mice were observed to produce less INF-γ than the infected control mice. This study shows the importance of IL-12 in the T_{H1} response in brucellosis. The reduced number and activity of macrophages in the spleen allows for nearly unlimited intracellular growth

of the *B. abortus in vivo.*

In one study, Jiang and Baldwin examined the effects of exogenously added IL-1, IL-2, IL-4, IL-6, and INF-γ on the intracellular growth of *B. abortus* S19, RB51, and 2308 in the murine macrophage-like cell line J774.A1 (52). IL-1, IL-4, and IL-6 did not appear to affect the intracellular growth of the S19 in the J774.A1 cells. However, IL-2 and INF-γ were observed to decrease the numbers of viable intracellular S19, RB51, and 2308. Using combinations of the other cytokines, no decrease in the numbers of intracellular brucella were observed. The authors also noted that the maximal inhibition of *Brucella* growth by INF-γ was achieved when this cytokine was present throughout the course of infection.

The role of IL-10 in brucellosis has been investigated (36). Mice were injected with an anti-IL-10 monoclonal antibody and then challenged with *B. abortus* strain 2308. These mice were observed to have a significant decrease in the number of viable strain 2308 in the spleen compared to control mice. *In vitro* cultures of lymphocytes from sensitized mice were observed to have an increase in the amount of INF-γ produced when the anti-IL-10 monoclonal antibody was present in the culture media. Conversely, when IL-10 was added to the culture media instead of the monoclonal antibody, there was an increase in the number of viable organisms recovered from peritoneal macrophages.

RATIONALE AND HYPOTHESIS

As cytokine production plays such an important role in the outcome of

brucellosis, antigens that have potential use in vaccine development should elicit an appropriate cytokine profile *in vitro* when confronted with lymphocytes from animals immunized with a protective vaccine like strain RB51. Moreover, the ability of such antigens to stimulate the appropriate CMI response *in vivo* and consequently decrease the number of bacteria present in the spleen after challenge should be evaluated.

MATERIALS AND METHODS

Bacteria Culture

B. abortus strains 2308 and RB51 were obtained from the collection of the Virginia-Maryland Regional College of Veterinary Medicine (Blacksburg, VA) and cultured on TSA at 37° C in 5% CO_2 for 72 hours.

Strain RB51 antigen preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): 1 X 10^8 strain RB51 were resuspended in 10 μL 10mM Tris (pH 7.4) followed by an equal volume of Lammeli 2X sample buffer (Sigma Chemical Co., MO). The sample was boiled in a boiling water bath for 5 minutes. The sample was allowed to cool to room temperature, spun at 8060 X g for 5 minutes, and the entire supernatant loaded into one lane of a SDS-PAGE gel.

Infections: strain RB51 was resuspended in trypticase soy broth (TSB) to a density of 4% transmittance (T) at 525 nm and frozen at -70° C in 500μL aliquots. An aliquot of the frozen preparation was thawed and ten-fold serially diluted to

determine the number of viable bacteria in the suspension. A dose corresponding to 1 X 10^8 viable strain RB51 organisms were injected into BALB/c mice intraperitoneally. Similarly, a dose corresponding to 6 X 10^4 strain 2308 were used to challenge mice where indicated.

Animals

BALB/c female mice 3-4 weeks of age (Charles River Breeding Laboratories, MA) were housed at the Non-Client Animal Facility at VPI & SU (Blacksburg, VA). The mice were allowed to acclimate to the facility for a minimum of 1 week prior to infection with *Brucella*.

Vaccination of Mice with *B. abortus* Strain RB51

Mice were vaccinated intraperitoneally with 1 X 10^8 RB51 by diluting a vial frozen at 4% T to the appropriate concentration with sterile 0.85% saline; the inoculated dose was verified by viable plate count as described. Mice were killed by CO_2 inhalation at 6 to 7 weeks post infection and their spleens removed for use in lymphocyte transformation assays or for cytokine response ELISAs.

B. abortus Strain RB51 Antigen Extract Preparation

Twenty trypticase soy agar (TSA) plates were streaked heavily with strain RB51 and incubated at 37°C in 5% CO_2 for 72 hours. The bacteria were harvested

from the plates by washing the surface several times with sterile distilled water and killed by adding an equal volume of acetone to the bacterial suspension for 3 hours at room temperature with stirring. The cells were pelleted by centrifugation (5600 X g for 10 minutes), washed twice with sterile distilled water, resuspended in 10 mL 10% NaCl, 4M urea, 0.001% β-mercaptoethanol (ME), and incubated overnight at 40°C with shaking. The suspension was centrifuged at 5600 X g for 10 minutes, the supernatant removed, dialyzed in 12,000-14,000 molecular weight cut-off (mwco) dialysis tubing at 4°C overnight with several changes of distilled water, and lyophilized. The pellet was washed twice with sterile distilled water and resuspended in 5 mL sterile distilled water. Five milliliters of a saturated ammonium sulfate solution, pH 7.4 (25°C) was added to the resuspended cells, the suspension vortexed well and incubated at room temperature for 48 hours with agitation. The suspension was centrifuged (5600 X g for 10 minutes), the supernatant was removed and designated S2. The pellet was resuspended in 5 mL sterile distilled water and designated P2. Both suspensions were dialyzed as described above, frozen at -70°C and lyophilized.

Immunization of Mice with S2 Antigen Extract and Virulent Challenge

Dimethyldioctadecylammonium chloride (DDA)(Eastman Kodak, NY) adjuvant was prepared by making a 2.5 mg/mL stock solution and heating at 80°C for 8 minutes (4). The solution was cooled to room temperature.

BALB/c mice were divided into three groups of six mice per group. Group 1 was designated the control group and received no treatment until challenged with strain 2308. Group 2 (adjuvant only) received 250μg DDA intraperitoneally in a volume of 200μl diluted in sterile 0.1M PBS, while Group 3 (S2 + adjuvant) received 100μg dry weight (approx 35μg protein) S2 in 250μg DDA (S2/DDA) intraperitoneally also in a volume of 200μl. Groups 2 and 3 were boosted at two weeks and four weeks following the initial immunization at day 0. Blood samples were taken from the retroorbital sinus at 13, 27, and 42 days. The blood samples were allowed to clot, centrifuged at 5600 X g for 3 minutes, the serum removed and stored at 4°C. Serum samples were diluted as indicated for Western blot and ELISA.

At 5 weeks following the final immunization, all three groups were challenged with 6 X 10^4 strain 2308 intraperitoneally. At 2 weeks post challenge, all groups of mice were killed by CO_2 inhalation, spleens aseptically removed and homogenized in sterile sand (fine granular, Fisher Scientific, GA) in 2 mL sterile 0.85% saline. The homogenate was then serially diluted, plated on TSA, and incubated at 37°C in 5% CO_2 for 72-96 hours. The colonies per dilution were enumerated and the CFUs per spleen determined. Statistical differences between groups was evaluated using the Students t-test (Microsoft Excel, Microsoft Corporation, USA).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Electroblotting

According to the method of Lammeli, 12.5% polyacrylamide gels with 2.5%

stacking gels were prepared (59b). Both mini-gel and standard sized gels were prepared and the protocols used were the same. The samples to be analyzed were diluted in 2X Lammeli sample buffer (Sigma Chemical Co., MO) and loaded into the appropriate lanes. A kaleidoscope molecular weight markers (BioRad Laboratories, CA) was used to estimate the molecular weights of the separated proteins. The gels were run at a constant current of 30mA until the dye front was approximately 1 cm from the bottom for a large gel or 0.5 cm for a mini-gel. The proteins in the gel were then electroblotted onto PH79 nitrocellulose (Schleicher & Schuell, MA) using a transphor unit following manufacture's instructions. Briefly, filter papers (Hoeffer Scientific, CA) were immersed in the appropriate anode or cathode buffers. The nitrocellulose membrane was immersed in distilled water. On the anode, the filters soaked in anode buffer #1 (Integrated Separation Systems Protein Electroblot Buffers, MA) were laid down, on top of which was layered the filter paper soaked in anode buffer #2 (Integrated Separation Systems Protein Electroblot Buffers, MA), followed by the sheet of nitrocellulose, the gel, and the filter papers soaked in cathode buffer (Integrated Separation Systems Protein Electroblot Buffers, MA). The cathode was then placed on top and a constant current of 100mA for a mini-gel or 200mA for a large gel was applied for 45 minutes. The proteins bound to the nitrocellulose were then available for protein staining or detection with specific antibodies.

Immunoblotting

The nitrocellulose membranes with the separated proteins bound to them were blocked with Tris buffered saline (0.04M Tris, 1M NaCl, pH 7.5, TBS) containing 2% bovine serum albumin (BSA) at room temperature for 1 hour with shaking. The membranes were cut into lanes when necessary, placed in the appropriately diluted antiserum in TBS-Tween 20 (0.04M Tris, 1M NaCl, 0.06% Tween-20, pH 7.5 TBST) and incubated overnight with agitation. The nitrocellulose was washed twice for 5 minutes in TBST at room temperature with shaking. The appropriate secondary antibody, conjugated with the enzyme horseradish peroxidase, was diluted in TBST, added to the washed nitrocellulose and incubated at room temperature for 45 minutes. The membrane was again washed twice in TSBT with shaking as described. The washed nitrocellulose was place in the enzyme substrate, prepared by dissolving 60 mg 4-chloro-1-naphthol in 10mL methanol, adding 100mL TBS and 600 μL H_2O_2 for 10 minutes with shaking. The enzyme reaction was then stopped by placing the nitrocellulose in distilled water and air drying on filter paper.

Colloidal Gold Total Protein Staining

To detect all of the proteins, the Colloidal Gold Protein Detection System (BioRad Laboratories, CA) was used as per manufacturer's directions. The nitrocellulose was placed in TBST for 1 hour with 3 changes of buffer on a tabletop shaker. The nitrocellulose was rinsed briefly in distilled water and placed in the

colloidal gold solution for 2 hours to overnight. If increased sensitivity (< 1ng) was necessary, the "enhanced protocol" was followed which layered silver on top of the colloidal gold bound to the proteins increasing detection to 1pg of protein.

Lymphocyte Transformation Assay

BALB/c mice of various treatment groups were killed by CO_2 inhalation and the spleens aseptically removed. The spleens were then pushed through stainless steel wire mesh into RPMI 1640 media. The cells were pelleted from suspension (225 X g for 6 minutes) and washed twice in 10ml aliquots of RPMI 1640 media. The red blood cells were then lysed in ACK Lysis Buffer (0.15M ammonium chloride, 0.1mM EDTA, 1M potassium carbonate, pH 7.2), and remaining splenocytes washed once in RPMI media. The splenocytes were then diluted to a cell density of 4 X 10^6 cells/ mL in RPMI media supplemented with 1mM HEPES, 2mM L-glutamine, 1.5mM nonessential amino acids, 0.001% β-ME, and 50μg/mL gentamicin. To the diluted antigens in triplicate in round-bottom 96-well plates (Corning Laboratory Sciences, NY), 100μl aliquots of the diluted splenocytes were added to each well giving a final volume of 4 X 10^5 cells/ well. The plates were then incubated at 37°C in 5% CO_2 for 48 hours and wells were pulsed with 1 μCi ^{3}H- thymidine and incubated for an additional 18 hours. The cells were harvested onto glass filter fiber using a PHD Harvester (Cambridge Technologies Inc., MA), scintillation fluid (Ecoscint, National Diagnostics, Inc., NJ)) was added to the sample vials, and incorporation of

radioactivity (cpms) determined in a Beckman liquid scintillation counter (Beckman Instruments, CA). Results were given as mean ($\bar{x}$) cpm ± standard deviation. The stimulation index (SI) was the calculated by dividing the $\bar{x}$ cpm of the antigen treated wells by the $\bar{x}$ cpm of the media only wells.

Cytokine ELISAs

Round-bottom 96-well plates (Corning Laboratory Science, NY) were set up with antigen and lymphocytes as described for LTA. The culture supernatant from the wells was collected in one of two ways following the 48 hour incubation with antigen. In the first, a parallel assay to the LTA was set up. The plates designated for cytokine testing were spun at 225 X g for 6 minutes to pellet the cells and 100 μl of the culture supernatant carefully removed from the top. Alternatively, 50 μl of the culture supernatant was carefully removed from the wells. The cells left in the wells were then pulsed as described. The supernatants from the three wells were pooled and subjected to slow speed centrifugation to remove any contaminating splenocytes. The supernatants were stored at -20°C if not used immediately in the cytokine ELISA.

INF-γ levels were determined using one of two different kits. The first kit used was a Cytoscreen Mouse INF-γ ELISA kit (Tago Immunochemicals, CA), as per manufacturer's instructions. All reagents were supplied in the kit. Results given in pg/mL INF-γ were determined based upon a standard curve. The detection range of

the kit is 1 -500 pg/mL INF-γ.

IL-2, IL-4, and INF-γ were detected using Endogen Mini Kits (Endogen, MA) for mouse cytokine detection. Nunc Maxisorb ELISA plates were used and the reagents prepared and the tests performed as per manufacturer's instructions. Concentration of cytokines were determined based generation of a standard curve upon standard curve using recombinant murine INF-γ, IL-2, or IL-4 as standards.

Proteinase K Digestion of S2 Extract

Lyophilized S2 antigen was dissolved to a concentration of 350 μg/mL in sterile distilled water. An aliquot of 100 μl of the diluted antigen was added to washed proteinase K linked agarose beads (Sigma Chemical Co, MO), vortexed well, and incubated at room temperature for 72 hours with rocking. The beads were removed from the suspension by centrifugation (5600 X g for 3 minutes) and the supernatant carefully removed off the top of the settled beads. A 30 μl aliquot (approximately 10.5 μg) was removed and diluted in 570 μl RPMI 1640 complete media and used in LTA. A 10 μl aliquot was added to 10 μl 2X Lammeli sample buffer (Sigma Chemical Co, MO) and run on SDS-PAGE. An identical aliquot was prepared as above, but no proteinase K beads were added, to serve as a control.

RESULTS

Colloidal Gold and Antiserum Detection of S2 Proteins

Colloidal gold and Goat 48 (a goat hyperimmunized with acetone-killed RB51, provided by Dr. G. Schurig, VPI & SU, Blacksburg, VA) staining of the S2 proteins separated by SDS-PAGE were found to visualize the same protein bands (Fig.19). Nine protein bands were detected of the following approximate molecular weights (in kDa): 68.4, 60.2, 42.2, 22.4, 17.4, 15, 13.2, 11.5, and 10.2. As observed in Fig. 20, the use of an anti-*B. abortus* Cu-Zn superoxide dismutase (SOD) serum identified the 17.4kDa band as the Cu-Zn SOD. The Cu-Zn SOD antiserum was kindly provided by Dr. Louisa Tabatabai (USDA, National Animal Diseases Center, Ames, IA). The preparation was similarly found not to contain the heat shock protein HtrA (antiserum provided by Dr. Schurig) or GroEL (Epicentre Technologies, WI). Serum from mice vaccinated with strain RB51 did not recognize any of the proteins in the S2 preparation.

In Vitro Responses of Sensitized BALB/c Lymphocytes to S2 Antigen

As can be seen in Table 1, sensitized splenocytes of BALB/c mice proliferate in response to various concentrations of the S2 antigen in triplicate wells. In contrast, nonsensitized splenocytes do not (in the media preparation without 0.001% β-ME) or at a low level (media with 0.001% β-ME). The sensitized splenocytes were induced to

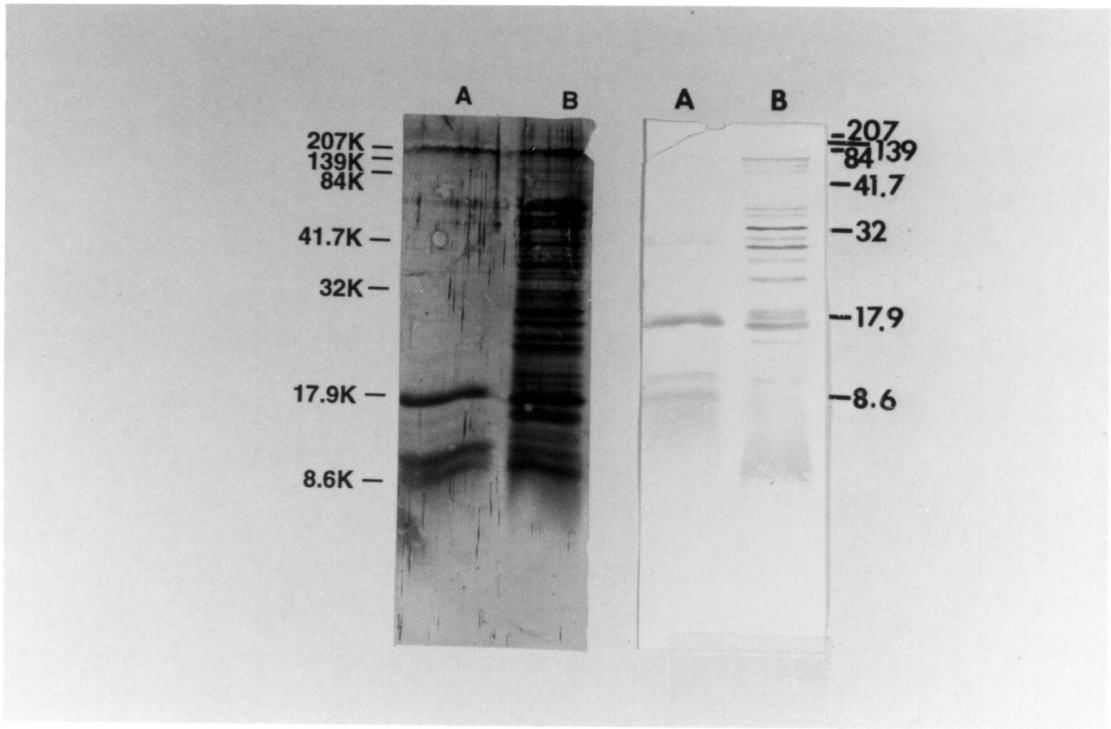


Fig. 19. SDS-PAGE of S2 proteins separated on a 15% polyacrylamide gel. Lanes A contain 17.5 μg S2 and lanes B contain 1 x 10^8 whole cell RB51. Lanes A and B on the left have been stained with Colloidal Gold Total Protein Stain enhanced protocol. Lanes A and B on the right are the results of immunoblotting with Goat 48 serum. Molecular weight markers are in kDa.

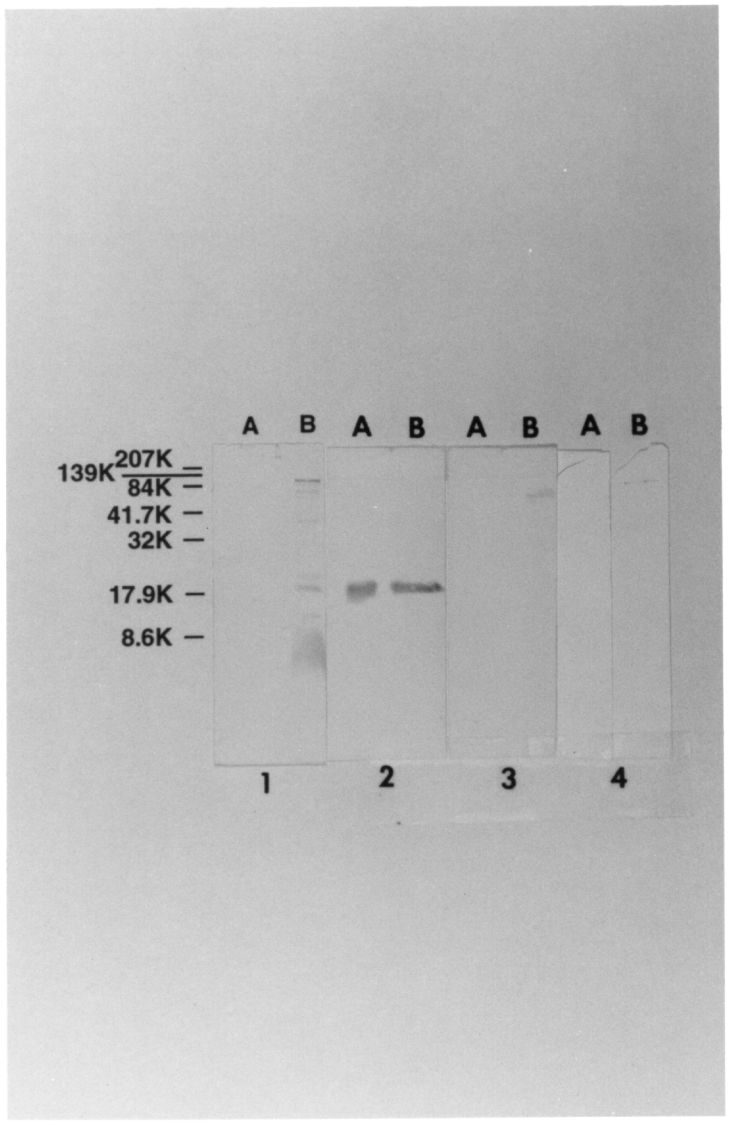


Fig. 20. Western blots of SDS-PAGE separated S2 (3.5μg/ lane) or whole cell (10^8) strain RB51. Lanes B contain whole cell (10^8) strain RB51. Lanes A contain S2 (3.5μg/ lane). Membrane #1 was probed with serum from mice vaccinated with strain RB51. Membrane #2 was probed with goat anti-*B. abortus* Cu-Zn-SOD serum. Membrane #3 was probed with mouse *B. abortus* anti-HtrA serum. Membrane #4 was probed with rabbit anti-GroEL. Molecular weight markers are given in kDa.

Table 1. Splenocyte responses to various concentrations of S2 antigen in lymphocyte transformation assay with and without the addition of β-ME in the culture media.

S2 Conc. (μg/mL)	Control SI[a]	Control INF-γ (pg/mL)[a]	Vaccinated SI no β-ME[b]	Vaccinated INF-γ (pg/mL) no β-ME[b]	Vaccinated +β-ME SI[c]	Vaccinated +β-ME INF-γ (ng/mL)[c]
0.035	1.0	0	4.9	0	3.8	4.9
0.175	0.9	0	4.8	0	4.1	12.7
0.35	1.2	0	7.4	26.6	4.7	22.8
1.75	1.3	0	22.6	292.4	10.2	45.8
3.5	1.6	0	24.1	119.8	11.0	41.8
17.5	2.0	0	11.6	253.3	12.9	0

SI= stimulation index.

[a]=control splenocytes in media without β-ME. Media cpm 149.0± 24.0. Concanavalin A SI=106.4

[b]=Media cpm 291.7± 32.2. Concanavalin A SI=191.6.

[c]= Media cpm 2273.9± 233.3. Concanavalin A SI= 59.8.

produce INF-γ, while the nonsensitized splenocytes did not induce INF-γ independently of the presence or absence of β-ME in the culture media. The optimal proliferative responses by the sensitized lymphocytes to the S2 antigen were obtained with 1.75μg/ml and 3.5 μg/ml and the optimal INF-γ response was observed with an S2 concentration of 1.75μg/mL. Therefore, 1.75μg/ml was used throughout subsequent assays.

At an optimal *in vitro* concentration of 1.75μg/well and testing pooled triplicate wells, the S2 extract was observed to induce a proliferative response, INF-γ and IL-2 production, but no IL-4 production by the strain RB51 sensitized splenocytes (Table 2). As the responses of the sensitized lymphocytes to the S2 extract elicited a T_{H1}-type cytokine profile, the S2 extract was then tested for it's ability to protect mice from a virulent challenge with *B. abortus* strain 2308.

Immune Responses of BALB/c Sensitized Lymphocytes to Proteinase K Treated S2

The optimal concentration of S2 (1.75 μg/well) was proteinase K digested with proteinase K bound to agarose beads. The beads were removed from suspension by centrifugation and the supernatant used directly in LTA (Table 2). The response of the sensitized lymphocytes to the S2 extract was dramatically reduced from an SI of 18 to 3.5 and the INF-γ production ceased following the proteinase K treatment of S2. Treatment of the S2 antigen with proteinase K eliminated the protein bands detected by the Goat 48 serum in Western blots. However, reactivity to LPS (6,000 to 9,000

Table 2. Proliferation and cytokine profiles of normal and strain RB51 immunized splenocytes to S2 with and without proteinase K (ProK) bead treatment.

Mouse	**Antigen (1.75μg)**	**SI**	**INF-γ (ng/ml)**	**IL-2 (pg/ml)**	**IL-4 (pg/ml)**
Control[a]	S2	1.3	0	315.5	0
Immunized[b]	S2	22.6	5.7	3020.0	0
Immunized[b]	S2	18.0	17.5	N/D	N/D
Immunized[b]	S2+ ProK	3.5	0	N/D	N/D

SI= stimulation index. N/D= not determined.

[a]= Media cpm 2634.7± 908.0. Concanavalin A SI= 37.8.

[b]= Media cpm 1251.9± 28.2. Concanavalin A SI= 108.6.

mw) is still observed (Fig. 21). These data suggest that the responses *in vitro* observed with splenocytes of sensitized mice to the S2 extract is largely due to a response to specific proteins.

Immunization and Subsequent Challenge of BALB/c Mice with S2 Antigen Extract

The *in vitro* responses of lymphocytes and humoral responses of mice immunized with 100μg S2 in 250μg DDA are reported in Table 3 and Fig. 22. In contrast with the mice treated with DDA, the mice treated with S2/DDA had lymphocytes which proliferated in response to the various concentrations of the S2 extract. The proliferative responses of the splenocytes from the S2/DDA treated mice were also observed to induce the production of INF-γ and IL-2. The production of IL-4 was not determined in this particular experiment. The response of the splenocytes from the DDA treated mice to S2 at higher concentrations may be indicative of contaminating LPS in the antigen extract. However, as was observed with splenocytes from BALB/c mice at 6 weeks post-strain RB51 vaccination, no INF-γ was produced by the splenocytes from the DDA treated mice in response to the S2 antigen.

Confirming the results reported in the literature, the antibody titer of the S2/DDA treated mice was low (4, 90). At all three bleeding time points, the humoral response to S2 antigens by Western blot was negative. Using whole cell strain RB51 as the antigen, the highest antibody titer (1:640) obtained by ELISA was at 2 weeks following the third boost (Fig. 22). The antibody titer dropped to 1:160 at final

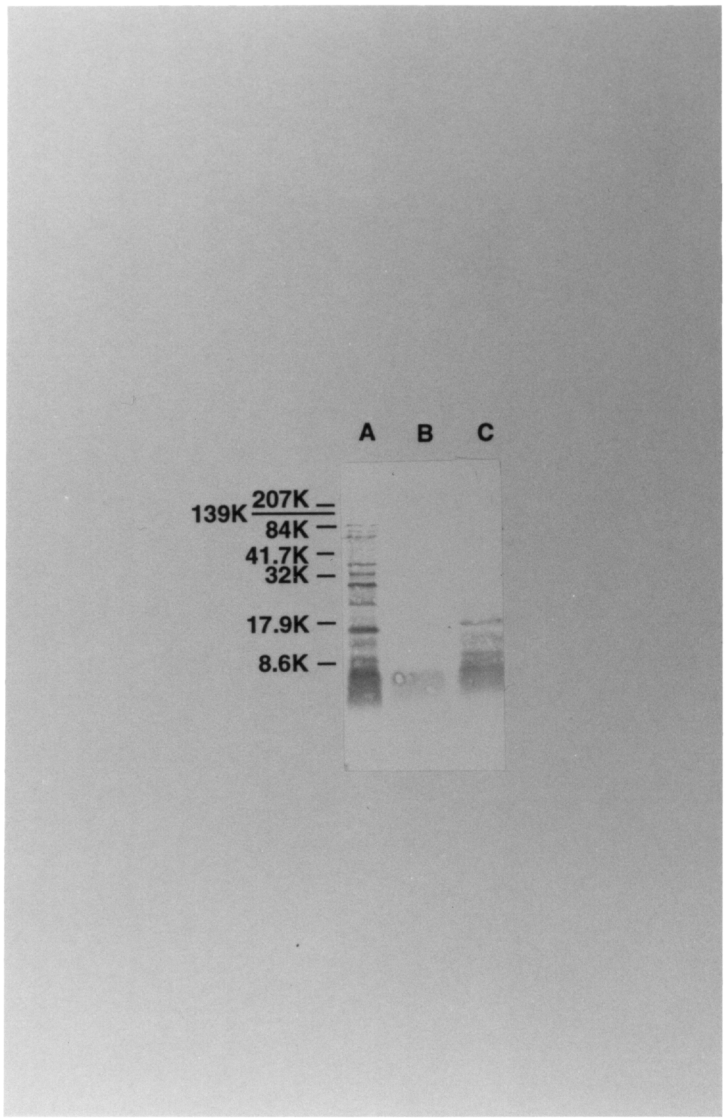


Fig. 21. Results of proteinase K digestion of S2 proteins separated by SDS-PAGE. Lane A- whole cell (10^8) strain RB51, lane B- proteinase K digested S2 (3.5μg), lane C- S2 (3.5μg). Lanes were probed with Goat 48 serum. Molecular weights are given in kDa.

Table 3. Immune responses and log colony forming units (CFU) from the spleens of control, DDA immunized, and S2/DDA immunized mice.

S2 Conc. (ug/ml)	DDA SI[a]	DDA INF-γ (pg/ml)[a]	S2/DDA SI[b]	S2/DDA INF-γ (pg/ml)[b]	S2/DDA IL-2 (pg/ml)[b]	Control
0.035	1.0	0	8.0	>500	>1300	ND
0.175	1.3	0	36.2	>500	>1300	ND
0.35	1.5	0	41.5	>500	>1300	ND
1.75	3.5	0	43.4	>500	>1300	ND
3.5	6.4	0	34.9	>500	>1300	ND
17.5	4.4	0	17.0	>500	>1300	ND
log CFU post-challenge from spleen	5.216 SD ± 0.372 p>0.05[c]		4.766 SD ± 1.21 p>0.05[c]			5.218 SD ± 0.508

ND=not determined. SI= stimulation index. Log_{10} CFU data are reported with ±SD (standard deviation) and Student's t-test probability (p) values and are representative of 6 mice per group. The stimulation indexes (SI) are representative of triplicate wells and the cytokine data reflective of a pool of triplicate wells.

[a]= Media cpm 416.6± 354.5. Concanavalin A SI=60.1.

[b]= Media cpm 1036.6± 310.7. Concanavalin A SI= 91.2.

[c]= not statistically significant as compared with controls.

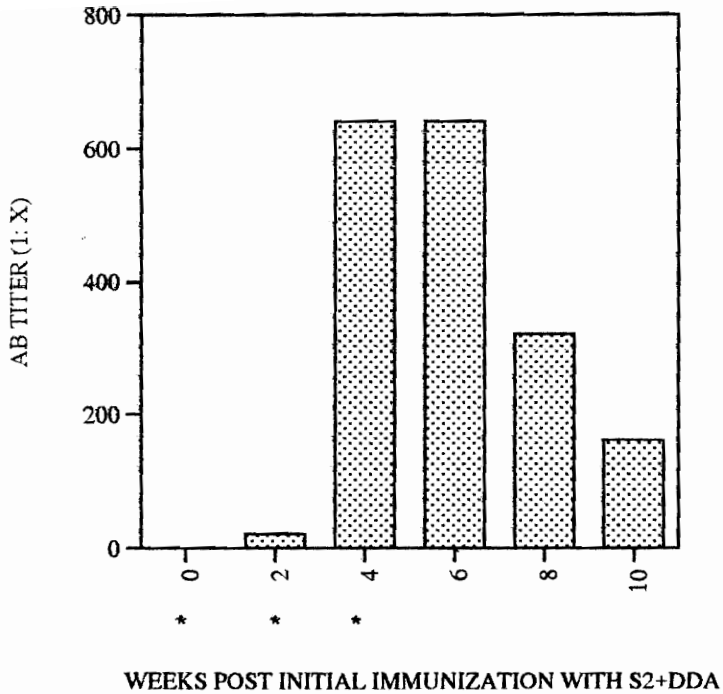


Fig. 22. Antibody titers of S2/DDA immunized mice to whole cell strain RB51 by ELISA over time. *= immunizations.

bleeding, which was two weeks post immunization with 100μg S2 without DDA. The DDA only and control mice were observed to have no antibody titer to S2 antigens at all time points by Western blot or ELISA.

Mice immunized with DDA, S2/DDA, or control mice were challenged with strain 2308 at five weeks post final immunization in order to minimize nonspecific protection induced by the adjuvant. All three groups were challenged with 6 X 10^4 strain 2308. At two weeks post challenge the mice were killed and the number of viable strain 2308 in the spleen enumerated. As can be observed in Table 3, there was no statistical difference in the number of 2308 per spleen among the three groups. Therefore, the 100μg S2/DDA did not confer any protection in mice, even though the S2 antigen stimulated a T_{H1}- type response by splenocytes of RB51 infected mice *in vitro*.

DISCUSSION

The results presented here partially characterize the S2 antigen as a whole. It contains 9 protein antigens in various concentrations as visualized by colloidal gold staining and immunoblots with Goat 48 antiserum. Using specific antisera, the S2 extract was determined to contain the Cu-Zn SOD, but not the HtrA or GroEL proteins of *B. abortus*. Although the serum (Goat 48) from a goat hyperimmunized with

acetone-killed RB51 reacted with all of the S2 antigens, serum from mice infected with RB51 did not. This supports the observation that infected mouse serum does not recognize the *B. abortus* Cu-Zn SOD (101). Furthermore, it suggests that there may be a family of proteins with a similar chemical character or nature which are not recognized by mice vaccinated with strain RB51, since all proteins in S2 are soluble in 50% ammonium sulphate.

Further characterization of the S2 antigen shows that mice vaccinated with strain RB51 have a cellular immune response to the antigen as indicated by the lymphoproliferative responses of sensitized lymphocytes to the antigen extract. Additionally, evidence that the responses are due to the proteinaceous components of the extract was demonstrated by the loss of proliferation and INF-γ production following proteinase K digestion of S2. The observation that a portion of the response is due to contamination of the extract with LPS will not be thoroughly addressed here as an entire subchapter has been devoted to this issue.

The splenocytes from strain RB51 immunized mice proliferated and produced INF-γ and IL-2 in response to *in vitro* stimulation by the S2 antigens at various concentrations suggesting that the response to S2 antigens is predominately a T_{H1} response. Exposure of these cells *in vitro* to the S2 antigens did not induce IL-4 production indicating that T_{H2} cells were not stimulated in the response. The *in vitro* CMI response of sensitized splenocytes from strain RB51 vaccinated mice is therefore indicative of a T_{H1} - type response, which is assumed to be important for the clearance

of *B. abortus* from an infected host as indicated by adaptive immune transfer experiments with $CD4^+$ and $CD8^+$ lymphocytes (6). It has been demonstrated that immunization of mice with RB51 induces a protective immune response and that this protective immune response is mediated by T cells (8). Since S2 antigens were stimulating *in vitro* the appropriate T cell population in splenocytes of RB51 vaccinated mice, it appeared reasonable to test whether direct immunization with the S2 antigens would lead to protective immunity. Following immunization of mice with S2 in DDA adjuvant it was found that the splenocytes from these immunized animals proliferated and produced INF-γ and IL-2 in response *in vitro* to the S2 antigen. The mice were not protected against virulent strain 2308 challenge, in contrast with strain RB51 vaccinated mice.

The *in vitro* stimulation findings contradict those observed by Zhan, *et. al.* where mice immunized with soluble *Brucella* proteins were observed to produce IL-2, but not INF-γ (115). The reasons leading to this difference observed with these antigen extracts are unclear, but could be explained by the many differences in the experimental designs of the individual studies. Zhan, *et. al.* used a 50% ammonium sulphate precipitate of a hot saline extract of strain 19 (115). This study employed the strain RB51, a rough strain lacking the O-chain polysaccharide antigen. In contrast, Zhan's study used smooth strain 19 possessing the O-chain polysaccharide antigen. Zhan's extract was precipitated in alum and mixed with 10^9 heat-killed *Bordetella pertussis* suspended in saline as an adjuvant. This study did not employ alum

precipitation and used DDA as the adjuvant. The choice of adjuvant and the isolation of different proteins from another strain of *Brucella abortus* which possesses smooth LPS by Zhan make it impossible to directly compare this study with Zhan's study.

The choice of DDA as adjuvant was an important decision. DDA has been reported in the literature to stimulate primarily a T_{H1} cellular immune response in immunized animals and it is known that the protective mechanisms induced by strain RB51 vaccination are T-cell mediated (4, 8, 90). It has also been reported that the $CD4^+$ cells induced with antigen and DDA were long-lived and, therefore, provided protection to immunized animals (4). Furthermore, DDA has the advantage of being a chemical rather than a bacterial adjuvant eliminating the question of potential cross reactivity of response to mycobacterial antigens. This is also the first report of the use of DDA as an adjuvant with *B. abortus* proteins.

In contrast with Andersen's study to *Mycobacterium tuberculosis* (4), the S2 antigen was not found to be protective. Andersen used short-term culture filtrates of *M. tuberculosis* mixed with DDA to immunize mice. The DDA was observed to induce a specific T cell response to two protein fractions which contained proteins in the molecular weight ranges of 5-12 kDa and 25-35 kDa. T cells from the short-term culture filtrate/DDA immunized mice could be passively transferred and confer protection against virulent challenge.

The S2 antigen did elicit lymphoproliferation and production of IL-2 and INF-γ, but not IL-4, by splenocytes from strain RB51 vaccinated mice (Table 1). These

responses are consistent with those reported for a T_{H1} type immune response (23). Furthermore, clearance of *Brucella* by an infected host is thought to be primarily by the cellular immune response (63). The data presented here indicate that, although individual antigens may elicit an appropriate response according to *in vitro* assays measuring proliferation and INF-γ, IL-2, and IL-4 production, the presentation of these antigens by live bacteria may be necessary to induce active protection. Also, it may be indicating that it is necessary to expand the *in vitro* assays on cytokine production to better predict the protective potential of antigens.

Others have reported the use of antigen extracts as potential vaccines in brucellosis (15, 33, 102). Bosseray and Dubray and Bézard looked at the efficacy of cell-wall antigens as vaccines (15, 33). Both observed that the protection provided was of short-term duration. Additionally, Tabatabai, *et. al.* observed short-term protection in lemmings vaccinated with *B. abortus* salt-extractable proteins (102). By four weeks post immunization, the lemmings were no longer protected from challenge with a virulent strain. Using three synthetic peptides based upon the amino acid sequence of the *B. abortus* Cu-Zn SOD, Tabatabai and Pugh observed that one of the synthetic peptides given in combination with monophosphoryl lipid A as an adjuvant to mice afforded two logs of protection upon virulent challenge with strain 2308 (101). The mice were given only one immunization with the synthetic peptide. The duration of this protective response by the synthetic peptide was not determined.

The use of LPS or antibodies against the O-chain-polysaccharide as a vaccine

has been investigated (14A, 25, 109). Cloeckaert, *et. al.* have used purified LPS of *B. melitensis* strain 16M to vaccinate BALB/c mice (25). They observed that these mice had low titers to LPS following immunization and were not significantly protected from virulent challenge with *B. melitensis* 16M. Bosseray, *et. al.* observed that immunization with *B. abortus* LPS did not confer protection against virulent challenge in guinea pigs (14a). In contrast, Winter, *et.al.* observed that passive transfer with a monoclonal antibody (IgG2a) to the O-chain polysaccharide conferred protection from virulent challenge in mice (109).

CHAPTER THREE

II. SEPARATION OF S2 PROTEINS AND CMI RESPONSES TO INDIVIDUAL S2 PROTEINS

INTRODUCTION

B. abortus has approximately 1000 individual proteins separable by 2-D gel electrophoresis (91). Many schemes have been devised in order to define which of these proteins induce CMI in brucellosis. One of the first studies examining the lymphoproliferation by sensitized lymphocytes in response to antigens of *B. abortus* was performed by Brooks-Worrell *et. al.* (16). *B. abortus* strain 19 antigens were separated by one-dimensional SDS-PAGE. The proteins were electroblotted onto nitrocellulose which was in turn cut into pieces and placed in triplicate into 96-well plates and the uptake of ^{3}H-thymidine by lymphocytes from nonvaccinated and strain 19 vaccinated bovines was compared. A similar method was performed by transferring proteins from 2-D gels of strain 19 to nitrocellulose and observing ^{3}H-thymidine incorporation. In this manner, the number of antigens capable of inducing ^{3}H- thymidine incorporation was narrowed to one protein spot. Lymphoproliferation by the sensitized bovine lymphocytes to the antigens separated by one-dimensional electrophoresis was observed with proteins of molecular weights (in kDa): 97-116, 43-87, 30-38, 23-25, 21, and 6. Lymphoproliferative responses to the proteins that were separated by 2-D gel electrophoresis were observed with proteins in the same

molecular weight ranges as identified by 1-D gel electrophoresis. Two proteins were found to be very stimulatory, a 12kDa and 31kDa protein. Partial amino acid sequences of these proteins were obtained, but did not to correspond to any proteins in the National Biomedical Research Foundation protein database (University of Washington Genetics Computer Group progam FIND).

Zhan, *et. al.* took another approach in investigating the CMI responses of sensitized murine lymphocytes to an antigen preparation of *B. abortus* strain 19 (115). The antigen extract was referred to as soluble brucella proteins (SBP) and was made by preparing a hot saline extract of strain 19 and precipitating the supernatant with 50% ammonium sulfate. The precipitate was then solubilized in phosphate buffered saline, dialyzed, and LTA performed. Additionally, the cytokines liberated into the supernantants from splenocytes in response to this antigen were also assessed. Lymphocytes from mice vaccinated with live strain 19 produced INF-γ and IL-2. However, lymphocytes from mice immunized with SBP extract did not produce INF-γ, but did produce IL-2 or IL-4. The authors speculated that only vaccination with live *Brucella* resulted in T cell production of INF-γ.

Using the reactivity of strain 19 sensitized bovine lymphocytes to blotted proteins as a means of detecting immunoreactive antigens, the *B. abortus ssb* gene product, *uvrA* gene product, and L7/L12 ribosomal proteins have been identified (78, 118).

RATIONALE AND HYPOTHESIS

In the previous section, it was demonstrated that S2 proteins induce a lymphoproliferation and production of INF-γ and IL-2 by splenocytes from strain RB51 vaccinated mice. Although this extract alone was not protective, the lack of protection may have been due to manner of presentation of the antigen in mice and therefore more effective if the proteins could be produced by a live organism as a vector. Therefore, it was still of interest to further investigate the S2 extract as it did induce a T_{H1} type response by splenocytes from RB51 vaccinated mice. Since S2 contains several proteins it was not clear which ones, either singly or synergistically, stimulated the responses *in vitro*. The individual S2 proteins were separated and tested for their ability to stimulate lymphoproliferation and induce the production of cytokines from splenocytes of strain RB51 vaccinated mice.

MATERIALS AND METHODS

Rotofor Separation of S2 Proteins

The S2 antigen was redissolved in a final concentration of 4M urea, 2% ampholytes (pH range 3-10; FMC Bioproducts, ME), as per manufacturer's instructions. The proteins of the S2 extract were separated for 4 hours at 12 W into 20 fractions based upon charge. The individual fractions were harvested and the pH of each fraction was determined. The individual fractions were dialyzed (12kDa-14kDa mwco) first against 1M NaCl overnight at 4°C, and then against several changes of

distilled water overnight. The dialyzed fractions were lyophilized and resuspended in 300μl sterile distilled water for testing . Ten μl aliquots of each fraction were mixed with an equal amount of 2X Lammeli sample buffer (Sigma Chemical Co., MO) and loaded in wells of a 12.5% SDS-PAGE. The individual proteins were visualized by colloidal gold total protein staining and immunoblotting with serum from a goat hyperimmunized with acetone-killed RB51 (Goat 48, provided by G. Schurig).

Fast Performance Liquid Chromatography (FPLC) Separation of S2 Proteins

Ten mg dry weight of S2 extract was resuspended in 0.2mM Tris-HCl (pH 7.8) and subjected to FPLC (Pharmacia, NJ) as per manufacturer's instructions. The column selected for the FPLC was a Mono Q (BioRad Laboratories, CA) column which binds negatively charged molecules. As the salt concentration through the column is increased, different proteins are eluted from the column into sample tubes. Two hundred μL aliquots of S2 extract was passed through the column per run, for a total of 10 runs. Equivalent fractions were pooled, samples were dialyzed (12kDa-14kDa mwco) at 4°C against several 4L changes of distilled water overnight and then lyophilized. Pooled fractions were reconstituted in 300μl sterile distilled water. BCA (Pierce, IL) total protein assay was performed on the samples to determine total protein concentration. However, the readings were below the detection limit of the assay (< 2.5 μg/mL). Ten μl aliquots of each fraction were mixed with an equal amount of 2X Lammeli sample buffer (Sigma Chemical Co., MO) and loaded in

wells of a 12.5% SDS-PAGE mini-gel. Following electrophoresis at 30mA for 1 hour, the individual proteins were visualized by colloidal gold total protein staining and immunoblotting with serum from Goat 48.

Preparation of Nitrocellulose Particles of the Individual S2 Proteins

Standard size 15% polyacrylamide gels were prepared as described in the previous subchapter. Aliquots of 17.5μg S2 per lane were separated by 1-D SDS-PAGE and transferred to PH79 nitrocellulose (Schleicher & Schuell, NH) sheets as described in the previous subchapter. One lane of S2 was colloidal gold stained to determine the location of the individual S2 bands. Individual bands were cut out with a scalpel blade. As a negative control, hen egg-white lysozyme was separated by SDS-PAGE. To serve as positive controls, nitrocellulose pieces from a nitrocellulose sheet subjected to a sham transfer were cut and spotted directly with 1.75μg S2 per piece or 1μg (dry weight) whole cell strain RB51 per piece. All pieces were air dried, and rinsed twice with RPMI 1640 complete media. Nitrocellulose particles were then prepared as described by Abou-Zeid *et.al.* (1). Briefly, the individual bands were placed in microcentrifuge tubes and 250μl dimethyl sulfoxide (DMSO) was added to solubilize the nitrocellulose. The nitrocellulose-DMSO solutions were allowed to incubate for 60 minutes at room temperature. The mixtures were then vortexed well and an equal volume (250 μl) carbonate-bicarbonate buffer (pH 9.6) was added dropwise, with frequent vortexing allowing for the precipitation of the nitrocellulose

particles from solution. The particles were then pelleted from solution at 8060 X g for 10 minutes, the supernatant discarded and the nitrocellulose particles washed 3 times in RPMI 1640 complete media. The particles were then resuspended in 1mL RPMI 1640 complete media and 50μl and 100μl of the resuspended particles were tested in triplicate in an LTA using lymphocytes from strain RB51 sensitized or nonsensitized BALB/c mice for an LTA as described in the previous subchapter.

Preparation of Nitrocellulose Pieces and Modification of LTA

SDS-PAGE and electroblotting were performed as described for the preparation of nitrocellulose particles. However, the washed individual bands were placed in individual wells of flat-bottom 96-well plates in triplicate and incubated at room temperature for 60 minutes in 100μl aliquots of RPMI 1640 complete media. The positive and negative controls were prepared as described for the nitrocellulose particles, but the nitrocellulose sections were placed in the individual wells following the washing as described for the individual S2 bands. The strain RB51 sensitized and nonsensitized lymphocytes were obtained and diluted as described for LTA, but the LTA was modified to allow more time for macrophages to remove the proteins from the nitrocellulose and process and present the antigens to the lymphocytes. The plates were incubated at 37°C in 5% CO_2 for 5 days. At days 2 and 4, the plates were removed from the incubator, placed on a tabletop rotary shaker and mixed for 5 minutes at a speed of 60 rpm. Following the 5th day of incubation, 50μl aliquots of

media were removed from the individual wells for IL-2 and INF γ testing as described. The wells were then pulsed with 1μCi ^{3}H-thymidine for 18 hours. The cells were harvested and mean ($\bar{x}$) cpms and SI determined as described previously.

Sequencing of the 10.2kDa and 11.5kDa Proteins of the S2 Extract

Standard-sized 15% polyacrylamide gels were prepared and incubated at 4°C overnight before use. Lanes containing 17.5μg S2 in 2X Lammeli sample buffer (Sigma Chemical Co., MO) were subjected to electrophoresis at 25mA for 6 hours. The proteins of the gel were transferred to Westran membranes (Schleicher & Schuell, NH) in CAPS transfer buffer (10mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0) in accordance with the method of Matsudaira (66). The proteins were visualized by staining with Coomassie blue, destained with several changes of 50% methanol, followed by several washes in distilled water. The 10.2kDa and 11.5kDa bands were cut from the Westran membrane and stored at -20°C until subjected to 15 cycles of microsequencing by the Protein Sequencing Lab at VPI & SU (Blacksburg, VA). The facility uses an Applied Biosystems 477A Protein Sequencer. The protein sequencing and amino acid determination were performed by Ms. Laura Sporakowski. A National Center for Biotechnology Information (NCBI) Blast search was then conducted to compare the two sequences with known sequences in an effort to identify the selected proteins.

Deduced Deoxyribonucleic Acid (DNA) Sequences Based Upon Amino Acid Sequences of the 10.2kDa and 11.5kDa S2 Proteins

Based upon the N-terminal 15 amino acids of the 10.2 kDa and 11.5 kDa proteins, putative DNA sequences were deduced. This was accomplished using a combination of the known genetic code tables, the G+C% content of *Brucella* spp. (48), and the published *ssb* and *uvrA* sequences of *B. abortus* (78).

RESULTS

Rotofor Separation of S2 Proteins

Pictured in Fig. 23 are the protein fractions of one of the resulting Rotofor fractionations. Unfortunately, these results were not reproducible. The amount of protein obtained in the individual fractions was insufficient and although some fractions appeared to contain only one band it was not possible to obtain enough of the protein to work with as the fractionations were irreproducible. These fractions were tested in LTA and the results compiled in Table 4. Ten microliters of fraction #5 containing 3 protein bands of approximate molecular weight of 17kDa, 9kDa, and 5kDa was stimulatory with an SI of 9.0.

FPLC Separation of S2 Proteins

FPLC separation of S2 proteins on the Mono Q column did yield fractions

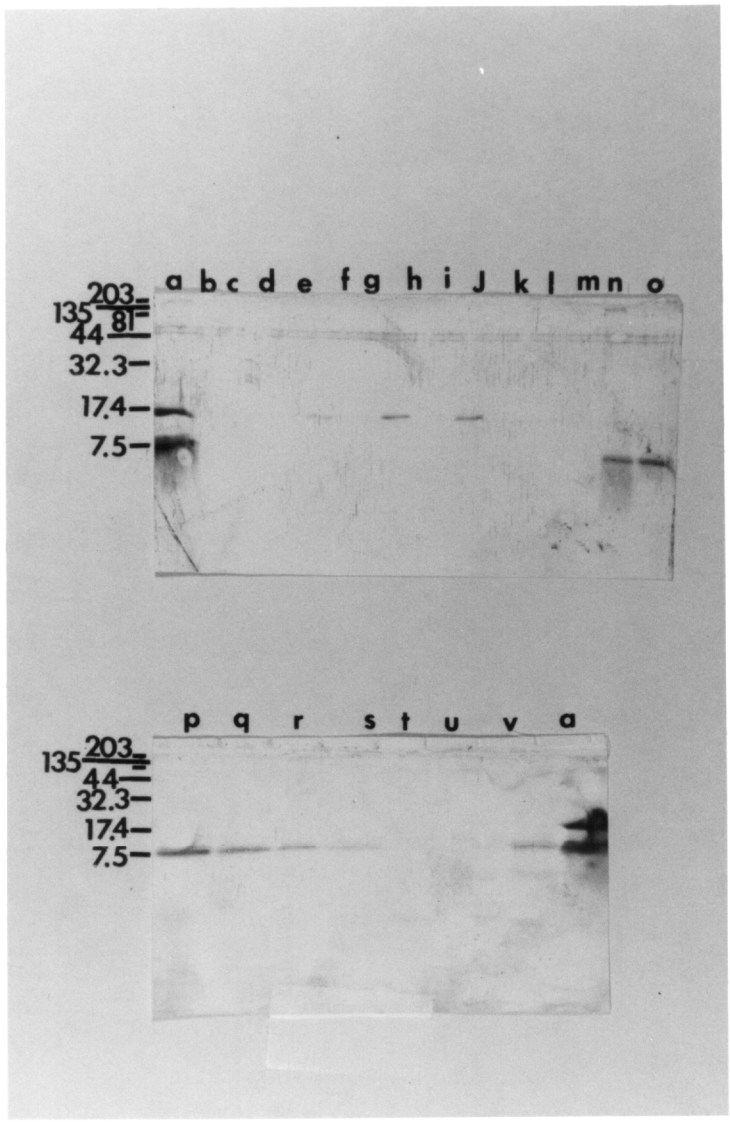


Fig. 23. Results of Rotofor fractionation of S2 proteins, stained with Colloidal Gold Total Protein Stain. Lanes contain samples of lanes a- S2, lanes b-v are fractions 1-20. Molecular weight markers are given in kDa.

Table 4. Rotofor fraction stimulation in LTA with strain RB51 sensitized splenocytes.

Fraction #	**pH**	**dilution of fractions which stimulate strain RB51 sensitized lymphocytes**	**Response of strain RB51 sensitized splenocytes (SI)[a]**
whole S2	ND*	1.75 ug protein	8.8
1	2.0	10ul, 1ul	36.4, 14.3
2	3.1	10ul	20.3
3	3.9	10ul	11.1
4	4.3	10ul	12.3
5	4.65	10ul	9.0
6	4.9	10ul	3.9
7	5.1	10ul	8.5
20	9.0	1ul	

*ND= not determined.

[a]= Media cpm without β-ME: 207.2± 21.3. Concanavalin A SI= 272.5.

which appeared to contain a single protein (Fig. 24). When tested in LTA, fractions 5 and 10 stimulated proliferation of strain RB51 sensitized lymphocytes, and induced the production of IL-2 and INF γ, but not IL-4 (Table 5). Fraction 10 was chosen for further investigation for the following reasons: the fraction induced strain RB51 sensitized splenocyte proliferation, stimulated IL-2 and gave the highest INF-γ production, but did not stimulate IL-4 production. The proteins associated with the activity were of low molecular weight, few in number (approximately 10 kDa, 9.5 kDa, and 7.2 kDa on a 1-D, SDS-PAGE mini gel), and were readily visible by colloidal gold total protein stain without the use of enhanced colloidal gold staining (Fig. 24).

In order to increase the yield of the proteins in fraction 10, FPLC with a higher initial concentration of S2 was attempted. However, separation of the proteins in the fraction was poor and the resulting fraction 10 contained 5 bands rather than three (Fig 25).

Due to the difficulties in obtaining individual bands with the Rotofor and FPLC methods, the SDS-PAGE/ electroblotting technique for separation was selected.

Lymphocyte Transformation Assay (LTA) of Nitrocellulose Particles of Individual S2 Proteins

The particle suspension was determined to be an unreliable means for eliciting a proliferative response from sensitized lymphocytes as evidenced by the responses

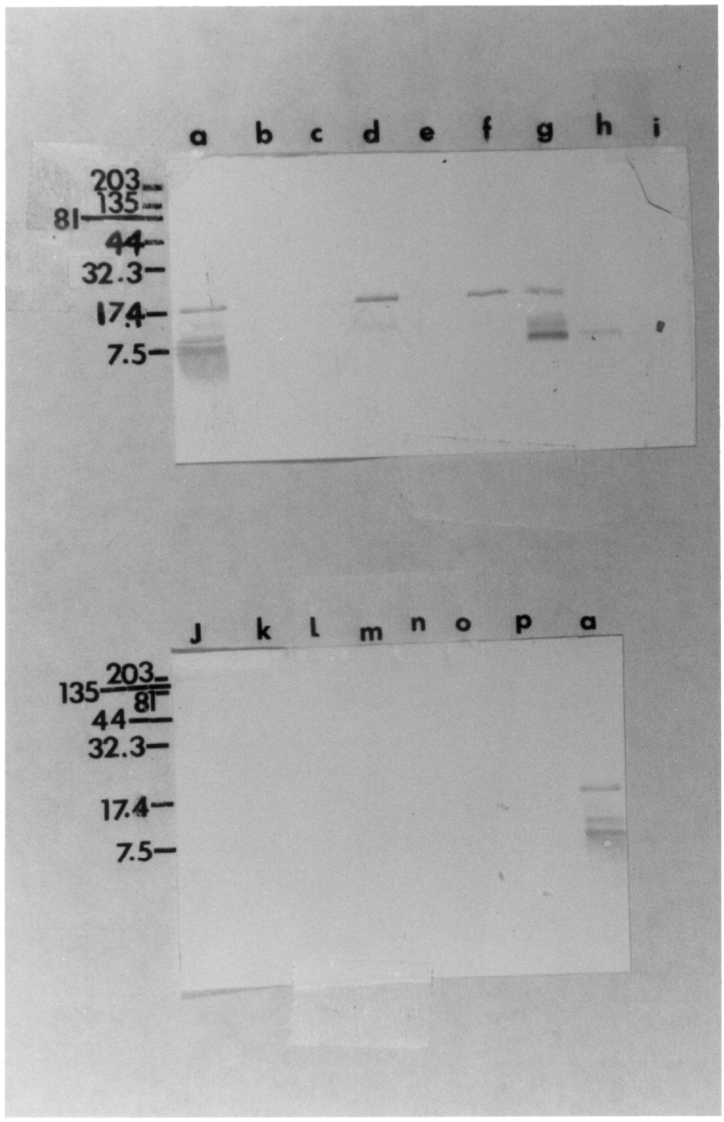


Fig. 24. FPLC fractions 4- 18 separated by SDS-PAGE. Lane a- S2, lanes b-p - fractions 4- 18, lanes 17 and 18- S2. Bands visualized based upon reactivity with Goat 48 serum. Molecular weights are given in kDa.

Table 5. Immune responses of splenocytes from sensitized (Imm.) and nonsensitized (Cont.) mice to FPLC fractions.

Anti-gen	Cont (SI)[a]	Cont. INFγ pg/ml	Cont. IL-2 pg/ml	Cont. IL-4 pg/ml	Imm. (SI)[b]	Imm. INFγ pg/ml	Imm. IL-2 pg/ml	Imm. IL-4 pg/ml
media	ND	0	461.0	0	ND	0	204.5	0
1.75ug S2	2.6	0	453.0	136.0	30.1	>500	3230.0	0
Fr. 5	0.4	0	440.5	101.8	7.8	104.8	838.5	0
Fr. 6	1.1	0	437.5	46.8	4.7	0	304.5	279.6
Fr. 7	0.9	0	581.0	212.0	6.5	2.4	429.5	0
Fr. 8	0.4	0	55.5	0	6.1	13.4	568.0	0
Fr. 9	0.3	0	225.0	114.3	4.8	47.7	401.0	100.6
Fr. 10	2.1	0	149.0	180.9	11.8	229.4	746.5	0

SI= stimulation index. ND= not determined.

[a]= Media cpm 3790.3± 963.9. Concanavalin A SI= 36.3.

[b]= Media cpm 1664.9±416.2. Concanavalin A SI= 91.6.

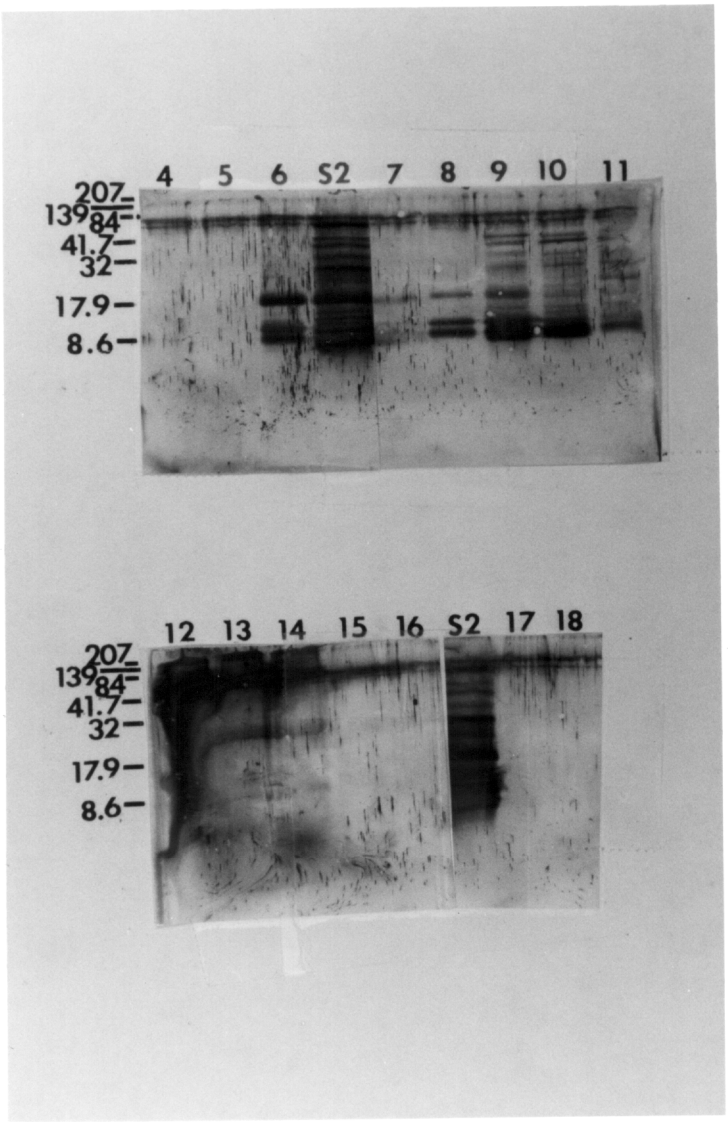


Fig. 25. SDS-PAGE separation of FPLC fractionated S2 proteins when a higher concentration of S2 added to the column. Lanes indicate fraction numbers as marked. S2 in lanes as indicated. Proteins visualized with Colloidal Gold Total Protein Stain. Molecular weights given in kDa.

observed in Table 6. Even the response of the lymphocytes to 1μg whole cell RB51 blotted onto nitrocellulose was poor, as the SI induced by the splenocytes of strain RB51 vaccinated mice was similar to that observed with the nonvaccinated control splenocytes.

Separation of S2 Proteins by SDS- PAGE and LTA of Nitrocellulose Pieces with Bound Protein

In this assay, the S2 antigen was separated by SDS-PAGE, transferred to nitrocellulose. The location of the protein bands was determined using colloidal gold and the stained proteins were cut out of the nitrocellulose. The nitrocellulose bands were cut in half and used to stimulate lymphoproliferation and INF-γ production (Table 7). The resulting cpms in the LTA were found to be very variable with standard deviations of the cpms greater than 20% between triplicates for most samples; as a result the SI values did not reflect overall significant lymphoproliferation. However, INF-γ production was elevated in the supernatants of strain RB51 sensitized splenocytes in response to the 10.2kDa, 11.5kDa, and 17.4kDa proteins (Table 7). The 17.4kDa protein was identified as the *B. abortus* Cu-Zn SOD, as determined by specific polyclonal serum from goats hyperimmuned with the *B. abortus* Cu-Zn SOD. All of the remaining S2 proteins elicited either none or low levels of INF-γ. In all cases, the splenocytes from the control mouse did not produce INF-γ in response to the antigens on the nitrocellulose. As INF-γ production was observed with the

Table 6. Immune responses of sensitized and nonsensitized splenocytes to Nitrocellulose (N/C) particles containing S2 proteins.

Protein band or N/C section	**Control SI[a]**	**Control INFγ (ng/ml)**	**Vaccinated SI[b]**	**Vaccinated INFγ (ng/ml)**
S2 particles	1.4	0	1.7	0
whole cell RB51 particles	1.9	0	1.4	0
10.2kDa	1.5	0	1.2	0
11.5kDa	1.5	0	1.4	0
17.4kDa	1.3	0	1.1	0
18-30kDa	1.0	0	1.3	0
30- 32kDa	1.2	0	1.2	0
32- 40kDa	1.2	0	1.1	0
>84kDa	1.4	0	1.2	0

SI= stimulation index. Results are of a representative experiment.

[a]= Media cpm 2634.7± 908.0. Concanavalin A SI= 37.8, S2 SI= 1.8, whole cell RB51 SI= 4.0.

[b]= Media cpm 1251.9± 28.2. Conconavalin A SI= 108.6, S2 SI= 18.0, whole cell RB51 SI= 28.0.

Table 7. Proliferation (SI) and INF-γ responses of strain RB51 sensitized (vaccinated) and nonsensitized (control) splenocytes to S2 antigens electroblotted onto nitrocellulose.

Antigen	**Control SI[a]**	**Control INFγ (ng/ml)**	**Vaccinated SI[b]**	**Vaccinated INFγ (ng/ml)**
S2-N/C	1.1	>13.1	1.9	>13.1
wcRB51 N/C	0.6	1.5	3.9	>13.1
10.2kDa	1.3	1.0	1.9	>13.1
11.5kDa	0.6	0	2.6	>13.1
11.5-15kDa	0.5	0	1.4	0
17.4 kDa	0.4	0	1.4	>13.1
18-35kDa	0.3	0	1.0	1.3
35- 45kDa	0.4	0	1.9	0
45- 84kDa	0.3	0	1.4	0
> 84kDa	0.4	0	2.3	0

One-half of a band or section of nitrocellulose was placed in each of triplicate wells as described in Methods and Materials. N/C= nitrocellulose. S2- and whole cell RB51 N/C= antigens spotted onto nitrocellulose pieces. SI= stimulation index.

[a]= Media cpm 2541.0± 1268.1. Concanavalin A SI= 18.7.

[b]= Media cpm 2036.7± 27.9. Concanavalin A SI= 31.3.

nitrocellulose pieces containing the 10.2kDa and 11.5kDa protein bands, these proteins were subjected to partial, N-terminal amino acid sequencing.

Microsequences of the 10.2kDa and 11.5kDa Proteins

The first 15 amino acids from the N-terminal end of the 10.2kDa protein were determined to be AFAKWWVTAMAWKKV. The sequence was not comparable to any in the NCBI protein database. Based upon the N-terminal amino acid sequences, several observations about the peptide were made. The protein is processed or cleaved during the extraction process, as the first amino acid is not a methionine. The deduced DNA sequence for this peptide is presented in Table 8. The N-terminal partial protein sequence of the 11.5kDa protein exhibited no homology to any other proteins in the NCBI protein database. These 15 amino acids of both proteins are primarily hydrophobic in nature.

Table 8. N-terminal amino acid (A.A.) sequences and deduced DNA sequences of the 10.2kDa and 11.5kDa proteins of the S2 extract.

Prot.	**N-terminal A.A. seq.**	**Deduced DNA Sequence**
10.2k Da	AFAKWWVTAMA-WKKV	GCXTTNGCXAAYTGGTGGGTXACXGCXATG GCXTGGAAYAAYGTX
11.5k Da	AQPMFIVLLYD-VVGV	GCXCAYCCXATGTTNATBGTXNTXNTXTAN GANGTXGTXGGXGTX

X= A,T,G, or C; N= T or C; Y= A or G; B= A, T, or C

DISCUSSION

The S2 extract was observed to induce lymphoproliferation and the production of T_{H1} cytokines by strain RB51 sensitized lymphocytes *in vitro*. In order to pinpoint the antigens responsible for the observed T_{H1} response to the S2 antigen, four methods for separating the individual antigens were attempted. Only methods based on SDS-PAGE with the concentration of the resolving gel increased to 15% were able to resolve the proteins. The extracted S2 proteins exhibit several similar characteristics. For instance, none of the proteins reacted with serum from RB51 vaccinated mice. As the Cu/Zn SOD was determined to be one of the isolated S2 proteins, the lack of a demonstrable humoral response found in this study is consistent with other reports (110). It was not possible to separate the proteins by methods involving separation by charge using the Rotofor and FPLC apparatuses to obtain fractions containing individual bands of sufficient quantity for testing. Separation of the proteins by molecular weight was difficult because of the similarity of their masses. However, their resolution was obtained by electrophoresis using 15% polyacrylamide gels.

Following dialysis of these fractions using 12-14 kDa mwco dialysis tubing, the 10.2 kDa and 11.5 kDa proteins remained in the preparation. The exact reason for this is unknown. However, there are many possibilities to explain this occurrence. In the case of the 11.5 kDa it may be retained by the tubing because it is so close in molecular weight to the 12kDa mwco of the tubing. In the case of the 10.2 kDa protein, it may actually be a subunit of a larger protein and therefore retained in the

tubing as it is of higher molecular weight and the 10.2 kDa band in the SDS-PAGE is present as the result of the denaturation of the larger protein. Alternatively, the 10.2 kDa protein may be a globular protein and therefore be unable to pass through the pores of the 12-14kDa mwco dialysis tubing.

In order to assess the CMI responses of sensitized and nonsensitized lymphocytes, individual proteins bound to nitrocellulose were presented to the macrophages for processing and presentation to the T cells in two different manners. The first involved the manufacture of nitrocellulose particles with the bound protein. Theoretically, the particles are easily phagocytosed by the macrophages, the bound proteins then processed and presented to the T cell populations in the wells (1). It was observed that the cpms between triplicates varied by greater than 20% by human lymphocytes to *M. leprae* protein nitrocellulose particles. In the study presented here, this method gives inconsistent results with ***Brucella*** proteins, including the responses to positive control antigens bound to the nitrocellulose.

Using the method of Brooks-Worrell *et. al.* combined with the protocol of Stevens *et. al.,* following a 5 day incubation of murine lymphocytes with antigen, cytokine responses to the antigens were obtained (16, 97). The proliferative responses were observed to have high degree of variation between triplicate wells and therefore did not yield significant differences among samples. One explanation for the variation may be the differences in the amounts of protein transferred to the nitrocellulose. The rationale for extending the incubation time in this assay to 5 days was to provide

ample time for the macrophages to effectively remove, process and present the proteins bound to the nitrocellulose and allow lymphocyte proliferation. Only the low molecular weight (10.2kDa, 11.5kDa, and 17.4kDa) antigens produced high levels of INF-γ (Table 7). Several authors have observed CMI activity in lower molecular weight proteins of *Brucella* antigens including animals vaccinated with RB51 (16, 94-97). Responses to the nitrocellulose from other portions of the S2 extract did not produce a response. Control mice did not respond to the bound antigens by either proliferation or detectable cytokine production, suggesting the responses are specific.

The identification of the *B. abortus* Cu/Zn SOD as the 17.4kDa protein suggests that this SOD may stimulate a T_{H1} response, although contamination with LPS may still be part of the observed immune responses *in vitro*. Tabatabai *et. al.* observed that a synthetic peptide based upon the Cu-Zn SOD amino acid sequence afforded mice a 2 log decrease in bacteria from virulent challenge (101). These data therefore suggest that epitopes of the *B. abortus* Cu-Zn SOD are capable of inducing an protective immune response.

The identities of the 10.2kDa and 11.5kDa antigens have not been reported in the literature thus far. Both proteins are hydrophobic within the first 15 amino acids from the N-terminus and both are processed proteins or cleaved during the extraction process as neither sequence begins with a methionine. The lymphocyte responses to the 10.2kDa and 11.5kDa antigens may be due to contamination with LPS, since the splenocyte population contains both T cells and B cells. *B. abortus* LPS has been

reported to act as a T-independent type 1 antigen in mice and is therefore capable of stimulating B cells in situations where few T cells are present (43). As a result, the use of *B. abortus* as a vaccine carrier for humans with immunodeficiency diseases is currently being explored (43). Both *B. abortus* rough and smooth LPS has been observed to elicit mitogenic responses by murine splenocyte cultures (76). In contrast, evidence in this study suggests that the splenocyte responses observed to the S2 extract were specific and the majority of the response was directed toward the protein components of the antigen. First of all, when the S2 antigen is digested by proteinase K, only a minimal proliferative response remains. Furthermore, the production of INF-γ is lost following proteinase K digestion of the S2 extract. These data would suggest that the major contribution of the response to the antigens is due to the proteins, not to contaminating LPS.

The putative DNA sequences for the 10.2kDa and 11.5kDa proteins reported here will be useful to find the genes encoding these two proteins (Table 8).

CHAPTER THREE

III. THE INTERACTION OF *BRUCELLA ABORTUS* LIPOPOLYSACCHARIDE WITH POLYMYXIN B

INTRODUCTION

In the Enterobacteriaceae, the lipopolysaccharide (LPS) structure consists of a lipid A moiety which is linked to the core oligosaccharide, which is in turn linked to the O-chain polysaccharide. The fatty acids of the lipid A moeity of the LPS are linked to the disaccharide backbone primarily by 3-hydroxy-myristic acid (67). In contrast, the *Brucella* lipid A moiety is composed primarily of acyloxyacyl residues (75). As a result of this difference in lipid A fatty acid composition, it was reported that *Brucella* LPS should not bind with polymyxin B (76). Polymyxin B is a compound produced by *Bacillus polymyxa* which is reported to bind the lipid A portion of Enterobacteriaceae LPS; it specifically binds to the lipid A linkage to the 2-keto-3-deoxyoctonate portion of the LPS (57).

Moreno, *et. al.* reported that polymyxin B does not bind *B. abortus* lipid A (76). Their study investigated the biological activity of both smooth and rough *B. abortus* LPS. *B. abortus* lipid A alone was observed to stimulate lymphocytes of C3H/HeJ mice, which are known to be resistant to the endotoxic effects of Enterobacteriaceae LPS. Furthermore, when the lipid A moiety was incubated with

polymyxin B and added to the C3H/HeJ lymphocytes, the activity could not be blocked. Additionally, treatment with polymyxin B did not block the ability of the *B. abortus* LPS to activate the complement cascade. In contrast, treatment with the polymyxin B was observed to eliminate or decrease the biological activities of *E. coli* LPS. Since the biological activity could not be eliminated, the authors concluded that the polymyxin B must not bind the *B. abortus* LPS. They felt that the unusual structure of the *Brucella* lipid A was the reason that it was able to stimulate the lymphocytes of the otherwise unresponsive C3H/HeJ mice.

RATIONALE AND HYPOTHESIS

Preliminary results with S2 antigen and intact cells of strain RB51 indirectly suggested that polymyxin B linked to agarose beads was capable of binding *B. abortus* LPS. As a result of the polymyxin B treatment, the biological activity of the LPS, to stimulate splenocyte proliferation should be inhibited. The experiments in this section were designed to test the ability of polymyxin B to inhibit LPS mitogenic stimulation of B cells.

METHODS AND MATERIALS

Polymyxin B Agarose Bead Treatment of S2 and 10% NaCl 4M Urea 0.001% β-ME Extract

S2 antigen and 10% NaCl, 4M urea, 0.001% β-ME extracts were prepared as described in a previous subchapter. Both extracts were resuspended at a concentration of 1mg dry weight/mL in sterile distilled water. The extracts were then incubated with washed polymyxin B agarose beads (Sigma Chemical CO., MO) at ratios (v/v) of 1:2, 1:5, and 1:10. Alternatively, the extracts were incubated with a similar volume of proteinase K-agarose beads to serve as negative control in the 2-keto-3-deoxyoctonate (KDO) assays. The extracts were incubated with the polymyxin or proteinase K beads for 1 hour at room temperature with shaking. The beads were pelleted by centrifugation (8060 X g for 3 min.) and the supernatants carefully removed. The supernatants were then tested for KDO content, reactivities in Western blot, proliferation of splenocytes by LTA, and production of cytokines.

2-keto-3-deoxyoctonate (KDO) Assay

KDO contents of the S2 and 10%NaCl, 4M urea, 0.001% β-ME strain RB51 extract were performed by the method of Karkhanis, *et.al.* (57). Briefly, 1ml of 0.2 N H_2SO_4 was added to 1 mg of each extract prepared as described above. A standard curve was prepared in the same manner using KDO concentrations of 0, 1, 3, and 9

μg. The preparations were boiled in a water bath for 30 minutes, allowed to cool, and centrifuged at 16,000 X g for 5 minutes. A 0.5 ml aliquot of the supernatants was removed from the tubes and used for the remainder of the assay. To these supernatants, 0.25ml of 0.04M HIO_4 in 0.125N H_2SO_4 was added, vortexed well, and incubated at room temperature for 20 minutes. Aliquots of 0.25 ml of 2.6% $NaAsO_2$ in 0.5N HCl were added, vortexed well and incubated at room temperature until the brown color was no longer visible. One half ml of thiobarbituric acid was added to the tubes, vortexed, and heated in a boiling water bath for 15 minutes. While the solutions were still hot, 1 ml dimethyl sulfoxide (DMSO) was added. The tubes were allowed to cool to room temperature and the optical density determined at 548nm. The KDO concentration of the samples was determined by comparing them to a standard curve obtained with known KDO concentrations.

RESULTS

The results of the biological activities of the S2 extract and the 10% NaCl, 4M urea, 0.001% β-ME extract are shown in Table 9. The amount of KDO decreased 73.5% following polymyxin B treatment of S2 extract, indicating that the beads were partially removing the LPS.

Additional evidence to suggest that the LPS is removed from the extract solutions was exemplified by immunoblot with a monoclonal antibody specific for *B.*

Table 9. KDO concentration and splenocyte responses of sensitized (strain RB51 vaccinated) or nonsensitized (control) mice to S2 antigen before or following treatment with polymyxin B agarose beads.

Vaccination	**strain RB51**	**strain RB51**	**control**	**control**
Antigen	S2 no treatment	S2 polymyxin B	S2 no treatment	S2 polymyxin B
LTA (SI)	22.6	20.8	7.6	1.5
INF-γ (ng/mL)	5.7	13.9	0	0
IL-2 (μg/mL)	3.0	2.7	0.45	0.36
IL-4 (μg/mL)	0	0	0	0
KDO concentration of sample (μg/mg)	3.4	0.9	3.4	0.9
Antigen treatment	10% NaCl extract no treatment	10% NaCl extract + polymyxin B	10% NaCl extract no treatment	10% NaCl extract + polymyxin B
LTA (SI)	20.8	9.6	10.7	2.2
INF-γ (ng/mL)	>13.1	>13.1	13.1	1.6
IL-2 (μg/mL)	ND	ND	ND	ND
IL-4 (μg/mL)	ND	ND	ND	ND
KDO concentration of sample (μg/mg)	34.4	5.1	34.4	5.1

Control splenocytes: media cpm 1143.5± 27.5. Concanavalin A SI= 117.5. Splenocytes from strain RB51 vaccinated mice: media cpm 1859.4± 226.8. Concanavalin A SI= 64.2. ND= not determined.

abortus rough LPS (Bru 48). When tested with the different preparations, reactivity of the monoclonal was only observed in the lanes containing the untreated extracts and the whole cell RB51 control (Fig. 26).

Regarding biological activity, treatment of S2 and the 10% NaCl, 4M urea, 0.001% β-ME extracts with polymyxin B beads decreased their abilities to stimulate of lymphocytes from both sensitized and nonsensitized mice. However, the SI of the sensitized splenocytes was still significantly above that of the nonsensitized (Table 9). The INF-γ levels produced by sensitized splenocytes to S2 treated with polymyxin B beads was 2 times higher than that of the untreated extract. The 10% NaCl, 4M urea, 0.001% β-ME extract is a very complex set of antigens and the amounts of INF-γ produced were all very high. Unfortunately, not enough of the supernatant remained to dilute and more precisely determine the amount INF-γ produced by the lymphocytes following polymyxin B treatment. However, in the control mice the amount of INF-γ produced was eliminated following polymyxin B treatment of the 10% NaCl, 4M urea, 0.001% β-ME extract.

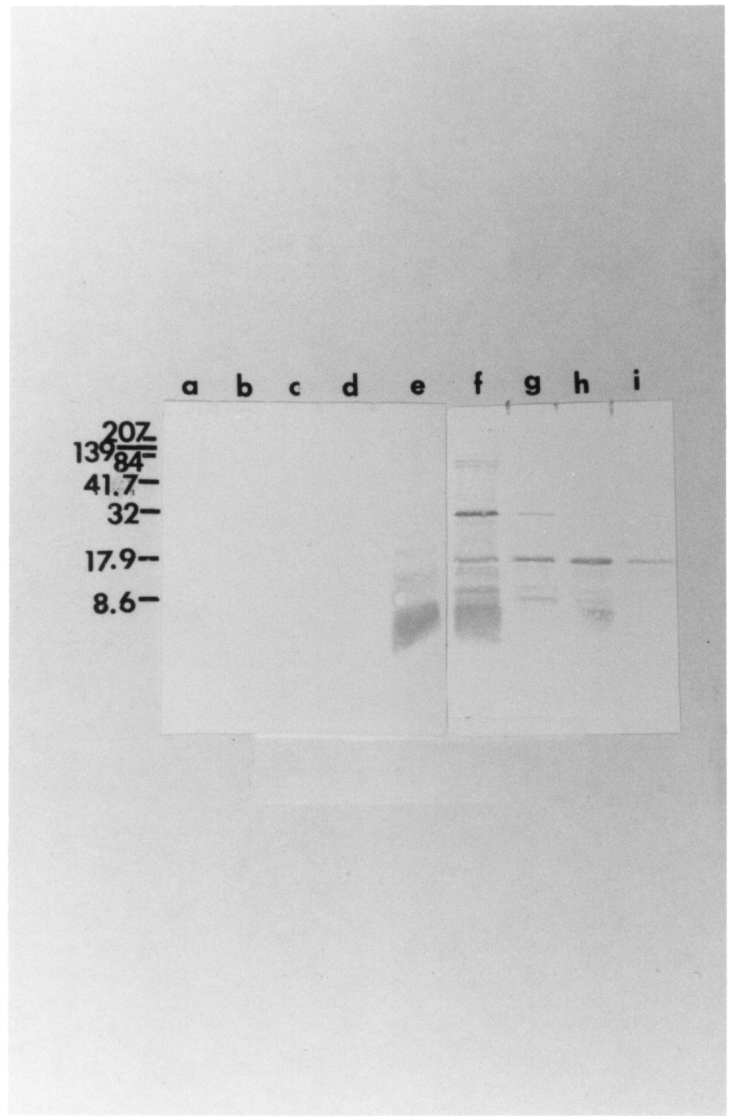


Fig. 25. SDS-PAGE separation of untreated and polymyxin B treated S2 and strain RB51 10% NaCl 4M urea 0.001% β-ME extract. Immunoreactivity with a monoclonal antibody (Bru 48) specific for *B. abortus* LPS to polymyxin B treated S2 (a= no treatment, b= 1:10 beads to extract, c= 1:5 beads to extract, d= 1:2 beads to extract, e= RB51 LPS), or Goat 48 serum to 10% NaCl 4M urea 0.001% β-ME strain RB51 extract (f= no treatment, g= 1:10 beads to extract, h= 1:5 beads to extract, i= 1;2 beads to extract). Molecular weight given in kDa.

DISCUSSION

The results presented here contradict the suggestion of Moreno, *et.al.* that polymyxin B does not bind *B. abortus* LPS (76). Although the lipid A composition is different from the Enterobacteriaceae, polymyxin B is still able to bind and remove a large portion of the contaminating *B. abortus* LPS from the antigen extracts, for example a decrease of 73.5% in the case of treated S2. The KDO assay was used as an indirect means of determining whether or not the amount of LPS following polymyxin B agarose treatment was decreased. The possibility that void volume or nonspecific binding to the agarose beads may be the cause for decreased KDO values was addressed by using similar amounts of proteinase K agarose beads as controls.

Immunoblots with both Goat 48 serum and the monoclonal antibody Bru 48 provided further evidence that the LPS was being removed from the extracts. The lack of reactivity of the treated S2 with the goat serum suggested that there was an optimal ratio of extract to polymyxin B beads for removal of the LPS. This observation could not be duplicated with the monoclonal antibody Bru 48. However, this may be due to the antigen specificity of the monoclonal antibody, for example it may not bind the lipid A portion of the rough LPS.

The splenocyte data provides strong evidence that the polymyxin B agarose did effectively remove stimulatory LPS from the extract. The loss of proliferative activity with the splenocytes from control mice is suggestive that there is a loss of *Brucella*

LPS mediated mitogenic activity for B cells (76). The increase in INF-γ production following polymyxin B treatment of the S2 extract could be explained in the following way: the elimination of the LPS (stimulation toward a T_{H2} response) allows for more of a T_{H1} response resulting from the protein portion of the extract (23). Moreover, treatment of the S2 fraction with proteinase K agarose eliminated the majority of the proliferative response and all of the INF-γ production by sensitized lymphocytes (Table 2). The remaining activity could primarily be attributed to the LPS left in the preparation, as most of the activity due to the protein component on the S2 antigen had been eliminated. These data confirm that the reactivity by the sensitized lymphocytes to the S2 fraction is primarily due to the proteins in the extract.

One reason that Moreno, *et. al.* concluded that the polymyxin B does not bind *B. abortus* LPS is because treatment with the polymyxin B did not inhibit the biological activity of the LPS, as indicated by the responses of splenocytes of C3H/HeAU mice (76). However, in their study the polymyxin B-LPS complex was not removed from the assay. In contrast with these experiments, the bound LPS was removed by centrifugation prior to testing in this study. These results suggest that the polymyxin B does bind to *B. abortus* LPS, but in relation to Moreno, *et. al.*, binding does not interfere with the stimulatory activity.

CONCLUSIONS

This work focused on a number of aspects of the interaction between *B. abortus* and the immune system of the host. The first portion of the work focused on the *Brucella* macrophage interaction. Using murine macrophage-like cell lines, various strains of *B. abortus* and *B. melitensis* were examined for their ability to replicate within the cell lines. Both the J774 and PU5-1.8 cell lines were found to support the intracellular growth and reproduction of *Brucella.* Although this has been reported in J774 cells, this was the first time that PU5-1.8 cells have been used as an infection model with *Brucella* spp. (52).

Based upon *Brucella* clearance studies from mice spleens, the J774 cell line more accurately reflected the clearance observed *in vivo.* However, in regard to ability to predict the degree of attenuation of vaccine candidates *in vitro,* the macrophage assay was unable to assess the degree of attenuation *in vivo.* Therefore, the *in vitro* model is not an accurate tool for assessing attenuation of *B. abortus* vaccine candidates.

During the course of the *in vitro* infection studies, it was noted that there was a consistent decrease in *Brucella* intracellular viability at 24 hours postinfection. The nature of this decrease was investigated and found to be due to the bactericidal activities of the macrophage, as the decrease at 24 hours postinfection did not occur with nonphagocytic Vero cells. The decrease in *Brucella* was not due to nutritional

shifts *in vitro* as there was no differences between strain 2308 grown in tissue culture media (DMEM) versus bacteriological media (TSA) at 24 hours postinfection.

In order to ascertain whether or not the decrease in intracellular *Brucella* at 24 hours postinfection in the macrophages was a selection process, *B. abortus* strain 2308 was grown intracellularly in PU5-1.8 cells. The harvested intracellular bacteria were then used to infect PU5-1.8 and P388D1 macrophage-like cell lines. Even though these bacteria had been subjected to the macrophage environment for 48 hours, the decrease in intracellular viability at 24 hours occurred similarly to that observed with TSA grown strain 2308. These data suggest that the survival of some of the *B. abortus* in the macrophage-like cell lines is not due to a selection process.

Electron microscopy of intracellular *B. abortus* strains 2308 and RB51 showed that both rough and smooth *B. abortus* are observed within vesicles and within the cytoplasm of the macrophage-like cell lines. To date, *Brucella* in macrophages have only been observed in vesicles (70). Alternatively, *Brucella* have been observed in the cytoplasm of murine trophoblast cells *in vivo* (105). The ability of the *Brucella* to localize in the cytoplasm of these murine macrophage-like cell lines raises the possibility of *Brucella* antigens being complexed with MHC class I, as well as MHC class II molecules (41, 74). Because of this distinct possibility, the role of $CD8^+$ T cells could be explored using the macrophage-like cell lines as antigen presenting cells (APC) to $CD8^+$ T cells from mice vaccinated with *Brucella.*

Antigen processing and presentation to T cells by macrophages and other

APC's is central to the host's ability to clear a *B. abortus* infection. Once the antigens have been processed and presented, the interactions between the macrophage and the T cell determine the resultant immune response by the host. A key in the quest to obtaining a new brucellosis vaccine is finding the antigen(s) which stimulate a protective immune response by the host. Such is the rationale for the partial characterization of the S2 antigens presented here.

Vaccination with live *B. abortus* strain RB51 is known to be protective in cattle and mice (24, 88). As strain RB51 is able to elicit a protective immune response, an antigen extract was prepared for further investigation. This antigen extract was observed to stimulate lymphoproliferation in splenocytes from mice vaccinated with strain RB51. Furthermore, these sensitized splenocytes additionally produced IL-2 and INF-γ, but not IL-4, in response to the S2 antigen. As CMI is important in clearing a *B. abortus* infection from a host, the cytokines specific for a T_{H1}, but not a T_{H2} response were observed (23). As a result of the combination of lymphoproliferation and cytokine profiles produced by sensitized splenocytes in response to the S2 antigen, the question arose as to whether the S2 antigens could provide protection against virulent challenge with strain 2308 in mice. Using the adjuvant DDA, mice were immunized with S2 in DDA with two additional boosters. Upon virulent challenge at 5 weeks post final immunization, the mice were not afforded any protection with the S2 antigens as compared with adjuvant only and untreated BALB/c mice. This study suggests that the whole, live bacteria may be

necessary in stimulating protective immunity against virulent *B. abortus* challenge in mice. The live bacteria may need to be localized specifically (either in the cytoplasm or in vesicles) within macrophages in order for the antigens to be processed and presented in such a way as to elicit a protective CMI (41). As this is an active process, it may be required that the S2 antigens be given to the mice using a live vaccine vector, such as vaccinia virus, before the role of protection for the S2 antigens can be accurately assessed.

In order to determine the specific proteins responsible for the observed immune responses of sensitized lymphocytes, the S2 proteins were separated. Use of the Rotofor and FPLC were observed to provide inconsistent results and yielded protein concentrations too low to work with effectively. Therefore, the S2 proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose, and tested for lymphoproliferative responses of strain RB51 sensitized and nonsensitized splenocytes. The lymphoproliferation in LTA was observed to vary greatly between triplicate wells. However, significant INF-γ production was observed in response to the 10.2 kDa, 11.5 kDa, and 17.4 kDa proteins. The 17.4 kDa was identified as the *B. abortus* Cu-Zn SOD. The N-terminal ends of the 10.2 kDa and 11.5 kDa proteins were sequenced; the identities of these two proteins could not be determined by comparing the amino acid sequences to the NCBI protein bank.

The role of LPS in the S2 preparation was also examined. Proteinase K treated S2 was observed to have a significantly decreased lymphoproliferative response

relative to untreated S2. This result suggested that the majority of the observed *in vitro* immune responses to S2 were due to the protein components. However, *B. abortus* proteins cannot be totally separated from contaminating LPS (76). It had been thought that polymyxin B is not able to bind *Brucella* LPS. In this study, polymyxin B linked to agarose beads was used to treat the S2 antigen extract, the bead-LPS complex removed from solution, and the supernatant tested in a variety of tests. Using a KDO test, the amount of KDO was decreased in S2 treated with polymyxin beads over S2 treated with proteinase K linked agarose beads as control.

In LTA, nonsensitized mouse splenocytes had decreased proliferation to polymyxin B treated S2. Strain RB51 sensitized splenocytes also had a decreased lymphoproliferative response to the polymyxin B treated S2 antigen. However, the response was still significantly above that observed with nonsensitized lymphocytes. The levels of cytokines also reflected this in that the INF-γ levels increased following polymyxin B treatment of S2 by strain RB51 sensitized controls. Additionally, monoclonal antibody Bru 48, specific for *B. abortus* rough LPS, was observed to react with untreated, but not polymyxin B treated, S2. Taken together, these data suggest that polymyxin B is capable of binding *B. abortus* LPS.

Currently, strain RB51 is considered to be a good candidate in the quest for a new *B. abortus* vaccine. These data have shown that strain RB51 is capable of localizing in the cytoplasm of murine macrophage-like cell lines, suggesting that RB51 antigens could be complexed with MHC class I molecules. An antigen extract from

RB51 (S2) is capable of stimulating a T_{H1}-type immune response *in vitro* as observed by lymphoproliferation, IL-2, and INF-γ production by strain RB51 sensitized splenocytes. However, S2 was not able to protect mice from virulent challenge with strain 2308 in the manner it was used to immune mice. Two proteins, a 10.2 kDa and 11.5 kDa, are capable of stimulating the production of INF-γ by strain RB51 sensitized lymphocytes. Polymyxin B was also observed to bind with strain RB51 LPS.

Future studies with the S2 antigens should focus on an alternate means of vaccinating mice. In this study, S2 antigens injected intraperitoneally were determined to not be protective in mice and yet the *in vitro* studies demonstrated that the S2 extract induced a T_{H1} type response by splenocytes of mice vaccinated with *B. abortus* strain RB51. It, therefore, may be that these antigens were not administered in a manner which allowed for a protective immune response to be induced. As the amino acid sequences of two of the proteins are known, it is possible to design labeled oligonucleotide primers based upon *Brucella* codon usage and locate the genes for the proteins in the *Brucella* genome. The genes could then be cloned into vaccinia virus (65A) or an attenuated *Salmonella typhimurium aroA* mutant (77A) and the live organisms used to express the proteins *in vivo*. In this manner, the antigen may be more effectively delivered to the host in order to evaluate their ability to induce a protective immune response.

REFERENCES

1. Abou-Zeid, C., E. Filley, J. Steele, and G.A.W. Rook. 1987. A simple new method for using antigens separated by polyacrylamide gel electrophoresis to stimulate lymphocytes in vitro after converting bands cut from Western blots into antigen-bearing particles. *J. Immunol. Meth.* 98: 5-10.

2. Adams, L. G. 1990. Development of live *Brucella* vaccines. In: Advances in Brucellosis Research. L.G. Adams, ed. Texas A & M University Press: College Station, TX. pp. 250-276.

3. Alton, G.G., L.M. Jones, R.D. Angus, and J.M. Verger. 1988. Techniques for the Brucellosis Laboratory: Institut National de la Recherche Agromique. pp. 34-60.

4. Andersen, P. 1994. Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted proteins. *Infect. Immun.* 62: 2536-2544.

5. Araya, L.N. and A.J. Winter. 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. *Infect. Immun.* 58: 254-256.

6. Araya, L.N., P.H. Elzer, G.E. Rowe, F.M. Enright, and A.J. Winter. 1989. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. *J. Immunol* 143: 3330-3337.

7. ATCC (American Type Culture Collection) catalog. 7th edition. 1992. pp. 291.

8. Bagchi, T. 1990. Immune mechanisms in murine brucellosis: Studies with strain RB51, a rough mutant of *Brucella abortus*. VA-MD Regional College of Veterinary Medicine, Blacksburg, VA. Accepted as doctoral dissertation, May, 1990.

9. Bang, B. 1906. Infectious abortion in cattle. *J Comp. Pathol.* 19: 191-202.

10. Barry, R.A., H.G.A. Bouwer, D.A. Portney, and D.J. Hinrichs. 1992. Pathogenicity and immunogenicity of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect. Immun.* 60: 1625-1632.

11. Berman, D.T. and R.S. Kurtz. 1987. Relationship of biological activities to structures of *Brucella abortus* endotoxin and LPS. *Ann. Inst. Pasteur/ Microbiol* 138: 98-101.

12. Betts, M., P. Beining, M. Brunswick, J. Inman, R.D. Angus, T. Hoffman, and B. Golding. Lipopolysaccharide from *Brucella abortus* behaves as a T-cell-independent type 1 carrier in murine antigen-specific antibody responses. *Infect. Immun* 61: 1722-1729.

13. Birmingham, J.R., and E.L. Jeska. 1981. Characterization of macrophage functions in mice infected with *Brucella abortus. Infect. Immun.* 32: 1079-1083.

14. Bosseray, N. 1983. Vaccine and serum-mediated protection against Brucella infection of mouse placenta. *Br J Exp Path* 64: 617-625.

14a. Bosseray, N., Plommet, M. and G. Dubray. 1978. Immunogenic activity of a cell wall fraction extract from *Brucella abortus* in guinea pigs. *Ann. Inst. Pasteur/Microbiol.* 129B: 571-579.

15. Bosseray, N. 1978. Immunity to *Brucella* in mice vaccinated with a fraction (F8) or a killed vaccine (H 38) with or without adjuvant. Level and duration of immunity in relation to dose of vaccine, recall injection and age of mice. *Br. J. Exp. Path.* 59: 354-365.

16. Brooks-Worrell, B.M., and G.A. Splitter. 1992. Antigens of *Brucella abortus* S19 immunodominant for bovine lymphocytes as identified by one- and two-dimensional cellular immunoblotting. *Infect. Immun.* 60: 2459-2464.

17. Brown, G.M., E.L. Love, D.E. Pietz, *et. al.* 1972. Characterization of *Brucella abortus* strain 19. *Am. J. Vet. Res.* 33: 759-764.

18. Buck, J.M. 1930. Studies of vaccination during calfhood to prevent bovine infectious abortion. *J. Agric. Res.* 41: 667-689.

19. Bundle, D.R., J.W. Cherwonogrodsky, M. Caroff, and M.B. Perry. 1987. The lipopolysaccharides of *Brucella abortus* and *B. melitensis. Ann. Inst. Pasteur/ Microbiol.* 138:92-98.

20. Canning, P. and J.A. Roth. 1989. Effects of in vitro and in vivo administration of recombinant bovine interferon-γ on bovine neutrophil responses to *Brucella abortus. Vet. Immunol. Immunopathol.* 20: 119-133.

21. Carmichael, L.E. and D.W. Bruner. 1968. Characteristics of newly recognized species of *Brucella* responsible for canine abortions. *Cornell Vet* 48: 579.

22. Caron, E., J.P. Liatard, and S. Köhler. 1994. Differentiated U937 cells exhibit increased bactericidal activity upon LPS activation and discriminate between virulent and avirulent *Listeria* and *Brucella* species. *J. Leuk. Biol.* 56: 174-181.

23. Cherwinski, H.M., J.H. Schumacher, K.D. Brown, and T.R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* 166: 1229.

24. Cheville, N.F., M.G. Stevens, A.E. Jensen, F.M. Tatum, and S.M. Halling. 1993. Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus*. *Am. J. of Vet. Res.* 54: 1591-1597.

25. Cloeckaert, A., I. Jacques, J.N. Limet, and G. Dubray. 1995. Host reponse to infection: immunogenic properties of *Brucella melitensis* cell-wall fractions in BALB/c mice. *J. Med. Microbiol.* 42: 200-208.

26. Corbel, M.J. 1987. Brucella phages: Advances in the development of a reliable phage typing system for smooth and nonsmooth isolates. *Ann. Inst. Pasteur/ Microbiol* 187: 70-75.

27. Corner, L.A., and G.G. Alton. 1981. Persistence of *Brucella abortus* strain 19 infection in cattle vaccinated with reduced doses. *Res. Vet. Sci.* 31: 342-344.

28. Cotton, W.E. and J.M. Buck. 1932. Further researches on Bang's disease. *J Amer Vet Med Assoc* 83: 342-355.

29. Dauer, C.C., R.F. Korns, and L.M. Schuman. 1968. Infectious and parasitic diseases. In: Infectious Diseases. Harvard University Press, Cambridge MA. pp. 21-24.

30. Detilleux, P.G., B.L. Deyoe, and N.F. Cheville. 1990. Entry and intracellular localization of *Brucella* spp. in vero cells: fluorescence and electron microscopy. *Vet. Pathol.* 27: 317-328.

31. Detilleux, P.G., B.L. Deyoe, and N.F. Cheville. 1990. Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro. *Infect. Immun.* 58: 2320-2328.

32. Dougan, G., L. Smith, and F. Heffron. 1989. Live bacterial vaccines and their application as carriers for foreign antigens. *Adv. Vet. Sci. Comp. Med.* 33: 271-300.

33. Dubray, G. and G. Bézard. 1980. Isolation of three *Brucella abortus* cell-wall antigens protective in murine experimental brucellosis. *Ann. Rech. Vét.* 11: 367-373.

33a. Dunne, M., B.K. al-Ramadi, S.W. Barthold, R.A. Flavell, and E. Fikrig. 1995. Oral vaccination with an attenuated *Salmonella typhimurium* strain expressing *Borrelia burgdorferi* Osp A prevents murine Lyme borreliosis. *Infect. Immun.* 63: 1611-1614.

34. Elzer, P.H., R.H. Jacobson, K.H. Nielsen, J.T. Douglas, and A. J. Winter. 1994. BALB/c mice infected with *Brucella abortus* express protracted polyclonal responses of both IgG2a and IgG3 isotypes. *Immunol. Letters* 42: 145-150.

35. Elzer, P.H., R.W. Phillips, M.E. Kovach, K.M. Peterson, and R.M. Roop II. 1994. Characterization and genetic complementation of a *Brucella abortus* high-temperature-requirement A (*htrA*) deletion mutant. *Infect. Immun.* 62: 4135-4139.

36. Fernandes, D.M. and C.L. Baldwin. 1995. Interleukin-10 downregulates protective immunity to *Brucella abortus. Infect. Immun.* 63: 1130-1133.

37. Fitzgeorge, R.B., M. Solotorovsky, and H. Smith. 1967. The behaviour of *Brucella abortus* within macrophages separated from the blood of normal and immune cattle by adherence to glass. *Br. J. Exp. Path.* 48: 522-528.

38. Fraser, C.M., ed. 1986. The Merck Veterinary Manual, 6th Edition. Merck & Co.: Rahway, NJ. pp. 639-645.

39. Freeman, B.A. and L.R. Vana.1957. Host-parasite relationships in brucellosis. I. Infection of normal guinea pig macrophages in tissue culture. *J Infect Dis* 102: 258-267.

40. Frenchick, P.J., R.J.F. Markham, and A.H. Cochrane. 1985. Inhibition of

phagosome-lysosme fusion in macrophages by soluble extracts of virulent *Brucella abortus*. *Am. J. Vet. Res.* 46: 332-335.

41. Germain, R.N. 1986. The ins and outs of antigen processing and presentation. *Nature* 322: 687-689.

42. Golding, B., S.P. Chang, H. Golding, R.E. Jones, K.L. Pratt, D.R. Burger, and M.B. Rittenberg. 1981. Human lymphocytes can generate thymus-independent as well as thymus-dependent anti-hapten plaque-forming cell responses in vitro. *J. Immunol.* 127: 220-224.

43. Goldstein, J., T. Hoffman, C. Frasch, E.F. Lizzio, P.R. Beining, D. Hochstein, Y.L. Lee, R.D. Angus, and B. Golding. 1992. Lipopolysaccharide (LPS) from *Brucella abortus* is less toxic than that from *Escherichia coli*, suggesting the possible use of *B. abortus* or LPS from *B. abortus* as a carrier in vaccines. *Infect. Imm.* 60: 1385-1389.

44. Graves, R.R. 1943. Story of John M. Buck's and Matilda's contribution to the cattle industry. *J Am. Vet. Med. Assoc.* 102: 193-195.

45. Halling, S.M., P.G. Detilleux, F.M. Tatum, B.A. Judge, and J.E. Mayfield. 1991. Deletion of the BCSP31 gene of *Brucella abortus* by replacement. *Infect. Immun.* 59: 3863-3868.

46. Harmon, B.G., L.G. Adams, and M. Frey. 1988. Survival of rough and smooth strains of *Brucella abortus* in bovine mammary gland macrophages. *Am. J. Vet. Res.* 49: 1092-1097.

47. Hinman, E.H. 1966. Eradication of animal diseases. In: World Eradication of Infectious Diseases. Charles C. Thomas, Publisher: Springfield, IL. pp.13.

48. Hoyer, B.H. and N.B. McCullough. 1968. Polynucleotide homologies of *Brucella* deoxyribonucleic acids. *J Bacteriol* 95: 444.

49. Hsieh, C.S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of T_H1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 260: 547-549.

50. Hsieh, C.S., A.B. Heimberger, J.S. Gold, A. O'Garra, and K.M. Murphy. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an $\alpha\beta$ T-cell-receptor transgenic system. *Proc. Natl. Acad. Sci.*

U.S.A. 89: 6065.

51. Jensen, A.E., N.F. Cheville, D.R. Ewalt, J.B. Payeur, and C.O. Thoen. 1995. Application of pulse-field gel electrophoresis for differntiation of vaccine strain RB51 from field isolates of *Brucella abortus* from cattle, bison, and elk. *Am. J. Vet. Res.* 56: 308-312.

52. Jiang, X. and C.L. Baldwin. 1993. Effects of cytokines on intracellular growth of *Brucella abortus*. *Infect. Immun.* 61: 124-134.

53. Jiang, X. and C.L. Baldwin. 1993. Iron augments macrophage-mediated killing of *Brucella abortus* alone and in conjunction with interferon-γ. *Cell. Immunol.* 148: 397-407.

54. Jiménez de Bagüés, M.P., P.H. Elzer, S.M. Jones, J.M. Blasco, F.M. Enright, G.G. Schurig, and A.J. Winter. 1994. Vacination with *Brucella abortus* rough mutant RB51 protects BALB/c mice against virulent strains of *Brucella abortus, Brucella melitensis, and Brucella ovis*. *Infect. Immun.* 62: 4990-4996.

55. Jones, L.M., V. Montgomery, and J.B. Wilson. 1965. Characteristics of carbon dioxide-independent cultures of *Brucella abortus* isolated from cattle vaccinated with strain 19. *J. Inf. Dis.* 115: 312-320.

56. Jones, S.M. and A.J. Winter. 1992. Survival of virulent and attenuated strains of *Brucella abortus* in normal and gamma interferon-activated murine peritoneal macrophages. *Infect. Immun.* 60: 3011-3014.

57. Karkhanis, Y.D., J.Y. Zeltner, J.J. Jackson, and D.J. Carlo. 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram- negative bacteria. *Anal. Biochem.* 85: 595-601.

58. Kaufmann, S.H.E. 1993. Immunity to intracellular bacteria. *Ann. Rev. Immunol.* 11: 129-163.

59. Kovacsovics-Bankowski, M. and K.L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267: 243-246.

59a. Lammeli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

60. Lin, J., L.G. Adams, and T.A. Fitch. 1992. Characterization of the heat shock response in *Brucella abortus* and isolation of the genes encoding the GroE heat shock proteins. *Infect. Immun.* 60: 2425-2431.

61. Lin, J. and T.A. Fitch. 1995. Protein synthesis in *Brucella abortus* induced during macrophage infection. *Infect. Immun.* 63: 1409-1414.

62. Lohoff, M., F. Sommer, W. Solbach, and M. Röllinghoff. 1989. Coexistence of antigen-specific T_H1 and T_H2 cells in genetically susceptible BALB/c mice infected with *Leishmania major*. *Immunobiol.* 179: 412-421.

63. Mackaness, G.B. 1964. The immunological basis of acquired cellular resistance. *J Exp Med* 120: 105-120.

64. Mackaness, G.B. 1954. The growth of tubercule bacilli in monocytes from normal and vaccinated rabbits. *Am. Rev. Tuber.* 69: 495-504.

65. Mackaness, G.B., N. Smith, and A.Q. Wells. 1954. The growth of intracellular tubercule bacilli in relation to their virulence. *Am. Rev. Tuber.* 69: 479-494.

65A. Mackett, M., T. Yilma, J.K. Rose. 1985. Vaccinia virus recombinants: expression of VSV genes and protective immunization of mice and cattle. *Science* 227: 433-435.

66. Madsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262: 10035-10038.

67. Martin, N.L. and R.E.W. Hancock. 1990. Function and structure of the major components of the outer membrane of gram-negative bacteria. In: Advances in Brucellosis Research. L.G. Adams, ed. Texas A & M University Press: College Station, TX. pp. 55-75.

68. Maximow, A.A. 1925. Tuberculosis of mammalian tissue in vitro. *J. Infect. Dis.* 34: 549-584.

69. McDonough, K.A., Y. Kress, and B.R. Bloom. 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. *Infect. Immun.* 61: 2763-2773.

70. Meador, V.P., B.L. Deyoe, and N.F. Cheville. 1989. Pathogenesis of *Brucella abortus* infection of the mammary gland and supramammary lymph node of the

goat. *Vet. Path.* 26: 357-368.

71. Merighi, A. and J.M. Polak. 1993. Post-embedding immunogold staining. In: Immunochemistry II. Cuello, C., ed. John Wiley & Sons: New York. pp. 238-264.

72. Meyer, M.E. and H.S. Cameron. 1968. Studies on the etiological agent of epididymitis in rams. *Amer J Vet Res* 64: 495.

73. Mingle, C.K., C.A. Manthei, and A.M. Jasmin. 1941. The stability of reduced virulence exhibited by *Brucella abortus* strain 19. *J. Am. Vet. Med. Assoc.* 78: 208-209.

74. Moore M.W., F.R. Carbone, and M.J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell.* 54: 777-785.

75. Moreno, E., D. Borowiak, and H. Mayer. 1987. *Brucella* lipopolysaccharides and polysaccharides. *Ann. Inst. Pasteur/ Microbiol.* 138: 102-105.

76. Moreno, E, D.T. Berman, and L.A. Boettcher. 1981. Biological activities of *Brucella abortus* lipopolysaccharides. *Infect. Immun.* 31: 362-370.

77. Nicoletti, P. 1980. The epidemiology of bovine brucellosis. *Adv Vet Science Comp Med* 24: 69-98.

77A. O'Callaghan,D., D. Maskell, F.Y. Liew, C.S.F. Eamson, abd G. Dougan. 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attenuation, persistence, and ability to induce protective immunity in BALB/c mice. *Infect. Immun.* 56: 419-423.

78. Oliveira, S.C., Y. Zhu, and G.A. Splitter. 1994. Recombinant L7/L12 ribosomal protein and γ-irradiated *Brucella abortus* induce a T-helper 1 subset response from murine $CD4^+$ T cells. *Immunology* 83: 659-664.

79. Plommet, M. and A.M. Plommet. 1983. Immune serum-mediated effects on brucellosis evolution in mice. *Infect Immun* 41: 97-105.

80. Plommet, M. 1987. Brucellosis and immunity: humoral and cellular components in mice. *Ann. Inst. Pasteur/ Microbiol.* 138: 105-110.

81. Portney, D.A., R.D. Schreiber, P. Connelly, and L.G. Tilney. 1989. Gamma interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. *J. Exp. Med.* 170: 2141-2146.

82. Prescott, J.F. and J.D. Baggot, eds. 1993. Polymyxins, glycopeptides, mupirocin, and bacitracin. In: Antimicrobial Therapy in Veterinary Medicine, 2nd ed., Iowa State University Press: Ames, Iowa. pp. 133-138.

83. Reynolds, E.S. 1963. The use of lead citrate at a high pH as an electron opaque stain in electron microscopy. *J. Cell. Biol.* 17: 208-212.

84. Rothbard, J.B. and W.R.Taylor. 1988. A sequence pattern common to T cell epitopes. *EMBO J* 7: 93-100.

85. Sangari, F.J., J.M. Garcia-Lobo, and J. Aguero. 1994. The *Brucella abortus* vaccine strain B19 carries a deletion in the erythritol catabolic genes. *FEMS Microbiol. Lett.* 121: 337-342.

86. Saunders, B.M., Z. Lui, Zhan, Y., and C. Cheers. 1993. Interleukin-6 production during chronic experimental infection. *Immunol. Cell. Biol.* 71: 275-280.

87. Schurig, G.G., C. Hammerberg, and B. R. Finkler. 1984. Monoclonal antibodies to *Brucella* surface antigens associated with the smooth lipopolysacchardie complex. *Am. J. Vet. Res.* 45: 967-971.

88. Schurig, G.G., R.M. Roop II, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan. 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. *Vet. Microbiol.* 28: 171-188.

89. Smith, H., A.E. Williams, J.H. Pearce, J. Keppie, P.W. Harris-Smith, R.B. Fitz-George, and K. Witt. 1962. Foetal erythritol: A cause of the localization of *Brucella abortus* in bovine contagious abortion. *Nature* 193: 47-49.

90. Snippe, H., M. Belder, and J.M.N. Willers. 1977. Dimethyldioctadecyl ammonium bromide as adjuvant for delayed hypersensitivity in mice. *Immunol.* 33: 931-936.

91. Sowa, B.A., K.A. Kelly, T.A. Fitch, and L.G. Adams. 1992. Virulence associated proteins of *Brucella abortus* identified by paired two-dimensional gel electrophoretic comparisons of virulent, vaccine and LPS deficient strains. *Appl. Theor. Electrophor.* 3: 33-40.

92. Splitter, G.A. and K.M. Everlith. 1989. *Brucella abortus* regulates bovine macrophage-T-cell interaction by major histocompatibility complex class II and interleukin-1 expression. *Infect Immun* 57: 1151-1157.

93. Stabel, T.J., Z. Sha, and J.E. Mayfield. 1994. Periplasmic location of *Brucella abortus* Cu-Zn superoxide dismutase. *Vet. Microbiol.* 38: 301-314.

94. Stevens, M.G., S.C. Olsen, and N.F. Cheville. 1995. Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or strain RB51. *Vet. Immunol. Immunopathol.* 44: 223-235.

95. Stevens, M.G., S.C. Olsen, G.W. Pugh, Jr., and D. Brees. 1995. Comparison of immune responses and resistance to brucellosis in mice vaccinated with *Brucella abortus* 19 or RB51. *Infect. Immun.* 63: 264-270.

96. Stevens, M.G., S.C. Olsen, and N.F. Cheville. 1994. Lymphocyte proliferation in response to immunodominant antigens of *Brucella abortus* 2308 and RB51 in strain 2308-infected cattle. *Infect. Immun.* 62: 4646-4649.

97. Stevens, M.G., S.C. Olsen, and G.W. Pugh, Jr. 1994. Lymphocyte proliferation in response to *Brucella abortus* 2308 or RB51 antigens in mice infected with strain 2308, RB51, or 19. *Infect. Immun.* 62: 4659-4663.

98. Stevens, M.G., G.W. Pugh, Jr., and L.B. Tabatabai. 1992. Effects of gamma interferon and indomethacin in preventing *Brucella abortus* infections in mice. *Infect. Immun.* 60: 4407-4409.

99. Suter, E. 1953. Multiplication of tubercule bacilli within mononuclear phagocytes in tissue culture derived from normal animals and animals vaccinated with BCG. *J. Exp. Med.* 97: 235-245.

100. Suter, E. 1952. The multiplication of tubercule bacilli within normal phagocytes in tissue culture. *J. Exp. Med.* 96: 137-150.

101. Tabatabai, L.B. and G.W. Pugh, Jr. 1994. Modulation of the immune responses in BALB/c mice vaccinated with *Brucella abortus* Cu-Zn superoxide dismutase synthetic peptide vaccine. *Vaccine* 12: 919-924.

102. Tabatabai, L.B., B.L. Deyoe, and J.M. Patterson. 1989. Immunogenicity of *Brucella abortus* salt extractable proteins. *Vet. Microbiol.* 20: 49-58.

103. Tatum, F.M., D.C. Morfitt, and S.M. Halling. 1993. Construction of a *Brucella abortus* RecA mutant and its survival in mice. *Microbial Pathogen.* 14: 177-185.

104. Tatum, F.M., N.F. Cheville, and D. Morfitt. 1994. Cloning, characterization and construction of *htrA* and *htrA*-like mutants of *Brucella abortus* and their survival in BALB/c mice. *Microbial Pathogen.* 17: 23-36.

105. Tobias, L. 1991. Pregnant mouse model of brucellosis: the pathology and protection studies comparing *Brucella abortus* strains 2308, 19, and RB51. VA-MD Regional College of Veterinary Medicine, Blacksburg, VA. Accepted as doctoral dissertation, May, 1991.

106. Tobias, L., G.G. Schurig, and D.O. Cordes. 1992. Comparative behavior of *Brucella abortus* strains 19 and RB51 in the pregnant mouse. *Res Vet Sci* 53: 179-183.

107. Tsolis, R.M., A.J. Bäumler, and F. Heffron. 1995. Role of *Salmonella typhimurium* Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages. *Infect. Immun.* 63: 1739-1744.

108. Winter, A.J., J.R. Duncan, C.G. Santisyeban, J.T. Douglas, and L.G. Adams. 1989. Capacity of passively administered antibody to prevent establishment of *Brucella abortus* infection in mice. *Infect. Immun.* 57: 3438-3444.

109. Winter, A.J., J.R. Duncan, C.G. Santisteban, J.T. Douglas, and L.G. Adams. 1989. Capacity of passively administered antibody to prevent establishment of *Brucella abortus* infection in mice. *Infect Immun* 57: 3438-3444.

110. Wright, P.F. and K.H. Nielsen. 1990. Current and future serological methods. In: Advances In Brucellosis Research. Adams, L.G., ed. Texas A & M University Press: College Station, TX. pp.305-320.

111. Young, E.J., M. Borchert, F.L. Kretzer, and D.M. Musher. 1985. Phagocytosis and killing of *Brucella* by human polymorphonuclear leukocytes. *J. Inf. Dis.* 151: 682-689.

112. Zhan, Y., A. Kelso, and C. Cheers. 1995. Differential activation of *Brucella*-reactive $CD4^+$ T cells by *Brucella* infection or immunization with antigenic extracts. *Infect. Immun.* 63: 969-975.

113. Zhan, Y. and C. Cheers. 1995. Differential induction of macrophage-derived cytokines by live and dead intracellular bacteria in vitro. *Infect. Immun.* 63: 720-723.

114. Zhan, Y. and C. Cheers. 1995. Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. *Infect. Immun.* 63: 1387-1390.

115. Zhan, Y., A. Kelso, and C. Cheers. 1993. Cytokine production in the murine response to brucella infection or immunization with antigenic extracts. *Immunology*. 80: 458-464.

116. Zhan, Y. and C. Cheers. 1993. Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. *Infect. Immun.* 61: 4899-4901.

117. Zhan, Y.F., E.R. Stanley, and C. Cheers. 1991. Prophylaxis or treatment of experimental brucellosis with interleukin-1. *Infect Immun* 59: 1790-1794.

118. Zhu, Y., S.C. Oliveira, and G.A. Splitter. 1993. Isolation of *Brucella abortus ssb* and *uvrA* genes from a genomic library by use of lymphocytes as probes. *Infect. Immun.* 61: 5339-5344.

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