IMMUNE MECHANISMS IN MURINE BRUCELLOSIS: STUDIES WITH STRAIN RB51, A ROUGH MUTANT OF *BRUCELLA ABORTUS*

by

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(ABSTRACT)

The roles of humoral and cell mediated immune responses in murine brucellosis were investigated in this study. B. abortus strain 19, the current vaccine strain, is known to induce an antibody as well as cell mediated immune responses, both of which protect mice against smooth strain 2308. B. abortus rough strain RB51 does not induce an O-side chain specific antibody response and yet protects mice against smooth strain 2308.

Passive transfer experiments using serum and nylon wool enriched T cells obtained from mice vaccinated with strain 19 and strain RB51 were carried out. Immune serum from strain 19 vaccinated mice protected against challenge with strain 2308 but not strain RB51. Nylon wool enriched T cells from strain 19 vaccinated mice protected recipient mice against challenge with both strains RB51 or 2308. Serum obtained from RB51 vaccinated mice did not protect recipient mice

against challenge with either strain RB51 or strain 2308. Nylon wool enriched T cells from the same vaccinated mice, however, protected mice against challenge with both strains.

Thioglycollate elicited mouse peritoneal macrophages could be activated by recombinant gamma-interferon to kill ingested B. abortus. This was true for both the rough strain RB51 and smooth strain 2308, although RB51 exhibited greater susceptibility to killing. Macrophages already invaded by either strain RB51 or strain 2308 retained their responsiveness to gamma-interferon activation and could kill either strain of B. abortus following activation by gamma-interferon. Results obtained in this investigation indicate that strain RB51 protects mice against strain 2308 by probably inducing a cell mediated immune response.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Brucellosis is a zoonotic disease affecting a wide range of animal species including cattle, dogs, goats, sheep and pigs. Manifestation of the disease may range from abortion to orchitis or epididymitis, depending on the infected host. The disease results in severe economic losses due to a reduced livestock and cattle production. Being a zoonotic disease, humans are frequently infected due to association with infected animals and in severe cases, may lead to the death of the individual. Brucellosis is caused by species of the genus Brucella and includes B. abortus, B. melitensis, B. suis, B. canis, B. ovis and B. neotomae. Like most other diseases, the response of the host's immune system to a Brucella infection results in the production of specific antibodies as well as development of cell mediated immunity (CMI). However, the precise contributions of either the antibody response or the cellular response toward eventual clearance of the bacteria remains inconclusive.

In both natural as well as experimental brucellosis, it is now well established that the host produces both IgM and IgG class of antibodies. While production of IgM antibodies constitutes the host's initial humoral response, eventually, antibodies of the IgG class are found to predominate (103). The humoral response of the host is directed against a variety of bacterial components which include both surface as well as non-surface antigens. These antigens include the lipopolysaccharide (LPS), protein antigens and a peptidoglycan linked

lipoprotein (30, 57, 121, 132, 139). The LPS appears to be the most dominant, and a humoral response to this molecule is used as an indication of infection or vaccination in agglutination and other serological tests (103). Although an antibody response to the above antigens have been documented, evidence of their role in protection has not been very conclusive. The most unequivocal results, have been obtained with antibodies to the O-side chain of the LPS. Several research groups have demonstrated the ability of anti O-side chain antibodies, both polyclonal and monoclonal, to provide some degree of protection against subsequent challenge with smooth virulent strains (83, 94).

Mackaness first demonstrated the importance of a cell mediated immune response for protection against intracellular pathogens (85, 86). This was later confirmed by others for various intracellular pathogens including Mycobacteria tuberculosis, Listeria monocytogenes and Salmonella typhimurium. Since the discovery of different T lymphocyte subpopulations by Cantor and Boyse (28, 29), considerable work has been done to demonstrate specific immune functions associated with each of these subpopulations. Broadly speaking, these subpopulations are T helper (Th) cells and cytotoxic T cells (CTLs).

T helper cells have been demonstrated to aid in the initiation of specific immune mechanisms particularly CMI and by providing help to B cells. These cells produce specific mediators called lymphokines which play an important role in specific immune induction. To be precise, they are known to produce interleukin 2 (IL-2), a T cell growth factor

and gamma-interferon (gamma-IFN), which activates macrophages enhancing their bactericidal activity. In addition, they are also known to produce other lymphokines like IL-4 and IL-5 that help in stimulating B cells to produce antibodies. More recently, however, the T helper subset has been further subdivided into Th1 and Th2 types based on their growth requirements as well as lymphokine secretion patterns (16, 55, 99, 112). It is now becoming apparent that Th1 cells which have been shown to produce gamma-IFN and IL-2, may have a role in the induction of delayed type hypersensitivity (DTH) and appear to play an important role in the host's defence against infectious agents. The Th2 subset which produces IL-4 and IL-5 on the other hand, is primarily responsible for providing help to B cells in mediating a successful, IgG mediated humoral response.

The other subpopulation of T cells, CTLs, have been primarily demonstrated to have cytotoxic effect, resulting in lysis of specific target cells recognized as 'non-self' either through a complex of antigen and self MHC class I molecule, or incompatible class I molecule. These target cells include virus infected normal host cells that exhibit viral antigens on their surface, macrophages presenting bacterial antigens, host cells that have undergone transformation to become tumor cells and in general, cells incompatible at the MHC locus (40, 77, 84, 134, 135, 147-149). Like Th1 cells, CTLs are also known to produce IL-2 and gamma-IFN which may aid in the induction of DTH reaction (22, 53, 58, 78).

The role of a cellular response in brucellosis has been explored

by several investigators in both humans and different animal models. These studies have included an analysis of how members of the genus Brucella interact with neutrophils and macrophages (25, 62, 63, 113, 115, 116). In addition, delayed type hypersensitivity (DTH) responses to Brucella antigens (68) as well as in vitro studies using lymphocyte transformation assays (6, 20, 70, 71, 110) have been carried out to demonstrate CMI in both cattle and mice. Furthermore, various research groups have demonstrated the ability of immune T cells to provide a certain level of protection to mice against subsequent challenge in adoptive transfer experiments (3, 4, 107).

The most common brucellosis vaccine in cattle utilizes B. abortus strain 19, which is a smooth, attenuated strain. Although it is attenuated, it can produce abortions when given in large doses at specific stages of pregnancy. Field trials have demonstrated that on an average, 65-70% of vaccinated cattle can resist challenge with virulent strain 2308 (103). The persistent problem with this vaccine is its ability to induce an O-side chain specific antibody response which cannot be serologically differentiated from infected cattle. Recent work from our laboratory has demonstrated the ability of a rough mutant of B. abortus to provide protection in murine brucellosis. When given intraperitoneally, strain RB51 is cleared rapidly from mice. In addition, mice vaccinated with strain RB51 do not produce anti-O-side chain antibodies and yet are protected against subsequent challenge with strain 2308 (23, 137).

In the light of the above information, the objectives of this

investigation were the following: To determine whether immune serum obtained in RB51 vaccinated mice would provide protection against a homologous RB51 or a heterologous smooth strain 2308 challenge. Being a rough strain essentially devoid of O-side chain, RB51 vaccination should not give rise to O-side chain specific antibodies and hence RB51 immune serum should not be able to protect against 2308 challenge. However, being devoid of the O-side chain, components on the surface of RB51 may be accessible to specific antibodies which might have a bearing on the survival of RB51.

In addition, it was also the aim of this investigation to determine whether immune T cells from RB51 vaccinated mice had the potential to protect mice against challenge with strain 2308. The fact that mice vaccinated with RB51 are protected against strain 2308 challenge suggests, that in the absence of O-specific antibodies and therefore serum mediated protection, the immune component responsible for protection could be T lymphocytes. Furthermore, because T lymphocytes are the effectors of enhanced macrophage bactericidal function, the ability of B. abortus to survive in gamma-IFN activated peritoneal macrophages was examined. The ability of gamma-IFN to activate macrophages already invaded by B. abortus was also examined to determine whether B. abortus could make macrophages refractory to the activation signals provided by gamma-IFN.

LITERATURE REVIEW

A diseased state in any animal can be perceived as a situation where the animal loses control over maintaining homeostasis. This could be due to causes that are both intrinsic and extrinsic. The former would include examples such as inherent genetic disorders leading to biochemical, physiological and other disorders or somatic mutations leading to malignancies, both of which are genetically dependent. External factors that cause imbalances in homeostasis are largely due to invaders that can take the form of both inert material or live forms such as bacteria, viruses and other organisms. The loss of control in this case is primarily due to the inability of the host's non-specific defenses and specific immune defenses to control the invasion by these external invaders. This is in spite of the fact that the target for these invasive organisms may not be components of the immune system per se.

Pathogenic organisms can be broadly classified into intracellular or extracellular pathogens, depending on whether they live and proliferate inside or outside of the host's cells. Extracellular pathogens are responsible for causing acute infections, while intracellular pathogens cause mostly systemic and chronic infections (47, 60). While members of Streptococcus, Hemophilus, Neisseria and Klebsiella, living outside of host cells are obligately extracellular, those living inside host cells may be either obligately intracellular (Rickettsiae tsutsugamushi) or facultatively intracellular (members of

Mycobacterium, Listeria, Brucella and Yersinia). It is now recognized that protection against extracellular pathogens is highly dependent upon phagocytes (neutrophils) and antibodies, complement, or a combination of these three. Protection against intracellular pathogens is dependent on activated macrophages, CTLs and to a certain extent depending on the parasite, on antibodies (140). Extracellular pathogens fail to survive in phagocytes and are better adapted to surviving outside these cells while many types of facultative or obligate intracellular pathogens seem to be well adapted for surviving inside phagocytic cells.

Quite appropriately therefore, virulence mechanisms employed by infectious organisms vary depending upon whether the organism has to combat host defence mechanisms prevailing outside or inside the eucaryotic cell. These include production of factors that help overcome anatomical barriers, release of toxins in the host and bacterial products that disrupt or avoid humoral factors including serum sensitivity. In the case of intracellular pathogens, virulence factors are primarily involved in avoiding or inactivating the various steps leading to intracellular killing. These include factors that interfere with accumulation of phagocytes at the site of infection, development of an oxidative burst and a normal functioning of lysosomes (47, 64, 91). One of these facultative intracellular pathogens, B. abortus, a causative organism of brucellosis, is the subject of this study.

BRUCELLOSIS

Brucellosis is a zoonotic disease affecting the human and several animal species. While in animals it is characterized by abortion, retained placenta, orchitis and epididymitis, in humans it causes undulant fever and may in some cases lead to the death of the host. The disease is caused by members of the genus Brucella and include B. abortus, B. melitensis, B. ovis, B. canis, B. suis and B. neotomae. Brucella organisms are small gram-negative coccobacilli which are 0.5 - 0.7 um wide and 0.5 - 1.5 um in length. They do not form spores or capsules and are non-motile or aflagellate (44). They are strict aerobes, having a respiratory metabolism although in some cases CO_2 is required for primary isolation.

Like other gram negative organisms, Brucella species have an LPS which is smooth in the presence of the O-side chain and rough in the absence of the O-side chain. The LPS of the smooth strains consists of lipid A, sugars including glucose, mannose, quinnovosamine and 2-keto-3-deoxyoctulosonic acid (KDO) constituting the core region, and an O-side chain. The O-side chain is a homopolymer of about 100 residues of N-formylated perosamine (4-formamido, 4,6-dideoxy D-mannose) (31). Depending on the species, two distinct epitopes are formed by the O-side chain, referred to as the 'A' and 'M'epitopes. The 'A' epitope is characteristic of B. abortus biovar 1 and consists of the N-formylated perosamine residues linked to each other by the C1 and C2 carbon atoms throughout the chain. The 'M' epitope present on B. melitensis biovar 1 also consists of N-formylated perosamine. In this latter case, the

O-side chain consists of repeating units of four 1,2-linked N-formylated perosamine residues and one 1,3- linked residue (24, 31). Although the exact structure of rough LPS is not known, it probably has a similar lipid A and core region, with the O-side chain either modified or absent (43).

HUMORAL IMMUNE RESPONSE

It is widely recognized that the predominant humoral immune response of a host to infection by B. abortus is against the LPS (139). Specifically, antibodies against the O-side chain of the LPS have been found consistently in animals infected with smooth B. abortus strains. In addition, there are reports indicating a humoral response against other antigens such as A1, A2, A3, (120, 121), outer membrane proteins (136), and salt extractable protein antigens (19, 132). Animals are also known to make antibodies to purified lipoprotein (57) and a purified 20 Kd protein which is now recognized as the Cu-Zn superoxide dismutase (12). Thus, although the O-side chain appears to be the antigen recognized by a large majority of the animals, they do show a humoral response to other antigens but in a less consistent manner. Reports in the literature indicate that while immune serum does not completely eliminate the bacteria, serum does confer some degree of protection. This however appears to be mainly mediated by antibodies to the O-side chain of the LPS (83, 94).

Various research groups have attempted to establish a protective role for immune serum in murine brucellosis. The immune serum used in

these investigations include polyclonal serum raised against specific Brucella fractions (9), vaccination with strain 19 or 2308 (4) as well as monoclonal antibody preparations against porins (94) and LPS (83, 94). An analysis of the results obtained by these groups indicate that only antibodies specific for the LPS of Brucella have the potential for protecting against challenge infection.

In their investigation, Bascoul et al (9) used three different phenol extracted fractions from B. melitensis for vaccinating mice. All three fractions namely 'PI', 'A4' and '5', contain proteins, lipoproteins, sugars, amino-sugars and trace nucleic acids, fractions 'A4' and '5' were depleted of 90% of the proteins contained in fraction 'PI'. In addition, all three fractions were known to contain peptidoglycan. Mice vaccinated with A4 had the best blood clearance on day 14 post vaccination. However, at 30 and 45 days post vaccination, mice immunized with 'PI' or '5' gave the best results. The effect of vaccination on splenic infection following challenge was also examined. It was observed that all three fractions were effective in reducing splenic infections. Fraction 'PI' was the best in regard to the extent of protection as well as sustaining this protective ability 45 days post vaccination. Their experiments with passive transfer of 'PI' immune serum demonstrated that blood clearance of the challenge Brucella was accelerated compared to controls but not as much as in actively immunized animals. Furthermore, passive transfer of fractionated immune serum demonstrated that humoral factors other than antibodies might also have a positive role in protection.

Montaraz and coworkers (94) examined the possibility of protecting mice against challenge with virulent B. abortus strain 2308, following passive transfer of specific monoclonal antibodies. Their experiments included transfer of monoclonal antibodies specific to porins as well as O-polysaccharide. They demonstrated that while the O-polysaccharide specific monoclonal antibody could transfer protection, the porin specific ones could not. In an effort to explain this difference they argued that the porin specific monoclonal antibody could not provide protection because of their inability to fix complement and this conclusion was supported by their data. In a similar experiment Limet et al (83) demonstrated that mouse monoclonal antibodies of isotype IgG1, IgG2a and IgG3 directed against the 'A' epitope of Brucella LPS could protect mice against challenge with B. abortus strain 544, a smooth virulent strain.

In a study by Araya and Winter (4) the effect of timing of passive transfer on the protective ability of immune serum was investigated. They demonstrated that mice receiving immune serum before challenge were protected better than when immune serum was received after challenge. In a separate study, Winter and coworkers (141) observed that when mice were challenged with opsonized Brucella organisms, splenic infection was dependent upon the level of challenge. In a majority of the animals, challenge with low numbers of bacteria resulted in prevention of infection. Higher challenge dose of opsonized bacteria resulted in higher spleen counts, although this was significantly lower than in mice that had received unopsonized

organisms.

CELLULAR IMMUNE RESPONSE

It is common knowledge that the chief effectors of the immune system include the macrophages, B lymphocytes and T lymphocytes. While macrophages play a central and multifunctional role, B cells are primarily associated with a humoral response and T cells are responsible for both cell mediated response (CMI) and providing help to B cells in humoral responses. A functional separation of different cell types has been possible on the basis of structural differences on their surfaces. While B cells have Ig molecules on their membrane surface, most mature T cells are known to have CD4 or CD8 markers, besides displaying the common T cell marker known as CD3. T lymphocytes bearing the CD4 marker (L3T4 in mice and T4 in humans), are referred to as helper T cells (Th) while CD8 bearing T cells (Lyt2 in mice and T8 in humans) are referred to as cytotoxic T lymphocytes (CTLs). Although overlaps are known to occur (17, 124), CD4 bearing T lymphocytes interact with antigen presenting cells in a MHC class II restricted manner, whereas CD8 T cells do so in a class I restricted manner. The Th cells are important in providing help to the immune system while the CTLs are responsible for cytotoxic activity against target cells including normal cells exhibiting viral antigens, macrophages presenting bacterial antigens and host cells that have become transformed or cells having altered class I antigen. Furthermore, CTLs are also known to secrete the Th1 pattern of

lymphokines such as IL-2 and gamma-IFN and therefore may also be responsible for a DTH reaction (22, 53, 58, 78).

Early evidence of cellular immunity to intracellular bacteria was obtained from studies on DTH (85). Development of DTH was observed with bacteria such as B. abortus, L. monocytogenes and M. tuberculosis. Mackaness (85) and coworkers had demonstrated that immunity to intracellular bacteria involved the cooperative action of two cell types; T lymphocytes acting as specific inducers and mononuclear phagocytes as non-specific effector cells. The function of the T cells was to recruit and subsequently activate mononuclear phagocytes for enhanced bactericidal activity. In the following years, the effectiveness of CMI in the elimination of intracellular pathogens has been recognized in many other systems. Both Th cells and CTLs have been shown to play a role in the elimination of pathogens such as L. monocytogenes, M. leprae, R. tsutsugamushi, and L. major (40, 45, 75, 79, 100, 123). However, in depth studies have been done only with a few organisms such as members of the genus Mycobacterium and L. monocytogenes; they are considered to be models of facultative intracellular pathogens as far as understanding the immune response to such organisms is concerned.

In recent years increasing evidence suggesting the involvement of the Th cells in functions associated with protective immunity has been obtained. Furthermore, results obtained from studies with cloned T cell lines demonstrate the existence of two distinguishable types of helper T cells, Th1 and Th2 (38, 50, 67, 96-98). Since most of these

studies were done by using *in vitro* cloning methods, the question still remained whether such differences were a manifestation of the different cloning conditions and techniques employed. Recent work done by Street et al (131) suggest that a commitment of the Th cells to either phenotype can occur *in vivo* as well. Using identical cloning conditions, they demonstrated that the frequency of either Th1 or Th2 type in cloned bulk cultures depended on the immune status of the donor animal. When mice were immunized with a *Brucella* antigen (used for *Brucella* ring test), the predominant type was Th1, while mice immunized with *Nippostrongylus brasiliensis* resulted in a predominantly Th2 type of clones.

While Th1 cells have been proposed to be necessary for immunity to intracellular pathogens, Th2 cells are believed to be required for an effective humoral immune response to extracellular pathogens (16, 99). Although both types of T cells bear the surface antigen CD3 (the invariant component of the T cell receptor complex) and CD4, it has been demonstrated that these two subsets have distinctive functions, lymphokine secretion profiles, growth requirements and activation pathways (38, 99). Th1 clones are known to secrete IL-2, gamma-IFN and lymphotoxin while Th2 clones secrete IL-4, IL-5, IL-6 and cytokine synthesis inhibitory factor. (50, 97, 98, 131). Lymphokines that are common to both these types of T cells include IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor (TNF) and a few others. Furthermore, investigators have also been able to correlate this difference in lymphokine secretion pattern with a

functional heterogeneity of the T cells (67, 96, 97).

Provision of help to B cells has been demonstrated in the case of both Th1 and Th2 clones (41, 56, 129). Recent work however (1) indicates that in the case of small resting B cells that have not been exposed to antigen, stimulation and proliferation can be induced by antigen and Th2 clones but not by antigen and Th1 clones. These results suggested that the failure of Th1 clones is not due to the inhibitory effect of gamma-IFN secreted by these cells but rather the lack of necessary stimulus for B cells. On the other hand, Th1 clones have been demonstrated to have the ability to help activated B cells, although to a lesser degree than Th2 clones. Another difference that has been observed in the helper function of these subsets in B cell response, lies in the phenomenon of isotype switching. While Th2 clones help induce IgE, IgA and IgG_1 responses, Th1 clones are known to have a preference for inducing IgG2a (129).

The relative contribution of each of the T cell subtypes towards DTH and other aspects of CMI have been examined by different groups. Using a T cell line specific for rickettsial antigen, Kodama et al (79) demonstrated the involvement of an $L3T4^+$ T cell subset in transferring protection. It was observed that the T cell line proliferated in an antigen specific and MHC restricted manner and produced gamma-IFN. The authors suggested that the gamma-IFN produced by these cells could activate macrophages, thereby resulting in the elimination of the bacteria. In other studies, it was demonstrated that Th1 clones and not Th2 have the ability to induce a DTH response.

This difference in a DTH response has been attributed to a difference in the pattern of gamma-IFN secretion (37, 66, 99). Along the same lines, investigations by Stout and Bottomly (130) revealed that only the gamma-IFN producing Th1 clones were able to activate macrophages in an antigen dependent manner, while the IL-4 producing Th2 clones are unable to do so.

Using protein blotting and T cell cloning, Mehra et al (89) and Lamb and Young (82) have established T cell clones with defined antigen specificity for different Mycobacteria species. Young and Lamb (144) were the first to transfer proteins onto nitrocellulose membranes and then use them in lymphocyte proliferation assays for identifying specific T cell epitopes. This technique avoided the use of monoclonal antibodies (which define B cell epitopes) for detection of potential T cell epitopes. Lamb and Young (82) were able to show specific T cell responses to SDS-PAGE separated proteins corresponding to various molecular weights. They demonstrated that while the M. tuberculosis specific clones proliferated in response to a soluble extract of M. tuberculosis, they did not do so in response to a non-specific M. bovis soluble antigen. Using a similar approach but slightly different technique, Mehra et al (89) identified different cell wall associated proteins of M. leprae that were able to stimulate specific T cell clones. Extending this approach to understanding human leishmaniasis, Melby & Sacks (88) were able to identify antigens that were specifically recognized by gamma-IFN producing $CD4^+$ human T lymphocyte clones.

More substantial evidence on the nature of protection and DTH was obtained from experiments involving adoptive transfer of specifically sensitized lymphocytes. With the help of cloned T cell lines, Kaufmann and Hahn (74) demonstrated that T cells of the helper type could proliferate in response to Listeria antigens in an MHC restricted fashion. Furthermore, they also demonstrated that these T cells could transfer both DTH and protection against live L. monocytogenes in vivo. Subsequent work by Kaufmann et al (76) demonstrated that in mice, protection against L. monocytogenes and DTH to listerial antigens depended on cooperation between Listeria specific $L3T4^+$ and $Lyt2^+$ T cells. Similar observations were made in the rat model by Chen-Woan and coworkers (36). They showed that DTH and acquired resistance to L. monocytogenes were mediated by different T cell subsets. While demonstrating cooperative interaction of the two subsets in resistance to L. monocytogenes, they showed that T cells responsible for acquired resistance failed to augment the expression of DTH but mediators of DTH enhanced the protective capacity of the other type. More recent reports (5) have demonstrated that in murine listeriosis, $CD4^+$ T cells are responsible for a DTH response while $CD8^+$ T cells mediate systemic anti-listerial immunity.

Parallel studies in the murine model by Czuprinski and Brown (45) showed that $Lyt2^+$ cells were responsible for anti-bacterial resistance while $L3T4^+$ T cells helped in the recruitment of inflammatory phagocytes. Recent studies by Lukacs and Kurlander (84) have extended earlier in vitro observations to an in vivo situation. The work of

Kaufmann and coworkers (73, 77) clearly demonstrated the ability of L. monocytogenes specific Lyt2$^+$ T cell lines to lyse Listeria infected macrophages in vitro. Lukacs and Kurlander have shown that when specific Lyt2$^+$ cells were adoptively transferred and the recipient challenged with L. monocytogenes, there was a decrease in the number of macrophages in the spleen of these infected animals. This result suggested that lysis of infected macrophages may lead to host resistance by eliminating a crucial niche for the bacteria.

In the case of R. tsutsugamushi, an obligate intracellular bacterium, resistance to infection could be transferred with immune T cells Rollwagen et al (117) have further shown that splenic lymphocytes obtained from mice infected with R. tsutsugamushi could specifically lyse target cells infected with the organism in vitro. Upon treatment of these lymphocytes with anti-Thy 1.2 and complement, this effect was eliminated, suggesting the involvement of T cells of the cytotoxic type. In the case of M. leprae, Chiplunkar et al (40) have demonstrated that T cell clones bearing the Lyt 2$^+$ phenotype do have the ability to lyse bone marrow derived macrophages rich in class I antigens and expressing M. leprae antigens. They therefore suggested that cytolysis of M. leprae infected macrophages could contribute towards protection against this organism.

Based on the information accumulated, activation of macrophage bactericidal function by helper T cells has been recognized as a major means of eliminating intracellular bacteria but, resistance against intracellular bacteria like M. leprae which escape the phagosome could

possibly depend on lysis of infected target cells.

There is an increasing consensus in assuming that resistance to B. abortus, as in other intracellular pathogens, requires a cell mediated immune response. Jones and Berman (68) examined DTH responses in guinea pigs vaccinated with either live or killed smooth strain 19 or rough strain 45/20 of B. abortus. They used a nucleoprotein rich brucellergen preparation for detecting DTH responses in a skin test on guinea pigs. It was observed that when animals were vaccinated with live organisms, the DTH response was similar for both strains 19 and 45/20. However, vaccination with heat killed preparations of either strain did not give a comparable response. In addition, when animals were vaccinated with killed bacterial preparations in the presence of adjuvant, the hypersensitivity reaction was larger and persisted longer than when adjuvant was not used.

The ability of B. abortus infected mice to resist infection with L. monocytogenes was examined by Halliburton and Hinsdill (61). Mice infected with B. abortus strain 19 could resist challenge with L. monocytogenes. This resistance, however, disappeared by 7 weeks post infection with B. abortus but could be recalled by subsequent injection with a killed B. abortus antigen preparation. The ability to recall was similar if mice were injected with antigenic preparations either from rough 45/20 or smooth 19 strains.

Cheers and coworkers (34) examined the pathogenicity and chronicity of Brucella infection in mice. They demonstrated that macrophages could be activated during the course of a Brucella

infection in mice. This was evidenced by the ability of Brucella infected mice to clear a subsequent L. monocytogenes infection. Therefore, they attributed the chronicity of brucellosis to the inability of macrophages to kill Brucella either because of reduced activation or replication of macrophage-resistant Brucella organism. Later they demonstrated (114) that macrophages from infected mice had certain inhibitory characteristics. Spleen cells from infected mice were shown to have a reduced response to mitogens and a Brucella antigenic preparation. This suppressive effect was abrogated, however, by removal of the adherent cells. The adherent cells from infected mice had the potential to suppress the proliferation of normal spleen cells as well, supposedly by a soluble factor. A similar situation was seen by Kaneene et al (72) in cattle. They observed that inclusion of indomethacin in their lymphocyte blastogenesis assays potentiated the response to B. abortus antigen in both normal and immune animals. The enhancement in immune lymphocytes was significantly greater than that in control lymphocytes. In addition, a Brucella specific significant enhancement was also observed in lymphocytes from anergic animals which had not shown a DTH response earlier.

The ability of murine immune T cells to provide protection against Brucella in adoptive transfer experiments has been demonstrated by a few groups. In brucellosis, the earliest indications of a T cell involvement was provided by Cheers and coworkers (107) in 1982. Spleen cells from infected and non-infected donor mice were treated in vitro with various lymphocyte specific antibodies and

complement. The resultant depleted population was transferred to recipient mice and challenged with B. abortus strain 19. It was shown that the ability to protect resided in lymphocytes which were Ly $1^{+}2^{+}$ T cells. In vivo depletion of specific T cell subsets led to some interesting observations. When anti-Ly-1 monoclonal antibody was injected on the day of Brucella infection, it had no effect on the bacterial numbers in the spleen. This treatment did, however, result in a complete suppression of IgG response. Injection of the antibody 5 days prior to Brucella infection resulted in an increase in the number of bacteria in the spleen but did not have any negative effect on a DTH response to Brucella antigen.

The most detailed and extensive analysis of CMI in brucellosis was not done until recently (3). Montaraz and Winter (93) demonstrated that immune T cells obtained from mice infected with strain 19 had the capacity to protect recipient mice against challenge with strain 2308. Since passive transfer of immune serum showed greater protection, it was argued that possibly at 1 week post challenge, protection was mainly due to an antibody response rather than a T cell mediated response. Recent work from Winter's group (3) demonstrated that both CD4 and CD8 subsets were important for protection. Although T cells developed the potential for protection only after 4 weeks of being exposed to B. abortus strain 19, immune serum from the same animal could transfer protection as early as 3 weeks. An interesting observation reported was the reduction in the number of CD4 and to a lesser degree, CD8 populations, at 2-4 weeks following infection with

Brucella. In mixing experiments, however, B cells depleted mononuclear cells obtained from this time period did not have any inhibitory effect on the protective capacity of immune T cells obtained from six week old donors.

Evidence of an involvement of bovine T cells has been presented by Splitter and Everlith (127). They demonstrated that monocytes were able to process and present Brucella antigens resulting in a T cell proliferation. This interaction was MHC restricted because antibody to MHC class II was able to block T cell proliferation. In an attempt to characterize the antigens that were recognized by immune T cells, they employed the technique of protein immunoblotting, which has been used for other pathogens (82, 88, 89, 144). It was shown that peripheral blood mononuclear cells could be specifically stimulated with pieces of nitrocellulose membrane containing proteins electroblotted on it following separation on SDS-PAGE. Proliferation of lymphocytes was observed in response to antigens corresponding to MW ranges of <25 kd, 25-45 kd and >45 kd. Using various combinations of these protein blot preparations, they established the specificity of these responses.

PHAGOCYTES

Once a microbe has breached the epithelial layer and is inside the host, its most important interaction is with the phagocyte. This encounter eventually decides the fate of the host and the pathogen. Broadly speaking, microbes susceptible to the bactericidal activity of phagocytes after ingestion are eliminated while those that resist,

persist for longer periods and cause disease. In other words, in order to cause disease, a microbe would have to avoid a direct encounter with the phagocyte or interfere with or prevent the functions of the phagocytes. Mechanisms such as inhibition of phagocytosis, resistance to killing by phagocytes or destruction of the phagocytes have been employed for eventual survival in the host.

The earliest step at which a microbe can alter its interaction with phagocytes is the mobilization of phagocytic cells. This phenomenon results in reduced sequestering of polymorphs and macrophages at the sites of infection. Bacteria which inhibit locomotion of polymorphs and macrophages include Staphylococcus aureus, and Clostridium perfringens. In order for a sensitive microorganism to avoid being killed by macrophages, it is important that phagocytosis is somehow prevented. This has been achieved by various bacteria in different ways. For example, the M protein of streptococci and the polysaccharide capsule of pneumococci are classic examples of antiphagocytic bacterial surface molecules. However, in the presence of antibodies to these factors, phagocytosis is achieved via the Fc receptor on phagocytes. Opsonization appears useful not only in the process of phagocytosis but also in the subsequent step of killing. For instance, in the absence of antibodies, Rickettsiae are phagocytosed and multiply inside macrophages. However, when infection proceeds and antibodies have formed, opsonized Rickettsiae are phagocytosed and killed.

The primary means of survival for an intracellular pathogen is in

its ability to be phagocytosed and then replicate in the phagocytes. The most common phagocyte used for this purpose is the macrophage. Pathogens such as M. tuberculosis, B. abortus, and L. monocytogenes are known to survive and replicate inside macrophages. It is quite obvious that survival of these organisms in the macrophages would depend on their ability to circumvent any of the steps that are eventually responsible for their elimination. M. tuberculosis is known to inhibit the fusion of phagosome and lysosome in infected macrophages. In their studies with a macrophage-like cell line, Chicurel et al (39) demonstrated the ability of M. tuberculosis derived proteins to modulate the lysosomal pH of macrophages. They suggested that the resulting alkalinization led to reduced hydrolytic activity of the lysosomal enzymes as well as inhibited phago-lysosomal fusion. Similar examples of inhibition of phago-lysosomal fusion are known in case of Toxoplasma gondii and Staphylococcus aureus. Escape from phagosome is another important mechanism for evading the killing activity of macrophages as demonstrated in various systems including M leprae, R. mooseri, Trypanosoma cruzi and L. monocytogenes.

Some pathogens like M. leprae can make macrophages unresponsive to the activation by gamma-interferon. Unlike normal macrophages, M. leprae infected macrophages would lose the ability to respond to activation by gamma-interferon. This was evident in their reduced microbocidal activity, reduced cytotoxicity to tumor target cells, reduced surface Ia antigen expression and O_2^- production, all of which are known to be enhanced by gamma-interferon treatment (125). Results

obtained in these studies indicated that this unresponsiveness was due to elevated levels of prostaglandin E_2 production by macrophages. It was also shown that treatment with indomethacin, a prostaglandin inhibitor, could alleviate this problem caused by M. leprae.

Investigators studying brucellosis have examined the interaction of members of the genus Brucella with polymorphonuclear leucocytes (PMNs) and macrophages. Reports indicate that while PMNs are able to ingest B. abortus organisms, they are unable to kill the bacteria. This was either due to a lack of stimulus for degranulation or an active inhibition of the degranulation process by the bacteria (80). This hypothesis has validity because granule extracts isolated from human PMNs have the ability to kill B. abortus organisms when exposed to these extracts in vitro (115). Similar effects of B. abortus on bovine PMNs was also observed by Lela et al (116). An analysis of the possible cause of this inhibitory effect has led to the finding that low molecular weight compounds produced by the bacteria are responsible for inhibiting phagosome-lysosome fusion (25, 54). These compounds were later identified to be GMP and adenine (26). Although PMNs have been shown to be relatively ineffective against B. abortus, their brucellacidal activity was enhanced following treatment with gamma-IFN (27).

In the mouse model, the chronic persistence of B. abortus infection has been attributed to the intracellular localization of the bacteria in the macrophages (34). This was suggested to be due to the macrophage bactericidal mechanism either being resistant or refractory

to activation. In ultrastructural analyses of the interaction between mouse peritoneal macrophages and B. melitensis, Filice et al (49) observed that phagolysosome fusion was affected in macrophages ingesting the bacteria. However, when the bacteria were treated with rifampicin prior to ingestion, there was no adverse effect on phagolysosome fusion. They therefore concluded that phagolysosome fusion is inhibited by B. melitensis and this inhibition is dependent on the active participation of the bacteria. Functions associated with macrophage activation were examined by Birmingham and Jeska (14) in murine brucellosis. They compared macrophage functions including spreading, Fc receptor expression, C3 receptor expression and chemotactic responses in uninfected and infected mice. It was observed that macrophages from infected mice showed enhanced spreading and an increase in Fc receptor and C3 receptor mediated phagocytosis. In addition, serum lysozyme, an indicator of macrophage activity, was higher in infected than in normal mice.

In cattle, the mammary gland is considered to be one of the sites of infection in chronic brucellosis. As a result, an understanding of how B. abortus interacts with mammary gland macrophages is very essential. In an in vitro study, Harmon et al (63) examined the relative ability of rough and smooth strains of B. abortus to survive in mammary gland macrophages. They observed that opsonization of both the rough and smooth strains was necessary for phagocytosis. Opsonized bacteria were able to induce an oxidative burst which was significantly higher than that for unopsonized bacteria. Furthermore,

the smooth strain 2308 was a better stimulator than the rough strain 45/20. However, examination of the intracellular survival of the two strains revealed that strain 45/20 survived better than the smooth strain. Extending these studies, Harmon et al (62) compared the functions of mammary gland macrophages from cows either resistant or susceptible to infection with B. abortus. While they did not find any differences in Fc receptor expression or lysosomal enzymatic activity between the two groups, differences in their oxidative burst capacities were evident. Macrophages from resistant cows produced higher oxidative burst activity compared to susceptible cows when infected with opsonized strain 2308. In addition, macrophages from resistant cows were also shown to have greater bactericidal activity against strain 2308 than did macrophages from susceptible cows.

The importance of CMI for the control of intracellular bacteria has been well documented. It has also been recognized that the effector mechanism of this protective role is an enhanced bactericidal activity of macrophages which eventually kill the ingested bacteria. This macrophage activating factor has been identified by Nathan et al (102) to be gamma-IFN. Detailed analysis of the effect of macrophage activation by gamma-IFN has demonstrated that enhanced antimicrobial activity of these macrophages is due to an increased oxidative burst resulting in the production of increased reactive oxygen intermediates. Macrophage activation by gamma-IFN has also shown to result in increased expression of MHC class II antigen, C3 component of complement and tumor necrosis factor (33). There is also evidence

indicating that activation of macrophages by gamma-IFN may result in enhanced oxygen independent mechanisms (51, 101) including increased phagosome lysosome fusion (69).

Organisms that are known to be susceptible to gamma-IFN activated macrophage include S. typhimurium (69), M. bovis and some strains of M. tuberculosis (51, 52), Coccidioidis immitis (11) and L. tropica (46, 105). There are reports, however, of instances where macrophage activation by gamma-IFN has not led to an increased killing of ingested organisms. One such example is M. leprae, where macrophage invasion by the organism results in an unresponsiveness to gamma-IFN activation as described earlier (125). In the case of M. tuberculosis, it has been shown that sulfatides obtained from the outer surface of the organism were able to block gamma-IFN mediated macrophage activation (106).

A possible role for IFN in brucellosis was documented in chickens by Youngner et al (145) in 1964 and in mice by Billiau et al (13) in 1970. Billiau et al demonstrated that when mice were injected intravenously with B. abortus smooth strain 19, there was increased IFN levels in the animals serum. Although the type of interferon was not characterized as gamma-IFN, they substantiated the IFN activity by associating this with an increase in resistance to vaccinia virus both in vivo and in vitro.

More recently, the effect of gamma-IFN on the interaction of bovine neutrophils with B. abortus has been examined by Canning and Roth (27). Neutrophils were shown to be activated by gamma-IFN even in

the presence of B. abortus. This was demonstrated by an increase in production of O_2^- as well as an enhanced myeloperoxidase-H_2O_2-halide activity. Although addition of GMP and adenine was inhibitory, there was significant difference between normal and gamma-IFN activated neutrophils. It was also shown that the survival of opsonized bacteria was reduced by about 10% of the control. The authors thereby concluded that gamma-IFN mediated activation of neutrophils resulted in a slightly enhanced ability to kill virulent B. abortus.

An interesting observation was made by Splitter and Everlith (126) in the bovine system with regard to the effect of gamma-IFN on macrophage-T cell interactions. They observed that in the presence of gamma-IFN and B. abortus smooth strain 2308, bovine monocytes were unable to induce a successful T cell proliferative response. It is well recognized that the expression of class II molecule as well as IL-1 are essential for a successful T cell response. Since both gamma-IFN and the smooth strain of B. abortus were shown not to reduce the expression of the class II molecule, they concluded that the down regulation of T cell response was not due to class II molecule expression. They observed that macrophages treated with gamma-IFN and B. abortus had reduced membrane IL-1 expression compared to macrophages treated with B. abortus or gamma-IFN alone. It was also shown that this negative effect on membrane IL-1 expression was restricted to gamma-IFN and O-polysaccharide since it could not be produced by alpha-IFN or rough strain 45/20 which has a reduced amount of O-polysaccharide. It was their contention that a combination of

gamma-IFN and O-polysaccharide of B. abortus LPS at a particular ratio and at an inappropriate time during the immune response resulted in down regulation of T cell proliferation which could not be restored by the addition of exogenous IL-1.

MATERIALS AND METHODS

MAINTENANCE AND GROWTH OF BACTERIA

All experiments involving use of live B. abortus organisms were carried out in the BL3 facility of the College of Veterinary Medicine. B. abortus strains used in this study were maintained and cultured on plates made from Trypticase soy broth supplemented with 1.5% agar (TSBA). B. abortus strain 2308 used as the challenge strain was obtained from Dr. A. J. Winter, NY State College of Veterinary Medicine, Ithaca, NY. The culture was grown on TSBA plates and frozen in skim milk. Counts of viable bacteria in the aliquots were assessed according to the method of Miles and Misra (90). Aliquots of frozen stock were thawed and used as required. B. abortus strain RB51 and other B. abortus strains used in this investigation were from stock cultures maintained at 4°C on TSBA slants. Prior to use, cultures were streaked on TSBA plates and incubated at 37°C for 48 hr in an atmosphere containing 5% CO_2 and processed according to requirements of particular experiments.

ANIMALS

Four to six week old female BALB/c mice were used. They were purchased from Dominion Labs, Dublin, Va or Charles River Labs, Wilmington, MA. Animals were held in the animal holding facility of the Veterinary Microbiology Research Laboratories and given food and water ad libitum. Infected carcasses were autoclaved at 121°C for 60

minutes prior to disposal.

INFECTIONS AND IMMUNIZATIONS

Cultures grown on TSBA plates were harvested in sterile 0.85% NaCl (saline) solution and resuspended to 10% transmittance at 525 nm (Bausch and Lomb Spectronic 20 spectrophotometer). Following this, ten-fold serial dilutions were made and viable counts were obtained for each dilution following the method of Miles and Misra (90). For strain RB51, each animal received approximately $1x10^8$ organisms intraperitoneally (i.p.) while strain 19 was given at approximately $5x10^4$ (i.p) per mouse. A frozen aliquot of strain 2308 was thawed and diluted in saline solution and injected at approximately $5x10^4$ organisms i.p. per mouse.

Mice were immunized with live strain 19 or strain RB51 bacteria with the numbers mentioned above. Animals used as a source of immune serum and immune cells were immunized in one of the following two ways: i) Two injections i.p. were given at 4 week intervals. Animals were bled intraorbitally at 2 weeks following second injection and serum was obtained from the blood samples. Four weeks after the second injection, mice were euthanized with CO_2 and spleens were processed for lymphocyte isolation. Non-immunized animals were processed in a similar way for use as controls. ii) Animal were injected once as mentioned above. Spleens were obtained from these animals 6 weeks post-injection and processed for lymphocyte isolation.

CELL CULTURE EXPERIMENTS

All cell culture experiments were done using medium consisting of the following and will be referred to as RPMI medium unless specified otherwise. RPMI 1640 was supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin, 50 ug/ml streptomycin, 10 mM Hepes, 2mM glutamine and non-essential amino acids. All incubations were done at 37^{o}C in a humidified atmosphere containing 5% CO_2 unless otherwise specified. Viability of cells was determined by the trypan blue exclusion method as described by Mishel and Shiigi (92).

PERITONEAL MACROPHAGES

Naive mice were injected with 1.5 ml of thioglycollate broth 72 hr prior to harvesting peritoneal macrophages. Mice were euthanized with CO_2 and peritoneal cavities were lavaged three times with 2 ml each of Ca^{++} and Mg^{++} free Hanks balanced salt solution (HBSS). The lavaged material was pooled and centrifuged at 300 x g for 10 min. The cell pellet was washed twice with RPMI medium and resuspended in the same. An aliquot of this suspension was taken to assess viability and estimate cell numbers. Resuspended peritoneal cells were plated at $2x10^5$ viable cells in 1 ml per well of a 24 well plate. Plates were incubated for 2 hr following which non-adherent cells were removed after gently flushing with RPMI medium with a 1 ml Eppendorf pipette. The wells were replenished with 1 ml of RPMI medium and plates incubated for another 24 hr before being used in bactericidal assays.

GROWTH OF MACROPHAGE LIKE CELL LINE

P388D1, a macrophage like cell line was obtained commercially (ATCC, Rockville, Maryland). Stocks of these were maintained under liquid nitrogen in a medium containing RPMI mediun supplemented with 5% dimethyl sulfoxide. Prior to use, a vial was thawed at 37^{o}C and immediately resuspended in 10 ml RPMI medium. The suspension was centrifuged at 300 x g for 10 min and used as follows. For routine maintenance, cells were grown in 260 ml Nunc tissue culture flasks in RPMI medium. They were seeded at 5x10^{5} per ml in 30 ml medium and incubated at 37^{o}C. To subculture, floater cells were centrifuged at 300 x g for 10 min and seeded in a new flask. For bactericidal experiments, cells were added into 24 well plates at 2x10^{5} cells in 1 ml per well and incubated until further use.

ELECTRON MICROSCOPY OF PHAGOCYTOSIS

The following monoclonal antibodies were used for opsonization of bacteria: i) BRU 48, a mouse monoclonal antibody for strain RB51 and ii) BRU 38, a rat monoclonal antibody for strains 19 and 2308. Bacteria were opsonized for 30 min at room temperature with sub-agglutinating concentrations of antibodies before adding to macrophage cultures.

All phagocytosis steps were done in the presence of RPMI medium lacking antibiotics. P388D1 cells plated in 24 well plates were given opsonized bacteria at a macrophage to bacteria ratio of 1:500. Opsonization was done for 30 min after which phagocytosis was allowed

for 2 hr. Following this, the medium was removed and the wells washed with RPMI medium three times. The cells were then fixed in glutaraldehyde, washed three times in 0.1 M cacodylate buffer (pH 7.5) and post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 hr at room temperature. Following this treatment, the sample was washed with 0.1 M cacodylate buffer and dehydrated with a series of cold ethanol rinses. Samples were treated with propylene oxide and embedded in Poly/Bed 812. Ultrathin sections of 60-90 nm thickness were stained with uranyl acetate and lead citrate and examined with a JEM 100 CX II electron microscope at the EM facility of the College of Veterinary Medicine.

MACROPHAGE BACTERICIDAL ASSAY

Peritoneal macrophages or P388D1 cells were always maintained in RPMI medium lacking antibiotics prior to bactericidal assays. Macrophages were given opsonized bacteria as mentioned above and phagocytosis was allowed to proceed for 30 min. Following this, medium in the wells was replaced with RPMI medium (containing antibiotics) and plates incubated for 30 min. This allowed for killing extracellular organisms in the wells which was initially examined in experiments with P388D1 and B. abortus cells. The antibiotic containing media was then removed and wells washed once by gentle rinsing with antibiotic free RPMI medium and this marked the '0' hr point of the assay. Wells were replenished with 1 ml antibiotic free media and incubated for various time points. Macrophages were lysed by

treating with distilled water for 5 min and aliquots of ten-fold serial dilutions of the lysate were spotted on TSBA plates. Colony forming units (cfu) were counted after 72 hr incubation at 37^{o}C.

CHLOROQUINE

A stock of chloroquine (0.2 M) (Sigma Chemicals, St Louis, MO) was made in sterile distilled water and stored at 4^{o}C. When required, macrophages were treated with RPMI medium containing 0.1 mM chloroquine in order to inhibit phagolysosome fusion. Macrophages were exposed to chloroquine 30 min prior to phagocytosis.

GAMMA-IFN

Recombinant murine gamma-IFN was obtained from Genzyme Corporation, Boston, MA and maintained at -80^{o}C. Prior to use, dilutions were made in RPMI medium lacking antibiotics to a final concentration of 50 U/ml of gamma-IFN. When required, wells were replenished with 1 ml of this preparation.

ISOLATION OF SPLENIC LYMPHOCYTES

Spleens obtained from control or immunized mice were placed in a 60 mm tissue culture dish containing RPMI medium. Splenic cells were obtained by teasing the spleen through a metal sieve (Sigma chemicals) of 50-mesh size. The suspension was then centrifuged at 300 x g for 10 min. Red blood cells were lysed by suspending the cell pellet in lysis buffer (1 ml for each spleen). Lysis buffer included TRIS buffered

ammonium chloride solution containing 90 ml of ammonium chloride and 10 ml of 0.17 M Tris-HCl pH 7.65 with the final pH adjusted to 7.2 with HCl. Lysis was achieved by incubating the cell suspension in a 37^{o}C water bath for 1 min. Spleen cells were then washed three times with RPMI medium and used as described for either mitomycin C treatment or nylon wool enrichment of T lymphocytes.

MITOMYCIN C TREATMENT

Splenic lymphocytes were treated with mitomycin C for use as antigen presenting cells in lymphocyte proliferation assays. Mitomycin C (Sigma Chemicals) was added to a final concentration of 25 ug/ml in RPMI medium and the cell suspension was incubated in a 37^{o}C water bath for 20 min. Following this, mitomycin C treated cells were washed five times with RPMI medium and held at 4^{o}C for further use.

NYLON WOOL ENRICHMENT OF T LYMPHOCYTES

Nylon wool obtained from DuPont (Westwood, MA) was teased apart to remove kinks and knots before packing into columns. Two and four-tenth gms of nylon wool was packed in a 60 ml syringe. The syringe was then filled with distilled water and the contents autoclaved for 15 min at 121^{o}C. The columns were used to obtain enriched T lymphocytes from the splenic lymphocyte population. The column was washed with 5 volumes of RPMI medium and equilibrated at 37^{o}C at least 1 hr prior to use. Three ml of spleen cell suspension at 1x10^{8}/ml were used for each column. The initial 3 ml were allowed to enter the column and the

column overlayed with 3 ml of RPMI medium and held in the incubator at 37^{o}C. After 20 min of incubation, the column was removed from the incubator, all media was allowed to pass through and the column was overlayed again with 3 ml of fresh warm RPMI medium. This was repeated twice and the column was finally washed with four bed volumes of warm RPMI medium. The eluted cells were centrifuged at 300 x g for 10 min and washed once with RPMI medium, then held at 4^{o}C for further use either in lymphocyte transformation assays or adoptive transfer experiments.

INDIRECT IMMUNOFLUORESCENT STAINING

Nylon wool enriched T cells obtained from control, non-immunized mice were quantitated by indirect immunofluorescent staining. Staining was performed for enumeration of lymphocytes expressing the L3T4 and Lyt2 surface antigens. Nylon wool enriched T cells were suspended in phosphate buffered saline (pH 7.2) supplemented with 5% fetal calf serum (PBS-FCS) and adjusted to $2x10^{7}$ per ml. One hundred microliters of this was centrifuged for 10 seconds in a microcentrifuge and the pellet was treated with 100 ul of antibody preparations specific for L3T4 or Lyt2 surface antigens. The anti-L3T4 antibody was obtained as direct culture supernatant of clone 53-6.72 (ATCC, Rockville, Maryland) grown in RPMI medium and the anti-Lyt2 antibody was obtained as direct culture supernatant of clone GK1.5 (ATCC, Rockville, Maryland) grown in RPMI medium. The culture supernatants of the above mentioned clones were a kind gift of Dr. P. K. Srivastava of Mount

Sinai Medical School, New York, NY. The cells were incubated on ice for 20 min with undiluted culture supernatants and then washed three times with PBS-FCS. Following this, they were suspended in 100 ul of a 1:100 dilution of FITC conjugated anti-rat IgG (Boehringer Mannheim, Indianapolis, IN) in PBS-FCS, and incubated on ice for 20 min. The cells were then washed three times in PBS-FCS, resuspended in 200 ul of PBS-FCS and analysed on a Coulter Epics V 752 flow cytometer at the Flow Cytometer Laboratory of the College of Veterinary Medicine.

IN VITRO ACTIVATION OF LYMPHOCYTES

In vitro activation of lymphocytes in the presence of various cytokines and T cell stimulating agents was carried out as described by Chen et al (35). Antigen used for in vitro activation and lymphocyte transformation assays was prepared as follows. Strain RB51 grown on TSBA plates was harvested in saline solution. The cells were washed once with saline solution and then resuspended in distilled water. The suspension was adjusted to 10% transmittance at 525 nm (Bausch and Lomb Spectronic 20 spectrophotometer) and autoclaved for 20 min at 121^{o}C. Nylon wool enriched T cells (1x10^{6}/ml) and mitomycin C treated splenic cells (5x10^{5}/ml) were incubated in RPMI medium with 15 ul/ml antigen, 20 u/ml IL-2, and 25 u/ml recombinant murine gamma-IFN. After 72 hr, the culture was centrifuged at 300 x g for 10 min. The cell pellet was resuspended in 4.5 ml RPMI medium and layered over 5 ml FICOLL (Histopaque 1083, Sigma Chemicals, St Louis, MO) and centrifuged at 1200 x g for 10 min at room temperature. The interface

containing live cells was collected and washed 3 times with RPMI medium. The cells were resuspended in RPMI medium in the presence of 20 U/ml IL-2, 25 U/ml gamma-IFN, 1 ng/ml phorbol myristic acetate (PMA) and 0.125 ug/ml ionomycin and incubated for a further 96 hr. Following incubation, they were harvested, washed twice with RPMI medium, resuspended in HBSS and used for adoptive transfer experiments.

LYMPHOCYTE PROLIFERATION ASSAYS

Proliferation assays were all done in triplicates in round bottom 96 well microculture plates (Nunclon, Gibco, Grand Islands, NY). Each well contained a total of 200 ul of medium. This included cells suspended to a concentration of $2x10^5$ in 100 ul of RPMI medium and an additional 100 ul of either medium control, or medium containing mitogen, antigen, PMA, Ionomycin, or a combination of PMA and Ionomycin. When required, recombinant murine IL-2 (Genzyme, MA) was added to a final concentration of 20 U/ml and gamma-IFN to a final concentration of 25 U/ml. Cultures were incubated for 72 hr and thereafter pulsed with 1 uCi of tritiated thymidine (methyl-^{3}H-thymidine, NEN) in 10 ul of RPMI medium and incubated for an additional 16 hr. Contents of each well were then harvested with an automatic cell harvester onto glass fiber filters. Individual filter disks were placed in vials with 2.5 ml of Ecoscint (National diagnostics, Manville, NJ) scintillation cocktail. The vials were then counted for 1 min in a liquid scintillation counter (Beckman LS 8100)

and the counts per minute (cpm) of each sample which reflected the incorporation of ^{3}H thymidine was obtained. The mean and standard deviation of the triplicate samples was then calculated.

ADOPTIVE TRANSFER EXPERIMENTS

Nylon wool enriched T lymphocytes were resuspended in HBSS at $3x10^{8}$/ml and 0.1 ml of the suspension was injected per mouse using the tail vein route. Specific experimental designs are described in the appropriate chapters. In vitro activated T cells were resuspended in HBSS at $4x10^{7}$/ml and 0.1 ml of the suspension was injected per mouse using the tail vein route.

WESTERN BLOT ANALYSIS

ANTIGEN PREPARATION

1) Whole cells (WC)

Bacteria grown on TSBA plates for 48 hr were harvested in distilled water and centrifuged at 12,000 x g for 20 min at 4°C. Bacteria were killed by resuspending the pellet in acetone (5 ml, for every plate harvested) and stirring the suspension for 3 hr at room temperature. The cells were then centrifuged at 12,000 x g for 10 min and the pellet resuspended in 10 mM Tris-HCl pH 8.0 at 8 ml buffer for every 10 plates harvested. One mg lysozyme was added to each 8 ml of the suspension and stirred overnight at room temperature. The lysozyme treated suspension was then centrifuged at 12,000 x g for 10 min and aliquots of the supernatant were frozen at -80°C until use. Prior to

SDS-PAGE analysis, an aliquot was thawed and an equal volume of 2x SDS buffer was added, the contents boiled for 5 min, centrifuged for 1 min in a microfuge and the supernatant used at 15 ul per lane.

2) Yersinia enterocolitica LPS

LPS from Y. enterocolitica serotypes O:8 and O:9 were isolated by modifications of procedures described by Moreno et al (95). Organisms were grown on TSBA plates as above and harvested in sterile distilled water using 2 ml for every plate harvested. The harvested cells were killed at 60°C for 30 min in a shaking water bath. The heat killed suspension was centrifuged at 10,000 x g for 10 min at 4°C. The pellet was resuspended in a mixture containing 55 parts of sterile distilled water and 45 parts of 90% phenol in distilled water. The contents were then agitated in a 68°C water bath for 40 min and centrifuged as above for separating the phenol and aqueous phases. The phenol phase was saved and the aqueous phase was further extracted by addition of 45 ml phenol, mixed and centrifuged as above. This was repeated three times following which the pooled phenol fractions were washed 10 times in an equal volume of warm (66°C) distilled water. LPS from the phenol fraction was precipitated using 5 volumes of cold methanol reagent (99 parts methanol, 1 part methanol saturated with sodium acetate) and stirred for 1 hr at 4°C. The precipitate was collected by centrifugation, dissolved in distilled water and dialyzed overnight with several changes of distilled water. The dialyzed material was then lyophilized and held until further use.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

One volume of sample was dissolved in an equal volume of 2X SDS-sample buffer. The SDS-sample buffer contained 0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue as the tracking dye. Electrophoresis was performed following the method described by Laemmli (81) with a 2.5% stacking gel and 12.5% resolving gel. Molecular weight standards (Pharmacia) consisting of phosphorylase b (m.w. 94,000), albumin (m.w. 67,000), ovalbumin (m.w. 43,000), carbonic anhydrase (m.w. 30,000), trypsin inhibitor (m.w. 20,100), and alpha-lactalbumin (m.w. 14,000) were also run alongside. Electrophoresis was performed at 25 milliamps until the tracking dye reached about 1 cm from the bottom of the gel. Following completion of electrophoresis, the gels were used for electroblotting onto nitrocellulose membranes.

WESTERN BLOTS

Antigens separated by PAGE were electroblotted onto nitrocellulose membrane in the presence of transfer buffer using a TE Series Transphor Electrophoresis Unit (Hoeffer Scientific Instruments). Transfer buffer consisted of 25 mM Tris, 192 mM glycine and 20% methanol. Prior to electroblotting, the gel was washed once in transfer buffer for 20 min. Nitrocellulose membrane and filter paper (Whatman #1) used for electroblotting were wetted with transfer buffer prior to use. The gel was sandwiched with a piece of nitrocellulose membrane in between 2 pieces of filter paper. This "sandwich" was then

placed in between two layers of Scotch Brite pads and the entire assembly was encased in a cassette. The cassette was immersed in transfer buffer contained in the electroblotting unit with the gel towards the cathode and the membrane towards the anode. Transfer was performed at 125 volts for 2 hr. Following transfer, the membrane was cut into strips corresponding to the gel lanes and processed for ELISA with specific serum samples or monoclonal antibody preparations. Membrane strips containing molecular weight standards were stained immediately for 5 min in Ponceau S stain, and then destained in distilled water. Antigen containing membrane strips were incubated for 60 min in blocking buffer consisting of 2% BSA in Tris-HCl (pH 7.2) buffered saline (TBS). The strips were then incubated overnight in a 1:100 dilution of antibody preparation in TBS. They were washed in TBS-Tween (TBST) 3 times, 10 min each and further incubated for 60 min in the appropriate peroxidase conjugated secondary antibody. Secondary antibodies used in this investigation included goat anti-mouse IgG (1:800) and goat anti-rat IgG (1:500). Following incubation in secondary antibody, the strips were washed thrice, 10 min each, in TBST and developed in 100 ml TBS, 10 ml methanol, 60 mg 4-chloro-1-naphthol and 0.6 ml 30% hydrogen peroxide. The reaction was stopped by immersing the strips in distilled water.

BIO DOT (TITER) ANALYSIS

Analysis of serum titers was performed using a BioDot chamber (Bio-Rad). A piece of nitrocellulose membrane was prewetted in TBS and

was placed in the BioDot chamber which was closed and sealed tightly. Strain RB51 organisms were suspended in distilled water and autoclaved for 20 min at 121^{o}C. The autoclaved material was resuspended in TBS, to 20% transmittance at 525 nm (Bausch and Lomb Spectronic 20 spectrophotometer). Thirty ul of RB51 antigen was added to each well and allowed to incubate at room temperature for 30 min before applying vacuum to the chamber. The vacuum was released and blocking solution containing 2% BSA was added to each well and incubated for 30 min. The blocking solution was drained with the help of vacuum and the wells were washed with TBST. After washing 3 times, serial dilutions of serum in TBS were added to the wells and incubated for 30 min. The wells were washed 5 times with TBST and dried under vacuum. Appropriate secondary antibody (as mentioned for Western blots) was added to each well, incubated for 30 min, washed five times with TBST and wells dried under vacuum. The nitrocellulose membrane was removed and developed in the same way as described for Western blots. The membrane was allowed to air dry in the dark and titers scored on the basis of coloring intensity of the spots developed on the membrane. Titers were scored visually and the dilution of negative serum showing no reaction was used as the cut off point. Coloring intensity stronger than the cut off point was scored as positive.

SERUM AGGLUTINATION TEST

B. abortus strain 19 diagnostic antigen used in this test was obtained from U.S.D.A. The antigen was diluted 1:100 in 0.5% phenolized saline. A 1:25 dilution of antisera was made for each sample and two fold dilutions in 0.5% phenolized saline carried out to a given end point. The tubes were then incubated at 37°C for at least 24 hr prior to reading.

COLONY BLOT

The colony blot ELISA method reported by Roop and coworkers (118) was followed. Briefly, B. abortus colonies were grown on TSBA plates and blotted onto nitrocellulose membrane disks. The disks were then submerged in 25 ml chloroform for 10 min in a desiccator to lyse the bacteria. Following this, they were removed and air dried for 15 min under an activated charcoal hood. The disks were then treated with agitation in blocking-digestion buffer for 45 min. Blocking-digestion buffer consisted of 0.02 M Tris-HCl pH 7.5, 0.005 M magnesium chloride, 0.15 M NaCl, 2 ug/ml DNase I, 80 ug/ml lysozyme and 3% bovine serum albumin. Disks were washed in TBST, incubated with monoclonal antibody preparations for 1 hr at room temperature. They were then washed again in TBST and incubated in the appropriate peroxidase conjugated secondary antibody for 1 hr. After washing in TBST, the disks were developed as mentioned for Western blots.

SERUM ABSORPTION

Immune serum obtained from RB51 vaccinated mice was absorbed with viable RB51 organisms. RB51 organisms grown on TSBA plates were harvested in sterile saline and brought to 1% transmittance at 525 nm (Bausch and Lomb Spectronic 20 spectrophotometer). One ml of this suspension was centrifuged in a Fisher Scientific Micro-Centrifuge for 2 min at room temperature and the supernatant discarded. The bacterial pellet was then resuspended in 1 ml of serum diluted 1:6 in saline, and mixed by rotating on a gyratory shaker for 1 hr. The samples were centrifuged at room temperature for 2 min as above and the supernatant saved at -20^{o}C until further use in Western blot analysis or passive transfer experiments.

STATISTICS

Mice experiments were performed twice. For mice experiments in chapters 1 and 2, groups of 5 mice were included for each treatment. Mean values for the control group were calculated. Log protection was determined as the mean of log differences in principal mice from the control value. Differences in the mean of different principal groups and control were tested by Students 't' test and data from chapter 2, experiment 5 was also tested by one-way analysis of variance following methods described by Zar (146).

Macrophage experiments in chapter 3 were done at least twice. Each treatment in every experiment included duplicate wells, and three samples were drawn from each well. Values given are the mean values

calculated from replicate wells of a single experiment. Standard deviation within replicates of an experiment are reported.

CHAPTER I

ROLE OF ANTIBODY RESPONSE IN MURINE BRUCELLOSIS

RATIONALE

An immune response to an invading pathogen usually results in the production of specific antibodies to the pathogen. These antibodies may be directed to antigens which are exposed on the surface of the pathogen as well as intracellular antigens. While the latter class of antibodies may not prove to have any protective function, antibodies raised against surface antigens do have the potential of being protective. In brucellosis, an immune response depends on the nature of the strain infecting the host. Infection by the smooth type of this organism results in an antibody response predominantly to the O-side chain of the LPS molecule (139) although antibodies to other antigens can also be detected (120, 121, 136). The rough form of this organism has been shown to elicit antibodies to antigens distinct from the O-side chain which can be part of the LPS molecule as well as other non-LPS antigens (23).

Several attempts have been made to demonstrate the role of these antibodies in providing protection to the host in the face of infection with Brucella (4, 9, 83, 94). These studies have been carried out primarily in the murine system and have employed both polyclonal and monoclonal antibody preparations. In these studies it has been repeatedly demonstrated that antibodies to the O-side chain of the LPS have the ability to give some degree of protection to the

host against challenge with the smooth strain (4, 83, 94).

Mice vaccinated with B. abortus rough strain RB51 are protected against challenge infection with either rough strain RB51 or smooth strain 2308 (23). It has been demonstrated that mice vaccinated with rough strain RB51 produce antibodies specific to antigens present in both rough and smooth strains of B. abortus (23). However, these animals do not make antibodies specific to the O-side chain of Brucella LPS. In an attempt to understand the immune mechanisms underlying the protective ability of strain RB51, experiments were carried out to determine if recipient mice could be protected against challenge infection with either strain RB51 or strain 2308, following passive transfer of strain RB51 immune serum.

EXPERIMENTAL DESIGN

Immune serum was obtained from mice vaccinated twice with either strain RB51 or strain 19 organisms. In these experiments, 4-6 week old BALB/c mice were vaccinated i.p. with viable $1x10^8$ strain RB51 or $5x10^4$ strain 19 organisms. Mice were vaccinated twice at 4 week intervals and 2 weeks following the second injection, serum was obtained which was then used for passive transfer to recipient mice.

Experiment 1 involved determination of titers of sera obtained from control mice and mice vaccinated with strain RB51 or strain 19. Titers were determined against RB51 WC antigen by the BioDot method, and strain 19 antigen, by serum agglutination test.

Experiment 2 was performed to determine the antigenic specificity

of serum obtained from mice vaccinated with strain RB51 or strain 19. The antigens tested were WC antigen obtained from strain RB51 and strain 2308 as well as LPS preparations from Y. enterocolitica serotypes O:8 and O:9. Antigens were separated by SDS-PAGE and transferred onto nitrocellulose membranes by Western blot. The membranes were then reacted with 1:100 dilution of the different serum samples and developed as described in Materials and Methods. In order to test for the presence of antibodies against surface antigens, immune serum obtained from strain RB51 vaccinated mice was examined by Western blot analysis following absorption with RB51 organisms. Serum was absorbed with viable strain RB51 organisms as described in Materials and Methods and tested against RB51 WC antigen as described above.

In experiment 3, serum obtained from vaccinated and control non-immunized mice was diluted 1:6 in sterile saline. The serum was then heat inactivated at 56°C for 30 min, filtered through 0.2 u filters and held at -20°C until injected into mice. Absorbed RB51 immune serum described under experiment 2 was also used in this experiment. Experiments using strain 19 immune serum and absorbed RB51 immune serum were performed twice, and those using unabsorbed RB51 immune serum were performed four times. Five mice per group were injected i.p. with 0.5 ml of the above serum preparations and challenged with either $1x10^8$ strain RB51 or $5x10^4$ strain 2308 2 hr thereafter. One week following challenge, spleens were cultured and the number of organisms per spleen determined.

RESULTS

The antibody titers of serum obtained from mice vaccinated with strain RB51 or 19 are given in Table 1. Serum obtained from mice vaccinated twice with strain RB51 did not react in the serum agglutination test with strain 19 antigen. When tested against strain RB51 WC antigen in the BioDot system, this serum demonstrated a titer of 1:1600. Mice vaccinated twice with strain 19 developed a reaction to RB51 WC antigen with a titer of 1:400 in the BioDot test and a tube agglutination test titer of 1:400.

Figure 1 depicts the results obtained in the Western blot analysis of the above serum samples. Immune serum obtained from RB51 vaccinated mice reacted with antigens present in RB51 WC and 2308 WC, but not with LPS from Y. enterocolitica serotypes O:8 and O:9 (panel A). It reacted with antigens in the core region of the LPS in the molecular weight (m.w.) range of 14.4 - 25 kd in addition to several antigens in the m.w. range of 45 - 94 Kd. Immune serum from strain 19 vaccinated mice reacted strongly with WC from strain 2308 and LPS from Y. enterocolitica serotype O:9 but not with LPS from Y. enterocolitica serotype O:8 (panel B). It also reacted weakly with RB51 WC antigen in the m.w. range of 67 - 94 Kd. Panel C shows the reactivity of RB51 immune serum before and after absorption with viable RB51 organisms. Absorption resulted in loss of reactivity to the "core" region of the LPS while retaining activity towards other antigens.

Results of passive transfer experiments are given in Figure 2, the data for which is presented in the appendix. Immune serum obtained

CHAPTER 1 - EXPERIMENT 1

Table 1.

TITERS OF SERA OBTAINED FROM STRAIN RB51 AND STRAIN 19 VACCINATED MICE

Immune serum	Antigen used	
	St 19 antigen (tube agglutination)	RB51 WC (BioDot)
Strain RB51	<1:25	1:1600
Strain 19	1:400	1:400

CHAPTER 1 - EXPERIMENT 2

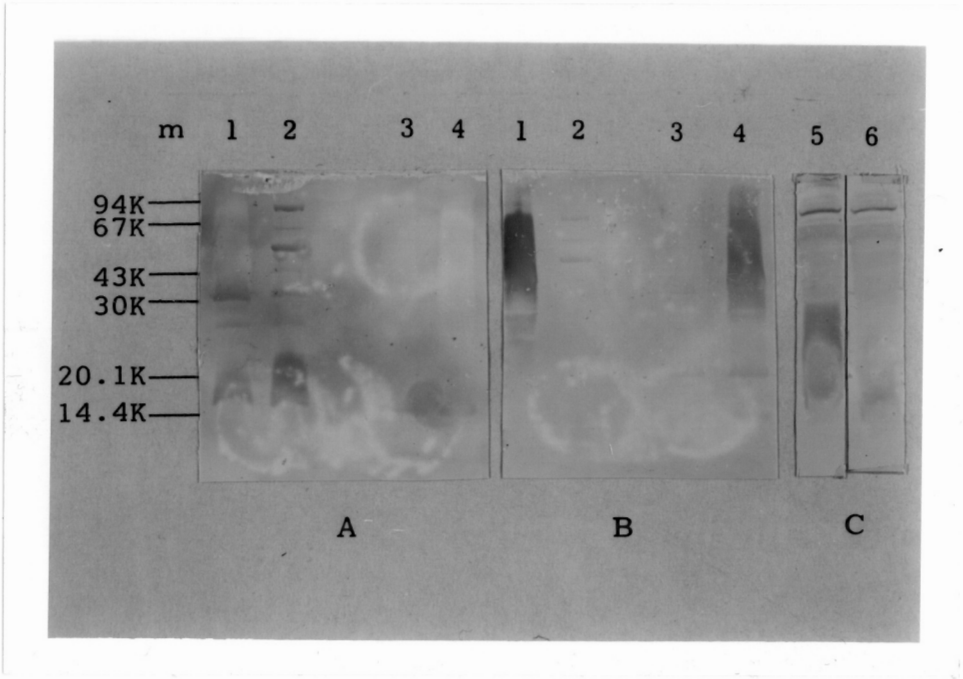

Figure 1.

Western blot analysis of strain RB51 and strain 19 immune serum. A= RB51 serum, B= strain 19 serum. Antigens tested were: lane 1. 2308 WC, 2) RB51 WC, 3) Y. enterocolitica serotype O:8 LPS 4) Y. enterocolitica serotype O:9 LPS. C= reaction of unabsorbed (lane 5) and absorbed (lane 6) RB51 serum with RB51 WC antigen. m- molecular weight marker.

CHAPTER 1 - EXPERIMENT 3

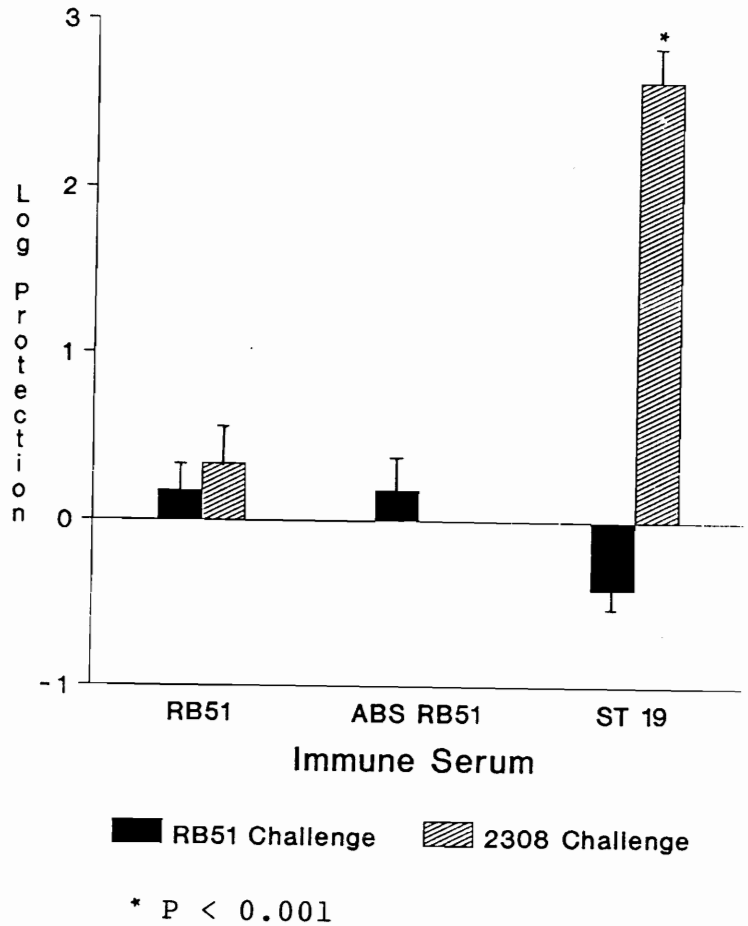

Figure 2.

Protection provided to BALB/c mice against challenge infection with B. abortus strains RB51 or 2308 by passive transfer of immune serum. Donors were immunized twice with either strain RB51 or 19 and immune serum obtained 2 weeks after the second injection. Transfers were made to groups of 5 mice and 2 hr later the animals were challenge infected. Log protection is the difference between mean log number of organisms in principal and corresponding control mice at 1 week p.i. Bars indicate S.D. within group. RB51 ABS = RB51 serum absorbed with RB51. Mean log number of RB51 cells per spleen= 4.674 and 2308 cells per spleen= 6.166.

from mice vaccinated twice with strain RB51 did not show any protection against challenge with either strain RB51 or 2308. This experiment was performed four times. Strain RB51 immune serum absorbed with viable RB51 organisms also did not protect mice which were challenged with strain RB51. On the other hand, when mice received immune serum from strain 19 vaccinated animals, they demonstrated a greater than 2 log protection against strain 2308 challenge. This was significantly different from control animals receiving non-immune serum ($p < 0.001$). Strain 19 immune serum was not able to protect mice against challenge with strain RB51.

DISCUSSION

Humoral immune response mediated protection against B. abortus smooth strains has been shown to be anti O-side chain specific (139). This investigation attempted to analyze if there was any role of the humoral immune response induced by strain RB51 in protecting against challenge with strain 2308. Immune serum obtained from strain RB51 vaccinated mice did not show any agglutinating antibodies toward strain 19 antigen. It however showed a strong titer against RB51 WC antigen (Table 1). Western blot analysis (Figure 1) indicated that RB51 immune serum contained antibodies directed to the "core" region of the LPS in addition to antigens in the m.w. range of 45 - 94 Kd. When RB51 immune serum was absorbed with viable RB51 organisms, the resultant serum preparation reacted with the high molecular weight antigens, but either did not, or reacted very weakly with the "core"

region. This demonstrated that mice vaccinated with strain RB51 do produce antibodies that recognize structures that are exposed on the surface of rough organisms. Absence of a typical LPS like reaction with WC antigen from strain 2308 and absence of titers in the agglutination test suggests that RB51 immune serum does not contain antibodies specific to the O-side chain of the LPS. This is also substantiated by the absence of any reaction with LPS from Y. enterocolitica serotype O:9, which is known to have an O-side chain similar to that of Brucella (33).

Immune serum obtained from strain 19 vaccinated mice had antibody titers against both RB51 WC antigen as well as strain 19 antigen (Table 1). This indicates that upon vaccination with strain 19, mice make antibodies not only to the O-side chain of the LPS but also to other antigens common to both rough and smooth strains. The fact that mice vaccinated with strain 19, produce antibodies specific to non-LPS antigens is also evident from Western blot analysis data (Figure 1) where serum is seen to react with antigens in the m.w. range of 67-94 Kd. However, the antibody response to the O-side chain is very prominent which is also evident from its reaction with Y. enterocolitica serotype O:9 LPS.

Passive protection experiments demonstrated that RB51 immune serum did not have the capacity to protect against either strain RB51 or strain 2308 challenge. Its inability to protect against strain 2308 is most probably due to the lack of O-side chain specific antibodies. The absence of protection against strain RB51 however, was quite

unexpected since absorption studies indicated that the serum reacted with surface structures of strain RB51. One possible reason to explain this phenomenon could be the large challenge dose of RB51 used in these experiments which may have overwhelmed potentially protective antibodies in the immune serum. It was not feasible to check lower doses of challenge because results from our laboratory (unpublished observations) indicated that challenge doses below $1x10^8$ bacteria per mouse resulted in inconsistent infection patterns. In addition, specific antibody isotypes important in protection may not have been induced by vaccination with strain RB51 or that isotypes which are not protective were induced (42).

An analysis of the protective ability of strain 19 serum reiterates the importance of the O-side chain, and antibodies to O-side chain in protection against brucellosis. Strain 19 serum could not protect against RB51 challenge because of the lack of O-side chain on RB51 LPS. At the same time, it showed significant protection against strain 2308 due to the presence of O-side chain antibodies as demonstrated by others (4, 83, 94).

CHAPTER II

ROLE OF IMMUNE T LYMPHOCYTES IN MURINE BRUCELLOSIS

RATIONALE

It is well established that in murine brucellosis protection is a function of both cell mediated and humoral responses (3, 4, 83, 93, 94, 107). All the studies that had been done so far, provided evidence to support the fact that cell mediated protection is provided by T cells of both CD4 and CD8 phenotype (3, 93, 107). These studies have been done using smooth strains as the vaccinating agent. Reports from our laboratory (23, 137) indicated that mice vaccinated with rough strain RB51 were protected against challenge with smooth strain 2308. Furthermore, mice were better protected when vaccinated twice with RB51 as compared to a single vaccination dose (23). It has been demonstrated by others (83, 94) that anti O-side chain antibodies play a role in protection against brucellosis. As demonstrated in chapter I, RB51 does not elicit an O-side chain specific antibody response.

Therefore, the main objective of this investigation was to determine whether vaccination with RB51 would result in the production of T cells with protective ability. The first step was to ask the question whether nylon wool enriched T cells obtained from strain RB51 vaccinated mice could protect recipient mice against challenge with the homologous strain. The second step, even more important than the first, was to determine whether these immune T cells could provide protection to recipient mice against challenge with the heterologous

and virulent strain 2308. Since the current vaccine, strain 19, has been demonstrated to induce immune T cells that can protect mice against challenge with strain 2308 (3, 4, 93), these studies also included strain 19 for the purpose of comparison. An additional objective of this investigation was to determine if the combination of specific antibodies and T cells have an additive, protective effect.

There are reports in the literature where investigators used *in vitro* activation of T cells with mitogens for enhancing the protective ability of these cells on adoptive transfer (7, 8, 15). It was demonstrated that when *L. monocytogenes* specific immune cells are activated *in vitro* with Con A, the adoptive transfer of protection in recipient animals is enhanced. Use of other stimulating agents in generating T cell clones has also been reported by Chen et al (35). Using a combination of PMA and ionomycin, they demonstrated high cloning efficiencies of T cells. More recently, Street et al (131) have demonstrated the use of stimulating agents such as PMA and Con A in developing T cell clones from bulk cultures of immune T cells. They used this technique for isolating clones specific for *N. brasiliensis* and *B. abortus*. Based on these observations, our final objective was to determine if lymphocytes that had been stimulated *in vitro* with specific antigens, could transfer protective immunity.

EXPERIMENTAL DESIGN

Two experiments were carried out for direct adoptive immune transfer. In both experiments 4-6 week old BALB/c mice were vaccinated

i.p. with viable $1x10^8$ strain RB51 or $5x10^4$ strain 19 organisms. In experiment 1, mice were vaccinated twice. Vaccination was given i.p. at an interval of 4 weeks. Four weeks after the second vaccination, spleens were obtained and processed for nylon wool enriched T cells. In experiment 2, mice were vaccinated once, and 6 weeks after vaccination spleens were used for T lymphocyte preparation. In both experiments 1 and 2, nylon wool purified splenic T lymphocytes were transferred to recipient mice at $3x10^7$ per animal. Transferred nylon wool enriched lymphocyte populations were more than 90% viable in all experiments as determined by the trypan blue exclusion test. Non-specific esterase staining of an aliquot of the T cell preparation indicated that macrophage population constituted less than 1%. Nylon wool enriched T cell preparations obtained from non-immune mice were quantitated by indirect immunofluorescent staining. Using anti-L3T4 and anti-Lyt2 specific monoclonal antibodies, it was determined that T cells constituted 85% of the nylon wool enriched lymphocyte population. Recipient mice were challenged i.p. 2 hr later with either $1x10^8$ strain RB51 or $5x10^4$ strain 2308 viable organisms in sterile saline. Control mice which received only HBSS-FCS were similarly challenged. Transfer of nylon wool enriched T cells from normal mice did not have a significant effect on bacterial growth in spleens of recipient mice challenged with either rough strain RB51 or smooth strain 2308 (unpublished observations). One week post infection, recipient mice were killed after obtaining blood samples, and numbers of Brucella (cfu) in the spleen was determined.

In experiment 3, immune serum was transferred i.p. followed by i.v. introduction of $3x10^7$ immune T cells and 2 hr later, mice were challenged with strain 2308 organisms. For the final activation experiments (experiments 4 and 5), spleen cells obtained from naive mice, and RB51 vaccinated mice were passed over nylon wool columns for T lymphocyte enrichment. They were then activated *in vitro* in the presence of mitomycin C treated syngeneic antigen presenting cells (APCs), IL-2 and gamma-IFN. Activation was carried out both in the presence and absence of an antigen preparation (as described in Materials and Methods) made from strain RB51. T lymphocytes were activated with the above combination for 72 hours following which, they were treated differently. For experiment 4, they were pulsed with tritiated thymidine and cpm incorporated was determined as described in Materials and Methods. For experiment 5, they were resuspended in an APC-free growth media. This growth medium consisted of PMA, ionomycin, IL-2, and gamma IFN. T lymphocytes were allowed to grow in this medium for 96 hours before they were used in adoptive transfer experiments. Nylon wool enriched T cells from non-immune donors were treated similarly. *In vitro* activated nylon wool enriched T cells were transferred i.v. to recipient mice at $4x10^6$ cells per mouse.

RESULTS

Data obtained from experiments in this chapter are presented in the appendix. In experiment 1, which was performed twice, immune cells obtained from RB51 vaccinated donors provided protection to recipient

mice challenged with either RB51 or 2308, when donors were vaccinated twice (Figure 1). Protection was significant ($p < 0.001$) when compared with corresponding control mice challenge infected with either strain RB51 or strain 2308. Donors vaccinated twice with strain 19 organisms also developed immune cells that could provide protection against challenge with strain 2308. Protection was significant ($p < 0.001$) when compared with control mice which did not receive immune T cells. These cells however, did not have the capacity to protect against challenge with the heterologous rough strain RB51. In this experiment, the levels of protection afforded by either RB51 immune cells or strain 19 immune cells against challenge with strain 2308 were not significantly different.

In experiment 2 it was observed (Figure 2) that immune T cells obtained from mice vaccinated once with strain RB51 could provide protection to recipient mice when challenged with either strain RB51 or strain 2308. The levels of protection provided against both challenge strains were significantly different from those of corresponding control mice ($p <0.001$). The level of protection afforded against the heterologous strain was not significantly different from that provided against the homologous challenge as observed in experiment 1. Similarly, immune cells from strain 19 vaccinated donors showed a protective ability against challenge with both strain RB51 as well as strain 2308 ($p <0.002$). There was no significant difference between the abilities of these cells to protect against either strain RB51 or strain 2308. This experiment was

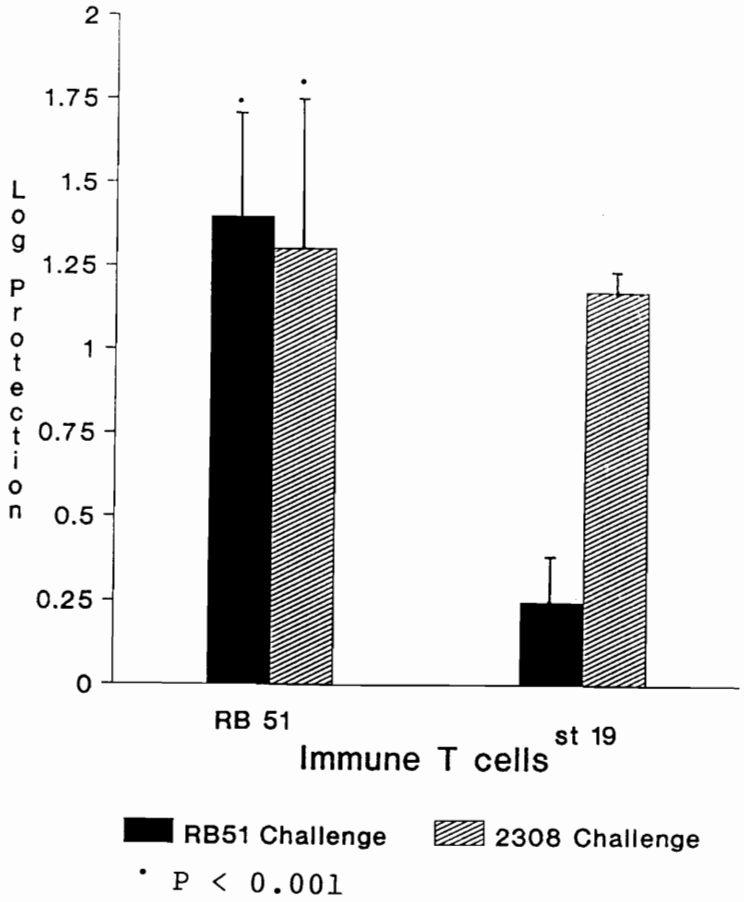

Figure 1.

Protection provided to BALB/c mice against challenge infection with B. abortus strains RB51 or 2308 by adoptive transfer of T cells. Donors were immunized twice with either strain RB51 or 19 and cells obtained 4 weeks after second injection. Transfers were made to groups of 5 mice and 2 hr later the animals were challenge infected. Log protection is the difference between mean log number of organisms in principal and corresponding control mice at 1 week p.i. Bars indicate S.D. within group. Mean log number of RB51 cells per spleen = 4.759 and 2308 cells per spleen = 6.209.

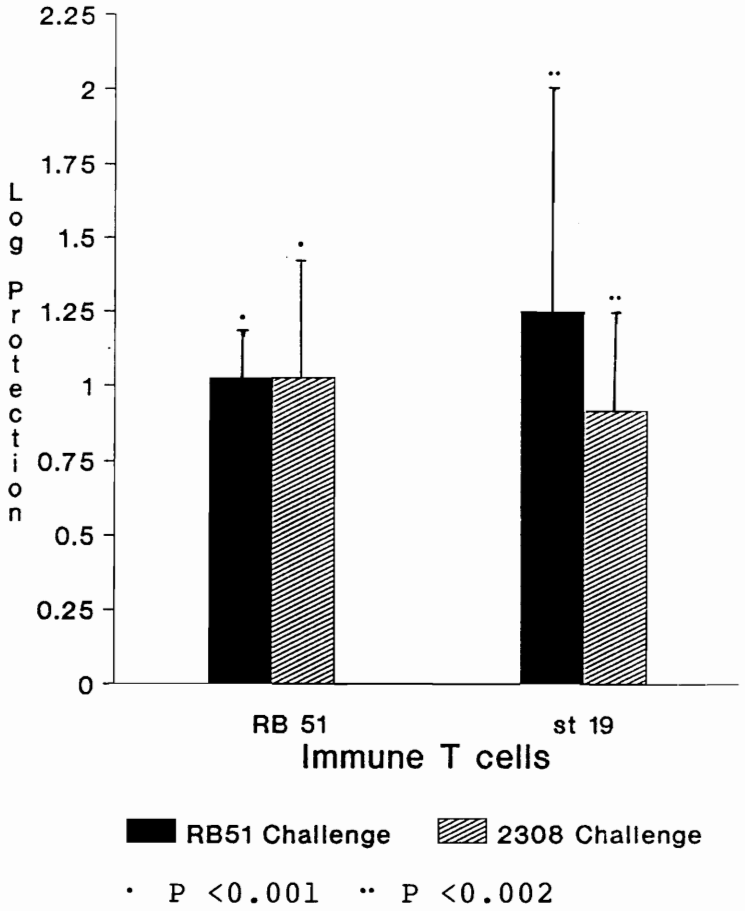

Figure 2.

Protection provided to BALB/c mice against challenge infection with B. abortus strains RB51 or 2308 by adoptive transfer of T cells. Donors were immunized 6 weeks earlier with either strain RB51 or 19. Transfers were made to groups of 5 mice and 2 hr later the animals were challenge infected. Log protection is the difference between mean log number of organisms in principal and corresponding control mice at 1 week p.i. Bars indicate S.D. within group.
Mean log number of RB51 cells per spleen= 4.631 and 2308 cells= 6.252.

performed twice and similar levels of protection was obtained in both experiments.

The possible role of antibodies produced by contaminating B cells in these adoptive transfer experiments was also indirectly investigated. Blood samples pooled from challenged mice at the time of sacrifice (1 week post challenge) was analysed by Western blot analysis for the presence of serum antibodies that might have contributed to the observed protection. Serum samples were reacted with WC antigen preparations from strains RB51 and 2308. Western blot analyses of these serum samples indicated that mice receiving strain 19 specific nylon wool enriched lymphocytes and challenged with strain 2308 developed a weak reaction to the O-side chain. The contribution of these trace amounts of antibodies towards protection is not clear because control mice also showed a similar reaction. Mice receiving strain RB51 specific nylon wool enriched lymphocytes and challenged with RB51 did not show any reaction to either RB51 WC or 2308 WC antigens.

Since immune serum from strain 19 vaccinated mice (chapter I, experiment 1) and immune T cells from strain RB51 vaccinated mice (experiment 1, this chapter) had demonstrated significant protection against strain 2308, we also examined (experiment 3) the possibility of enhanced protection in mice receiving both cells and serum and being challenged with strain 2308. It was observed that (Figure 3) while both serum and T cells could independently provide protection as in above mentioned experiments, a combination of the two had an

CHAPTER 2 - EXPERIMENT 3

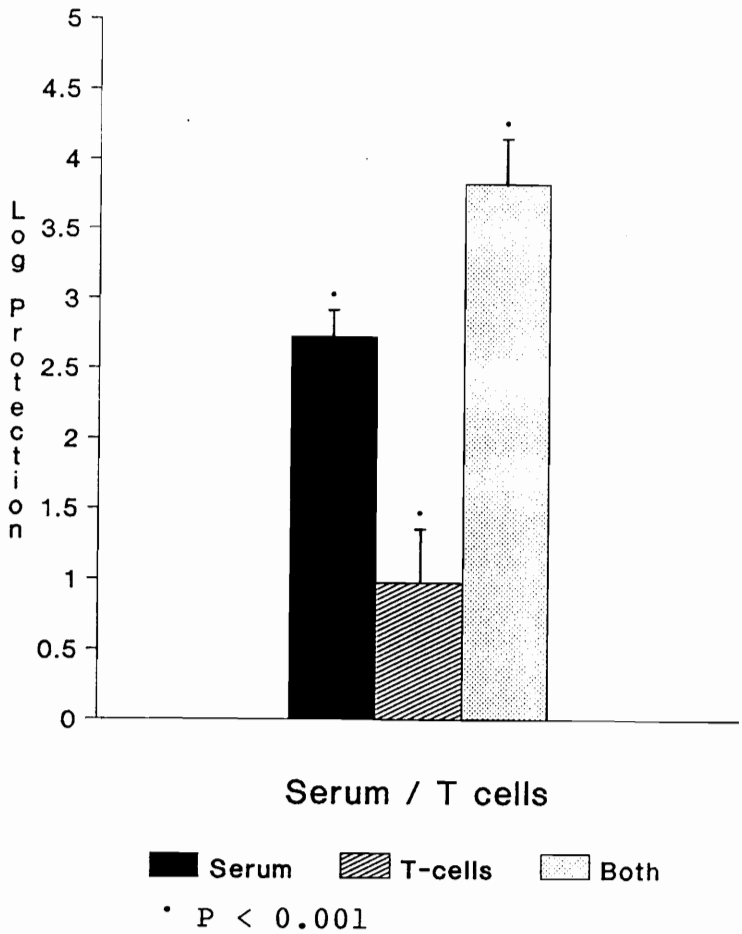

Figure 3.

Protection provided to BALB/c mice against challenge with B. abortus strain 2308 by transfer of immune serum and T cells. Donors were immunized twice. Strain 19 immune serum was obtained as in chapter 1 experiment 3, and RB51 immune T cells as in this chapter, experiment 1. Transfers were made to groups of 5 mice and 2 hr later the animals were challenge infected. Log protection is the difference between mean log number of organisms in principal and corresponding control mice at 1 week p.i. Bars indicate S.D. within group. Mean log number of 2308 cells per spleen = 6.379.

additive effect. Mice receiving serum and T cells had significantly better protection than mice receiving serum or cells alone ($p < 0.001$).

In order to establish that antigen specific lymphocytes were being selected for in our *in vitro* activation experiments, lymphocyte transformation assays (experiment 4) were carried out with both control and immune T cells in the presence of RB51 antigen preparation. Table 1 demonstrates that there is an antigen specific proliferation of strain RB51 immune T cells when compared to T cells from control, non-immunized mice. The results also show that RB51 T cells proliferate in response to the antigen even in the absence of added APCs. This is probably due to the presence of small amounts of macrophages in the nylon wool enriched lymphocyte populations as determined by non-specific esterase staining.

Results (Figure 4) obtained from the *in vitro* activation experiment (experiment 5) demonstrated that activated lymphocytes had an enhanced potential of providing protection to recipient mice. Non-immune lymphocytes stimulated *in vitro* with PMA and ionomycin in the absence of initial antigen stimulation, did not show any protective ability when compared to mice which did not receive T cells. When non-immune T cells were stimulated in the presence of antigen, they protected mice against challenge with strain 2308. Protection was significantly different ($p < 0.001$) from control mice that had not received any T cells. Strain RB51 immune T cells stimulated *in vitro* with PMA and ionomycin without prior antigen specific stimulation were also able to transfer protection to recipient mice against challenge

CHAPTER 2 - EXPERIMENT 4

Table 1.

Lymphocyte proliferation responses cpm +/- S.D. x 10^{-3}

Responding cells	RB51 antigen	cpm +/- S.D. x 1000	SI[a]
APC	-	0.10 +/- 0.019	
	+	0.10 +/- 0.016	1.0
Non-immune T cells	-	2.15 +/- 0.19	
	+	5.09 +/- 1.4	2.37
RB51 T cells	-	4.20 +/- 0.58	
	+	21.5 +/- 2.39	5.12
Non-immune T cells + APC	-	1.90 +/- 0.23	
	+	5.00 +/- 1.71	2.63
RB51 T cells + APC	-	6.30 +/- 0.87	
	+	29.12 +/- 1.76	4.62

a Stimulation Index = $\frac{\text{cpm with antigen}}{\text{cpm without antigen}}$

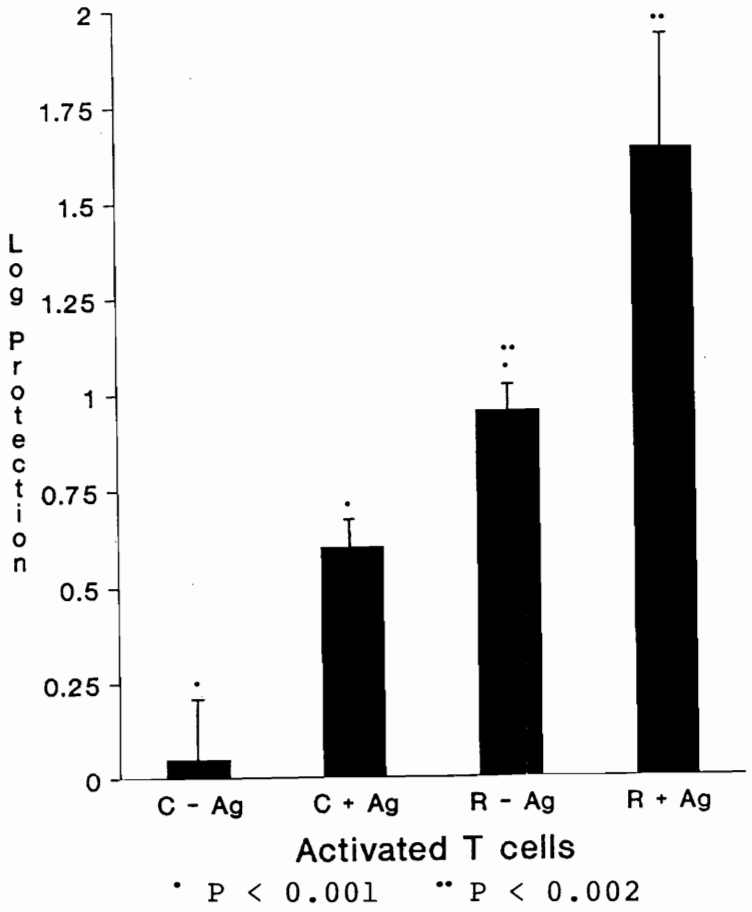

Figure 4.

Protection provided to BALB/c mice against challenge infection with B. abortus strain 2308 by passive transfer of in vitro activated T cells. Non-immune (control) or strain RB51 immune T cells were activated in the presence or absence of antigen as described in Materials and Methods. Transfers were made to groups of 5 mice and 2 hr later the animals were challenge infected. Log protection is the difference between mean log number of organisms in principal and corresponding control mice at 1 week p.i. Bars indicate S.D. within group. C=control T cells. R= RB51 immune T cells. Ag= RB51 antigen. Mean log number of 2308 cells per spleen = 6.244.

with strain 2308. The level of protection provided was significant ($p < 0.001$) when compared to control mice. In addition, when these immune T cells were activated *in vitro* in the presence of the antigen, the protective capacity was enhanced by at least half a log value and was significantly different ($p < 0.001$) from control mice which had not received any T cells.

Table 2 gives results obtained from a one-way analysis of variance test performed on the observations made in experiment 5. Protection provided by RB51 immune T cells activated in the presence of antigen was greater than protection provided by the same cells activated in the absence of antigen. Protection provided by the latter was greater than protection provided by non-immune T cells activated in the presence of antigen. There was significant difference ($p < 0.05$) between the levels of protection provided by the different *in vitro* activated T cell populations.

DISCUSSION

The protective role of T lymphocytes has been demonstrated in a variety of diseases caused by intracellular pathogens. These include pathogens such as *L. monocytogenes*, *M. leprae*, *L. major*, and *R. tsutsugamushi* for which T cell clones of both CD4 and CD8 phenotypes have been developed *in vitro* (40, 45, 75, 79, 100, 123). Adoptive transfer of such clones has been demonstrated to afford protection against challenge infection (36, 74, 76, 117).

In brucellosis, the vaccine strain 19 used against infection with

CHAPTER 2 - EXPERIMENT 4

Table 1.

Log protection against strain 2308 provided by *in vitro* activated T cells

One-way Analysis of Variance

Treatment	Log protection
Non-immune T cells activated without antigen	0.046* (a)
Non-immune T cells activated with antigen	0.602 (b)
RB51 T cells activated without antigen	0.955 (c)
RB51 T cells activated with antigen	1.642 (d)

* log protection followed by different letters in parenthesis differ significantly ($p < 0.05$)

the smooth virulent strain 2308, is by itself a smooth strain. Investigations in cell mediated immunity to murine brucellosis have established that mice vaccinated with strain 19 do develop immune T cells which can protect recipient mice upon adoptive transfer (3, 4, 93). The class of the protective T cell population has been established to be of both CD4 and CD8 phenotype (3, 107). This has been demonstrated by showing protection in recipient mice by transfer of a particular phenotype or conversely by depleting a phenotype and showing lack of protection.

Vaccination of mice with live strain RB51 organisms has shown to protect mice against challenge with either the homologous rough strain or the heterologous smooth strain 2308 (23). Experiment 1 demonstrated that the levels of protection provided by immune cells from either strain RB51 or strain 19 vaccinated mice is similar when recipient mice are challenged with strain 2308 (Figure 1). This is in contrast to other findings where mice vaccinated with different smooth strains i.e. strain 19 or strain 2308 showed different protective capacities in adoptive transfer experiments (4). It was observed that protection provided by transfer of immune T lymphocytes was better when the challenge strain was the same as that used for immunizing the donor. Regardless of these observations, transfer of RB51 sensitized lymphocytes results in protection against strain 2308.

It appears from the literature that an analysis of the temporal development of protective T cells is critical to the understanding of murine brucellosis. In BALB/c mice splenic infection following

challenge with strain 2308 reached peak levels by 2 weeks post infection, remained at about that level for 8 weeks after which it gradually declined (93). Strain 19 has similar kinetics in the beginning, reaching a higher peak at 2 weeks, following which there is a sharp decline (93). Although splenic infections starts declining after 2 weeks, Araya et al (3) have reported that the development of immune T cells with significant protective ability is not apparent until 4 weeks post infection. They also demonstrated that by 3 weeks post infection, there is a sharp drop in the CD4 population and a comparatively nominal drop in CD8. The number of CD8 cells did not change very much throughout the period of their study, but the protective ability of unfractionated immune T cells did not manifest itself until 4 weeks post immunization at which time CD4 cells reached their lowest level (3). Protection at 6 weeks post-immunization was significantly better which coincided with the highest level of CD4 cells.

In our experiments using twice vaccinated mice as donors, strain 19 immune T cells obtained 4 weeks after the second immunization develop the capacity to protect against strain 2308 (Figure 1). They however failed to protect mice against challenge with strain RB51. This difference in the protective potential of strain 19 immune cells against challenge with rough and smooth strains is not apparent in immune cells obtained from mice immunized 6 weeks before transfer (Figure 2). In contrast, strain RB51 immune cells obtained after similar vaccination schedules do not show these differences and are

able to protect against challenge with both the rough and smooth strain. These experiments were performed twice and the data was reproducible.

It is quite possible however, that the protective abilities induced by strain RB51 and strain 19 might not rest in the same subset of T cells. In other words, the subset responsible for protection in strain RB51 immune T cells may not be the same as those in strain 19 immune cells. Another possibility could be that immune T cell subsets responsible for transferring protection when mice are vaccinated twice with strain 19 may be different from those obtained from mice that have been vaccinated only once. In addition, it is also possible that the epitopes recognized by the immune cells are different and that at different stages of infection, macrophages may be presenting different sets of antigens. This hypothesis can be reconciled with observations made by Orme in a different system (104). In his study with M. tuberculosis (104), he demonstrated evidence that different population of protective T cells were developed at different stages of infection. He further hypothesized that emergence of different populations of protective T cells could be due to the availability of different classes of antigens at different stages of infection.

There are no reports of rough B. abortus sensitized T cells capable of protecting against challenge with smooth virulent strain 2308. Halliburton and Hindsill (61) had examined non-specific resistance to L. monocytogenes, a correlate of CMI response, in mice immunized with B. abortus. They observed that it was possible to

recall this resistance to L. monocytogenes in immunized mice by using an antigen extract of strain 45/20 considered to be a rough organism.

It is important to ascertain the above mentioned possibilities in order to obtain a better understanding of CMI in murine brucellosis. A reasonable approach would be to examine the proliferative responses of T cells obtained from both strain RB51 and strain 19 vaccinated mice from different stages post-vaccination. These responses should be evaluated with regard to antigen specific stimuli provided by splenic macrophages from infected mice. In order to discern any differences in antigen presentation during the course of an infection, such macrophages may be obtained from infected mice at different stages in the course of infection. In addition, an analysis of lymphokines secreted as a result of the above specific proliferative responses may throw some light on the nature of the T cells that are being stimulated. Such analyses have revealed that in Leishmania major infection, adoptive transfer of different antigen specific T cell lines can lead to either exacerbation of infection or protection (123), depending on whether Th1 or Th2 cells have been transferred.

The combination of RB51 immune cells and strain 19 immune serum gave better protection to mice challenged with strain 2308. This protection was greater than that with either immune cells or immune serum alone. It is therefore possible that in the event of vaccinating animals with strain RB51, immune T cells would complement the protective effect of O-side chain specific antibodies present in strain 2308 infected animals.

In vitro stimulation of immune T cells obtained from strain RB51 vaccinated mice was seen to enhance the protective ability of these cells (Figure 4). In this investigation, activation was initially carried out in an antigen specific manner employing a heat killed preparation of strain RB51. Following this, activated lymphocytes were further cultured in the presence of IL-2, gamma-IFN, PMA and ionomycin. Reports have indicated that while IL-2 and gamma-IFN are required for the *in vitro* development of CTLs (87), use of gamma-IFN preferentially selects the Th1 type of cells (55).

T cells from naive mice activated *in vitro* in the presence of IL-2, gamma-IFN, PMA and ionomycin, were able to transfer a low level of protection against strain 2308 only when they were activated in the presence of antigen. Such low levels of protection significantly different from control values could be attributed to antigen-specific selection of protective cells from a normal, antigen specific, pre-existing T cell repertoire in naive mice. An increased level of protection is observed when strain RB51 immune T cells are stimulated in the absence of antigen. Although there is no strain RB51 antigen driven selection in this case, *Brucella* specific immune Th1 cells and/or CTLs already present in the population in higher numbers than in control populations could have been favored due to the presence of IL-2 and gamma-IFN in the medium. This is a distinct possibility because IL-2 and gamma-IFN have been shown to be necessary lymphokines for the development of such cells *in vitro* (35). A further enhancement in protection is observed if strain RB51 immune cells are stimulated

in the presence of heat killed strain RB51 antigen. It is possible that specific antigen responsive T cells are further expanded by antigen specific in vitro activation and therefore are able to provide better protection than antigen unstimulated immune cells.

Such enhancement of the protective ability of immune T lymphocytes has been documented in studies with L. monocytogenes. Barry and Hinrichs (7, 8) demonstrated an increase in the level of protection afforded by transferred T cells that were stimulated in vitro with Con A. They also noted that this increase was only with respect to systemic clearance of the bacteria and showed no increase in a Listeria specific DTH response. This suggests that in vitro activation had led to enrichment of a class of T cells with protective ability but lacking the capacity to induce a DTH response. In a subsequent report (15) Bishop and Hinrichs demonstrated that while immunity provided by unstimulated immune cells was short lived and required both CD4 and CD8, protection by stimulated cells was enhanced and long lasting and mediated only by the CD8 phenotype.

CHAPTER III

ROLE OF MACROPHAGES IN MURINE BRUCELLOSIS

RATIONALE

Since the early work of Mackaness in the 1960's, the importance of the macrophage has been well documented in many instances. Organisms like M. tuberculosis, L. tropica, S. typhimurium, and L. monocytogenes have been shown to be susceptible to the killing activity of macrophages (46, 51, 52, 69, 105, 109). Work with B. abortus has been carried out using macrophages in both the murine as well as bovine systems. The salient features of these findings are that B. abortus needs to be opsonized for phagocytosis (63). Furthermore, they are able to survive better in macrophages from susceptible cows than those in resistant cows (62, 113).

Available evidence in the literature suggests that in the case of intracellular pathogens, an interaction between macrophages and T cells could be mediated either by Th cells, CTLS, or both. An important outcome of the interaction between macrophages and Th cells is the production of gamma-IFN by these T cells. Gamma-IFN is then responsible for "activating" the macrophages i.e. enhancing the bactericidal capacity of these cells leading to killing of ingested bacteria.

Reports have indicated instances where invasion of macrophages by an intracellular pathogen has made the macrophage unresponsive to activation signals provided by gamma-IFN (11, 125). Therefore, a part

of this investigation dealing with macrophage-B. abortus interactions was also to determine whether invasion of macrophages by B. abortus would make macrophages refractory to subsequent treatment with gamma-IFN.

In this investigation, experiments with mouse peritoneal macrophages were carried out to answer the following questions: i) Will recombinant gamma-IFN enhance the ability of macrophages to kill ingested Brucella organisms and if so, is there any difference in their ability to kill rough versus smooth strains of B. abortus ? ii) Will macrophages already invaded by B. abortus still be responsive to recombinant gamma-IFN and therefore kill the ingested bacteria ? These experiments would give us a clear indication if the "activation" route is important in Brucella immunity and it would also tell us if B. abortus can make macrophages refractory to the activation effects of recombinant gamma-IFN. If macrophages become refractory this would suggest that B. abortus probably evolved mechanisms which abrogate the effector function of the Th cells at the level of macrophage activation.

Macrophage bactericidal activity is exhibited via either an oxygen-dependent mechanism or an oxygen-independent mechanism. Oxygen-dependent mechanisms which takes place primarily in the phagosomes are a result of the oxygen burst which occurs following phagocytosis. On the other hand, bactericidal activity of oxygen-independent mechanisms is located in the phago-lysosome, which occurs following fusion of phagosomes with the lysosomes. In studies related with functions of

phagosomes and lysosomes, both in the case of intracellular pathogens and antigen presentation, investigators have made use of lysosomotropic drugs that are known to inhibit phagosome-lysosome fusion (2, 51, 59, 128). Use of these drugs, which include monensin and chloroquine, has helped in the dissociation of functions associated with the lysosomes from the combined effect of phagosomes-lysosomes. In order to determine whether the effect of macrophage activation by gamma-IFN was restricted to the phagosomes or otherwise, macrophage-Brucella interactions were analysed in the presence of chloroquine.

Several investigators have used in vitro models using phagocytic cell lines in order to gain a better understanding of host-parasite relationship. The primary reason for taking this approach has been the ability to work with a homogenous cell population which is readily propagated and available in the laboratory. These studies have included a variety of organisms such as N. gonorrhoeae (138), Coxiella burnetti (59), Histoplasma capsulatum (142), Pseudomonas aeruginosa (10) and M. tuberculosis (39). Using this approach investigators have been able to analyze in some detail the characteristics of the pathogen which contribute to its virulence properties. In the case of Legionella pneumophila, Pearlman and coworkers (108) have been able to demonstrate the differences in susceptibility of a virulent and avirulent strain to a human macrophage-like cell line. Similarly, a protein extract from M. tuberculosis was shown to modulate macrophage lysosomal pH, in a study to explain phagosome-lysosome inhibition by

this organism (39). Buchmeier and Heffron (21) have used such *in vitro* studies for characterizing virulence genes of *Salmonella*. Several transposon mediated mutants of *S. typhimurium* were developed and their relative ability to survive within various types of macrophages including J774, a human macrophage cell line was examined.

In efforts to further our understanding of the virulent nature of smooth strain 2308, and the reduced virulence of rough strain RB51, this investigation used a similar *in vitro* approach. There is no evidence in the literature that indicates the use of a cell line with macrophage like properties for the study of *Brucella*-macrophage interactions. P388D1, a macrophage like cell line which has been used in similar studies (10, 142) was chosen for these studies.

EXPERIMENTAL DESIGN

In experiment 1, western blot analysis of BRU 38 and BRU 48 was performed with antigen preparations of strain RB51 whole cells and LPS isolated from smooth strain 2308 and *Y. enterocolitica* O:9. These antigens were separated by PAGE following which they were electroblotted onto nitrocellulose membranes. The membranes were then reacted with the above monoclonal antibody preparations to determine their antigenic specificities.

Experiment 2 was performed to demonstrate the location of the antigen recognized by BRU 48, on the surface of strain RB51. Viable strain RB51 and 2308 were used separately and absorbed with BRU 48 preparation. These absorbed preparations were then analysed in a

colony blot ELISA technique (118) to demonstrate that BRU 48 is absorbed by viable strain RB51, but not by strain 2308.

Thioglycollate elicited peritoneal macrophages were obtained from naive mice for use in experiments involving gamma-IFN activation of macrophages. Following separation of the non-adherent cells from the adherent macrophage population, the macrophages were incubated overnight before using them for bactericidal assays. Bactericidal assays were always performed in medium lacking antibiotics except briefly at a stage following phagocytosis where antibiotic containing medium was used to kill extracellular bacteria.

B. abortus strains were opsonized with different monoclonal antibody preparations in order to facilitate phagocytosis. B. abortus strain RB51 was opsonized with Bru 48, and strain 2308 with BRU 38. Overnight cultures of peritoneal macrophages were allowed to ingest opsonized bacteria at a ratio of 1:500. Phagocytosis was allowed either in the presence or absence of 50 U/ml gamma-IFN. Following phagocytosis for 30 min, extracellular bacteria were killed by incubating the cultures for 30 min in the presence of medium containing antibiotics. After washing out the antibiotic containing medium, and replacing it with antibiotic free medium with or without gamma-IFN, the survival of ingested bacteria was determined at various time intervals. The number of B. abortus organisms present at zero time in gamma-IFN treated and untreated macrophages were taken as 100%.

Experiments 3 and 4 were carried out with strains RB51 and 2308

respectively in order to assess the effect of gamma-IFN activated macrophages on these organisms. In these experiments, 50 U/ml gamma-IFN was included in the media at the time of phagocytosis. In order to examine whether the growth of B. abortus inside macrophages had any abrogative effect on the function of gamma-IFN, experiments 5 and 6 were carried out similar to those mentioned above. In these experiments, however, 50 U/ml gamma-IFN was added to macrophage cultures at 24 hr following the start of the assay.

Experiments 7 and 8 were performed to examine the effect of chloroquine on the ability of gamma-IFN activated macrophage to kill B. abortus. These were again similar to experiments 3-6 except for the inclusion of chloroquine in the culture medium. Macrophage cultures were treated with antibiotic free RPMI 1640 medium containing 100 uM chloroquine 30 min prior to phagocytosis. At the time of phagocytosis, 50 U/ml gamma-IFN was added to the required wells. The number of bacteria surviving in these macrophages was observed for upto a period of 12 hr. Control experiments indicated that during this period, chloroquine had no effect on the viability of macrophages as evidenced by trypan blue exclusion.

Experiments using P388D1 cells were performed similar to those done using peritoneal macrophages. The only difference was that with P388D1, the assay was terminated at 8 hr only and no gamma-IFN usage was involved. Experiments 9 and 10 were performed to determine the ability of P388D1 cells to ingest both rough and smooth strains of B. abortus.

In experiment 9, rough strain RB51 was opsonized with BRU 48 and cultured with P388D1 cells. Following phagocytosis, the macrophages were fixed with glutaraldehyde and processed for electron microscopic observation to determine the invasion of these cells by the bacteria. Similarly, smooth strain 19 was opsonized with BRU 38 and the ingestion of these organisms by P388D1 cells was examined by electron microscopy (experiment 10).

The ability of P388D1 cells to kill ingested B. abortus was studied in experiments 11 and 12. Chloroquine treated or untreated P388D1 cells were allowed to ingest opsonized rough or smooth B. abortus. Following killing of extracellular bacteria with antibiotics, the number of rough (experiment 11) or smooth (experiment 12) B. abortus surviving inside the macrophages was determined at various time intervals.

RESULTS

Data obtained from experiments in this chapter are presented in the appendix. Figure 1 depicts the results of western blot analysis of BRU 38 and BRU 48. BRU 38 reacts with the O-side chain of LPS obtained from strain 2308 and Y. enterocolitica but not with whole cell antigen from strain RB51. BRU 48, however, reacts with the core region of strain RB51 WC as well as LPS preparations obtained from strain 2308 and Y. enterocolitica.

Results of absorption experiment are shown in Figure 2. It is evident that when unabsorbed BRU 48 is used, it reacts with both the

CHAPTER 3 - EXPERIMENT 1

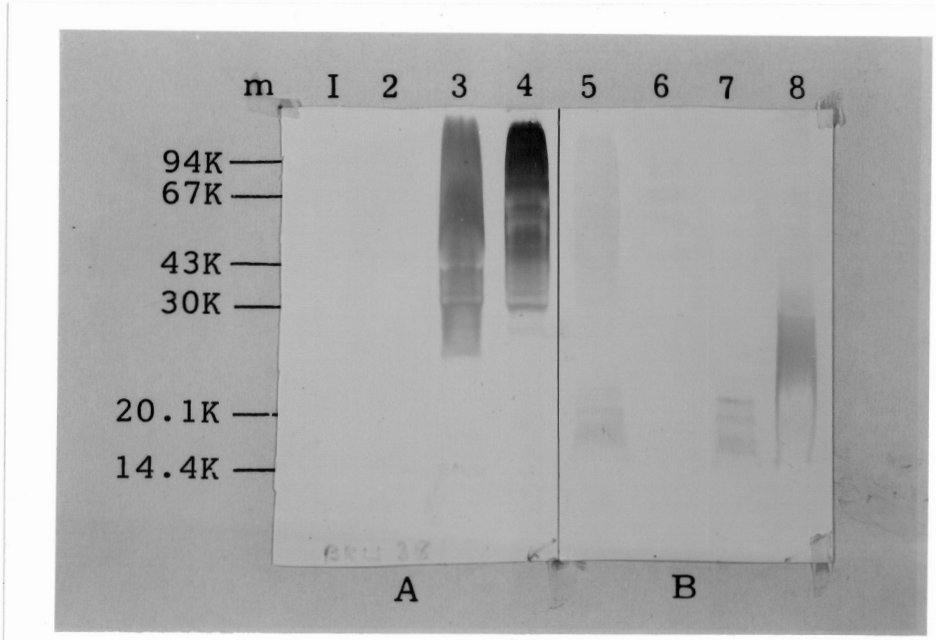

Figure 1.

Western blot analysis of monoclonal antibodies BRU 38 and BRU 48. Lanes: 1 and 5 - RB51 WC, 2 - Y. enterocolitica serotype O:8 LPS, 3 - Y. enterocolitica serotype O:9 LPS 4 and 8 2308 WC, 6 - strain 19 WC, 7 - strain 45/20 WC, m - molecular weight markers. A= BRU 38, B= BRU 48.

CHAPTER 3 - EXPERIMENT 2

A B C

Figure 2.

Colony blot ELISA showing reactivity of monoclonal antibody BRU 48 before and after absorption.
A - unabsorbed BRU 48. B - BRU 48 absorbed with strain RB51. C - BRU 48 absorbed with strain 2308. Different strains of B. abortus were grown on TSBA plates, blotted onto nitrocellulose membranes, and processed as described in Materials and Methods.

rough and smooth strains in a colony blot ELISA. When BRU 48 is absorbed with live strain RB51 organisms, the absorbed reagent does not react with either rough or smooth colony types. However, when BRU 48 is absorbed with smooth strain 2308, it does not lose the ability to react with either strain.

Figure 3 demonstrates the effect of gamma-IFN on the survival of strain RB51 in gamma-IFN treated macrophages as observed in experiment 3. The survival of strain RB51 organisms drops to about 40% in macrophages not treated with gamma-IFN. However, in macrophages treated with 50 U/ml of gamma-IFN, the survival of RB51 organisms drops to about 10% during the same period. RB51 organisms were allowed to grow in macrophages for an additional 12, and 24 hr post ingestion. When the numbers of surviving B. abortus were determined at the end of these incubation periods, there were more surviving inside gamma-IFN untreated macrophages than in treated macrophages. In 12 hr, the number of bacteria increased from 40% to 50% in untreated macrophages whereas in macrophages treated with gamma-IFN, the number was even less than that found at 12 hr.

In a similar experiment (experiment 4) with strain 2308 organisms, their ability to survive in gamma-IFN treated macrophages was determined. In untreated macrophages (Figure 4), the number of smooth strain 2308 decreases to 60% within the first 12 hr. During the same time period, macrophages treated with gamma-IFN, demonstrate fewer strain 2308. At the end of 24 hr incubation, strain 2308 replicates in untreated macrophages and their numbers increase to

CHAPTER 3 - EXPERIMENT 3

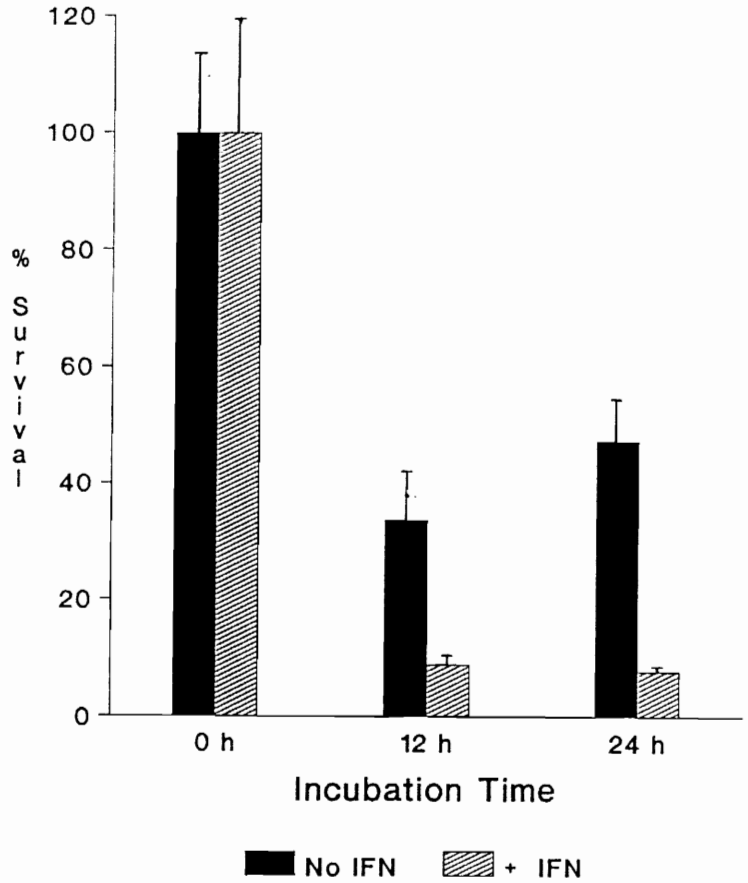

Figure 3.

Mean (+/- S.D.) percentage of strain RB51 organisms surviving in peritoneal macrophages treated with 50 U/ml gamma-IFN at the time of phagocytosis. Survival percentage was calculated on the basis of 100% at 0 hr. Bars indicate S.D. within replicates.

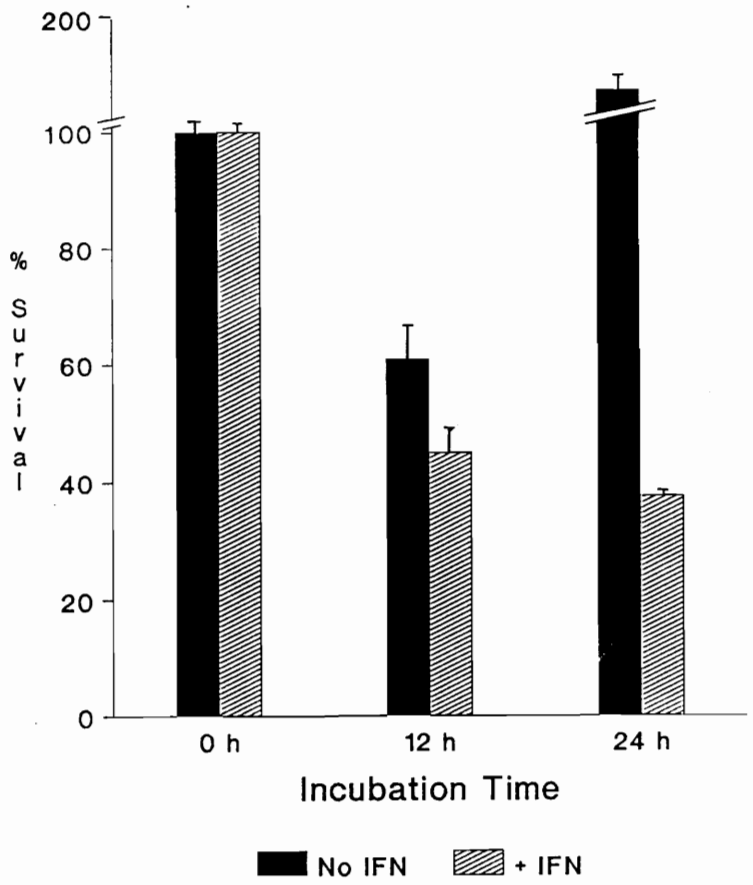

Figure 4.

Mean (+/- S.D.) percentage of strain 2308 organisms surviving in peritoneal macrophages treated with 50 U/ml gamma-IFN at the time of phagocytosis. Survival percentage was calculated on the basis of 100% at 0 hr. Bars indicate S.D. within replicates.

136%. Treatment of macrophages with gamma-IFN, however, does not allow this growth, reducing the numbers of surviving bacteria to about 40% (Figure 4).

In experiment 5, following ingestion, strain RB51 survived to a level of about 30% in untreated macrophages by 24 hrs. These numbers increased to more than 150% by 36 hr. When gamma-IFN was added to these macrophages at 24 hr (Figure 5), the effect of gamma-IFN on the survivability of ingested bacteria is evident. Whereas in the absence of gamma-IFN treatment bacteria inside macrophages replicated to a level of more than 500%, the growth of bacteria in macrophages treated with gamma-IFN was restricted to less than 10% within the same period. The numbers of strain RB51 organisms remained at those levels for the duration of the experiment following treatment with gamma-IFN.

A similar trend (experiment 6) was observed with strain 2308. In the initial 24 hr following phagocytosis, the number of strain 2308 inside mouse peritoneal macrophages increases to about 241%. When gamma-IFN was added to macrophage cultures at this stage, growth of bacteria is restricted to about 50% at 12 hr and to about 60% at 24 hr (Figure 6). However, in the absence of gamma-IFN treatment, the corresponding values are 340% and 500%.

Figure 7 shows the effect of chloroquine on the ability of gamma-IFN activated macrophages to kill ingested strain RB51 organisms (experiment 7). When macrophages are treated with 0.1 mM chloroquine alone, there is no effect on the survival percentage of B. abortus. Treatment of macrophages with 50 U/ml gamma-IFN restricted the growth

CHAPTER 3 - EXPERIMENT 5

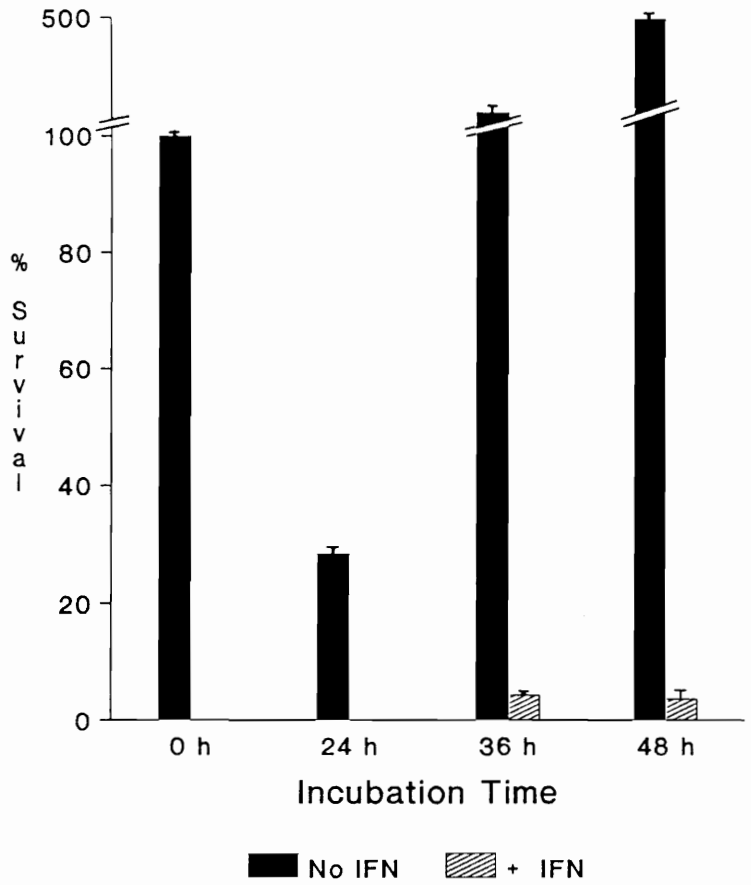

Figure 5.

Mean (+/- S.D.) percentage of strain RB51 organisms surviving in peritoneal macrophages treated with 50 U/ml gamma-IFN. Gamma-IFN treatment was initiated 24 hr following phagocytosis. Survival percentage was calculated on the basis of 100% at 0 hr. Bars indicate S.D. within replicates.

CHAPTER 3 - EXPERIMENT 6

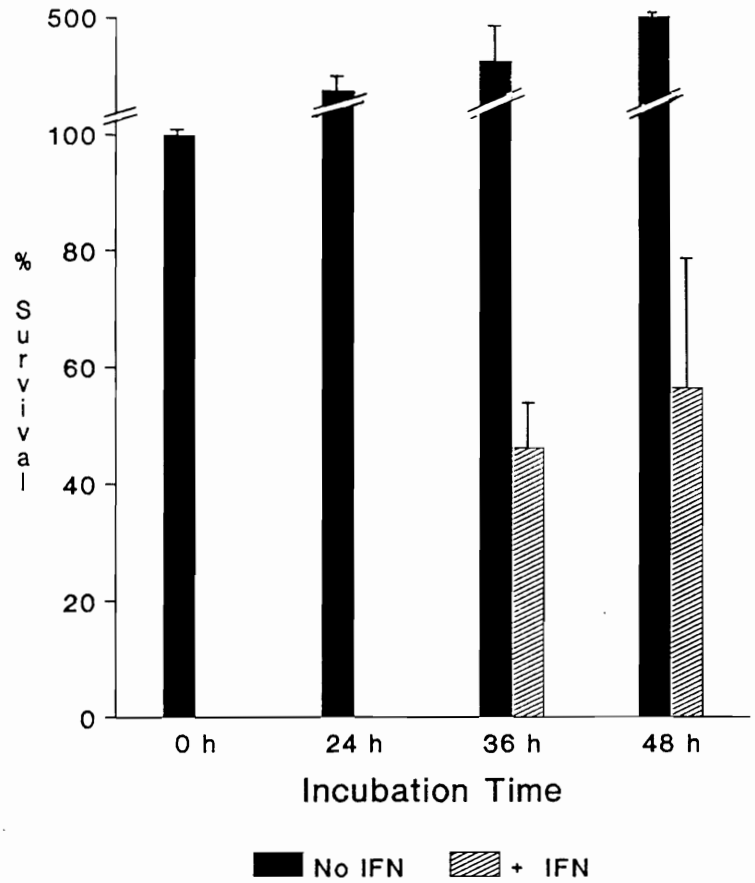

Figure 6.

Mean (+/- S.D.) percentage of strain 2308 organisms surviving in peritoneal macrophages treated with 50 U/ml gamma-IFN. Gamma-IFN treatment was initiated 24 hr following phagocytosis. Survival percentage was calculated on the basis of 100% at 0 hr. Bars indicate S.D. within replicates.

CHAPTER 3 - EXPERIMENT 7

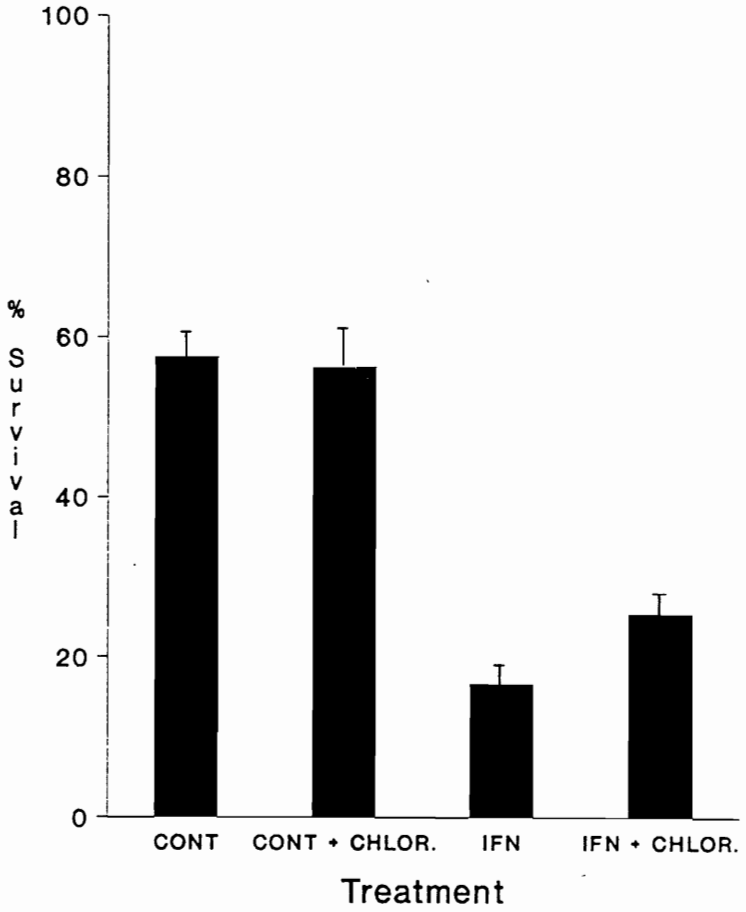

Figure 7.

Effect of inhibition of phagosome-lysosome fusion on the ability of gamma-IFN activated macrophages to kill ingested strain RB51 organisms. Macrophages were treated with chloroquine 30 min prior to phagocytosis and 50 U/ml gamma-IFN was added at the time of phagocytosis. Percentage of organisms surviving at 12 hr following gamma-IFN treatment was determined. Bars indicate S.D. within replicates.

of strain RB51 to about 17% of zero hr values. When gamma-IFN was added to macrophages pretreated with chloroquine, the effect of macrophage activation is still obvious. Strain RB51 organisms are seen to be susceptible to gamma-IFN activation even in the presence of chloroquine. The level of survival, however, is about 26% which is greater than that in macrophages not treated with chloroquine.

The effect of chloroquine on the ability of gamma-IFN activated macrophages to kill ingested strain 2308 organisms (experiment 8) is depicted in Figure 8. A trend similar to that observed with strain RB51 is seen here. Chloroquine does not have adverse effects on the growth of strain 2308 in peritoneal macrophages. Results obtained also indicate that gamma-IFN activated macrophages are equally effective at restricting the growth of strain 2308 whether or not they are treated with chloroquine. When macrophages are treated with gamma-IFN alone, the number of Brucella organisms surviving are about 43%; and when chloroquine treated macrophages are activated with gamma-IFN, the survival percentage is about 41% of the corresponding control values.

The ability of P388D1 cells to ingest both rough and smooth strains of B. abortus have been observed in this investigation. Figure 9 is an electron micrograph of a P388D1 cell with ingested strain RB51 organisms. Apparently intact bacterial cells can be seen inside membrane bound vesicles of the macrophages. Similarly, ingestion of smooth strain 19 is depicted in the electron micrograph shown in Figure 10. It was not possible to determine if the apparent rupture of the phagosome seen in Figure 10 was due to an artifact or direct

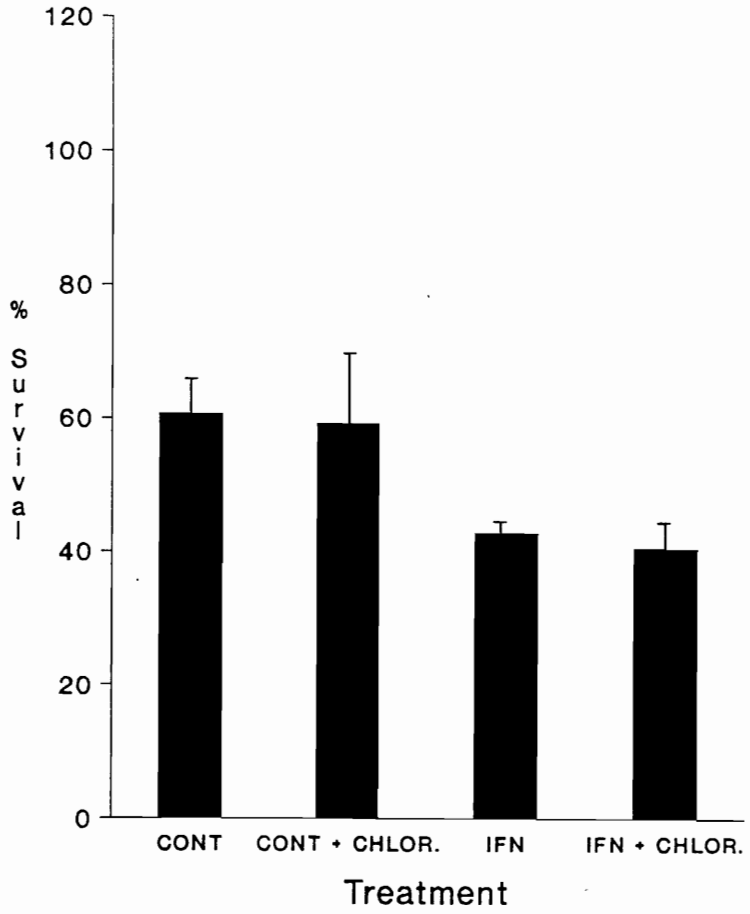

Figure 8.

Effect of inhibition of phagosome-lysosome fusion on the ability of gamma-IFN activated macrophages to kill ingested strain 2308 organisms. Macrophages were treated with chloroquine 30 min prior to phagocytosis and 50 U/ml gamma-IFN was added at the time of phagocytosis. Percentage of organisms surviving at 12 hr following gamma-IFN treatment was determined. Bars indicate S.D. within replicates.

CHAPTER 3 - EXPERIMENT 9

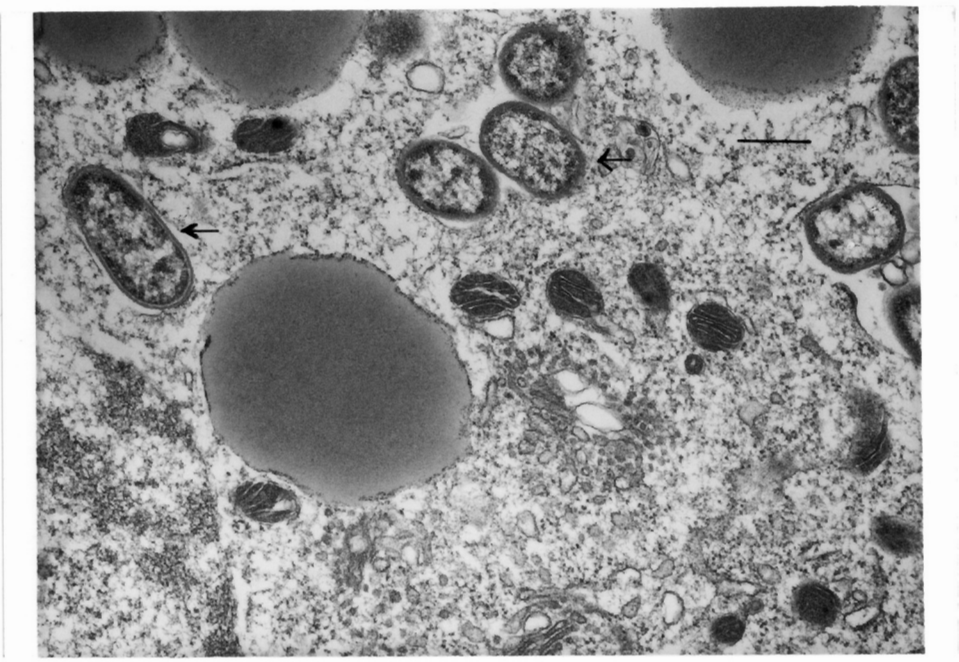

Figure 9.

Electron micrograph showing ingestion of B. abortus strain RB51 (⟶) organisms by P388D1 cells. (magnification= 19 000 X). Bar represents 1000 nm.

CHAPTER 3 - EXPERIMENT 10

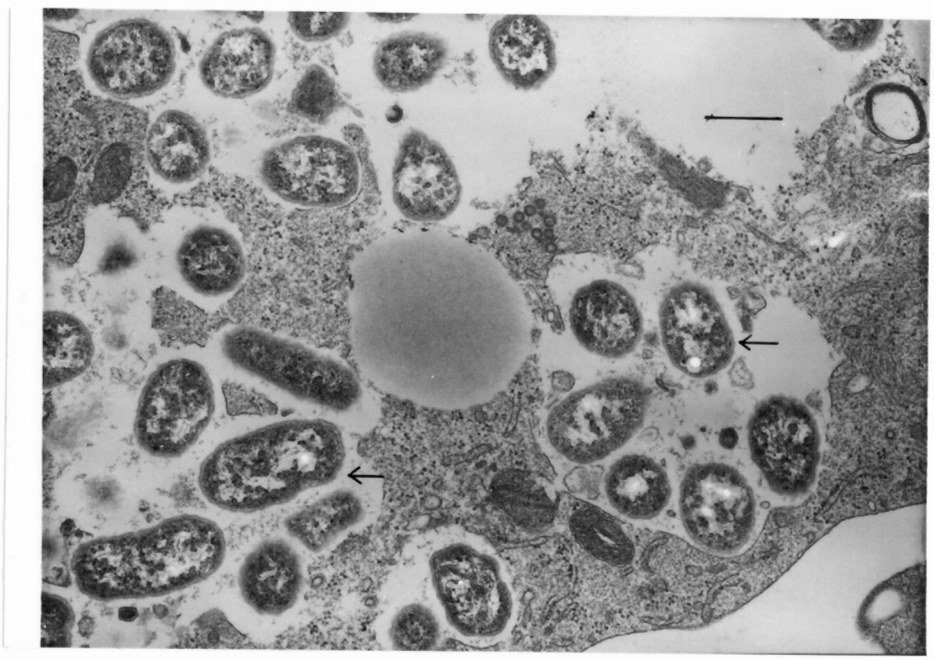

Figure 10.

Electron micrograph showing ingestion of B. abortus strain 19 (⟶) organisms by P388D1 cells. (magnification= 19 000 X). Bar represents 1000 nm.

action of B. abortus strain 19.

The ability of P388D1 cells to kill both rough and smooth strains of B. abortus are shown in Tables 1 and 2. Table 1 shows the number of rough organisms surviving inside these cells at various intervals upto a period of 8 hr following phagocytosis. In the absence of chloroquine, the number of rough organisms surviving are very low even at the start of the assay. This difference in the numbers of surviving bacteria persists up to 8 hr following phagocytosis. Survivability of the rough organisms is affected even in the presence of chloroquine throughout the assay.

Similar results were obtained with strain 2308 as depicted in Table 2. P388D1 cells are able to kill the smooth organisms at the 0 hr interval of the assay. At the end of 8 hr, the macrophages continue to kill more numbers of the bacteria in the absence of chloroquine. A reduction in the number of surviving organisms is also seen in the presence of the drug at all intervals of the assay.

DISCUSSION

Opsonization of Brucella has been shown to be necessary for phagocytosis (63). In these experiments, monoclonal antibodies BRU 48 and BRU 38 were used to opsonize rough and smooth strains respectively. Western blot analysis (Figure 1) demonstrates that BRU 48 reacts with the core region of RB51 WC antigen.

The surface location of the core on strain RB51 is further substantiated by the absorption experiments (Figure 2). Absorption of

CHAPTER 3 - EXPERIMENT 11

Table 1.

NUMBER (S.D.) OF COLONY FORMING UNITS OF STRAIN RB51

HOURS AFTER PHAGOCYTOSIS	CHLOROQUINE +	CHLOROQUINE -
0	12500 (3415)	3000 (1080)
2	4925 (1374)	1000 (405)
4	2500 (1248)	875 (475)
8	1750 (957)	325 (200)

CHAPTER 3 - EXPERIMENT 12

Table 2.

NUMBER (S.D.) OF COLONY FORMING UNITS OF STRAIN 2308

HOURS AFTER PHAGOCYTOSIS	CHLOROQUINE +	CHLOROQUINE -
0	17750 (4573)	2325 (880)
2	8750 (577)	790 (210)
4	7750 (3500)	415 (175)
8	4750 (957)	75 (35)

BRU 48 by live strain RB51 abolishes reactivity with both rough and smooth strains in the colony blot ELISA; this suggests that BRU 48 reacts with an antigen(s) on the surface of strain RB51. On the other hand, absorption with live strain 2308 does not result in a similar loss of BRU 48 activity. This suggests that the antigen recognized by BRU 48 is not accessible on the surface of smooth organisms. In spite of the inaccessibility of this antigen, BRU 48 reacts with smooth strain as well as it does with the rough strain. A possible explanation for this could be that in this technique, smooth colonies are lysed by chloroform and therefore expose the antigen which is otherwise naturally exposed in the case of the rough organisms.

Macrophage activation is one of the mechanisms by which T cells mediate their protective function. Activation is primarily achieved by the action of gamma-IFN which is secreted by T lymphocytes in response to an antigen specific stimulation. The importance of gamma-IFN in Brucella immunity was clearly demonstrated when peritoneal macrophages were treated with gamma-IFN at the time of phagocytosis. The growth of strain RB51 was restricted to about 10% of the original numbers at zero time. Rough strain RB51 appears to be highly susceptible to the activation of macrophages by gamma-IFN because at 24 hr, no recovery of surviving organisms was observed compared to the number of organisms in untreated controls (Figure 3) in which the population of strain RB51 started to increase. In the experiments with smooth strain 2308, the effect of gamma-IFN is also evidenced by a restricted growth of strain 2308 in gamma-IFN treated macrophages (Figure 4). This

restriction however, is not as severe and does not persist as in the case of strain RB51. It is apparent that the ability of activated macrophages to kill B. abortus depends on the virulence of the strain under investigation and suggests that activation of macrophages via gamma-IFN is not enough to protect from or eliminate the virulent organisms.

The presence of activated macrophages in murine and bovine brucellosis has been reported by others (14, 113, 114). These studies have included an analysis of both physical as well as functional characteristics including brucellacidal activity of macrophages. There are however no reports on the direct brucellacidal capacity of gamma-IFN activated macrophages. Canning and Roth (27) had looked at the effects of recombinant gamma-IFN in the bovine system both in vitro and in vivo. They noted an enhancement in the capacity of gamma-IFN treated polymorphonuclear leukocytes (PMNs) to kill ingested B. abortus. It was found that PMNs from animals treated with gamma-IFN had better brucellacidal ability than control ones. Similar in vitro activation of macrophages to inhibit growth of intracellular parasites has been observed in other systems like M. tuberculosis, L. monocytogenes, and S. typhimurium (51, 52, 109).

In their investigations using bovine mammary gland macrophages, Adams and coworkers (62, 63, 113) have examined the survival of both rough and smooth strains of B. abortus. They observed that the intracellular survival rates for the smooth virulent strain 2308 was better than the rough strain 45/20. The ability of smooth strains of

B. abortus to replicate better than rough strains within macrophages was also observed in the guinea pig system (18, 65).

This investigation has also demonstrated that invasion of macrophages by B. abortus does not make macrophages refractory to activation by gamma-IFN (Figures 5 and 6). Addition of gamma-IFN to macrophages 24 hr following invasion by B. abortus results in reduced growth for both the smooth and rough strains. In agreement with our earlier experiments, survival of the smooth strain has been found to be much better than the rough strain. This enhanced restriction of strain RB51 growth by IFN activated macrophages may explain why specific T cells are efficient in protecting against RB51 challenge (chapter 2) in the absence of a protective function of specific antibodies (chapter 1).

In fact, the growth of rough strains appears to be severely restricted, suggesting that the activation of strain RB51 infected macrophages may be enough to eliminate this organism. In contrast, although macrophages suppress the replication of B. abortus for a while, the activation of strain 2308 infected macrophages is not enough for their elimination and would call for additional mechanisms for their eventual control. Lysis of infected macrophages by CD8 T lymphocytes may therefore be an important component in this aspect.

Macrophages mediate their bactericidal effects through an oxygen-dependent or oxygen-independent mechanism. Activation of macrophages by gamma-IFN can result in enhanced killing of intracellular pathogens by either an oxygen dependent (109) or oxygen independent (11, 51, 69)

mechanism. Results from this investigation suggested that both the rough strain RB51 and smooth virulent strain 2308 are susceptible to an oxygen-dependent killing by gamma-IFN activated macrophages. Similar observations were made by Canning and Roth (27) with bovine PMNs where they conclude that gamma-IFN activation leads to enhanced killing of B. abortus by an oxygen-dependent mechanism.

P388D1 cells were shown to ingest both rough strain RB51 and smooth strain 2308 following opsonization. Use of such a cell line may aid in analyzing different isotypes of antibodies specific to B. abortus which may have a role in macrophage-B. abortus interactions. The ability of P388D1 cells to kill both strains RB51 and 2308 is in sharp contrast to that observed with peritoneal macrophages. Killing of B. abortus is inhibited by use of chloroquine, suggesting that the lysosome plays an important role in brucellacidal activity of P388D1 cells. This is in contrast to what was observed with peritoneal macrophages where gamma-IFN activation resulted in an oxygen-dependent killing of both rough and smooth strains. The mechanisms employed by P388D1 lysosomes, however, does not differentiate between the rough strain RB51 and the virulent smooth strain 2308.

SUMMARY

Vaccination of mice with B. abortus rough strain RB51 is known to protect against challenge infection with B. abortus smooth strain 2308 (23). In order to understand the mechanisms involved in this protection, the contributions of humoral and CMI responses induced by strain RB51 were analysed. This study included examination of the ability of strain RB51 immune serum to passively protect against both the homologous rough strain and the heterologous smooth strain 2308. Immune serum obtained from mice vaccinated twice with strain RB51 was transferred to recipient mice which were then challenged with either strain RB51 or strain 2308. Experiments were also performed using serum from mice infected with strain 19, the current vaccine strain, for comparing its protective abilities with that of strain RB51.

The results demonstrated that RB51 immune serum did not have the ability to protect against challenge with either strain RB51 or 2308. Immune serum obtained from mice vaccinated with strain 19 did not show any protection against strain RB51 but protected against challenge infection with strain 2308. Therefore, the conclusion made by others (4, 83, 94) with regard to the protective ability of strain 19 immune serum and particularly, O-side chain specific antibodies, against strain 2308 is supported by these results. The lack of protection of strain RB51 immune serum against challenge with strain 2308 can be explained by the lack of O-side chain antibodies; however, the lack of protection against strain RB51 remains unexplained. A possible reason

could be that the large challenge dose used, could have overshadowed the protective ability of this serum. It is also possible that specific antibody isotypes important in protection may not have been induced by vaccination with strain RB51 or that isotypes which are not protective were induced (42).

In the absence of strain RB51 immune serum providing protection against challenge with strain 2308, the CMI mechanisms induced by strain RB51 were investigated. This included examination of nylon wool enriched T lymphocytes obtained from mice vaccinated with strain RB51. When mice were vaccinated twice with strain RB51, immune T cells were able to transfer protection against both strain RB51 and 2308. In comparison, mice vaccinated twice with strain 19 developed protective immune cells against strain 2308 and not against strain RB51. In contrast, mice vaccinated once, 6 weeks prior to transferring immune cells, developed immune cells with the ability to protect against both strain RB51 and 2308. This difference in the protective ability of strain 19 immune T cells could be due to one of the following two reasons: i) The type and/or specificity of strain 19 immune T cells providing protection against strain 2308 may be different from those needed to provide protection against strain RB51. ii) the antigens presented by macrophages of challenge infected recipients may be different in the two situations.

Mice receiving strain 19 immune serum and strain RB51 immune cells were protected when challenged with strain 2308. The protection observed was found to be an additive effect since it was greater than

that observed with either immune serum or immune cells alone. Enhanced protection provided by immune cells following *in vitro* activation has been noted by others (7, 8, 15, 35). In this investigation, strain RB51 immune T cells were activated *in vitro* with antigen, cytokines like IL-2 and gamma-IFN, and stimulating agents like PMA and ionomycin. Activation of non-immune control T cells in the presence of RB51 antigen, cytokines and stimulating agents resulted in significant protection against strain 2308, over non-activated control cells. Similarly, strain RB51 immune T cells activated with PMA, ionomycin, gamma-IFN, and IL-2 showed significant protection which was greater than that observed with stimulated non-immune cells. In addition, when strain RB51 immune cells were activated in the presence of RB51 antigen, PMA, ionomycin, gamma-IFN, and IL-2, the level of protection was the highest and was significantly different from protection provided by RB51 cells activated in the absence of the antigen.

One of the mechanisms by which T cells provide protection against intracellular pathogens is by gamma-IFN mediated activation of macrophages. This study examined the brucellacidal activity of macrophages which were activated *in vitro* with recombinant gamma-IFN. When macrophages were activated with gamma-IFN, they could kill ingested *B. abortus* organisms. This was true for both rough strain RB51 and smooth virulent strain 2308. However, activated macrophages did exhibit differences in their ability to contain the intracellular growth of the two strains. The rough strain RB51 was shown to be more susceptible to macrophage killing than was the smooth virulent strain

2308. Furthermore, gamma-IFN activated macrophages retained their Brucella killing ability even after treatment with chloroquine. This suggested that macrophages can kill either rough or smooth B. abortus strains by an oxygen-dependent mechanism. However, as in other experiments, the smooth strain survived better than the rough strain. This suggests that the difference in intracellular survival ability among the strains could be one of the reasons why strain 2308 continues to persist in infected animals while strain RB51 is efficiently cleared.

The possibility that invasion of macrophages by B. abortus could render the macrophages unresponsive to gamma-IFN mediated activating stimuli was also tested. Macrophages were allowed to ingest both rough and smooth strains of B. abortus and the ingested bacteria were allowed to grow unrestricted for 24 hr. Following this, macrophages were activated with gamma-IFN and the resultant effect on intracellular survival of the two strains was examined. Results demonstrated that in spite of intracellular growth of B. abortus for 24 hr, peritoneal macrophages were still responsive to the gamma-IFN activation signals. On treatment with gamma-IFN, these macrophages were able to kill both rough and smooth strains of B. abortus although the rough form was more susceptible than the smooth strain

This investigation also included a study of Brucella-macrophage interactions using a macrophage like cell line P388D1. Results demonstrated that P388D1 cells were able to ingest both strain RB51 and 2308. Furthermore, unlike in the case of peritoneal macrophages,

these cells were able to kill both the rough and smooth organisms following ingestion and there was no difference in the abilities of the two strains to survive in these cells. It therefore appears that B. abortus organisms are definitely susceptible to macrophage bactericidal effects but the exact mechanism remains to be explored.

It is possible to conclude from this investigation that strain RB51, a rough strain of B. abortus protects against a homologous and heterologous challenge by inducing a CMI response. Experiments on macrophage-Brucella interactions carried out in this study suggest that the effector arm of this CMI response, at least in the case of RB51 challenge, could possibly be the activation of macrophages by gamma-IFN. This mechanism nevertheless may not be able to account for the total elimination of strain 2308.

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APPENDIX

Table 1. Data from experiment 3, chapter 1

Log number of bacteria per spleen

Serum X Challenge

Control X RB51	RB51 serum X RB51	Abs. RB51 serum X RB51
4.954	4.748	4.684
4.710	4.778	4.390
4.721	4.329	4.622
4.787	4.857	4.572
4.623	4.437	4.691

Control X 2308	RB51 serum X 2308
6.278	5.687
5.899	5.811
5.928	5.783
6.000	5.783
6.093	5.811

Control X RB51	Strain 19 serum X RB51
4.539	5.222
4.587	4.903
4.650	4.892
4.572	4.941
4.595	4.928

Control X 2308	Strain 19 serum X 2308
6.204	3.609
6.315	3.342
6.286	3.710
6.355	3.548
6.301	3.514

Table 2. Data from experiment 1, chapter 2

Log number of bacteria per spleen

T cells X Challenge

Control X RB51	RB51 T cells X RB51
4.954	3.518
4.710	3.427
4.721	2.826
4.787	3.427
4.623	3.669

Control X 2308	RB51 T cells X 2308
6.426	5.392
6.623	5.342
6.222	5.426
6.146	5.125
6.477	5.186
6.278	4.301
5.899	5.028
5.928	3.820
6.000	5.050
6.093	4.669

Table 3. Data from experiment 2, chapter 2

Log number of bacteria per spleen

T cells X Challenge

Control X RB51	Strain 19 T cells X RB51
4.968	3.903
4.934	3.934
4.724	4.000
4.663	3.778
4.602	2.000
Control X 2308	**Strain 19 T cells X 2308**
6.415	4.700
6.286	5.693
6.315	5.514
6.487	5.699
6.380	5.301
6.146	5.447
6.124	5.342
6.053	4.602
6.146	5.539
6.124	
Control X RB51	**RB51 T cells X RB51**
4.496	3.522
4.514	3.602
4.467	3.527
4.602	3.427
4.505	3.670
4.522	3.477
4.861	3.185
4.616	3.643
4.531	3.690
4.458	3.565
Control X 2308	**RB51 T cells X 2308**
6.505	5.426
6.166	5.355
6.329	4.778
6.125	5.286
6.186	5.362

Table 4. Data from experiment 3, chapter 2

Log number of bacteria per spleen

Serum/T cells X Challenge

Control X 2308	Strain 19 serum X 2308
6.426	3.758
6.623	3.587
6.222	3.609
6.146	3.477
6.477	3.426
RB51 T cells X 2308	**Strain 19 serum + RB51 T cells X 2308**
5.392	3.777
5.342	3.601
5.426	3.952
5.125	3.952
5.186	3.555

Table 5. Data from experiment 5, chapter 2

Log number of bacteria per spleen

T cells X 2308 Challenge

Control	Control T cells activated without antigen	Control T cells activated with antigen
6.125	6.400	5.531
6.330	6.082	5.616
6.270	6.330	5.727
6.125	6.450	5.681
6.368	6.400	5.656

RB51 T cells activated without antigen	RB51 T cells activated with antigen
5.186	4.602
5.342	4.938
5.315	4.824
5.315	4.523
5.286	4.125

Table 6. Data from experiment 3, chapter 3

Number of colony forming units of strain RB51 in peritoneal macrophages

Time	Gamma-IFN	
	-	+
0 hr	468000	366000
	382800	498000
12 hr	119400	45000
	166200	31800
24 hr	222000	28200
	183000	38400

Table 7. Data from experiment 4, chapter 3

Number of colony forming units of strain 2308 in peritoneal macrophages

Time	Gamma-IFN	
	-	+
0 hr	16980	14280
	14640	12600
12 hr	10323	5640
	9000	6480
24 hr	22800	4980
	20100	5162

Table 8. Data from experiment 5, chapter 3

Number of colony forming units of strain RB51 in peritoneal macrophages

Time	Gamma-IFN	
	-	+
0 hr	700000	
	533333	
24 hr[a]	166667	
	183333	
36 hr	933333	34800
	1116667	31800
48 hr	2666667	28320
	2500000	26400

a. 50 U/ml gamma-IFN was added at this stage

Table 9. Data from experiment 6, chapter 3

Number of colony forming units of strain 2308 in peritoneal macrophages

Time	Gamma-IFN	
	-	+
0 hr	170000	
	148333	
24 hr[a]	316667	
	450000	
36 hr	716667	66667
	366667	83333
48 hr	816667	66667
	800000	116667

a. 50 U/ml gamma-IFN was added at this stage

Table 10. Data from experiment 7, chapter 3

Number of colony forming units of strain RB51 in peritoneal macrophages

Treatment	0 hr	12 hr
Control	250000	143333
	180000	100000
+ Chloroquine	256667	143333
	189999	106667
+ Gamma-IFN	250000	57777
	180000	50000
+ Chloroquine Gamma-IFN	256667	35000
	189999	39999

Table 11. Data from experiment 8, chapter 3

Number of colony forming units of strain 2308 in peritoneal macrophages

Treatment	0 hr	12 hr
Control	16800	10200
	14400	9000
+ Chloroquine	14100	10200
	12600	6300
+ Gamma-IFN	16800	5700
	14400	6000
+ Chloroquine Gamma-IFN	14100	5280
	12600	5520

VITA

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