THE PREGNANT MOUSE MODEL OF BRUCELLOSIS: THE PATHOLOGY AND PROTECTION STUDIES COMPARING *BRUCELLA ABORTUS* STRAINS 2308, 19 AND RB51

by

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(ABSTRACT)

Brucellosis caused by *Brucella abortus* is an important zoonotic disease characterized in cattle by placentitis, fetal death and abortion. Brucellosis research employing the pregnant mouse model has been limited to the bacteriology and immunology of the disease.

Studies reported in this dissertation characterized the pathology and serology of one virulent (2308) and two attenuated (19 and RB51) strains of *B. abortus* in the pregnant mouse. When $10^{5.7}$ strain 2308 organisms were administered intraperitoneally to BALB/c mice in midgestation, by 9 days post-inoculation they consistently produced a severe, necro-suppurative placentitis frequently asociated with fetal death. Sequential examination of the placenta during the second half of gestation revealed infection of the trophoblast giant cells and visceral yolk sac. As occurs in the cow, brucellae localized within the rough endoplasmic reticulum of the trophoblast cells. An inoculum of $10^{7.5}$ strain 19, the current vaccinal strain, produced a similar lesion. Both strains 2308 and 19 induced antibodies against lipopolysaccharide O-side-chain as monitored by serum agglutination and western blot analysis. Inoculation of $10^{9.5}$ strain RB51 brucellae, a stable rough organism, produced minimal placentitis

and did not induce fetal death or anti-O-side-chain antibodies as assessed by serum agglutination or Western Blot analysis.

Mice vaccinated prebreeding with 10^6 strain 19 demonstrated excellent protection against midgestational challenge by $10^{5.7}$ strain 2308 organisms as assessed by lesion development and infection of spleens and placentas. Vaccination with 10^8 strain RB51 produced a lesser, although significant, degree of protection against infection and prevented severe lesions and fetal death. Two inoculations of strain RB51 improved protection against placental infection.

The intravenous transfer of strain 19 antiserum or monoclonal antibodies directed against the O-side-chain one hour before challenge with brucellae provided protective immunity to the pregnant mouse. In contrast, antiserum against strain RB51 was not protective, indicating the importance of cell-mediated rather than humoral immunity in protection afforded by vaccination with strain RB51.

These experiments suggest that the pregnant mouse is an appropriate model to study the immunopathology of brucellosis. It also supports the development of strain RB51 as a vaccine for bovine brucellosis, offering protection against infection without eliciting anti-O-chain antibodies that confound the serodiagnosis of infection with virulent smooth strains.

DECLARATION OF WORK PERFORMED

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

Western blot analysis was performed by Ms. Jan Simmers at the Veterinary Medical Research Center. Protein analysis of the monoclonal antibody, BRU 38, was performed by Ms. Patsy Dillon-Long, also at the Research Center. Placental samples for light microscopy were collected by myself and processed by technicians at the Histopathology Laboratory with both H & E and Giemsa stains. Ms. Amelia Walton at that laboratory modified and performed the immunohistochemical staining techniques used for this study. Placental samples for electron microscopy were collected and processed by me but sectioned both by myself and by Ms. Kathy Hayman at the EM facility of the Veterinary College. Immunogold staining on grids was performed by technicians under the direction of Dr. Norman Cheville, National Animal Disease Center in Ames, Iowa.

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CHAPTER 1

INTRODUCTION:

THE HISTORICAL PERSPECTIVE AND HYPOTHESIS

1.1 Historical perspective

The bacterium, Brucella abortus is the principal cause of bovine brucellosis in which chronic infection is characterized by abortion and decreased milk yield in the cow and orchitis and epididymitis in the bull. Brucellosis in human beings is a serious, debilitating disease characterized by fever with diurnal variation, myalagia and arthritis (Marston, 1861; Bruce, 1889; Huddleson, 1943; Spinks, 1956; Madkour, 1989). Human disease is an accidental expression of a much more widespread disease in animals and is always traceable to exposure to infected animals or their products. Although bovine brucellosis has been erradicated or controlled in many developed countries, by huge expenditures of effort and funding, its prevalence has actually increased in many developing countries as they have attempted to build-up dairy industries without implementation of brucellosis control programs (Thimm, 1982). Attempts to estimate the true prevalence of human brucellosis world-wide are frustrated because adequate information on the occurrence of the disease is not available in many countries but the incidence of human brucellosis now probably shows little net improvement on a world-wide basis over the situation thirty years ago (Corbel, 1989). In a recent comprehensive review on brucellosis in man and animals, Nicoletti (1990) concludes that brucellosis continues to have global importance, remaining a highly

relavent human health problem and annually causing serious losses in the livestock industry in affected areas.

Though according to Hughes (1897), a disease consistent with brucellosis had been described as early a 460 BC by Hippocrates, the first accurate description of what was called "Mediterranean gastric remittent fever" was made by Marston in 1861. An assistant surgeon in the British army stationed in Malta, he wrote a clear and detailed account based on personal experience after he contracted the disease himself. Human brucellosis had many synonyms derived from its resemblance to typhoid fever and malaria (intermittent typhoid, typho-malarial fever), its remittent character (undulant fever), its duration (common continued fever), and its geographic location (Malta fever, Mediterranean Fever, Cyprus fever).

Epidemic abortion in cattle was recognized in the United States as early as 1864 and was regarded as a contagious disease (Huddleson, 1943). In 1895 Bernard Bang, a Danish veterinarian, recovered the bacterium then described as Bacillus abortus from the placenta of an aborted cow. He and his associate Stribolt showed that the inoculation of pregnant heifers with this organism induced abortion (Bang, 1897). At the time, Bang's discovery was of interest only to veterinarians and livestock producers and no correlation was made between "Bang's Disease" in cattle and what was popularly called "Malta fever" in humans. The disease was first recognized as a zoonosis by Zammit in 1905, who discovered that goats were the source of infection for Malta fever. In 1918 the name "brucellosis" was suggested for both diseases by Alice Evans, an American microbiologist, upon identifiying Bacillus abortus in cow's milk and

reporting the close relationship of this bacterium to Micrococcus melitensis, which had been earlier described by David Bruce in 1887 as the cause of "undulant fever". Evans proposed the genus Bacterium for both bacteria as she noted no distinction could be made between the two on the basis of the agglutination test. This relationship was confirmed by Meyer and Shaw (1920) who were the first to suggest the current nomenclature for the genus, Brucella, to recognize the work of David Bruce.

Brucellosis in pregnant ruminants is characterized by placentitis, intracellular replication of Brucella abortus in trophoblasts, large numbers of B. abortus in placental tissues, fetal death and abortion (Smith, 1919; Hallman, 1924; Payne, 1959; Molello, 1963a; Alexander et al., 1981; Jubb et al., 1985). The characteristic location of B. abortus within the chorioallantoic trophoblast cells of cattle was first reported by Smith in 1919 and later further described in cattle (Hallman, 1924; Payne, 1959; Meador and Deyoe, 1989) sheep (Molello, 1963a) and goats (Anderson et al., 1986 a & b). The pathology of the gross and histologic lesions of ruminant placentitis due to B. abortus have been well described (Hallman, 1924; Huddleson, 1943; Payne, 1959; Molello et al., 1963a; Anderson et al., 1986 a & b).

In bovine brucellosis B. abortus establishes infection both within the pregnant reproductive tract as well as within tissues which contain a high proportion of phagocytic cells of the reticuloendothelial system (Doyle, 1935; Payne, 1959; Jubb, et al. 1985; Enright, 1990). Anderson and associates (1986 a & b) examined more closely the pathogenesis of the reproductive tract lesions in the ruminant. The intracellular location of brucellae colonizing the placenta of the goat was described, revealing

an apparently unique relationship to the host enabling it to survive and multiply preferentially within the rough endoplasmic reticulum of the trophoblast cell. This same intracellular location was later described in the cow as well (Meador and Deyoe, 1989). Mechanisms of cell selectivity, cell entry and intracellular localization remain speculative.

The mouse experimental model of brucellosis has been used extensively to investigate both the immune response to and the pathogenesis of systemic infection in the non-pregnant animal (Feldman and Olsen 1935; Young et al., 1979; Ho and Cheers 1982; Phillips et al., 1989b; Pugh et al., 1989). Although the pregnant mouse model offers an economic and convenient alternative which has been employed by investigators of other placental pathogens (Buzoni-Gatel and Rodolakis 1983; Redline and Lu, 1987, 1988, 1989; Tuffey et al., 1987) research into the immunopathology of brucellosis in the pregnant animal primarily has utilized the ruminant. The pregnant mouse model of brucellosis has been used only to study some aspects of the bacteriology and immunology involved with infection (Bosseray, 1980, 1982, 1983a). In 1957 Payne described placental lesions in the rat given unquantified doses of B. abortus during gestation. Later studies reporting the preferential growth of B. abortus within the murine placenta described the bacteriology but did not include histologic examination of infected placentas and concluded that infection did not alter the course of pregnancy in the mouse (Bosseray, 1980, 1982, 1983a).

Researchers have shown that protective immunity to systemic infection by brucellae involves both humoral and cell-mediated immunity (Cheers, 1984; Winter et al., 1988; Araya et al., 1989, 1990). However, the relative and temporal contribution of the antibody or cellular reponse

toward protection and eventual elimination of the bacteria remains unclear. The immune response within the environment of the gravid uterus is exquisitely tuned to allow the survival of the fetal allograft. Recent investigation of listeriosis in the pregnant mouse suggested that local immune suppression to prevent fetal rejection may also suppress the immune response to this intracellular pathogen (Redline and Lu, 1987). Unchecked preferential growth of this intracellular pathogen occured within the placenta and resulted in trophoblast cell necrosis and fetal death. In contrast to the predominantly mononuclear cell infiltration of most organs associated with systemic Listeria monocytoges infection, placental inflammatory cell infiltrates were primarily neutrophilic, again suggesting modification of the immune respone at the feto-maternal interface. Study of the local immune response to placental infection by Brucella may help clarify factors involved in fetal survival as well as those pertinent to effective disease control, particularly host susceptibilty and resistance to disease.

A significant proportion of the current research effort on bovine brucellosis is directed at defining the constituents of protective immunity in order to develop a more effective vaccine. Strain 19, an attenuated smooth strain of B. abortus, is widely used as a live vaccine in brucellosis control programs. Though use of this vaccine has significantly reduced the level of brucellosis in the United States and other developed countries, strain 19 has a number of disadvantages which have prompted further efforts at vaccine development. These include the ability to cause abortion in pregnant cattle (Birch et al., 1943) and serious disease in those who are accidently infected when administering

the vaccine (Gillman, 1944; Reviah, et al., 1961), incomplete protection of vaccinates against virulent field strains (Manthei, 1959), and possession of antigens in common with virulent field strains resulting in confusing serologic reactions (Nicoletti, 1981). Vaccination of cattle over one year of age with strain 19 may result in persistent antibody titers that cannot be distinguished easily from those resulting from natural infection when tested by serological tests commonly used to monitor cattle herds for Brucella infection (Subcommittee on Brucellosis Research, 1977).

The antibody response monitored by the serum agglutination tests is directed almost totally against the specific O-side-chain of the lipopolysaccharide of B. abortus (Diaz et al, 1968; Schurig et al., 1981). Recently, a stable rough mutant, strain RB51, has been developed which is virtually devoid of O-side-chain polysaccharide (Schurig et al., 1991). Intraperitoneal inoculation of mice with this strain does not induce sufficient quantities of antibodies against the O-side-chain to interfere with the interpretation of serum agglutination tests. Rough strain variants of Brucella spp., including partially rough strains of B. abortus and the fully rough species, B. ovis and B. canis, are less virulent than smooth organisms that possess the entire O-side-chain (Meyer, 1990). Strain RB51 has reduced virulence in the non-pregnant mouse and protects non-pregnant mice against challenge with virulent, smooth B. abortus strain 2308 (Schurig et al., 1991). Preliminary in vitro work with bovine placental explants suggested reduced virulence of strain RB51 for the reproductive tract of cattle (Enright, 1990) and limited vaccine trials in cattle indicated that strain RB51 may also protect cattle against challenge by strain 2308 (Enright et al., 1990b). Strain

RB51, therefore, appears to have significant potential as a vaccine for bovine brucellosis. In light of the current emphasis on the study of the immunopathology of brucellosis aimed particularly at vaccine development, investigations were made to test several hypotheses.

1.2 Hypothesis

The hypotheses to be tested were:

1. That the pregnant mouse would be an appropriate model of bovine brucellosis caused by virulent, smooth B. abortus with similarities in pathology, microbiology and immunology.

2. That immunization with B. abortus strain RB51 does not induce anti-O-side-chain antibodies in the pregnant mouse and will protect mice against a virulent smooth strain inoculated in midgestation.

To test these hypotheses, the following experiments were planned:

a. characterize the placental colonization by virulent B. abortus strain 2308 and associated anatomic lesions and investigate their pathogenesis,

b. define aspects of the serological response of the pregnant mouse to Brucella infection,

c. compare the serology, virulence and pathogenicity of B. abortus strains 2308, 19 and RB51 in the pregnant mouse,

d. examine the protection afforded the pregnant mouse by vaccination with attenuated strains 19 or RB51 against challenge with virulent strain 2308.

CHAPTER 2

REVIEW OF THE PATHOLOGY OF BRUCELLOSIS IN THE MOUSE AND OTHER SPECIES

Infections which interfere with the establishment or continuation of a healthy pregnancy, and the subsequent well-being of the young, are of considerable interest and importance in both human and veterinary medicine. In human medicine, the social and humane consequences of these infections have received greatest consideration, while in veterinary medicine the effect on animal production, economics and transmissibility to people are the more important concerns. Indeed, zoonotic diseases bridge these considerations and so an understanding of the pathegenesis of a disease in one species is frequently of importance in solving similar problems in another.

The spectrum of micro-organisms able to affect the reproductive performance of domestic animals is wide and includes viral, fungal, protozoal and bacterial agents. Susceptibility or resistance to a pathogen is determined by acquired specific immunity superimposed on nonspecific or innate host resistance constrained by genotype and modified by a myriad of factors such as nutrition, stress, age, sex and stage of gestation. The outcome of exposure of a susceptible host to a pathogen is also related to factors pertaining to the organism itself, including the route and duration of exposure and dose and virulence of the microbe. Once established within the host, the pathogenicity and tissue tropism of the organism

acting in concert with the immune response of the host, determine the outcome of the infection.

2.1 Brucellosis-The Brucella species

Many successful bacterial pathogens have adopted an intracellular habitat to aquire needed environmental trophic factors for replication and to avoid the immune defenses of the host. The brucellae are facultative intracellular pathogens of a number of wild and domestic animal species and human beings. World-wide in distribution, brucellosis is an important cause of reproductive failure in domestic food animals and also causes serious chronic human illness (Subcommittee on Brucellosis Research, 1977). Currently, the genus Brucella is not subsumed to any family of bacteria but is defined as a group of cocco-bacilli ranging from 0.6 to 1.5 microns long by 0.5 to 0.8 micron wide, with the coccoid forms predominating in vivo (Corbel and Brinley-Morgan, 1984). The genus is currently divided into six species on the basis of metabolic patterns; colony morphology; requirement of CO_2 and serum demand for growth in vitro; susceptibility to bacteriophage infection, and host preference and range. Of the six recognized species, three are pathogenic in a single host reservoir; B. canis in dogs, B. ovis in sheep, and B. neotomae in the desert wood rat. In contrast, the three "classical" species of Brucella, (B. abortus, B. melitensis and B. suis) are pathogenic in the preferred reservoirs of cattle, goats and pigs respectively, but occasionally infect and cause disease in other species. All but B. ovis and B. neotomae have been reported causing human disease (Subcommittee on Brucellosis Research, 1977, Meyer, 1990).

Recent research on the genome of this genus utilizing such techniques as DNA/DNA hybridization (Verger et al., 1985) and ribosomal nucleic acid comparison (DeLey et al., 1987) have revealed an exquisite closeness in the genetic relationships between all Brucella species. The suggestion has been made that all are biovars of a single species and should be renamed to reflect this. However, the nomenclature is well entrenched in the literature and the current taxonomic status of the genus remains based on the aforementioned biochemical and epidemiological factors. A reflection of this close genetic relationship among the five species that infect domestic animals is the similarity in the pathogenesis of brucellosis amongst different host species. Brucellosis in domestic animals (and humans) is a chronic disease in which the host develops a systemic granulomatous inflammatory response typically located in the spleen, lymph nodes and other tissues with a prominent reticuloendothelial component. A common theme for the Brucella species is the preferential localization within the male and pregnant female reproductive tracts through which the bacteria exit the body and become available for transmission of the disease. The clinical presentation and pathology varies among the host reservoirs, and is also slightly different for each species of brucellae.

The ultrastructural morphology of the brucellae is similar to other Gram-negative bacteria (Cherwonogrodsky et al., 1990). The cell envelope of this non-motile, non-sporulating organism is composed of a cytoplasmic membrane, a periplasmic space, a peptidoglycan layer and an outer cell membrane composed of phospholipid, protein and lipopolysaccharide (Fig. 1a). The lipopolysaccharide (LPS) molecule, also known as endotoxin, is

a particularly important component of the outer membrane and responsible for many of the biological properties of Gram-negative bacteria. LPS consists of lipid A, a glycophospholipid which is the active principle (and the toxic fraction), a constant polysaccharide core region and a specific O-side-chain polysaccharide region of repeating oligosaccaride units (Figs. 1b, 2a). The O-side-chain is situated on the surface of the outer cell membrane and is primarily responsible for the antigenic specificity of the bacterium (Fig. 2a). The component monosaccharide of the Brucella O-polysaccharride antigens is 4-amino-4,6-dideoxy-D-mannose, which exists in both A and M antigens as the N-formyl derivative (Fig. 2b) (Miekle et al., 1989). The A antigen is a linear homopolymer of essentially alpha 1,2-linked 4-6-dideoxy-4-formamido-D-manno-pyranosyl residues (Caroff et al., 1984b) in contrast to the M antigen which is a linear pentasaccharide repeating unit composed of four alpha 1,2- and one alpha 1,3-linked sugars (Bundle et al., 1987 a & b, 1989). The O-side-chain polysaccharide of smooth strain of B. abortus consists of 98 percent A antigen with only a small number (2 percent) of alpha 1,3 linkages (Miekel et al., 1989). Strains incapable of completing the "O" antigen polysaccharide or core are referred to as rough strains because they tend to form non-smooth or rough colonies in vitro (Zinsser, 1988). Of the six species, B. abortus, B. melitensis, B. suis and B. neotomae occur primarily in the smooth form, while B. canis, and B. ovis have the rough morphology. The polysaccharide component of the LPS apparently plays an important role in virulence in vivo because smooth isolates tend to have a broader host range than rough or semi-rough variants. This may be due to differing abilities of the variant strains to penetrate

mucosal surfaces of the host, or to resist the immune mechanisms of the host as influenced by the composition of the surface of the cell envelope (Rulter, 1988). The biological activities of endotoxin are diverse and likely pertinent to the pathogenesis of disease caused by the brucellae and will be discussed in conjunction with the pathology of brucellosis.

2.2 Epidemiological importance of Brucella abortus

Brucella abortus is the principal agent of brucellosis in cattle and has been isolated also from goats, sheep, pigs, dogs, horses and a number of wildlife species including bison, elk, caribou and numerous Asian and African ungulates (see Davis, 1990; Crawford et al., 1990 for recent extensive reviews of the epidemiology of B. abortus). Human infection with B. abortus is a major health problem wherever the organism is prevalent in the domestic animal population. It occurs world-wide except for a few countries where the bovine disease has been eradicated. Human beings usually acquire the infection through direct or indirect contact with infected animals or their products and are generally considered terminal hosts not involved in transmission of the disease (Nicoletti, 1989). Cattle or other bovidae are the usual reservoir host and source of infection for people and occupational contact with the products of abortion or ingestion of raw dairy products are the most common means of acquiring the disease.

2.3 Epidemiology and pathogenesis of B. abortus in several species

The bacteria responsible for bovine brucellosis or "Bang's disease" was originally isolated and described as a Bacillus by Bang and Stibolt (Bang, 1897). The organism was recovered as an almost pure culture from

the exudate in the intervillous space of a placenta from an aborted cow. Further, they showed that the inoculation of pregnant heifers with this organism induced abortion. Since that time, the pathogenesis of disease due to this organism, later named Brucella abortus by Meyer and Shaw (1920), has been studied by numerous workers (Hallman, 1924; Huddleson, 1943; Payne, 1959; Molello et al., 1963a; Anderson et al., 1986 a & b). In its reservoir host, B. abortus infection is characterised by abortion and reduced milk yield in the cow and orchitis and epididymitis in the bull (Huddleson, 1943; Subcommittee on Brucellosis Research, 1977). The following summary of the pathogenesis of brucellosis will be limited to manifestations of the the disease in the cow, the species gender most important in the transmission and maintainance of the infection.

The exposure of susceptible cows to B. abortus is the epidemiological genesis of the disease process. Compared to many other non-sporulating bacteria, B. abortus has a substantial ability to survive and persist in the environment under suitable conditions (up to six month in cool, shaded soil) (Wray, 1975). Infected cows shed very large numbers of bacteria in uterine discharges at parturition. Bacteria are also shed at abortion and in the milk (Alexander et al., 1981). Ingestion from a contaminated environment is considered the most common means of exposure and infection for cattle (Nicoletti, 1984). Conjunctival infection may be important also under conditions of confinement, as with intensively housed cattle (Russel, 1977). Congenital infection is also of major epidemiologic importance with as many as 20% of live calves born to infected cows persistently infected (Catlin and Sheehan, 1985; Crawford et al., 1986). Although B. abortus infection in bulls may result in the

shedding of bacteria in the semen, the risk of transmitting the disease to cows by natural service is believed small (Rankin, 1965).

As with all pathogens, whether infection results from exposure is determined by a range of factors influencing host susceptibility but a few are particularly noteworthy in brucellosis. Susceptibility is correlated strongly with the onset of sexual maturity. After sexual maturity, age per se, as distinct from age-related exposure to infection, does not appear to be a factor. The effect of gender has not been as well studied but it appears that relative susceptibilty may be that the least susceptible are immature calves, followed by mature bulls and mature cows (Crawford et al., 1990). The non-pregnant bovine uterus is relatively resistant to infection by B. abortus (Bang et al., 1933, Subcommittee on Brucellosis Research, 1977), with susceptibility increasing markedly with advent of pregnancy (Fitch et al., 1939). Susceptibility increases further as the stage of gestation progresses (Crawford et al., 1988). Genetic factors are likely to be involved in the resistance of cattle to brucellosis. Innate differences in susceptibility of various strains of mice to brucellosis have been documented (Ho and Cheers, 1982; Cheers and Ho, 1983; Cannat and Serre, 1984) and experimental trials in cattle involving a variety of bacterial strains and challenge doses have established that a proportion of cattle are naturally resistant to B. abortus infection (Price et al., 1990; Harmon et al., 1985, 1989; Enright, 1990).

Regardless of the route of infection, the general mechanism of pathogenesis is similar. B. abortus must first penetrate a mucosal surface, usually in the digestive tract, to establish infection in the regional lymph nodes within the host. A study investigating the trans-

epithelial migration of B. abortus in ligated ileal loops in calves revealed that the bacteria entered this region of the digestive tract principally by dome lymphoepthelial cell endocytosis and transport to the lamina propria. Direct invasion of the enterocytes did not occur (Ackerman et al., 1988). Passage of the organism through the epithelial barrier results in an acute regional lymphadenitis (Payne, 1959). It is not known if invading brucellae which escape the submucosa to arrive at the regional nodes are carried within phagocytic cells or arrive as free organisms (Enright, 1990). Further evasion of host defenses within these draining nodes results in persistent infection and eventually, in bacteremia. Brucellae are able to survive within neutrophils (Riley and Robertson, 1984) and non-activated macrophages (Harmon et al., 1988) and this intracellular location helps protect them from humoral and cell-mediated bacteriocidal mechanisms during hematogenous spread. Although B. abortus may localize in a variety of tissues during bacteremia (renal cortex, synovial membranes, bone marrow, liver), infection most frequently occurs in the lymphoid tissues, pregnant uterus and mammary gland of the cow (Schroeder and Cotton, 1916; Bang et al., 1933; Doyle, 1935; Ministry of Agriculture and Fisheries, 1977; Bracewell and Corbel, 1980). In the pregnant uterus, infection of the chorionic epithelium leads to a severe, ulcerative placentitis with an associated purulent exudate (Payne, 1959). This exudate is present in the inter- and periplacentomal utero-chorionic spaces and at the base of cotyledonary villi. Chorioallantoic membranes are markedly edematous and display segmental fibrinous vasculitis and multifocal erosion of periplacentomal chorionic epithelium. Trophoblasts lining the base of cotyledonary villi and periplacentomal regions are

filled with brucellae (Meador and Deyoe, 1989). In non-reproductive tract organs, infection results in multifocal granulomas composed primarily of macrophages, lymphocytes and plasmacytes with fewer neutrophils (Payne, 1959).

As Brucella may survive and multiply within cells of the reticuloendothelial system, the persistence of infection within these cells is of primary importance in the evolution of the granulomatous reaction (Thoen and Enright, 1986). In vitro studies show that the brucellae are ingested by mononuclear and polymorphonuclear phagocytes but are partially protected within the phagosome by several mechanisms. The resistance to killing by neutrophils has been correlated with a reduced oxidative response of the phagocyte due to the presence of bacterial lipopolysaccharide in the cell envelope (Kreutzer et al., 1979) or to the release of nucleotides by the bacterium that compromise nuclear function of neutrophils (Canning et al., 1986). Water extracts of virulent B. abortus have been shown to inhibit phagosome-lysosome fusion in unelicited murine macrophages following ingestion of yeast (Frenchick et al., 1985). A recent study suggests that Brucella abortus may lead to a decline in membrane interleukin-1 in co-cultured macrophages and concomitant down regulation of T-lymphocyte proliferation (Splitter and Everlith, 1989). It is well accepted that the eventual clearance of intracellular pathogens involves cell-mediated immunity which depends on the appropriate interactions between specifically sensitized T lymphocytes and macrophages. The mechanisms allowing brucellae to establish chronic infection have yet to be fully defined and are currently still under investigation.

Brucella abortus has a marked predilection for the ruminant placenta. In the acute infection of pregnant cows, up to 85% of the bacteria are located in the cotyledons, placental membranes and allantoic fluid (Smith et al., 1961). Numbers of B. abortus in placental tissue can reach 10^{10} organisms per milliliter of allantoic fluid or 10^{11} to 10^{13} organisms per gram of cotyledon (Alexander et al., 1981). As early as 1919, Smith described the characteristic intracellular location of B. abortus in the chorionic trophoblast cells of the placenta of the cow (Smith, 1919). Intratrophoblastic localization and replication occurs also in B. abortus infection of sheep and goats (Molello et al., 1963a), in B. ovis infection of sheep (Molello et al. 1963b), in B. melitensis infection of sheep (Molello et al. 1963c), in B. suis infection of pigs (Manthei, 1948) and in infection of the dog with B. canis (Carmichael and Kenny, 1968).

This remarkable predilection of B. abortus for the gravid reproductive tract was originally thought to be the result of a specific tropism of the bacterium due to the high concentration of erythritol found in the reproductive tracts of ruminants and swine (Smith et al., 1962; Williams et al., 1962; Keppie et al., 1965). While erythritol does stimulate the in vitro growth of some strains of Brucella, B. abortus strain 19, the current vaccine strain and B. ovis, a closely related pathogen of sheep, do not metabolize this sugar as an energy source but still localize and grow preferentially in the ruminant placenta (Keppie et al., 1965; Berman, 1977). In addition, recent investigations on the kinetics of brucellosis in the pregnant mouse revealed preferential growth of B. abortus in the gravid uterus (Bosseray, 1983a). Erythritol has been detected in very low concentrations in placental extracts from a variety of rodents,

including rats and guinea pigs (Smith et al., 1962). It seems unlikely therefore that a single growth factor is the cause of placental localization of the Brucella species.

Another class of compounds which may be involved in the localization and preferential growth of B. abortus in the placenta is the steroid hormones. Progesterone, synthesized in the smooth endoplasmic reticulum of both the ruminant and the murine trophoblast cell (Keppie et al., 1965, Britton, 1967; Sherman, 1983), enhances the growth of B. abortus in vitro (Misra et al., 1976).

In addition to various compounds which may promote the growth of B. abortus within the placenta, there may be a local "permissive" environment at the feto-maternal interface that allows the unchecked growth of certain placental bacterial pathogens. This does not imply that mechanisms have evolved to make the feto-placental unit more susceptible to intracellular bacterial infection, but rather that local immunosuppression permiting the survival of the fetus may prevent optimum functioning of the antibacterial immmune response. Thus the mechanisms protecting the fetus from maternal rejection may allow the survival of certain pathogens uniquely adapted to this situation.

The effect of pregnancy on the local immune environment is complex and worthy of some comment. The ability of an allogenic graft such as the fetus to survive without eliciting maternal rejection has interested researchers in the field of immunopathology and transplantation and has lead to a number of proposed mechanisms to explain the success of the fetal allograft. Mechanisms investigated have focused on the roles of the trophoblast cell, maternal antigen presenting cells in the decidua,

maternal suppressor cells and immunosuppressive factors from the endometrium, decidua and the developing fetus (Hill, 1990). Recent research in this area has been limited almost exclusively to the human or rodent placenta. Though species differences are apparent even between these two groups which share placental anatomic similarites (both hemochorial, Kirby, 1965; Bjorkman, 1970), there are likely similar principles involved in the maintenance of pregnancy in non-hemochorial placental species such as the ruminants with their epitheliochorial placentas.

2.4 Local immune suppression and the pathogenesis of placental infection

The fetal trophoblast cell appears to play a key role in the success of pregnancy and it is also the primary target cell infected by several intracellular bacterial pathogens (*Listeria monocytogenes*, *Brucella spp.*, *Coxiella burnetii*, *Campylobacter fetus* and *Chlamydia psittaci*). Trophoblasts are the principal cells of the fetal component of the feto-maternal placental interface. An absence of maternally recognized antigen on these cells would be a convenient explanation for the lack of maternal rejection of the fetus. The expression of Class I major histocompatibility complex (MHC) antigen has been reported in some populations of trophoblast cells in the mouse (Colavincenzo and Lala, 1985; Zuckerman and Head, 1986 a & b; Redline and Lu, 1989) and the rat placenta (Billington and Burrows, 1986; Saito et al., 1990) but the regulation and differential expression of Class I antigen and the implications for the feto-maternal immune relationship are still in question. Though the presence of Class I antigen on human trophoblast cell populations (Redman et al., 1984) is uncertain, there is some evidence for trophoblast specific or trophoblast

associated antigen in this species as well (Davies and Brown, 1985; McIntyre et al., 1983). There also apears to be species variabilty in the susceptibility of the trophoblast cell to immune destruction (Billington, 1989). This resistence to lysis in the presence of apparently appropriate target structures suggests therefore that suppressor mechanisms are important at the decidua-trophoblast interface in the species studied to date.

Suggested roles for the trophoblast cell include the production of immunosuppressor proteins and steroid hormones in local concentrations far greater than are measurable systemically (Siiteri and Stites, 1982), recruitment of suppressor cells and the promotion of blocking factors (antibodies) that bind to the placenta on an antigen-specific basis (Hill, 1990). There is growing evidence for the involvement of soluble factors synthesized by mouse (Chaouat, 1987) and human (Bardos et al., 1987) trophoblast. Protein A secreted from isolated trophoblast cells has been found to be highly immunosuppressive and also has the capacity to generate or recruit suppressor lymphocytes (Sanyal et al., 1989). A variety of studies performed *in vitro* and *in vivo* have demonstrated the effects of progesterone on the immune response, influencing regulation of immune cell traffic within the gravid uterus, T lymphocyte activation and macrophage function (Siiteri et al., 1977; Heap et al., 1983; Lloyd, 1983; Stites and Siiteri, 1983). Progesterone therefore, may have a local suppressive effect on the immune response as well as a stimulatory effect on certain bacterial pathogens of the placenta such as the brucellae.

Other proposed mechanisms of local immunosuppression include the following: (1) Decidual cells and placental macrophages may produce sol-

uble messengers such as prostaglandin E2, which can suppress T-lymphocyte function and natural killer (NK) cell activation through interference with IL-2 production and detection (Parhar et al., 1989). (2) Bone-marrow derived suppressor cells may be recruited to the endometrium and decidua by factors derived from trophoblast or decidual cells. A novel non-T-lymphocyte suppressor cell population at the site of implantation may exist within the decidua allowing these maternal cells to prevent anti-paternal cytotoxic T-lymphocyte generation by releasing a soluble factor that blocks the response of T-lymphocytes to interleukin-2, a T-lymphocyte growth factor (Clark et al., 1984, 1986a; Daya et al., 1989). (3) A contribution towards the local immunosuppression may also be derived from the fetus itself. It has been demonstrated that supernatants of _in vitro_ fertilized embryos suppress lymphocyte proliferation _in vitro_ (Daya and Clark, 1986). Alpha feto-protein, synthesized primarily within the liver of the fetus, has been proposed as interfering with maternal immune function (Lloyd, 1983; Lu et al., 1984). This list is not meant to include all proposed mechanisms of immunosuppression protecting the fetus from rejection but rather to demonstrate the complexity of the interactions within this local environment which may influence the growth of placental pathogens. Intracellular bacteria targeting the trophoblast cell seem to be in the ideal position to take advantage of local immunosuppression.

As previously mentioned, the overwhelming majority of studies investigating immunoregulation at the feto-maternal interface have utilized human or rodent tissue and the mechanisms by which the ruminant fetus is protected from immunologic attack are not well characterized (Low and

Hansen, 1988). Studies examining the immunology of epitheliochorial placentation support the theory that immunosuppressive mechanisms are involved in the success of the fetal allograft. The trophoblast cells of the cow's placenta are known to produce steroids including progesterone (Reimers et al., 1985) as well as prostaglandins (Shemish et al., 1984) especially PGE2, compounds thought to participate in immunoregulation in hemochorial placentas. PGE2 has been found to inhibit bovine lymphocyte function in vitro and has been proposed as having a direct effect on maternal lymphocytes, modulating immune function at the feto-maternal interface in the ruminant (Low and Hansen, 1988). The role of progesterone is however, less clear, and, rather than regulating immune function by direct inhibition of lymphocyte function, it may exert its influence by indirect means (Low and Hansen, 1988) such as modulation of prostaglandin release (Vagel et al., 1987) and induction and secretion of immunosuppressive molecules from the uterus (Murray and Chenault, 1982).

A study on the lymphocyte population in the bovine uterus during estrus and early gestation revealed a decrease in the number of lymphocytes in the epithelium of the gravid uterus 19-27 days post-conception. No further characterization of these cells was carried out (Van Der Weilen and King, 1984). A more recent study using immunohistochemical staining techniques reported major differences in the immune response to the fetus in the placentomal and the interplacentomal regions of the uterus of the sheep (Gogolin-Ewens et al., 1989). In the sheep as with other ruminants, trophoblast invasion of maternal tissue occurs only in the placentome (King et al., 1982). In interplacentomal regions, invasion does not occur and uterine epithelium remains intact throughout most of pregnancy. In

the non-pregnant uterus, lymphocytes were uniformly present in both the caruncular and intercaruncular uterine epithelium (Lee et al., 1988). In the placentome fewer lymphocytes were observed compared to either the non-pregnant caruncle or the pregnant interplacentomal region. In addition, subsets of lymphocytes differed in these regions with fewer CD4+ lymphocytes present in placentomal regions. These observations suggest that different mechanisms for preventing immune rejection may operate within placentomes where trophoblast invasion of maternal tissue occurs as opposed to the interplacentomal regions where the maternal/fetal interface is less intimate (Gogolin-Ewens et al., 1989). This variability in the immunomodulation of pregnancy may in turn have implications on site susceptibility to placental pathogens in non-diffuse placentation.

Redline and Lu have recently investigated allograft tolerance and immunosuppression within the murine placenta and its implications for the local susceptibility to intracellular pathogens and cellular traffic within the placenta (Redline and Lu 1987, 1988, 1989; Redline et al., 1988). The bacterium Listeria monocytogenes was chosen to probe the immune response of the placental region, as it is known that this intracellular pathogen resists antibody killing. To destroy Listeria, macrophages and T lymphocytes must enter infected tissues (Hahn and Kaufman, 1981). Study with this pathogen in the pregnant mouse model revealed that when a small threshold number of Listeria reached the placenta, overwhelming bacterial multiplication and infection occurred (Redline and Lu, 1987), a situation similar to that noted by Bosseray with B. abortus infection of the mouse placenta (Bosseray, 1983a). The level of infection and the nature and extent of the anti-listeria response in the maternal liver,

lung and spleen were unaffected by pregnancy. These findings suggested that local immunoinhibitory factors were responsible for the unchecked bacterial growth noted in the placentas (Redline and Lu, 1987, 1988).

2.5 The murine model of listeriosis

Prior to discussing the histologic findings of murine placental listeriosis, a brief description of the discoid, hemochorial placenta of the mouse is relevant. The mouse placenta is composed of four principal zones: the chorionic plate; the labyrinth; the spongiotrophoblast or junctional zone, and the decidua basalis. The chorionic plate is the region closest to the fetus and is the site of insertion of the visceral yolk sac as well as the site and the origin of the umbilical vessels. From here fetal capillaries enter the labyrinth region and interlace with a network of maternal blood sinuses which are surrounded by three layers of trophoblast cells. The junctional zone or spongiotrophoblast is a compact region composed of fetally-derived spongiotrophoblast cells, is perfused only by maternal venous sinuses and together with an incomplete lining of trophoblast giant cells, juxtaposes the decidua basalis. The decidua basalis is maternally-derived and underlies the metrial gland, which is a transient uterine structure associated with pregnancy in the rodent (Fib. 3b) (Enders, 1967; Redline and Lu, 1987; Theiler, 1972, 1983; Zuckerman and Head, 1986b).

Histological features of murine placental listeriosis were distinct, being predominantly suppurative in contrast to the typically granulomatous response noted in other organs such as the liver, lung and spleen of pregnant mice (Hoffman, 1984). While the endometrium and myometrium

contained substantial numbers of MHC class II antigen (IA) bearing macrophages and mature T-lymphocytes, the decidua basilis and metrial gland were almost devoid of these cells. The only leukocytes in the decidua basalis were granulated metrial gland cells which bore phenotypic similarities to natural suppressor cells. Recent in vitro studies have suggested that granulated metrial gland cells have a role in non-specific intrauterine immunosuppression (Croy and Kassouf, 1989). The deficiency of macrophages and T-lymphocytes noted in infected placentas suggested to these investigators that specific defects in the anti-listerial immune response in the pregnant uterus, perhaps related to the physiologic immunosuppression, might account for the exquisite sensitivity of the placenta to this intracellular pathogen (Redline and Lu, 1989).

The studies of Redline and Lu, orignially designed as an alternative method for investigating the existence of local placental immunoinhibition in vivo, also gave new insights into the possible pathogenesis of intracellular placental pathogens. These studies support a local immunosuppression within the gravid uterus rather than a generalized immunosuppression due to the systemic effects of pregnancy, since this would be detrimental to the survival of the mother. Systemic alteration in the cell-mediated response during pregnancy and lactation is postulated as involved in increased susceptiblility to a limited number of bacterial, viral, protozoal and helminth parasites (Lloyd, 1983) and the contribution of altered local versus systemic immunity during pregnancy is still under investigation. Contradictory reports on the immune function during pregnancy and lactation may be related to the use of different species, breeds and strains of animals and pathogens as well as the use of different

immunologic assays with accompanying technical difficulties. The complexity of factors affecting the immune response within a species to a particular pathogen makes it difficult to generalize on the subject of immunomodulation during pregnancy.

2.6 Local uterine immunity and pathogenesis of ruminant brucellosis

The susceptibility to brucellosis does increase during pregancy but the mechanisms of immune suppression are unclear and may operate at both the systemic and local levels. Two recent studies have examined the immune response of pregnant cattle to Brucella antigens. Manak (1982) tested the blastogenic response to the lectins concanavalin A (Con A), phytohemagglutinin (PHA) and poke weed mitogen and concluded that lymphocyte responses from pregnant heifers during the latter half of pregnancy were greater than those of ovariectomized, non-pregnant animals. However, sera from 3-7 month pregnant heifers produced a suppression of mitogenic activity. A later study comparing the lymphocytes of pregnant and normal, rather than ovariectomized, heifers found no significant difference in the reactivity to PHA and Con A during the last seven months of gestation. This study concluded that the magnitude, quality and duration of the humoral and cell-mediated immune response to Brucella vaccination, with few exceptions, were uninfluenced by pregnancy (Winter et al., 1986). The explanation for the increased susceptibility of pregnant cattle to brucellosis therefore is far from complete and is now regarded as having a multifactorial basis (Weinberg, 1984; Brabin, 1985) likely encompassing both the systemic and local immune reponses to infection.

It seems likely therefore, that a variety of immunomodulating factors work in concert resulting in the successful colonization of the ruminant placenta by B. abortus. Recent studies using a goat model of B. abortus infection detailed the placental lesions and suggested the route of entry of the bacteria into the placenta (Anderson et al., 1986b). This study demonstrated that the initial site of Brucella infection within the placenta of this species is the erythrophagocytic trophoblast cells of the placentome. Subsequently, large numbers of brucellae were present within periplacentomal chorioallantoic trophoblasts. Though the mechanism of trophoblast cell entry is still in question several possible routes are proposed. Trophoblasts at the initial site of placental infection are actively engaged in the uptake of maternal erythrocytes (Anderson et al., 1986a) and may engulf brucellae during the bacteremic state but the active penetration by the bacteria cannot be ruled out (Anderson et al., 1986b). Though distinct erythrophagocytic areas are not present in the placenta of the cow, the bovine placentome does contain hematomas at the tips of maternal septa. Neighboring chorionic trophoblasts may be infected by cell to cell transfer through lateral plasmalemma as well as directly by endocytosis of bacteria that are free within the uterine lumen following rupture of infected trophoblasts (Anderson et al., 1986b). Ruminant trophoblasts are known to endocytose endometrial secretions important in fetal nutrition (Boshier and Holloway, 1977).

Recent evidence supports the receptor mediated uptake of brucellae. Coated pits were noted in close proximity to brucellae attached to the surface of Vero cells in an in vitro study of cell entry and localization (Detilleux et al., 1990b). Similar coated pits are seen in association

with adherent invasive Yersinia enterocolitica (Miller and Falkow, 1988) and Shigella flexneri (Hale, 1986) but not with non-invasive Escherichia coli (Miller and Falkow, 1988). In addition, monodansylcadaverine, an inhibitor of receptor mediated endocytosis, prevented the infection of Vero cells by B. abortus (Detilleux et al., 1990b).

An unusual adaptation to intracellular existence is the selective localization of B. abortus to the cisternae of the rough endoplasmic reticulum (RER) when infecting cells particularly permissive to its growth. In ruminant trophoblast cells, B. abortus replicates specifically within the RER (Anderson et al., 1986a) and a similar intracellular location is seen with its infection of chicken embryo mesenchymal cells, yolk endodermal and hepatic cells (Detilleux et al., 1988) and in vitro Vero cells (Detilleux et al., 1990a, 1990b). Within the RER of these host cells Brucella replication is brisk. Electron microscopy revealed trophoblast cells infected with B. abortus have dilated membrane bound cisternae containing massive numbers of bacteria (Anderson and Cheville, 1986; Anderson et al., 1986a). Replication within the RER is apparently a unique mechanism of epithelial cell intracellular parasitism, other intracellular protozoan and bacterial pathogens being either free in the cytoplasm or within membrane bound phagosomes (Anderson et al., 1986a). Only Legionella pneumophila may have a similar intracellular location since it inhabits organelles indistinguishable from the RER in monocytes and macrophages. Its exact intracellular location is still controversial and some authors prefer to call this location a ribosome studded phagosome (Horwitz, 1983).

The large number of brucellae present within the RER of ruminant trophoblast and Vero cells suggests that transfer of the bacteria to the RER is necessary for rapid bacterial multiplication (Detilleux et al., 1990a, 1990b). Transfer to the RER, not internalization, has been suggested as the limiting step for replication in the Brucella spp. and the difference in pathogenicity between rough and smooth strains may correlate with their ability to gain access to the RER. The mechanism by which the brucellae gain access to the RER is unclear but since brucellae reside in the RER of different cell types it has been suggested that this process is bacteria-induced and may involve receptor-mediated vesicular tranfer. Both rough and smooth brucellae gain access to the RER, albeit in different proportions, suggesting that the recognition signal is not related to the O-side-chain antigen of the LPS molecule but more likely involves outer membrane proteins (Detilleux et al., 1990a, 1990b) which are similar and conserved within the genus (Santos et al., 1984).

Bacterial localization and replication within the RER is accompanied by significant hypertrophy of this organelle without concommitant hypertrophy of the Golgi apparatus. This suggests that B. abortus may utilize the RER of the trophoblast cell for the synthesis of bacterial proteins to enhance its growth. Another possible explanantion of this specific localization is the uptake of proteins from the cisternae and the incorporation of aminoacids from the breakdown of these proteins into bacterial proteins (Anderson et al., 1986a). Most strains of brucellae require complex media containing several aminoacids for in vitro growth (Corbel and Brinley-Morgan, 1984). L. pneumophila, which inhabits organelles similar

to the RER, also requires aminoacids as a source of carbon and energy for growth in culture (Hoffman, 1984).

In contrast to the situation in trophoblast cells, B. abortus is not seen within the RER of phagocytes in which their replication is more limited. In macrophages and neutrophils brucellae are located and replicate within phagosomes and phagolysosomes (Karlsbad et al., 1964; Detilleux et al., 1988; Harmon et al., 1988; Meador and Deyoe, 1989).

B. abortus does not uniformly infect all bovine trophoblast cells in vivo. In the goat model of placental infection, chorioallantoic cytotrophoblasts had large numbers of B. abortus, while binucleate trophoblast cells (giant cells) were not seen to contain brucellae. These binucleate cells were never infected even when adjacent to heavily infected cytotrophoblasts (Anderson et al., 1986a). The reason for this selective parasitism of trophoblast cell types is unknown.

Massive replication of B. abortus within the trophoblast cell, perhaps stimulated in some cases by erythritol and progesterone and fostered by the use of the host RER, eventually leads to trophoblast cell dysfunction and death. In an electron microscopic study of the infected caprine placenta, trophoblasts filled with brucellae often displayed features characteristic of degenerate cells, being swollen with electron lucent cytoplasm, lipid droplets and dilated membranous cisternae devoid of ribosomes (Anderson et al., 1986a). The brucellae do not appear to be highly cytopathic as minimal cell degeneration was noted in some heavily infected cells. However, trophoblast cell necrosis is undoubtedly linked with intracellular bacterial replication. In detached, necrotic infected cells, Brucella-filled cisternae occupied more than 36% of the trophoblast

cytoplasm while in non-degenerate, or minimally degenerate trophoblasts still attached to the chorioallantoic membrane, the Brucella-filled cisternae occupied only 24-36% of the cytoplasm (Anderson and Cheville, 1986). Trophoblast cell death may be related to physiologic disruption due to the massive numbers of bacteria within the RER or perhaps also may involve the direct action of bacterial endotoxin either within the cell or present in the environment.

Bacterial endotoxin has a diverse range of biological properties which may be important in the pathogenesis of placental disease due to Gram-negative bacteria. The products of LPS-activated macrophages (including tumor necrosis factor alpha and transforming growth factor beta) but not LPS itself have been reported to modify DNA synthesis by the rat trophoblast cell (Hunt et al., 1989). Thus some products of Brucella LPS-activated macrophages may have a detrimental effect on trophoblast cell survival. Placental vasculitis, which is seen later in the course of the infection (Anderson et al., 1986b), may also produce anoxic conditions damaging trophoblast viability.

The most prominent placental lesions present in the Brucella-infected placenta are in the chorioallantoic membranes and are characterized by the diffuse filling of chorioallantoic trophoblast cells with bacteria, subsequent trophoblast cell necrosis and ulceration of these membranes with accompanying necro-suppurative exudation. B. abortus does not infect the endometrial epithelium or lamina propria (Anderson et al., 1986b). However, the majority of fetuses associated with infected placentas are themselves infected and display a variety of histologic lesions including suppurative bronchopneumonia, non-suppurative perivascular

hepatitis and marked macrophage proliferation in the spleen and lymph nodes (Payne, 1959; Enright et al., 1984; Jubb et al., 1985; Anderson et al., 1986b). The proximity of placental capillaries to the ulcerated chorioallantoic membrane may allow access of brucellae-filled exudate to the fetal circulation resulting in the hematogenous spread of the bacteria to the fetus (Anderson et al., 1986a).

In the cow the inflammatory infiltrate associated with Brucella infection of the placenta is primarily suppurative, with fewer macrophages and plasma cells in contrast to the predominantly mononuclear infiltrate that accompanies infection of non-reproductive tract organs. This is not inconsistant with the proposed restriction of cell trafficing related to mechanisms of local immunosuppression associated with the survival of the fetus. Both neutrophils and macrophages may be attracted by non-immune mechanisms (i.e. without specific lymphocyte direction) that include chemo-attractant factors liberated by bacterial organisms (F Met peptides) and by activated complement factor C5A (Sell, 1987). Both smooth and rough Brucella LPS is known to be able to activate the complement cascade (Moreno et al., 1981). Necrotic cellular debris in itself also contains factors attractive to phagocytic leukocytes. The histologic findings of experimental and natural cases of ruminant brucellosis do not conflict with the theory that local immunosuppression may play a part in the pathogenesis of this placental pathogen. Degenerate phagoctyes containing intact brucellae prevalent in the chorionic villi (Anderson et al., 1986a) also concur with reports that non-activated macrophages may not be able to arrest the multiplication of facultative intracellular pathogens such as Brucella (Collins and Campbell, 1982). The histologic lesions also

support the finding that *in vitro* *B. abortus* may inhibit the bacteriocidal mechanisms of ruminant neutrophils (Riley and Robertson, 1984).

2.7 Pathogenesis of Brucella-induced abortion

The exact mechanism of *Brucella*-induced abortion is at present controversial but likely involves the interaction of several factors including fetal stress, placental insufficiency and the effects of endotoxin. The extent of the placentitis is variable, from minimal focal lesions to widespread destruction of placentomes and fetal membranes. Abortion may occur in either case (Enright, 1990). Thus it is difficult to accept the theory that abortion is due solely to placental insufficiency, that is, inadequate transfer of gases, nutrients and clearance of fetal waste products (Thoen and Enright, 1986).

Several studies suggest that *Brucella* endotoxin may be responsible for abortion (Payne, 1957; Anderson et al., 1886a). The endotoxins of various Gram negative bacteria including *B. abortus* can induce placental hemorrhage and abortion in laboratory mice (Zahl and Bjerknes, 1943; Moreno et al., 1981). *B. abortus* crude endotoxin caused abortion in guinea pigs 24-36 hours after inoculation (Pennel and Huddleson, 1937). The same material inoculated into a pregnant heifer produced abortion 3 to 4 days post-inoculation.

Endotoxin induces changes in endothelial cells and results in vascular lesions characterized by increased permeability and/or thrombosis (Hardie and Kruse-Elliott, 1990). Vasculitis was a prominent feature in the placentitis noted in the goat model of brucellosis and was felt to contribute greatly to the placentomal lesions, the separation of maternal and fetal

epithelium as well as to fetal death (Anderson et al., 1986a). Endotoxin is also known to induce platelet aggregation and fibrin deposition and to activate the complement cascade (Hardie and Kruse-Elliot, 1990) which in combination with vasculitis may have resulted in trophoblast ischemic damage. As previously mentioned, endotoxin may have a more direct effect on trophoblast DNA synthesis through a macrophage intermediary (Hunt et al., 1989).

The maintenance and termination of pregnancy in ruminants is dependent on a complex interplay of hormonal interactions and prominent among these hormones is the steroid, progesterone. In cattle and sheep, progesterone produced by the placenta and fetus maintains pregnancy in the latter half of gestation so that diseases affecting either the fetus or the placenta at this time may result in the termination of pregnancy. The shift from production of progesterone to estrogen results in the production of PGF2 which can initiate parturition (Enright, 1990). In a study investigating the hormonal effects of Brucella infection in the ruminant, fetal stress resulted in the elevation of fetal cortisol levels which lead to decreased progesterone production and increased estrogen production by the placenta. Increased levels of maternal PGF2 followed, as did abortion (Liggins, 1981). This and other studies strongly suggest that fetal infection and the resultant fetal stress may induce hormonal alterations that are associated with abortion or premature delivery (Enright et al., 1984). Brucella-induced abortion therefore is likely due to a combination of factors affecting both the fetus and the placenta. The relative importance of specific causes or inducers is presently unclear and may vary with

each individual event as influenced by both host and pathogen determinants.

2.8 The mouse as a model for brucellosis

Though the anatomy, pathology and bacteriology of brucellosis have been well characterized in the ruminant, large scale studies are difficult to conduct in this species due to prohibitive cost of animal procurement and care in isolation facilities. Historically, the mouse has been used to study the immunopathology in the non-pregnant animal (Fabyan, 1912; Feldman and Olsen, 1935; Young et al., 1979; Ho and Cheers, 1982; Phillips et al., 1989b; Pugh et al., 1989). The murine model permits the economical and convenient analysis of several aspects of brucellosis: pathogenesis; immunology, vaccine screening; and the course of acute and chronic infection. There has been recent extensive progress in the immunologic response of the mouse (including the pregnant mouse) (Bosseray, 1983b; Cheers and Young, 1987; Bosseray and Plommet, 1988; Phillips et al., 1989b; Pugh et al., 1989) to B. abortus infection and several investigations concerning placental colonization kinetics and protection studies in the pregnant mouse (Bosseray, 1980, 1983 a & b) have been reported.

Experiments on the bacteriology of virulent strains of B. abortus in the non-pregnant BALB/c mouse have shown that the liver and spleen support moderate bacterial growth that peaks in approximately two weeks. The bacterial growth displays a plateau phase which lasts approximately six weeks. This is followed by the slow clearance of the bacteria over the next four to five months (Ho and Cheers, 1982). The course of chronic infection does differ however between strains of inbred mice (Pugh et al., 1989).

Studies challenging outbred mice during gestation with a virulent strain of B. abortus (strain 544) found that placental infection increased to high levels over the course of pregnancy, suggesting that bacterial replication is poorly controlled by the immune response within this organ (Bosseray, 1980, 1983a). It was determined that the sensitivity of the mouse placenta was not due to an early localization of bacteria but rather to the placenta's becoming the site of intense bacterial proliferation. Study of bacterial infection in various organs of the pregnant mouse revealed markedly different colonization kinetics in the spleen and placenta. Only a few bacteria (less than 1% of the inoculum) initiallly became associated with the placenta but within this organ bacteria multiplied rapidly with an estimated generation time close to previous in vitro estimates (Richardson and Holt, 1964). In contrast, the spleen, which retained the largest number of bacteria immediately post-challenge, had a much slower subsequent bacterial growth rate. The level of splenic infection of pregnant mice was not significantly different from that of non-pregnant mice, suggesting that pregnancy did not alter the susceptibility of the pregnant mouse to systemic infection by B. abortus. Brucellosis did not appear to modify the course of pregnancy in mice; the conception rates, litter size and the viability of the fetuses did not differ from control mice in this study. Histologic examination of infected placentas, however, was not included in review of materials from this study (Bosseray, 1983a).

The rate of splenic and placental infection in the pregnant mouse challenged with B. abortus was related to the dose and route of challenge, with the most marked colonization occurring with high intraveneous

inoculation. Placentas were susceptible to infection at all the times tested during gestation (day 4 to day 15) with the highest levels of placental infection occurring in mice receiving the challenge dose in mid-gestation (days 7 to 11) (Bosseray, 1980, 1983a).

A study of the mother-to-young tranmission of murine brucellosis established that transplacental infection occurred frequently in association with heavy colonization of the murine placenta with B. abortus (Bosseray, 1982). The majority of fetuses associated with heavily infected placentas were infected when the dams were killed just prior to parturition. Sixty percent of mice born to dams inoculated with high doses of Brucella on day 7 of gestation were infected at birth, bacteria being isolated from the spleens, livers and lungs of the neonatal mice. Congenital infection persisted at least 30 days. Brucellae were isolated from the mammary glands of 80% of infected dams 3 weeks post-partum. Transmission of infection from infected nurse mice to adopted suckling pups from control mice was, however, rare.

Thus despite the anatomical differences in placentation, the pregnant mouse model of brucellosis offers significant similarities to infection in cattle. In both the ruminant and mouse, unchecked bacterial multiplication occurs in the placenta, resulting in congenital infection of the newborn. In the cow, the mammary gland is a frequent site of chronic infection and persistent shedding of brucellae. Similarly, pregnant mice infected with B. abortus often develop mammary gland infection which results in the shedding of bacteria in the milk. The pathology of Brucella infection of these two organs in the mouse has not been reported.

The pregnant mouse has been used previously as an experimental model of a number of bacterial (Buzoni-Gatel and Rodolakis, 1983; Bosseray 1980, 1982, 1983a; Tuffey et al., 1987; Redline and Lu 1987, 1988, 1989) and viral (Spertzel et al., 1972; Aaskov et al., 1981; Milner and Marshall, 1984) pathogens that affect the course of pregnancy. The recent research utilizing the histologic examination of infected placentas in tandem with bacteriologic culture in the pregnant mouse model of listeriosis documented the preferential growth of the organism within the placenta (Redline and Lu, 1987, 1988). This study, originally designed to investigate the immune mechanisms operating at the feto-maternal interface, also led to the definition of placental pathology associated with Listeria colonization of the murine placenta. Listeria were first detected extracellularly adjacent to the maternal venous sinuses of the decidua basalis in the absence of an inflammatory response. Later, bacteria were observed within trophoblast and other fetally-derived tissues, accompanied in some cases by a diffuse neutrophilic infiltration of the lower decidua. Placental infection had devastating consequences to the fetus, resulting frequently in the infection of viable fetuses followed by death of the fetus. The bacterial colonization kinetics in this model shares several similarities with the previously reported kinetics of brucellosis in the pregnant mouse model (Bosseray, 1980, 1982, 1983a): 1. L. monocytogenes grew unchecked within the placentas of mice; 2. fetal transplacental infection occurred subsequent to severe placental infection; 3. splenic bacterial multiplication was comparably more modest than placental growth and 4. the severity and duration of splenic infection in the

pregnant mouse infected with L. monocytogenes did not differ significantly from non-pregnant infected mice.

Although brucellosis is primarily a reproductive tract disease in the natural host, studies involving the histology of murine brucellosis have been limited to the systemic lesions of the non-pregnant mouse. As early as 1912, Fabyan reported macroscopic lesions in non-pregnant mice after the inoculation of B. abortus. Microscopic description of the characteristic histiocytic lesions were first reported in detail by Feldman and Olson in 1935. Nodular to diffuse granulomatous lesions were noted in the lung, liver, kidney, spleen and testes of male mice receiving an intraperitoneal injection of a suspension of B. abortus. Subsequent reports have confirmed the granulomatous nature of the tissue response in the non-pregnant mouse (Young et al., 1979; Cheers, 1984; Phillips et al., 1989b; Pugh et al., 1989; Enright et al., 1990a).

Payne in 1957 reported changes in the rat placenta and fetus subsequent to the experimental infection of the pregnant rat with B. abortus isolated from an aborted cow. Following the intraperitoneal injection of an undetermined dose of B. abortus in rats (0.2 mls of a two day growth suspended in saline to faint opacity) on day 12 or 13 of gestation, both acute and subacute effects were noted. Within 24 hours post-inoculation, extreme placental congestion and hemorrhage occured, an effect which may be similar to the injection of Gram negative endotoxin in the pregnant mouse (Zahl and Bjerknes, 1943). The endotoxin of B. abortus is known to be abortifacient in pregnant mice (Moreno et al., 1981) but the histologic lesions associated with this process were not described. The acute response of pregnant rats to Brucella inoculation, likely a result of

profound vascular damage from massive endotoxemia, caused placental lesions (necrosis of the spongiotrophoblast and labyrinth) which lead to the death of a percentage of the fetuses. The placental lesions which occurred as a result of the massive doses of endotoxin administered hindered the subsequent interpretation of the subacute lesions associated primarily with the localization and multiplication of B. abortus within trophoblast cells. Acute initial changes did not appear to be related to large numbers of bacteria in the placenta, though some bacteria were demonstrated both extracellularly at the feto-maternal interface and intracellularly within occasional trophoblast cells. Rats maintaining pregnancy for several days after the challenge did develop lesions associated with the presence of numerous bacteria in the maternal tissue adjacent to the spongiotrophoblast. B. abortus was thought to multiply initially and primarily within spongiotrophoblast cells, leading to cell necrosis and subsequent neutrophil infiltration. Small, secondary "abscesses" occurred in the labyrinth near the insertion of the fetal umbilical vessels. This study suggested that B. abortus retains its predilection for multiplication within trophoblast cells in the hemochorial discoid placenta of the rodent as occurs in the epitheliochorial, cotyledonary placenta of the ruminant. The precise intracellular location of B. abortus in the rat trophoblast was not reported.

At the present time, though it is well established that the pregnant mouse infected with B. abortus displays bacterial proliferation kinetics in the spleen and placenta similar to the pregnant mouse model of listeriosis, the details of the pathogenesis of placental infection are obscure. Payne's study on the consequences of Brucella infection in the

pregnant rat suggest that growth of this bacterium within the rodent placenta is associated with significant placental pathology. It would now seem prudent to conduct the histologic examination of the murine feto-placental unit during the course of Brucella infection to aid in an increased understanding of both the pathogenesis and the immunology of brucellosis in pregnant animals. The following investigation was designed to identify and characterize lesions of the placenta induced by the inoculation of the pregnant mouse with a virulent strain of B. abortus and to examine the pathogenesis of such lesions.

CHAPTER 3

PLACENTAL PATHOLOGY OF THE PREGNANT MOUSE INOCULATED WITH *BRUCELLA ABORTUS*

Brucella abortus is a facultative, intracellular, Gram negative bacterium with a marked affinity for the pregnant reproductive tract of ruminants. The organism produces a chronic infection in pregnant cattle, replicating preferentially within the chorioallantoic cells of the placenta and resulting in placentitis, fetal death and abortion (Thoen and Enright, 1986; Enright, 1990). The murine model, particularly the non-pregnant mouse, has been employed extensively to study some aspects of the pathogenesis of brucellosis (Bosseray, 1978; Cheers, 1984; Enright et al., 1990a). Although brucellosis is primarily a reproductive tract disease in the natural host (cattle), studies in the pregnant mouse have been limited to investigating only the bacteriology and immunology of the infection (Bosseray, 1980, 1982, 1983a, 1983b; Bosseray and Plommet, 1988). The placental pathology of the pregnant mouse infected with *B. abortus* has not been reported.

In 1957 Payne reported pathologic changes in the rat placenta and fetus subsequent to experimental intraperitoneal infection with a virulent strain of *B. abortus* isolated from an aborted cow (Payne, 1957). Following the injection of an undetermined dose of the bacterium, *B. abortus* was thought to localize within trophoblast cells leading to necrosis acompanied by neutrophil infiltration and, occasionally, fetal death. In a later study by Bosseray, pregnant mice challenged by several routes at different stages of gestation with virulent *B. abortus* strain 544 suffered no

abortions or fetal death (Bosseray, 1980). It was concluded that brucellosis in mice did not modify pregnancy although preferential placental colonization occurred. Histologic examination of placentas was not included in this study. One could speculate that brucellae may infect mouse trophoblast cells since Listeria monocytogenes, a pathogen of trophoblast cells of ruminants leading to abortion in this species, has been shown to infect murine trophoblast cells in vivo. Overwhelming placental infections with Listeria often lead to severe, diffuse neutrophilic infiltration of the lower decidua and resulted in fetal death (Redline and Lu, 1987).

A preliminary study in our laboratory had revealed that doses of 10^8 organisms or greater of B. abortus strain 2308 organisms administered intraperitoneally to BALB/c mice in mid-gestation produced a severe, necro-suppurative placentitis associated with fetal death. This study was designed to determine the minimum dose of this virulent strain of B. abortus able to produce consistent placental lesions in BALB/c mice and to investigate the pathogenesis of these lesions in order to evaluate the pregnant mouse as a model for the placental pathology of brucellosis. It was part of a wider evaluation of the efficacy of attenuated strains of B. abortus to immunize the pregnant animal.

MATERIALS and METHODS

1. Animals

Two- to four-month-old BALB/c female mice, reared in our animal holding facilility at Virginia Tech, were individually mated to four- to eight-month-old BALB/c male mice. Parents of the female mice and all male mice were obtained from Dominion Laboratories, Dublin, V.A.. Day 1 of

gestation was the day the vaginal plug was observed. The normal gestational time for these mice was 19 days. All uninfected and infected non-pregnant female mice were housed conventionally, five mice per cage. Infected pregnant mice were housed individually in microisolator cages. Male mice were also housed individually but in standard cages. All mice received food and water ad libitum until killed by CO_2 inhalation at the times indicated in the protocol. Sentinel mice killed at the conclusion of the experiment were found free of antibodies to six major murine bacterial and viral pathogens (Mycoplasma pulmonis, Mouse Hepatitis Virus, Sendai Virus, Minute Virus of Mice, Pneumonia Virus of Mice and Murine Encephalomyelitis Virus).

2. Bacterial Cultures

B. abortus strain 2308 organisms used for mouse inoculations originated from stock cultures held in the P3 facility at Virginia Tech. Trypticase soy plates were streaked with organisms from a stock slant and incubated for 48 hours in air atmosphere supplemented with 5% CO_2 at 37°C. Bacteria were harvested from plates in trypticase soy broth, brought to 10% transmittance at 525 nm in a Bausch and Lomb Spectronic 20 spectrophotometer and held at -70° in 0.5 ml aliquots until used. Viable counts were performed prior to inoculation in order to establish the numbers of organisms in the frozen aliquots. Three frozen aliquots were thawed and a series of ten fold dilutions in sterile saline carried out. Fifty microliters of each dilution were plated on trypticase soy plates in 10 microliter drops and plates were incubated for 72 hours under the conditions previously described. Colony forming units (cfu) were then counted and

the mean number of the three plates was used to determine the number of viable bacteria per milliliter of frozen broth. This allowed precise administration of known doses of B. abortus to mice.

3. Experimental Design

Three groups of mouse inoculation experiments were conducted. All inoculations were administered intraperitoneally with a tuberculin syringe and a 27 gauge needle. Doses of live organisms were confirmed after inoculation by viable counts of the residue of each inoculum.

Group 1 minimum dose experiments. Determination was made of a minimum dose of B. abortus strain 2308 producing consistently severe placental lesions. Groups of five, timed-pregnant mice received either $10^{4.7}$, $10^{5.7}$ or $10^{6.7}$ cfu of B. abortus strain 2308 on day 9 of gestation. Doses were varied by $\log_{10}$ increments to determine a minimum inoculum producing placental lesions with a histologic score of 2.0 or greater (see Light Microscopy) when placentas were examined nine days post-inoculation (day 18 of pregnancy).

Group 2 minimal dose follow-up experiments. Once a minimum dose producing severe placental lesions was determined, ten pregnant mice were inoculated with this same dose on day 9 of gestation and killed on day 18 of gestation. Ten non-pregnant mice were inoculated with this dose and were killed nine days later. Additionally, five pregnant mice received an equal dose of heat-killed B. abortus bacteria on day 9 of gestation and were killed on the same schedule.

Group 3 pathogenesis experiments. Serial pathogenesis. To study the pathogenesis of bacterial colonization and the development of placental

lesions, four groups of five, timed-pregnant mice received $10^{6.7}$ cfu of B. abortus strain 2308 organisms on day 9 of gestation and were killed sequentially on days 12, 14, 16 and 18 of gestation. This dose was chosen to ensure early infection with a number of brucellae sufficient to allow early detection of the bacteria by avidin-biotin immunohistochemical stains. It has been reported that approximately 10^6 organisms per gram of tissue is the limit of sensitivity for this determination of bacterial localization (Meador et al., 1986). Twenty pregnant mice received sterile saline IP in midgestation and were killed on the same schedule.

4. Necropsy

All mice were subjected to a complete necropsy. Blood was collected immediately post-mortem from the heart with a sterile glass pipette and allowed to clot at room temperature for one hour. Sera were separated by centrifugation and stored at $4^{o}C$ until needed. Spleens and two placentas per mouse (pregnant mice) or the uterus (non-pregnant mice) were removed aseptically for Brucella culture.

5. Bacterial Culture

Spleens, placentas and uteri were weighed and placed in sterile glass tubes with 2 mls sterile saline, homogenized with sterile sand and serially diluted. Dilutions were plated on trypticase soy plates and cfu per organ or gram of tissue were determined. The minimum number of bacteria detectable by this method was 40 per organ. B. abortus colonies were identified by colony morphology, growth characteristics and colony blot ELISA (Roop et

al., 1987; Appendix) using monoclonal antibodies specific for the O-side-chain of B. abortus (Schurig et al., 1984).

6. Histology

Placentas not used for bacterial culture were fixed in situ within uteri in 10% neutral buffered formalin, and processed routinely for histologic examination and scoring using hematoxylin and eosin or Giemsa stains. One mid-sagittal section was processed for each placenta. Placental sections were coded and examined as unknowns and scored on a scale of 0 to 3 according to the severity of the lesion (0 = no change, 1 = peripheral placental inflammation associated with Reichert's membrane, 2 = grade 1 plus inflammation/necrosis of the spongiotrophoblast, 3 = grade 2 associated with fetal death; see also Fig. 3). Examination of gestational-age matched placentas from uninfected mice allowed the establishment of baseline scores. The mean placental score for each mouse was determined then a mean score for the entire group was determined to minimize the effect of litter size (placentas within litters tended to receive similar grades). The histopathology of spleens, livers and placentas was characterized in 10 pregnant mice that received $10^{5.7}$ brucellae on day 9 of gestation. Uteri of five non-pregnant mice infected with $10^{5.7}$ brucellae were collected for histologic examination.

Specific labeling of B. abortus in placental sections was performed by standard immunoperoxidase techniques utilizing biotinylated secondary antibody and avidin-biotin-peroxidase complex (ABC) techniques (Meador et al., 1986; Appendix). Serum obtained from a rabbit hyper-immunized with multiple injections of killed, whole B. abortus strain 2308 organisms

served as the primary polyclonal antibody (Appendix). Biotinylated anti-rabbit IgG and the avidin-peroxidase-complex were obtained from Vector Laboratories, Inc., Burlingame, CA. Deparaffinized, endogenous peroxidase blocked, trypsinized placental sections were immunostained by following the manufacturer's directions except that 3% sodium cloride was added to the TRIS buffer used to prepare the ABC reagent to reduce non-specific background staining. Controls were used to assess specificity of the anti-B. abortus primary antibody. For each infected tissue, one slide was processed using pre-immunization rabbit serum as the primary antibody. As another control, non-infected murine placental sections and tissue containing other bacteria were processed with the ABC technique using post-immunization rabbit sera. Paraffin embedded sections of ovine lung containing visible colonies of Actinomyces pyogenes and Pasteurella hemolytica which had been identified by culture served as the bacterial controls.

7. Electron Microscopy

To determine the ultrastructural localization of bacteria, placental samples were obtained from mice infected with $10^{6.7}$ cfu of Brucella and non-infected control pregnant mice killed on day 18 of gestation. Placental disks were cut in half and, to facilitate sectioning of the fragile tissue, chilled 30 minutes at $2^{o}C$ by immersion in a solution of 5% glutaraldehyde and 3% formalin in a 0.05M Na cacodylate buffer with 2.5% picric acid. A 1 millimeter thick sagittal section was then cut from the placental hemi-disk and fixed in the same solution, refridgerated, for 48 hours. Samples were post-fixed in 1% osmium tetroxide, embedded

in epoxy resin (POLY/BED 812, Polysciences, Inc., Warrington, PA), sectioned at 1 micron and stained with toluidine blue and saffarin. Ultrathin sections of appropriate areas were then cut and routinely stained with uranyl acetate and lead citrate or processed for immunogold labeling (courtesey of N. F. Cheville, National Animal Disease Center, Ames, IA; Appendix). Stained sections were examined on a JEOL 100 cx-III STEM.

8. Serology

The standard tube agglutination test with B. abortus strain 19 Diagnostic Antigen from the USDA, was performed on all infected and selected control serum samples, starting at 1:25 dilution (Alton et al., 1975). Titers are expressed as the $\log_{10}$ value of the reciprocal (Appendix).

9. Statistical Analysis

Analysis of variance was used to compare the level of splenic infection in pregnant versus non-pregnant mice receiving $10^{5.7}$ B. abortus strain 2308 organisms (Snedecor, 1956). All data was expressed as mean +/- standard deviation and statistical differences between groups with $p<0.05$ were considered significant.

RESULTS

All mice remained clinically normal by visual assessment for the duration of the experiments and no abortions were observed. At necropsy, placentas of non-infected mice were dark red, firm, of a uniform size within a litter and supported a live fetus. In Brucella-infected mice, gross lesions of

the placenta were seen only in mice receiving $10^{5.7}$ cfu or greater in the mimimum dose study, and on days 16 and 18 of gestation in the pathogenesis study. These placentas varied in size and color. Most were dark red and firm and supported viable fetuses; some were pale, shrunken and associated with an edematous or autolysed fetus. Mildly affected placentas with viable fetuses had a yellow rim of material at the periphery. The weight of infected placentas was considerably less than non-infected placentas but this decrease was not noticably altered by dose in the range tested (Table 1). In the serial pathogenesis study, placental weights of control mice were similar to mice receiving $10^{6.7}$ organisms until day 14 of gestation. After that time, the weight gain of infected placentas declined sharply in contrast to the weight of non-infected placentas which continued to increase steadily and in contrast to the weights up to day 12 (Fig. 4 & Table 2). Marked splenomegaly was present in all infected mice with the largest spleens associated with doses equal to or greater than $10^{5.7}$ brucellae (Table 1). In the serial pathogenesis study, splenic weight of control mice remained fairly stable throughout gestation in contrast to the splenic weight of mice infected with $10^{6.7}$ organisms which rose sharply in the latter part of pregnancy (Fig. 4 & Table 2). Livers of pregnant mice were often pale and slightly yellow. All other organs examined (non-gravid uterus, lungs, heart, kidneys, gastrointestinal tract) were grossly normal.

Histology of day 12 placentas (that is, three days post-infection with $10^{6.7}$ organisms), revealed minimal evidence of Brucella infection (Fig. 5 a & b). Occasional trophoblast giant cells (TGCs) in all three areas of the decidua-(capsularis, basalis and parietalis) had slightly

hyperchromatic, granular cytoplasm suggestive of intracellular bacteria. Inflammatory cells were not noted in association with these infected cells. Immunostained sections (Fig. 6 a-d) confirmed the presence of bacteria within numerous TGCs along the entire periphery of the placenta. Bacteria were also located within neutrophils associated with the regressing decidua capsularis or free within the newly formed uterine lumen adjacent to this region of the placenta.

In day 14 placentas (five days post-infection) there were few small foci of necrosis within the spongiotrophoblast zone (Fig. 7 a & b). Infected TGCs were frequent in this region and ABC staining revealed the presence of Brucella antigens as an almost unbroken ring adjacent to the entire decidua (Fig. 8 a-d). At this stage of infection, bacteria were localized exclusively within TGCs or maternal phagocytic cells, primarily neutrophils, associated with the regressing decidua. Where bacteria were located solely within TGCs, there was an absence of inflammatory response.

By day 16 of gestation (seven days post-infection and 3 days from term), there was a moderate to severe, multifocal to coalescing necrosis of the spongiotrophoblast zone of the placenta. Minimal to moderate neutrophilic infiltration was noted in association with the necrotic debris (Fig. 9 a & b). Prominent extracellular bacterial colonies as well as occasional infected TGCs were present in this region, which extended from Reichert's membrane at the periphery of the disk to the interior. Throughout the necrotic regions, ABC (Fig. 10 a-d) and Giemsa stains (Fig. 11) revealed massive bacterial colonization and phagocytosis of brucellae by neutrophils. Occasionally, there was also positive

staining of endoderm cells of the long fronds of the visceral yolk sac adjacent to the remnants of Reichert's membrane.

Lesions noted in day 18 placentas (9 days post-infection) from mice receiving at least $10^{5.7}$ brucellae were similar to those noted in day 16 placentas except for increased to near total involvement of the spongiotrophoblast zone, some extension of infection up into the decidual basalis and more extensive neutrophilic inflammation (Figs. 3a, 12 a & b, 13, 24 b). ABC stains revealed marked involvement of the spongiotrophoblast zone (Fig. 14 a-d), Reichert's membrane (Figs. 15 & 16a) and the visceral yolk sac endoderm (Fig. 16 a & b). There was a paucity of TGCs in severely affected placentas. In some placentas, thrombosis of the uterine vessels in the junctional zone resulted in infarction of the labyrinth zone (Fig. 17 a & b). Fetuses associated with such placentas were autolyzed (Fig. 17b). Except for occasional infarction however, the labyrinth region of the placenta did not become involved in the course of infection. Viable fetuses appeared histologically normal.

A dose of $10^{5.7}$ cfu of brucellae was determined to be the minimum inoculum used that produced consistent, severe placental lesions (histologic score of 2.0 or greater, Table 1). Day 18 placentas from mice inoculated with $10^{4.7}$ cfu of brucellae had only occasional peripheral or junctional inflammation and were associated with viable fetuses (Table 1).

The granulomatous inflammation associated with brucellosis of non-reproductive tract organs has been well described (Young et al., 1979; Pugh et al., 1989; Enright et al., 1990a). These typical findings were present in the liver and spleen sections of all infected mice. A mild degree of hepatic lipidosis was often present in the livers of both infected and non-

infected pregnant mice. Sections of non-gravid uteri from infected mice and sections of spleens and placentas from mice receiving killed B. abortus or saline were histologically normal.

Ultrastructural examination of selected junctional zones of day 18 placentas confirmed the intracellular infection of trophoblast giant cells with B. abortus (Fig. 18 a-d). Membrane bound cisternae lined discontinuously on the cytoplasmic side with ribosomes contained numerous bacteria which stained positively with immunogold labeling for brucellae (Fig. 19). These cisternae were continuous with normal rough endoplasmic reticulum (RER) and the perinuclear envelope. Infected trophoblast cells were associated with focal areas of placental necrosis heavily infiltrated by neutrophils. In neutrophils brucellae were present within phagosomes rather than the cisternae of the RER (Fig. 20). Extracellular bacteria were abundant within necrotic foci and in maternal vascular channels (Figs. 20 & 21). Occasionally, colonies of bacteria were associated with Reichert's membrane on the periphery of the placental disk (Fig. 22).

Culture of infected spleens consistently yielded moderately heavy growths of B. abortus, the levels of which were independent of inoculation doses in the range tested (Table 1). There was no statistically significant difference in the degree of splenic infection between pregnant and non-pregnant mice receiving $10^{5.7}$ strain 2308 organisms (Table 3). The level of placental infection and the frequency and severity of placental lesions increased with dose (Table 1). At all doses tested, placental infection was approximately three logs greater per gram of tissue than in spleens. During the course of infection the level of splenic colonization

remained constant in contrast to the placental infection which had increased tenfold as pregnancy progressed (Fig. 23 & Table 2). Culture of non-gravid uteri yielded markedly lower numbers of B. abortus organisms per gram ($10^{2.2}$) as compared to placentas ($10^{9.6}$). Culture of spleens and placentas from mice receiving killed B. abortus were negative.

Circulating antibody titers to B. abortus, as measured by the Standard Tube Agglutination test, were detected only in mice infected with live brucellae at nine days post-infection (at all doses tested). Titers of infected pregnant (2.30) and infected non-pregnant mice (2.25) receiving $10^{5.7}$ strain 2308 organisms were similar.

DISCUSSION

This work indicates that there is preferential growth of B. abortus in the gravid uterus, specifically within the rough endoplasmic reticulum of the trophoblast cell in the mouse, confirming previous work that indicated this was true in the cow (Bosseray, 1980; Meador and Deyoe, 1989). Though factors responsible for this preferential growth have not been determined, erithrytol, a sugar alcohol synthesized in the ungulate placenta and known to stimulate the growth of virulent strains of B. abortus, has long been given credit for the preferential localization of this bacterium within the placenta of ruminants (Smith et al., 1962; Keppie et al., 1965). However B. abortus strain 19, the current vaccine strain and B. ovis, a closely related pathogen, do not metabolize this compound (and are in fact inhibited by it), but still localize and grow preferentially in the ruminant placenta (Keppie et al., 1965). Although low concentrations of erythritol are found in the placentas of rodents

(Smith et al., 1962), B. abortus will localize and proliferate preferentially in the placentas of rats and mice as described by others (Payne, 1957; Bosseray, 1980, 1983a) and corraborated in this study. Therefore it seems unlikely that a single growth factor is the cause of placental localization of Brucella species in both the bovine and murine species.

The interactions that result in Brucella-induced abortion are complex and poorly understood. Functional insufficiency of the placenta resulting from chorioallantoic necrosis and vasculitis have been suggested as a cause of fetal death in the ruminant infected with B. abortus (Payne, 1959). Placental vasculitis as well as chorioallantoic necrosis was a prominent feature in the goat model of brucellosis (Anderson et al., 1986b). In this study impairment of fetal circulation may have occurred as a direct result of placental vascular damage and inflammation or may have been due to the effect of Brucella endotoxin since endotoxin from other Gram-negative bacteria is known to induce alterations in endothelial function and coagulation (Stern et al., 1988; Schlessinger and Schaechter, 1989). Brucella lipopolysaccharide has induced abortion in a variety of species, including cattle (Pennel and Huddleson, 1937) and mice (Moreno et al., 1981). Fetal infection and resultant fetal stress-induced maternal hormonal changes have also been suggested as a cause of abortion (Kennedy, 1971; Enright et al., 1984; Enright, 1990). The majority of aborted bovine fetuses are infected and have a variety of gross and histological lesions (Enright et al., 1984; Jubb et al., 1985; Thoen and Enright, 1986).

The extent and severity of placentitis caused by B. abortus in both the ruminant and in experimental murine brucellosis is highly variable (Payne,

1959; Anderson et al., 1986b; Enright, 1990). Brucella infection in ruminants often results in extensive, widespread damage to placental tissues but abortion may also occur where only minimal, focal placental inflammation and damage is noted (Enright, 1990). It is therefore unlikely that placental damage alone is the sole cause of abortion in the ruminant. We found that in the mouse model however, dead fetuses were always associated with extensive placental damage, suggesting that placental insufficiency was an important cause of fetal death in this species. Autolyzed fetuses were most often paired with extensive necrosis of both the spongiotrophoblast and labyrinth, interpreted as placental infarction as a result of thrombus formation. Occasionally, thrombi were observed within the uterine spiral artery and maternal sinuses of the decidua basalis and spongiotrophoblast. Large bacterial colonies were frequently observed in close proximity to these vascular channels, suggesting a role for endotoxin induced endothelial damage, platelet activation and resultant thrombus formation in the pathogenesis of this lesion. Transplacental infection is known to occur frequently in heavily colonized placentas of the mouse but the level of fetal infection is low (Bosseray, 1982) and fetal microscopic lesions were not apparent, suggesting that placental damage rather than fetal infection with B. abortus was more important in determining fetal death in the pregnant mouse. In this study abortion did not occur in the mouse, perhaps due to the short duration of infection or because placentas in a uterus were not uniformally affected leaving some viable fetuses in each litter to support the pregnancy and prevent abortion of more severely affected ones.

Mice that received killed B. abortus did not suffer placental damage. This suggests that local bacterial replication is necessary to induce the placental lesions noted in this study. The consequent build-up of endotoxin may or may not play a role in the pathogenesis of the lesions.

In both the ruminant and the rodent placenta, selected populations of trophoblast cells are actively phagocytic during gestation (Wimsatt, 1950; Parks, 1952; Moskalewski et al., 1974; Myagkaya and Vreeling-Sindelarova, 1976; Jollie, 1981). In ruminants, erythophagocytic trophoblast cells are infected early in the course of Brucella infection (Molello et al., 1963a, 1963b, Anderson et al., 1986b, Meador and Deyoe, 1989). In the mouse the trophoblast giant cell (TGC), known to be actively phagocytic for a significant portion of gestation in the rodent (Jollie, 1965, 1981) was most susceptible to Brucella infection. The trophoblast cells in both species are extremely active physiologically and synthesize a wide variety of factors, including progesterone (Allen, 1975; Heap et al., 1983; Sherman, 1983) which is known to stimulate Brucella growth in vitro (Misra et al., 1976). In this study, the TGCs around the entire periphery of the mouse placenta were early sites of bacterial localization and replication, perhaps due to the phogocytic capabilities of this cell population. Physiologic degeneration of the decidua capsularis and parietalis and associated TGCs resulted in the localization of the infection to the remaining TGCs in the junctional zone of the placental disk. The lysis of infected TGCs resulted in the release of massive numbers of brucellae into the placenta and later, the uterine lumen as it reformed below the fetus. This shedding of bacteria into the local environment with concomitant replication likely resulted in the spread

of infection to other trophoblast cells, the fetus and eventually to the yolk sac endoderm cells.

As in avian placentation, the yolk sac placenta is an important component of the murine placenta. The rodent yolk sac is active in the uptake and transport of a variety of macromolecules such as maternal antibodies, B vitamins and iron to the fetus (Padykula et al., 1966; Lambson, 1968; Beaudin, 1980). In the chicken embryo infected with B. abortus, intracellular replication of the bacteria closely resembles that in experimental infection of the ruminant trophoblast cells and occurs within the RER of the yolk sac endoderm cells (Detilleux et al., 1988). The morphologic appearance of the free surface of the yolk endoderm cells of the rodent suggest motility and it is likely that the long microvilli present assist in the active uptake of material (Padykula et al., 1966) and perhaps are sites of attachment and entry of brucellae. In midgestation, with the degeneration of the parietal wall of the yolk sac, the visceral endoderm is directly exposed to the contents of the uterine cavity (Theiler, 1972, 1983), and thus may have been infected in this study through direct contact with uterine fluids or via the blood stream during bacteremia, this being a highly vascular membrane.

The nature, course and extent of the inflammatory reaction of murine brucellosis in this study resembles the infection of the pregnant mouse with Listeria monocytogenes (Redline and Lu, 1987). In the non-reproductive organs of the mouse, both bacteria elicit a predominantly granulomatous reaction, characterized by multifocal microaggregates of mononuclear cells predominantly occurring in the spleen, liver and kidney. Early in the course of the placental infection with either organism, the inflammatory

reaction is quite meager despite fairly heavy intracellular infection of the trophoblast cells. Once trophoblast cell necrosis occurs, neutrophilic infiltration is intense. These models support the theory that local immune regulation required at the fetal/maternal interface to prevent fetal rejection may influence the nature of the local inflammatory reaction to bacterial infection (Redline and Lu, 1988; Redline et al., 1988). Both Listeria and Brucella are intracellular pathogens, which typically require macrophages and lymphocytes to enter an infected organ for effective bacterial elimination (Hahn and Kaufman, 1981; Collins and Campbell, 1982). The uncontrolled growth of Listeria and Brucella in the placenta may be due to an impairment of function of these inflammatory cells due to local immune suppression which occurs during pregnancy (Redline and Lu, 1988; Redline et al., 1988). Trophoblast cells produce a variety of substances known to inhibit lymphocyte and macrophage function, including alpha feto-protein (Lu et al., 1984) and progesterone (Siteri et al., 1977; Lloyd, 1983). These are in high concentration locally and may inhibit macrophage infiltration and killing of these pathogens. Decidual cells also have been demonstrated as inhibiting lymphocyte activation by secreting substances that inhibit response to Il-2 (Clark et al., 1985) or by secreting PGE2, a known inhibitor of lypmhocyte stimulation and mitogenesis (Synder et al., 1982). Murine trophoblast cells also have been shown to inhibit the effector function of natural killer cells (Chaouat and Kolb, 1985). There does not appear to be preferential localization of either organism in the murine placenta but rather the preferential growth of these intracellular bacteria once localized within the area (Bosseray, 1983a; Redline and Lu, 1987). In

this study, preferential localization and growth of B. abortus was noted solely within the RER of TGCs. Localization specifically within the RER of the trophoblast cell may be necessary for effective replication of B. abortus, as has been suggested by the intracellular growth of this bacterium within the RER of Vero cells (Detilleux et al., 1990a, 1990b).

Data from this and other studies (Kenny and Diamond, 1977; Ward et al., 1978; Winter et al., 1986) suggest that pregnancy does not cause widespred generalized suppression of the immune response to the organism. The level of Brucella infection in the spleen of pregnant and non-pregnant mice given the same challege dose is approximately equivalent. Pregnant mice without overwhelming Listeria infection also controlled Listeria growth in their spleens and livers equally as well as non-pregnant controls (Reline and Lu, 1987). Pregnant mice in this study developed the same level of circulating antibodies as non-pregnant mice as assessed by the Standard Tube Agglutination test. A study utilizing heifers concluded that the magnitude and duration of the humoral and CMI response to brucellosis vaccination were, with few exceptions, unaffected by pregnancy (Winter et al., 1986). It is likely, therefore, that the exquisite sensitivity of the pregnant animal to brucellosis is not due to a generalized suppression of immunity but rather involves the combination of local suppression of the immune response with a susceptible cell population perhaps uniquely suitable for Brucella colonization and replication.

As occurs in cattle, so in murine listeriosis (Redline and Lu, 1987) and murine brucellosis (Bosseray, 1982) the placenta does not constitute an effective barrier protecting the fetus from infection. In murine brucellosis, the percentage of infected fetuses lags behind that of the

placentas and fetuses are never infected in the absence of placental infection (Bosseray, 1982), thus it is unlikely that bacteria first infect the fetus and later the placenta.

It is concluded that despite anatomical differences in the placentation between the ruminant and the rodent (epithelial vs. hemochorial), that there are significant similarities in the pathogenesis of placental infection of both groups with Brucella abortus. In the cow and the mouse, infection with brucellae during gestation results in the preferential growth of the organism specifically within the RER of the trophoblast cell of the placenta. Intratrophoblastic and probably some extracellular replication of brucellae results in placental damage, fetal infection and fetal loss in both species. The pregnant mouse, therefore, is a convenient model for the study of the pathogenesis of ruminant placental infection with Brucella abortus. This study also suggests that events within the placental microenvironment may strongly influence and perhaps hinder the local immune response, resulting in a predominantly neutrophilic inflammatory cell reponse. Thus physiologic impairment of cell-mediated immunity may lead to uncontrolled local bacterial replication and fetal wasteage. Further use of the pregnant mouse model for intracellular bacterial placental pathogenesis appears warranted and protection studies employing this model may prove to mimic the situation in cattle.

CHAPTER 4

A REVIEW OF THE

IMMUNOLOGY OF BRUCELLOSIS

As with most other bacterial pathogens, infection with B. abortus results in the production of specific antibodies as well as the development of cell-mediated immunity. The precise contribution of either response towards immunity against brucellosis remains inconclusive. Facultative intracellular bacteria are capable of multiplying within phagocytic cells, being protected from extracellular factors such as complement, specific antibodies and antibiotics. By resisting the bacteriocidal activities of phagocytes, elimination of virulent strains of Brucella depends on a cell-mediated response involving both T-lymphocytes and macrophages. A better understanding of the importance and interaction of these two arms of the immune response in protection against Brucella infection would be helpful in developing a more effective vaccine.

4.1 Humoral response

Control programs for brucellosis are based primarily on the immunization of cattle and the identification and slaughter of naturally infected cattle. Although the immune response is most often measured by serum antibody titer, the role of antibody in protection against brucellosis is unclear. Antibody may be involved in the complement-mediated killing of the organism during the extracellular phase of infection (McCullough, 1970) and/or may play a role in the opsonization and intracellular

killing of the bacteria by phagocytes (Cheers and Ho, 1983; Canning et al., 1988). Numerous investigators have shown that passive immunization of mice with immune sera (Plommet and Plommet, 1983; Montaraz and Winter, 1984; Bosseray and Plommet, 1988; Araya et al., 1989) or monoclonal antibodies (Montaraz et al., 1986; Limet et al., 1987; Phillips et al., 1989a; Winter et al., 1989) directed against the O-side-chain of the LPS molecule affords a degree of protection against subsequent challenge with a virulent strain of B. abortus. There is evidence that the humoral response is particularly effective early in the course of infection, most likely due to the extracellular habitat of organisms at that time exposing the bacteria to circulating antibodies, but that other immune mechanisms are necessary during the later stages of infection (Araya et al., 1989; Philips et al., 1989a).

The role of antibodies in extracellular killing is still controversial. Sera from agammaglobulinemic calves, or unexposed adult cattle or from adult cattle in the early phases of infection have equal abilities to kill B. abortus in vivo, indicating that complement-mediated killing can occur in the absence of either natural or acquired antibodies. Since virtually no killing activity remained after treatment with ethylene glycol-tetraacetic acid and magnesium chloride (EGTA-$MgCl_2$ chelates calcium, inactivating C1, to inhibit the classical but preserve the alternative complement pathway), it is likely that classical complement pathway activation occurs (Corbeil et al., 1988). This is consistent with data showing that B. abortus LPS does not activate the alternate pathway of complement (Hoffman and Houls, 1983). It has been reported that lipid A from B. abortus can activate the complement cascade (Moreno et al., 1981) and

that lipid A of other Gram negative bacteria binds and activates C_1 to initiate the classical pathway of complement (Betz and Isliker, 1981). Serum from cattle early in infection did not inhibit or increase complement-mediated killing of bacteria whereas killing was significantly decreased in serum from infected cattle later in infection. The first samples taken after infection contained increased levels of IgM but by the time killing decreased, IgM had increased along with IgG1 & IgG2. In later samples when IgG antibodies predominated, bacterial killing was negligible and the addition of purified IgM to normal bovine serum produced no significant change in killing. In contrast, the addition of IgG 1 & 2 anti-smooth LPS antibodies blocked serum bacteriocidal activity. The lack of enhanced killing when IgM was added is not clear and may be related to the capacity of B. abortus to bind to the Fc portion of a subpopulation of IgM (Corbeil et al., 1988). The cause of the blocking action of the IgG isotypes is also unknown as both have been reported to fix bovine complement (McGuire and Musoke, 1981). Thus complement-mediated killing may occur principally early in infection and the role of antibody may be principally directed at opsonization. Later in infection, IgG antibodies which block serum killing may also protect the host against the release of excessive quantities of endotoxin (Corbeil et al., 1988).

Further obscuring the role of antibody in protective immunity is the lack of evidence correlating the level of agglutinating antibodies with protection againist brucellosis (Rasooly et al., 1968). In addition, another study comparing inherent resistance and susceptibility to brucellosis revealed that susceptible vaccinated cattle were found to have initially higher and more prolonged total antibody response, significantly higher

IgG1 responses and preferential expression of IgG2a allotypes (Butler et al., 1986) upon challenge than resistant vaccinated cattle. A recent investigation of natural resistance to Brucella infection in cattle found that naturally resistant cattle developed low circulating antibody titers and did not abort, in contrast to naturally susceptible cattle which developed high titers and aborted their fetuses. (Price et al., 1990).

In a murine study of protection induced by subcellular vaccines, there was a proportional relationship between the ability of a vaccine to induce antibodies against the O-side-chain antigen and its ability to induce protective immunity against splenic infection upon challenge with virulent strain 2308. However, this relationship did not hold in all cases and examples of mice with a high level of protection in the absence of anti-O-antibody and others in which protection was marginal or absent despite raised levels of anti-O-antibody makes it unclear as to the importance of O-side-chain antigen in protective immunity (Winter et al., 1988).

4.2 Cell-mediated response

Although antibodies may play a role in protective immunity against brucellosis, the bacteriocidal phase in murine brucellosis is correlated with the onset of specific cell-mediated immunity. (Ho and Cheers, 1982). A recent study in mice on the temporal development of protective cell-mediated and humoral immunity to Brucella infection found that serum antibodies conferred significant protection at 3 weeks post-infection whereas protection by T-lymphocytes was not evident until 4 weeks post-infection (Araya et al., 1989). The combined transfer of immune serum

and T-lymphocytes enhanced protection over that provided by either cells or serum alone. This study also examined the specific T-lymphocyte lineages involved in protective immunity and suggested that the development of resistance to B. abortus in mice is the result of independent and probably interactive effects of antibody and T-effector-lymphocytes of both the CD4 and the CD8 phenotypes. The initial decline in bacterial numbers which occurred in the absence of detectable cell-mediated immunity was ascribed to the effects of antibody and non-immune stimuli responsible for the increased formation, attraction and activation of macrophages. The kinds and relative importance of effector functions mediated by CD4 and CD8 cells, including the activation of macrophages and direct cytolytic effects, remains to be established as do the interactive effects between antibodies and immune T-lymphocytes. It is likely that opsonization coupled with enhanced intracellular killing (Harmon et al., 1989) constitute the principle protective role of antibody against B. abortus effective early in the extracellular phase of infection but that eventual resistance depends on the successful collaboration between specifically sensitized T-lymphocytes and macrophages (Splitter and Everlith, 1986).

4.3 B. abortus strain 19

A better understanding of the critical components of pathogen antigenicity and host response would allow the development of a more effective vaccine for bovine brucellosis. B. abortus strain 19, an attenuated smooth strain, is the organism used widely as a live vaccine in control programs. This strain has several disadvantages as a vaccine: it is known to protect only 65-75 percent of recipients (Manthei, 1959); it

may cause abortion if administered to pregnant cattle (Corner and Alton, 1981); it remains a health hazard to those who administer it (Reviah et al., 1961) and a proportion of cattle vaccinated with this strain maintain agglutinating antibody titers which impede clear distinction between naturally exposed and vaccinated animals on serological examination (Nicoletti, 1981). As the agglutinating antibodies detected in standard serological tests for brucellosis are almost exclusive for the O-side-chain of the LPS molecule, (Diaz et al., 1968; Schurig et al., 1981) the use of a stable rough variant which lacks the O-side-chain potentially could overcome the serologic diagnostic problems associated with vaccination. Use of such a vaccine strain would be an improvement over the current vaccine strategies if it were able to induce protective immunity in the host while only replicating for a limited amount of time. Whether or not immunologic response to the O-side-chain is an essential element in protective immunity is presently unclear.

In many Gram negative organisms, virulent strains tend to form surface components associated with a smooth colony morphology. This appears to be associated with the polysaccharide chains on the outer membranes of the Enterobacteriaceae and brucellae (Davis, 1980). In vitro studies examining the pathogenecity of various strain of B. abortus indicate that rough strains are more susceptible to host bacteriocidal mechanisms than are smooth strains. The lack of the O-side-chain was correlated with increased killing of rough strains of B. abortus as compared to smooth strains by bovine serum (Corbeil et al., 1988) and is consistant with data on the serum resistance of other rough and smooth Gram-negative bacteria including the Enterobacteria (Taylor, 1983). Reduced survivabiltiy

of rough strains of B. abortus was also documented both within bovine polymorphonuclear leukocytes (Riley and Robertson, 1984) and macrophages (Harmon et al., 1988). This decrease in survival was proposed to relate to cell surface components. Smooth strains of B. abortus also replicate more rapidly in guinea pig monocytes than do less virulent rough strains (Braun et al., 1958). These findings are consistent with a study which revealed that smooth Brucella cell wall components are more resistent to digestion by hydrolytic enzymes than rough strains (Kreutzer et al., 1979). Thus use of a rough strain of Brucella as a vaccine might offer both decreased virulence and freedom from serologic confusion between vaccinates and field infections.

4.4 B. abortus strain 45/20

B. abortus strain 45/20 is a rough variant that was first isolated from a cow in 1922 (McEwen and Priestly, 1938) and later studied extensively as a potential vaccine. This strain appeared promising since it was rough as judged by colony morphology, and reactivity with crystal violet and acriflavine and was shown to have good immunizing properties in cattle and guinea pigs (McEwen, 1940). Various studies have now concluded that rough strain 45/20 is unstable since it reverts to a smooth virulent mutant (Edwards et al., 1945; Wilson Taylor and McDiarmid, 1949) and is not totally devoid of the O-side-chain of the LPS molecule (Corbel and Bracewell, 1976; Schurig et al., 1984, 1991). The strain was used for some years as a killed vaccine but the limited immunogenicity of the non-replicating bacteria required multiple injections to provide maximum protection and the 45/20 adjuvant bacterins often caused a marked local

reaction at the site of injection. With the development of the reduced dosage strain 19 vaccine which decreased the problem of the seroconversion of vaccinates, use of strain 45/20 has declined (Nicoletti, 1990).

4.5 B. abortus strain RB51

B. abortus strain RB51 was developed by the in vitro passage of virulent field strain 2308 on rifampin (Schurig et al., 1991). This rifampin resistant rough mutant has been found to be stable in vivo and in vitro and is highly deficient in the O-side-chain of the LPS molecule. Rabbits, mice, goats and cattle hyperimmunized with sonicates or live suspensions of strain RB51 develop antibodies to B. abortus specific antigens but do not develop antibodies specific for the O-side-chain.

Recent studies with strain RB51 indicate that this strain has reduced virulence in the non-pregnant mouse model as well as in preliminary in vivo and in vitro studies in the ruminant. BALB/c mice infected with strain RB51 by the intraperitoneal route clear the organism within four weeks (Schurig et al., 1991). This is in contrast to attenuated strain 19 and virulent strain 2308 which may be isolated at six and 24 weeks post-infection respectively (Montaraz and Winter, 1986). A study examining the pathogenicity of strains 19 and RB51 in fetal goats revealed that fetuses inoculated with strain 19 were aborted 11 days post-infection while fetuses receiving strain RB51 were carried to term and born alive. While fetal infection occurred with both strains resulting in gross and histologic lesions, this study suggests that strain RB51 may be less pathogenic to the feto-maternal unit. To explore further the differences in pathogenicity of various strains of B. abortus to the ruminant, mid-gestational bovine

chorionic membrane explants were also infected with either strain 2308, 19 or RB51. All explants were inoculated with equal numbers of one of these strains. Histologic examination of tissue harvested 12 hours post-inoculation with strain 2308 revealed near total destruction of chorionic trophoblast cells. In contrast, both strains 19 and RB51 caused only slight necrosis of trophoblast cells, despite the fact that these two strains were cultured in higher numbers from the explant tissue than the virulent strain (Enright, 1991).

Preliminary experiments with strain RB51 indicate that this strain confers significant protection to non-pregnant mice against challenge with virulent B. abortus strain 2308 as assessed by splenic bacterial colonization (Schurig et al., 1991). Adoptive transfer experiments with nylon wool purified splenic lymphocytes obtained from RB51 immunized mice demonstrated protection (Bagchi and Schurig, 1990) at a level comparable to that obtained with lymphocytes from strain 19 immunized mice. Preliminary experiments with adult cattle have indicated that strain RB51 immunization can protect this species against challenge with strain 2308 and that this protection is superior to that induced by strain 19 (Enright et al., 1990b).

Thus limited trials with this organism suggest that strain RB51 has the potential of being used as a protective, live, attenuated vaccine which would not elicit the O-side-chain specific humoral immune response that interferes with standard serologic tests for brucellosis. If these limited trials are predictive of the behavior of strain RB51 when used to immunize cattle herds against bovine brucellosis, development of this

strain as a vaccine would be a significant contribution towards the control of brucellosis world-wide.

To further evaluate the potential of strain RB51 as a vaccine, the following investigation was carried out utilizing the pregnant mouse model of brucellosis. The pathogenicity of strains 19 and RB51 were compared to the previously examined virulent strain, B. abortus 2308, to determine if the attenuation of virulence noted in the non-pregnant mouse model and in bovine trials would be reflected in the pregnant mouse model. Histology of placentas infected with each strain was included to compare and contrast the pathogenecity of various strains of B. abortus for the murine placenta and to examine the inflammatory reponse to infection. The serologic response of the pregnant mouse to infection was examined to determine if, as previously reported in the non-pregnant mouse, infection with strain RB51 did not result in the production of anti-O-side-chain antibodies sufficient to interfere with standard agglutination tests used to monitor cattle herds for brucellosis.

CHAPTER 5

COMPARATIVE BEHAVIOR OF BRUCELLA ABORTUS STRAINS 19 AND RB51 IN THE PREGNANT MOUSE

In cattle, brucellosis is an important cause of reproductive failure characterized by abortion and decreased fertility (Enright, 1990). Brucella abortus, the principal causative agent of bovine brucellosis, may also cause a serious human illness commonly known as undulant fever (Acha and Szyfres, 1980). Field strains and virulent laboratory adapted strains of B. abortus occur in the smooth phase and contain a homopolymer of perosamine as the O-side-chain of the lipopolysaccaharide (LPS) molecule (Bundle et al., 1985). A major portion of the humoral immune response is directed against this antigen and conventional tests used for the serologic diagnosis of brucellosis are almost exclusively based on the detection of antibodies specific for this O-side-chain (Diaz et al., 1968; Cherowonogrodsky et al., 1990). Strain 19 is the current vaccine strain used widely in brucellosis control and eradication programs. This strain is an attenuated smooth organism and will cause a proportion of vaccinates to develop and maintain agglutinating titers which confound serodiagnosis of field cases (Subcommittee on Brucellosis Research, 1977). This problem becomes particularly serious if cattle over one year of age are vaccinated (King and Frank, 1961). Strain 19 may also cause abortion in pregnant cattle (Corner and Alton, 1981; Becket and McDiarmid, 1985), will protect only 65-75% of vaccinates (Manthei, 1959; Confer et al., 1985) and is a health hazard to people who administer it (Reviah et al., 1961). B.

abortus strain RB51 is a stable rough organism that lacks or contains only minimal amounts of O-side-chain and therefore does not induce a humoral immune response to this antigen when used as a live vaccine (Schurig et al., 1991). Strain RB51 is less virulent than strain 19 in the non-pregnant mouse model as assessed by level and duration of splenic infection and has been found to protect non-pregnant mice against challenge with strain 2308 (Schurig et al., 1991), a virulent strain used widely in brucellosis research. An in vitro study assessing the pathogenic potential of B. abortus strains 2308, 19 and RB51 for the bovine placenta confirmed the reduced virulence of strain RB51. Histologic examination of strain 2308 infected uterine explants revealed near total destruction of the chorionic trophoblasts while infection with strain 19 or RB51 resulted in minimal damage to trophoblast cells (Enright, 1990).

The present study describes the use of the pregnant mouse for comparing the pathogenecity of B. abortus strains 19 and RB51 to strain 2308. The pathology of the pregnant mouse inoculated with strain 2308 has been recently described as having significant similarities to the disease in pregnant cattle (Chapter 3). In the cow and the mouse, B. abortus localizes and preferentially multiplies within the rough endoplasmic reticulum of the trophoblast cell (Meador and Deyoe, 1989; Chapter 3). In both hosts Brucella colonization of the gravid reproductive tract can lead to severe placental damage, fetal infection and fetal death (Payne, 1959; Bosseray, 1982; Chapter 3). A dose of $10^{5.7}$ B. abortus strain 2308 given intraperitoneally (IP) to BALB/c mice on day 9 of gestation of the 19 day gestational interval (day 1 = day vaginal plug observed) consistently produced a severe, necro-suppurative placentitis often associated with

fetal death when the mice were killed and examined on day 18 of gestation. (Chapter 3).

Material and Methods

Groups of 6 to 10, timed-pregnant BALB/c mice received varying doses of either strain 19 or RB51 on day 9 of pregnancy and were killed 9 days thereafter (day 18 of pregnancy). Blood was collected by cardiac puncture immediately post-mortem for serologic examination. The Standard Tube Agglutination test (STA) was performed on all sera according to the methods described by Alton et al. (1975; Appendix). Non-agglutinating titers of strain RB51-infected mice were determined by BioDot ELISA (enzyme linked immunosorbant assay) (Burhman, 1989; Appendix). Titers are expressed as the $\log_{10}$ value of the reciprocal. Selected sera underwent western blot analysis for detection of antibodies against O-side-chain of LPS and other Brucella antigens (Schurig et al., 1991; Appendix). Spleens and two placentas per mouse were removed aseptically and cultured for the detection of Brucella by homogenation in sterile saline, serial dilution and plating on trypticase-soy plates (Schurig, et al., 1991). The remaining placentas were fixed in situ within uteri in 10% neutral buffered formalin for light microscopy. After routine histologic processing, placental sections were stained either with hematoxylin and eosin (H & E) or, for more sensitive localization of bacterial colonization, received a standard avidin-biotin immunohistochemical stain (ABC-Appendix). Primary antibody for the ABC stain consisted of rabbit polyclonal serum against either strain 19 or strain RB51 as appropriate (Appendix). H & E stained sections of placentas were coded and examined as unknowns and

scored on a scale of 0 to 3 (0 = no change, 1 = peripheral inflammation, 2 = lesions of grade 1 plus spongiotrophoblast necrosis, 3 = lesions of grade 2 plus fetal death, Chapter 3). Examination of gestational age-matched placentas from non-infected mice allowed the establishment of baseline scores. Infective doses of strains 19 and RB51 were adjusted by $\log_{10}$ increments to determine the minimum inoculum necessary to produce a mean placental score of 2.0, similar to that caused by an IP injection of $10^{5.7}$ strain 2308 organisms (2.2 +/- 0.4, Chapter 3,). When this minimum dose for both strains was determined, ten non-pregnant mice received that dose IP and were killed 9 days later. Blood was collected for serology and spleens cultured for Brucella.

Results and Discussion

A minimum dose of $10^{7.5}$ B. abortus strain 19 was required to produce lesions consistently similar to those caused by $10^{5.7}$ strain 2308 organisms, as measured by a histological score of 2.3 +/- 0.3 (Figs. 24 a-c., 25 a & b, Tables 4 & 6). A dose as high as $10^{9.5}$ strain RB51 organisms failed to produce comparable lesions and resulted in only minimal bacterial colonization, trophoblast necrosis and inflammation within the spongiotrophoblast (Figs. 26 a-d & 27 d) or associated with the recoiled Reichert's membrane (Fig. 27 a-c, Tables 5 & 6, scored 0.8 +/- 0.6). Both strains 19 and RB51 localized primarily within the giant trovhoblast cells (Figs. 24 c, 27 c & d) located at the placental maternal/fetal interface as did virulent strain 2308 (Fig. 24 a). Placental damage and colonization by strain RB51 was markedly less than with either strain 19 or strain 2308 even at the high dose given (Table 6). Higher doses of

strain RB51 were not administered in an attempt to produce a more severe placental lesion as it was felt that greater challenge would increase the amount of endotoxin to levels which by themselves would be detrimental (Moreno et al., 1981) and that reduced virulence of strain RB51 as compared to strain 19 had already been demonstrated with the doses used.

Brucella cultured from the placentas were characterized according to colony type by: visual inspection; autoagglutinating capacity (Braun and Bonestall, 1947; Appendix); absorption of crystal violet (White and Wilson, 1951; Appendix) and reactivity in a colony blot ELISA using the monoclonal antibody, BRU 38, which recognizes the O-side-chain of the B. abortus lipopolysaccharide (Roop et al., 1987; Appendix). Colonies recovered from mice receiving strain RB51 absorbed the crystal violet stain, a suspension of these organisms autoagglutinated and colonies failed to react with BRU 38, confirming stability of rough colony morphology. In contrast, bacterial colonies recovered from placentas of mice inoculated with strain 19 did not take up crystal violet stain nor did these bacteria autoagglutinate and they reacted strongly with the monoclonal antibody for the O-side-chain of the LPS as is expected from a smooth type Brucella strain.

Based on colony counts, the level of splenic infection resulting from inoculation of $10^{7.5}$ strain 19 or $10^{5.7}$ strain 2308 organisms was similar while a dose of $10^{9.5}$ strain RB51 organisms produced approximately one $\log_{10}$ less splenic colonization (Table 6). This pattern of relative virulence for the three strains in the placenta of the pregnant mouse is consistent with previous splenic colonization findings in the non-pregnant mouse at comparable times post-infection (Montaraz and Winter, 1986; Schurig,

1991). The virulence of strains 19 and RB51 in the non-pregnant BALB/c mouse have been found to reflect their pathogenicity in cattle (Enright, 1990; Enright et al., 1990; Schurig et al., 1991).

Strain 19 infected mice showed strong seroconversion in the Standard Tube Agglutination test. In contrast, the titer of the sera from strain RB51 infected mice did not differ from the titer of sera obtained from control seronegative mice (Table 6). Sera from strain RB51-infected mice did not react against the O-side-chain as demonstrated by a negative reaction against Yersinia enterocolitica O:9 LPS, which has an essentially identical O-side-chain structure B. abortus (Caroff et al., 1984a; Cherowonogrodsky et al., 1990) or LPS from B. abortus strain 2308 by western blot analysis. These sera did react weakly against a variety of antigens distinct from the O-side-chain present in strain RB51 (Fig. 28) and had non-agglutinating titers of 2.6 +/- 0.2 in a BioDot ELISA, demonstrating that RB51 will induce a humoral immune response in the pregnant mouse. Sera from strain 19-infected mice reacted with the LPS of both the above bacteria and showed some reaction to non-O-side-chain related strain RB51 antigens (Fig 28). Thus, strain RB51 retained both antigenic and morphologic stability with in vivo passage in the pregnant mouse as had been demonstrated previously in the non-pregnant mouse (Schurig et al, 1991).

Ten non-pregnant mice received either $10^{7.5}$ strain 19 or $10^{9.5}$ strain RB51 IP and were killed nine days later. Splenic colonization and serum agglutinating titers did not differ significantly from those of pregnant mice (ANOVA $p<0.05$, Table 3). These findings are in aggreement with other studies which concluded that reproductive status does not alter the humoral response to this organism in mice (Chapter 3) or cattle

(Winter et al., 1986) or susceptibility to systemic bacterial infection in mice (Tobias et al., 1991a; Ward et al., 1978).

This study, together with the previously demonstrated protective abilities of strain RB51 in non-pregnant mice (Schurig et al., 1991) and cattle (Enright et al., 1990b), supports the ongoing efforts in developing strain RB51 as a live vaccine for bovine brucellosis. It confirms the decreased pathogenicity and virulence of this strain as compared to the current strain 19 vaccine. The lack of seroconversion of strain RB51-inoculated mice as assessed here by the STA test and absence of O-side-chain antibodies confirmed by western blot analysis demonstrates that this strain will not induce significant levels of agglutinating antibodies in pregnant as well as non-pregnant animals. Studies examining the protective abilities of both strain RB51 and strain 19 in the pregnant mouse system are to be reported.

CHAPTER 6

A REVIEW OF THE USE OF THE PREGNANT MOUSE MODEL IN BRUCELLOSIS PROTECTION STUDIES

The non-pregnant mouse has been used extensively as a model to examine the components of protective immunity in brucellosis (Cheers, 1984; Phillips et al., 1989a; Winter et al., 1989; Araya et al., 1990) and to aid in vaccine development (Bosseray 1978, Montaraz and Winter 1984, Winter et al., 1988, Beuclair and Khansar, 1990). Recent research carried out with this model, reviewed in Chapter 4, has not yet established the respective roles of humoral and cell-mediated mechanisms intervening in the resisitance to systemic infection, though it appears that both arms of the immune response participate in protective immunity, likely in a temporal fashion. The mechanisms involved in protection of the gravid reproductive tract are currently unknown. Increased susceptibility of the pregnant animal to brucellosis may be linked to the high susceptibility of the placenta to infection by this pathogen. The mechanisms involved in placental and in systemic immunity may be different in nature or efficacy and these differences may be pertinent to the development of effective vaccines for bovine brucellosis.

Brucella research employing the pregnant mouse model to examine the immune mechanisms of brucellosis has been limited and has not involved the histopathology of placental infection. Bacteriologic examination of the spleen, placenta and fetus was employed to assess protection offered by vaccination prior to pregnancy or by the transfer of immune sera or

splenic lymphoid cells. Mouse serum raised against Brucella peptidoglycan (PG) or LPS fractions and splenic lymphoid cells from peptidoglycan fraction vaccinated mice when given prior to challenge transferred passive or adaptive immunity against Brucella infection to pregnant mice. Immunity was expressed by lower frequency and/or level of infection in spleens, placentas and fetuses (Bosseray and Plommet, 1988).

Vaccination one month prior to pregnancy decreased the frequency of placental colonization and the number of brucellae per placenta and spleen. Vaccination with protein-bound cell wall PG fractions extracted from smooth brucellae protected pregnant mice as well as did vaccination with killed virulent B. abortus strain H38 or living attenuated B. abortus strain B19. The LPS fraction of the cell wall was comparatively less protective when used as a vaccine. Interestingly, anti-LPS, anti-PG or anti-killed smooth Brucella sera protected pregnant mice as well as did vaccination, as evaluated by culture of the spleens and placentas (Bosseray, 1983b).

Thus humoral immunity appears to act efficiently against placental brucellosis in the pregnant mouse model. Slight decreases in the intensity or duration of the bacteremia may decrease the probability of blood borne-brucellae reaching the placenta and the fetus. Supporting this hypothesis, decreased protection was observed when serum was transferred after challenge (Bosseray, 1983b). Macrophages are present within the murine placenta (Moskalewaski et al., 1974) but the ability of these cells to restrict Brucella multiplication, especially once the pathogen localizes within the trophoblast cells in the unique immune setting of the fetoplacental interface, is unknown.

Histologic characterization of the lesions of murine placental brucellosis will allow refinement of the use of this model to study various aspects of immunity as they relate to the pregnant animal. The degree of protection afforded the pregnant mouse by passive or active immunization may now be assessed by the level and frequency of splenic and placental infection, the degree of lesion development in the placenta and the frequency of fetal death.

The following investigation combined the use of bacteriology, serology and histology to evaluate protection afforded by circulating antibodies or by vaccination in the pregnant mouse. To continue exploring the use of strain RB51 as an effective vaccine, mice were immunized with either strain 19 or strain RB51 before challenge in mid-gestation with virulent strain 2308. In addition, immune sera raised in mice against either strains 19 or RB51 or a monoclonal antibody specific for the O-side-chain of the LPS molecule was administered immediately before mid-gestation challenge to evaluate the role of humoral immunity in protection provided the pregnant mouse by these two strains.

CHAPTER 7

BRUCELLA ABORTUS STRAINS 19 AND RB51;

COMPARATIVE PROTECTION IN THE PREGNANT MOUSE MODEL

Brucellosis remains an important cause of abortion in cattle (Thoen and Enright, 1981) and a serious human disease worldwide (Corbel, 1989). Attenuated Brucella abortus strain 19, the currently used vaccine strain, can cause abortions in pregnant cows (Corner and Alton, 1981; Becket and Mcdiarmid, 1985), induces agglutinating and complement fixing antibodies to lipopolysaccharide O-side-chain (Subcommittee on Brucellosis, 1977), protects only 65-75% of vaccinates (Confer et al., 1985; Manthei, 1959) and remains a health hazard to those who handle it (Reviah et al., 1961). B. abortus strain RB51, a stable rough mutant derived from virulent strain 2308, appears to have vaccine potential (Schurig et al., 1991). In the non-pregnant mouse, strain RB51 is of low virulence, protects against challenge by virulent strain 2308 as assessed by splenic bacterial colonization, and does not elicit antibodies detectable by standard agglutinating tests used to monitor cattle herds for brucellosis. This is due to its inability to induce anti-O-side-chain antibodies. An in vitro study examining the pathogenic potential of B. abortus strains 2308, 19 and RB51 for the bovine placenta confirmed the reduced virulence of strain RB51 by histologic evaluation of uterine explants infected with these strains. Strain 2308 infection resulted in near total destruction of chorionic trophoblast cells while infection with either strain 19 or RB51 produce only minimal damage to these cells (Enright, 1990).

The murine model of brucellosis has been used extensively to study the immunology, bacteriology and pathogenesis of the disease, though the vast majority of studies have used the non-pregnant mouse (Feldman, 1935; Young et al., 1979; Ho and Cheers, 1982; Phillips et al., 1989b; Enright et al., 1990a). The pregnant mouse model has been largely ignored despite the fact that brucellosis is most importantly a reproductive tract disease. We have found that an intraperitoneal injection of $10^{5.7}$ B. abortus strain 2308 given in midgestation to BALB/c mice consistently produces a severe, necro-suppurative placentitis often associated with fetal death (Chapter 3). As in cattle (Meador and Deyoe, 1989), murine placental infection involves the localization and replication of B. abortus within the rough endoplasmic reticulum of the trophoblast cell, resulting in placental damage, transplacental infection and fetal death (Bosseray, 1982; Chapter 3). Strain RB51, as compared to strains 19 and 2308, has reduced virulence in the pregnant (Chapter 5) and non-pregnant mouse (Schurig et al., 1991), as assessed by splenic and placental infection and placental lesions. Confirming studies in the non-pregnant mouse (Schurig et al., 1991), strain RB51 remains stable after in vivo passage and does not induce agglutinating antibodies in the pregnant mouse (Chapter 5). The purpose of the study reported here was to examine the protective abilities of strains 19 and RB51 in the pregnant mouse against challenge with virulent strain 2308. Both active immunity induced by living vaccines and passive immunity obtained by the transfer of immune sera against the two strains were tested for efficacy.

Material and Methods

1. Animals: Two- to six-month-old BALB/c female mice raised at the animal holding facility at the Veterinary Medical Research Center at Virginia Tech were mated individually to four- to ten-month-old male BALB/c mice. Male mice and the parents of the female mice were obtained from Dominion Laboratories, Dublin, V.A.. Sentinel mice killed at the conclusion of the experiment were found free of antibodies to major murine pathogens (Tobias et al., 1991a). The BALB/c strain was chosen for this study due to its widespread use in brucellosis research (Montaraz and Winter, 1986; Phillips et al., 1989b; Pugh et al., 1989; Araya et al., 1990; Phillips et al., 1990a; Enright et al., 1990a), including previous work in our laboratory with the pregnant mouse model (Tobias et al 1991a, 1991b). Clearance of B. abortus strains 2308 (Phillips et al., 1989b; Enright et al., 1990a), 19 (Montaraz and Winter, 1986; Araya et al., 1989) and RB51 (Schurig et al., 1991) in BALB/c mice corresponds closely to their respective virulence in cattle (Nicoletti and Milward, 1983; Nicoletti, 1990; Schurig et al., 1991), suggesting that this inbred strain of mouse is an appropriate choice for initial vaccine trials.

2. Bacterial cultures: B. abortus strains 19 and RB51 grown on trypticase soy plates for 48 hours at 37°C in 5% CO_2, both from stock cultures held at the P3 facility of the Veterinary Microbiology Research Laboratories, were used as live vaccine strains and for the production of antisera for passive immune transfer. The vaccine dose for strain RB51 was 10^8 bacteria since fewer organisms produce inconsistent infections in mice (personal communication, G. G. Schurig). Strain 19 vaccinates received

10^6 organisms, a dose used in a previous protection study involving pregnant mice (Bosseray, 1983b). Virulent B. abortus strain 2308, also from stock cultures, was used for all challenge infections at a dose of $10^{5.7}$ organisms per mouse, an inoculum that consistently causes severe placental lesions in naive BALB/c mice (Chapter 3). For vaccination and challenge, cultures of predetermined concentrations of viable bacteria were held suspended in a trypticase-soy broth at -70°C until used. They were thawed, diluted in sterile saline to the desired concentration at the time of inoculation and administered intraperitoneally. Exact doses delivered were confirmed retrospectively by determining colony forming units (cfu) as described elsewhere (Chapter 3).

3. Serology: The Standard Tube Agglutination (STA) test was performed according to the method of Alton et al. (1975) on all samples (Appendix). Non-agglutinating titers of strain RB51 immunized mice were determined by BioDot enzyme-linked immunosorbant assays (ELISAs) using whole RB51 bacteria as antigen (Burhman, 1989; Appendix) and selected samples were analyzed by western blot for antibodies to the O-side-chain of the LPS (Schurig et al., 1991; Appendix). Titers are expressed as the $\log_{10}$ value of the reciprocal.

4. Antibodies for passive transfer: Sera were obtained from mice actively immunized with the above doses of strain 19 or RB51. These mice were bled from the retro-orbital space four weeks post-vaccination and the sera of each group pooled for passive transfer. Sera from strain 19 vaccinates had a titer of 2.60 by STA test and a titer of 2.51

by Biodot ELISA while strain RB51 vaccinates had no detectable titer in the STA test (<1.39) but had a titer of approximately 3.41 to strain RB51 antigens as determined by Biodot ELISA (Table 9). Sera from unvaccinated mice served as a control for both serology and passive tranfer. BRU 38, a monoclonal antibody recognizing the O-side-chain of B. abortus lipopolysaccharide (LPS), was also used in the passive tranfer study. BRU 38 is a rat IgG2a secreted by a hybridoma resulting from the fusion of a mouse SP2/0 myeloma cell and a rat B cell (Schurig et al., 1984; Appendix). Supernatants from BRU 38 hybridoma were collected and applied to a column filled with beads coupled to goat antibodies against rat IgG (#A6542, Sigma Chemical Co., St. Louis, MO). Beads were washed and monoclonal antibodies desorbed with glycine buffer (pH 2.2) following the instructions of the manufacturer. The desorbed monoclonal antibodies were held at $4^{o}C$ and concentrated (Amicon Minicon concentrator, Amicon Corporation, Lexington, MA) to 115 micrograms protein per milliliter phosphate buffered saline (BIO-RAD protein determination assay, BIO-RAD, Richmond, CA: Appendix).

5. Active Immunization: Groups of forty, eight-week-old female mice received 10^{6} strain 19, 10^{8} strain RB51 or sterile saline intraperitoneally and were mated ten weeks later. Twenty bred mice from each group were challenged IP on day 9 of gestation (day 1 being the day the vaginal plug was observed) with $10^{5.7}$ B. abortus strain 2308. Remaining bred mice from each group received sterile saline IP on day 9 of gestation. Previous studies have determined that the doses of strains RB51 and 19 employed in this study are cleared systemically four weeks (Schurig et

al., 1991) and eight weeks (Montaraz and Winter, 1986) post-inoculation respectively, therefore an eleven-week period between vaccination and challenge was deemed sufficient to prevent vaccinal recrudesence at pregnancy. A fourth group of twenty mice were vaccinated twice with 10^8 Strain RB51, first at eight weeks of age and again six weeks later. Previously unreported studies determined that this dose of strain RB51 given twice will be cleared by ten days after the second inoculation (personal communication, G. G. Schurig). These mice were bred ten weeks after the second vaccination and challenged with strain 2308 nine days later.

6. Passive Transfer of Immunity: Groups of five timed-pregnant mice, 2- to 4-months-old, received 0.1 milliliter of immune serum from strain RB51 or strain 19 vaccinated mice, normal mouse serum or monoclonal antibody (BRU 38) intraveneously via the tail vein on day 9 of gestation, one hour before challenge with strain 2308.

7. Collection and Handling of Samples: Unchallenged pregnant mice were housed conventionally, five to a cage. Pregnant mice challenged with strain 2308 were housed individually in micro-isolator cages. All mice were killed on day 18 of gestation. Blood for serology was collected by retroorbital bleeding of vaccinated mice four weeks post-inoculation for titer determination and use in the passive transfer study. At necropsy, blood was collected again immediately post-mortem by cardiac puncture and sera were stored at $4^{o}C$ until needed.

The spleen and two placentas were removed aseptically from each mouse. These tissues were weighed and homogenized in 2mls of sterile

saline. Ten-fold dilutions of the homogenate were plated onto trypticase-soy plates in five 10ul drops. Plates were incubated for 72 hours at 37°C with 5% CO_2 at which time colony forming units (cfus) were determined. The minimum number of bacteria detectable by this method was 40 cfu per organ. Bacteria recovered from strain 19 vaccinates were also cultured in the presence of 1% erythritol to ensure the isolates were strain 2308. Growth of strain 19 on media with erythritol is inhibited due to this strain's inability to utilize erythitol as an energy source (Nicoletti, 1990) while strain 2308 grows vigorously. Roughness of bacteria recovered from strain RB51 vaccinates was determined by autoagglutination (Braun and Bonestall, 1947; Appendix), uptake of crystal violet (White and Wilson, 1951; Appendix) and confirmed by colony blot ELISA (Roop et al., 1987; Appendix).

Placentas not used for bacteriology were fixed in situ within uteri in 10% neutral buffered formalin and processed routinely for histologic examination utilizing hematoxylin & eosin stain. All placentas sampled were coded and examined as unknowns by light microscopy and scored on a scale of 0 to 3 based on lesion development: 0 = no change, 1 = peripheral inflammation, 2 = grade 1 lesions plus junctional (spongiotrophoblast) inflammation, 3 = grade 2 lesions plus fetal death (Chapter 3). Examination of gestational age-matched placentas from non-infected mice allowed the establishment of baseline scores. Placental scores for each mouse were averaged then a grade for the entire experimental group was determined as an average of all mice. In this manner, all mice, regardless of the number of fetuses carried, were given equal weight, as the fetuses from

one individual tended to secure a similar score but number of fetuses per mouse varied from one to fourteen.

8. Statistical Analysis: All data is expressed as mean +/- standard deviation. When bacteria were not detected in placental tissues, one less than the minimum value detectable (39 cfu) was used for statistical analysis (Winter et al., 1989). Analysis of variance was used to compare splenic and placental infections and lesion grades of placentas (Snedecor, 1956). Statistical differences between groups with $p < 0.05$ were considered significant. Results of active and passive transfer experiments were expressed as logs of protection (mean $\log_{10}$ number of organisms of the principal group minus the mean $\log_{10}$ number of organisms in the corresponding control group).

Results

1. Protection-Active Immunization:

No bacteria were cultured from unchallenged mice immunized with strain 19 or strain RB51. Bacteria recovered from all mice either vaccinated or controls challenged with strain 2308 were strain 2308. One intraperitoneal inoculation of 10^6 viable strain 19 organisms given eleven weeks prior to challenge in mid-gestation with $10^{5.7}$ strain 2308 organisms provided 3.6 logs of protection to splenic infection and 6.9 logs of protection to placental infection (Table 7). Strain 2308 was detected in less than 3% of placentas from these mice. One inoculation of 10^8 viable strain RB51 organisms given as above provided approximately 1.5

logs of protection to splenic infection and 1.7 logs of protection to placental infection. Mice inoculated twice with the same dose of strain RB51 administered six weeks apart and challenged eleven weeks after the second vaccination were provided with approximately 2.7 logs of splenic protection and 3.2 logs of placental protection. Strain 2308 was detected in 88.2% of placentas from mice vaccinated once with strain RB51 and in 70.0% of placentas from mice receiving two vaccinations. Strain 2308 was detected in 100% of placentas from challenged non-immunized mice.

Though superior protection, as assessed by cfu recovered and histologic scoring of placentas, was provided by strain 19 versus one or two inoculations of strain RB51, all three groups of immunized mice were afforded a significant degree of protection against splenic and placental colonization and lesion development as compared to non-vaccinated mice. It is important to note that in all cases protection provided by strains 19 and RB51 was sufficient to prevent fetal death (Table 7). A second dose of strain RB51 did provide additional statistically significant protection against bacterial colonization of the placenta over that afforded by one dose of the vaccine. Splenic bacterial colonization post-challenge did not differ significantly between mice vaccinated once or twice with strain RB51.

2. Protection-Passive Tranfer:

Transfer of 0.1 ml of strain 19 immune serum intraveneously one hour before challenge with $10^{5.7}$ strain 2308 provided pregnant mice in mid-gestation with approximately 2.8 logs of protection to splenic infection and 6.7 logs of protection to placental infection (Table 8). Transfer of 0.1 ml of 115 ug protein/ml Bru 38 monoclonal antibody provided 2.3

logs of splenic protection and 5.6 logs of placental infection. Strain 2308 was detected in 30% of placentas from strain 19 antiserum recipients as compared to 60% placentas from Bru 38 recipients. Transfer of serum from either non-immunized or strain RB51-immunized mice did not provide significant protection against splenic or placental infection with strain 2308 when compared to the challenged, non-immunized mice. All placentas cultured from mice receiving strain RB51 immune serum or normal mouse serum were infected post-challenge.

Antisera from both strain 19 vaccinates and Bru 38 provided complete protection against the development of placental lesions (Table 8). Placentas from challenged mice that had received either normal mouse or strain RB51 immune serum were severely damaged and received grades similar to challenged control mice.

3. Splenic Weights:

In general, the greatest degree of splenomegaly was noted in the most heavily infected spleens. The heaviest spleens were noted in strain 2308 challenged mice that had not been actively immunized or had received either normal mouse serum or anti-serum to strain RB51 (Tables 7 & 8).

4. Serology:

Active Immunization: Mice immunized with strain 19 displayed a strong agglutination titer of 2.60 one month post-immunization which declined to 2.00 in non-challenged vaccinates at necropsy (Table 9). After challenge with strain 2308, strain 19 vaccinated-mice displayed an

anamnestic response, with the titer rising again to 2.60 nine days post-challenge. In contrast, mice immunized once with strain RB51 did not develop an agglutinating titer (<1.39) until challenged with strain 2308. Mice immunized once or twice with strain RB51 developed an agglutinating titer of 2.30 only after challenge. This titer was lower than the titer of strain 19 immunized mice after challenge and similar to that of strain 2308 challenged control mice. One month post-vaccination with strain RB51, mice showed a strong antibody response of 3.41 against RB51 organisms as measured by Biodot ELISA. By the time they were necropsied this titer declined to 2.60 in unchallenged mice, while the titer in mice challenged with strain 2308 rose to 4.11. The titer of mice to RB51 organisms that had received two strain RB51 vaccinations to RB51 organisms was 4.20 post-challenge with strain 2308. Biodot ELISAs performed on unchallenged, strain 19 immunized mice showed a low response (2.50), which did not increase with subsequent challenge. Unchallenged control mice did not display an antibody response to strain RB51 antigens (<1.00) while challenged, non-immunized mice had only a meager response (1.00) in the Biodot ELISA.

Western blot analysis of pooled sera obtained four weeks post-RB51 immunization did not reveal antibodies against the O-side-chain as demonstrated by a negative reaction to Yersinia enterocolitica 0:9 LPS or B. abortus strain 2308 organisms. This serum did react against a variety of antigens present in strain RB51 and non-O-side-chain related antigens of strain 2308 (Fig. 29). Sera from strain 19 vaccinates reacted strongly to both Y. entercolitica LPS and strain 2308 organisms with minor reactions to strain RB51 antigens.

Sera obtained at necropsy from strain 2308 challenged control mice primarily reacted against the LPS of Y. enterocolitica and strain 2308 organisms with only a weak reaction to antigens of strain RB51 (Fig. 30). Sera from mice vaccinated once with strain RB51 and challenged with strain 2308 reacted strongly to both strain RB51 antigens and the O-side-chain as demonstrated by a positive reaction against strain RB51 organisms, Y. entercolitica LPS and strain 2308 organisms (Fig. 31). An anamnestic response against non-O-side-chain B. abortus antigens was again demonstrated by a relatively stronger reaction of the serum from challenged strain RB51 vaccinates to strain RB51 organisms when compared to the reaction of sera from unchallenged strain RB51 vaccinates or challenged control mice to RB51 antigens. A western blot analysis of the sera from mice immunized twice strain RB51 post-challenge resembled that of the sera from mice once vaccinated with strain RB51 and challenged with strain 2308 (Fig. 31). The sera from mice vaccinated with strain 19 and challenged with strain 2308 reacted weakly to some non-O-side-chain antigens of strain RB51 while reacting strongly to both Y. enterocolitica LPS and strain 2308, demonstrating an anamnestic response primarily directed against the O-side-chain of LPS (Fig. 32).

In summary, vaccination of the non-pregnant mouse with strain RB51 did not induce the development of agglutinating antibodies during subsequent gestation but did induce a strong antibody reponse to non-O-side-chain antigens. RB51 vaccinates showed an anamnestic response to these non-O-side-chain antigens when exposed to a virulent smooth strain during pregnancy and this response did not inhibit a response to O-side-chain antigens present on the challenge strain. Vaccination of the non-pregnant

mouse with strain 19 induced the production of antibodies against some non-O-side-chain antigens as well as against O-side-chain antigens. After challenge with a virulent smooth strain during pregnancy, the anamnestic response of strain 19 vaccinates was directed primarily against the O-side-chain of the LPS molecule.

Passive Transfer: Two mice receiving 0.1 ml of strain 19 serum had a tube agglutination titer of 1.40 and 1.70 respectively one hour post-transfer. Two mice receiving 0.1 ml of strain RB51 immune serum each had an anti-RB51 titer of 1.90 one hour post-transfer. At necropsy, both strain 19 immune sera and Bru 38 recipients had an average agglutinating titer of less than 1.39 while strain RB51 and control serum recipients had average agglutinating titers of 2.0 and 2.15, respectively.

Discussion

Recent research using the murine model has established that systemic immunity to *B. abortus*, like other intracellular pathogens, involves the participation of both humoral (Montaraz and Winter, 1986; Araya et al., 1989, 1990; Winter et al., 1989; Phillips et al., 1989a) and cell-mediated (Cheers, 1984; Araya et al., 1989, 1990) immune responses. Neither the nature of the protective immunity nor the mechanisms involved in protection of the gravid reproductive tract against these pathogens is understood. In non-pregnant and pregnant mice, either active immunization (Montaraz and Winter, 1986; Bosseray, 1983b) or passively transferred immune serum (Bosseray, 1983b; Bosseray and Plommet, 1988; Phillips et al., 1989a; Winter et al., 1989; Araya et al., 1990) or immune T-lymphocytes

(Araya et al., 1989, 1990; Bosseray and Plommet, 1988) from mice infected with smooth strains can provide some degree of protection from infection for both spleens and placentas. The combined transfer of serum and T-lymphocyte enriched spleen cells in the non-pregnant mouse enhances protection over that provided by serum or cells alone, suggesting an interplay of humoral and CMI reponses (Araya et al., 1989).

Agreement has not been reached on whether the O-side-chain of the LPS molecule of smooth strains of B. abortus is an indispensible element of an effective vaccine for bovine brucellosis, since it is the immunodominant antigen to which the humoral response is directed. Monoclonal antibodies directed against this molecule are able to confer a significant degree of protection in both the non-pregnant (Phillips et al., 1989a) and pregnant mouse (this study). However, antibody titers in vaccinated cattle are still regarded as undesirable because they intefere with the interpretation of serodiagnostic tests. The rough strain B. abortus RB51, which contains insufficient quantities of O-side-chain to cause sero-conversion of vaccinates, has the ability to protect both pregnant (this study) and non-pregnant (Schurig et al., 1991) mice against challenge with virulent smooth strain 2308. Protection is evidenced by decreased degree of splenic infection in both groups and by decreased severity and frequency of placental infection, decreased development of placental lesions and protection against fetal loss in pregnant mice. Confirming previous reports on strain RB51 infection in the pregnant (Chapter 5) and non-pregnant mouse (Schurig et al., 1991), pregnant mice in this study developed antibodies against several B. abortus antigenic components but not antibodies against the O-side-chain were not induced as measured

by the Standard Tube Agglutination test or the more sensitive western blot analysis. When challenged months later with strain 2308 during pregnancy, mice immunized with strain RB51 developed a strong anamnestic response to these non-O-side-chain antigens but also developed antibodies to the O-side-chain as indicated by agglutination tests and western blot analysis. Active immunization with strain 19 or strain RB51 provided signigicant protection to the pregnant mouse as assessed by a decreased level of splenic and placental infection, decreased placental lesions and protection from fetal death. This study also suggests that two vaccinations with strain RB51 may result in greater protection. Such an observation was made in a previous unreported study done with non-pregnant mice (G.G. Schurig, personal communication).

It is important to notice the rather large standard deviation in the data for cfu of challenge organisms in the placentas and spleens of strain RB51 vaccinated mice as compared to strain 19 vaccinated mice. This has been a recurrent finding in our vaccination trials with strain RB51 and is the reason for inconsistencies in the degree of protection afforded by this strain in different experiments (from low degrees of protection to very high; G. G. Schurig, personal communication). This variable response may be due to the known autoagglutination tendency of strain RB51 which may induce a major variation in the effective vaccination dose every individual mouse receives in a specific group, inducing different levels of immunity. Experiments in which strain RB51 is suspended in a dispersing vehicle that reduces its tendency to autoagglutinate are in progress, and may decrease the presently observed high standard deviation values of strain RB51-induced protection levels.

Transfer of immune serum against strain 19 or of monoclonal antibody directed against the O-side-chain afforded a lesser degree of protection than active immunization. Passive transfer of monoclonal antibodies or antibodies against strain 19 depressed subsequent antibody production to the O-side-chain in strain 2308 challenged mice. This interference with the production of antibodies against Brucella has been noted in young pigs (Hoerlin, 1957) and rats (Halliday, 1968) following passive antibody transfer. The specific opsonization of the challenging bacterial cells enhancing phagocytosis of B. abortus by neutrophils and macrophages and complement-mediated killing may decrease the effective challenge dose and resultant immune response. Also, the presence of specific antibodies may result in a negative feedback mechanism.

Passively transfered antibodies directed against strain RB51 did not provide a significant level of either splenic or placental protection and did not prevent a humoral response to the O-side-chain of the virulent smooth strain. Antibodies to strain RB51 will not specifically opsonize smooth strains of B. abortus, probably due to steric hindrance of the O-side-chain of the LPS located on the surface of smooth strains (Bagchi, 1990). Therefore, strain RB51 immune serum alone has little activity against smooth strains and cell-mediated immunity (CMI) must be essential in protection afforded by this strain. The existance of such a protective CMI was demonstrated by splenic lymphocyte (obtained from strain RB51 immunized mice) transfer experiments (Bagchi, 1990).

Active immunization with strain 19 or the passive transfer of antibodies to the O-side-chain gave markedly superior protection against placental infection than vaccination with strain RB51 (Tables 7 & 8). This observation

added to the lack of protection confered by anti-RB51 serum suggests that antibodies to the O-side-chain play a crucial role in the protection of murine placentas against Brucella infection. The mechanisms involved in placental protection may differ from the situation in the spleen, where the difference in protection afforded by the two strains was not as great. It has been postulated that the uncontrolled placental growth of another intracellular pathogen, Listeria monocytogenes, may be in part due to the altered immune response at the feto-maternal interface required physiologically to permit fetal survival. Though there is not a general suppression of the immune response in the pregnant animal, there is substantial evidence that within the local environment of the placenta, cell-mediated immunity is attenuated to some degree (Hunziker et al., 1984; Clark et al., 1986 a & b; Redline and Lu, 1987, 1988). This may explain in part the uncontrolled growth of B. abortus seen in both the ruminant and the murine placenta and alludes to the importance of humoral response for local immunity within the gravid reproductive tract.

Placental and splenic tissues are exposed essentially simultaneously to infection during the bacteremic phase of brucellosis with subsequent preferential, unchecked growth of B. abortus occurring within placental trophoblast cells in the cow and the mouse. Decreasing the duration or extent of this bacteremia might decrease the level or frequency of infection. In a previous study of the placental pathogenicity of B. abortus strain 2308, we observed a positive relationship between the level of placental colonization in the mouse and subsequent placental damage resulting in fetal loss (Chapter 3). Thus immunization procedures that

limit infection might effectively result in placental and fetal protection without totally preventing infection of the gravid uterus. This is apparently the case with strain RB51 in which immunization significantly protects against placental damage and fetal death but does not provide complete protection from placental infection.

It has been postulated that opsonization coupled with enhancement of intracellular killing constitutes the principal protective role of antibody against B. abortus in the systemic immune response (Winter et al., 1989; Price et al., 1990). The essential role of the macrophage-T-lymphocyte system is exemplified histologically by the mononuclear cell inflammatory response typically noted in most organ systems with Brucella infection. However, in the placental environment where cell-mediated immunity appears modified, the response to infection, even with this intracellular pathogen, is predomimantly suppurative (Chapter 3). Immunization with strain RB51, which does not induce protective levels of antibody as assessed by passive tranfer alone, does provide an effective level of placental protection by mechanisms which are unclear at this time. It may be that vaccination limits bacteremia sufficiently via the induced CMI response so that an initial level of trophoblast infection sufficient to result in placental damage is prevented. In adoptive transfer experiments, nylon wool purified spleen cells obtained from strain RB51 immunized mice were found capable of transfering protection to non-pregnant mice at levels of protection comparable to those obtained with lymphocytes from strain 19 immunized mice (Bagchi and Schurig, 1990).

The entry of Brucella into the trophoblast cell may be receptor mediated, as has been suggested with study of Brucella entry into Vero cells by the proximity of coated pits to sites of attachment (Detilleux et al., 1990b). Preliminary in vitro studies utilizing a human trophoblast cell line have suggested that opsonization of Brucella with specific antibodies may prevent cell uptake or entry, perhaps through antibody mediated interference of receptor sites (G.G Schurig, personal communication). Protection against placental infection by antisera to strain 19 or monoclonal anti-O-side-chain may be achieved by decreasing the bacteremic load which prevents a large number of organisms from reaching the placenta and/or by preventing those reaching the placenta from entering the trophoblasts efficiently. Thus, it appears that several different factors may be involved in placental infection as compared to the systemic immune response for prevention and clearance of generalized infection. Further study of the immune response within the placental environment as well as clarification of the mechanisms of Brucella entry into the trophoblast cell appears warranted to aid in the development of more effective vaccines.

The results of this study investigating the protective ability of strain RB51 in the pregnant mouse support the findings of previous studies with the non-pregnant mouse (Schurig et al., 1991). A preliminary study with adult cattle also indicates that strain RB51 immunization can protect this species against abortion by strain 2308 and will not induce a humoral response to the O-side-chain (Burhman, 1989; Enright et al., 1990b). It appears that strain RB51 has significant potential for use as a protective live vaccine that will not induce agglutinating antibodies

that interfere with standard tests used to monitor cattle herds for brucellosis. This would greatly aid brucellosis control programs where descrimination between vaccinal titers and titers from natural infections are critical for disease erradication.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Reference was made to the wide importance of brucellosis in human and animal health and as a source of economic loss in cattle. In addition to previous studies of bovine brucellosis, other investigators showed that inoculation of the non-pregnant mouse with smooth virulent strains of Brucella abortus produced a chronic infection which resulted in granulomatous lesions in a variety of organs, most notably in the spleen and the liver. The research that employed the pregnant mouse model had been limited to and directed towards bacteriology and immunology.

The hypothesis put forth that the pregnant mouse would be an appropriate model of bovine brucellosis caused by virulent, smooth B. abortus was proved true through investigations which characterized the pathology, microbiology and serology of a virulent, laboratory adapted strain of B. abortus in a inbred strain of mice. The results demonstrated that infection of the pregnant mouse with a virulent smooth strain of B. abortus produced a severe, necro-suppurative placentitis that was often associated with fetal death. The inoculation of $10^{5.7}$ B. abortus strain 2308 organisms produced severe placental lesions when administered intraperitoneally to BALB/c mice at midgestation. Three days post inoculation with B. abortus strain 2308, brucellae were identified within trophoblast giant cells around the entire periphery of the placenta as well as within and around neutrophils associated with the regressing decidua capsularis. As previously reported in the bovine placenta, B.

abortus was specifically located within the rough endoplasmic reticulum of the trophoblast cell and within phagosomes of neutrophils within the murine placenta. As the course of pregnancy and the infection proceeded, bacteria localized and proliferated at the feto-maternal interface, specifically at the junction of the decidua basalis and the spongiotrophoblast zone. The early inflammatory reaction to the intracellular infection of trophoblast cells within this junctional zone was negligible but became intensely suppurative with the necrosis of infected cells. Intra- and extra- cellular bacterial proliferation in the spongiotrophoblast zone in severely affected placentas resulted in the infarction of the underlying labyrinth zone and associated fetal death. The severity of placental lesions was associated with the level of placental infection. In placentas near the end of pregnancy, brucellae were also noted within the visceral yolk sac endoderm cells. B. abortus has been previously reported as infecting the yolk sac endoderm of the chicken embryo as well.

The predominantly junctional distribution of lesions reported in this mouse study resemble the lesions of the rat placenta infected with B. abortus (Payne, 1957). However, the secondary abscesses noted in the labyrinth zone of the rat placenta were not observed in the infected mouse placenta. Payne made no mention of the infection of the visceral yolk sac endoderm but appreciation of intracellular infection within these cells would have been difficult without immunohistochemical staining techniques.

The pregnant mouse model of brucellosis has striking similarities to the disease in cattle. In both species, the preferential growth of the

organism within the RER of the trophoblast cells of the gravid uterus results in marked placental lesions, transplacental infection, and frequently in fetal death. The cause of fetal loss in each species appears to differ however, as in the mouse model fetal death is always associated with severe placental damage, while in cattle the cause of abortion appears multifactorial and abortion may occur in the absence of histologically severe placental damage. In both the mouse and the cow, infection of the mammary gland occurs after challenge during pregnancy and Brucella organisms are present in the milk of both species. Lesions associated with murine mammary brucellosis and sites of chronic infection in the post-partum mouse have not been reported.

As in murine placental listeriosis, the predominantly neutrophilic inflammatory reponse noted in the placenta infected with B. abortus contrasted with the mononuclear inflammatory reaction induced by these intracellular pathogens in non-placental organs. This supports the theory of Redline and Lu that the immune suppression present at the feto-maternal interface involved in the maintainance of pregnancy may influence local immune cell trafficing and the subsequent course of infection. Further immunohistochemical investigation of the specific types of inflammatory cells within the murine placenta infected with B. abortus could help clarify the immune response of the gravid uterus to brucellae. The pregnant mouse model of brucellosis offers a convenient avenue of study to examine the immunopathology of brucellosis in particular but could be also relevant to the immune mechanisms involved at the site of the fetal allograft and lead to a better understanding of the mechanisms of protective immunity specifically to brucellosis within the gravid reproductive tract.

The understanding of immunity to infection within the placenta may be of importance in developing more effective vaccines for brucellosis and other significant placental pathogens.

Interestingly, the degree of placental protection provided by either vaccination with strain 19 or transfer of anti-strain 19 or anti-O-side-chain antibodies was markedly superior to splenic protection provided by these manipulations in this experiment and also in those of Bosseray and Plommet's with the pregnant mouse. This may be a reflection of differing mechanisms of immunity operating at these sites. An understanding of the critical elements involved in protective immunity both systemically and within the unique immune arena of the placenta would seem relevent to the production of more efficient vaccines for placental pathogens.

Experiments also compared the serologic response and placental pathology associated with infection of the pregnant mouse with two attenuated strains of B. abortus, strains 19 and RB51. Strain 19, the current widely used vaccine strain, produced a severe placentitis similar to that caused by $10^{5.7}$ strain 2308 organisms when inoculated into the pregnant mouse at a dose of $10^{7.5}$ organisms. Both strain 19 and strain 2308 induced a serologic reaction in the pregnant mouse primarily directed against the O-side-chain of the LPS molecule which could be monitored by standard serum agglutinating tests for brucellosis. Inoculation of the pregnant mouse with $10^{9.5}$ B. abortus strain RB51, a stable rough organism with possible vaccine potential, produced only minimal placental inflammation and did not result in fetal loss or in the development of anti-O-side-chain antibodies as assessed by standard agglutination tests, ELISAs or western blot analysis.

These trials confirm strain RB51's reduced virulence and absence of significant amounts of O-side-chain antigen noted in previous studies in the non-pregnant mouse and in limited bovine trials. To examine further this strain's potential as an attenuated live vaccine for bovine brucellosis, mice were vaccinated with either strain RB51 or strain 19 and challenged in mid-gestation with a dose of virulent strain 2308 previously shown to induce severe placental lesions and fetal death. Both bacteriology and histology were utilized to compare the protective abilities of these two strains in the pregnant mouse model. Mice vaccinated with strain 19 demonstrated excellent protection against challenge by strain 2308 as determined by development of placental lesions and culture of maternal spleens and placentas. Protection against placental infection was particularly striking with strain 19 immunization. Immunization with a single dose of strain RB51 produced a lesser, although significant, degree of protection against placental and splenic infection and prevented severe placental damage and fetal death. Two inoculations of strain RB51 given four weeks apart improved protection against placental infection significantly. This study indicated that although strain 19 may offer more effective protection in the pregnant mouse model of brucellosis, strain RB51 also confered significant protective immunity as had been previously reported in the non-pregnant mouse and limited pregnant cow studies. These trials with strain RB51 thus led to the acceptance of the second hypothesis that immunization with this stable rough strain will not induce anti-O-side-chain antibodies in the pregnant mouse and will protect mice against a virulent smooth strain inoculated in mid-gestation.

The passive transfer of immunity by either serum raised to strain 19 or monoclonal antibodies specific to the O-side-chain provided protective immunity to the pregnant mouse, and confirmed several previous studies in the non-pregnant mouse and limited trials in the pregnant mouse. As had been reported in the non-pregnant mouse, strain RB51 immune serum did not protect the pregnant mouse against challenge with strain 2308. Thus the conclusion made by others with regards to the protective ability of strain 19 immune serum and particularly anti-O-side-chain antibodies against strain 2308 is supported by these results. Whether these antibodies are critical in protective immunity against bovine brucellosis, however, is still unclear.

As evaluated by immunohistochemical staining techniques, both attenuated strains localized and replicated within the same murine trophoblast cell population as did virulent strain 2308. The mechanisms of entry of brucellae into cells are speculative and further use of this model may support or refute the receptor mediated uptake of this pathogen and may help identify specific components associated with the cell wall critical to this process. The specific intracellular location of either attenuated strain was not determined in this investigation but would be of interest in studying the causes of reduced virulence of some strains of Brucella. It has been proposed that transfer to the RER is the limiting step in the infection of permissive host cells by B. abortus and that the difference in infectivity between smooth and rough strains may be correlated with their ability to gain access to the RER. An examination of the situation in the mouse placenta might help clarify this point.

Though certainly the final test of the potential of strain RB51 as a vaccine for bovine brucellosis awaits large scale studies in cattle, studies in both the pregnant and non-pregnant mouse support the continued development of this strain as a vaccine alternative. The pregnant mouse model also appears to have considerable potential for research into the immunopathology of this and other placental pathogens as well as the intricate mechanisms of the immune response critical to the survival of the fetus.

TABLES

TABLE 1

Effect on spleen and placenta of various doses of B. abortus strain 2308 inoculated intraperitoneally into BALB/c mice[a] on day 9 of gestation

Infection Dose (Log_{10})	Spleen		Placenta		
	Mean Wt. (g.±SD[b])	Mean bact./organ (Log_{10} ± SD)	Aver.Wt. (g.±SD[b])	Mean bact./organ (Log_{10} ± SD)	Histology Score (Mean ± SD)
6.7	0.61 ± 0.14	6.0 ± 0.4	0.13 ± 0.05	8.8 ± 0.3	2.5 ± 0.4
5.7	0.68 ± 0.09	6.1 ± 0.3	0.12 ± 0.03	8.7 ± 0.1	2.2 ± 0.4
4.7	0.28 ± 0.14	5.9 ± 0.1	0.13 ± 0.04	8.6 ± 0.2	1.1 ± 0.8
Uninfected Control	0.13 ± 0.02	0	0.18 ± 0.02	0	0

[a] = 5 mice per group

[b] = grams ± standard deviation

TABLE 2

Pathogenesis Study - B. abortus Strain 2308[a]
Effect on Spleen and Placenta

Days of Gestation	12[b]		14		16		18	
	I[c]	C[d]	I	C	I	C	I	C
Mean Bacteria/Spleen (Log_{10} ± SD)	6.4 ± 0.2	NA[e]	6.4 ± 0.2	NA	6.3 ± 0.4	NA	6.4 ± 0.3	NA
Mean Splenic Weight (Grams ± SD)	0.35 ± 0.04	0.18 ± 0.02	0.32 ± 0.08	0.16 ± 0.03	0.39 ± 0.07	0.14 ± 0.02	0.78 ± 0.15	0.13 ± 0.02
Mean Bacteria/Placenta (Log_{10} ± SD)	7.5 ± 0.2	NA	7.8 ± 0.9	NA	8.4 ± 0.3	NA	8.5 ± 0.3	NA
Mean Placental Weight (Grams ± SD)	0.06 ± 0.02	0.06 ± 0.01	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.16 ± 0.02	0.14 ± 0.04	0.18 ± 0.02

[a] each mouse received $10^{6.7}$ bacteria IP on day 9 of gestation
[b] 5 pregnant mice/group
[c] infected
[d] control
[e] NA = not applicable
Values expressed as mean ± standard deviation.

TABLE 3

Effect of B. abortus strains 2308, 19 and RB51 on spleens of non-pregnant BALB/c mice[a] killed 9 days post IP inoculation.

	Strain 2308	Strain 19	Strain RB51
Inoculum (Log_{10})	5.8[b]	7.5[b]	9.5[c]
Mean Bacteria/ Spleen ($Log_{10} \pm SD$)	5.4 ± 0.6 [6.1 ± 0.3][d]	5.8 ± 0.2 [6.0 ± 0.4][d]	4.9 ± 0.3 [4.9 ± 0.2][d]

[a]10 mice/group
[b]minimum dose producing placental scores ≥ 2.0 when given to pregnant mice IP day 9 of gestation
[c]maximum dose of this strain given to pregnant mice
[d][mean bacteria/spleen in pregnant mice given same dose IP on day 9 of gestation] Values not significantly different from non-pregnant mice ($p < 0.05$)
Values expressed as mean ± standard deviation.

TABLE 4

Trials to determine the minimum inoculum of B. abortus strain 19 producing severe placental lesion (score $\geq$ 2.0) when administered IP to BALB/c mice on day 9 of gestation.

	Trial I	Trial II*
Inoculum (Log_{10})	6.5	7.5
Mean Bacteria/Spleen ($Log_{10} \pm$ SD)	6.0 $\pm$ 0.4	6.0 $\pm$ 0.4
Mean Weight of Cultured Spleens (Grams $\pm$ SD)	0.41 $\pm$ 0.02	0.69 $\pm$ 0.06
Mean Bacteria/Placenta ($Log_{10} \pm$ SD)	8.2 $\pm$ 0.3	8.6 $\pm$ 0.2
Mean Weight of Cultured Placentas (Grams $\pm$ SD)	0.19 $\pm$ 0.03	0.14 $\pm$ 0.05
Mean Placental Histology Score ($x \pm$ SD)	1.6 $\pm$ 0.6	2.3 $\pm$ 0.3

*minimum inoculum
Values expressed as mean $\pm$ standard deviation.

TABLE 5

Trials to determine the minimum inoculum of B. abortus strain RB51 producing severe placental lesions (score $\geq$ 2.0) when administered IP to BALB/c mice on day 9 of gestation.

	Trial I	Trial II
Inoculum (Log_{10})	8.5	9.5
Mean Bacteria/Spleen ($Log_{10} \pm$ SD)	3.8 $\pm$ 0.13	4.9 $\pm$ 0.2
Mean Weight of Cultured Spleens (Grams $\pm$ SD)	0.26 $\pm$ 0.08	0.70 $\pm$ 0.11
Mean Bacteria/Placenta ($Log_{10} \pm$ SD)	4.8 $\pm$ 2.1	6.9 $\pm$ 0.3
Mean Weight of Cultured Placentas (Grams $\pm$ SD)	0.23 $\pm$ 0.04	0.19 $\pm$ 0.02
Mean Placental Histology Score ($x \pm$ SD)	0.2 $\pm$ 0.3	0.8 $\pm$ 0.6

Values expressed as mean $\pm$ standard deviation.

TABLE 6

Histologic, bacteriologic and serologic findings of BALB/c mice[a] inoculated with B. abortus strain 2308, 19 or RB51 intraperitoneally on day 9 of gestation

Infection Strain	Infection Dose Log_{10}	Placental Histology	Placental Score ± SD[b]	Mean bacteria/organ(Log^{10} ± SD) Spleen	Mean bacteria/organ(Log^{10} ± SD) Placenta	Reciprical STA[c] Log_{10} ± SD
2308	5.7	Severe placentitis Occ. fetal death	2.2 ± 0.4	6.1 ± 0.3	8.7 ± 0.1	2.1 ± 0.2
19	7.5	Severe placentitis Occ. Fetal death	2.3 ± 0.3	6.0 ± 0.4	8.6 ± 0.2	2.3 ± 0.0
RB51	9.5	Minimal placentitis No fetal death	0.8 ± 6	4.9 ± 0.2	6.9 ± 0.3	<1.4 ± 0.0

[a] - Seven mice per group

[b] - SD = Standard deviation

[c] - STA = Standard Tube Agglutination test

Table 7

Protection against placental and splenic infection with strain 2308[a] as well as placental damage by vaccination

Vaccination Status	Spleen		Placenta	
	Log_{10} cfu Mean ± SD[b]	Weight Mean grams ± SD	Log_{10} cfu Mean ± SD	Score Mean ± SD
Control[c] N=20	6.2 ± 0.2	0.70 ± 0.06	8.6 ± 0.3	2.0 ± 0.4
(Unchallenged control) N=10	(0)	(0.12 ± 0.02)	(0)	(0)
Strain 19[d] N=19	2.6 ± 0.5[f]	0.14 ± 0.04[f]	1.7 ± 0.5[f]	0 ± 0[f]
Strain RB51[e] 1x N=17	4.7 ± 0.8[f]	0.51 ± 0.21[f]	6.8 ± 2.7[f]	0.8 ± 0.5[f]
Strain RB51[e] 2X N=5	3.5 ± 2.0[f]	0.24± 0.04[f]	5.4 ± 2.9[f,g]	0.6 ± 0.6[f]

a $10^{5.7}$ IP day 9 of gestation
b SD = Standard deviation
c 0.2 ml saline IP
d 10^{6} B. abortus strain 19 IP
e 10^{8} B. abortus strain RB51 IP
f Significantly different from control values ($p < 0.05$)
g Significantly different from 1x RB51 vaccination

Table 8

Protection against placental and splenic infection with strain 2308 as well as placental damage by transfer of immune sera and monoclonal antibodies[a] before challenge[b]

Antibodies	Spleen		Placenta	
	Log_{10} cfu mean ± SD[c]	Weight Mean grams ± SD	Log_{10} cfu Mean ± SD	Score Mean ± SD
Control sera N=6	6.2 ± 0.2	0.70 ± 0.06	8.4 ± 0.3	1.8 ± 0.3
Anti-Strain 19 N=5	3.4 ± 0.2[d]	0.15 ± 0.06[d]	1.7 ± 0.3[d]	0 ± 0[d]
Anti-Strain RB51 N=6	6.1 ± 0.2	0.68 ± 0.06[d]	8.5 ± 0.2	1.9 ± 0.3
Monoclonal BRU 38 N=5	3.9 ± 0.3[d]	0.13 ± 0.04	2.8 ± 1.5[d]	0 ± 0[d]

a 0.1 ml administered IV 1 hour before challenge
b $10^{5.7}$ strain 2308 IP day 9 of gestation
c SD - standard deviation
d Significantly different from control values ($p < 0.05$)

Table 9

Serum titers[a] of challenged[b] and unchallenged vaccinates against B. abortus O-side-chain antigens (STA[c]) and B. abortus strain RB51 organisms (BIODOT[d])

Group	STA			BIODOT		
	4 weeks Post Vaccination	At Necropsy		4 weeks Post Vaccination	At Necropsy	
		No Challenge	With Challenge		No Challenge	With Challenge
Strain 19[e]	2.60	2.00	2.60	3.10	2.50	2.50
Strain RB51[f] 1x	<1.39	<1.39	2.30	3.41	2.60	4.11
Strain RB51[f] 2x	NA[h]	NA	2.30	NA	NA	4.20
Control[g]	<1.39	<1.39	2.30	<1.00	<1.00	1.00

a Titers expressed as $\log_{10}$ value of reciprical
b $10^{5.7}$ B. abortus strain 2308 IP day 9 of gestation
c Standard Tube Agglutination test
d BioDot ELISA (Enzyme linked immunosorbant assay)
e $10^{6.0}$ B. abortus strain 19 IP
f $10^{8.0}$ B. abortus strain RB51 IP
g 0.2 mls saline IP
h NA - not available

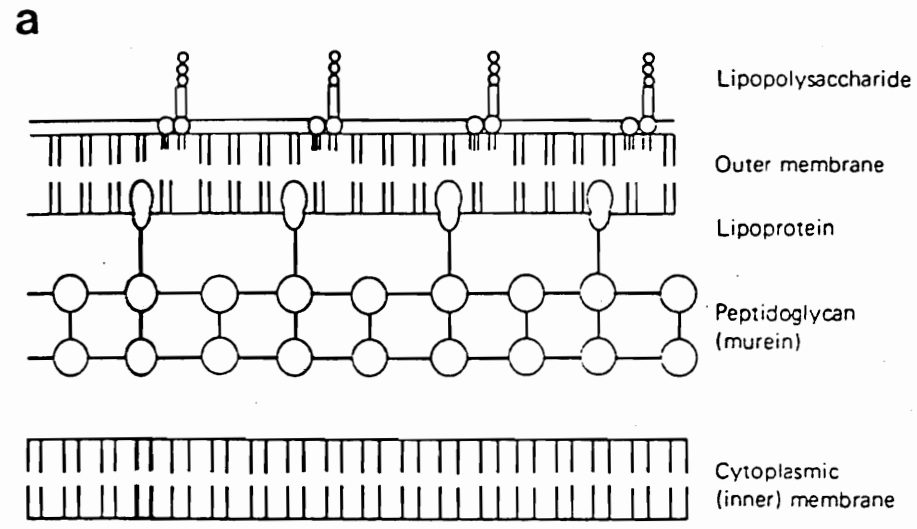
FIGURES

Figure 1. The Gram negative cell envelope.

a. Schematic of the major constituents of the cell envelope.

b. Schematic of a segment of the lipopolysaccharide polymer.

(from Jawetz, E., Melnick, J. L., and Adelberg, E. A., eds. Review of medical microbiology. 11th edition. Figs. 2-19, 2-20, pp. 18 & 20. Lange Medical Publications, Los Altos, CA., 1980)


a
Lipopolysaccharide
Outer membrane
Lipoprotein
Peptidoglycan
(murein)
Cytoplasmic
(inner) membrane

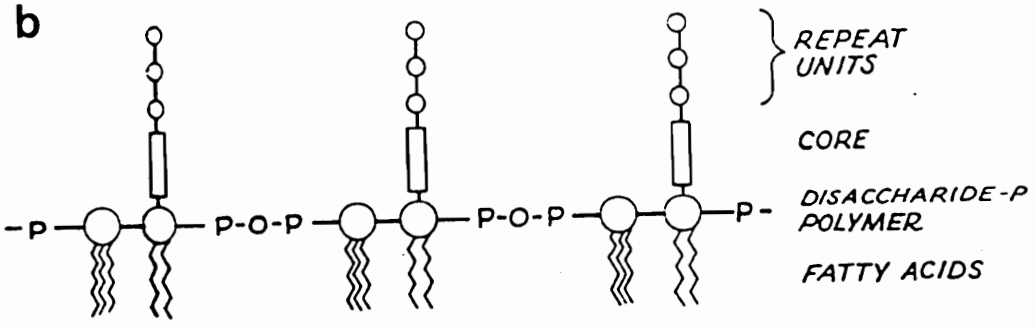
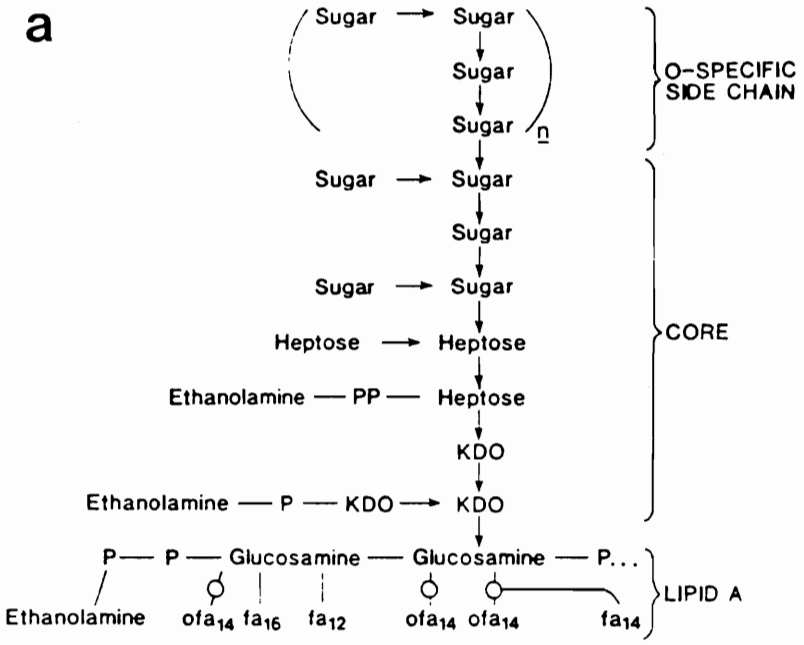

b
REPEAT
UNITS
CORE
DISACCHARIDE-P
POLYMER
FATTY ACIDS
-P
P-O-P
P-O-P
P-

Figure 2. The lipopolysaccharide polymer.

a. Schematic of a typical LPS molecule. KDO-ketodeoxy-octulonate, fa-fatty acid.

(from Ingrham, J., Maaloe, O., and Neidhardt, F. C. Growth of the bacterial cell. Fig. 9, P. 16. Sinauer Associates Sunderland, MA. 1983)

b. Chemical structure of the Brucella prototype A (1) and M (2) antigen. (from Bundle et al., 1989)


a
Sugar
Sugar
Sugar
Sugar
n
O-SPECIFIC SIDE CHAIN
Sugar
Sugar
Sugar
Sugar
Sugar
Heptose
Heptose
Ethanolamine — PP —
Heptose
KDO
Ethanolamine — P — KDO
KDO
CORE
P — P — Glucosamine — Glucosamine — P...
Ethanolamine
ofa14 fa16 fa12 ofa14 ofa14 fa14
LIPID A

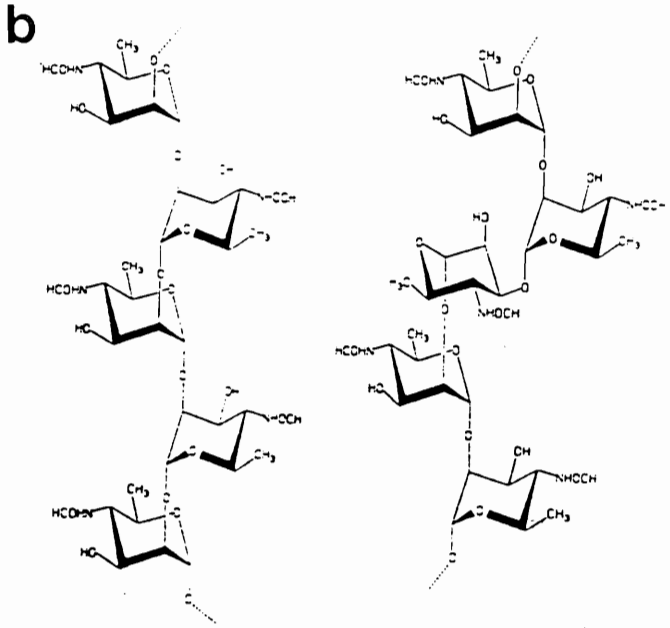

b

Figure 3. Schematic cross-section of the mouse uterus, day 18 of gestation.

a. Colonization of the placenta 9 days post-infection with *B. abortus* strain 2308.

b. Normal anatomy.

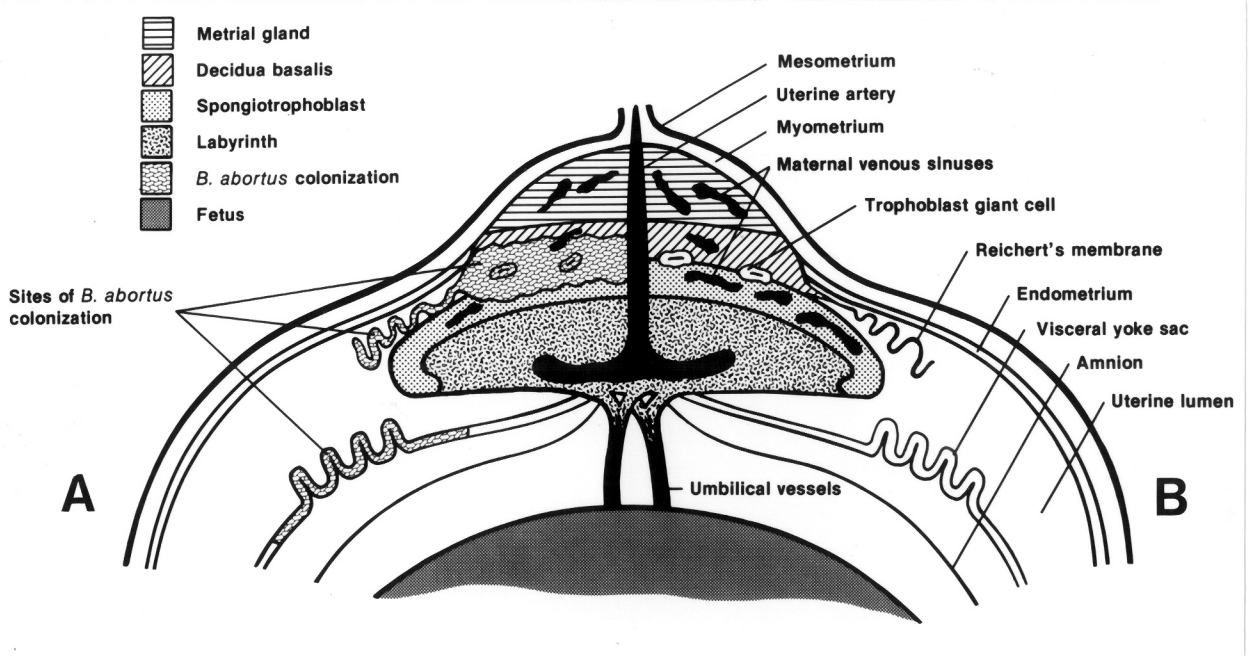

Metrial gland
Decidua basalis
Spongiotrophoblast
Labyrinth
B. abortus colonization
Fetus
Sites of B. abortus colonization
A
Mesometrium
Uterine artery
Myometrium
Maternal venous sinuses
Trophoblast giant cell
Reichert's membrane
Endometrium
Visceral yoke sac
Amnion
Uterine lumen
Umbilical vessels
B

Figure 4. Effect of $10^{6.7}$ B. abortus strain 2308 organisms on the weight of spleen and placenta at various days of gestation. (see Table 2 for numerical values)

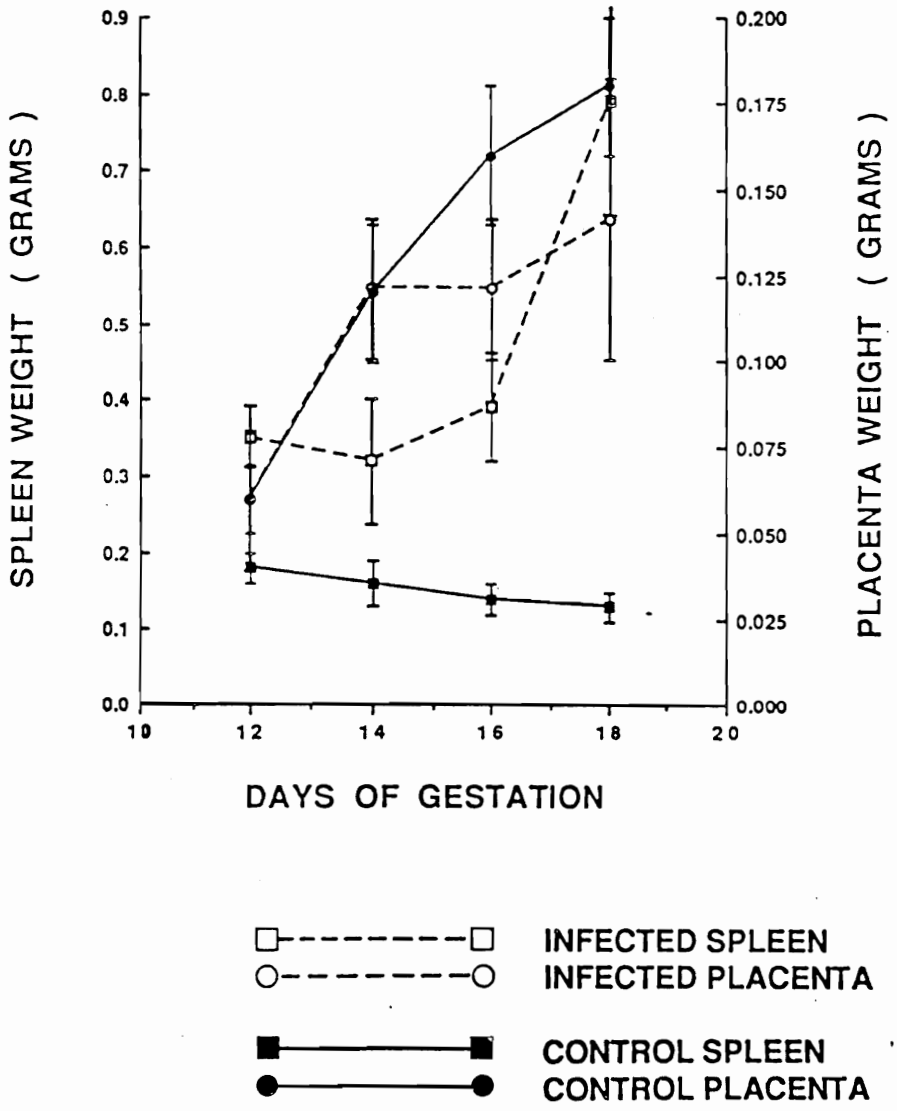
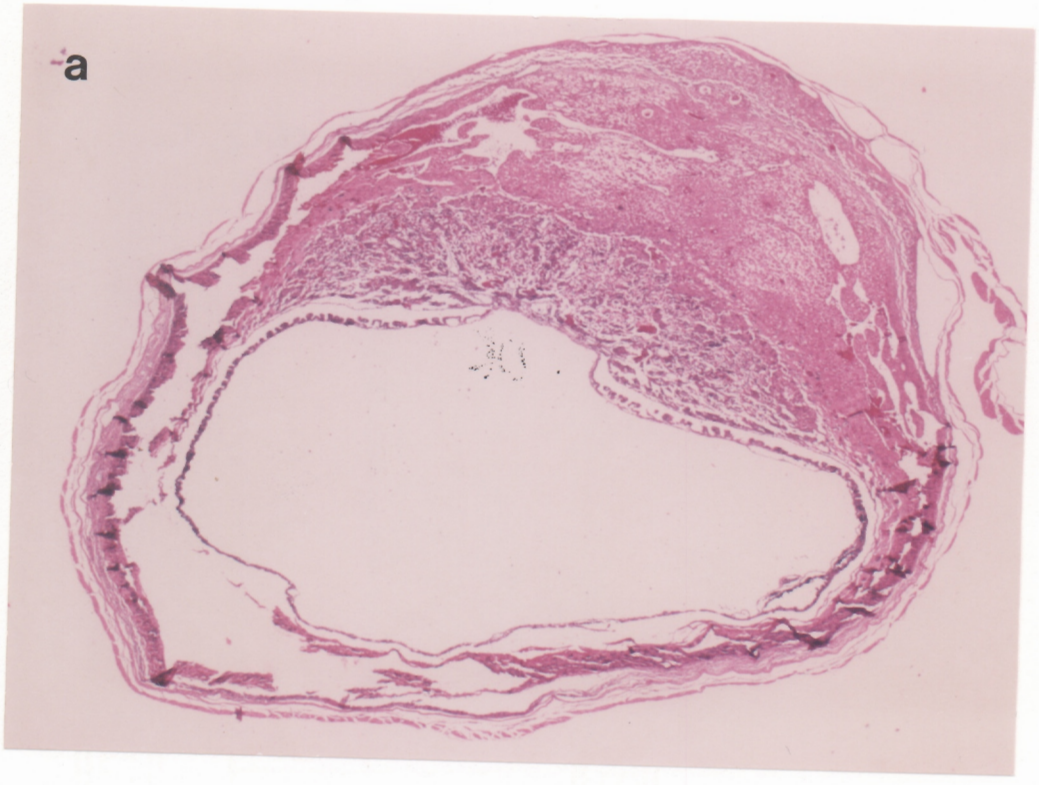

SPLEEN WEIGHT (GRAMS)
PLACENTA WEIGHT (GRAMS)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
10
12
14
16
18
20
DAYS OF GESTATION
INFECTED SPLEEN
INFECTED PLACENTA
CONTROL SPLEEN
CONTROL PLACENTA

Figure 5. Cross-sections of the mouse uterus at 12 days of gestation. H & E stain.

a. Control. 18x.

b. Mouse received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. At this time post-infection, changes due to infection are not readily appreciable with this stain. 18x.


a

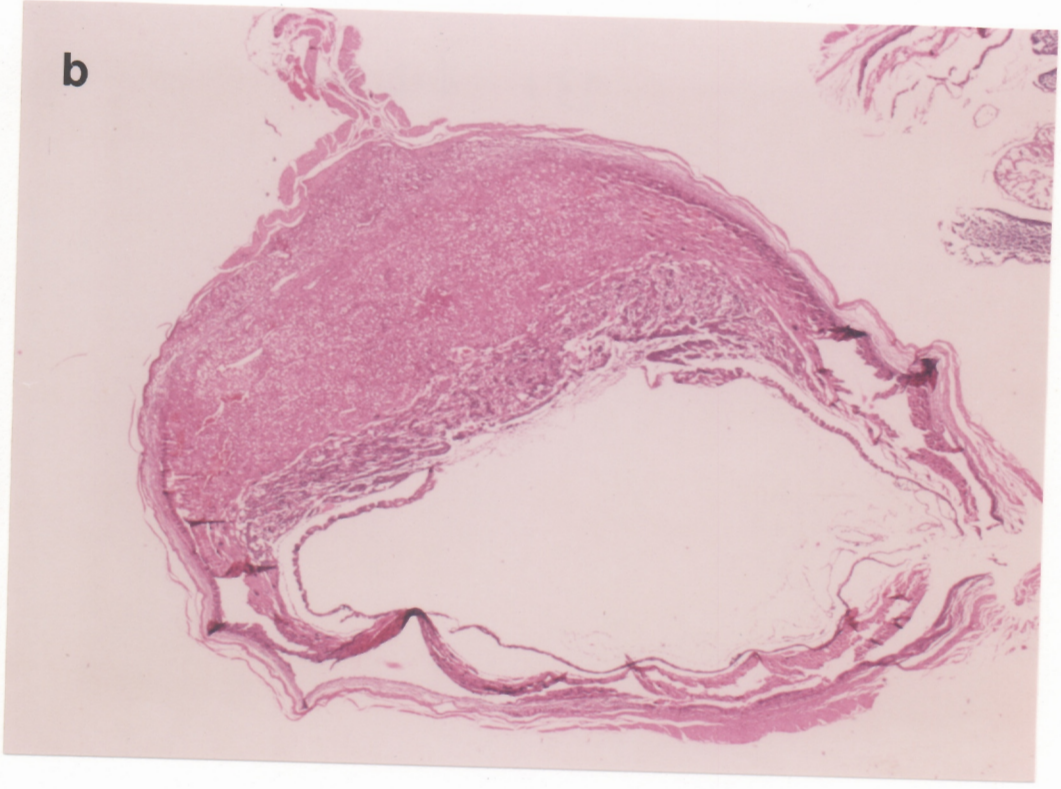

b

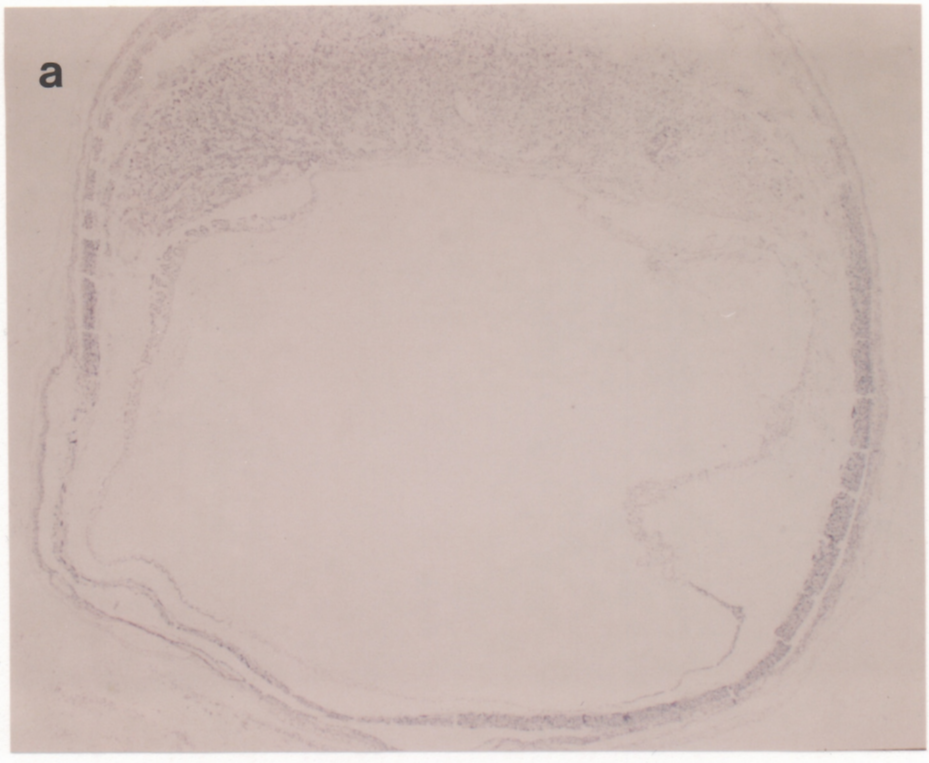
Figure 6. Mouse placenta at 12 days of gestation. ABC stain.

a. Cross-section of control uterus. Specific primary antibody. 18x.

b. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Specific primary antibody. Note darkly staining cells around the periphery of the placenta (arrowheads) indicative of Brucella infection. 18x.

c. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Normal serum as primary antibody. Note lack of non-specific staining. 18x

d. Higher magnification of b. taken at the junctional zone of the placenta. Note trophoblast giant cells (arrowhead 5) with positively staining cytoplasm revealing Brucella infection. 360x.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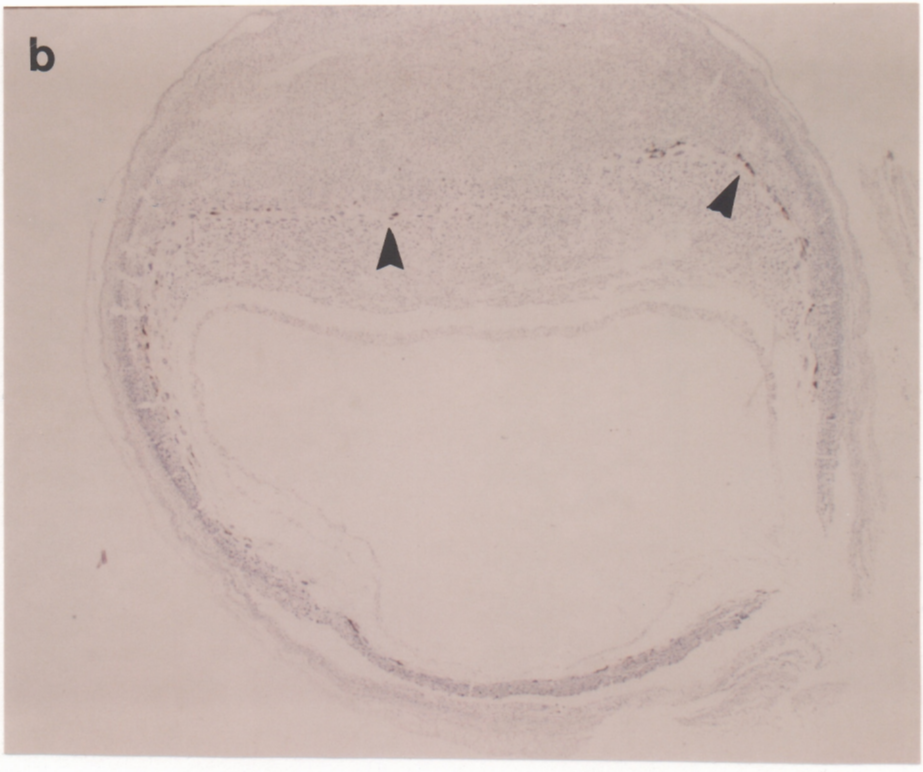
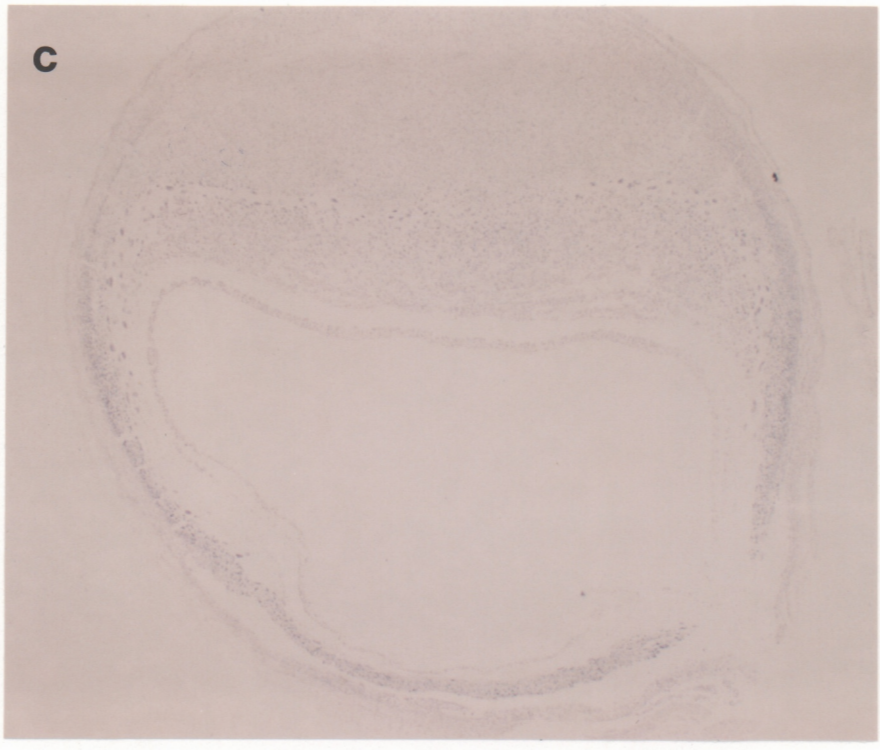

b


c

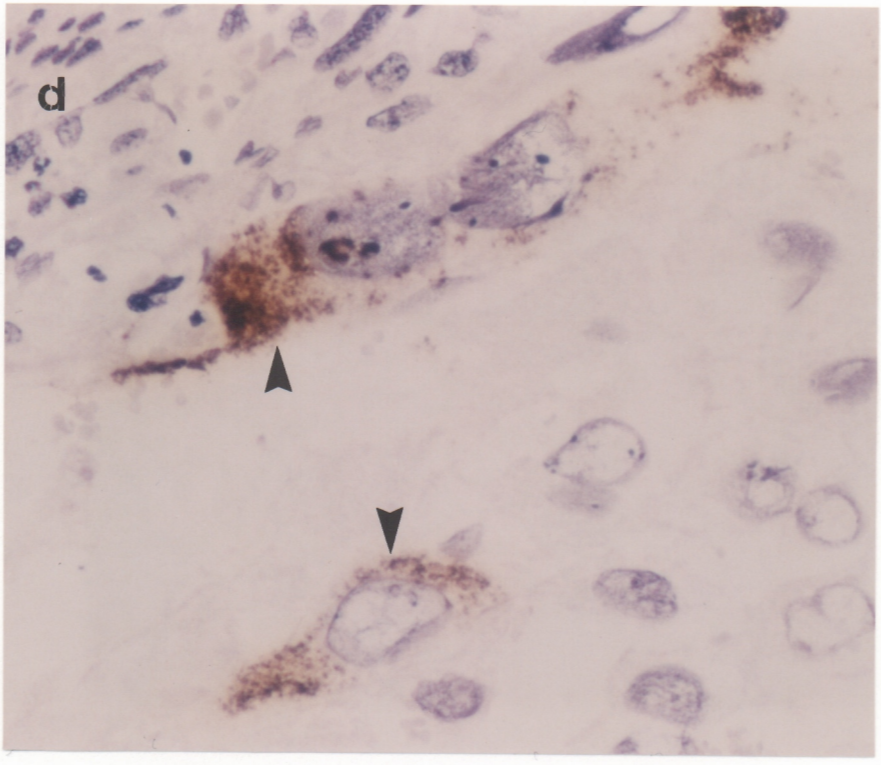
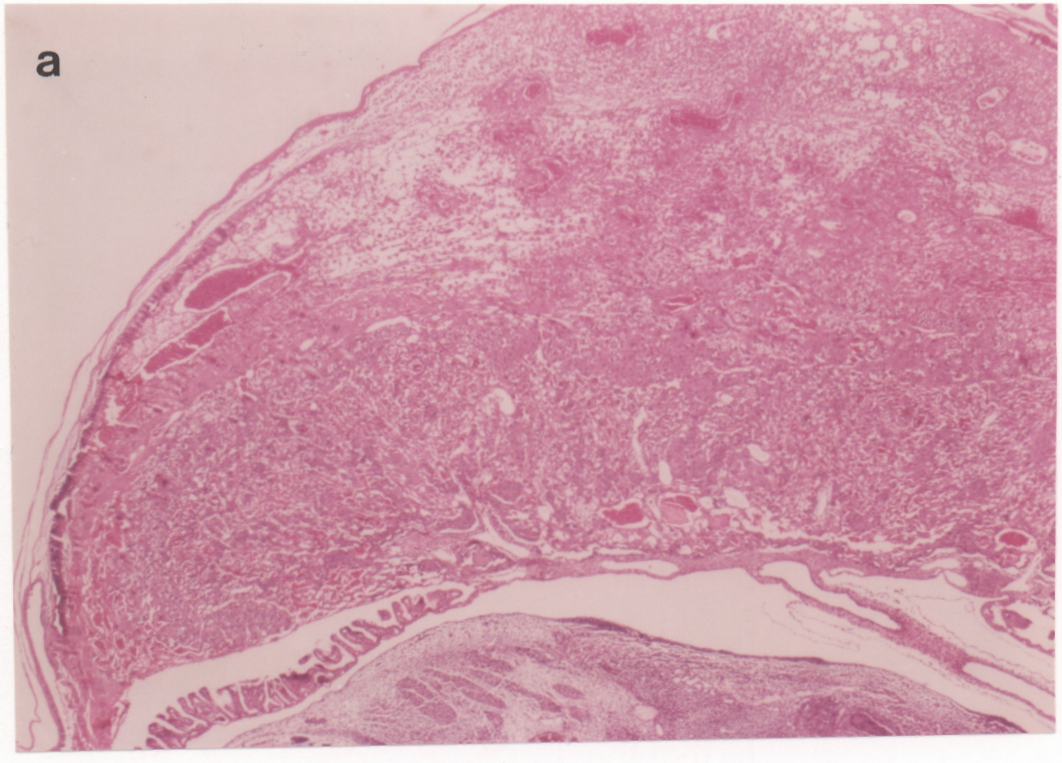

d

Figure 7. Cross-sections of the mouse uterus at 14 days of gestation. H & E stain.

a. Control. 24x.

b. Mouse received $10^{6.7}$ B. abortus strain 2308 organisms day 9 day of gestation. Note single small focus of inflammation (arrowhead). 18x.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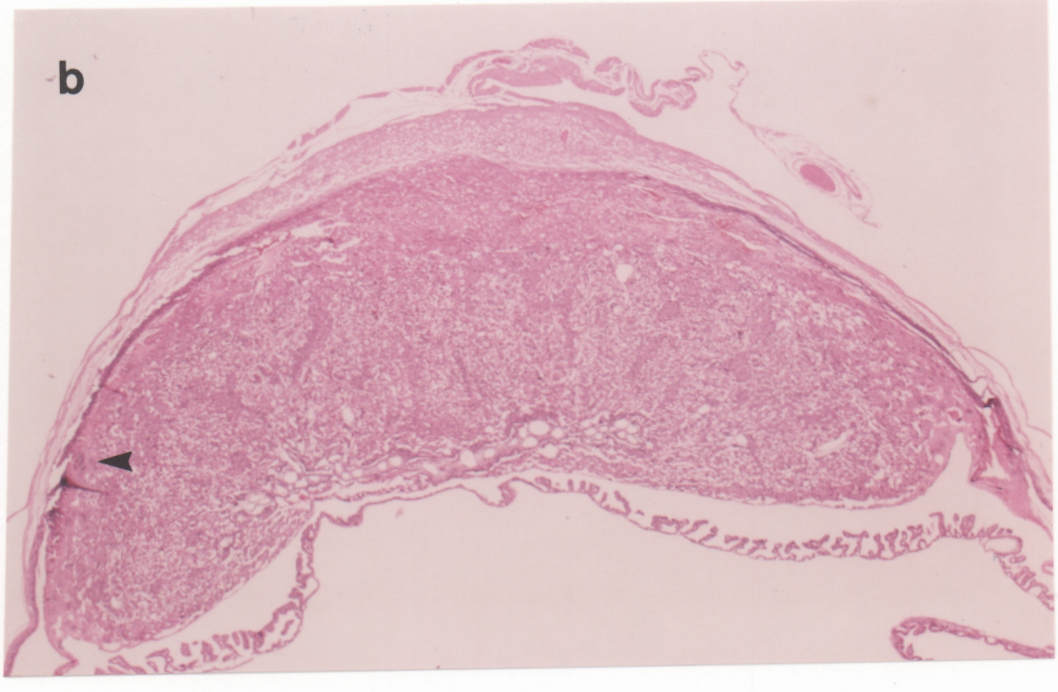

b

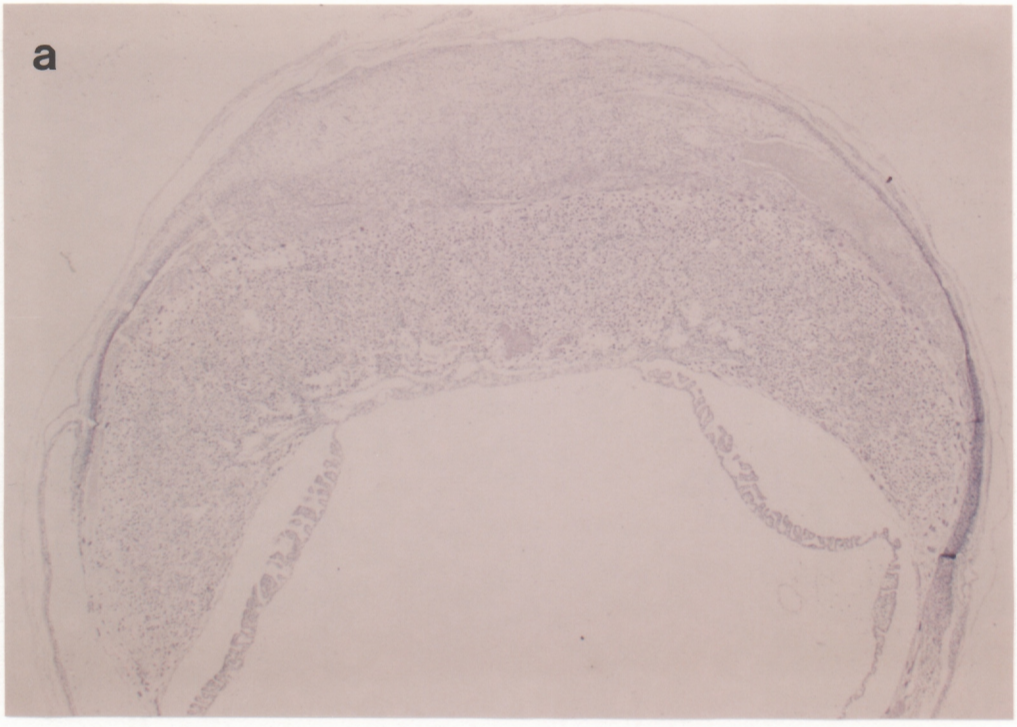
Figure 8. Mouse placenta at day 14 of gestation. ABC stain.

a. Cross-section of control uterus. Specific primary antibody. 18x.

b. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Specific primary antibody. Note coalescing ring of positively stained around the entire periphery of the placenta. 18x.

c. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Normal serum as primary antibody. Note lack of non-specific staining. 18x

d. Higher magnification of b. taken at the junctional zone. Note heavy staining of the cytoplasm of trophoblast giant cells. MG-metrial gland, DB-decidua basalis, MVS-maternal venous sinus, T-trophoblast giant cell, S-spongiotrophoblast or junctional zone, L-labyrinth. 90x.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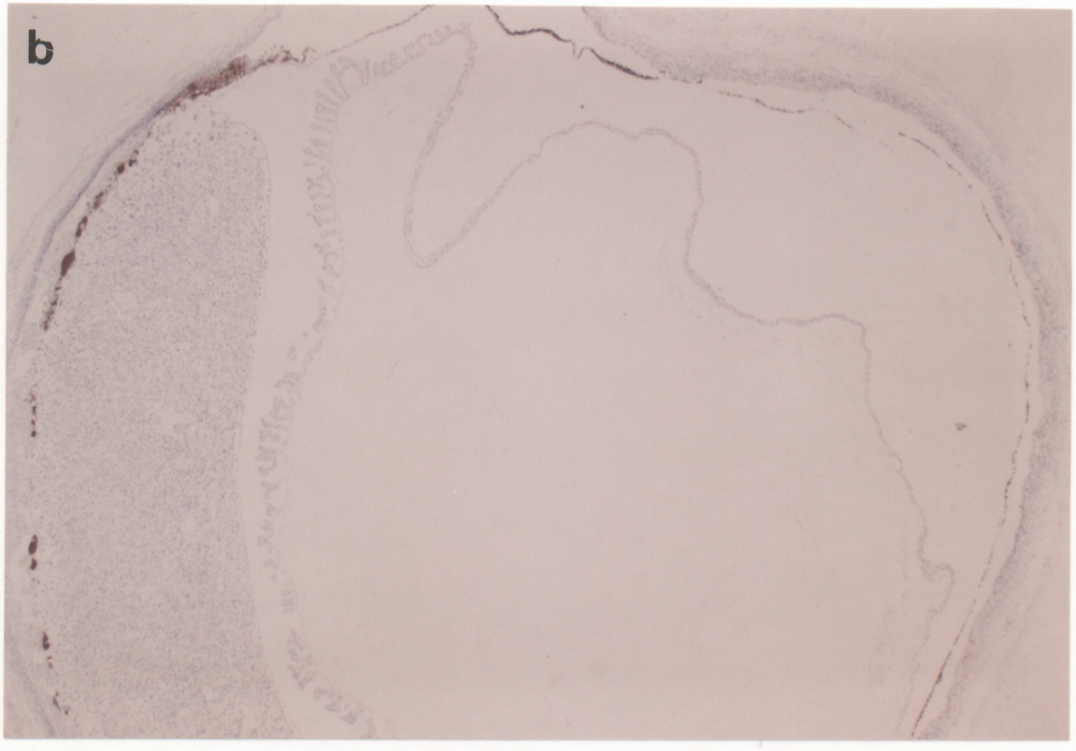
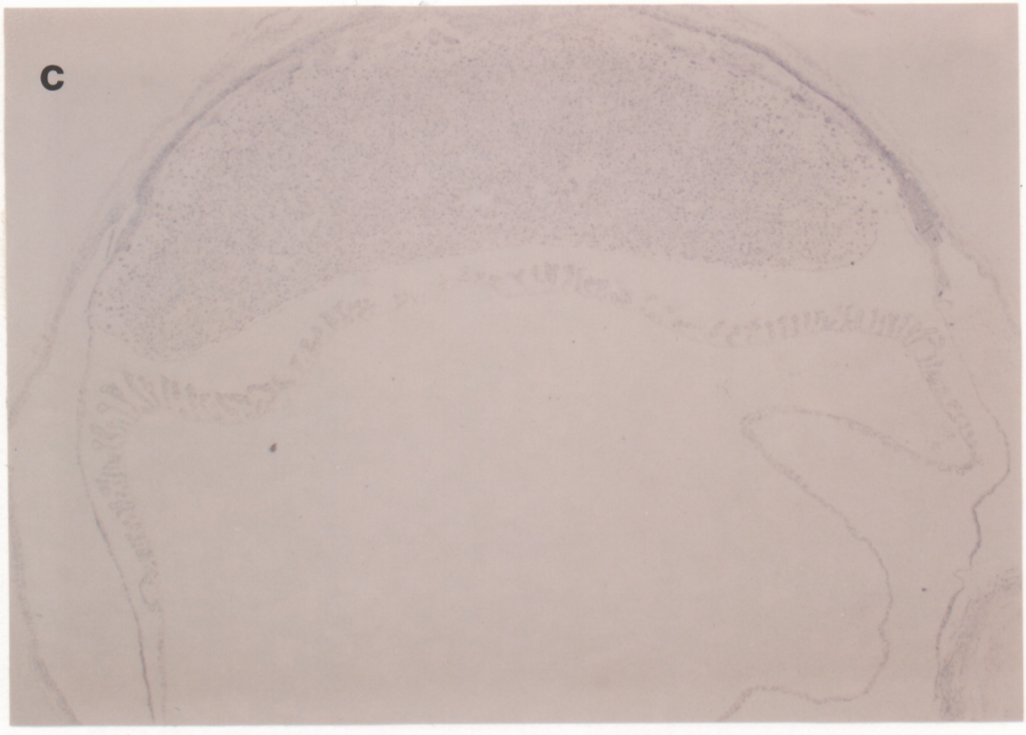

b


c

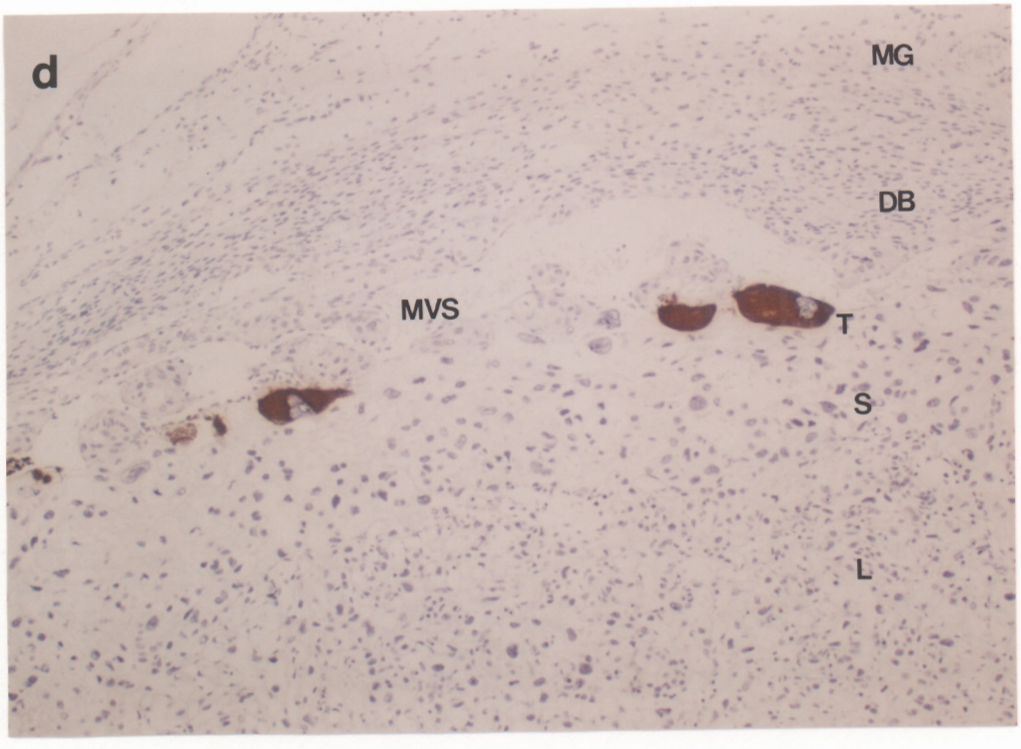
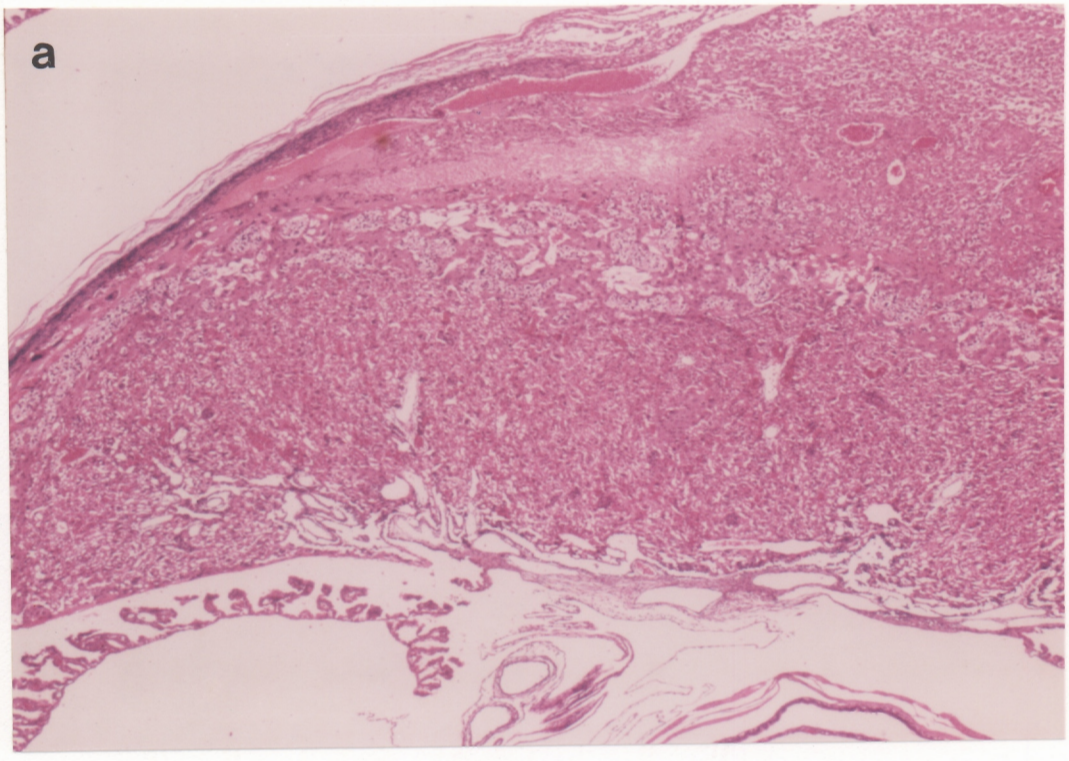

d
MG
DB
MVS
T
S
L

Figure 9. Cross-section of the mouse uterus at day 16 of gestation. H & E stain.

a. Control. 29x.

b. Mouse received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Note multiple, small foci of inflammatory cells (arrowheads) within the spongiotrophoblast. 36x.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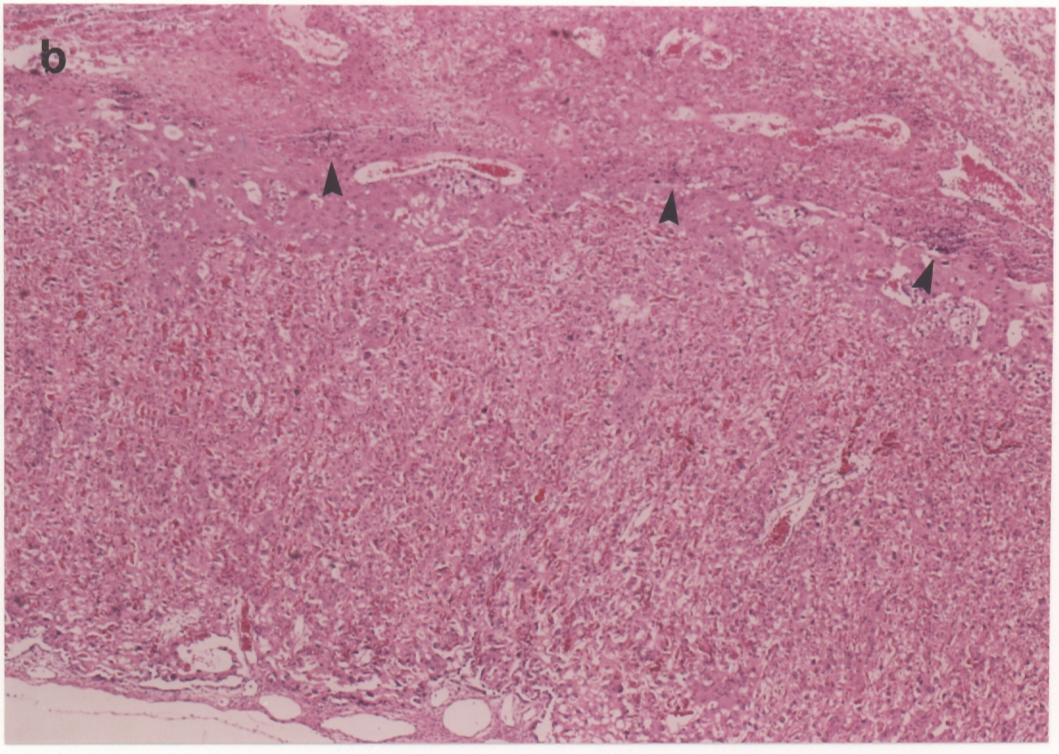

b

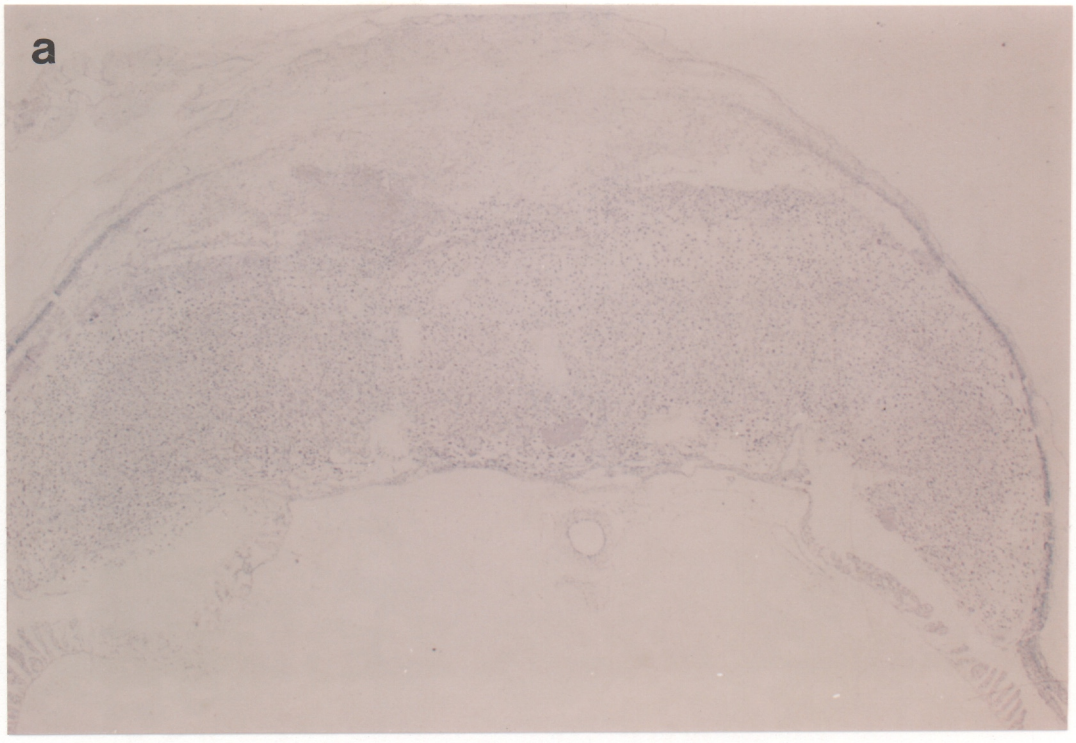
Figure 10. Mouse placenta at 16 days of gestation. ABC stain.

a. Cross section of control uterus. Specific primary antibody. 18x.

b. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Specific primary antibody. Note extensive staining of the spongiotrophoblast zone with minor extension to the overlying decidua. 18x

c. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Normal serum as primary antibody. Note lack of non-specific staining. 18x.

d. Higher magnification of b. taken at the spongiotrophoblast zone. Note trophoblast giant cell in which the cytoplasm is entirely filled with Brucella organisms adjacent to a small focus of neutrophils and cellular debris also staining positively for Brucella antigen. 360x.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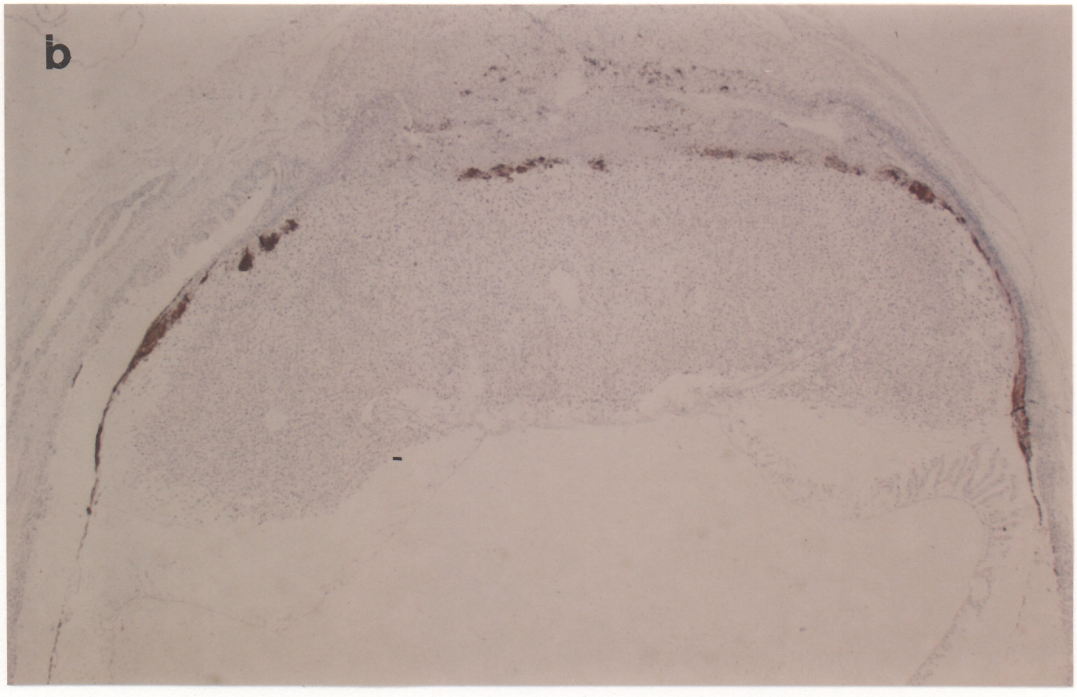

b


c

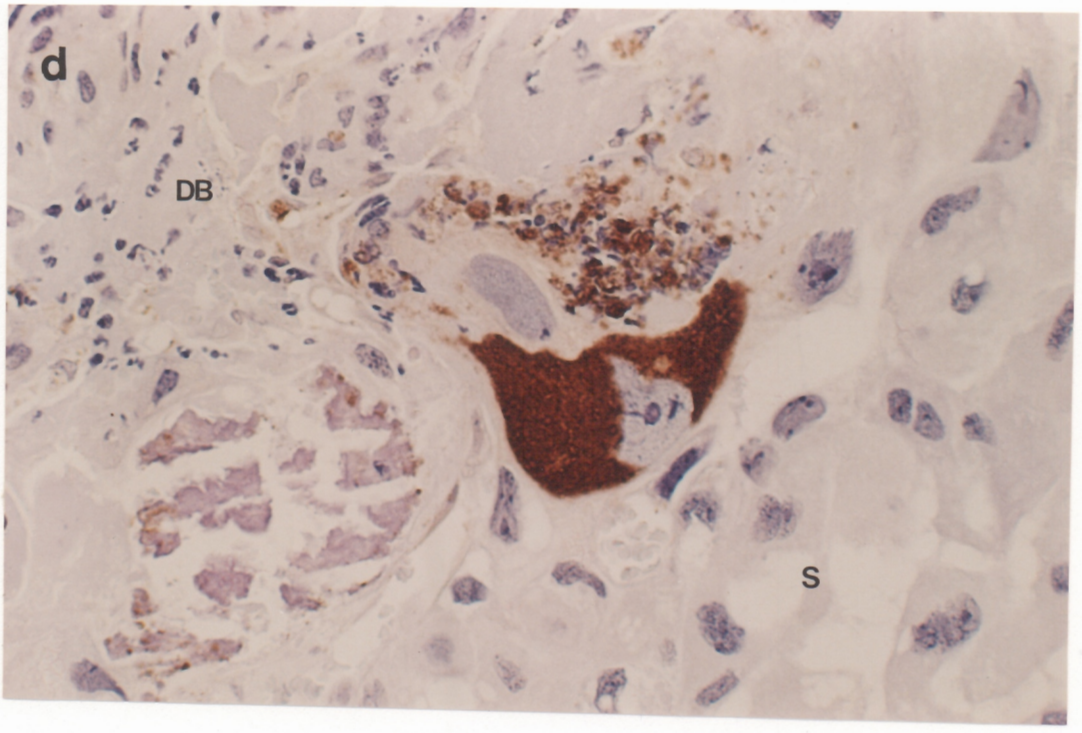

d
DB
S

Figure 11. Junctional zone of the day 16 placenta from a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Giemsa stain. Note trophoblast giant cell (arrowhead) staining dark purple due to the presence of intracellular brucellae. It is surrounded by several foci of positively staining neutrophils. S-spongiotrophoblast, L-labyrinth. 180x.

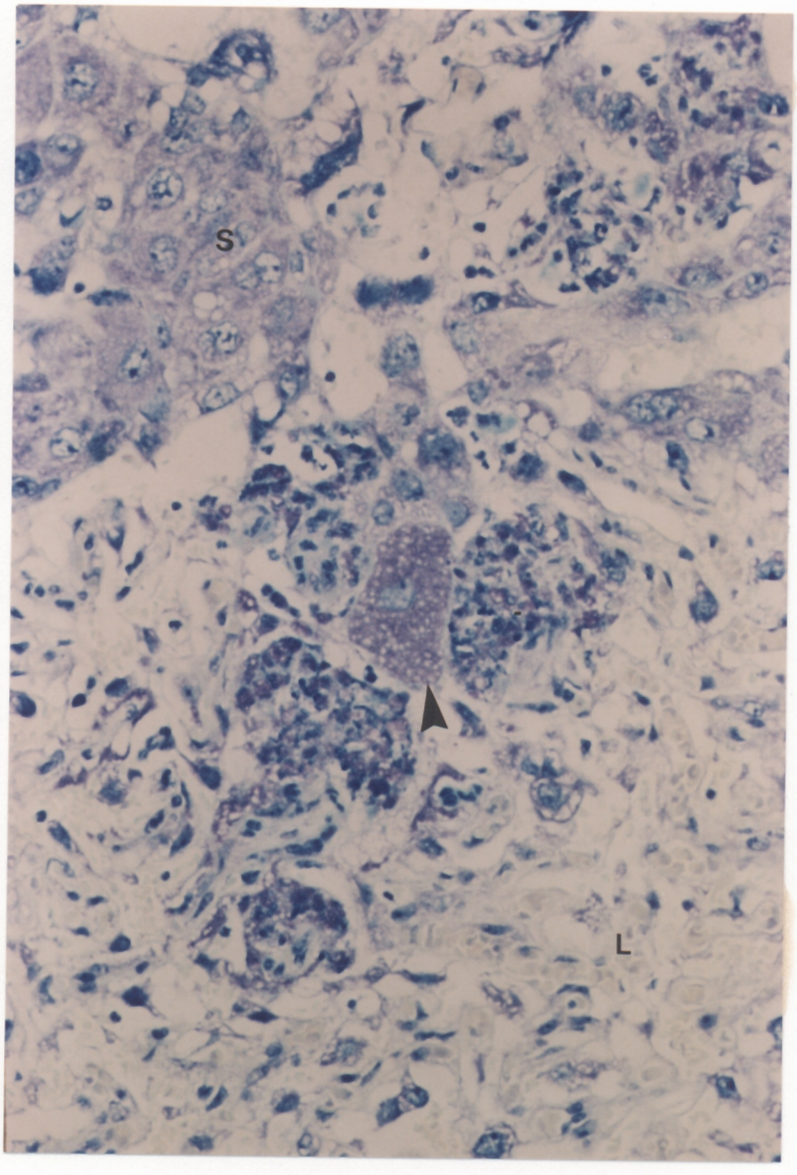
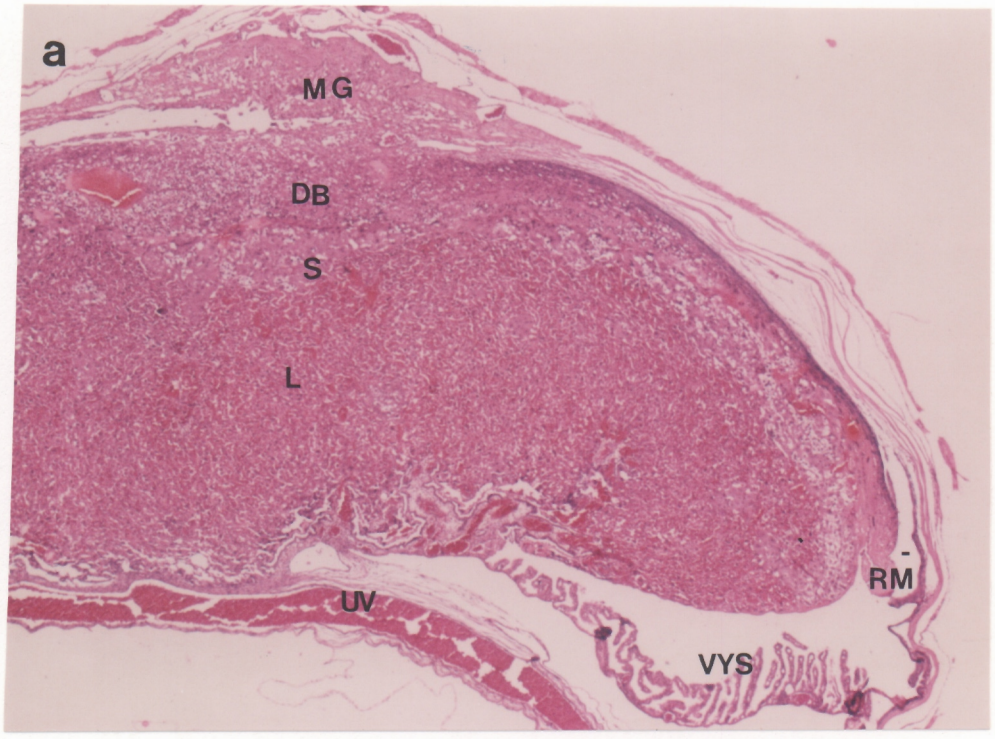

S
L

Figure 12. Cross-section of the mouse uterus at 18 days of gestation. H & E stain.

a. Control. MG-metrial gland, DB-decidua basalis, S-spongiotrophoblast or junctional zone, L-labyrinth, RM-Reichert's membrane, VYS-visceral yolk sac, UV-umbilical vessels. 18x.

b. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Note coalescing foci of inflammation within the spongiotrophoblast zone (arrowheads) and inflammation associated with Reichert's membrane (arrow). 29x.


a
MG
DB
S
L
UV
RM
VYS

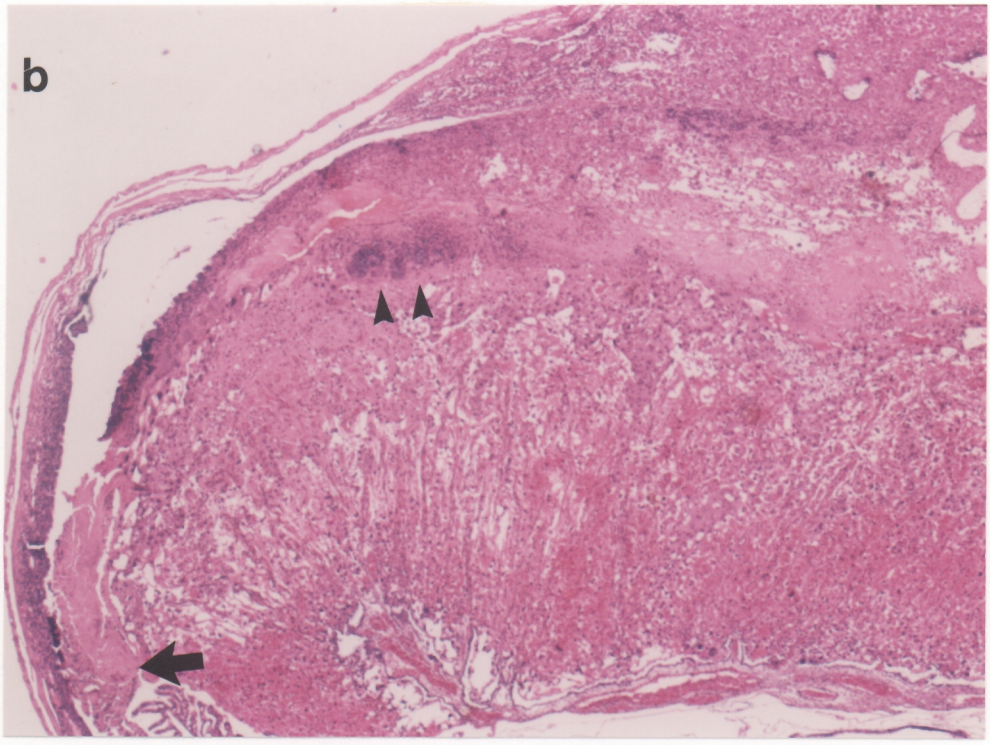

b

Figure 13. Spongiotrophoblast zone of the day 18 placenta from a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Giemsa stain. Note numerous trophoblast giant cells staining dark purple due to intracellular brucellae (arrowheads), a large extracellular colony of brucellae (dark arrow) and foci of positively staining neutrophils (open arrow). 180x.

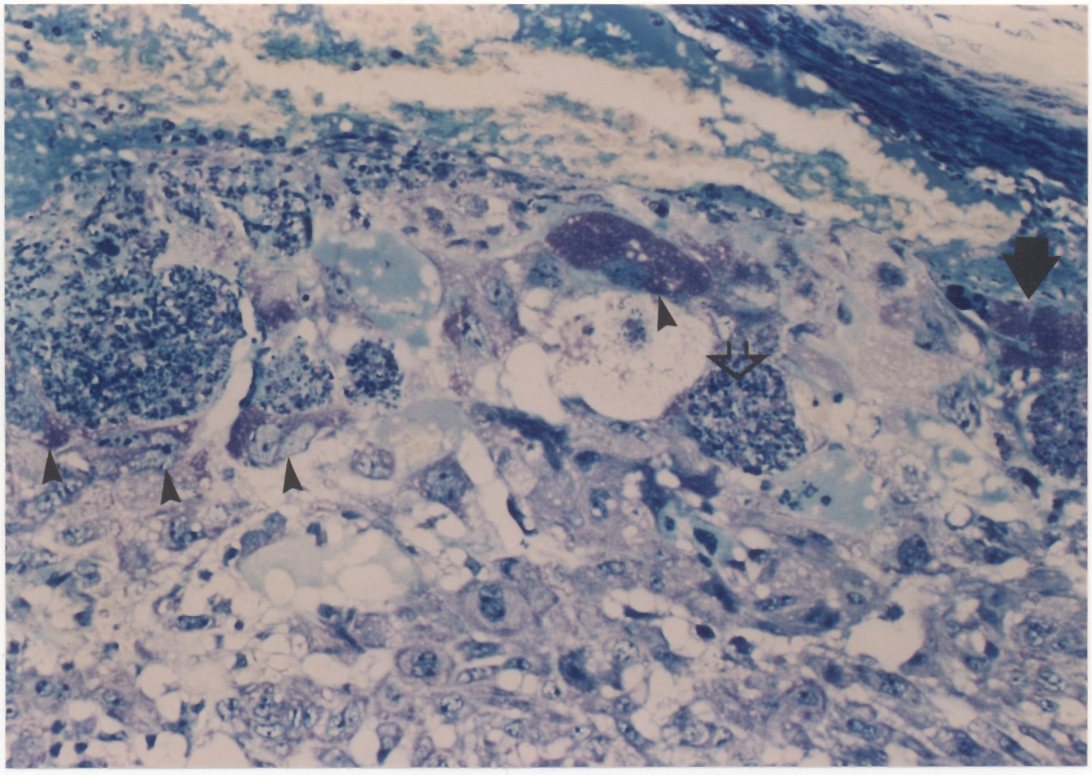


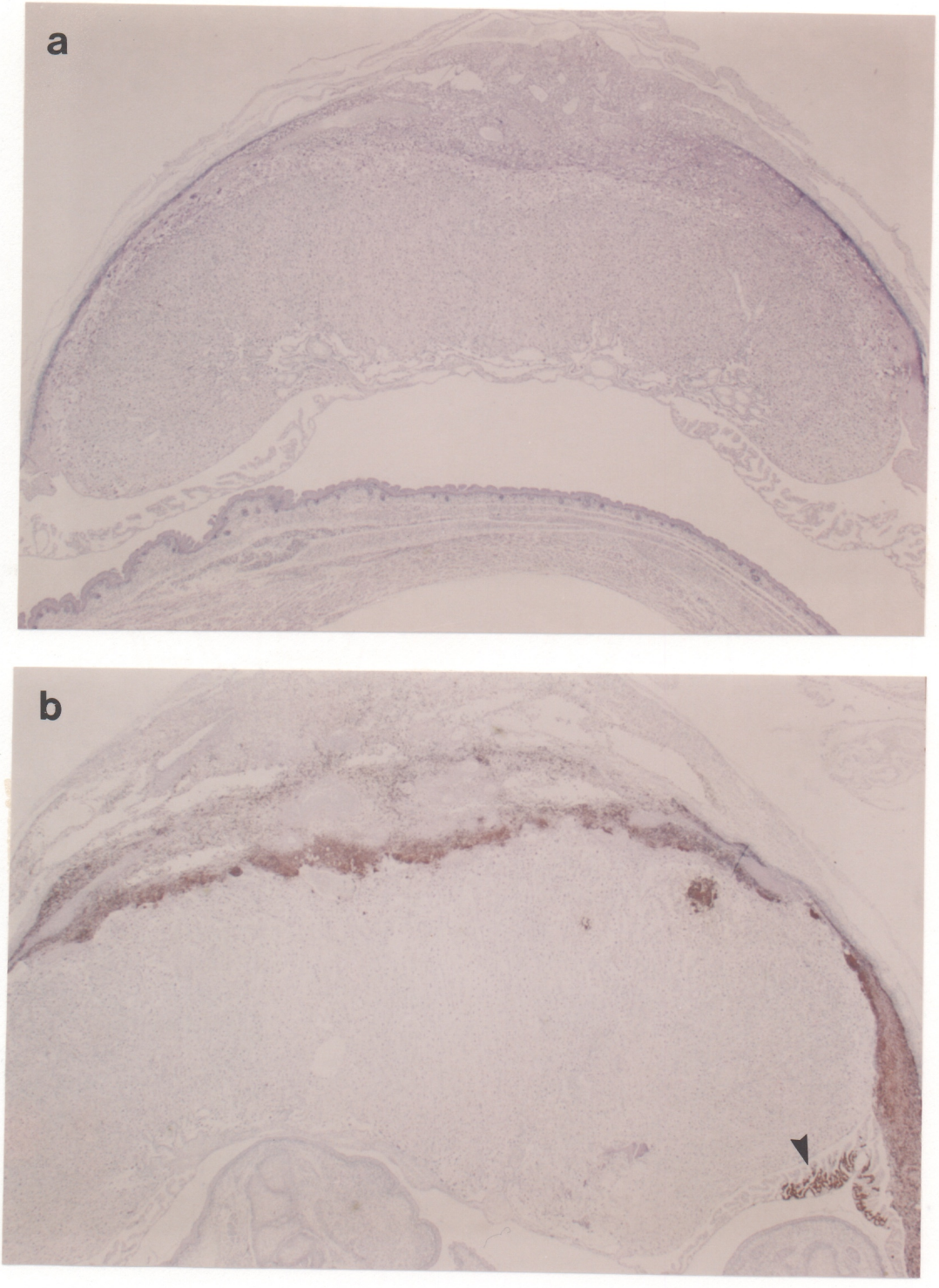
Figure 14. Mouse placenta at day 18 of gestation. ABC stain.

a. Cross-section of control uterus. Specific primary antibody. 18x.

b. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Specific primary antibody. Note extensive staining of the spongiotrophoblast zone, Reichert's membrane (which is thickened by marked inflammatory cell infiltration) and the visceral yolk sac (arrowhead). 18x.

c. Cross-section of the uterus of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Normal serum as primary antibody. Note lack of non-specific staining. 18x.

d. Higher magnification of b. taken at the junctional zone. Note heavily infected trophoblast giant cell (arrowhead) in a band of necrotic cellular debris and neutrophilic inflammation. 240x.


a
b


c

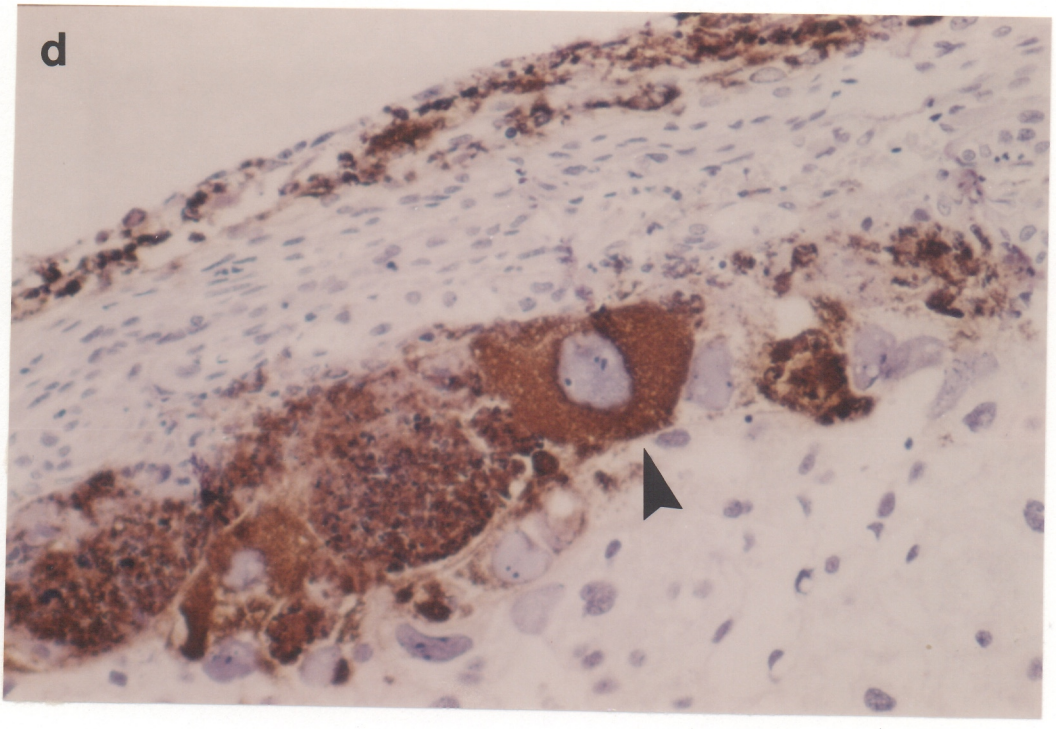

d

Figure 15. Periphery of a day 18 placenta from a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms om day 9 of gestation. ABC stain. Specific primary antibody. Note Reichert's membrane (arrowhead) associated with positively staining neutrophilic inflammation. L-labyrinth. 180x.

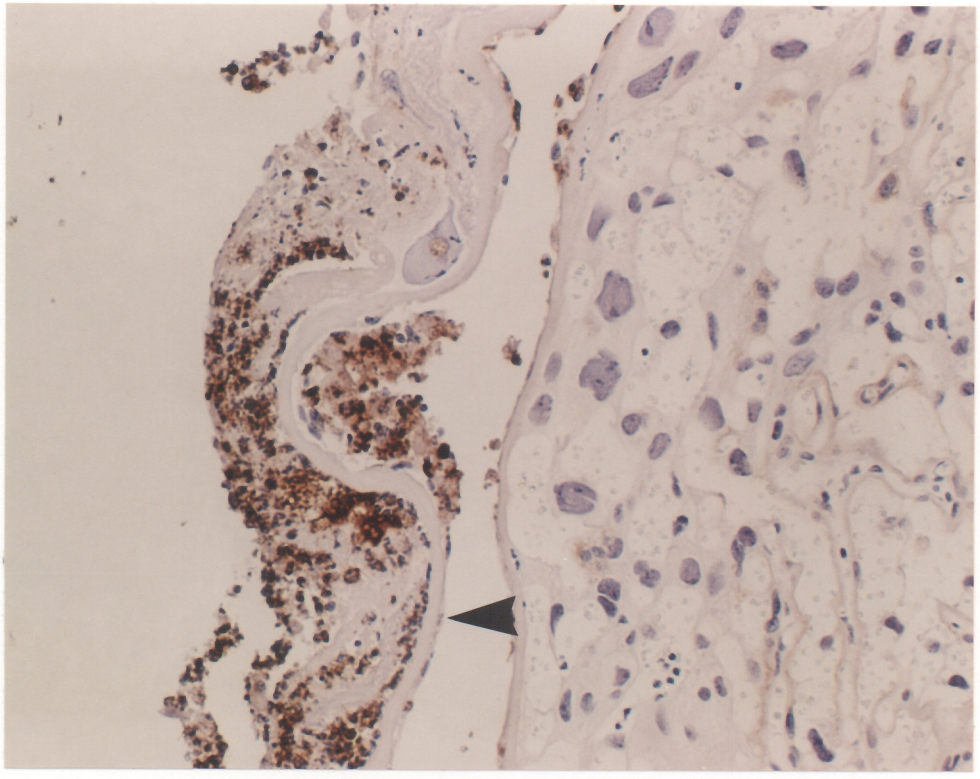
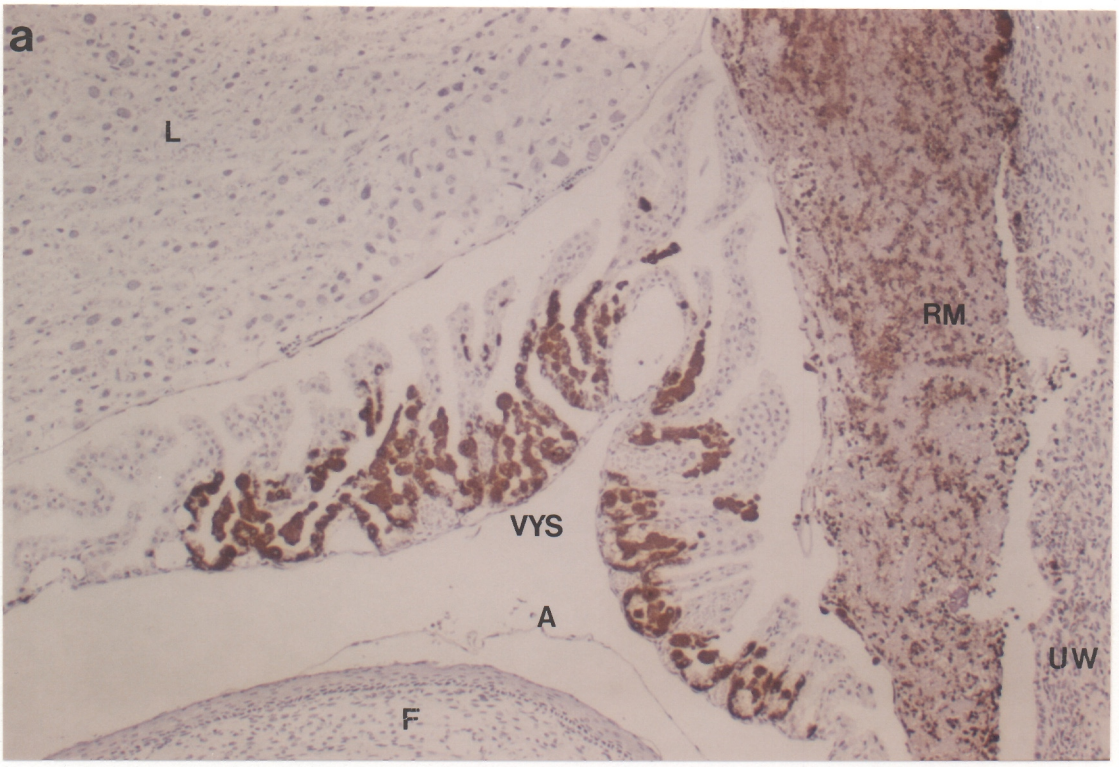


Figure 16. Day 18 placenta from a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. ABC stain. Specific primary antibody.

a. Periphery of placenta. Note positive staining of the visceral yolk sac and extensive neutrophilic inflammation associated with Reichert's membrane. L-labyrinth, VYS-visceral yolk sac, RM-Reichert's membrane, UW-uterine wall, A-amnion, F-fetus. 90x.

b. Visceral yolk sac. Note the heavily stained cytoplasm of numerous endoderm cells that are often distended due to the presence of intracellular brucella. 360x.


a
L
RM
VYS
A
UW
F

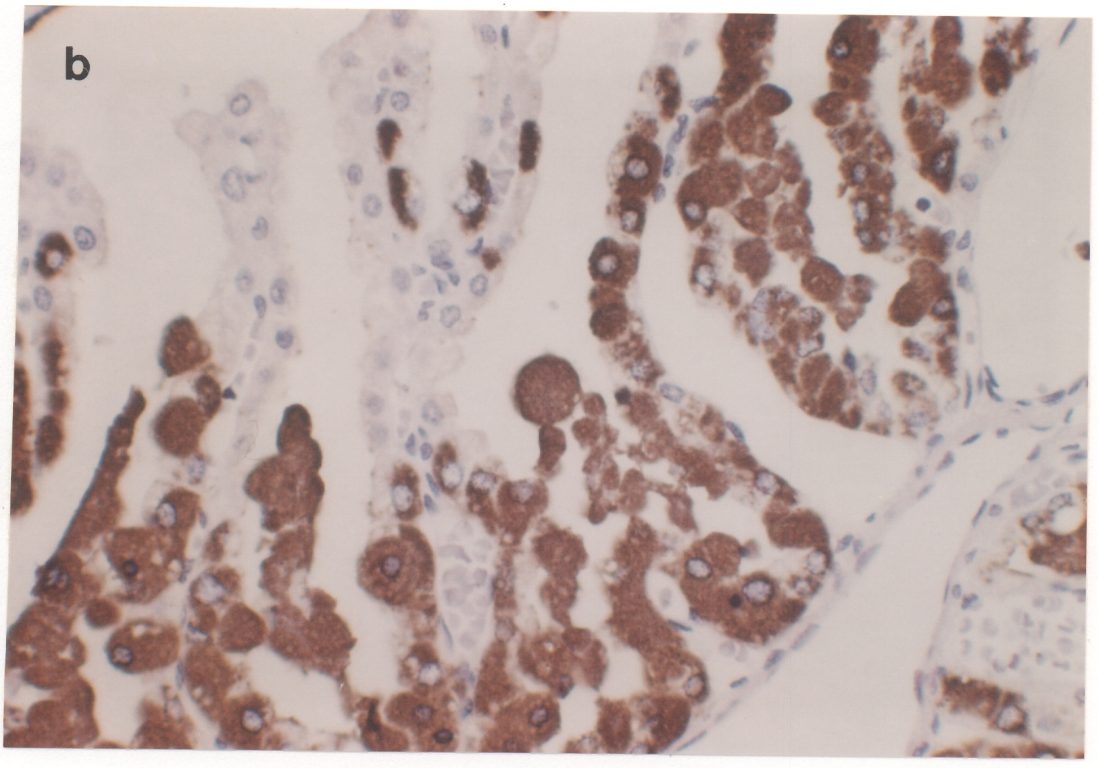
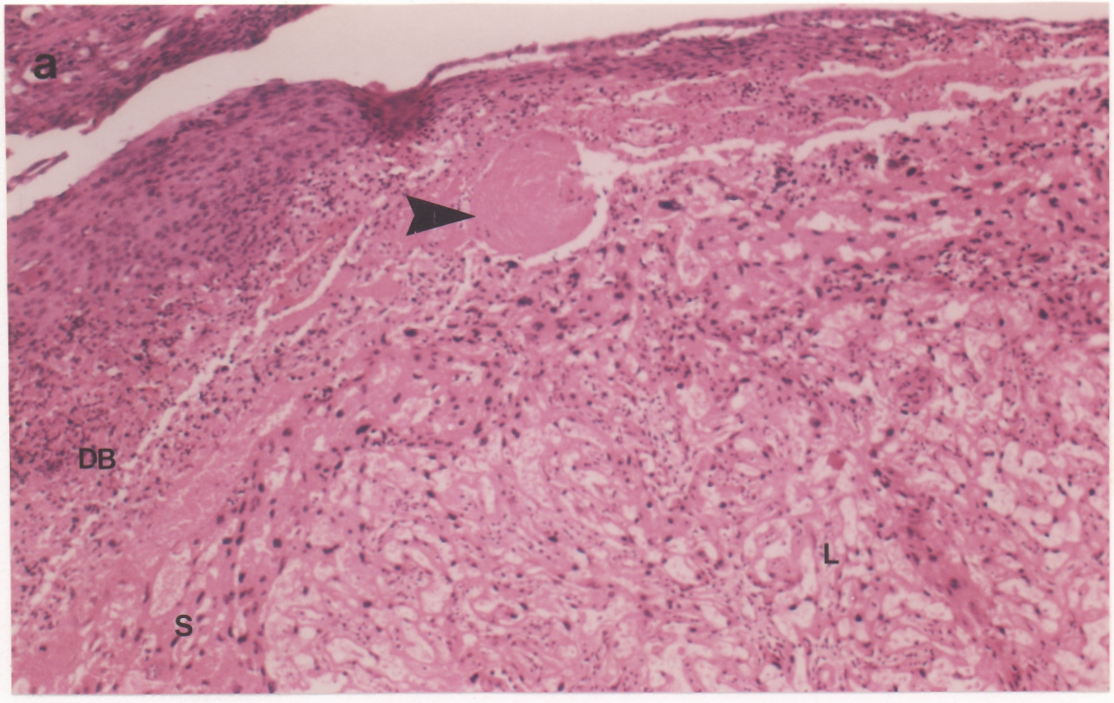

b

Figure 17. Cross-section of the uterus on day 18 of gestation from a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. H & E stain.

a. Note thrombus within maternal sinus (arrowhead). The underlying labyrinth zone is devoid of maternal blood. DB-deciua basalis, S-spongiotrophoblast, L-labyrinth. 72x.

b. The labyrinth of this placenta is also infarctedand the associated fetus stains poorly due to autolysis. Note band of suppurative inflammation within the spongiotophoblast (arrowheads). UA-uterine artery, DB-decidua basalis, S-spongiotrophoblast, L-labyrinth, F-fetus. 36x.


a
DB
L
S

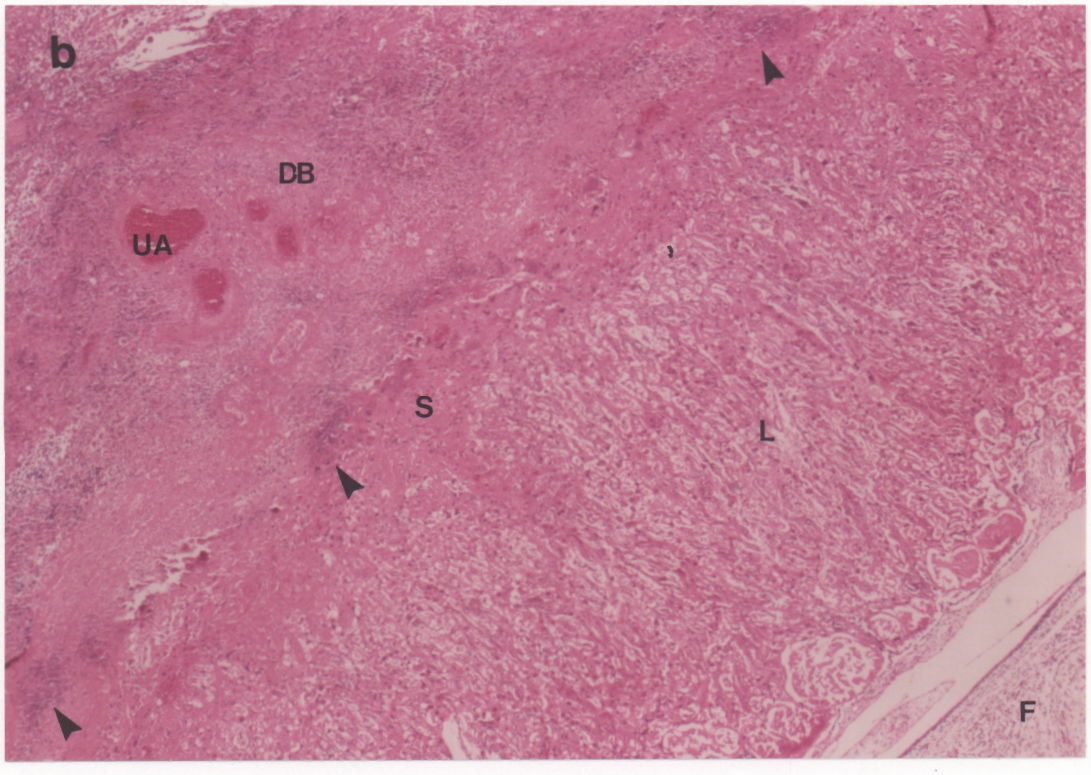

b
DB
UA
S
L
F

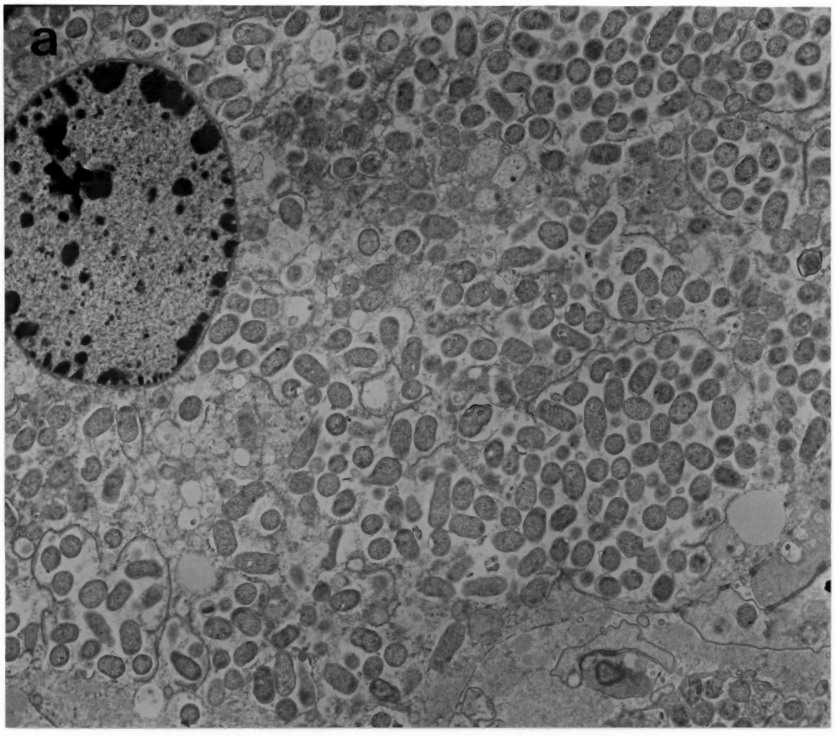
Figure 18. Infected trophoblast giant cells of the day 18 placenta from a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation.

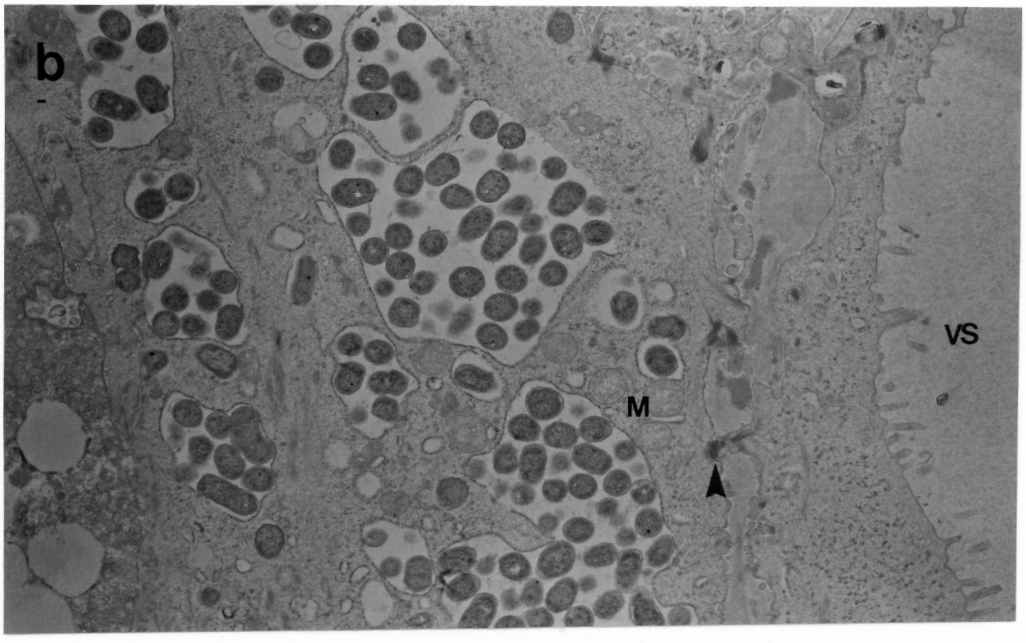
a. Trophoblast giant cell with dilated cisternae of the r o u g h endoplasmic reticulum containing numerous Brucella organisms. 6300x.

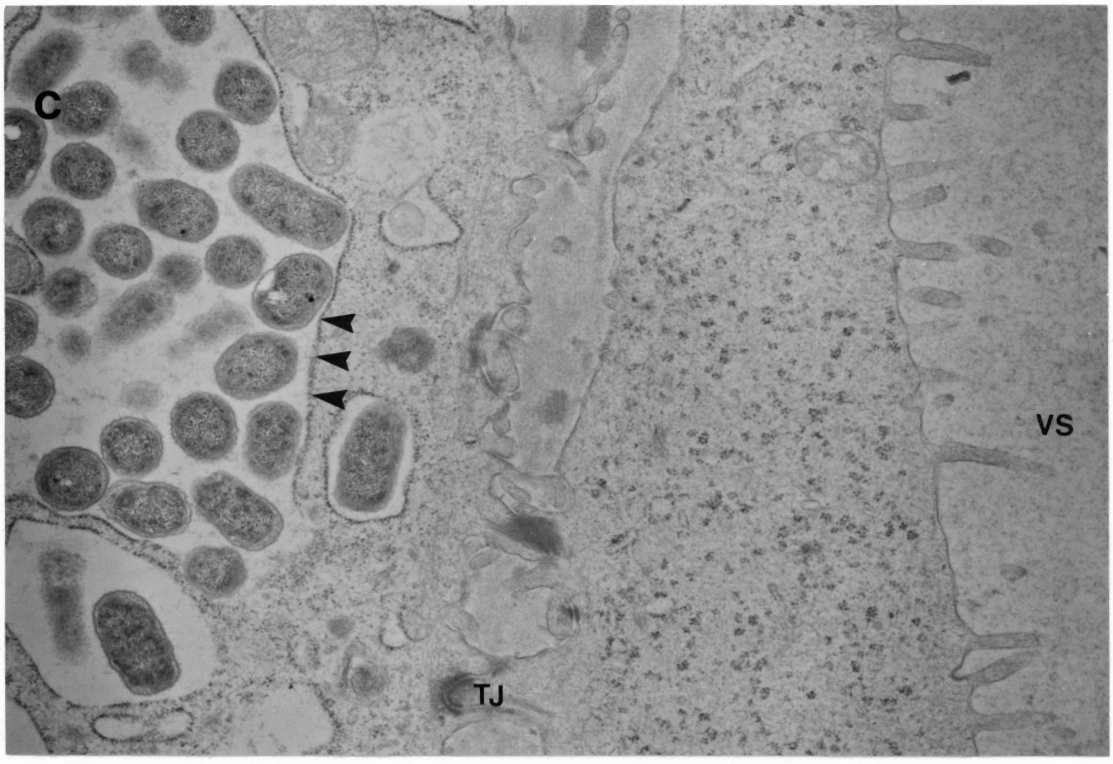
b. The cisternae of this cell also contain numerous brucellae. Note mitochondria (M), adjacent tight junction (arrowhead) and venous sinus (VS). 7600x.

c. Higher magnification of b. showing details of RER (arrowheads), tight junction (TJ) and venous sinus (VS). Note microvilli extending into the sinus. 16500x.

d. Cisternae of the RER containing many Brucella organisms.At this magnification it is possible to distinguish ribosomes lining the outside of the endoplasmic reticulum (arrowheads) and to easily contrast the ultrastructure of mitochondria (M) to the Brucella organisms (B). 44800x


a


b
VS
M


C
VS
TJ

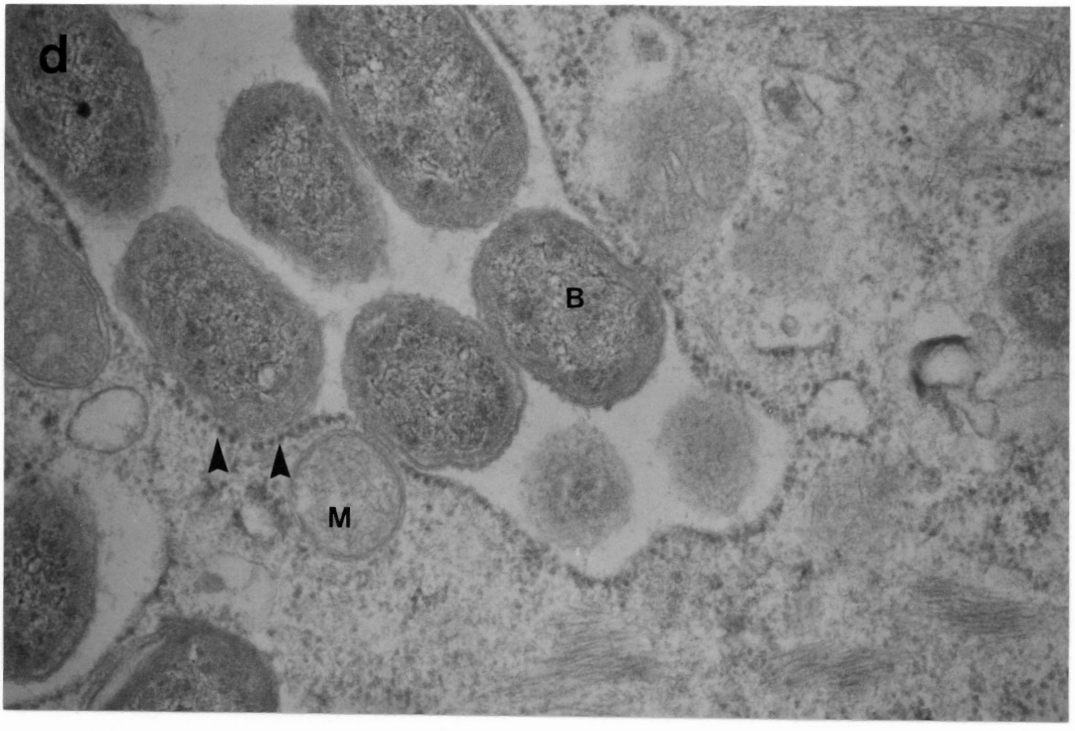

d
B
M

Figure 19. Electron micrograph of a trophoblast giant cell from the day 18 placenta of a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Immunogold stain. The cisternae of the RER contains a bacterium that is labeled by 15-20 nanometer gold particles (arrowheads) that had been conjugated onto Brucella-specific antibodies. 76000x.

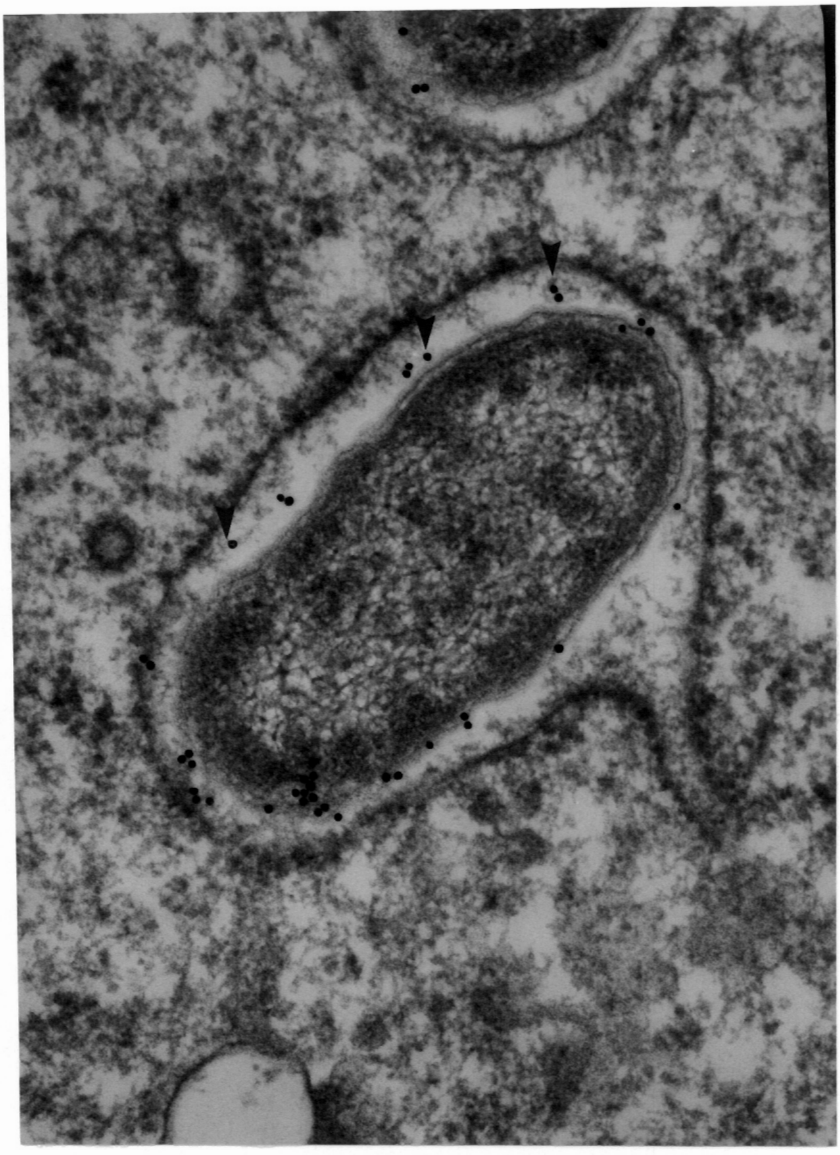


Figure 20. Inflammatory infiltrate associated with Reicherts's membrane from the day 18 placenta of a mouse infected with $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Note neutrophil (arrowhead) with intracellular brucellae and numerous extracellular bacteria free within the uterine lumen (arrows). 7200x.

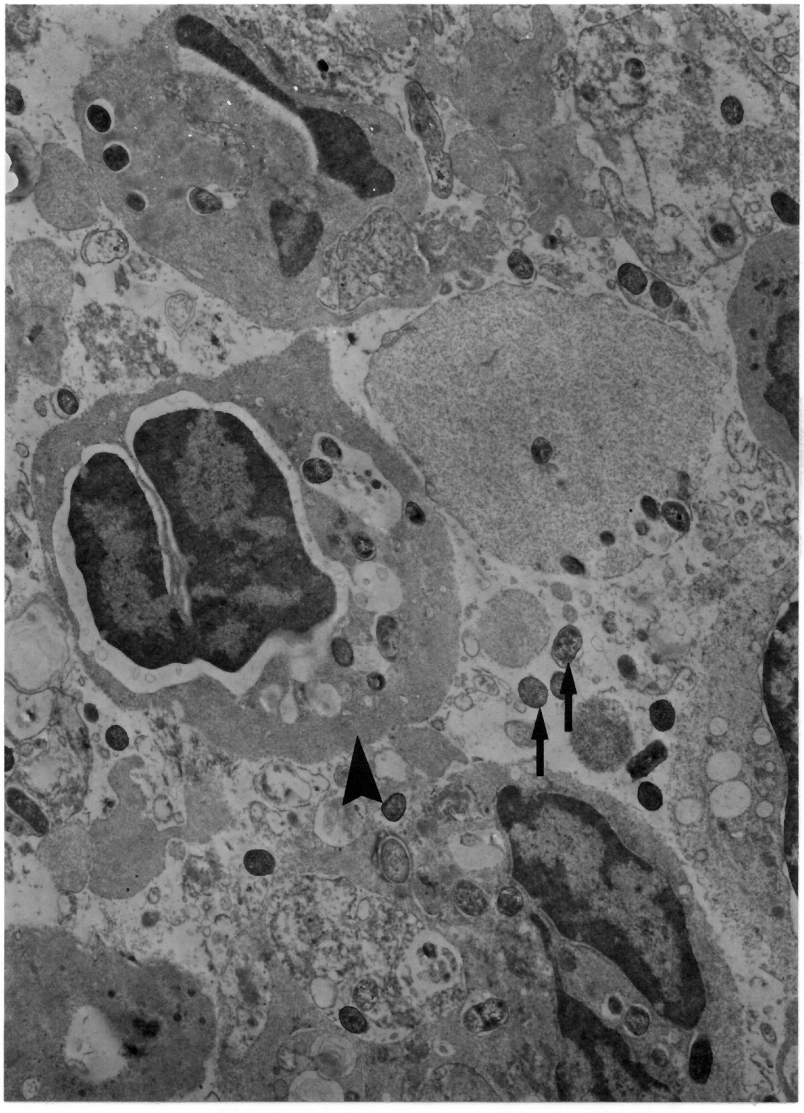


Figure 21. Electron micrograph of the spongiotrophoblast zone of the day 18 placenta from a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Note extracellular brucellae (arrowhead) free within the venous sinus (VS). RBC-red blood cell, T-trophoblast giant cell. 15700x.

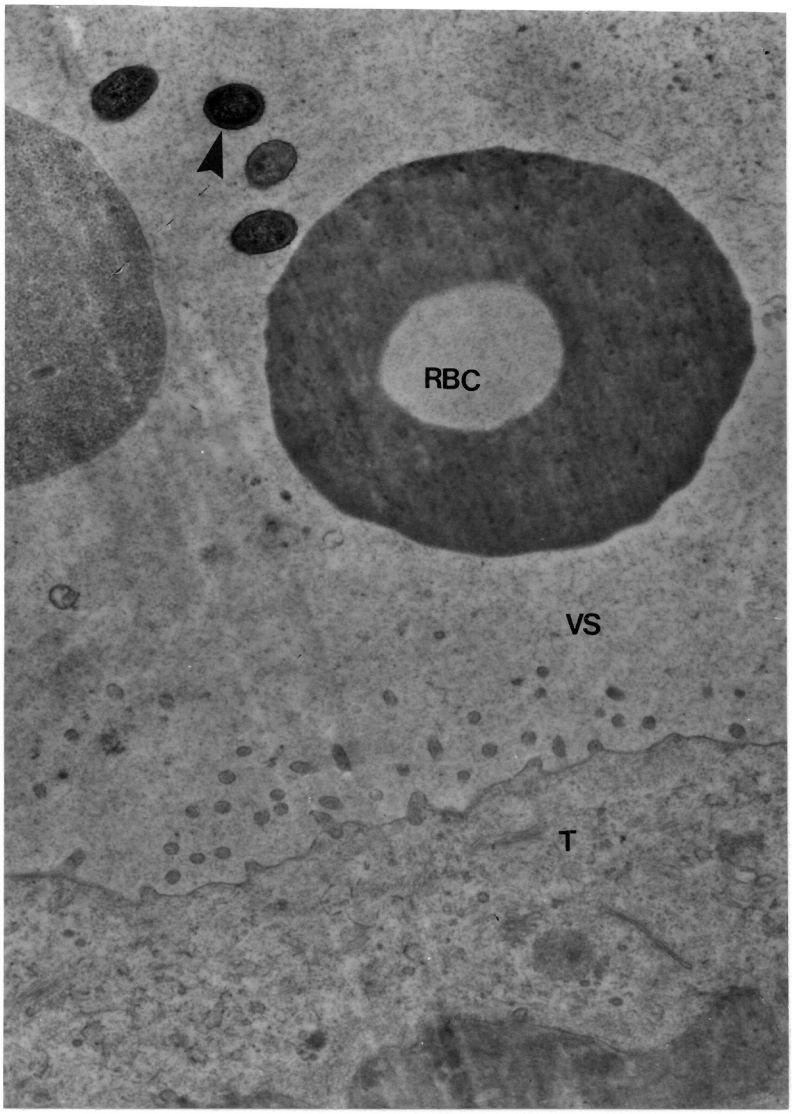

RBC
VS
T

Figure 22. Electron micrograph of Reichert's membrane of the day 18 placenta from a mouse that received $10^{6.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Note a colony of Brucella organisms (B) associated with Reicherts's membrane RM. 4700x.

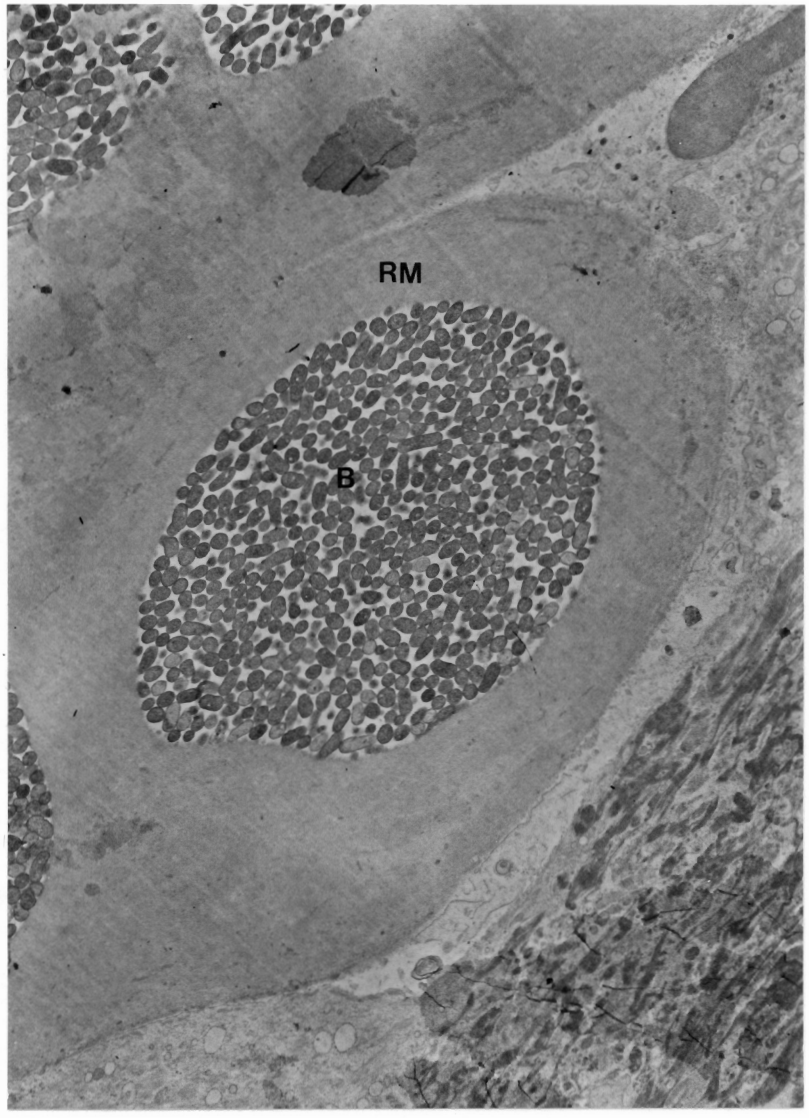

RM
B

Figure. 23 Replication of B. abortus strain 2308 organisms in spleen and placenta of BALB/c mice at various days of gestation. (See Table 2 for numerical values)

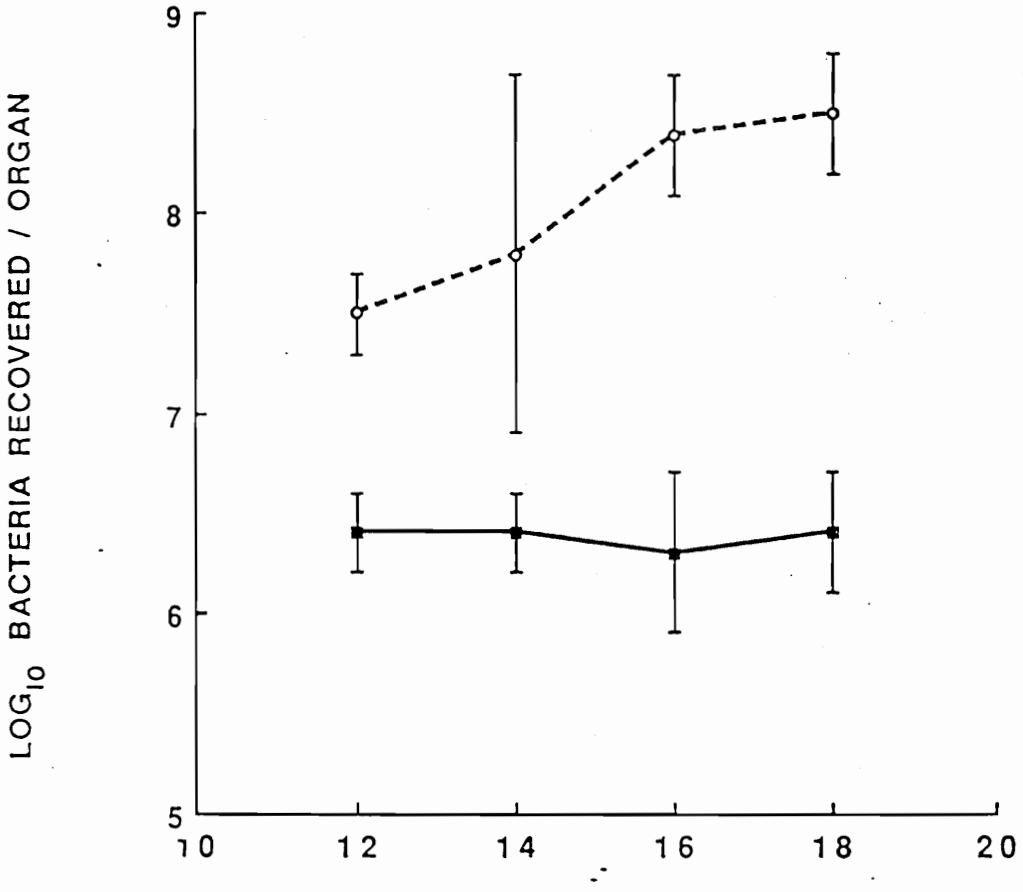
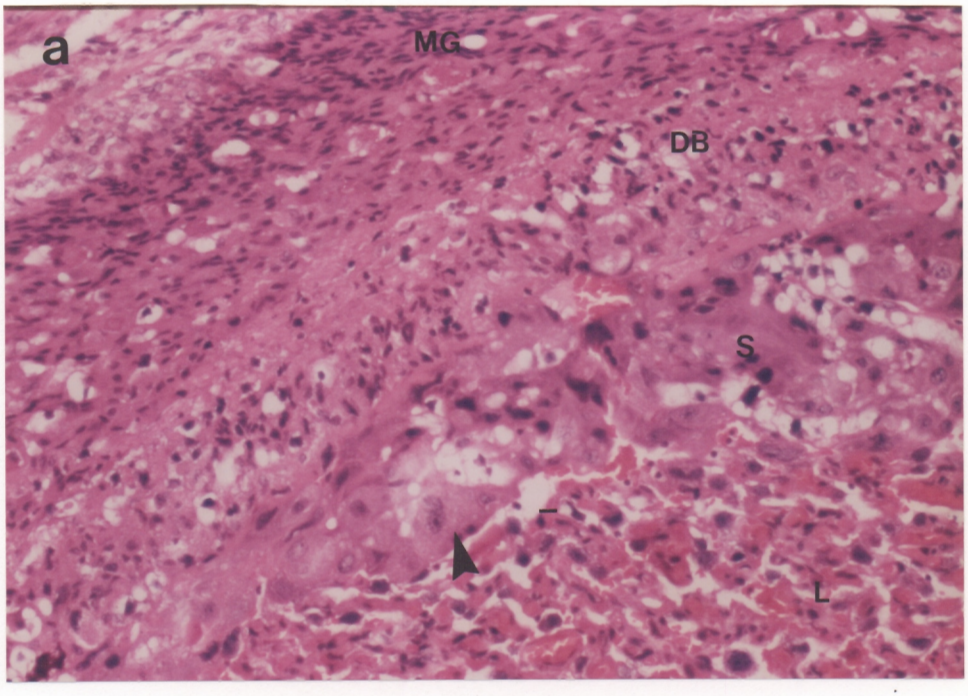

LOG10 BACTERIA RECOVERED / ORGAN
9
8
7
6
5
10
12
14
16
18
20
DAYS OF GESTATION
SPLEEN
PLACENTA
* INFECTED DOSE 10 6.7
BARS INDICATE STANDARD DEVIATION

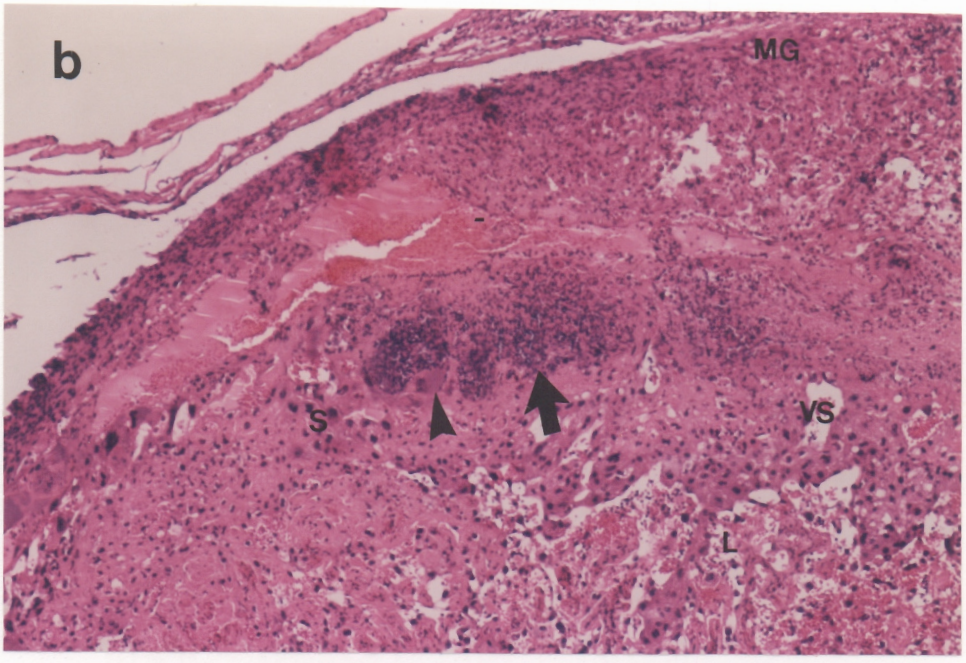
Figure 24. Cross-section of the day 18 mouse placenta. H & E stain.

a. Control. MG-metrial gland, DB-decidua basalis, S-spongiotrophoblast. L-labyrinth. 24x

b. Placenta from a mouse that received $10^{5.7}$ B. abortus strain 2308 organisms on day 9 of gestation. Note extensive neutrophilic inflammation (arrow) within the spongiotrophoblast and trophoblast giant cell (arrowhead) with hyperchromic cytoplasm due to intracellular brucellae. MG-metrial gland, DB-decidua basalis, S-spongiotrophoblast, VS-venous sinus, L-labyrinth. 72x.

c. Placenta from a mouse that received $10^{7.5}$ B. abortus strain 19 organisms on day 9 of gestation. Note extensive neutrophilic inflammation (arrow) within the spongiotrophoblast (S) and hyperchromic trophoblast giant cell (arrowhead) infected with brucellae. L-labyinth. 90x.


a
MG
DB
S
L


b
MG
S
VS
L

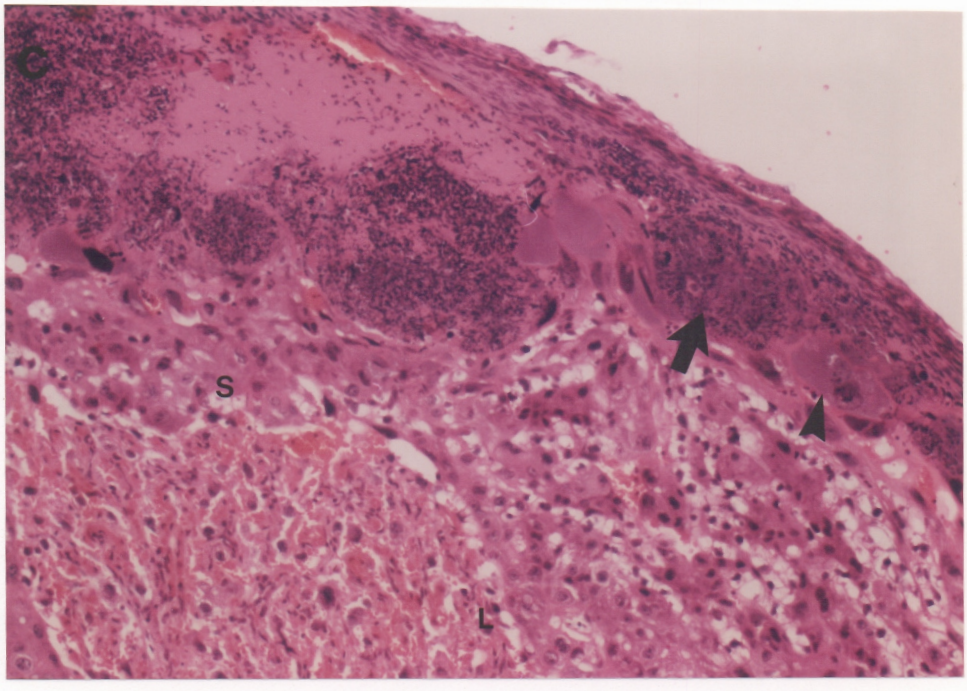
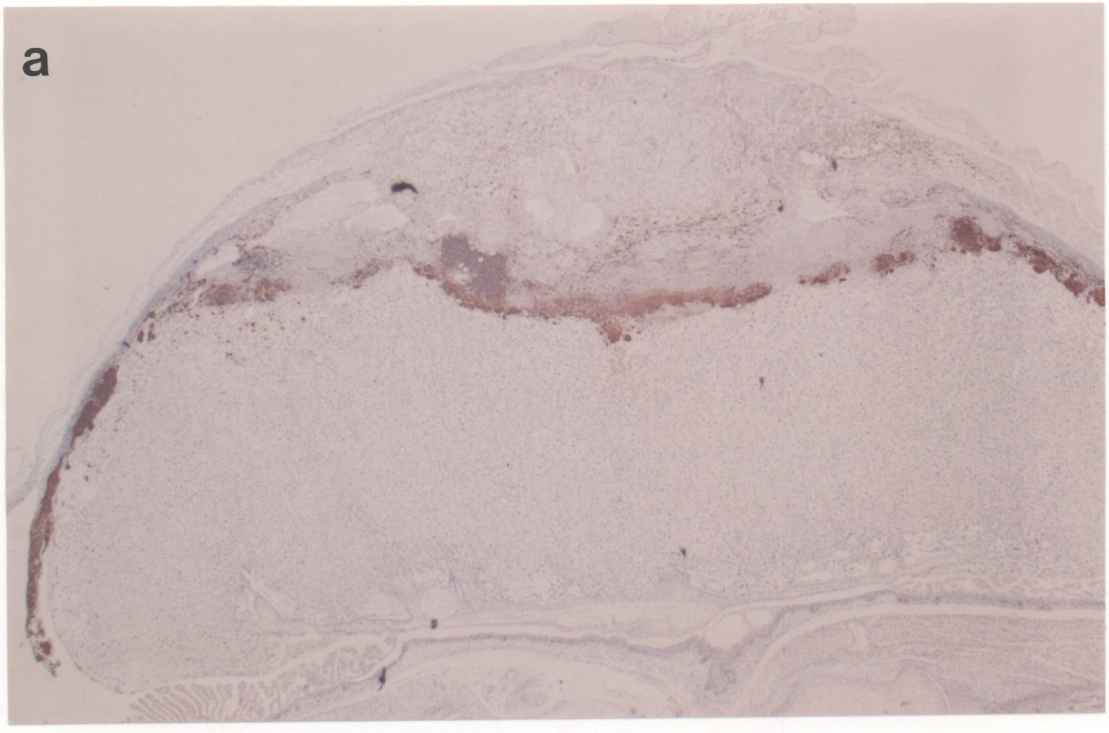

C
S
L

Figure 25. Cross-section of the day 18 uterus of a mouse that received $10^{7.5}$ B. abortus strain 19 organisms on day 9 of gestation. ABC stain.

a. Specific primary antibody. Note extensive staining of the spongiotrophoblast extending to the periphery of the placenta. 18x.

b. Normal serum used as primary antibody. Note lack of non-specific staining. 18x.


a

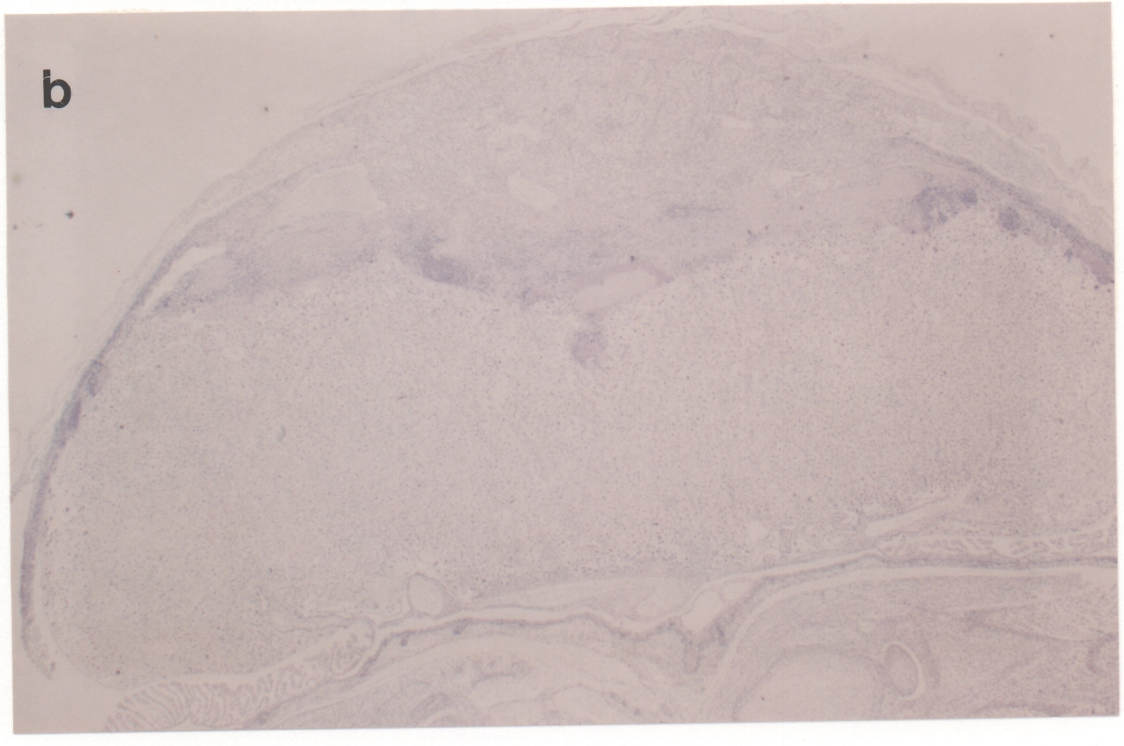
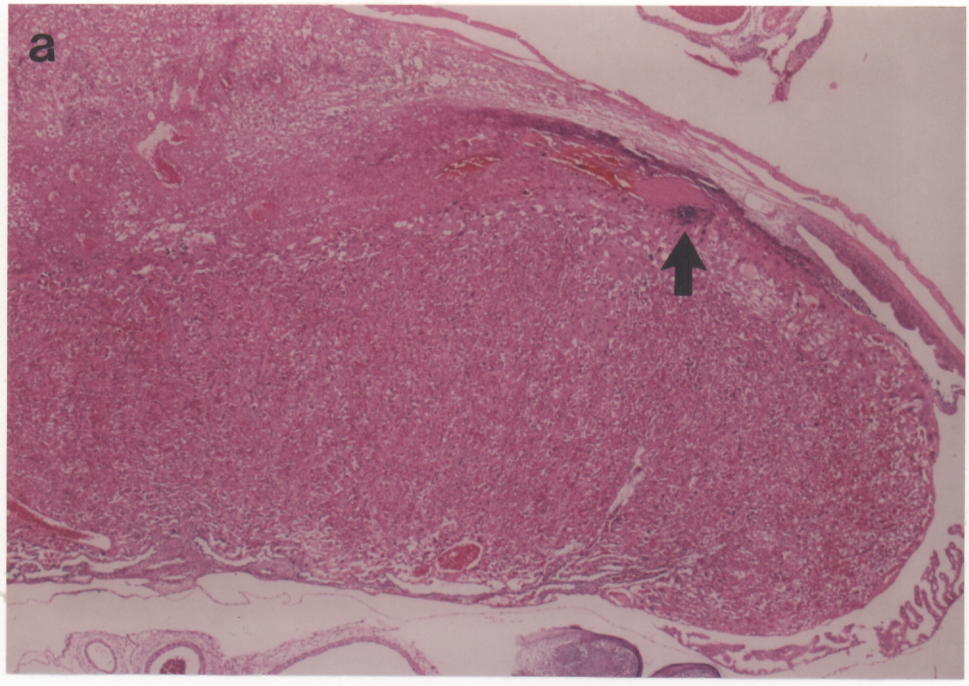

b

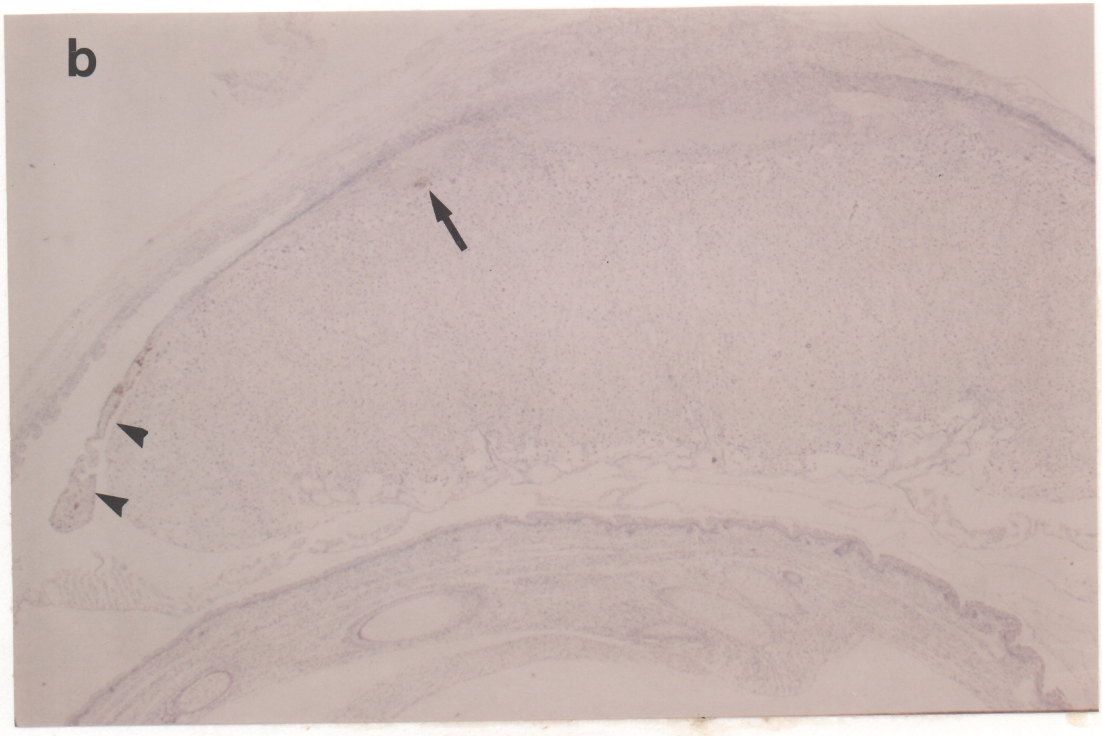
Figure 26. Cross-section of the uterus of a mouse that received $10^{9.5}$ B. abortus strain RB51 organisms on day 9 of gestation.

a. H & E stain. Note small focus of neutrophilic inflammation within the spongiotrophoblast (arrow). 24x.

b. ABC stain. Specific antibody. Note faint staining of the spongiotrophoblast (arrow) and associated with Reichert's membrane (arrowheads). 18x.

c. ABC strain. Normal serum used as primary antibody. Note lack of non-specific staining. 18x.


a


b

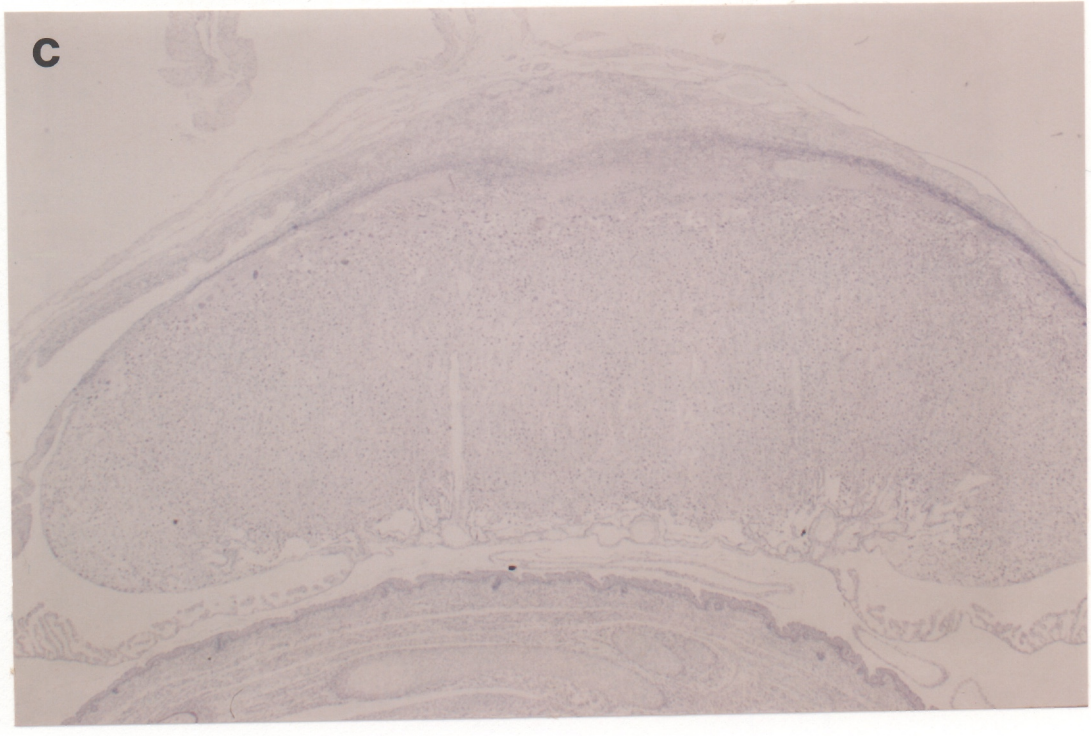

c

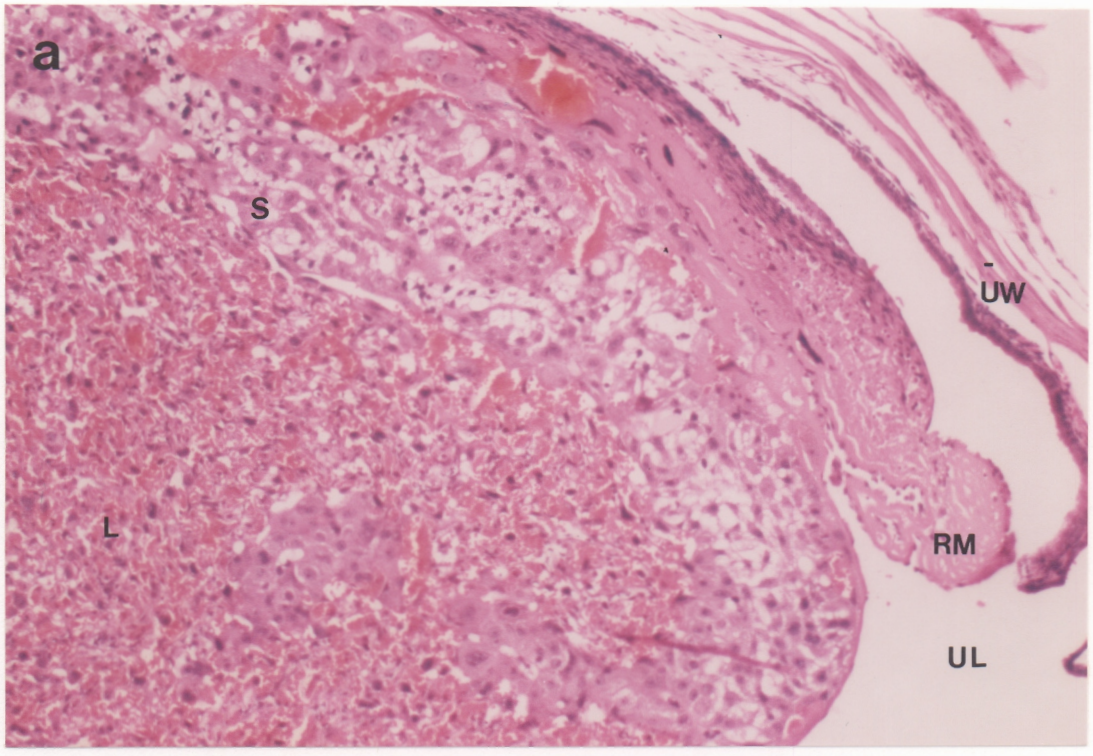
Figure 27. Cross-section of the day 18 mouse placenta.

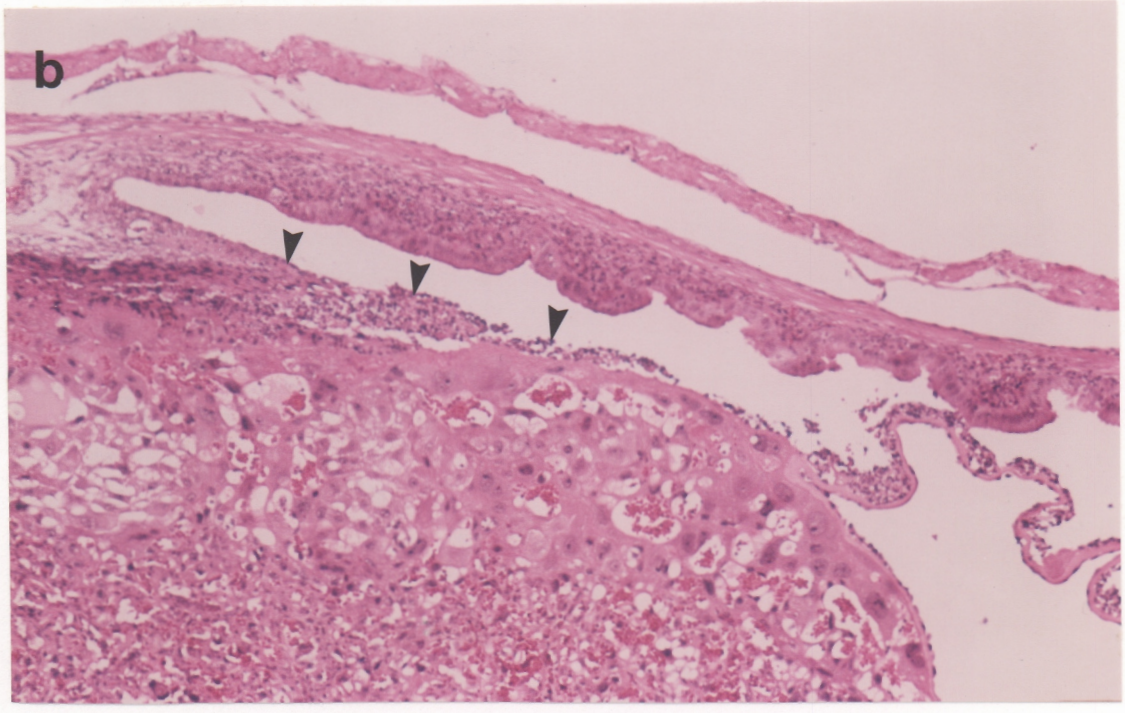
a. Reichert's membrane. Control. H & E stain. UW-uterine wall, RM-Reichert's membrane, S-spongiotrophoblast, L-labyrinth, UL-uterine lumen. 90x.

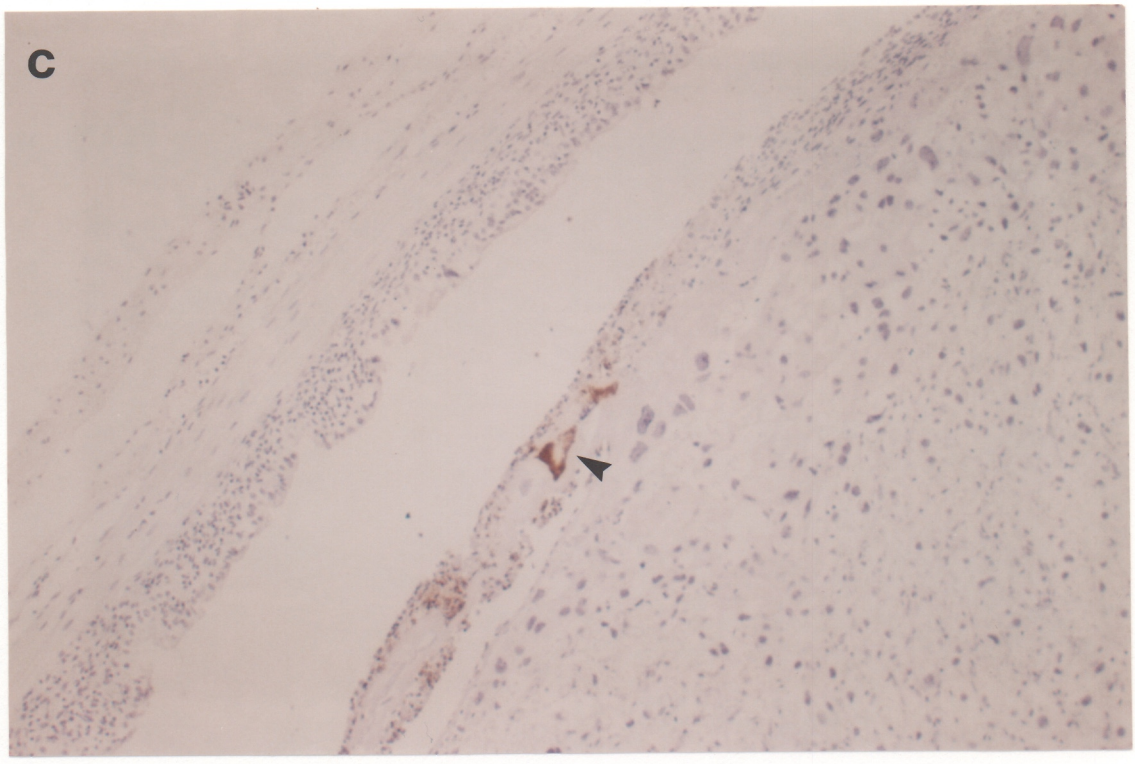
b. Mouse that received $10^{9.5}$ B. abortus strain RB51 organis on day 9 of gestation. H & E stain. Note neutrophilic inflammation associated with Reichert's membrane. 72X.

c. Reichert's membrane of a placenta from a mouse that received $10^{9.5}$ B. abortus strain RB51 organisms on day 9 of gestation. ABC stain, specific antibody. Note infected trophoblast giant cell (arrowhead) adjacent to inflammation associated with Reichert's membrane. 90x.

d. Spongiotrophoblast zone of a mouse that received $10^{9.5}$ B. abortus strain RB51 organisms on day 9 of gestation. ABC stain, specific antibody. Note small focus of positively staining inflammatory cells (arrowhead) within the spongiotrophoblast (S) adjacent to maternal venous sinuses (VS). 180x.


a
S
UW
L
RM
UL


b


C

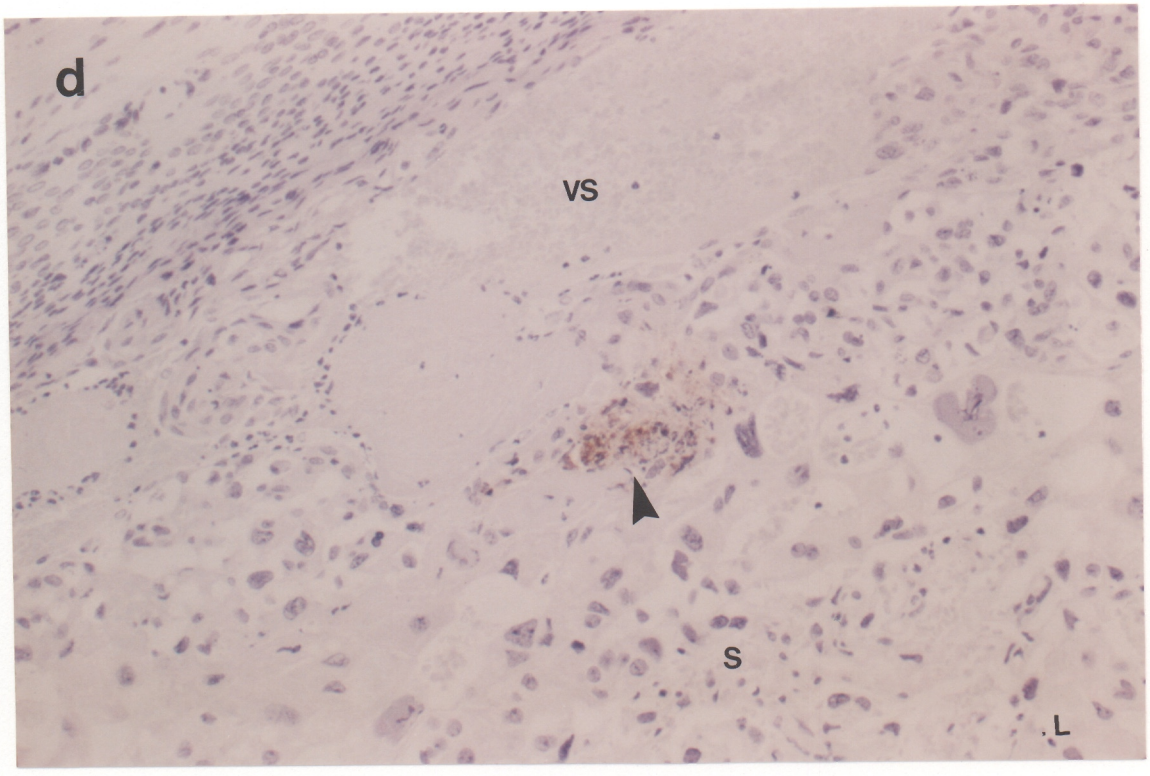
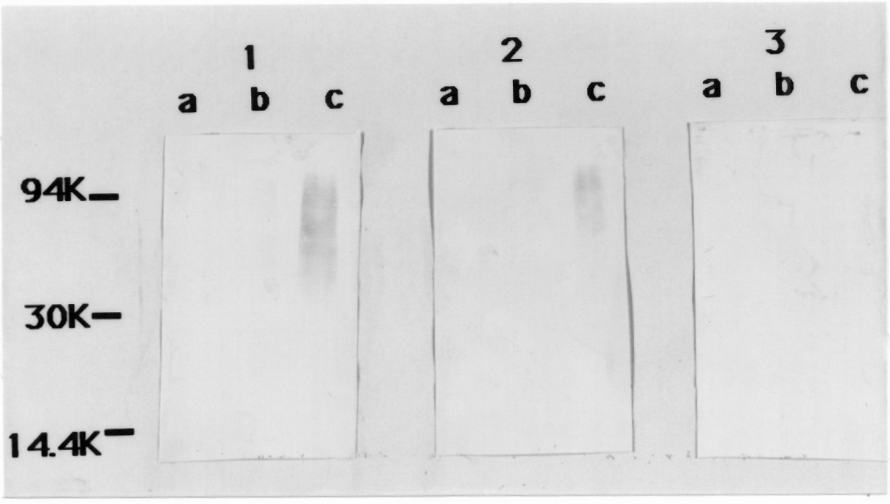

d
VS
S
L

Figure 28. Western blot analysis of Day 18 sera from mice infected with strain 2308, strain 19 or strain RB51 on day 9 of gestation.

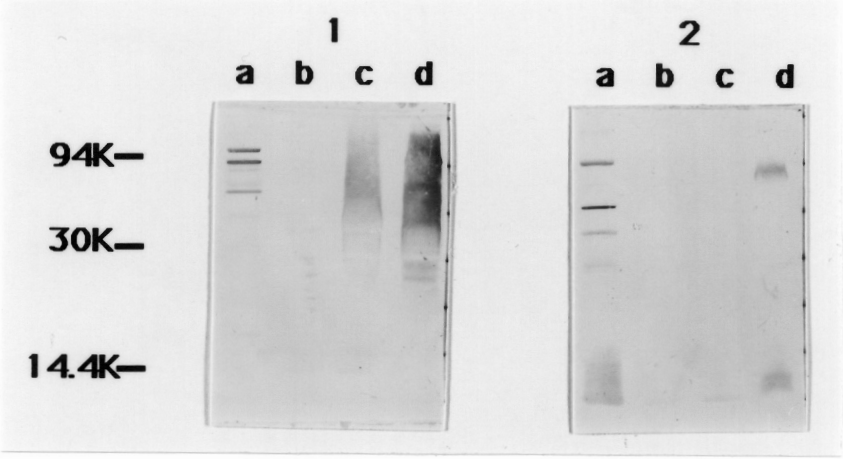
WESTERN BLOT ANALYSIS OF SERA FROM MICE INFECTED WITH STRAIN 2308, STRAIN 19 OR STRAIN RB51 ON DAY 9 OF GESTATION



1-mice received $10^{5.7}$ strain 2308, 2-mice received $10^{7.5}$ strain 19, 3-mice received $10^{9.5}$ strain RB51. Antigens tested were: lane a-strain RB51 (whole cell); lane b- Y. enterocolitica 0:9 LPS; lane c-strain 2308 (whole cell). Molecular weight standards to the left.

Figure 29. Western blot analysis of sera from strain RB51 and strain 19 immunized mice four weeks post-vaccination.

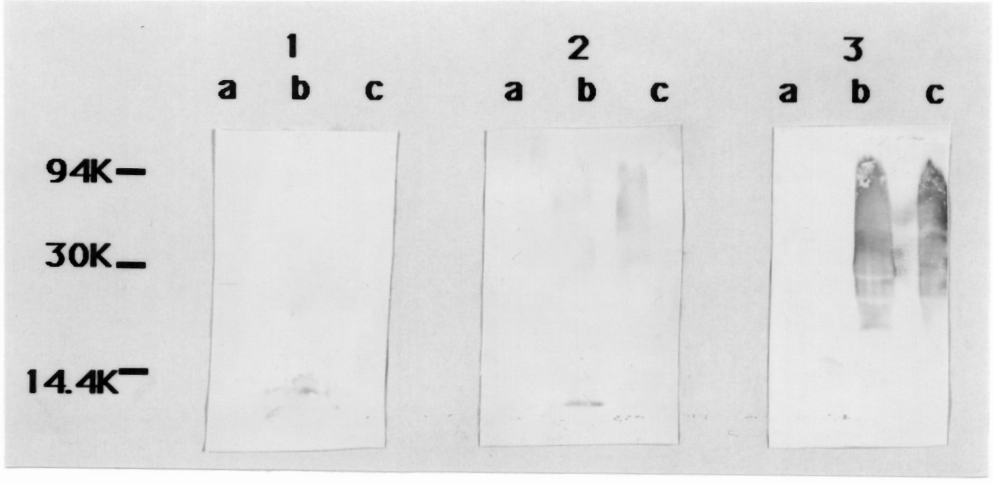
WESTERN BLOT ANALYSIS OF SERA FROM STRAIN RB51 AND STRAIN 19 IMMUNIZED MICE FOUR WEEKS POST-VACCINATION



1-strain 19 antiserum, 2-strain RB51 antiserum. Antigens tested were: lane a-strain RB51 (treated with DMSO-pellet); lane b-Y. enterocolitica 0:8 LPS; lane c-Y. enterocolitca 0:9 LPS; lane d-strain 2308 (whole cell). Molecular weight standards to the left. Note the absence of reactivity of strain RB51 immunized mice to Y. enterocolitica 0:9 LPS indicating the absence of anti-O-side-chain antibodies.

Figure 30. Western blot analysis of Day 18 sera from control mice with or without strain 2308 challenge and BRU 38.

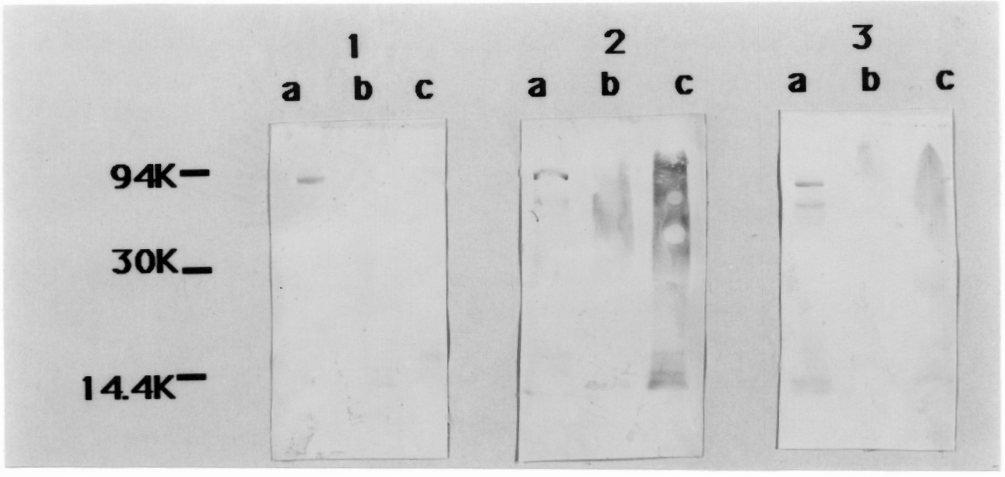
WESTERN BLOT ANALYSIS OF SERA FROM CONTROL MICE WITH OR WITHOUT STRAIN 2308 CHALLENGE AND BRU 38



1-control, no challenge, 2-control with strain 2308 challenge, 3-BRU 38. Antigens tested were: lane a-strain RB51 (whole cell); lane b-Y. enterocolitica 0:9 LPS; lane c-strain 2308 (whole cell). Molecular weight standards to the left.

Figure 31. Western blot analysis of Day 18 sera from strain RB51 immunized mice with or without strain 2308 challenge.

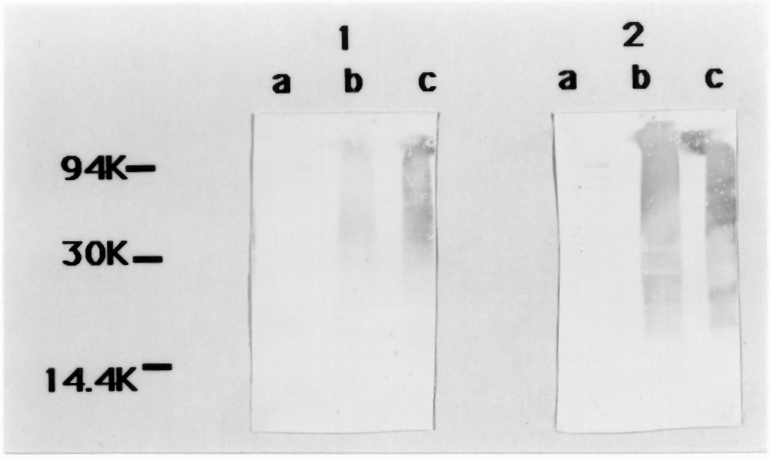
WESTERN BLOT ANALYSIS OF SERA FROM STRAIN RB51 IMMUNIZED MICE WITH AND WITHOUT STRAIN 2308 CHALLENGE



1-1X strain RB51 vaccination, no challenge, 2-1X strain RB51 vaccination with strain 2308 challenge, 3-2X strain RB51 vaccination with strain 2308 challenge. Antigens tested were: lane a-strain RB51 (whole cell); lane b-Y. entercolitica 0:9 LPS; lane c-strain 2308 (whole cell). Molecular weight standards to the left.

Figure 32. Western blot analysis of Day 18 sera from strain 19 immunized mice with or without strain 2308 challenge.

WESTERN BLOT ANALYSIS OF SERA FROM STRAIN 19 IMMUNIZED MICE WITH AND WITHOUT STRAIN 2308 CHALLENGE



1-strain 19 vaccination, no challenge, 2-strain 19 vaccination with strain 2308 challenge. Antigens tested were: lane a-RB51 (whole cell); lane b-Y. enterocolitica 0:9 LPS; lane c-strain 2308 (whole cell). Molecular weight standards to the left.

APPENDIX

List of Abbreviations

ABC	-	avidin - biotin - complex
ANOVA	-	analysis of variance
ELISA	-	enzyme linked immunosorbant assay
LPS	-	lipopolysaccharide
MHC	-	major histocompatibility complex
RER	-	rough endoplasmic reticulum
SAT	-	serum agglutination test
SD	-	standard deviation
STA	-	standard tube agglutination
TBS	-	tris buffered saline
TBST	-	tris buffered saline with Tween
TEMED	-	tetramethylethyl enediamine
TGC	-	trophoblast giant cell
TRIS	-	(hydroxymethyl) aminomethane

Mouse Husbandry

Initially, we had great difficulty in achieving satisfactory breeding and conception rates using the BALB/c strain of inbred mouse. As this is a strain heavily utilized in brucellosis research, we persevered with this strain, changing management techniques until we were able to obtain satisfactory breeding results in timed matings. Essential to the procedure were:

1) female mice at least eight-weeks-old at breeding
2) experienced male mice
3) lighting: 14 hours light, 10 hours dark
4) put shavings from male cage into female's cage 24 hours before breeding
5) Put one female into a male's cage at breeding (one female to one male)

With this protocol, approximately 25% bred/night over three consecutive nights. Conception rates ranged from 50-95% with usually 3-10 pups/mouse.

CRYSTAL VIOLET STAINING

The method of White and Wilson (1951) was used for differentiation of smooth and nonsmooth colonies. A stock solution of crystal violet was prepared by suspending 2 g crystal violet and 0.8 g ammonium oxalate in a solution of 20% ethanol. This was further diluted to a working solution by diluting it to 1:40 in distilled water. Colonies on plates were flooded with working solution for 20 seconds then observed for staining. Stained colonies are considered of rough morphology.

ACRIFLAVINE AGGLUTINATION

This assay was performed according to a modified method of Braun and Bonestell, 1947 to differentiate smooth and non-smooth colonies. One mg acriflavine was suspended in 1 ml distilled water. Approximately 50 ul of this solution was placed on a slide and a small amount of bacterial growth was mixed into it with a loop. The slide was then gently rocked back and forth for 2 minutes and checked for agglutination. Rough organisms will agglutinate.

STANDARD TUBE AGGLUTINATION TEST (STA)

This assay was performed based on the methods of Alton et al. (1975) to determine agglutinating titers. Brucella St. 19 Diagnostic antigen was received from the U.S.D.A. and diluted to the working dilution by adding 1 ml of stock to 99 mls 0.5% phenolized saline for

a working O.D. of 0.25 at 525 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. 1:25 dilutions of antisera were made for each sera sample in phenolized saline and double dilutions carried out to a given end point. The tubes were incubated at 37^{o} for at least 24 hours before reading. Titers were expressed as the Log_{10} value of the reciprocal (example: titer = 1/100, reciprocal = 100, Log_{10} 100 = 2.00).

Preparation of Rabbit Primary Polyclonal Antibody

for ABC Technique

1. Incubate trypticase plates, 1 each, with B. abortus strain 2308 or RB51.
2. Harvest in distilled H_2O, incubate with acetone 3 hours to kill, spin down (discard supernatant), wash pellet 2 times with distilled H_2O.
3. Suspend pellet in sterile saline to 10% T at 525 nm.
4. Mix 3 mls of each with a) 3 mls Freunds complete adjuvant (FCA) and b) 3 mls Freunds incomplete adjuvant (FIA).
5. Inoculate 1 rabbit (#3187) with 1 ml strain 2308 plus FCA subcutaneously in cervical region.
6. Inoculate 1 rabbit (#3189) with 1 ml strain RB51 plus FC subcutaneously in cervical region.
7. 14 days later: boost -
   a. strain 2308 - inoculated rabbit receives 1 ml of strain 2308 and FIA.
   b. strain RB51-inoculated rabbit receives 1 ml of strain RB51 and FIA.

   All rabbits bled from ear vein immediately prior to the first inoculation and 10 days after the second inoculation. Rabbits were killed by exsanguination 22 days after the second inoculation.

Standard Tube Agglutination Test		Log_{10} of recipricol
Rabbit 3187 - (St. 2308 inoculated)	1st bleed < 1/25	<1.39
	2nd bleed + 1/400	2.60
Rabbit 3189 - (St. RB51 inoculated)	1st bleed < 1/25	<1.39
	2nd bleed < 1/25	<1.39

Biodot ELISA

Antigen - RB51 whole cells acetone killed to 30% in TBS

30 ul TBS per well room temperature 30 minutes

Washes - TBST

Sera samples - 1:20 in TBST + 2% BSA (bovine serum albumen)

Conjugate - anti-rabbit 1/400 in TBS + 2% BSA

Rabbit #3187 (St. 2308 inoculated)	Log_{10} of recipricol
1st bleeding < 1/20 (pre-inoculation)	< 1.30
2nd bleeding +1/160 (post-inoculation)	2.20
Rabbit #3189 (St. RB 51 inoculated)	
1st bleeding < 1/20 (pre-inoculated)	< 1.30
2nd bleeding + 1/1280 (post-inoculation)	3.11

Used serum from 2nd bleeding of rabbit #3187 as primary antibody for murine placentas infected with either B. abortus strain 2308 or 19.

Used serum from 2nd bleeding of rabbit #3189 as primary antibody for murine placentas infected with B. abortus strain RB51.

Tissue Preparation Protocol for Transmission Electron Microscopy (TEM)

A. Standard TEM processing

1. Fix tissue in 5% glutaraldehyde and 3% formalin in 0.05 M sodium cacodylate buffer with 2.5% picric acid.
2. Wash 2 x in 0.1 M cacodylate buffer.
3. Post-fix in 1% osmium tetroxide in 0.05 M sodium cacodylate.
4. Wash in buffer 2 x, 10 minutes each.
5. Dehydrate in graded alcohols, 15 minutes each (15%, 30%, 50%, 70%, 95%, 100%).
6. Place in propylene oxide, 15 minutes.
7. Infiltrate in 50:50 propylene oxide resin (POLY/BED 812, Polysciences, Warrington, PA) for 12 hours.
8. Infiltrate in pure resin 12 hours.
9. Change resin, cure at 60^{o}C 48 hours.

B. Cutting and Staining Procedure for Thick Sections

1. 1 micron thick sections were cut on a Reichert-Jung Ultracut E and collected on glass microscope slides.
2. These were stained with 1% Toluidine blue and 1% safranin O.

C. Cutting and Staining Procedure for Thin Sections

1. 60-90 nm (600Å-900Å) thick sections were cut on a Reichert-Jung Ultracut E and collected on copper grids (standard TEM) or nickel grids (immunogold labeling).

2. For standard TEM examination, thin sections on copper grids were stained 12 minutes with 2% uranyl acetate and 5 minutes with lead citrate (lead citrate, sodium borate and water) and sodium hydroxide.

3. For immunogold labelling - Direct labelling. Thin sections on nickel grids were etched in saturated sodium metaperiodate- 30 minutes; blocked in 1% bovine serum albumin - 10 minutes; incubated with bovine polyclonal antibody directly conjugated with 15-20 nn colloidal gold, jet washed. Counter stained with uranyl acetate and lead citrate as for standard TEM examination.

Preparation of colloidal gold, antibody/gold probe and immunogold labelling was performed by Mrs. Judy Stasko, a laboratory technician working with Dr. Norman F. Cheville at the Midwest National Animal Disease Center, P.O. Box 70, Ames, Iowa, 50010.

Procedure for Avidin-Biotin-Complex Immunohistochemical Staining for Light Microscopy

4 μm, paraffin embedded sections on Elmer's glue coated slides were immunostained using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). All steps, unless otherwise noted, were carried out at room temperature. Fresh phosphate buffered saline, pH 7.4 (Sigma Chemical Co., St. Louis, MO) was used for each washing step. To the final buffer rinse each time, before the addition of serum, 1% bovine serum albumin (Sigma) was added to prevent tissue drying.

1. Deparaffinize and hydrate tissue sections through xylenes and graded alcohol series.
2. Rinse 5 minutes in distilled water.
3. Incubate for 30 minutes in 0.3% H_2O_2 in methanol.
4. Wash in buffer for 20 minutes.
5. To unmask antigen sites, incubate in waterbath with 0.1% trypsin in 0.1% calcium chloride, pH 7.8 for 30 minutes at 37°C.
6. Wash in buffer 20 minutes.
7. Incubate sections with normal goat serum at 1:10 dilution for 30 minutes.
8. Blot excess serum from sections.
9. Incubate 90 minutes with appropriate primary mouse antiserum diluted 1:8000 for 90 minutes (see below for preparation of

primary antisera).

10. Wash slides for 10 minutes in buffer.
11. Incubate sections for 30 minutes with diluted biotinylated goat-anti-mouse antibody solution.
12. Wash slides 10 minutes in buffer.
13. Incubate 60 minutes with VECTASTAIN ABC reagent with 3% sodium chloride added to the tris buffer to decrease non-specific labelling.
14. Incubate sections for 1-2 minutes in peroxidase substrate solution.
15. Wash sections for 5 minutes in tap water.
17. Counter stain with Gills' #2 hematoxylin for 30 seconds.
18. Wash for 5 minutes in running tap water.
19. To dehydrate, pass through graded alcohols, clear with xylene.
20. Mount in permount.

Biodot Enzyme-Linked Immunosorbant Assay (ELISA) Procedure

A Biodot ELISA procedure was used to determine serum titers to strain RB51.

1. Wet nitrocellulose with 1 x TBS.
2. Place nitrocellulose in Biodot chamber (BioRad).
3. Thirty ul of antigen added to wells. Antigen was autoclaved or acetone killed whole cell B. abortus strain RB51 suspended to 30% transmittance at 525 nm in TBS in Bausch and Lomb Spectronic 20.
4. Dry under vacuum 1-2 minutes, release vacuum.
5. Add 100 ul of 2% BSA in TBS, let penetrate by gravity 30 minutes.
6. Dry under vacuum, wash 1 x with TBST (TBS + 0.3% Tween 2D), release vacuum.
7. Make serial dilutions of sera to be tested and apply 100 ul of each dilution to wells. Let penetrate by gravity 30 minutes.
8. Dry under vacuum, wash 5 x with TBST, release vacuum.
9. Add 10 ul of appropriate secondary antibody (1/1000 goat anti-mouse) in 2% BSA in TBS. Let penetrate by gravity 30 minutes.
10. Dry under vacuum, wash 5 x in TBST, dry under vacuum. Release vacuum.
11. Develop nitrocellulose sheet in
    1. 60 mg H-cloro-1-naphthol in 10 mls methanol.
    2. Add 0.6 mls H_2O_2 to 100 mls TBS. Mix 1 and 2.

12. Stop reaction by submerging sheet in distilled H_2O.

Titer was determined by visual scoring, therefore, a negative and a positive serum sample was included in each assay. The dilution of the negative serum showing no reaction was selected as the cut of point. Color reactions stronger than the cut off point were scored as positive. Due to the visual assessment, this method of titer determination is somewhat subjective, but is the best test presently available to examine the serologic response to strain RB51. Normal mouse serum used as negative control. The positive control was a mouse hyperimmunized with strain RB51. Titers were expressed and the Log_{10} value of the reciprocal (example: titer = 1/100, reciprocal = 100, Log_{10} 100 = 2.00).

Colony Blot Enzyme-Linked Immunosorbant Asasy (ELISA) Procedure

A colony blot enzyme-linked immunosorbent assay procedure developed by Roop and coworkers (Roop, et al., 1987) was used to identify bacterial isolates from the placentas of pregnant mice.

1. Isolates grown 72 hours on trypticase-soy plates are blotted onto sterile nitrocellulose disks.
2. Submerge disks in 25 mls chloroform for 10 minutes, and allow to dry 10 minutes at room temperature under an activated charcoal hood.
3. Place disks in 25 mls of a blocking-digestion buffer consisting of 0.15M sodium chloride, pH 7.5. The buffer was supplemented with 2 ug/ml DNase I, 80 ug/ml lysozyme, and 3% bovine serum albumin. Agitate disks for 45 minutes.
4. Wash in TBST.
5. Incubate with appropriate monoclonal antibody diluted at 1/100 in TBS for 1 hour monoclonal antibody used was BRU38. BRU38 is a rat immunoglobulin G produced by a hybridoma obtained from the fusion of SP2.0 myeloma cells with B cells derived from spleens of rats immunized with B. abortus strain 19. BRU38 reacts with the o-side-chain of the Brucella LPS complex.
6. Wash in TBST.
7. Incubate with appropriate secondary antibody (horseradish peroxidase conjugated goat IgG fraction anti-rat at 1/500 in TBS) for 1 hour.

8. Wash in TBST.

9. Develop in:

   a. 60 mg 4-cloro-1-naphthol in 10 mls methanol.

   b. Add 0.6 mls H_2O_2 to 100 mls TBS.

   c. Mix a & b.

10. Stop reaction by submerging disks in distilled water.

Purification of Monoclonal Antibodies (BRU 38)

BRU 38 (MoAb against O-side-chain) used in passive transfer protection study and colony blot ELISA.

Ascites fluid or cell culture supernatants may be used for purification. The affinity column is filled with anti-rat IgG bound to sepharose beads. When the sample is filtered through the rat IgG binds to the anti-rat on the beads in the column. An acid buffer (glycine) is used to disassociate the Ab-Ag bond, and the antibodies are collected. Dialyze antibody sample for 48 hours agains PBS (dialysis tubing MW cutoff 6-8,000). Purified monoclonal antibody is filtered and stored (may be necessary to store in 1% BSA).

Materials:
1) Anti rat IgG - A6542, Sigma Chem. Co., St. Louis, MO
2) Anti rat IgG - 76B-0081, CAPPEL from Cooper Biomedical Inc., Scientific Division, West Chester, PA
3) Waste breaker
4) 10 ml glass test tubes and test tube rack
5) pasteur pipettes
6) dialysis tubing (may be necessary to treat tubing)
7) spectrophotometer cuvette
8) Glycine - pH 2.2
9) PBS (phosphate buffered seline)

10) PBS-NaN_3

11) Affinity Column - BIO-RAD Affinity Column
BIO-RAD Econo-Colomn, Chromatography Column
#373, 2202, Richmond, CA

Procedure:

1. Turn spectrophotometer on. Single sample, 6 position holder, wavelength 280 nm.

2. Blank machine to PBS buffer.

3. Drip PBS-NaN_3 from column. Wash with PBS until Abs is below 0.02.

4. Add material to be purified, allow to drip through column. DO NOT LET THE COLUMN RUN DRY.

5. Wash column with PBS until absorbance is less than 0.02. This indicates that all the nonbound-anti-mouse proteins in the sample have been washed away.

6. Reblank the spectrophotometer to the glycine buffer.

7. Elute the sample with the glycine buffer. Begin collecting samples immediately after glycine is added to column. Retain any sample with an absorbance above 0.1. Continue to wash the column until absorbance is below 0.02. Note: the absorbance of the first sample off the column following addition of glycine may be low, collect tree or four more samples before deciding that nothing bound to the column.

8. Wash the column with PBS-NAN_3 until the pH returns to pH 7 (use pH paper). Store column at 4°C.

9. Immediately after collecting the eluted antibodies (antibodies are in samples with absorbance above 0.1) add to dialysis tubing and begin dialyzing. Dialyze at 4°C against PBS.

10. Dialyze for 48 hours, changing PBS after 24 hours.

11. Centrifuge the sample, read absorbance (remember to blank the machine to PBS)

12. To determine the amount of protein per sample: Protein determination Procedure:

BIO-RAD

1414 Harbour Way South

Richmond, CA

13. It may be necessary to store the purified monoclonal antibodies in PBS + 1%BSA.

14. Concentrate 5 Fold with Amicon Minicon concentrator B-15, Amicon Corporation, Lexinton, MA.

Procedure for

Gel electrophoresis

samples to be prepared for Western blot analyses:

1. For lyophilized cell wall preps:

    a) 1 mg lysozyme/1 ml 10 mM Tris pH 8.0

    b) 2.5 mg cell wall/0/95 ml 10 mM Tris pH 8.0

    Add 50 ul of "a)" to all of "b)". Incubate for 2 hours, 37°C.

    After the 2 hour incubation, store in 4°C until ready for use.

or 2. Grow 10 plates of Brucella, 48 hours, 37°C, 5% CO_2.

    Harvest in distilled water, pool plates and divide into 2 corex tubes.

    Spin and discard supernatant.

    Kill with acetone (mix for 3 to 6 hours in acetone to kill cells).

        Suspend pellets in acetone, pool in beaker, and stir for 3 hours at room temperature. Total volume of acetone should be around 50 ml.

    Centrifuge and discard supernatant, leave pellet as dry as possible.

    Suspend pellets in total volume of 8 ml of 10 mM Tris, pH 8.0.

    Pool suspended pellets and add 1 mg lysozyme. Leave mixing at room temperature overnight.

    Centrifuge, discard pellet and save the supernatant.

Aliquot into 250 ul volumes and freeze at -70°C, using minifuge tubes.

Label and use these as stock antigens.

When ready for use:

a) Thaw 1 tube.

b) Add 250 ul 2x Sample buffer.# (for small gel, use 15ul + 15ul, save rest)*

c) Boil 5 minutes.

d) Run 150 ul big gels/lane, and 15 ul small gels/lane.

e) Date the tube, store at 4°C.

*For large gels, add 250 ul 2x Sample buffer to the 250 ul antigen.

For small gels, take 15 ul of antigen stock and add 15 ul of 2x Sample buffer.

Samples with 2x Sample buffer are kept at 4°C for 5 days, then disposed of.

Samples of antigen without the 2x Sample buffer are stored for 10 days, then discarded.

#Recipe for 2x Sample buffer:

0.0625M Tris, pH 6.8

2% SDS

10% glycerol

5% 2-MCE: (Mercaptoethanol)

0.001% bromo-phenol blue

Procedure:

1. Wash glass and white metal plates well with 70% ethanol. Make sure that the plates are dried before putting together.
2. Put plates together, one white metal plate behind one glass plate, with the spacers between, notched edge of spacers to side edges of plates. Clamp the plates to the gel apparatus. Place the apparatus on a large square glass. This will serve as a movable platform once the agarose plug is formed.
3. Melt agarose* needed for plug between the bottom of the plates. With a pasteur pipette carefully add the agarose to the bottom edge of the plates, making sure that the agarose draws up between the plates to form a small line for a plug. Let stand until the agarose hardens. DO NOT move the apparatus on the platform once the agarose plug has hardened - move only the platform, not the apparatus. If the apparatus is moved away from the platform, the agarose plug

will be broken and no seal will be present for pouring the resolving gel.

4. Make the resolving gel solution, adding the TEMED only when ready to pour. If the TEMED is added before ready and allowed to sit, the solution will polymerize quickly in the beaker! Add the TEMED and pour or pipette resolving gel solution between the glass plates-make sure that the agarose plug does not leak. Leave enough room at top of plates for the stacking gel to be poured. (When pouring a stacking gel, there should be stacking gel below the comb in order to form the wells).

   Carefully add distilled water to the top of the resolving gel before it polymerizes. This will help the gel polymerize more evenly and prevents any evaporation. If the distilled water is added too quickly and too aggressively, the power will cause indentions to form in the gel.

   Let the resolving gel stand for approximately 1 hour before adding the stacking gel. This assures polymerization.

5. Make the stacking gel solution, again waiting until ready to pour to add the TEMED. Draw the distilled water off the resolving gel and place the comb between the top of the plates. Add the TEMED to the stacking gel solution and pour or pipette around the comb -- do not allow air bubbles to form around the comb.

   If air bubbles do form, shift the comb around until the air bubbles disappear.

Let stand until the stacking gel polymerizes well. Carefully pull the comb straight up and out from between the plates, leaving unharmed wells.

6. Wash the wells carefully with distilled water (add distilled water then draw out of wells). Repeat 2 more times. Fill the wells and the back of the plates with reservoir running buffer after placing the apparatus in its tray.

7. Prepare the samples, cool after boiling, and load the wells. Usually a minigel gets 15 to 30ul of sample per a well, a large gel 150 to 200ul per well.

   With our stock antigens, we add 15ul/well on the minigel, and 150ul/well large gels.

8. Hook up the apparatus to a cooling bath (circulation). Add reservoir running buffer to the apparatus tray. Place lid on the apparatus, making sure that the reservoir buffer level is well above the samples. If the reservoir buffer level drops below the level of the top of the wells, the samples will not run.

   Plug the apparatus into a power pack and run at 25milliamps/gel minigel until the blue dye level reaches near the agarose plug. Do not run the dye front beyond the bottom of the gel or the samples will run off! The run on an average takes 1½ to 2 hours, but varies at times.

   A large gel is run at 25 mA for 1 hour, then 30 mA for 1 hour, then 35 mA for the remaining time. Large gels usually run 4 to 5 hours, but also varies at times.

* Agarose recipe:

99 ml 1x Reservoir buffer + 1g agarose I

Heat to dissolve, aliquot into glass screw cap tubes while hot, cool, store at 4°C.

Boil to melt when ready for use.

Procedure for Western Blot

Western blot analysis was conducted to determine antibody response (presence or absence of anti-O-side-chain antibodies).

1. Electrophorese gel by previous procedure.
2. After gel electrophoresis, remove the gel from glass plates. Handle gel carefully, it is easy to tear. Cut off stacking gel and agarose plug layer.
3. Nick one corner of gel for face up orientation. (It is usually best to nick the lower corner below the molecular weight lane).
4. Equilibrate the gel in transfer buffer 30 to 60 minutes. Cool the rest of the transfer buffer in 4°C until ready for use.

   Transfer buffer recipe:

25mM Tris	6.06g Tris
192mM glycine	=28.8g glycine
20% methanol	400ml methanol
	up to 2 liters with distilled water

5. Prewet 2 Scotch-Brite pads, 2 pieces Whatman 1 filter paper, and 1 piece of nitrocellulose per gel in transfer buffer. (Cut corner of nitrocellulose to correspond with nick in the gel).
6. Set up BioRad transfer chamber:
   a) Hook up to cooling circulation bath, set to 5°C.

b) Fill chamber with transfer buffer to the bottom of the electrode peg. (Transfer buffer should be precooled to 4°C).

c) Carefully add stir bar - do not drop in, or may crack bottom plate.

7. Assemble the cassette:

a) minigel: place one holder for bottom

large gel: open holder so grey side is at the bottom

b) place one scotch-brite pad

c) place one Whatman 1 filter paper

d) place gel, rub out air bubbles underneath

e) wear gloves and place nitrocellulose over the gel

Make sure that there are no air bubbles between the gel and the nitrocellulose.

Rub hand across the nitrocellulose surface to remove any air bubbles.

Keep wet with transfer buffer.

f) place one piece of Whatmen 1 filter paper

g) place one scotch-brite

8. Add holder for top of minigel set-up, or for large gel close the cassette.

Insert the "sandwich" in chamber so that the nitrocellulose (top side) is facing the anode- (red disk electrode). The transfer takes place from the gel into the nitrocellulose,

therefore it must be set up so that the gel is behind the nitrocellulose, and the nitrocellulose faces the anode. Transfer occurs from the cathode (black) towards the anode (red). Place lid accordingly.

9. Hook apparatus to power unit and begin electroblotting.
   constant voltage: 100V for 3 hours or 125V for 2 hours
   voltage and time is the same for both mini- and large gel transfers.
   Use the BioRad 250/2.5 power source, the model we use for gel electrophoresis does not have the required amp capacity needed for the transfer.

10. When transfer is completed, disconnect the power and disassemble the cassette.

11. If staining molecular weight marker, mark the top of the lanes lightly with pencil using the comb for orientation. Cut off the molecular weight lane in a strip and stain immediately.
   We use unstained molecular weight markers which we stain with Ponceau S stain for 5 minutes, then destain 1 to 2 minutes with distilled water - pencil mark lines before drying (sometimes the stained lines disappear upon drying).
   Ponceau S stain recipe: 0.5g Ponceau S + 1ml glacial acetic acid + 98.5ml d H_2O.

12. If needed, cut the nitrocellulose into strips corresponding to the lanes. Place strips into a blocking solution for 1 hour with agitation at room temperature.

For blocking solution:

a) if using bovine serum for primary antibody, use a solution of 0.25% gelatin in TBS for blocking. Do not use 2% BSA here, or you will get false positive results in the end.

b) if using anything but bovine for primary antibody, use a solution of 2.0% BSA (bovine serum albumin) in TBS for blocking.

13. Dilute primary antibody in TBS (Tris buffered saline). Place the strips in desired antibody and leave agitating overnight at room temperature.

14. Wash the strips 5x in TBST (TBS + Tween 20). Place strip in fresh TBST each time and agitate for 2 minutes.

15. Place strips in secondary antibody of appropriate dilution in TBS --- use an anti-antibody IgG conjugated with horseradish peroxidase. Agitate for 1 hour at room temperature.

16. Wash strips 5x in TBST as in step 14.

17. Develop strips.

Developer recipe:

Make "a)" and "b)" separately, then add together:

a) 60 mg 4-chloro-1-naphthol + 10 ml methanol

b) 100 ml TBS + 0.6 ml H_2O2

Develop until positive control shows purple lines or smears.

18. Stop developing step with a large volume of distilled water. Blots continue to develop in distilled water, so don't develop too long. Dry on paper towel.

19. Photograph blots when wet, i.e. have them shot immediately for best contrast.

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