Brucella abortus strain RB51 infection of bovine trophoblast epithelial cells: characterization of receptor signaling and bacterial outer membrane proteins

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Brucella abortus strain RB51 infection of bovine trophoblast epithelial cells: characterization of receptor signaling and bacterial outer membrane proteins.

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

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CHAPTER 1: GENERAL INTRODUCTION

Introduction

Bovine brucellosis, caused by *Brucella abortus*, is an economically important disease that causes abortion and infertility. Control of bovine brucellosis has relied on vaccinating cattle with *B. abortus* strain 19, and the serologic testing and removal of cattle with brucellosis from vaccinated herds. However, serologic testing of cattle with brucellosis is complicated because strain 19 vaccine like field strain isolates, induces antibodies to the lipopolysaccharide (LPS) O-antigens of *B. abortus* that are detected by agglutination tests used for surveillance.

*Brucella abortus* strain RB51 is a LPS O-antigen-deficient mutant of the virulent strain 2308. Strain RB51 vaccine protects cattle against infection and abortion, but does not induce antibodies to the LPS O-antigens which interfere with the serological diagnosis of brucellosis (40, 102, 155, 156). The USDA Animal Plant and Health Inspection Service has recently accepted strain RB51 as an official calfhood brucellosis vaccine.

Seroconversion in strain RB51-vaccinated cattle can be detected by a dot enzyme-linked immunoabsorbent assay that uses killed (gamma-irradiated) intact RB51 bacteria as an antigen. However, the results are subject to individual interpretation and cannot be automated.

Intravenous inoculation of pregnant cattle with strain RB51 causes placentitis and, occasionally, premature birth. Invasion and replication of *Brucella* spp. in trophoblastic
epithelial cells of bovine placenta is a key event in the pathogenesis of brucellosis. However mechanisms of attachment to and phagocytosis of bacteria by trophoblastic cells and consequences of placentitis on trophoblastic cell populations are still poorly understood.

The purposes of the studies presented in this dissertation were 1) to isolate antigenic outer membrane proteins from B. abortus strain RB51 to be used in a diagnostic test, 2) to identify the receptor and intracellular mediators of uptake of strain RB51 in trophoblastic cells, and 3) to study the effect of placentitis on the binucleate cell population.

**Literature review**

**History of brucellosis**

In 1859, Marston reported an illness in humans on the island of Malta which was characterized by malaise, anorexia, fever, and profound muscular weakness. David Bruce isolated the organism responsible for the disease from spleens of infected patients and reproduced the disease by inoculating monkeys. Bruce named the disease Mediterranean fever and the etiologic agent *Micrococcus melitensis* for the island of Malta (23, 24, 25). A large epidemiological study initiated by the British government in 1904 to study Mediterranean fever on Malta found that 40% of the goat serum collected reacted positively in agglutination reactions using *Micrococcus melitensis* antigen (75). Because the organism was isolated from the milk of goats, it was concluded that humans were infected after ingestion of contaminated milk (153).
Other members of the genus *Brucella* were isolated during the 19th century. In 1862, Nocard reported a bacterium in fetal membranes from an aborted bovine fetus. Later in the end of that century, Bang described large amounts of exudate between the uterine wall and fetal membranes of a cow and an aborted fetus, isolated a bacterium from the exudate, and reproduced abortion by inoculating cows with the organism (10). Bang designated the organism as *Bacillus abortus*. Alice Evans, a bacteriologist, recognized the relationship between *Bacillus abortus* and *Micrococcus melitensis* (61). Meyer and Shaw confirmed her results and suggested to name this new genus of bacteria *Brucella* in honor of David Bruce (108). The bacilli of Bruce and Bang were then reclassified as *Brucella melitensis* and *Brucella abortus* respectively. In 1914, Traum isolated a third member of the genus, *Brucella suis*, from aborted piglets (161). Four additional species of the genus *Brucella* have now been isolated: *Brucella canis* (dogs), *Brucella ovis* (sheep), *Brucella neotomae* (desert rats), and *Brucella spp* (unnamed) (marine mammals) (12, 13, 62).

**The organism**

*Brucella* are gram-negative, non motile, non-spore-forming, coccobacillary rods with a size of 0.5 to 0.7 μm by 0.6 to 1.5 μm. They are strict aerobes, capnophilic, catalase-positive, oxidase-positive (except for *B. ovis* and *B. neotomae*), and urease-positive (except for *B. ovis*). They grow slowly in culture, reaching a maximum colony size only after 5 to 7 days. *B. abortus* and *B. ovis* require 5 to 10% CO₂ for growth (46, 176). *Brucella* are
classified as smooth or rough based, respectively, on total or partial expression of the O-side
chain of their LPS.

Bacterial identification of Brucella species and biovars is based on physiologic, antigenic, and genetic tests: CO₂ requirement, H₂S production, growth in the presence of basic fuchsin and thionin, utilization of amino acids and carbohydrates, gas liquid chromatography of fatty acid esters, phage lysis, agglutination with monospecific antisera A (abortus) and M (melitensis), polymerase chain reaction, and pulse gel electrophoresis (20, 21, 46, 80, 115, 177).

Brucella abortus strains are classified in 8 biovars (115). Brucella abortus strain 2308, a virulent strain and B. abortus strain 19, the vaccine currently used in cattle, are smooth biovar 1 strains, but the latter is characterized by a stable low pathogenicity, high immunogenecity and antigenecity (109, 115, 169). Brucella abortus strain RB51 is a rough biovar 1 strain derived from strain 2308 by serial passage on tryptose agar containing varying concentration of rifampin or penicillin (147).

Antigens of Brucella

The envelope of Brucella spp. consists of a cell membrane surrounding the cytoplasm, a peptidoglycan layer, and an outer membrane. The cell membrane or inner membrane is a lipid bilayer composed of phospholipids, proteins, and lipoproteins. The outer membrane is composed of LPS, phospholipids, glycolipids, and proteins. The inner and outer membranes are separated by the periplasmic space (41, 58, 59, 162).
Polysaccharides

Lipopolysaccharides from smooth strains of *Brucella* are composed of an O-polysaccharide attached to lipid A via a core oligosaccharide. The LPS of *B. abortus* and *B. melitensis* are respectively named antigen A and M (28, 39). The O-polysaccharide of *B. abortus* is a linear polymer of alpha-1,2-linked 4,6-dideoxy-4-formamido-D-mannopyranosyl that contains the major antigenic epitopes of the LPS (34). Because both field strains and the strain19 vaccine have complete expression of the LPS O-side chain, sera from infected or vaccinated animals have antibodies directed against these major epitopes and cross react in the standard tube agglutination test (14, 40, 102 114). Therefore, it is impossible to determine the vaccination status of cattle based on serologic detection. However, because *Brucella abortus* strain RB51 has only reduced expression of the O-side chain of the LPS, sera from inoculated animals do not react in the standard tube agglutination test (155, 156). Therefore, strain RB51 is used in a new commercial vaccine that has largely replaced use of strain 19 in the United States.

Cross reactions in the standard tube agglutination test are also expected with other genera of bacteria that have LPS structures similar to *Brucella*. Because, the O-polysaccharide of *Brucella* is similar to that of *Yersinia enterolitica*, sera from *Yersinia*-infected animals can cross react in *Brucella* agglutination tests. Because some bacteria like *B. abortus* also have N-acyl derivatives of 4-amino-4, 6-a-D-mannopyranose containing LPS, they can also induce false positive reactions with the same test (54, 123, 129).
Polysaccharide B is a low molecular weight carbohydrate isolated from rough strains of *Brucella* (83). It belongs to a family of non-reducing cyclic 1,2-linked polymers of beta-D-glucopyranosyl residues that are not antigenic (29).

**Protein antigens of *Brucella abortus***

The outer membrane proteins (OMPs) from *B. abortus* are separated into group 1 proteins (84 to 94 kDa), group 2 porin proteins (35 to 40 kDa), group 3 proteins (25 to 30 kDa), and lipoproteins (8 kDa) (58, 71, 162, 170). Group 2 and group 3 proteins correspond respectively to the OMPF and OMPA of *Escherichia coli* (162). The OMPs of 25-27, 31-34, and 36-38 kDa are closely associated, perhaps covalently bound, to peptidoglycan (43). The cell wall also contains proteins referred to as *Brucella* protective protein antigens (Bpa) (57). Only a single fraction from these Bpa has been characterized after SDS-PAGE and is composed of 2 OMPs of 36 to 38 kDa and 25 to 27 kDa (57).

Purified group 2 and 3 proteins, when injected intradermally into cattle induce the production of antibodies directed against OMPs and LPS (163). When the Bpa of 36 to 38 kDa and 25 to 27 kDa are injected into mice they induce both humoral and cellular immune responses (57). Therefore they could be candidates for vaccine.

Sera from cattle infected with *B. abortus* strain 2308 were tested to determine humoral responses to these proteins. According to the specificity of a competitive ELISA, the OMPs useful for the detection of infected animals are 10, 16.5, 17, 19, 25 to 27, 31, and 36 to 38 kDa proteins (43). These OMPs were shown to be more accessible to monoclonal antibodies on rough than on smooth bacteria. Therefore each of these could be used as
component for serologic diagnostic tests of cattle vaccinated with a rough *Brucella* such as *B. abortus* strain RB51.

**Epidemiology of brucellosis**

Brucellosis in livestock is a disease that occurs mostly in the Mediterranean, Arabian peninsula, Indian subcontinent, and parts of Mexico, Central, and South America (174). *B. melitensis* is probably the most threatening species today because it has reached epidemic proportions especially in the middle east. In the United States, bovine brucellosis caused by *B. abortus* has been eradicated in most states. Only 13 states remain designated as Class A (no more than 0.25 percent of all herds infected) which is the last step before to being designated Class Free (26).

*Brucella abortus* infects primarily cattle but can also cause disease in sheep, goats, horses, dogs, and wild mammals such as bison, elk, moose, reindeer, foxes, coyotes, hares, and humans beings (113, 114). In cattle, spread of the disease is mostly due to the introduction of contaminated animals into non-infected herds. Clinical disease in cattle occurs only in sexually mature animals and is characterized by abortion, stillbirth, epididimitis, and arthritis. Infection in pregnant cattle is associated with high number of organisms in fetal and placental membranes, and milk (101). Transmission to other animals occurs mainly via oropharyngeal lymphoid tissue from licking of the infected membranes. *Brucella abortus* can also be transmitted by conjunctival, genital, and respiratory mucosa. Pregnant heifers typically abort after infection, but seldom abort in subsequent gestations.
Most calves infected in utero clear the organism, but occasionally after maturity, will shed the organism during their first pregnancy.

Humans are infected by ingestion of contaminated dairy products or by direct mucosal contact, inoculation, or inhalation of the organism (174). Disease is most common in veterinarians, ranchers, abattoir workers, and laboratory personnel. The symptoms are fever, sweating, anorexia, weight loss, fatigue, joint pain, and depression. Cases of human abortions following Brucella infection have also been reported (125).

Diagnosis of brucellosis is based on the isolation of the organism and on detection of antibodies using agglutination tests (113, 154). Because Brucella spp. grow slowly and the rate of isolation is low, many laboratories rely on the standard tube agglutination test (STAT) for diagnosis. The antibody response to Brucella is characterized by an initial increase of IgM during the first weeks post-infection followed by a rise in IgG after 2 weeks (2, 37). The STAT detects both IgM and IgG production. Because IgM is sensitive to 2ME or DTT treatments, the STAT test can be repeated following incubation of the serum with 2ME or DTT to detect IgG and therefore extrapolate levels of IgM which is a better indicator of acute infection (3, 88). Reactions with STAT test should be interpreted along with clinical observations because cross reactions can also occur with Vibrio cholerae, Francisella tularensis, and Yersinia enterolitica infections (115).
Pathogenesis of Brucella abortus infection

Infection with *B. abortus* has been shown to occur via the alimentary tract, conjunctivae, respiratory tract, genital tract, mammary gland, and skin. Experimentally, intestinal infection can involve uptake of bacteria by M cells. After infection of the mucosa, organisms are phagocytized by macrophages and neutrophils and carried to regional lymph nodes (159). Replication in lymph nodes leads to diffuse lymphoid depletion of the outer cortex and hystiocytosis in the inner cortex (40). The organism then spreads hematogenously in macrophages, neutrophils, or free to secondary sites of localization (18). Chronic infection in susceptible animals results in intermittent bacteremia.

In pregnant cows with brucellosis, bacteria can be found in the uterus, placenta, fetus, mammary glands, lymph nodes, spleen, liver, and kidney (47, 101). In males, bacteria are located in the testis, accessory glands, and lymphoid organs (92). The affinity of *Brucella* for the reproductive system has been attributed to the presence of erythritol in these organs (121, 151). This four-carbon sugar is used as an energy source by *B. abortus* in vitro. Erythritol is present in the placenta and seminal vesicles of cattle, sheep, goats, swine, and deer (87). However, some isolates of strain 19 that are inhibited in vitro by erythritol still induce placentitis, and rats, rabbits, and guinea-pigs that do not have erythritol in their placenta, are susceptible to *Brucella*-induced placentitis (17, 87, 152). Therefore, the presence of erythritol in the placenta does not totally explain the affinity of the bacteria for that organ; other mechanisms, such as the presence of a specific receptor on trophoblasts, are probably involved.
Pathogenesis of placentitis

Embryology and anatomy of the placenta

Fertilization of the ovum by sperm generates a diploid cell. This cell sequentially divides into a mass of 16 cells enclosed in the zona pellucida; together they form the morula. These cells will differentiate into the blastocyst. At that stage, a layer of cells (mural trophoectoderm) surrounds a central cavity, the blastocele, and leaves at one pole of the blastocyst a group of undifferentiated cells named the inner cell mass. The inner cell mass gives rise to the endodermal and mesodermal components of the placenta and the embryo, and also produces mitogens for the trophoblasts. The nature of these mitogens is unknown but the addition of a second inner cell mass within the blastocele results in a second zone of proliferative trophoectoderm (68).

The mural trophoectoderm will become the trophoblasts. As the trophoectoderm forms, trophoblasts from sheep and cattle produce trophoblastic interferon (135, 136). Subsequently, they express E-cadherin at their surfaces and redistribute Na\(^+\)-K\(^+\)-adenosine triphosphate at their basolateral surfaces in order to generate a sodium gradient that accumulates fluid in the blastocele (94, 167).

In the lumen of the uterus, the blastocyst will be released from the zona pellucida and will begin its implantation in the uterine wall. Implantation has been prepared by priming of the uterus with estrogen and progesterone (166). Estrogen stimulates uterine epithelium to produce four epidermal growth factors (EGF, transforming growth factor (TGF), heparin-binding EGF, amphiregulin) and leukemia-inhibiting growth factor (LIF) (51, 157).
Epidermal growth factor is not required for implantation; mice deficient for the EGF-receptor or for TGF still have implantation of the blastocyst (99). However, LIF seems to have a central role in the implantation because null mutations of this gene result in non-implantation of the blastocyst in the uterus of mice (157). The trophoblasts also prepare for implantation by the production of interleukin-1β (IL-1β). In mice treated with anti-IL-1 receptor molecules, the blastocyst will not implant into the uterine wall (149).

During implantation, adhesion of the trophoblasts to uterine epithelium is mediated by receptors. Trophoblasts express carbohydrates such as sialylated Lewis X antigen that binds to uterine lectins (22). Uterine epithelial cells express heparan sulfate-binding proteins, that interact with trophoblastic proteoglycans, chondroitin sulfate and hyaluronic acid that participate in adhesion (79, 128). Integrins are also involved in cell to cell adhesion and trophoblasts to extracellular matrix adhesion. The first integrin to be expressed is αvβ3 which binds to vitronectin (96); then, α7β1 integrin that binds to laminin (158).

Following implantation, uterine blood vessel permeability increases, resulting in infiltration of inflammatory cells and production of proinflammatory cytokines by the uterus (165).

Invasion of trophoblasts in the uterine epithelium is mediated by the production of proteinases, such as gelatinase B (a matrix metalloproteinase) and urokinase-type plasminogen activator, and the differential expression of integrin receptors (97, 127). In humans, cytotrophoblasts express different integrins depending on their stage of invasiveness. Villous stem cells express α6β4 integrin and E cadherin (49, 89). As the trophoblastic epithelial cells progress along the trophoblastic columns they decrease the
expression of α6β4 integrins and increase the expression of α5β1 integrins. Distal
trophoblastic epithelial cells still express α5β1 integrins but also increase the expression of
α1β1 integrins. Alpha 5 beta 1 integrin decreases invasion whereas α1β1 integrin increases it
(6, 16, 50, 100, 158, 164). Abnormal expression of integrins at the surface of trophoblasts can
result in pathology of pregnancy. In human preeclamptic pregnancy, decreased invasion of
the cytotrophoblast into the uterine wall and blood vessels, results in reduced blood flow and
growth retardation of the fetus. Immunohistochemistry of the placenta shows that
trophoblasts of the cell columns produce a non invasive pattern of integrins; e.g., they stain
for α6β4 and α5β1 integrins, but not for α1β1 (175).

The bovine placenta is epitheliochorial, villous, cotyledonary, and non-deciduate.
Placentomes (cotyledons) are separated by the intercotyledonary placenta which is composed
of a single avillous layer of trophoblasts. The placentome is formed by the fetal cotyledon
that interdigitates into the endometrial caruncle. The fetal cotyledon is formed from chorionic
villi covered by trophoblasts that invade the endometrial caruncles. The resulting placentome
is a barrier composed of six layers: maternal capillary endothelium, maternal connective
tissue, maternal epithelium, fetal chorionic trophoblasts, fetal connective tissue, and fetal
capillary endothelium. The chorionic trophoblasts are classified into two types: the
columnar cytotrophoblasts and the binucleate cells. The columnar cytotrophoblasts function
for placental attachment, nutrition of the fetus, steroid and protein hormone secretion, and as
progenitors of binucleate cells. Binucleate cells produce the hormones placental lactogen,
estrogen, and progesterone (32, 104, 130, 172). An electron microscopic study has shown
that binucleate cells migrate distally from the chorionic villi to deliver the placental lactogen to the maternal epithelial cells by fusion (171).

**Pathogenesis of Brucella-induced placentitis**

Placentitis is caused by systemic dissemination to the placenta, invasion, and replication of the bacteria in the rough endoplasmic reticulum of mononuclear trophoblasts. Intracellular replication of *Brucella* leads to necrosis of infected trophoblasts and release of bacteria into the fetal circulation.

In 1959, Payne inoculated 11 cows intraconjunctivaly with *B. abortus* to study the pathogenesis of placentitis (120). During 6 weeks post-infection, cows were necropsied every week, and various tissues were cultured for *Brucella* and fixed for histopathologic examination. The uterine lesions were observed as soon as 21 days post-infection and were characterized by periglandular inflammation of the endometrium. This initial lesion was later complicated by accumulation of neutrophils into the uterine glands and release of the exudate into the lumen of the uterus resulting in intercotyledonary infection and ulceration. The cotyledons eventually became infected and intracellular bacteria were present in the chorionic trophoblasts. The trophoblastic epithelium was then ulcerated and bacteria contaminated the chorionic connective tissue and the fetus.

Similar experiments were repeated in pregnant sheep using *B. abortus, B. melitensis*, or *B. ovis*. In these animals, bacteriemia was followed by phagocytosis and replication of bacteria in trophoblasts that caused trophoblast necrosis. The necrotic trophoblasts released
the bacteria into the lumen of the uterus and the fetus was infected by bacterial dissemination into capillaries underneath the ulcerated trophoblastic epithelium.

Pathogenesis of placentitis was also studied in goats given *B. abortus* intravenously into the jugular vein and the uterine arteries (4, 5). Placentomes from small ruminants progressively develop hematomas during gestation. Anderson et al. postulated that *Brucella* entered the placenta in these hematomas. The bacteria are then phagocytized by erythrophagocytic trophoblasts and after replication, *Brucella* invaded the chorioallantoic trophoblasts. Anderson et al. hypothesized that surrounding chorionic trophoblasts could be infected by cell to cell transfer or by lysis of infected trophoblasts and dissemination to the surrounding trophoblasts and the uterine lumen. Necrosis of trophoblasts would result in intravascular invasion of the chorionic villi followed by infection of the fetus.

**Pathogenesis of abortion**

**Immunosuppression and the placenta**

Although both paternal and maternal genes are involved in the formation of the placenta, there is normally no rejection by the pregnant animal. This is due to the unique cell surface of trophoblasts, the production of progesterone, and the high Th2/Th1 lymphocyte ratio that together generate an immunosuppressed environment.

Trophoblastic epithelial cells escape the immune system by three different mechanisms: inhibition of immune system, production of non-classical MHC molecules, and unresponsiveness to immune system stimulation. In vitro experiments demonstrate that
trophoblasts inhibit the immune system function by the production of hormones such as progesterone, human placental lactogen, prolactin, estrogens, pregnancy associated alpha 2 glycoprotein, pregnancy associated plasma protein, and alpha feroprotein, and the release of cytokines like IL-10 that reduce the proliferation of cytotoxic T helper cells (55, 137). Human, rat, and horse trophoblasts produce a specific population of MHC class 1 molecules (90). These nonclassical MHC class 1 b molecules expressed by human invasive cytотrophoblasts are named HLA-G. HLA-G is associated with β 2-microglobulin and binds to CD8 (90, 146). However, unlike HLA-A, B, and C, there is only limited polymorphism in the gene coding for HLA-G. This lack of diversity protects the trophoblast from the cytotoxic effect of natural killer cells (90). Additionally, trophoblasts do not express MHC class 2 molecules and therefore are not recognized by antigen presenting cells (105). Finally, trophoblasts are unresponsive to stimulation by the immune system of the expression of these MHC. Attempts in mice to stimulate the expression of MHC class 2 molecules by INF-γ and attempts in humans to stimulate MHC 1 expression by INF-α and INF-γ have been unsuccessful (105).

Progesterone blocks myometrial contractions, prevents rejection of the fetus, suppresses the production of prostaglandins (PG), maintains uterine growth, and also has anti-inflammatory effects (35, 141). In vitro assays have shown that progesterone inhibits T cell proliferation and in vivo subcutaneous implants of progesterone inhibit granulomatous reactions (42, 150). These effects are due to binding of progesterone to the glucocorticoid receptor on endothelial cells that stimulates the steroid pathway and reduces the production of PGE, a vasodilator, and IL-8, a chemotactic cytokine (85). This results in decreased influx
of neutrophils into the tissue. Progesterone is produced during all stages of gestation by the corpus luteum of the ovary. In ruminants, trophoblasts produce IFN-\(\tau\), a unique antiluteolytic interferon that acts on the uterus to prevent the production of PGF2\(\alpha\) and thereby to maintain ovarian production of progesterone (95). Bovine placenta also produces progesterone but only for a limited period between 150 and 250 days of gestation (107).

Studies in mice suggest that the immune system of the placenta has increased humoral and decreased cellular responses. This is due to increased activity of Th2 lymphocytes that produce cytokines that stimulate the humoral immunity (IL-4, 5, 6, 10, 13) and reduced activity of Th1 lymphocytes that produce cytokines that stimulate the cellular immunity (IL-12, TNF\(\beta\), INF\(\gamma\)) (110). The reduced activity of Th1 cells is further enhanced by the production of IL-10 by the trophoblasts (111). This results in the formation of an environment beneficial for the maintenance of pregnancy because Th1 cytokines such as IL-12, TNF\(\beta\) and INF\(\gamma\) lead to abortion in mice, whereas Th2 cytokines such as GM-CSF and IL-3 increase trophoblastic growth in vitro (8).

**Pathogenicity of bacterial-induced abortion**

Normal parturition is stimulated by the release of hypothalamic corticotropin-releasing factor and arginine vasopressin (AVP) that induce secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary (36). ACTH stimulates cortisol production by the adrenal glands that in turn activates the 17 alpha hydroxylase/17,20-lyase, a rate limiting enzyme that transforms pregnelonone into estrogen and therefore depletes the C21 precursor for the synthesis of progesterone (64). During gestation, progesterone decreases the
production of prostaglandin (PG) by the amnios and the myometrium by: 1) supressing PG synthase, 2) increasing production of prostaglandin dehydrogenase, an enzyme that degrades prostaglandins, and 3) decreasing production of cyclooxygenase 2 (1, 160). Therefore decreased levels of progesterone at parturition results in the transformation of arachidonic acid into PGD2 by the enzyme cyclooxygenase and subsequently the transformation of PGD2 into other PGs by the enzyme PG synthase. PGs are uterotonic and reinforce signals of parturition by stimulating directly and synergistically with AVP the production of ACTH (38).

During bacterial-induced placentitis, release of bacterial products and infiltrate of inflammatory cells stimulate anticipated parturition. Proliferation of gram-negative bacteria in the placenta is accompanied by release of LPS. LPS indirectly induce myometrial contractions by stimulation of platelet activating factor (PAF) production and inhibition of the enzyme prostaglandin dehydrogenase (PGDH) that results in increased half life of PGs (74, 176). LPS also cause vasculitis directly or indirectly by PAF (176). Finally, LPS stimulate production of TNFα and IL-1α that trigger release of PGE and IL-8 that increase the recruitment of neutrophils (27).

Chemotaxis and diapedesis of neutrophils and macrophages into infected placenta is mediated directly by IL-8 and indirectly by IL-1α, IL-1β, IL-6, and TNFα that stimulate endometrial cells to produce PGs (86). Recruitment of neutrophils by PGs is explained by a two mediator theory in which neutrophils are first chemoattracted by IL-8 and then diapedesis occurs due to the vasodilatation induced by PGE (86). Once neutrophils are in the extravascular tissue, their degranulation is stimulated by IL-8. Therefore, there is a
synergistic effect of PGE and IL-8. Similarly, monocytic infiltrate would depend on the synergistic action of both PGE and monocyte chemotactic factor (MCP-1).

Extravasated leukocytes stimulate myometrial contractions directly by PAF or indirectly by IL-1, IL-6, and phospholipases that increase the production of TNFα and PGs (12). They also causes ripening of the cervix, required during normal parturition, by the release of proteases that destroy the cervical and uterine glycosaminoglycan and collagen (84, 93). Leukocytes secrete IL-2 that stimulates NK cell cytotoxicity towards trophoblasts.

The PGs produced by the leukocytes can also decrease uterine resistance to bacterial infection by modifying the lymphocyte subsets and the activity of the natural killer cells present in the placenta. They invert the ratio of the T cell population towards increased production of Th2 cells and decreased production of Th1 cells, therefore abolishing cell mediated immune response of the uterus against intracellular bacteria such as Brucella. In addition, PGs switch immunoglobins production towards IgG-1 and IgE that do not efficiently fix complement resulting in decreased opsonisation of the bacteria (15, 124). They also decrease the natural killer cell activity and therefore reduce the killing of infected cells.

**Intracellular survival and Brucella**

In the infected host, Brucella invade and replicate in a variety of mesodermal and ectodermal cells; they invade macrophages, neutrophils, and trophoblasts. Bang was the first to recognize intracellular bacteria within trophoblastic epithelial cells from infected placenta.
Electron microscopic studies have shown that Brucella are located within cisterna of the rough endoplasmic reticulum of these cells (4).

In vitro, Brucella invade Vero cells, MDBK cells, ROS cells, Pk-15 cells and Jeg-3 cells and replicate within the rough endoplasmic reticulum of Vero cells (52, 126). Replication within the RER of macrophages and neutrophils is still to be determined.

Intracellular survival of the bacteria has better been studied in macrophages and neutrophils. Macrophages, without stimulation by cytokines, kill intracellular bacteria by superoxide dismutase and hydrogen peroxide; in-vitro addition of catalase or SOD inhibited the killing of the bacteria (69). Since the production of reactive oxygen species is important for the killing of intracellular bacteria, Gay et al. studied the localization of NADPH oxidase after phagocytosis of Brucella. Although, it was rarely detected in Brucella containing phagosomes, when the NADPH oxidase was present, it was closely associated with the bacteria (69).

Opsonisation of bacteria by antibody alters the survival in macrophages in vitro. In bovine mammary gland macrophages, antibody opsonisation stimulates the oxidative burst and the bacteria cannot inhibit phagolysosomal fusion anymore (73).

Macrophages activated by cytokines during infection in vivo have increased: 1) resistance to Brucella, 2) spreading, 3) expression of Fc-receptor, 4) phagocytosis of sheep red blood cells mediated by C3 receptors, 5) chemotaxis, and 6) random locomotion (19). Cytokine-induced activation of macrophages can be divided in two phases: 1) recruitment, and 2) activation. IL-1, by induction of colony stimulating factor (CSF), increases the number of neutrophils and myeloperoxidase positive macrophages in the spleen of infected
mice (56). At the site of inflammation, macrophages activated by interferon-γ and interleukin-2 have enhanced anti-bacterial properties. Macrophages treated with these cytokines inhibit the replication of Brucella. Interferon-γ induced anti-Brucella activities are inhibited by the addition of superoxide dismutase, catalase, and by the addition of TGF-β and anti-TNF antibody. This suggest that interferon-γ increases killing of Brucella by the generation of oxygen radicals (82).

Brucella spp. can survive intracellular killing by escaping from the lysosomes. Virulent strains of B. abortus produce cyclic nucleotides adenine and guanosine monophosphate that inhibit the degranulation of neutrophils and therefore provide a way for the bacteria to avoid the myeloperoxidase-hydrogen peroxide halide system and the oxidative burst (91, 133). Brucella abortus strain 2308 is also able to inhibit phagolysosome fusion in macrophages (33). Finally, intracellular localization of B. abortus within the rough endoplasmic reticulum of Vero cells and trophoblasts allows the bacteria to survive and replicate outside phagolysosomes (52). Other structural and non structural components of Brucella spp. also function as virulence factors. For example, smooth strains of Brucella are more resistant to intracellular killing than rough strains (133). SOD, catalase, and heat shock proteins can also enhance the intracellular survival of the bacteria in phagocytes (11, 98).

**Nonopsonic phagocytosis of bacteria by mammalian cells**

Nonopsonic attachment of bacteria to mammalian cells is mediated by normal cell receptors such as integrins, cadherins, epidermal growth factor receptors, and lectins. In vitro
studies have shown that clustering of receptors at the cell surface is required for the attachment of *Brucella abortus* to mammalian cells: infection of Vero cells by *Brucella* was abolished by monodansylcadaverine, amantadine, and methylamine; all three inhibit receptor mediated endocytosis (53).

**Integrins**

Integrins are heterodimeric membrane receptors on the surface of a variety of mammalian cells that specifically bind to bacterial surface proteins. They are composed of one α and one β subunit that bind cells to extracellular matrix proteins such as laminin, fibronectin, and collagen and mediate cell-to-cell adhesion such as adhesion of leukocytes to endothelial cells (45) (Table 1). Twenty-one integrins are identified based on the possible associations between fourteen different α and eight different β chains (76). The binding site of the integrin is specific for each association and shared between a cation-binding region present on both the α and the β subunits. For most integrins, binding to this site is dependent on the presence of negatively charged amino acids (aspartic or glutamic acid) in the ligand protein (143).

A sequence frequently recognized by some integrins is Arg-Gly-Asp (RGD) (Table 1). Although both α and β subunits have intracytoplasmic tails, binding of the integrin to its ligand induces actin polymerization only around the intracytoplasmic tail of the β subunits (30). The actin fibers articulate with the intracytoplasmic domain of the integrin by focal adhesion plaques which are composed of structural proteins such as talin, vinculin, and α actinin, and kinases. Binding of the ligand to the integrin stimulates one of three different
small G proteins, Rho, Rac, or Cdc42, that sequentially induce actin polymerization. The nature of the kinases present in the focal adhesion depend upon which one of these three small G proteins has been activated (115). Rho-induced focal adhesions, are inhibited by the protein kinase C-inhibitor, staurosporin, and the tyrosine kinase-inhibitor, genistein. Because tyrosine kinases pp60 src and pp125fak are both present in focal adhesions, they could be involved in the Rho pathway. Rho induces polymerization of actin stress fibers by triggering actin-myosin binding (31). RhoA activates a Rho-kinase that phosphorylates and inactivates the myosin binding subunit of the light chain phosphatase that normally inhibits phosphorylation of myosin light chain by kinases. Therefore, inhibition of light chain phosphatase by Rho-kinase causes phosphorylation and activation of myosin light chain that polarizes the actin-myosin bundles to make the actin stress fibers. Rac and Cdc42 pathways are less understood (116). They both induce formation of focal adhesions independently of Rho. These focal adhesions also contain a serine-threonine kinase and a tyrosine kinase (Plaxin) that could participate in their formation. Actin polymerization by Rac and Cdc42, respectively, generates the formation of lamellipodia and filopodia on the cell membrane.

β1 integrins. Yersinia pseudotuberculosis and Y. enterolitica produce a protein, invasin, that binds to α 3,4,5,6, and β1 integrins on surfaces of mammalian cells; invasin is encoded by the bacterial chromosome (78). Binding results in phosphorylation of a protein kinase called focal adhesion protein kinase or pp125fak (140). Phosphorylation of this kinase results in polymerization of actin fibers. After initial binding and polymerization of actin fibers, bacteria inhibit phagocytosis by plasmid encoded Yersinia outer membrane proteins (Yops) (67, 72). Yops are divided in YopE a cytotoxin that depolymerizes the actin fibers, YopH a
protein tyrosine kinase, and YopA a protein kinase. The transcription of these genes is activated by temperatures above 37°C and inhibited by millimolar concentrations of calcium. To inhibit phagocytosis, Yops are transferred to the cytoplasm of the cell by a specialized organelle, that moves from the bacteria to the cell membrane, and accessory proteins that translocate the Yops inside the cytoplasm (48). This process is regulated by bacterial LcrQ proteins that act as repressor of the Yops expression. Once inside the cytoplasm, Yops reverse the action of the pp125fak by dephosphorylation of its tyrosine and therefore inhibit further phagocytosis of the bacteria by the cell.

β 2 integrins. β 2 integrins are found mostly on leukocytes. One of the β 2 integrins, the αmac β 2 integrin, is the receptor for complement fragment C3bi, the adhesion molecule ICAM-1, and for opsonised and non-opsonised bacteria. Binding of organisms to this receptor without antibody results in phagocytosis but not in the production of H2O2 (173). For example, Bordetella pertussis binds to αmac receptor without opsonisation by a unique bacterial surface protein, the filamentous hemagglutinin (FHA) (131). FHA has 2 RGD repeats in its sequence, one of which inhibits the adhesion when mutated. E. coli type 1 pili also bind to the same receptor (70). In contrast, Legionella pneumophila require opsonisation by complement components in order to attach to the αmac and CR1 receptors (13).

β 3 integrins. Borrelia burgdorferi binds to the αIIbβ3 integrin (44). This binding is inhibited by the RGD peptide and desintegrin from snake venom that also bind to the αIIbβ3 integrin.
Table 1: Integrin family

<table>
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<tr>
<th>β chain</th>
<th>Names</th>
<th>α chain</th>
<th>Names</th>
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**Cadherin**

Cadherins are calcium dependent receptors involved in cell to cell adhesion, cell differentiation, cell polarization, intracellular junctions, and maintenance of the adult tissue architecture. They are divided into N-, R-, M-, P-, and E-cadherins. N-cadherin is expressed in the nervous system, skeletal and cardiac muscle; R-cadherin is expressed in the retina and the nervous system; M-cadherin in skeletal muscle; P-cadherin in placenta; and E-cadherin, which is expressed in epithelial tissue (skin, digestive tract) and in the liver. On enterocytes, the E-cadherin is mostly expressed at the basolateral surface.

*Listeria monocytogenes* enters epithelial cells by binding of the bacterial internalin to the E-cadherin (106). Cadherins have an intracytoplasmic carboxyterminal domain connected directly to actin fibers by two cytoplasmic proteins, α- and β-catenin. Like *Yersinia*, the actin polymerization is mediated by tyrosine kinases, but unlike *Yersinia*, broad inhibition of protein kinases with staurosporin does not inhibit the uptake of *Listeria* (106).

**Epidermal growth factor receptor**

Binding of growth factors to their cellular receptors induces cytoskeletal rearrangements, ruffling of the cell membrane, and increased intracellular calcium concentration (132). Similarly, binding of *Salmonella* spp. (carrying the INV gene) to an unknown receptor on mammalian cells induces these events by triggering the EGF-receptor (63, 66, 119). Initial binding of bacteria stimulates a growth factor-activated protein kinase that activates phospholipase A2 (PLA2). PLA2 produces arachidonic acid that is transformed
by lipooxygenase into leukotriene D4 (LTD4). LTD4 opens Ca\textsuperscript{2+} channels resulting in increased intracellular Ca\textsuperscript{2+} concentration that stimulates the release of proteins such as gelsolin, villin, or fombrin from actin and causes subcortical-actin depolymerisation with generation of free actin monomers that are used to polymerize cytoskeletal proteins involved in membrane ruffling. However, increased intracellular level of Ca\textsuperscript{2+} alone cannot induce membrane ruffling. Stimulation of EGFr also results in translocation of phospholipase C-\gamma to the plasma membrane accompanied by the release of profilin that induces actin polymerization.

Salmonella can also enter cells that do not have EGF-receptor. In this case the uptake is mediated by the activation of phospholipase C-\gamma that results in the production of inositol triphosphate and releases intracellular Ca\textsuperscript{2+} reserves to triggers the cytoskeletal changes (144).

Lectins

Lectins are glycoproteins that bind specifically to carbohydrate residues. They participate in non-opsonic attachments of bacteria by two different mechanisms. First, lectins present on the bacterial surface bind to cell surface carbohydrates. Secondly, lectins present on the surface of mammalian cells can bind bacteria.

Some *E. coli* have a lectin on type 1 fimbriae that recognize in affinity chromatography oligomannose residues present on Gp150, gp100, gp70/80 surface glycoproteins from neutrophil membranes (112, 138). Gp150 and Gp100 are identical to the
α and β subunits from the C3bi receptor. C3bi would bind to the peptidic unit of the receptor while type I fimbriae would bind the carbohydrate residue (118). After attachment to this receptor, enteropathogenic *E. coli* encodes a genomic protein, intimin, that has homology to the *Yersinia* invasin and mediates the release of intracellular Ca²⁺ (81). The exact mechanism of intracellular release of Ca²⁺ is not completely understood but it is proposed that activation of phospholipase C-γ increases intracellular inositol triphosphate that releases Ca²⁺ from intracellular compartments (9). After this initial step, a second bacterial chromosomal protein, cfn, is released and activates cytoskeletal rearrangements, phosphorylation of proteins, and increased calcium (139). Finally, binding of the lectin to mannose residues on the host cell surface activates the killing of intracellular and extracellular bacteria by macrophages (122).

Type 2-fimbriated *Actinomyces* spp. have fimbrial lectins that bind to β-galactoside residues present on neutrophil plasma membrane (145). Binding to these residues is also followed by phagocytosis and killing of the organism. However, phagocytosis is increased when neutrophils are pretreated with sialidase that unmasks galactose residues on the surface of neutrophils.

Three types of lectins are detected at the surface of mammalian cells: mannose-specific lectins present on macrophages (168), galactose-specific lectins present on Kupffer cells, hepatocytes, and subpopulations of peritoneal macrophages (148), and a fucose specific lectin present on hepatocytes (7). These lectins clear bacteria from the blood such as non-fimbriated *E. coli* and type II group *Streptococci*. 
Dissertation organization

This dissertation is presented in an alternate format. The next three chapters are formatted as journal papers. Following the third paper is a general conclusions chapter.

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CHAPTER 2: IDENTIFICATION OF 35 kDa ANTIGENIC OUTER MEMBRANE PROTEIN FROM BRUCELLA ABORTUS STRAIN RB51 BY LECTIN AFFINITY CHROMATOGRAPHY

A paper to be submitted to Infection and Immunity

Dominique J. Brees, Steven C. Olsen,
Mark G. Stevens, and Norman F. Cheville

Abstract

A bovine brucellosis vaccine containing Brucella abortus strain RB51 was introduced in 1995 because it was shown to be immunogenic and has lipopolysaccharides deficient in the O-side chain, e.g., it does not induce production of lipopolysaccharide antibodies which are detected by standard serodiagnostic tests for brucellosis. However, antibody responses to surface components of strain RB51 have not been characterized, and there is no objective serologic test, such as ELISA, which detects antibodies which are specific for RB51-vaccinated cattle. In this paper, we report the isolation of diagnostic immunoreactive outer membrane proteins (OMPs) from Brucella abortus strain RB51 based on their affinity for binding to lectins. Strain RB51 OMPs were separated on SDS-PAGE and reacted with seven different lectins. Of these lectins, Concanavalin A (Con A) had the highest reactivity with the OMPs and was used in the procedure to isolate immunoreactive OMPs. Strain RB51 OMPs
were separated by isoelectric focusing and those with an isoelectric point of 4.19 to 4.55 were further purified by Con A affinity chromatography. The eluted proteins were analyzed by western blotting using serum from strain RB51-vaccinated cattle. Nine of the isolated proteins reacted with pooled antisera. From these nine, a 35 kDa protein reacted to every individual serum from strain RB51 and 2308 infected cattle. These results indicate that Con A affinity chromatography permitted the isolation of nine antigenic OMPs from RB51. These proteins may be useful candidate antigens in developing a specific test for detecting antibody to RB51 in RB51-vaccinated cattle. For this purpose, a 35 kDa protein was further characterized by amino acid sequencing.

**Introduction**

*Brucella abortus* is a gram negative, intracellular pathogen that causes chronic infections, placentitis, and abortions in cattle. Control of infection has traditionally relied on vaccinating cattle with *B. abortus* strain 19, and the serologic testing and removal of cattle with brucellosis from vaccinated herds. Serologic identification of cattle with brucellosis is complicated since strain 19 vaccine induces antibodies to the lipopolysaccharide (LPS) O-antigens of *B. abortus* that are detected by serodiagnostic tests for field cases of brucellosis.

*Brucella abortus* strain RB51 is a lipopolysaccharide (LPS) O-antigen-deficient mutant of the virulent strain 2308. Strain RB51 vaccine induces immunity to infection and abortion in cattle at a level that is similar to that obtained with strain 19 vaccine, but it induces no antibodies to the LPS O-antigens which interfere with the serological diagnosis of
brucellosis (1, 8, 14, 15). Consequently, RB51 vaccination may facilitate the identification and removal of cattle with brucellosis from vaccinated herds. The USDA Animal Plant and Health Inspection Service has recently designated strain RB51 as an official calfhood brucellosis vaccine.

Seroconversion in strain RB51-vaccinated cattle can be monitored by a dot enzyme-linked immunoabsorbent assay using killed (gamma-irradiated) intact RB51 bacteria as an antigen. This assay measures antibody responses to the surface antigens of RB51 and presumably some of these responses are to RB51 outer membrane proteins (OMPs). Although dot ELISA is a useful screening test, the results are based on subjective interpretation and it cannot be automated. The OMPs from Brucella are termed class 1 (88 to 94 kDa), class 2 (35 to 40 kDa), and class 3 (25 to 30 kDa) based on molecular mass (2, 3, 4, 12, 17). Class 2 and 3 OMPs are the major OMPs of B. abortus and the most antigenic following infection or vaccination of cattle with B. abortus. The OMPs are tightly bound to certain carbohydrates such as the peptido-glycan layer and therefore can be isolated by lectin affinity chromatography because lectins are glycoproteins that bind to specific carbohydrates. The purpose of this study was to identify B. abortus OMPs that are associated with carbohydrates and to test the feasibility of using lectin affinity chromatography to isolate them and test their antigenicity with sera from cattle that have been vaccinated with B. abortus strain RB51 or infected with B. abortus strain 2308.
Materials and Methods

Reagents. A biotinylated lectin kit containing Concanavalin A (Con A), Ricinus Communis agglutinin (RCA), Peanut agglutinin (PA), Soybean agglutinin (SA), Ulex Europaeus agglutinin (UEA-I), Wheat Germ agglutinin (WGA), Dolichos Biflorus agglutinin (DBA), and an avidin labeled peroxidase kit were purchased from Vector Laboratories, Inc. (Burlingame, CA). Immobilized Con A was purchased from EY Laboratories, Inc. (San Mateo, CA). The polypropylene chromatography columns were obtained from PIERCE (Rockford, IL). Monoclonal rabbit anti bovine IgG (H and L chain specific) peroxidase conjugate was purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). 3-(cyclohexylamino)propanesulfonic acid (CAP) buffer was purchased from Calbiochem-Novabiochem international (La Jolla, CA). Sera taken from thirteen cattle at 4 to 10 weeks after vaccination with B. abortus RB51 (10^{11} CFU/ml given subcutaneously) and sera from three cattle infected with B. abortus 2308 (10^{11} CFU given intraconjuctivally) were provided by Dr. S. Olsen at the National Animal Disease Center. Hipure was purchased from Norland (New Brunswick, NJ).

Identification of carbohydrate associated proteins. Strain RB51 OMPs were isolated as previously described (16) and separated by a 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electroblotted on to immobilon membranes in 3-(cyclohexylamino)propanesulfonic acid (CAP) buffer. Membranes were blocked by incubating at 4^\circ C overnight in PBS (0.01 M, pH 7.2) containing 1% Hipure and 0.05% Tween 80. A separate membrane was reacted with 5 ug of each of the seven
biotinylated lectins for 1 hr at room temperature. The membranes were washed twice for 5 minutes in PBS (0.01 M, pH 7.2) containing 1% bovine serum albumin (BSA) and then incubated with avidin-labeled peroxidase for 30 minutes at room temperature. After 2 washings in PBS (0.01 M, pH 7.2) 1% BSA for 5 min at room temperature, the reactive proteins were revealed by incubation in a solution containing 100 ml of PBS (0.01 M, pH 7.2), 10 ml of 60 mg of chloro-L-napthol in 95 % methanol, and 0.6 ml of H2O2.

**Lectin affinity chromatography.** Strain RB51 OMPs were separated based on their isoelectric point using a rotofor apparatus (BioRad, Hercules, CA) following the manufacturer's instructions. Western blots of each isolated OMP fraction were performed using sera from RB51-vaccinated cattle to identify the most antigenic fractions. These fractions had an isoelectric point 3.68 to 5.15 and they were refocused a second time using the rotofor apparatus. After Western blot analysis of these newly isolated fractions, the most antigenic fraction had an isoelectric point which ranged from 4.11 to 4.55. This fraction was concentrated in a speed vacuum and diluted in a Tris buffer (0.05 M, pH 7.6) containing 0.15M NaCl and 0.004M CaCl2. The proteins were added to Con A lectin affinity chromatography columns as described by the manufacturer. Unbound proteins were washed from the column by adding 3 times the gel volume of Tris buffer (0.05 M, pH 7.6). Bound proteins were eluted from the Con A column by passing 0.2 M mannose (2 to 3 times the gel volume) through the column. The eluted proteins were concentrated by membrane filtration by centrifugation in centricon 3 (3,000 MW cutoff) concentrators (Amicon, Beverly, MA).

**Antigenicity of the eluted proteins.** The proteins eluted from the Con A column were separated by SDS-PAGE and blotted on to immobilon membranes as described above. The
membranes were blocked by incubating for 2 hr in a 10% skim milk solution. Membranes were then incubated overnight at 4°C with pooled sera from four strain RB51-vaccinated cattle or individual sera from either strain RB51-vaccinated cattle or strain 2308-infected cattle which had been diluted 1:50 in Tris buffer (0.05 M, pH 7.6). Membranes were washed twice for 20 min in Tris buffer (0.05 M, pH 7.6) and incubated for 3 hr with a rabbit anti-bovine IgG (H and L chain specific) peroxidase conjugate at concentration of 1/200000. Membranes were washed twice in Tris buffer (0.05 M, pH 7.6) for 20 min at room temperature. Peroxidase reactions were revealed as described above. Negative controls consisted of testing sera obtained from cattle either before vaccination with strain RB51 or before infection with strain 2308.

Protein sequencing. Eluted proteins were electrophoresed in a 12% SDS-PAGE gel followed by a transfer in CAP buffer to a Immobilon membrane. One lane was cut off the membrane and reacted with pooled cattle sera to identify reactive proteins. The rest of the membrane was coomassie stained and 35 kDa was cut and sequenced at Iowa State University protein facility.

Electron microscopy of lectin stained bacteria. B. abortus strain RB51 was grown for 48 hr at 37°C on tryptose agar. Bacteria were removed from the agar by aspiration using PBS buffer (0.01M, pH 7.2) and washed 3 times in PBS (0.01M, pH 7.2) by centrifugation at 2500 x g for 15 min. The bacteria were diluted to a final concentration of 10^{11} cells/ml by spectrophotometric analysis at 600 nm. A one ml aliquot was placed in a microcentrifuge tube and centrifuged at 2500 x g for 15 min. The supernatant was removed and replaced with 1 ml of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After overnight fixation
at 4°C, the tubes were centrifuged at 2500 x g for 15 minutes and the fixative replaced with
0.1 M sodium cacodylate buffer at 4°C. The bacteria were later fixed in osmium tetroxide,
infiltrated and embedded in an epoxy resin, and sectioned at 70 to 90 nm. Sections were
incubated for 15 min with PBS buffer, incubated 1 hr at room temperature with 20 ug/ml of
Con A, jet-washed, incubated for 30 min at room temperature with a 1:30 dilution of gold
labeled horseradish peroxidase (15 nm) (EY, Inc.), jet-washed again, and examined by
electron microscopy. Negative controls were performed by inhibiting lectin affinity with 0.1
M of mannose.

Results and Discussion

Con A, PA, SA, UEA-1, WGA, and DBA, at a concentration of 5 ug/ml, reacted with
multiple B. abortus strain RB51 OMPs (Fig. 1). OMPs were placed into three groups based
on their affinity for lectins: groups include a 35 and 50 kDa OMPs which bound to Con A
and WGA, possibly by attachment to D-mannose and N-acetyl-glucosamine carboxy
residues, a group of 10 to 18 kDa OMPs that reacted with PA, SA, DBA, and UEA, possibly
by attachment to galactose, galactosamine, or fucose carboxy terminal moieties; and a group
of 18 kDa and 80 kDa OMPs that apparently possess all carbohydrate moieties described
above since these two OMPs bound to all lectins except RCA.

Con A had the highest binding activity with OMPs and was chosen for isolating
OMPs by lectin affinity chromatography. Efficient elution of the OMPs from Con A affinity
chromatography column occurred only when the OMPs were diluted in PBS prior to their
addition to column. This indicated that OMPs at their isoelectric point do not bind to Con A which may have resulted from a low affinity of non charged OMPs to interact with lectins or a change in the spatial conformation that decreased exposure of the OMPs-carbohydrate to binding by the lectin. Western blot analysis revealed that seven proteins which were eluted from the Con A column reacted with pooled sera from strain RB51 vaccinated cattle (Fig. 2). Among the seven proteins, a 35 kDa protein was particularly reactive and sera from all strain RB51-vaccinated and all strain 2308-infected cattle reacted with this protein (Fig. 3).

Analysis of the first 20 n-terminal amino acids of the 35 kDa protein revealed a sequence of A-E-V-D-V-S-V-F-P-D-A-Y-D-N-E-G-I-G-V-L. Search of the protein data bank indicated no homology with previously characterized proteins from all species of Brucella. However, the sequence did have a 65% homology with the sequence of chicken antitrypsin. Antitrypsin could either bind trypsin at its reaction site and inhibit its function or bind outside the reaction site and use it to invade extracellular matrix. Korhonen et al. have shown that bacteria have a plasminogen receptor that binds plasmin and use it to degrade the extracellular matrix and basement membrane; this results in increased bacterial migration into tissues (5). Binding to trypsin could be a virulence factor used by Brucella to reach placenta. Once in placenta, B. abortus induces necrosis and ulceration of the trophoblastic epithelium and invades fetal blood vessels. Therefore B. abortus could use antitrypsin or a trypsin receptor to cross connective tissue and vascular basal membranes to infect the fetus.

To identify the cellular localization of mannose residues in B. abortus strain RB51 we used electron microscopy histochemistry with gold labeled Con A (Fig. 4). Binding of gold-labeled Con A was prominent on the cell wall and in the cytoplasm of strain RB51. The
presence of mannose inside the cytosol of bacteria results from uptake of mannose by bacteria. Mannose is used as a catabolite to generate energy or as a metabolite in the biosynthesis of more complex carbohydrates or carbohydrate associated molecules. These metabolites function as intracellular substrates, enzymes, or architectural components, or are translocated to the cell wall of bacteria. For instance, Halobacteriae glycoproteins are assembled by translocation of glycosylated lipids from the cytoplasm to the bacterial membrane, where the carbohydrate moieties of glycolipids are exchanged with membrane proteins.

Separation of strain RB51 OMPs by SDS-PAGE followed by Western blot with lectins, showed several proteins that reacted with one or more lectins. This indicates the presence of carbohydrates which are strongly associated with the OMPs. These carbohydrates can be covalently or non-covalently associated with the proteins. Glycoproteins are proteins covalently linked to carbohydrates by O-or N-glycosyl bounds. Non-covalent bonds can be made through weak ionic, hydrogen, or Van-der-Walls bonds between proteins and carbohydrates, glycolipids or lipopolysaccharides. Because noncovalently bound carbohydrates can migrate with proteins in SDS-PAGE gels, lectin staining after SDS-PAGE is not indicative of glycoproteins per se (6, 7). Currently, bacterial glycoproteins have only been described in archaeabacteria (Halobacterium salinarium and halobium) where biochemical analysis of these proteins has shown covalently linked carbohydrates (6, 7, 9, 10, 11). Some reports have hypothesized the presence of glycoproteins in eubacteria. However, none of these reports studied biochemically the nature of the bond between proteins and carbohydrates.
The presence of carbohydrate linked to protein in SDS-PAGE gels of Brucella OMPs does not prove the existence of actual glycoproteins in the bacterial wall. Brucella peptidoglycan has the same structure as E. coli peptidoglycan and they are both composed of N-acetyl muramic acid and N-acetylglucosamine. Because lectins used in this experiment recognized a wider range of carbohydrates than these two, there must be other associations than peptidoglycan-OMPs to explain lectin reactivity or cross reactivity. Nevertheless, the link between OMPs and carbohydrates, allowed the isolation of OMPs using lectin affinity chromatography.

Western blots with pooled sera from animals vaccinated with RB51 revealed 7 antigenic proteins. The isolated proteins can be placed in 3 groups according to their molecular weight: high molecular weight (60 to 85 kDa), medium molecular weight (30 to 35 kDa), and low molecular weight (< 10 kDa). This last protein could correspond to a 8 kDa lipoprotein previously described as being covalently linked with the peptidoglycans. The high molecular weight proteins are too small to belong to group 1 OMPs (1). The medium molecular weight proteins could belong to either group 2 or group 3 OMPs.

35 kDa protein reacted to all sera from RB51 or 2308 challenged animals. Therefore, after we isolate efficiently a large quantity of 35 kDa protein, it could be used as a serologic diagnostic test to detect Brucella positive animals. We propose that serologic detection of Brucella positive animal sera would be done in 2 steps. First the sera would be tested in standard tube agglutination test. A positive reaction would mean that the sera are either from 2308 and strain 19 infected animals (true positive) or from Yersinia, Vibrio cholerae, Salmonella and E. coli infected animals (false positive). In the second step, sera would be
tested in ELISA using the 35 kDa protein. Positive reactions would be interpreted as RB51, 2308, and probably strain 19 infected animals whereas negative reactions would probably indicate non Brucella infection. However, because we did not test sera from strain 19, Yersinia, Vibrio cholerae, Salmonella and E. coli infected animals, we cannot predict the result to this test.

Isolation of bacterial proteins using ConA lectin affinity chromatography identified a 35 kDa protein that reacted to sera from strain RB51 and strain 2308 infected animals. Sequencing of the n-terminal of the protein showed 65% homology with chicken antitrypsin. Further isolation of the gene coding for that protein and its expression are underway in order to produce sufficient amounts of the protein for additional testing as a diagnostic antigen.

References


Fig. 1. Lectin blot, using Concanavalin A (Con A), Peanut agglutinin (PA), Soybean agglutinin (SA), Ulex Europaeus agglutinin (UEA-I), Wheat Germ agglutinin (WGA), and Dolichos Biflorus agglutinin (DBA), of outer membrane proteins from *Brucella abortus* strain RB51.

Fig. 2. Western blot of proteins isolated by Con A lectin affinity chromatography (Con A) and total OMPs reacted with pooled sera from RB51 infected cattle.
Fig. 3. Western blot of 35kDa protein (arrow) with sera from 13 strain RB51 and 3 strain 2308 infected cattle.
<table>
<thead>
<tr>
<th>B. abortus</th>
<th>B. abortus</th>
<th>B. abortus</th>
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<td>RB51</td>
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</tr>
<tr>
<td>1 2 3 4 5 6 7</td>
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<td>1 2 3</td>
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Fig. 4. Electron micrograph. Note the presence of mannose residues on the surface and in the cytoplasm of *Brucella abortus* strain RB51. Gold labeled horseradish peroxidase (15 nm). Bar = 690 nm.
Abstract

*Brucella abortus* invades and replicates within trophoblastic epithelial cells of bovine placenta, the most critical event in the pathogenesis of brucellosis. The purpose of this study was to identify the receptor that is involved in adhesion and the signal pathway that mediates cellular uptake of *B. abortus* strain RB51 to bovine trophoblastic epithelial cells in vitro; a bioassay and double immunofluorescence labeling were used to differentiate intracellular and extracellular bacteria. In bioassays, adhesion of *B. abortus* to trophoblastic cells was inhibited by fibronectin and RGDS peptide but not by collagen or laminin. The alpha 5/beta 1 integrin is a high affinity receptor for fibronectin. Antibody to alpha 5 or beta 1 integrin subunits inhibited adhesion of strain RB51 by 42% or 63% respectively. When combined, both antibodies inhibited 98% of the adhesion. This result was confirmed by double fluorescence. Uptake of bacteria was inhibited by genistein, an inhibitor of tyrosine kinases, but not by exoenzyme C3, an inhibitor of Rho small G protein. Ultrastructurally, intracellular bacteria were located in the rough endoplasmic reticulum or in phagolysosomes of trophoblasts.
These results suggest that *B. abortus* strain RB51 binds to the trophoblastic epithelial cell surface by an alpha 5 beta 1 integrin, that uptake is mediated by tyrosine kinases, and confirm reports that bacteria replicate intracellularly within the rough endoplasmic reticulum.

**Introduction**

Members of the genus *Brucella* cause acute febrile disease and chronic persistent infection in mammals. During asymptomatic phases of infection, *Brucella* are sequestered in lymphoid tissue, but are stimulated by hormones of pregnancy to replicate and infect the placenta. *Brucella abortus*, which causes bovine and human brucellosis, replicates within the rough endoplasmic reticulum of the trophoblastic epithelial cell to cause placentitis and abortion with dissemination of *Brucellae* in placental fluids, the critical event in the transmission of bovine brucellosis (1, 2, 21). The presence of a specific receptor for adherence of *Brucella* spp. on trophoblastic epithelial cells has not been established but could explain the unique tropism of these bacteria for the placenta.

Receptors at the cell surface are required for the attachment of *B. abortus* in vitro; infection of Vero cells by *Brucella* was abolished by monodansylcadaverine, amantadine, and methylamine; compounds that inhibit receptor mediated endocytosis (5). Adhesion of virulent, smooth *B. abortus* strain 2308 to bovine macrophages has been shown to be inhibited in some animals by fibronectin and RGD peptides suggesting that integrins might participate to the attachment process (4). *Brucella* spp. attach to bovine trophoblastic cells in vitro that normally express a variety of integrins on their plasma membrane (27, 29, 36).
Integrins are heterodimeric cellular receptors composed of one of 14 different α and one of 8 different β subunits that mediate cell-to-cell and cell-to-extracellular matrix adhesion by binding of their extracellular domains to extracellular matrix proteins such as fibronectin, collagen, and laminin (12, 33). Because different associations of α and β subunits bind these proteins with different affinity, inhibition of bacterial binding to the integrin using these proteins could indicate to which specific integrin *B. abortus* is binding.

The initial binding of integrin to extracellular matrix is followed by an intracellular cascade of secondary messengers that causes the formation of focal adhesions and actin polymerization, respectively, by protein kinases and three small G proteins; Cdc 42, Rac, and Rho that consecutively and respectively induce the formation of filipodia, lamellipodia, and actin stress fibers (20). Two types of protein kinases are phosphorylated during focal adhesion formation: protein kinase C and tyrosine kinase. Inhibition of protein kinase C by staurosporin and actin filament polymerization by cytochalasine D inhibit uptake of *B. abortus* strain RB51 by trophoblastic cells (32). The effect of tyrosine kinase or small G protein inhibition on bacterial uptake has not been studied.

Because fibronectin, a ligand for several integrins, inhibited binding of *B. abortus* to macrophages (4) and because invasion of *Brucella* into trophoblastic epithelial cells causes actin fiber polymerization (32), we hypothesized that binding of *B. abortus* strain RB51 to bovine trophoblastic cells is mediated by a fibronectin-binding integrin that could then activate tyrosine kinases and Rho to phagocytize bacteria. The goals of this study were to 1) identify the receptor that mediates the adhesion of the *B. abortus* on trophoblastic cells. 2) demonstrate that Rho GTPase and tyrosine kinases induce phagocytosis of *Brucella*, 3)
identify specific organelles associated with adhesion and uptake of *Brucella* by transmission electron microscopy, and 4) quantitate the rate of intracellular replication of *B. abortus* strain RB51 within a bovine trophoblastic cell line during a 24 hour infection.

**Materials and Methods**

**Materials.** Insulin, transferrin, selenium, epidermal growth factor (EGF), gentamycin, deoxycolate, FITC-labeled mouse anti-rabbit antibody, FITC-labeled goat anti-mouse antibody, mouse anti-β1 integrin subunit antibody, collagen, fibronectin, laminin, and RGDS peptide were purchased from Sigma Chemical Co. (St Louis, MO). MEM and F12 medium, L-glutamine, and antibiotic-antimycotic (ABAM) were obtained from Gibco Laboratories (Grand Island, NY). Exoenzyme C3 and genistein were purchased from Calbiochem (La Jolla, CA). The 4 well chamber slides were obtained from Nunc Inc (Naperville, IL) and 24 well plates were from Becton Dickinson and Company (Lincoln Park, NJ). Mouse anti-α5 subunit antibody was purchased from Dako Corporation (Carpintaria, CA). The 0.45 um culture plate insert Millicell-HA were obtained from Millipore (Bedford, MA)

**Bacterial cultures.** *Brucella abortus* strain RB51 was grown on tryptose agar culture plates with 5 % bovine serum for 2 days at 37°C in 7.5 % CO₂ atmosphere; 5 ml of PBS (0.01 M, pH 7.2) was added to each plate to resuspend the organisms. Bacteria were washed 3 times in PBS and centrifuged at 2500 x g for 15 minutes at room temperature. Pellets were resuspended in 5 ml of PBS and bacterial concentrations were determined by
spectrophotometry at 600 nm. Final concentrations were adjusted with PBS to $10^{11}$ bacteria per ml.

**Cell cultures.** A primary bovine trophoblastic cell line from 5 month old bovine placenta was grown on 50% MEM-50% F12 media (19). This medium was supplemented with 1% heat inactivated fetal bovine serum, 5 ug/ml insulin, 5 ug/ml transferrin, 5 ng/ml selenium, 10 ng/ml EGF, 200 mM L-glutamine, 100 U penicillin, 100 ug/ml streptomycin, and 0.25 ug/ml amphotericin B. $10^5$ Cells were inoculated and grown to confluence in 4 well chamber slides or in 24 well plates.

**Infection and intracellular replication of Brucella in trophoblastic cells.** Invasion assays were conducted as follows (7): medium without ABAM was added to 24 well plates overnight before inoculation. One hour prior to addition of bacteria, medium was removed and replaced by 1 ml of fresh medium without antibiotic. Ten ul of bacterial suspension ($10^9$ bacteria/ml final concentration) were added to each well and incubated for 4, 8, 16, and 24 hours. Except for the 4 hour wells, medium from all wells was removed 8 hours post-inoculation. The cell cultures were washed twice in $37^\circ$C PBS and two ml of medium containing 50 ug/ml of gentamycin was added for the rest of the incubations or for 2 hours in the 4 and 8 hour cultures. This step killed extracellular bacteria. To determine numbers of intracellular bacteria, gentamycin-containing medium was removed and monolayers were washed with $37^\circ$C PBS. One ml of 0.1% deoxycholate diluted in distilled water was added for 15 minutes to lyse cells and release intracellular bacteria. Dilutions of lysates were made in PBS and plated on tryptose agar plates containing fetal bovine serum and incubated 2 days
at 37°C with 5 % CO₂. The number of intracellular bacteria was counted and analyzed by Students’ T-test. This experiment was repeated 4 times.

**Double immunofluorescence.** Trophoblastic cells infected by *Brucella* strain RB51 were examined by a modification of Detilleux’s technique of double immunofluorescence to differentiate intra and extracellular bacteria (7). Trophoblastic cells were inoculated to 4 well chamber slides. Medium without antibiotic was added overnight and replaced 1 hour before inoculation of bacteria with 1 ml of medium without antibiotic. Ten ul of bacteria were incubated for 4, 8, 16, and 24 hours with the cells. Except for the 4 hour wells, medium from all wells was removed at 8 hours post-incubations. Cell cultures were washed twice in 37°C PBS and two ml of medium containing 50 ug/ml of gentamycin were added for the remaining incubations or for 2 hours in the 4 and 8 hour cultures. This step killed the extracellular bacteria. One hour before removal of antibiotic, polyclonal rabbit anti-RB51 antibodies were added to a final concentration of 1:1000. Because the cells were not fixed when the primary antibody was added, only extracellular bacteria were labeled. The cells were washed in cold PBS 5 times and fixed for 10 minutes in 4°C acetone. Slides were air dried and rehydrated in PBS for 5 minutes. Secondary FITC labeled mouse anti-rabbit antibody was added for 1 hour at 1:500 concentration to label extracellular *Brucella*. The slides were washed twice in cold PBS and incubated for 5 minutes with propidium iodine at room temperature. Propidium iodine labeled DNA from intracellular bacteria and trophoblastic cell nuclei. Unreacted propidium iodine was removed by washing slides with H₂O for 5 minutes. Slides were coverslipped with fluorescence vectastain medium and examined with a Zeiss fluorescent microscope. The percentage of infection and the amount of infection were determined by a
semi-quantitative technique. Ten random sites from each slide were examined following a diagonal at a magnification of 40X. In each site, the amount of infection was graded: non infected as A, less than 5 intracellular bacteria as B, more than 5 intracellular bacteria located under the plasma membrane as C, bacteria diffusely located in the cytoplasm as D, bacteria that effaced the normal cellular architecture as E. The experiment was repeated three times.

**Immunofluorescence detection of integrins.** Immunofluorescent labeling was used to show cross reactivity of mouse anti-human integrin antibodies with bovine trophoblasts. Cells, grown in chamber slides, were fixed in cold acetone for 10 minutes followed by 5 minutes rehydration in PBS. They were blocked for 15 minutes with goat serum at room temperature. A 1:10 dilution of mouse anti-α5 or 1:50 dilution of mouse anti-β1 were added for 1 hour at 37°C. Slides were washed twice in PBS for 5 minutes and 1:1000 of FITC-labeled goat anti-mouse antibody was added for 1 hour at room temperature. The slides were washed twice in PBS for 5 minutes and were incubated with propidium iodine for 5 minutes at room temperature. Unreacted propidium iodine was washed in H₂O for 5 minutes and slides were mounted with fluorescence vectastain medium and examined for fluorescence.

**Inhibition.** In invasion assays, cells were incubated with 1 ml of medium containing 100 ug of collagen, fibronectin, laminin, and RGDS peptide or 1 mg of RGDS peptide one hour prior to the addition of the bacteria to inhibit bacterial adhesion. Ten ul of bacteria were added for 4 hours and the number of intracellular bacteria was determined as described above. Because adhesion of strain RB51 was inhibited by fibronectin and RGDS, but not collagen or laminin, we decided to test the hypothesis that α5β1 integrin was the receptor for *Brucella* and inhibited uptake of the bacteria with monoclonal antibodies directed against α5
or and β1 subunits of the integrin in the bioassay. Cells were incubated for 1 hour with 1:10 dilution of anti-α5 integrin and 1:50 dilution of anti-β1 integrin followed by the bacteria. Antibody inhibition was also confirmed by double fluorescence in chamber slides and graded as above.

**Electron microscopy.** 10⁵ trophoblastic cells were inoculated onto 0.45 um Millicell-HA and grown for 1 week before inoculation with Brucella. Media without antibiotic was added overnight prior to inoculation and replaced with 1 ml of antibiotic free media 1 hour prior to the addition of 10⁹ final concentration of bacteria. Cells were incubated with the bacteria for 4, 8, and 24 hours. Except for 8 hours incubations, the medium from all cell cultures was removed and replaced with 2 ml of medium containing 50 ug/ml of gentamycin for 40 minutes in 4 or 8 hour incubation samples or for the rest of the incubation in 24 hours samples. The cells were fixed in 2.5% glutaraldehyde for 1 hour and stored in cacodylate buffer until processing.

**Protein kinase and RHO inhibition.** Rho GTPase and tyrosine kinases were inhibited by exoenzyme C₃ from Clostridium botulinum and genistein, respectively. Cells were grown in 24 well plates and medium was replaced by antibiotic-free medium 12 hours before inoculation (22). Before addition of exoenzyme C₃, the cell culture medium was replaced with fetal bovine serum free medium to increase uptake of the exoenzyme by the cells. Cells were incubated for 1 hour with either 10 ug/ml of exoenzyme C₃ or 250 μM of genistein prior to the addition of the bacteria. Bacteria were added for 4 hours at 10⁹ bacteria final concentration. The number of intracellular bacteria was determined in bioassay as described above.
Results

Inhibition. Adhesion of *B. abortus* strain RB51 to trophoblastic epithelial cells was inhibited by fibronectin and RGDS peptide but not by collagen or laminin at concentration of 0.1 mg/ml (Table 1). When the concentration of RGDS peptide was increased to 1 mg/ml, inhibition of adhesion was raised to 98%. By immunofluorescent labeling, both anti-human α5 and -β1 antibodies cross reacted with bovine trophoblasts (Fig. 1a-b). When added individually to the bioassay, both antibodies inhibited adhesion of *Brucella* to trophoblasts (Table 1). When added in association, the inhibition was almost complete (Table 1).

To confirm these results, we repeated the experiment using double immunofluorescent technique (Fig. 2 a-b). When both anti-α5 and -β1 antibodies were used to inhibit *Brucella* uptake, the cells were not infected or infected with a number of bacteria smaller than 5 whereas the control cells were infected with high number of bacteria diffusely located under the cytoplasmic membrane (Fig. 3). Therefore, the decreased number of bacteria indicated by the bioassay was due to decreased number of uptake and not increased intracellular killing.

Protein kinase and Rho inhibition. Inhibition of Rho by exoenzyme C3 did not decrease the number of intracellular bacteria. However, inhibition of tyrosine kinase with genistein almost totally decreased the number of intracellular bacteria (Table 2).

Electron microscopy. Binding sites of bacteria to trophoblasts were characterized by filopodia-like processes from the plasma membrane that partially or entirely surrounded the bacteria, and by 8 to 10 nm electron dense thickening at the cytoplasmic surface of the
plasma membrane that diffusely or segmentally surrounded the forming phagosomes (Fig. 4a-b-c). Phagosomes were associated with coated pits and vesicles characterized by thick electron-dense membranes (Fig. 4a-c). After 4 hours of incubation, most intracellular bacteria were in phagosomes but occasional bacteria were present in cisternae of rough endoplasmic reticulum (Fig. 4d). After 24 hours incubation, the number of intracellular bacteria had increased. Approximately 95% of the bacteria were located in phagosomes, and ultrastructurally the bacteria were intact or in various stages of degeneration. Only 5% of bacteria were within the cisternae of the rough endoplasmic reticulum and all bacterial cells were intact. Myelin figures, granular debris, and other evidence of bacterial degradation were present in Brucella-containing phagosomes (Fig. 4e).

**Intracellular replication.** In bioassays, numbers of bacteria increased from 4 to 8 hours. After addition of antibiotic, the number of live intracellular bacteria did not increase significantly at 16 hours and decreased to a low at 24 hours (Fig. 5). Double immunofluorescence allowed to differentiate green extracellular bacteria and red intracellular bacteria (Fig. 2a). At 4 hours most intracellular bacteria were located diffusely at the periphery of the cytoplasm (Fig. 7). At 8 hours, most cells had large numbers of bacteria located diffusely in the cytoplasm. Occasionally the cellular architecture was effaced by large number of bacteria that partially covered and effaced nuclei. At 16 hours, numbers of cells with bacteria located diffusely in the cytoplasm or effacing part of the nuclei were 48 and 45% respectively (Fig. 7). At 24 hours, most trophoblastic cells lost their architecture and were filled with bacteria that effaced partially or completely the nuclei (Fig. 6a-b). Therefore.
there was a constant increase number of intracellular bacteria from 4 to 24 hours but very few of them were viable.

Discussion

This study suggests that an α5β1 integrin, also referred to as a fibronectin receptor, mediates the attachment of *B. abortus* strain RB51 to bovine trophoblastic epithelial cells by binding to the amino acid sequence RGD that is present in bacterial outer membrane proteins. Binding of *Brucella* strain RB51 to this receptor is competitive; i.e., increasing concentrations of RGDS increases the inhibition of bacterial adhesion. The binding site of integrins is shared between cation-binding regions present on both the α and the β subunits that recognized negatively charged amino acids (aspartic or glutamic acid) in the ligand protein (33). Similarly, *Brucella* required both the α and β subunits of the integrin to adhere completely because antibodies used individually only partially inhibited binding whereas when both the anti-α5 and -β1 antibodies were present, the inhibition was total.

Strain RB51 must express a protein that is a ligand for the α5β1 integrin. Since strain RB51 is a laboratory derived mutant of strain 2308, RB51 might also express the outer-membrane protein 2b of *B. abortus* strain 2308 that contains the amino acid sequence RGD and is therefore a potential ligand for the receptor (9). Further studies are needed in order to characterize other RGD-containing outer membrane proteins present on the bacterial surface. *Yersinia* also bind to α3, 4, 5 and v β1 integrins via invasin, a unique RGD containing protein (13).
In this study, binding of *B. abortus* strain RB51 to trophoblasts may only be mediated by the α5β1 integrin because association of anti-α5 and β1 antibodies strongly inhibited the adhesion of the bacteria. Additional integrin receptors, such as α3 and α4β1, also bind to fibronectin. However, α3β1 also binds to collagen and laminin, both of which did not inhibit bacterial adhesion, and α4β1 is expressed predominantly on leukocytes but has not been reported on trophoblasts (27).

β2 integrin could also mediate the attachment of *B. abortus* to macrophages and neutrophils. Adhesion of *B. abortus* to macrophages and neutrophils could also be mediated by α5β1 integrin since this receptor is present at the surface of these cells (10, 15, 24, 35). However, macrophages and neutrophils also express the C3 receptor, an αmacβ2 integrin (CD11b/CD18), that mediates non-opsonic attachment of bacteria by an RGD amino acid sequence; *Bordetella pertussis* filamentous hemagglutinin has an RGD sequence that binds to αmacβ2 integrin (25). Therefore, *Brucella* outer-membrane 2 b protein could also bind to this receptor.

Attachment of *Brucella* to α5β1 integrin stimulates filopodia-formation in the plasma membrane and activates tyrosine kinases that induce focal adhesion plaque formation. We believe that the electron dense thickenings of plasma membrane present at binding sites of *Brucella* on electron micrographs, are focal adhesions. Similar structures were also described in Vero cells infected with *Brucella* (7). Binding of extracellular matrix proteins to integrin causes the formation of focal adhesions associated to the intracellular domain of the β subunits of the integrins by structural proteins such as talin, vinculin and α actinin and protein kinases (18, 30). Binding of integrins to their ligand induces the phosphorylation of 3
tyrosine kinases present in focal adhesion, paxillin, tensin, and FAK. Therefore the inhibition of phagocytosis of \textit{Brucella} by genistein could result from the inhibition of one or several of these kinases. \textit{Yersinia} which also binds to $\alpha3$, 4, 5 and $\nu\beta1$ integrins via the unique RGD containing protein invasin, induces the phosphorylation of FAK (13). Formation of focal adhesions is followed by activation of Rho, Rac, and Cdc42, small G proteins that induce actin polymerization (18, 30). We showed that \textit{Brucella}-induced actin polymerization was independent of Rho, but that binding of bacteria resulted in formation of filopodia. Because the formation of filopodia is mediated by Cdc42, this small G protein could be a cellular target activated during phagocytosis of \textit{B. abortus}.

Coated vesicles may carry the intracellular signals necessary for the phagosomes to fuse with the rough endoplasmic reticulum. Subsequent to attachment and polymerization of actin, intracellular \textit{Brucella} follow one of two trafficking pathways: first they are taken into phagosomes that either fuse with rough endoplasmic reticulum and promote bacterial replication, or second they fuse with lysosomes and bacteria are killed in the new phagolysosome (7). Coated vesicles were also associated with \textit{Brucella} containing phagosomes from Vero cells (7). Coated vesicles are involved in receptor-mediated endocytosis (9, 35). They are composed of triskellion molecules that polymerize in areas of endocytosis to invaginate the plasma membrane and tag the phagosome with molecular signals that mediate intracellular trafficking.

The ability of \textit{Brucella} to move to and replicate in cisternae of the rough endoplasmic reticulum determine its intracellular survivability in Vero cells; bacteria contained in phagolysosomes are destroyed whereas bacteria that reach the rough endoplasmic reticulum
replicate (7). The present study shows that intracellular bacteria replicate in trophoblasts during all the infection; although the number of live bacteria decreases from 16 to 24 hours, the total number of bacteria continues to increase in the double immunofluorescence assay. Because on the immunofluorescent micrographs from 16 and 24 hours post-infection, the trophoblasts had large numbers of intracytoplasmic bacteria that effaced the nuclei, we believe that the bacteria replicate in rough endoplasmic reticulum or perinuclear envelope as described in Vero cells (7). These findings were confirmed by electron microscopy. Data show that 24 hours post incubation, phagolysosomes contained degenerate bacteria and myelin figures. Myelin-figure-containing phagolysosomes were also described in Vero cells in which they represent remnants of bacterial membranes. Therefore, bacteria within phagolysosomes are unable to replicate and their killing is responsible for the decreased number of viable bacteria at 24 hours post-infection (7). Because the bacteria present in the rough endoplasmic reticulum were still intact after 24 hours, we hypothesize that only bacteria located in rough endoplasmic reticulum or perinuclear membranes are able to replicate (7).

References


10) Gao JX, Issekutz AC. 1995. Polymorphonuclear leukocyte migration through human dermal fibroblast monolayers is dependent on both beta 2-integrin (CD11/CD18) and beta 1-integrin (CD29) mechanisms. Immunology 85:485-94.


Table 1. Inhibition of *Brucella abortus* strain RB51 adhesion to trophoblastic epithelial cells by extracellular matrix proteins and antibodies (n=4)

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<thead>
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<th>Treatments</th>
<th>% inhibition</th>
<th>p value</th>
<th>Significance</th>
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Table 2. Inhibition of *Brucella abortus* uptake into trophoblastic epithelial cells by exoenzyme C₃ and genistein (n=4)

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<td>Genistein</td>
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Figs. 1a-b. Light microscopy of bovine trophoblastic cells labeled with anti-α5 (a) or anti-β1 (b) antibodies. Note the diffuse granular green cytoplasmic stain. No bacteria are labeled by the antibodies. Bar = 10 μm (1a) and 50 μm (1b).
Figs. 2a-b. Light microscopy of bovine trophoblastic cells 4 hours after infection with *Brucella abortus* strain RB51. Monolayers were stained by double immunofluorescence. a. Control: Note large number of red intracellular bacteria present in trophoblast cytoplasms (arrow) and green-yellow extracellular bacteria (arrow head). Bar = 50 μm. b. Treatment with antibodies anti-α5 and -β1 integrin: Note trophoblastic cell cytoplasms with very few intracellular bacteria. Bar = 50 μm.
Fig. 3. Results of the semi-quantitative analysis of numbers of intracellular of *Brucella abortus* strain RB51, 4 hours post-inoculation, in trophoblastic cells after inhibition with anti-α5 and -β1 antibodies in chamber slides and staining of monolayers by double immunofluorescence. Each bar represents the percentage of total cells in each category from control and treated samples: non infected as A, less than 5 intracellular bacteria as B, more than 5 intracellular bacteria located under the plasma membrane as C, bacteria diffusely located in the cytoplasm as D, bacteria that effaced the normal cellular architecture as E.
Fig. 4a. Electron micrograph of trophoblasts 4 hours after inoculation with *Brucella abortus* strain RB51. Note filopodia surrounding bacteria at the cell surface (arrow head) and coated pit vesicles associated with phagosome or close from the binding site of the bacteria (arrow).

Bar = 1.6 µm.
Figs. 4b-c. Electron micrographs of trophoblasts 4 hours after inoculation with *Brucella abortus* strain RB51. 4 b. Note electron dense thickened areas under the phagosome (arrow). 4 c. Note similar electron dense areas (arrow head) and the accumulation of vesicles around the binding site of bacteria (arrow). Bar = 600 nm. Bar = 500 nm.
Fig. 4d. Electron micrograph of trophoblasts 24 hours after inoculation with *Brucella abortus* strain RB51. Note bacteria within the rough endoplasmic reticulum (arrows). Bar = 676 nm.
Fig. 4e. Electron micrograph of trophoblasts 24 hours after inoculation with *Brucella abortus* strain RB51. Note intact bacteria, and bacterial debris (arrow). Bar = 2.2 μm.
Fig. 5. Kinetics of uptake and intracellular replication of *Brucella abortus* strain RB51 in trophoblastic cells determined by bioassay. The graph represents million of CFU/ml of intracellular bacteria at 4, 8, 16, and 24 hour post-infection. Numbers of bacteria peak at 8 and 16 hour post-infection and decrease at 24 hour.
CFU intracellular bacteria (million/ml)

0  2  4  6  8  10  12  14  16  18  20  22  24  26  28  30  32  34  36  38  40

4
8
16
24

Hours post-infection
Figs. 6a-b. Light microscopy of bovine trophoblastic cells 24 hour after infection with *Brucella abortus* strain RB51. Monolayers were stained by double immunofluorescence. The normal cell architecture, nuclei and cytoplasms, is effaced and replaced by large numbers of intracellular red bacteria. Bar = 50 um (6a) and 25 um (6b).
Fig. 7. Semi quantitative count of intracellular bacteria by double fluorescence at 4, 8, 16, and 24 hours post-infection. Results expressed as percentage of cells in each categories: non infected as A, less than 5 intracellular bacteria as B, more than 5 intracellular bacteria located under the plasma membrane as C, bacteria diffusely located in the cytoplasm as D, bacteria that effaced the normal cellular architecture as E.
CHAPTER 4: BINUCLEATE CELL DEPLETION IN BRUCELLA ABORTUS STRAIN RB51 INFECTED BOVINE PLACENTA

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Abstract

*Brucella abortus* causes placentitis and abortion in pregnant cattle. The consequences of placentitis on the trophoblastic binucleate epithelial cell population have not been reported. Binucleate cells were stained with Dolichos Biflorus agglutinin lectin and numbers were quantified, by morphometric analysis, in placentomes from non-infected and *Brucella abortus* strain RB51-infected pregnant cattle. There was a diffuse decrease in numbers of binucleate cells in placentomes with purulent exudate in the arcade zone. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) and terminal deoxy dUTP nick end-labeling (TUNEL) showed reduced proliferation of progenitor mononuclear cytotrophoblasts in the arcade zone but increased programmed cell death was not demonstrated. Therefore, binucleate cell depletion is likely due to reduced proliferation of progenitor cytotrophoblasts, but not due to increased programmed cell death. Because binucleate cells produce placental lactogen, a hormone required for normal fetal
development, decreased numbers of these cells, and consequently of the hormones, could induce abortion in the B. abortus-infected placenta.

**Introduction**

Bovine brucellosis is caused by *Brucella abortus*, a gram negative intracellular bacteria that replicates within mononuclear cytotrophoblastic epithelial cells from arcade zones of placentomes and causes placentitis (1, 2). Mononuclear cytotrophoblastic epithelial cells from arcade zones are progenitor cells for binucleate or multinucleate trophoblastic epithelial cells that have a central role in the homeostasis of the placenta by the production of estrogen, progesterone, and lactogen (28, 29). Placentitis, by the production of cytokines that decrease trophoblastic cell growth and by necrosis of the progenitor cells, could lead to decreased numbers of binucleate trophoblastic cells that would result in hormonal imbalance and possibly contribute to abortion (30).

The purpose of this study was to quantitate, by image analysis, numbers of binucleate trophoblastic epithelial cells in normal and infected placenta and to clarify the mechanisms of cellular depletion. First, numbers of *Brucella* infected and non-infected binucleate cells in arcade zones, upper and lower placentomes were quantified using double histochemical labeling with Dolichos biflorus agglutinin (DBA) lectin and anti-*B. abortus* strain RB51 antibody; DBA lectin specifically labels hormone-containing granules in binucleate trophoblastic cells of sheep and cattle (15). Second, numbers of mononuclear progenitor
trophoblastic cells from arcade zones in G1, S, and G2, active phases of the cell cycle were quantified using immunohistochemical detection of the proliferating cell nuclear antigen (PCNA). PCNA is an auxiliary protein of DNA polymerase that is expressed at increasing levels during G1, peaks at S, decreases at G2 and is present only at low levels in M and G0 phases of the cell cycle (4, 16). Third, numbers of trophoblastic epithelial cells undergoing programmed cell death were quantified in arcade zones, and upper and lower placentomes using terminal deoxy deoxydUTP nick end-labeling (TUNEL). TUNEL detects blunted ends of DNA caused by calcium dependent endonucleases that cut double stranded DNA between nucleosomes during programmed cell death (14). Finally, the ultrastructural morphology of binucleate cells was examined to detect changes in hormone-containing granules.

Materials and Methods

Animals

Eight Hereford heifers, serologically negative for brucellosis by standard tube agglutination test, were inseminated with semen from a serologically negative bull (18). At 6 month gestation, the animals were inoculated intravenously in the jugular vein with $1 \times 10^{10}$ CFU of \textit{B. abortus} strain RB51 suspended in 10 ml of sterile saline (0.85% NaCl). At 8 weeks post-infection, 5 heifers were euthanized and necropsied. The last three animals were examined at term. Three random placentomes were collected from each animal and fixed in 10% neutral buffered formalin for 12 hours. Three sections from each placentomes were
processed by routine paraffin embedment techniques and cut in 4 to 6 μm thick sections. One section from each placentome was randomly selected and used in this study. Two non-infected term gestation and one late last trimester gestation heifers were used as controls and tissues from these animals were processed as described above.

**DBA and Brucella antigen immunohistochemistry**

Placentomes were stained for the presence of binucleate cells and *Brucella* antigens by a double labeling procedure. Formalin fixed, paraffin embedded sections were deparaffinized and rehydrated. They were incubated with 0.15 g of trypsin (Difco laboratories, Detroit, MI) in 250 ml of Tris buffer pH 7.4 for 30 minutes at 37°C, washed twice in Tris buffer for 5 minutes, and incubated in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature to quench endogenous peroxidase. Sections were washed once in Tris buffer for 5 minutes and in Tris buffer with 1% BSA, 0.1 mM Ca^{++}, and 0.1 mM Mg^{++} for 5 minutes. Tissues were incubated with biotinylated DBA lectin (Vector laboratories, Burlingame, CA) diluted at 1/3500 in Tris buffer with 1% BSA and Ca^{++}/Mg^{++} for 1 hour at room temperature. Sections were washed twice in Tris buffer and incubated with horse radish peroxidase avidin (ABC kit Vector laboratories, Burlingame, CA) for 30 minutes at room temperature. After 2 washes in Tris buffer, sections were incubated with DAB chromogen (Kirkegaard and Perry laboratories (KPL), Gaithersburg, MD) for 5 minutes, washed twice in Tris buffer and blocked for 15 minutes with goat serum. They were incubated with polyclonal rabbit anti-RB51 antibody at 1/1000 in Tris 1% goat serum for 1
hour at room temperature. Sections were washed twice in Tris buffer and incubated with biotin labeled goat anti-rabbit monoclonal antibody (KPL, Gaithersburg, MD) for 30 minutes at room temperature. Excess antibody was removed by 2 washes in Tris buffer and alkaline phosphatase streptavidin complex (KPL, Gaithersburg, MD) was added for 30 minutes at room temperature. Unreacted streptavidin was washed twice in Tris buffer and slides were incubated for 10 minutes with Vecta Red (Vector laboratories, Burlingame, CA). Sections were then washed, counterstained with Gill's hematoxylin, dehydrated, and coverslipped. Negative controls for lectin were performed by incubating DBA lectin with the inhibitor sugar N-acetyl galactosamine, at a concentration of 0.1 M for 20 minutes.

**Programmed cell death immunohistochemistry**

The TUNEL technique was used to detect programmed cell death in trophoblastic epithelial cells in formalin-fixed, paraffin-embedded placentomes. The TUNEL technique uses a terminal deoxynucleotidyl transferase that adds fluorescein labeled nucleotides to the 3' end of blunt ends of DNA. The incorporated nucleotides are revealed by biotinylated anti-fluorescein antibodies. Sections were deparaffinized, rehydrated and incubated with 5 mg proteinase K (Boehringer Mannheim, Indianapolis, IN) in 250 ml of Tris buffer pH 7.4 for 30 minutes at 37°C. After 2 washes in Tris buffer, endogenous peroxidase was quenched in 0.3 % hydrogen peroxide in methanol for 30 minutes at room temperature. Sections were labeled with a programmed cell death kit (Boehringer Mannheim, Indianapolis, IN) according to manufacturer recommendations. Briefly, sections were washed twice in Tris buffer and
incubated with the enzyme terminal deoxynucleotide transferase, fluorescein labeled nucleotides and buffer for 1 hour at 37°C under a coverslip. Negative controls were incubated without enzymes. Unreacted nucleotides and enzymes were washed in Tris buffer twice for 5 minutes. Peroxidase labeled anti-fluorescein antibodies were added at dilution of 1/5 for 1 hour at room temperature under a coverslip and washed twice in Tris buffer. DAB peroxidase substrate (KPL, Gaithersburg, MD) was added for 5 minutes and washed in distilled water for 5 minutes. Slides were counterstained with hematoxylin, dehydrated and coversliped.

Proliferating cell nuclear antigen (PCNA) immunohistochemistry

Number of proliferating trophoblastic epithelial cells in arcade zones was determined by immunohistochemistry with anti-PCNA antibodies. Slides were deparaffinized, rehydrated, and incubated for 15 minutes in a vegetable steamer (Black and Decker) for antigen retrieval. They were cooled for 5 minutes at room temperature and were blocked with goat serum for 10 minutes, followed by 10 minutes with 0.25 % fish gelatin Tris buffer at room temperature. Sections were incubated with mouse anti-PCNA antibody at a dilution of 1/30 (Dako, Carpinteria, CA) in Tris buffer with 1% goat serum and 1% BSA for 1 hour at room temperature. Unbound antibodies were washed twice in Tris buffer with Tween 20 and 1% BSA for 5 minutes and biotinylated goat anti-mouse (KPL, Gaithersburg, MD) was added for 30 minutes at room temperature. Antibody excess was washed twice in Tris buffer and alkaline phosphatase labeled streptavidin (KPL, Gaithersburg, MD) was added for 30 minutes at room temperature. After 2 washes, sections were incubated with vecta red for 5
minutes. Slides were washed 5 minutes in distilled water, counterstained with hematoxylin, dehydrated and coverslipped.

**Morphometry**

Each section of placentome was divided into the arcade zone and the upper half and lower half the body. Five columns of trophoblastic epithelial cells from the arcade zone were randomly traced and the number of PCNA, DBA, and PCD positive cells were determined by point counting image capture on morphometry software Vidas with an IBM 386 computer. Results were expressed as number of positive cells per 10000 um² surface area. The body of the placentome is composed of interdigitating columns of trophoblastic epithelial and maternal epithelial cells. When observed in cross section, trophoblastic cells form nests surrounded by maternal epithelial cells. In the upper and lower half of the body, 6 random regions composed of nest of trophoblasts within maternal epithelium were selected. The surface inside the maternal epithelium was measured and numbers of *Brucella*, PCD, and DBA positive cells were determined by computer. Data were checked by protected least square difference and analyzed by ANOVA.

**Electron microscopy**

Sections were fixed in 2.5 % glutaraldehyde in 1.0 % cacodylate buffer for 24 hours and stored in cacodylate buffer. They were processed by routine procedure, embedded in epoxy resin and cut in 200 nm sections. They were examined with a Phillips 110 electron microscope.
Results

Double labeling

Binucleate cells were identified by their marked brown granular cytoplasmic staining in the double labeling technique (Fig. 1). In normal placentas of control animals, binucleate cells were diffusely present in placentomes and numbers were not statistically different between arcade zones, and upper and lower areas of placentomes (Fig. 2, 3 and 15). Fusion of binucleate cells with maternal epithelium and trophoblastic DBA positive granules within maternal epithelial cells were present in multiple foci (Fig. 1).

At 8 week post-infection, sections from only one animal had placentitis; placentomes from this animal were characterized by large amounts of purulent exudate in arcade zones and necrosis of trophoblastic villi. Binucleate cells in sections of placentomes without placentitis were present diffusely throughout the three areas and numbers were not statistically different from control animals (Fig. 15). Sections of placentomes with placentitis had statistically reduced numbers of binucleate cells in all three areas. However, the depletion was more pronounced in arcade zones and upper halves than in lower halves of placentomes. Sections from placentomes with placentitis were also characterized by multifocal intense intracytoplasmic staining for Brucella antigen in mononuclear cytotrophoblasts.

Placentomes that were sampled at twelve weeks post-infection, were characterized by a marked, diffuse, decrease of binucleate cells in all three areas, large amounts of purulent exudate that expanded the arcade zone, multiple foci of Brucella antigen in the cytoplasm of
mononuclear cytotrophoblastic cells, and necrosis of trophoblastic villi of progenitor cells (Fig. 4 and 5). Numbers of binucleate cells were statistically different from control animals (Fig. 15). The depletion was more severe in arcade zones and upper halves than in lower halves of placentomes.

**Programmed cell death**

There were no statistical differences between numbers of PCD positive cells in the three areas of the placentomes, or between control and infected animals (Fig. 16). PCD positive cells were characterized by round to oval nuclei with large 1 to 2 um dense, dark, perinuclear clumps of chromatin and open central nucleolosms (Fig. 7 and 8). Occasional cells had remnants of small nuclei spread into an irregular shaped cytoplasm that had left a lucent oval area (4 to 6 um in diameter) between normal trophoblastic cells (Fig. 9). PCD positive cells were not associated with inflammatory infiltrate of macrophages or neutrophils. Features of programmed cell death were most often seen in mononuclear cytotrophoblasts although occasional binucleate cells (Fig. 7) and even multinucleated cells also had positive staining (Fig. 8).

**PCNA**

Control placentomes were characterized by large numbers of PCNA positive nuclei especially in trophoblastic villi (Fig. 10). Numbers of replicating cytotrophoblasts in sections of placentomes eight weeks post-infection without inflammation were not statistically
different from controls (Fig. 17). However, 8 weeks and 12 weeks post-infection sections of placentomes which had purulent exudate in the arcade zone had statistically significant decreased numbers of positive nuclei in mononuclear cytотrophoblastic cells compare to controls (Fig. 11 and 17).

Electron microscopy

Because cells with binucleate morphology were occasionally present in infected placentomes, but did not stain with DBA lectin, we studied their ultrastructural characteristics (Fig. 6). The morphology of granules within binucleate cells was similar between infected or non infected placentomes (Fig. 12). Some granules contained dense tubular inclusions composed of an outer wall with a clear empty center identified as crystalline precipitations of proteins commonly associated with endocrine secretion (Fig. 13) (8). Numbers of granules and the amount of golgi apparatus were mildly depleted in binucleate cells from placentomes that contained inflammatory exudate (Fig. 14).

Discussion

This study showed that 1) there was severe, diffuse depletion of binucleate trophoblastic epithelial cells in arcade zones, upper halves, and lower halves of sections of placentomes with placentitis, and 2) this depletion was likely due to a decreased replication
rate and necrosis of progenitor cytotrophoblasts in the arcade zone but not due to increased programmed cell death. This is the first report of binucleate cell depletion during placentitis.

Production of cytokines by inflammatory cells recruited during *Brucella*-induced placentitis might reduce numbers of progenitor trophoblastic epithelial cells in the active phase of cell cycles in arcade zones of placentomes. In vitro, addition of cytokines such as interleukin-1α, interleukin-1β, or tumor necrosis factor (TNF) reduced growth of malignant trophoblastic cells (27, 30). A previous study, that used the same animals, showed that 3 additional 8 weeks post-infection animals had multifocal mild to moderate areas of placentitis close from the sections sampled in this study (18). In all vaccinated animals, even those with mild focal areas of placentitis, TNF was diffusely present within the cytoplasm of mononuclear cytotrophoblasts (19). In this study, all sections of placentomes without placentitis, irrespective of the status of adjacent sections, had normal index of proliferation in arcade zones. This indicates that in vivo production of TNF by inflammatory cells has a very local effect on the cell cycle of trophoblastic cells. Levels of TNF in the serum of these animals were similar to controls (19). Additionally, replication of *Brucella* within the rough endoplasmic reticulum of trophoblastic epithelial cells could deprive cells of their metabolites and block them into resting phase of the cell cycle. However, this later hypothesis is improbable because the infection of progenitor cells was multifocal whereas the cell cycle arrest was diffuse in the arcade zones.

Necrosis and decreased proliferation of progenitor cells may lead to decreased production of binucleate cells. Binucleate cells normally migrate from the upper to the lower
placentomes where they fuse with maternal epithelial cells and release their granules. Therefore, abnormal production of binucleate cells should result in a gradual cellular depletion more severe in arcade zones than in upper, and finally in lower, parts of placentomes. Data confirm this hypothesis: all 12 weeks post inoculation placentomes had increased numbers of binucleate cells from arcade zones to lower halves of placentomes. The only 8 weeks post infection placentomes that had placentitis had marked depletion in arcade zones and upper parts but not in lower parts of placentomes; indicating that at this early stage of infection, depletion in lower halves of placentomes had not yet occurred.

Because inflammatory cells produce oxygen radicals and tumor necrosis factor, we hypothesized that *Brucella*-infected placentas would have increased PCD of binucleate cells. Programmed cell death is initiated by a variety of stimuli that include reduction in growth factors, exposure to cytokines (TNF), bacterial toxins, oxygen radicals, irradiation, corticosteroids, and toxicity (26). However, data show that there was no statistical differences between normal and infected placenta. Because the exudate was located exclusively in arcade zones, trophoblastic epithelial cells present in the rest of the placentomes could be distant enough not to be influenced by the production of oxygen radicals or TNF.

Depletion of binucleate cells was due to decreased proliferation of progenitor cytотrophoblasts but not increased programmed cell death. Additionally, this depletion was possibly enhanced by the diffuse necrosis of trophoblastic villi which provide the extracellular matrix framework for the trophoblasts to differentiate and migrate into the body of the placentome.
The reduction or absence of labeling with DBA lectin of the remaining binucleate cells present in infected placentomes may be due to abnormal levels of hormone production. Autoradiographic studies showed that binucleate cells actively transfer galactose and fucose residues to secretory hormones; bovine placental lactogen has O-linked and N-linked carbohydrates (5, 7, 29). Therefore, intracytoplasmic labeling of binucleate cells by DBA lectin could be attributed to glycosylated hormone contained in secretory granules as well as galactosamine present in cisterna of the golgi apparatus. In this study, ultrastructural examination of binucleate cells from 12 weeks post-infection animals revealed a mild decrease in the golgi apparatus and number of normal shaped granules compare to normal placenta that could account for decreased affinity of the lectin; however further morphometric studies are needed to quantify the amount of golgi and granules within these cells. Additionally, reduction in hormone glycosylation by the golgi could also explain decreased lectin affinity for binucleate cells.

Reduction of binucleate cell function by reduction of their numbers, their hormonal production, or abnormal hormone glycosylation could decrease hormone levels of progesterone, estrogen and placenta lactogen (17, 22). Perhaps a reduction in the estrogen and progesterone production by binucleate cells could be compensated by increase synthesis in mononuclear trophoblasts and ovary. However, reduction of placental lactogen has no compensatory pathways. Placenta lactogen is normally released in fetal and maternal circulation, from where it is redistributed to target organs in which it has prolactin-like and somatogenic activities. In mammary glands, placental lactogen is necessary for the
development of lobules and alveoli (24). In ovaries, although mouse and rats placental lactogen are luteotrophic, studies in sheep showed that the hormone neither affects the production of progesterone nor protects against luteolysis of the corpus luteum by prostaglandin F2 alpha (25). Finally, ovine placental lactogen regulates the supply of nutrient necessary for fetal growth; in vitro, the hormone stimulates amino acid transport in fetal rat diaphragm myocytes, glycogen synthesis of rat hepatocytes, and glycogenolysis in ovine fetal hepatocytes (10, 11, 12). In cows, maternal plasma concentration of placenta lactogen rises four months after mating, remains stable throughout the rest of the gestation and decreases before parturition (3, 6). A similar decrease occurs in the fetal circulation of lambs 2 weeks before parturition (13).

Because placental lactogen is luteotropic, regulates fetal growth, and decreases days before parturition, premature decreased levels of the hormone associated with placentitis could cause abortion. Virulent strains of Brucella abortus cause placentitis sooner and are more cytotoxic to trophoblastic epithelial cells than the vaccine strain RB51 (21, 23). Animals infected intraconjunctivaly with virulent strain 445 developed placentitis as soon as 21 days post-inoculation and abort on average 6 weeks after. In this study, animals inoculated intravenously with strain RB51 consistently developed placentitis 12 weeks post-inoculation and but one of them had premature delivery. Therefore delayed placental colonization and decreased cytotoxicity of Brucella abortus strain RB51, could delay binucleate cell depletion and therefore reduce the number of abortions. Sub cutaneous vaccination with Brucella abortus strain RB51 does not induce placentitis (20).
References


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Fig. 1. Double labeling immunohistochemistry. Control placentome. Note intense brown granular cytoplasms of binucleate cells stained by DBA lectin (arrow). Note also Binucleate cell in the maternal epithelium (arrow head). Bar = 50 um.

Fig. 2. Double labeling immunohistochemistry. Control placenta. Note large density of binucleate cells in the lower placentome. Bar = 100 um.
Fig. 3. Double labeling immunohistochemistry. Control placenta. Note binucleate cells present the arcade zone and upper placentome. Bar = 200 um.
Fig. 4. Double labeling immunohistochemistry. 12 weeks post infection. Note the intense inflammatory infiltrate that thickens the arcade zone. There is diffuse severe depletion of binucleate cells in the arcade zone and upper body of the placentome and large amount of red brucellar antigen in trophoblastic cells from the arcade zone. Bar = 100 um.

Fig. 5. Double labeling histochemistry. 12 weeks post infection. Note large amount of red brucellar antigen in the cytoplasm of trophoblastic cells and the marked contrast between the brown DBA staining and the red antigen. Bar = 50 um.
Fig. 6. Double labeling immunohistochemistry. 12 weeks post-infection. Note remaining binucleate cells with decreased affinity for the lectin. Bar = 50 μm.
Fig. 7. PCD immunohistochemistry. Note binucleate cells with intense perinuclear clumping of chromatin. No inflammatory cells surround the cell. Bar = 25 µm.

Fig. 8. PCD immunohistochemistry. Note the multinucleate cell with the morphologic characteristic of PCD. Bar = 25 µm.
Fig. 9. PCD immunohistochemistry. Note trophoblastic cell cytoplasms containing lucent oval areas with weak staining. Bar = 50 um.
Fig. 10. PCNA immunohistochemistry. Arcade zone from control placentome. Note large number of red positive nuclei. Bar = 100 um.

Fig. 11. PCNA immunohistochemistry. Arcade zone 12 weeks post-infection. Note depletion in numbers of nuclei positive for PCNA and large amount of inflammatory exudate. Bar = 100 um.
Fig. 12. Electron micrograph of a binucleate cell from a control animal. Note the large number intracytoplasmic secretory granules. Bar = 2.2 um.
Fig. 13. Electron micrograph of a binucleate cell from a control animal. Note longitudinal and cross sections of tubular protein inclusions within granules (arrows). Bar = 832 nm.
Fig. 14. Electron micrograph of a binucleate cells from 12 weeks post infection animal. Note depletion of intracytoplasmic secretory granules. Bar = 5.6 um.
Figure 15. Numbers of binucleate cells in arcade zones, upper and lower placentomes.
Figure 16. Numbers of PCD positive cells in arcade zones, upper, and lower halves of placentomes.
Figure 17. Numbers of PCNA positive cells in arcades zones.
CHAPTER 5: GENERAL CONCLUSIONS

*Brucella abortus* strain RB51 is the main component of a new bovine brucellosis vaccine accepted in 1995 in the United-States by the USDA Animal Plant and Health Inspection Service. Because lipopolysaccharides from strain RB51 are deficient in the O-side chain they do not induce production of lipopolysaccharide antibodies which are detected by standard serodiagnostic tests for brucellosis.

However, there is no objective serologic test, such as ELISA, which detects antibodies specific for RB51-vaccinated cattle and intravenous inoculation can cause placentitis.

Con A lectin affinity chromatography allowed the isolation of a 35 kDa diagnostic immunoreactive outer membrane protein from *B. abortus* strain RB51. That protein may be useful candidate antigen in developing a specific test for detecting antibody to RB51 in vaccinated cattle. For this purpose, the protein was further characterized by amino acid sequencing. The sequence should allow cloning of the gene coding for the protein and expression of large quantity of protein to be used in ELISA diagnostic test. However, because cross-reactions to sera from *Yersinia, Vibrio cholerae, Salmonella* and *E. coli* infected animals can lead to false positives in standard diagnostic tests, further studies are needed to determine the results of sera from these animals to the ELISA test. Isolation of bacterial proteins by lectin affinity chromatography indicates the presence of bonds between proteins and carbohydrates. The nature of these bonds would need to be studied by biochemistry. The amino acid sequence from the 35 kDa protein had 65% homology with antitrypsin and could
be a virulence factor. Therefore, the role of this protein in *Brucella* outer membrane also deserves to be studied more intensively.

*Brucella abortus* strain RB51 binds to α5β1 integrins present on the surface of bovine trophoblastic epithelial cells. Binding stimulates uptake of the bacteria by tyrosine kinases, Rho independent signaling pathway, and translocation of bacteria to cisternea of the rough endoplasmic reticulum (RER). Additional studies are needed to identify which specific protein kinases and small G proteins are involved in uptake of the bacteria and which signals stimulate translocation of phagocytized bacteria to the RER. Binding of *Brucella* to macrophages may also involve β2 integrins which are not expressed at the surface of trophoblastic cells. Finally, smooth strains of *B. abortus*, such as 2308, could also bind to lectin receptor because they express complete O-side chain which is composed of carbohydrates.

Depletion of binucleate trophoblastic cells in *Brucella abortus* strain RB51 infected placenta could cause abortion due to decreased levels of placental lactogen produced by these cells. Placental lactogen levels should therefore be monitored in serum from pregnant and infected cattle to confirm this hypothesis. Additionally further studies are needed to quantitate numbers of binucleate trophoblastic cells in placentomes from *B. abortus* field strains-infected cattle.
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