Placental pathology, immune responses, bacteriologic findings and clinical signs in pregnant cattle vaccinated with Brucella abortus strain RB51

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Placental pathology, immune responses, bacteriologic findings and clinical signs in pregnant cattle vaccinated with *Brucella abortus* strain RB51

by

Mitchell Van Palmer

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

Major: Veterinary Pathology

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ABSTRACT

To determine the placental tropism, abortigenicity, immunogenicity and effect on TNF-α levels, pregnant cattle were vaccinated IV (n=10) or SC (n=5) with the vaccine *Brucella abortus* strain RB51 (SRB51), SC with strain 19 (S19) (n=5), or saline (n=2) at 6 months gestation. Eight of ten IV vaccinated heifers developed placentitis and fetal infection. Strain RB51 was cultured from all tissues in which lesions were seen. No lesions were seen in SC vaccinated cattle and SRB51 was isolated from 2/5 superficial cervical lymph nodes in the area draining the site of SC vaccination. One premature calf born to an IV vaccinated heifer had mild interstitial pneumonia and disseminated SRB51 infection. No lesions were seen in other calves from IV vaccinated heifers, heifers vaccinated SC with SRB51 or S19, and neither SRB51 nor S19 could be recovered from calves of SC vaccinated cattle. Maternal PBMC from SRB51 vaccinates and S19 vaccinates showed proliferative responses to both γ-irradiated SRB51 and S19 which were greater than controls. Strain RB51 vaccinates did not develop antibodies detected by the standard tube agglutination test, but did develop antibodies to SRB51 that reacted in a dot ELISA test with irradiated SRB51. Radioimmunoassay for bovine plasma TNF-α revealed insignificant differences (P>0.05) between SRB51 vaccinates, S19 vaccinates and controls. Similarly, TNF-α levels in amniotic or allantoic fluids from vaccinated cattle were not different from placental fluids of controls (P>0.05). Immunohistochemistry for TNF-α revealed increased immunoreactivity within trophoblastic epithelial cells which was most extensive in IV vaccinated cattle with vaccine-induced placentitis. These results indicate that SRB51 is less abortifacient than previously published reports with S19; however, SRB51 can infect the bovine placenta and fetus, can induce placentitis, and in some cases, lead to preterm expulsion of the fetus. Undesirable effects of SRB51 are dependent on dose and route of administration as SC vaccination with a lower dose does not result in placental or fetal infection.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Brucellosis is an economically important abortifacient disease of cattle. Since 1940, the United States has been actively engaged in a brucellosis eradication effort. A cornerstone of this effort has been the vaccination of young female calves with the live brucellosis vaccine *Brucella abortus* strain 19 (S19). Strain 19 has been effective in decreasing the incidence of bovine brucellosis; consequently, the goal of the United States Department of Agriculture (USDA) is the eradication of bovine brucellosis from the United States by the end of 1998. Although effective in the prevention of brucellosis, S19 is not without limitations. An obstacle to the eradication effort is the inability to serologically distinguish S19 vaccinates from naturally infected cattle. Additionally, infrequent but problematic sequelae of S19 vaccination include abortion in pregnant cattle, arthritis, anaphylaxis, and persistent infection.

In April, 1996, for the first time in half a century a new brucellosis vaccine was approved for use in cattle. The vaccine, *Brucella abortus* strain RB51, is as effective as S19 in the prevention of brucellosis, but allows the serologic distinction of vaccinates from naturally infected animals.

When brucellosis infected herds have been detected, the USDA has implemented the practice of whole herd vaccination, where all female cattle, regardless of age or pregnancy status are vaccinated. In such situations, safety of SRB51 in pregnant cattle would be necessary to avoid widespread abortion. The studies presented in this dissertation were
designed to determine; 1) the tropism of SRB51 for the bovine placenta, 2) the ability of SRB51 to colonize the placenta, induce placentitis and cause abortion, 3) the immunogenicity of SRB51 vaccination in pregnant cattle, measuring both humoral and cell mediated responses, and 4) the effect of SRB51 vaccination on levels of the potent proinflammatory cytokine, TNF-α, in plasma and placental tissues.

Literature Review

Historical Background

Human brucellosis was known possibly as early as the time of Hipocrates who described the symptoms of prolonged, intermittent fever; however, the earliest accurate description of the disease in man was made in 1861 when Marston designated it as Mediterranean fever and described the disease as “a preliminary stage of dyspespsia, anorexia, nausea, headache, feeling of weakness, lassitude, and inaptitude of exertion, mental or physical; chills, muscular pains, and lastly a fever having a long course 3 to 5 or 10 weeks, marked by irregular exacerbations and remissions, great derangement of the assimilative organs, tenderness in the epigastric region, and splenic enlargement. It is prone to relapses, has a protracted convalescence, and is frequently marked by rheumatism.” The name “undulant fever” was given to the disease in humans by Hughes in 1897. In 1887 Colonel David Bruce discovered a microorganism isolated from the spleen of fatal cases of Malta fever, also known as Mediterranean fever or undulant fever, in soldiers on the island of Malta. The causative organism, now known as Brucella melitensis, was named
*Streptococcus melitensis* by Hughes in 1892 and *Micrococcus melitensis* in 1893 by Bruce. Further confirmation of Bruce's conclusions were made in 1897 when it was shown that the organism that Bruce identified was agglutinated by sera from afflicted patients. The association of the disease in man with that seen in goats was recognized in 1905 when local goats on the island of Malta, which were going to be used as experimental animals, were found to have preexposure agglutinating antibodies to the organism. Subsequent culture of milk from the goats yielded the causative organism of Malta fever.

As early as 1864 epizootics of abortion were known to occur in regions of Louisiana and the Mississippi River. In 1864 in *Cattle and their Diseases*, Richard Jennings describes epizootics of abortion which he hypothesized were of a sympathetic or empathetic nature; that is, when one cow observed another cow aborting she would also abort in a few days or a few weeks. As erroneous as his etiologic diagnosis was, his recommended treatment of isolation of cows during calving so other cows could not observe an abortion, did help in controlling the disease. In 1862, Nocard, a French bacteriologist, first recognized the presence of bacteria between the fetal membranes and the wall of the uterus of a pregnant cow following abortion. Similarly, in 1897, Bang, a Danish veterinarian, assisted by Stribolt, noted a thick yellow exudate between fetal membranes and the uterus from an abortion. From this exudate they isolated a microorganism which when injected into pregnant cows caused abortion. The organism was reisolated from the abortion materials and injected into pregnant cows, reproducing abortion. Bang and colleagues were also able to produce abortion in pregnant sheep injected with the organism. Bang designated the organism,
Bacillus abortus and the disease came to be known as Bang's disease. The diseases of Malta fever in man and contagious abortion in cattle were studied independently and no connection between the two diseases was made until 1918. Evans made this connection by demonstrating the close morphological, cultural and serological relationship between the two bacteria causing the disease in man and sheep, *B. melitensis* and *B. abortus*, respectively, which are now known to be very closely related.28

In Rhodesia, Duncan was the first to recognize and report brucellosis in man due to *B. abortus*.25 The first case of brucellosis in America that was reported as being caused by other than *B. melitensis* was recognized in 1924,40 however, it was later found to be caused by *B. suis* rather than *B. abortus*.

Description of the genus, *Brucella*

*Brucella abortus* is of the Order Eubacteriales, Family Brucellaceae. The genus *Brucella* is unrelated to other pathogens but is closely related to the agrobacterium-rhizobium complex. Seven species of *Brucella* exist with preferential host relationships: *B. abortus* (cattle), *B. melitensis* (goats), *B. suis* (swine), *B. neotomae* (desert wood rat), *B. canis* (dogs), *B. ovis* (sheep) and a poorly characterized *Brucella* sp. isolated from dolphins.29 All *Brucella* are closely related and share greater than 90% DNA homology.88 Some authors have suggested that only one species be recognized, *Brucella melitensis*, and that other specific epithets previously used as species names be used to designate biovars (e.g. *Brucella melitensis* biovar Abortus).88
Brucella organisms are Gram-negative, coco-bacilli, arranged singly, or occasionally in pairs or small groups. True capsules are not detected and Brucella do not form spores and are nonmotile. Brucella are aerobic and many strains require supplemental CO₂ for optimal growth. Growth can be stimulated by the addition of 5 to 10% normal serum. Colonies generally become visible after 3 days incubation; however, growth may require up to 10 days in some cases. Brucella colonies after 3-5 days incubation are round, 1-2 mm in diameter with smooth margins, translucent and a pale honey color when viewed from through a transparent medium using a low power microscope and obliquely transmitted light. Viewed from above, colonies are convex and pearly white.³

Description of B. abortus strain RB51

Brucella abortus strain RB51 (SRB51) is a rifampin-resistant strain of B. abortus derived by repeated passage of strain 2308 on trypticase soy (TS) broth supplemented with varying concentrations of rifampin.⁷⁵ SRB51 is highly deficient in the O-side chain component of LPS when compared to the parental 2308, but may contain small amounts of the perosamine homopolymer which is the O-chain component of B. abortus.⁷⁵ Rough morphology is characterized by absorption of crystal violet, autoagglutination and lack of the O-chain component in LPS extracted from SRB51 on SDS - PAGE gels. Furthermore, Western blot analysis with the monoclonal antibody BRU 38, specific for the perosamine homopolymer O-chain of smooth Brucella LPS indicates that SRB51 is highly deficient in the O-chain compared to 2308.⁷⁵ Biochemically, SRB51 resembles 2308 in the ability to
metabolize erythritol.\textsuperscript{75} This ability is linked to the presence of the \textit{eri} gene which is present in both SRB51 and 2308, but absent in \textit{B. abortus} strain 19 (S19).

\textit{B. abortus} strain RB51 as a vaccine

Vaccination with SRB51 is protective in mice and cattle which are challenged after vaccination with virulent \textit{B. abortus}.\textsuperscript{75,20} Cattle vaccinated with SRB51 remain serologically negative when tested by conventional brucellosis surveillance tests including tube agglutination, particle concentration fluorescence immunoassay (PCFA), card test and complement fixation (CF).\textsuperscript{81} These tests measure antibody responses to the LPS O-side chain of \textit{B. abortus}\textsuperscript{62} and, therefore, do not measure anti-SRB51 antibodies.

SRB51 is cleared more rapidly than S19 or S2308 in both murine spleens and bovine lymph nodes.\textsuperscript{19,64,87} In mouse spleens, SRB51 was not cultured beyond 4 weeks after infection while S19 and S2308 were present at 10 and 20 weeks after infection, respectively.\textsuperscript{82} In bovine lymph nodes, SRB51 was not recovered from lymph node biopsy specimens at 6 weeks after vaccination while S19 and S2308 were cultured at 8 weeks after vaccination.\textsuperscript{19}

Strain 19 induced abortion

The eradication of bovine brucellosis has centered on the use of \textit{Brucella abortus} strain 19 (S19), an attenuated, live vaccine introduced into the federal brucellosis program in 1940. Although S19 has been an effective tool in the control of bovine brucellosis for over
50 years it has significant limitations. These limitations include not only the production of antibodies indistinguishable from those resulting from natural infections, but also the ability to induce abortion in pregnant cattle. As early as 1937 it was noted that experimental subcutaneous injection in pregnant cattle of a dose of $2 \times 10^{11}$ S19 organisms may produce typical clinical signs and lesions of brucellosis with the discharge of large numbers of the organisms in uterine material at the time of parturition. Cattle in which S19 vaccination induced weak or dead calves were in late gestation when vaccinated. However, one cow vaccinated at 4 months gestational age and challenged with a virulent strain 32 days later gave birth to a weak calf with S19 being isolated from placental and fetal tissues.

Experimental intravenous inoculation of S19 leads to infection of the placenta and abortion. Two separate studies in the United States and United Kingdom utilized experimental intravenous inoculation of S19 in pregnant cattle in larger than recommended doses. S19 induced abortion in 100% of the cattle tested in both experiments. Abortions occurred between 16 days and 42 days after inoculation.

In pregnant cattle inoculated by the traditional subcutaneous route, the incidence of abortion is less. In a Canadian study, the effects of subcutaneous S19 vaccination on pregnant cattle were investigated. Of 112 cattle vaccinated between one and nine months of gestation, 16 (14.29%) aborted. However, it was noted that of 30 cows between six and nine months of gestation, four (13.33%) aborted and S19 could not be recovered from colostrum or uterine exudate in any of the four.
Not only is route of vaccination important but also vaccine dose. In cattle vaccinated during pregnancy with 1/20th the recommended dose of \(1.12 \times 10^{11}\) and challenged with a virulent strain 10 weeks later, S19 was recovered in 4/9 (44%) following parturition with one abortion and one premature birth.\(^2\) Vaccination with 1/400th the recommended dose caused no abortions and isolation of S19 in colostrum from 1/9 (11%) cows.\(^17\)

Some researchers have associated S19 colonization of the bovine placenta and abortion with erythritol utilizing mutants.\(^21\) In 4/9 (44%) pregnant cattle, vaccinated with 5.8 \(x 10^8\) S19 organisms, S19 was isolated from cotyledonary tissues and in 2 cows colonization led to abortion or premature calving. These two isolates were determined to be erythritol utilizing mutants of S19.\(^21\) Similarly, 307 head of cattle, three to five months pregnant were vaccinated with a dose of 3 \(x 10^8\) S19 organisms and abortion occurred in 15 (4.9%) of the cows with four of the isolates being erythritol utilizing variants of S19.\(^8\) In spite of these findings which suggest that erythritol utilization is responsible for the placental tropism of S19 and other Brucella strains there is evidence to the contrary. Species which do not synthesize erythritol in placental tissues, i.e. humans, rats, mice, rabbits and guinea pigs can become infected with Brucella sp. and develop placentitis.\(^65,42\) Moreover, rate of erythritol metabolism has not been correlated with increased virulence in Brucella sp.\(^53,54\) Therefore, it is likely that although erythritol may enhance Brucella growth, it is not essential for growth or placental infection.

Large numbers of pregnant cattle can be vaccinated with S19 with minimal concern for large numbers of vaccine-induced abortions. In a Florida field trial, cows in late gestation
were vaccinated with S19 at a dose of $10^{11}$ organisms / animal. Vaccination with S19 induced abortion in less than 1% of over 10,000 cows.61

A small percentage of cows vaccinated with S19 as calves may retain S19 infection into adulthood and in some instances this persistent infection may be responsible for abortion.86 A study in the United Kingdom showed that of 34 isolates of bovine origin determined to be S19, 11 were from abortion material.86 Calfhood vaccination was the only known exposure to S19 in all cases.

Virulence Factors

*Brucella* with smooth colony morphology are more virulent than *Brucella* with rough colony morphology,73 and this suggests a significant role for LPS in the virulence of *B. abortus*. Both smooth and rough *Brucella* are able to enter host cells; however, rough *Brucella* may enter host cells more effectively.13 Smooth *Brucella* survive and even multiply while the rough bacteria are eventually eliminated.36 Survival of *Brucella* has been related to various factors including their ability to inhibit phagosome-lysosome fusion,70,16 resist destructive effects of lysosomal enzymes after fusion has occurred,70 and inhibit TNF-α secretion by phagocytic cells.17-18

An important property of *Brucella* sp. is the ability to survive and multiply intracellularly in host phagocytes. Extracts from the smooth virulent *B. abortus* strain 2308 are able to suppress phagosome-lysosome fusion in murine peritoneal macrophages.32 In bovine neutrophils, extracts of *B. abortus* strain 2308 show a concentration dependent
inhibition of primary granule release.\textsuperscript{10} This inhibition may be due to the production of 5' guanosine monophosphate (GMP) and/or adenine which are found in the culture supernatant of live \textit{Brucella} but not \textit{S. epidermidis} or \textit{E. coli}.\textsuperscript{15} Adenine and GMP have been shown to inhibit the ability of neutrophils to iodinate proteins.\textsuperscript{15} Macrophage lysosomal release has also been shown to be inhibited by adenosine and other nucleosides and nucleotides most likely by inhibition of methylation of membrane proteins or phospholipids important in membrane fusion.\textsuperscript{69} Protein iodination and lysosomal release are important antibacterial mechanisms employed by phagocytes. Persistence of \textit{Brucella} in phagolysosomes of nonactivated macrophages is thought to provide safety from host antibacterial factors. However, activated macrophages are able to phagocytose and kill \textit{Brucella}.

\textit{B. abortus} has a predilection for the placental cotyledons, specifically the trophoblast.\textsuperscript{77} Pathogenesis studies have shown that in placental infection, \textit{B. abortus} is first seen in phagosomes of erythrophagocytic trophoblastic epithelial cells of the placentome. Following replication within erythrophagocytic trophoblastic cells, \textit{B. abortus} next replicate within the rough endoplasmic reticulum cisternae and nuclear envelope of adjacent chorioallantoic cells of the trophoblast.\textsuperscript{4} Hematogenous spread to chorionic villi and the fetus follow necrosis of trophoblast and ulceration of the chorioallantoic membrane. It remains unclear if \textit{Brucella} are passively phagocyted by the trophoblastic cells or if there is a more selective receptor-ligand interaction between the bacteria and trophoblastic cells. However, indiscriminate phagocytosis of \textit{Brucella} would not account for the remarkable numbers of
bacteria within trophoblastic cells when few bacteria are seen free in the extracellular space, suggesting a more specific tropism for the placental trophoblast.

*Brucella sp.* synthesize several proteins which have been determined to be important in protecting bacteria against the damaging effects of host cell enzymes including superoxide dismutase, catalase, and high temperature resistant stress proteins. Construction and testing of genetically altered *B. abortus* mutants deficient in the Cu-Zn superoxide dismutase gene, *htrA* heat shock gene, catalase gene, urease gene, *recA* gene or a gene encoding a 31-kDa protein of unknown function have not resulted in mutants of decreased virulence.

Iron chelating siderophore synthesis is a virulence mechanism utilized by various pathogens to survive in the low iron environment of the host. In animal cells and body fluids free iron levels are low. Rather, iron is bound to host proteins such as transferrin and lactoferrin. Bacteria have developed proteins (siderophores) which scavenge iron from host iron binding proteins in low iron environments. *Brucella abortus* has been shown to produce the siderophore 2,3-dihydrobenzoic acid when grown under iron-limiting situations *in vitro*. However, the importance of siderophores *in vivo* has not been established.

Enzymes such as nitric oxide are of limited importance in host defense against brucellosis. Addition of L-arginine, an inhibitor of nitric oxide synthesis, to *Brucella*-infected macrophages *in vitro* has only minor affects on killing of *Brucella*. Nitric oxide has been shown to be important in resistance to infection with the intracellular parasite *Leishmania*.47
Placental lesions in cattle with brucellosis

Early descriptions of gross lesions of brucellosis consisted of an abundant yellow exudate with a slimy, somewhat lumpy character found between the uterine mucosa and the allantochorion. This exudate contained numerous short bacilli. Later microscopic descriptions reported necrosis of trophoblast cells and large numbers of intracellular bacilli within trophoblast cells. Uterine mucosal epithelial cells as well as amniotic epithelial cells were reported to be unaffected. More recent and complete descriptions describe placental cotyledons with marked inflammation characterized by cell debris, intact and degenerate neutrophils and bacteria lying between the maternal caruncle and the allantochorion. These changes in the placental cotyledon were limited to the periphery of the cotyledon early in infection (3-4 weeks after infection) and did not extend to the interior of the cotyledon until 5 weeks after infection. Close examination of the trophoblast layer showed masses of bacteria within the trophoblast. Inflammation extended to the connective tissues of the allantochorion which was edematous and contained numerous neutrophils. Endothelial cells lining fetal blood vessels contained large numbers of intracellular bacilli. Maternal tissues of the placentome were largely unaffected. Additionally, a mild interstitial lymphoplasmacytic mastitis with acinar lumina variably filled with neutrophils was described in experimentally infected cattle.
Development and anatomy of the placenta

Placental development begins early during the morula stage as the outer layer of ectodermal cells differentiate into trophoblastic cells. Trophoblastic epithelium is differentiated to physically connect the developing embryo with the uterus. Differentiation includes the redistribution of intercellular adhesion molecules such as E-cadherin and Na⁺/K⁺ ATPases to the basolateral aspect of the trophoblastic epithelial cell. Cadherins and ATPases are present on all cells in the morula, but redistribution occurs only in trophoblastic epithelial cells. The Na⁺/K⁺ ATPase may function in ion and fluid fluxes necessary to create the cavity of the blastocyst known as the blastocoel. Cytokeratins characteristic of epithelial cells are detectable in differentiating trophoblastic epithelial cells at this early morula stage.

Remaining cells of the morula differentiate at one pole into the inner cell mass. Endodermal and mesodermal components of the placenta later develop from portions of the inner cell mass. The continued development of the remaining inner cell mass into the embryo does not proceed until the first structures of the placenta have been formed. Early in placental development and coincident with implantation, maternal recognition of pregnancy is required. In a broad sense, maternal recognition is the sequence of events required to ensure that the conceptus is not rejected by the maternal immune system. It is hypothesized that release of cytokines selectively modulates the immune cells at the implantation site. In some species, the release of factors from the placenta act in a paracrine or endocrine fashion to
ensure continued pregnancy. In ruminants the secretion of type I interferons from trophoblastic epithelial cells plays a key role in maternal recognition of pregnancy.

Progesterone is required during pregnancy to maintain uterine endometrium in a secretory and receptive state which is optimal for embryonal growth and development. Therefore, it is vital during pregnancy to ensure the continued secretion of progesterone. The major source of progesterone is the postovulation ovarian structure the corpus luteum (CL). Since the gestational period for most species is longer than the functional period of the normal corpus luteum, during pregnancy it becomes necessary to extend the functional life of the CL. Different mammals utilize different strategies to extend CL function. Primates and horses produce a luteotrophic substance, chorionic gonadotropin, which supports the CL by supplementing the action of luteinizing hormone from the anterior pituitary. In ruminant species, a type I interferon, IFN-\(\tau\), is produced by mononucleate trophoblastic epithelial cells. IFN-\(\tau\) inhibits prostaglandin \(F_2\alpha\) (PGF\(_{2\alpha}\)).\(^7\) Prostaglandin \(F_2\alpha\) is produced by the uterus and serves to lyse the CL in nonpregnant animals. The expression of IFN-\(\tau\) mRNA occurs as early as postconception day 7, is maximal around days 16-19, and is limited to the mononucleate trophoblastic epithelial cells and has not been detected in binucleate trophoblastic cells, embryonic disc cells or embryonic endodermal cells.\(^7\)

Implantation of the conceptus proceeds differently among different species. In rodents and primates, implantation occurs soon after the blastocyst hatches from the zona pellucida. In ruminants, swine and horses the conceptus remains free in the uterine lumen for several days before implantation. In rodents, the study of implantation has shown that
trophoblast - uterine attachment may be initiated by carbohydrate - lectin interactions. Mouse blastocysts have been shown to express several carbohydrate structures including the lectin ligand, sialyl lewis X which is also important in lymphocyte - high endothelial cell interaction in lymph node postcapillary venules.

The maternal response to the implanting embryo includes vascular changes such as angiogenesis, vasodilation and increased permeability. Hypercellularity occurs as inflammatory cells (large granular lymphocytes, macrophages and γδ+ T cells) are recruited to the implantation site. Several proinflammatory cytokines are produced in the uterus and increased angiogenesis is noted. In primates and rodents the uterine epithelium is lost, and poorly characterized stromal cells, decidual cells, undergo transformation and proliferation. This decidua contains numerous inflammatory cells such as macrophages, lymphocytes, natural killer cells and lymphocytes of the γδ subtype. γδ T-cells have been shown to play a protective role on some mucosal surfaces and may be especially important in infections due to intracellular bacterial pathogens.

Early steps in placental development and cell differentiation may proceed similarly between species; however, the degree of invasion of uterine tissues and the final form the placenta takes are quite variable. In primates and rodents the trophoblast invades maternal vasculature in the submucosa creating a hemochorial placenta. In the pig, the trophoblast is noninvasive and forms a diffuse apposition to the uterine epithelium resulting in a diffuse epitheliochorial placenta. In ruminants, the trophoblastic epithelium is noninvasive; however, some binucleate trophoblastic cells fuse with uterine epithelial cells forming
syncytia which then release granules of placental lactogen into the maternal circulation. Syncytia formation is more pronounced in small ruminants such as sheep and goats than in cattle. Intimate contact between chorionic trophoblast and uterine epithelium occurs in ruminants in specialized structures termed placentomes. The maternal contribution, the uterine caruncles, occur in four rows more or less evenly spaced in the uterus and are present in both uterine horns. The bovine uterus contains approximately 100-160 caruncles. The size of the placentome is dependent on stage of pregnancy and position in the uterus. Nonpregnant, multiparous females and virgin heifers have well-defined caruncles composed of small cells with small, dense oval or spindle-shaped nuclei. Ultimately, these cells are the source of growing maternal connective tissue during the development of the placentome.

The expanding chorioallantoic membrane of the early embryo is apposed closely to the uterine wall, and in those areas apposing caruncles, the chorioallantoic membrane enters narrow spaces within the caruncle. The chorioallantoic membrane associated with caruncles forms thickenings described as "milky patches". These fragile attachments between caruncle and chorioallantois occur as early as 33 days after ovulation. The mesoderm of these patches contains thin walled allantoic blood vessels with primitive nucleated erythrocytes. The caruncular tissue forms a crypt system composed of numerous deep and extensively branched crypts which open toward the chorioallantoic membrane. Initially the crypts are filled with cell debris and erythrocytes while being lined by uterine epithelial cells. The corresponding chorioallantoic membrane forms villi with loose mesenchymal cores which are well vascularized by allantoic blood vessels. Villi are covered by a simple layer of
trophoblastic epithelium. Trophoblastic epithelium comprises a heterogeneous population of erythrophagocytic, placentomal and chorioallantoic trophoblastic cells that differ in location and function. Chorioallantoic villi penetrate into a network of caruncular crypts. Villi advance through the caruncle until their tips reach the base of the caruncle. The trophoblastic and cryptal cells have surface microvilli which interdigitate with each other, thus increasing the surface area of contact between fetal and maternal tissues. Primary chorionic villi run deep and straight to the base of the placentome, decreasing slightly in diameter toward the base. Branches of secondary and tertiary villi form, and interdigitate with maternal crypts of primary, secondary, and higher order. The bases of the primary villi form arcades around club-shaped endings of maternal septa. Further growth of the placentome comes from enlargement of the diameter and length of existing villi and/or from extensive villus branching and increased surface corrugation.

In ruminants, the placentomal arcade zone is the area between the base of primary chorionic villi and the tips of maternal septa. Within this space there is often extravasated blood, autolyzed cells and uterine secretions. This material is often resorbed by the adjacent trophoblast cells. In sheep, this zone has been studied extensively where extravasation of blood is significant and can be seen grossly. In the bovine placenta the amount of extravasation is not as great; however, small deposits of blood are first noted in the third month of gestation and increase in volume during gestation. Erythrophagocytic chorionic trophoblastic epithelial cells in this zone contain erythrocytes in various degrees of degeneration as well as hemoglobin derived pigment. Erythrophagocytosis is thought to
be a major pathway of iron acquisition for the fetus as well as an important factor in the pathogenesis of placentitis and fetal infection in brucellosis infection in goats and sheep.

The intercotyledonary area is situated between placentomes and is comprised of a maternal side of glandular uterus lined on the surface by simple epithelium of varying height. The fetal side is represented by the allantochorion which is sometimes folded. The surface facing the maternal side is lined by trophoblast. Trophoblastic epithelial cells opposite the surface openings of uterine glands are also highly phagocytic, ingesting uterine secretions.

Pathogenesis of bovine abortion

Both maintenance and termination of pregnancy involve a complex interplay between fetus, placenta, uterus and various fetal and maternal endocrine control mechanisms. Any of these factors must be considered a potential target of any abortifacient. Further complicating the scenario are species differences concerning known abortifacients. For example, the abortifacient activity of PGF$_{2\alpha}$ in rats is largely due to the disturbance of luteal function while in guinea pigs, PGF$_{2\alpha}$ causes abortion due to oxytocic effects on uterine contractility.

The definitive mechanism of abortion in brucellosis is unknown. Fetal death and abortion may be due to interruption of nutrient and oxygen transfer by the placenta and subsequent fetal hypoxia, however, rats infected with *Brucella abortus* or *Listeria monocytogenes* showed large areas of placental necrosis but no fetal death. However, if placental destruction was extreme, survival was not possible. These findings suggest that more than disruption of the fetal maternal interface is involved in brucellosis abortion.
Another hypothesis involves the toxic effects of endotoxin, including increased uterine sensitivity to oxytocin following exposure to *Brucella* endotoxin.\(^{24}\) As early as 1943 endotoxin was found to be a potent abortifacient. Placental hemorrhage and abortion in mice and rabbits was demonstrated following intraperitoneal injections with *Shigella paradysenteriae*.\(^{93}\) It was hypothesized that decidual and placental capillaries have a much lower threshold of damage due to endotoxin than vessels of other organs.\(^{93}\) Investigators later described fibrinoid swelling in the placental interstitium in addition to placental hemorrhage and abortion in mice and rabbits following administration of endotoxin. These findings led to the suggestion that endotoxin caused a Shwartzmann-like reaction in the placenta resulting in abortion. This idea was later discounted when it was found, by other researchers, that there were areas of hemorrhage in the decidua basalis in untreated mice at term as well as endotoxin-treated mice.\(^{67}\) This finding suggested that this histologic change represented a mechanism by which normal placental separation proceeded and not a change specific for endotoxin-induced abortion. A finding which was not seen in control mice; however, was vascular engorgement of the placental labyrinth.

Cattle given LPS during gestation abort within 10 days; however, the fetus does not appear to play a significant role in endotoxin-induced abortion.\(^{31}\) Several parameters were evaluated in pregnant cattle following intravenous infusion of *Salmonella typhimurium* LPS using catheterized fetal and placental structures.\(^{31}\) Maternal responses included increases in PGF\(_{2\alpha}\), TNF\(\alpha\), ACTH, cortisol and decreases in leukocytes and progesterone with rapid clearance of endotoxin from maternal plasma. Fetal changes included increases in ACTH
and cortisol with delayed increases in PGE2 in amniotic and allantoic fluids and no elevations of fetal TNFα levels and no detection of LPS in fetal plasma. No gross or microscopic lesions were seen in the fetuses and fetal motion and blood O2 levels were normal up to the time of abortion. These findings do suggest; however, a possible role for increases in PGF2α, TNFα or cortisol in endotoxin induced abortion.

The luteolytic effect of PGF2α may play a more important role in early first trimester abortion rather than in abortions which occur during the second or third trimester, more typical of brucellosis.33 In cattle, pregnancy during early gestation is maintained primarily by progesterone secreted by the CL. In advanced gestation, extraluteal sources of progesterone, such as the placenta, predominate.51 One report showed cows in the first trimester of pregnancy are more sensitive to the abortigenic effects of LPS than are cows in the second or third trimester.33 The increased sensitivity of first trimester cows may be attributable to the lack of extraluteal sources of progesterone. The oxytocic effects of PGF2α; however, could play a role in abortion at any stage. In humans, LPS inhibits prostaglandin dehydrogenase, the prostaglandin inactivating enzyme creating a net increase in prostaglandins.74

Gram negative bacterial invasion of placental and uterine tissues in humans is characterized by the release of bacterial LPS and phospholipases from bacterial and placental sources. Phospholipases cause the release of arachidonic acid and subsequent elevations in prostaglandins as well as the induction of cytokines.78 Metabolism of PGE2 from human amnion cells has also been shown to increase during term as well as pathogen -induced
labor. The role of prostaglandins in the initiation of term and preterm parturition are supported by several observations: 1) prostaglandins cause cervical ripening and uterine contractions; both necessary preparatory changes for fetal expulsion, 2) the precursor of prostaglandins, arachidonic acid, is found in increased levels in the amniotic fluid of laboring women, 3) injection of arachidonic acid into the amnion stimulates labor, and 4) inhibitors of prostaglandin synthesis inhibit parturition.

In addition to the effects of LPS mediated through prostaglandins, maternal endotoxemia results in the stimulation of both the fetal and maternal hypothalamic-pituitary-adrenal axes. This results in a sharp rise in fetal and maternal adrenocorticotrophic hormone (ACTH) and cortisol. Activation of this response in normal term pregnancies in ruminants is thought to initiate the physiologic process of parturition.

Increased cortisol values have been noted in fetuses inoculated with virulent B. abortus as early as PID 6; therefore, this may play a role in premature labor as Brucella organisms multiply within trophoblastic cells. As trophoblastic cells are shed into the inflammatory exudate, high numbers of organisms can potentially be achieved. As local LPS and prostaglandin concentrations increase, parturition may be induced.

A strong correlation between prostaglandin levels with TNF-α and IL-1 levels in amniotic fluid has been noted in women during preterm labor. It is well known that LPS can cause the release of cytokines such as TNF-α and IL-1 from macrophages; furthermore, TNF-α stimulates prostaglandin synthesis by several cell types. The significance of uteroplacental TNF-α is not entirely clear, but it likely involves the following; regulation of
cell proliferation at the uteroplacental interface (TNF-α inhibits DNA synthesis of
trophoblastic cells), regulation of expression of MHC-I antigens necessary for the binding of
many hormones and growth factors, and elevated levels of TNF-α have been implicated in
the initiation of preterm labor associated with infection in women.5,92

In humans epidemiological, bacteriologic and histologic evidence for the association
of bacterial infection and preterm labor exists. Prenatal association of specific infectious
agents and preterm labor are suggestive but inconsistent. These agents include, *Neisseria
gonorrhoea*, group B streptococci, *Chlamydia trachomatis*, *Mycoplasma hominis*, *Ureaplasma
urealyticum*, and *Trichomonas vaginalis*.52

Histologic evidence of an association of infection and inflammation with preterm
labor has been reported.43 Histologic findings such as these, as well as bacteriologic findings
can be debated on the argument that bacteriologic and tissue changes may have occurred after
labor. However, studies in which measurements of the onset of uterine contractions and
membrane integrity were controlled, show that histologic and microbiologic changes occur
prior to the initiation of parturition.44

A potential model is hypothesized in which bacterial infection of uterine and/or
placental tissues occurs. Bacterial factors such as LPS activate the host monocyte
macrophage system to produce TNF-α as well as other proinflammatory cytokines. TNF-α
and other cytokines stimulate the release of prostaglandins from intrauterine tissues which
signal the initiation of parturition.71 This model is supported by the detection of measurable
TNF-α in >90% of women with preterm labor and amniotic fluid from which bacteria were
cultured. TNF-α was not detected in amniotic fluid from women during the second and third trimesters who were not in labor.⁷¹,⁷² This model is not without opponents. Other researchers have found that amniotic fluid TNF-α levels increase during pregnancy and normal parturition as well as preterm labor.⁴⁵ This same study showed no detectable changes in plasma TNF-α of pregnant women at any time during gestation.

The cellular origin of uteroplacental TNF-α is also not completely understood. In vitro studies have shown the release of TNF-α by decidual and chorionic villi explants, cytotrophoblast cells in culture, dispersed amniochorion cells and macrophages isolated from chorionic and decidual tissues.⁴⁵

In ruminants, TNF-α levels rise following the intravenous infusion of endotoxin.¹,⁴¹ Chronic administration of endotoxin to cattle results in depression, cachexia, and diarrhea with renal hemorrhage and interstitial nephritis, thymic atrophy, myocardial atrophy and subendocardial hemorrhage, skeletal muscle atrophy, serous atrophy of fat, lymphoid depletion, hepatic necrosis and vacuolar change, pancreatic necrosis and ileal villus atrophy.⁶³ In periparturient cattle, isolated mononuclear cells are more responsive to the effects of endotoxin, producing more TNF-α, than those of cows in mid to late lactation.⁷⁹ Furthermore, supramammary lymph node mononuclear cells were more responsive to endotoxin than those from the peripheral blood.⁷⁹ The role of increased mononuclear cell responsiveness to the effects of endotoxin in periparturient diseases such as late term abortion, endometritis and mastitis is unclear; however, it may provide insight into the high prevalence of these periparturient diseases of cattle.
In addition to the uterine synthesis of prostaglandins stimulated by proinflammatory cytokines, the luteolytic action of PGF$_{2\alpha}$ may also be mediated through TNF-$\alpha$. Using a procedure known as continuous flow microdialysis, it has been shown \textit{in vivo}, that TNF-$\alpha$ is increased in luteal tissue coincident with a decline in progesterone.\textsuperscript{76} Immunohistochemical studies have localized the source of luteal TNF-$\alpha$ to macrophages within the CL. These findings suggest that the luteolytic action of PGF$_{2\alpha}$ may, in part, be mediated through TNF-$\alpha$.

Tumor necrosis factor is increased in the milk of dairy cattle near parturition.\textsuperscript{68} Milk TNF-$\alpha$ levels peak 4 to 6 weeks prior to parturition and decrease to undetectable levels at parturition. Following parturition, TNF-$\alpha$ levels increase rapidly and maintain a midlevel concentration throughout lactation.\textsuperscript{68} It is not noted if plasma TNF-$\alpha$ levels follow a similar pattern as those seen in the milk; however, it is assumed that the source of the TNF-$\alpha$ is the increased number of macrophages in milk which parallel the increase in TNF-$\alpha$.\textsuperscript{68}

Dissertation Organization

This dissertation is prepared in the alternate format and includes manuscripts prepared and submitted to refereed scientific journals. The format used in the individual chapters is consistent with the scientific journal to which the manuscript was submitted. The general introduction and general conclusion chapters are prepared in a format consistent with \textit{Veterinary Pathology}. Literature cited in the general introduction and general conclusion sections appears at the end of the respective section.
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CHAPTER 2. EXPERIMENTAL INFECTION OF PREGNANT CATTLE
WITH THE VACCINE CANDIDATE BRUCELLA ABORTUS STRAIN RB51:
PATHOLOGIC, BACTERIOLOGIC AND SEROLOGIC FINDINGS

A paper published in Veterinary Pathology 1996;33:682-691

Mitchell V. Palmer, Norman F. Cheville, and Allen E. Jensen

Abstract-
To determine the placental tropism and abortigenicity of the vaccine candidate, B. abortus strain RB51 (SRB51), a rough mutant of the virulent strain 2308, 10 Polled Hereford heifers were inoculated intravenously in the sixth month of gestation. Heifers were euthanatized and examined at 8 weeks p.i. (n=5) or at full term (n=5). Four of five infected heifers sampled at 8 weeks p.i. and 3/4 infected heifers at term had placentitis while reproductive tissues of 3 normal cows used for comparison had no placentitis. Numerous macrophages, immunoreactive for SRB51 antigen, as well as neutrophils, fibrin and cell debris filled the arcade zone between chorion and maternal septae. Trophoblastic epithelium of the placentomal arcade zone had intracellular bacteria that were immunoreactive for SRB51 antigen. The tips of maternal septae had a lymphoplasmacytic infiltrate with small multifocal erosions and ulcerations of maternal epithelium. SRB51 was cultured from all tissues in which lesions were seen. Placentae of one cow from each group had no placentitis and contained no SRB51. In mammae, interstitial lymphoplasmacytic infiltrates and suppurative infiltrates within alveoli and intralobular ductules were seen in 2/5 heifers at 8 weeks p.i. SRB51 was cultured from liver, spleen, lung and bronchial lymph nodes in 4/5 calves at 8 wks. p.i. and 3/4 full term calves, but no lesions were seen. One term heifer had disseminated infection, placentitis, lymphoplasmacytic endometritis, and delivered a
premature weak calf. These results establish that SRB51 is less abortifacient than previously published reports with strain 19, in that only 1/4 heifers delivered prematurely following intravenous inoculation with SRB51 while intravenous inoculation with strain 19 leads to 100% abortion. However, it also shows that SRB51 can infect the bovine placenta, mammary gland and fetus; can induce placentitis and in some cases, lead to preterm expulsion of the fetus.

Introduction-

The eradication of bovine brucellosis has centered on the use of *Brucella abortus* strain 19 (S19), an attenuated, live vaccine introduced into the federal brucellosis eradication program in 1940. Although effective and important in the control of bovine brucellosis, S19 has significant limitations including virulence for humans, ability to induce abortion in pregnant cattle, and the development of agglutinating antibodies indistinguishable from those seen in naturally infected animals.

Experimental intravenous infection of pregnant cattle using S19 typically leads to placentitis and abortion; e.g. in two separate studies abortions occurred in 100% of the cows. Abortion occurred as early as 16 days and as late as 42 days postinoculation. Additionally, in one of the preceding studies, *B. abortus* strain 45/20, given intravenously caused abortion in 100% of the cows between days 9 and 47 after inoculation. A separate study utilizing *B. abortus* strain 544 resulted in abortion in 100% of the cows in 3-4 weeks when given intravenously. *B. abortus* S19 has also been shown to induce abortion in a small percentage of pregnant cattle vaccinated subcutaneously with the normal vaccine dose.

*B. abortus* strain RB51 (SRB51) is a stable rough mutant of *B. abortus* strain 2308 (S2308), which lacks much of the LPS O-side chain, and has been investigated as an alternative vaccine to S19. A major concern in evaluating SRB51 is the possibility of
tropism for the bovine placenta and subsequent abortion. In mice, SRB51 has been shown to produce a mild to minimal placentitis which is not associated with fetal death. Although SRB51 did localize in the giant trophoblast cells of the mouse placenta similar to S19 and the virulent S2308, placental damage and colonization by SRB51 were markedly less than with either S19 or S2308. Further testing has shown that SRB51 did not induce abortion in pregnant goats when fetuses were inoculated intramuscularly with SRB51. SRB51 was found to colonize bovine chorioallantoic membrane explants in a manner similar to S19 and S2308; however, the degree of cytotoxicity associated with SRB51 infection was much less than that measured for S2308 infection and not significantly different from that seen with S19.

The abortifacient potential of SRB51 in pregnant cattle has not been determined. It is essential that SRB51 or any other vaccine candidate be tested in cattle to determine the ability to not only cause abortion but any other pathologic changes which may be considered detrimental. The purpose of this study is to determine the potential for abortion, placental infection, placentitis and fetal infection in pregnant cattle infected with SRB51.

Materials and Methods-

Ten virgin Polled Hereford heifers used in the experiment were obtained from nonvaccinated, brucellosis-free herds. Six heifers were 27-months old while the remaining 4 were 16 months old at breeding. All cattle were serologically negative for brucellosis by standard tube agglutination. Upon arrival, cattle were kept on pasture and bred by natural service using a Polled Hereford bull which was also serologically negative for brucellosis by standard tube agglutination. During challenge, animals were housed individually as described in concrete isolation rooms with positive air flow from a clean corridor and negative air flow to a contaminated corridor.
Normal tissue for comparison was obtained from a meat packing plant. Three reproductive tracts containing fetuses estimated by fetal crown-rump length to be approximately 240 days gestation were collected, processed for light microscopy and examined.

At 6 months of gestation, heifers were injected intravenously in the right jugular vein with $10^{10}$ CFU *B. abortus* strain RB51 suspended in 10 ml sterile saline (0.85% NaCl). At postinfection week 8 (PIW), 5 heifers (3 from older group and 2 from younger group) were randomly selected, euthanatized and examined. The remaining cattle were examined at term (n=3) or following premature delivery (n=1). Samples of maternal uterus, placentomes, intercotyledonary placenta, mammary gland, supramammary lymph node, liver, spleen, and fetal liver, spleen, lung and bronchial lymph node were obtained for bacteriologic culture, histopathology and immunohistochemical staining for SRB51 antigen. Maternal blood, milk, vaginal swab, allantoic and amniotic fluids and fetal blood, cerebrospinal fluid, abomasal contents and rectal swab, were also collected for bacterial culture. All cattle were euthanatized by intravenous administration of sodium pentobarbital.

Following a premature delivery, heifer (#9) was treated with oxytetracycline (Liquamycin LA-200, Pfizer, New York, N.Y.), 11mg/kg, SID for 5 days prior to necropsy. Treatment of cows aborting or delivering premature calves has been standard protocol in our laboratory as a matter of personnel safety due to the zoonotic potential of *B. abortus* and the unknown virulence of SRB51 for humans.

*Histologic and Immunohistochemical analyses*- Tissue specimens were placed in 10% neutral buffered formalin. Tissues were processed by routine paraffin embedment techniques, cut 4-6 μm thick and stained with hematoxylin and eosin. Unstained sections were used for immunohistochemical staining of SRB51 antigen using an avidin-biotin-alkaline phosphatase complex staining technique as described and a commercially
available kit (Histomark, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

Primary antibodies were prepared by hyperimmunization of rabbits with whole cell, irradiated SRB51. Anti-SRB51 antisera was used at a dilution of 1:1000. Optimal dilutions for the primary antibody had previously been determined using formalin-fixed, paraffin-embedded sections of SRB51-infected murine spleens or bovine lymph nodes. Control slides were stained with serum from clinically normal rabbits. Sections of SRB51-infected BALB/c mouse spleen and liver were used as positive control slides. Staining of bovine macrophages using the monoclonal antibody EBM 11 (anti-CD68) (Dako, Carpenteria, CA) was done as described.

Samples of placentome and intercotyledonary placenta were also collected, embedded in O.C.T. compound (Tissue Tek, Miles Scientific, Naperville, IL) and immediately frozen by immersion in liquid nitrogen. Frozen sections of intercotyledonary placenta and placentome were processed and stained with monoclonal antibodies to CD4, CD8 and γ/δ lymphocyte surface markers as described.

Bacteriologic analysis - B. abortus strain RB51 was used in this study. SRB51 was prepared from master seed stock of B. abortus RB51 (ARS-1) at the Brucellosis Research Unit, National Animal Disease Center, Ames, IA. SRB51 was propagated from working seed stock on tryptose agar and incubated at 37°C for 48 hours. The surface of the agar was flooded with saline and the suspended bacteria were collected by aspiration, twice washed by centrifugation and resuspended in saline. Bacterial suspensions were subsequently diluted to the appropriate concentration prior to inoculation.

Tissue samples to be tested for SRB51 were processed as described using tryptose agar with 5% bovine serum, cyclohexamide (30μg/ml), bacitracin (7.5 U/ml), polymyxin B sulfate (1.8 U/ml) and ethyl violet (1:300,000). Brucella colonies were identified as described previously.
Collection and handling of blood for culture- At time periods corresponding to postinoculation day (PID) 1-6 and postinoculation week (PIW) 1-12, 100 ml of blood was collected aseptically for culture from jugular veins on alternating sides beginning with the side contralateral to the initial injection. The samples consisted of one 125 ml serum vial containing 50 ml of blood and 50 ml of blood culture media, and 5 - 20 ml tubes each containing 10 ml of blood and 10 ml of blood culture media. Upon receipt, 5 ml of blood was drawn aseptically from the 125 ml serum bottle and 1 ml was inoculated on to each of 5 plates containing tryptose agar with 5% bovine serum. These plates were incubated at 37°C and checked at 24 hrs., 3, 7, 10 and 14 days for bacterial growth. Any suspect growth was subcultured and processed for SRB51 identification. The remaining sample in the 125 ml serum vial was incubated at 37°C and subcultured as above on days 3, 10, 20, 30 and 40. The 5 serum tubes were immediately placed at -20°C and kept until placed in biphasic flasks. Frozen blood was thawed at 37°C and transferred to biphasic culture flasks containing tryptose agar and processed as described. These flasks were checked for bacterial growth at 24 hrs., 3, 7, 10, 20, 30 and 40 days. Suspect growth was subcultured and processed for SRB51 identification. Calf blood was examined similarly at necropsy.

Serological analysis- Twenty milliliters of blood was collected by venipuncture from each heifer prior to inoculation and PIW 1, 2, 4, 6, 8, 10 and 12. Blood was collected into sterile 10-ml tubes, allowed to clot for 6-24 hrs. at 4°C, and centrifuged. Serum was divided into 2 ml aliquots, frozen, and stored at -70°C. Blood was similarly collected from the calves at necropsy. Samples were later thawed and tested. The standard tube agglutination test was performed on all samples. Antibodies to SRB51 were measured by use of an antibody dot blot assay using anti-bovine IgG, heavy chain specific and anti-bovine IgM.
antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) at dilutions of 1:1,000 and 1:100 respectively.

Results -

Clinical Signs - Eight of ten cattle were febrile during the first 24-48 hours after inoculation. Elevated temperatures ranged from 39.1°C to 41.1°C (normal range 38.5±0.5°C). Six of eight heifers returned to normal body temperature by 24-48 hours after inoculation; however, two heifers (#4 and #9) had elevated body temperatures which persisted for 72 hrs., being normal by 96 hours after inoculation. Febrile responses were not accompanied by other notable clinical signs. All cattle remained on feed and no obvious signs of depression were noted.

One heifer (#9) delivered a premature, small, weak calf, one month prior to the expected calving date (PID 59). The calf was unable to stand or nurse and had a weak suckling reflex. The dam was afebrile at the time of delivery.

Bacterial Culture of Blood - SRB51 was detected in blood in one or more sampling times up to 6 days after inoculation in 8/10 cattle, however, no organisms were cultured beyond that time (Table 1). Two of ten heifers showed no SRB51 in the blood at any sampling time. Calf blood culture revealed 2/10 calves contained SRB51 in the blood (Table 2).

Bacterial Culture of Tissues and Fluids - Four of five heifers (80%) sampled at PIW 8 had SRB51 in placentomes, intercotyledonary placenta, uterus and spleen (Table 3). Mammary tissue and supramammary lymph nodes from 2/5 (40%) yielded SRB51. One
Table 1. Isolation of *B. abortus* strain RB51 from blood\(^a\) of pregnant cattle inoculated i.v. at six months of gestation.

<table>
<thead>
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<th>ID</th>
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<th>PID</th>
<th>PID</th>
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<tbody>
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<td>+</td>
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<td>ND</td>
</tr>
<tr>
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<td>-</td>
<td>+(^c)</td>
<td>-</td>
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<td>10</td>
<td>-</td>
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</tr>
</tbody>
</table>

\(^a\) Unless otherwise stated, *B. abortus* RB51 isolation was successful only after culture enhancement (see text for details).

\(^b\) ND = cow not tested on specified day.

\(^c\) *B. abortus* RB51 isolated from direct plating, \(4.16 \times 10^2\) CFU/ml blood.

\(^d\) *B. abortus* RB51 isolated from direct plating, one colony.
heifer was free of SRB51 by PIW 8. The calf from this heifer was also free of SRB51 in all tissues and fluids cultured. Bacteriologic culture of 4/5 (80%) calves from heifers euthanatized at PIW 8 showed SRB51 in liver, spleen, lung and bronchial lymph node (Table 2).

Three of four heifers (75%) which went to full term had SRB51 in samples of uterus, placenta and placentome (Table 3). One heifer which went to full term was negative for SRB51 in all tissues and fluids cultured. The calf from this heifer was similarly free of SRB51. All other full term calves had SRB51 in spleen and bronchial lymph nodes. Two of four calves (50%) also had SRB51 in lung (Table 2).

After the premature delivery of a weak calf, SRB51 was found in milk samples from all four mammary quarters of heifer #9. A vaginal swab and samples of expelled placenta also contained SRB51. After oxytetracycline therapy for 5 days, SRB51 was still recovered from intercaruncular uterus and caruncle. No SRB51 was found in milk or mammary tissue following antibiotic therapy (Table 3). The premature weak calf was euthanatized within 8 hours of delivery and examined. All samples cultured from the calf contained SRB51 except cerebrospinal fluid and a rectal swab specimen (Table 2).

Serology—All heifers and calves had no antibody titers as determined by standard tube agglutination at all time points sampled. Serologic results measured by dot blot assay are reported in Table 4. No calf from either group had an antibody titer, as measured by the dot blot assay, for anti-SRB51 IgG antibody. The premature calf (#9) had an IgM titer to SRB51 of 1:160 at necropsy. Other calves had no anti-SRB51 IgM antibody titer. Anti-IgM titers were not evaluated on the heifers.
Table 2. Summary of culture results to isolate *B. abortus* RB51 from calves of pregnant heifers inoculated i.v. with RB51 at six months of gestation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PIW 8</th>
<th>Full term</th>
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<tbody>
<tr>
<td></td>
<td>Animal Number</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1  2  3  4  5  Total</td>
<td>6  7  8  9  10  Total</td>
</tr>
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<td>Liver</td>
<td>-    +    +    +  ND* 3/4</td>
<td>+   -   -   +   + 3/5</td>
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<tr>
<td>Spleen</td>
<td>+    +    +    +    - 4/5</td>
<td>+   -   -   +   + 3/5</td>
</tr>
<tr>
<td>Lung</td>
<td>+    +    +    +    - 4/5</td>
<td>+   -   -   +   - 2/5</td>
</tr>
<tr>
<td>Bronchial LNb</td>
<td>+    +    +    +    - 4/5</td>
<td>+   +   -   +   + 4/5</td>
</tr>
<tr>
<td>Blood</td>
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<tr>
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<tr>
<td>Abomasal</td>
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<td>-   -   -   NAd 1/4</td>
</tr>
<tr>
<td>Contents</td>
<td>Rectal Swab</td>
<td>-    -    +    -    - 1/5</td>
</tr>
</tbody>
</table>

*Not done.*

b Tracheobronchial lymph node.

c Cerebrospinal fluid.

d Not available for culture.
Table 3. Summary of culture results to isolate *B. abortus* RB51 from pregnant heifers inoculated i.v. at six months of gestation and examined at PIW 8 or at full term.

<table>
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Table 3 (continued). Summary of culture results to isolate *B. abortus* RB51 from pregnant heifers inoculated i.v. at six months of gestation and examined at PIW 8 or at full term.

<table>
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<tr>
<td>VS*</td>
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</tbody>
</table>

Pre and post samples refer to oxytetracycline treatment given after premature delivery of a weak calf.

NA = not available for culture.
ND = not done.
Mamm = mammary gland tissue.
LN = supramammary lymph node.
Allantoic fluid.
Amniotic fluid.
Vaginal swab
**Gross Lesions** - Mild placentomal edema was present in 4 of 5 cattle at PIW 8. The chorioallantois covering caruncles was mildly thickened, but translucent. Placentomes of 4/5 cattle at PIW 8 contained a small amount of tan, creamy exudate found between the chorioallantois and maternal tissues. This exudate was most prominent over caruncles although it was found less frequently in intercotyledonary areas. Gross lesions were not present in other organs. Amniotic and allantoic fluids appeared normal.

Placentae from 3/4 cows examined at full term were more edematous than those examined at PIW 8 with thick gelatinous edema of the intercotyledonary placenta which was slightly opaque but translucent. Extensive areas of creamy, tan exudate were between the chorioallantois and uterine epithelium within placentomes and intercotyledonary areas. Allantoic and amniotic fluids appeared normal.

The cow delivering a premature calf had cotyledons covered with a brown creamy exudate while the intercotyledonary areas were normal. Postparturient lochia contained small amounts of brown, creamy exudate.

**Microscopic Lesions** -

**PIW 8** - Placentitis was present in 4/5 cattle examined at PIW 8 (Figures 1,2). Infiltrates of numerous macrophages, neutrophils, necrotic cell debris and fibrinohemorrhagic exudate was seen in the placentomal arcade zone. Inflammatory infiltrate separated the tips of maternal septa from the trophoblast layer. Epithelial cells of the trophoblast contained abundant intracytoplasmic bacteria which were immunoreactive with anti-RB51 antibody (Figure 3). Many trophoblastic epithelial cells with intracellular bacteria were detached from the chorion and present in the inflammatory infiltrate. In some areas of intense inflammation, the trophoblast layer of the chorioallantois was flattened with cuboidal to squamous rather than columnar epithelial cells. Immunostaining for SRB51 showed intense staining within trophoblastic epithelial cells as well as within macrophages as a
Figure 1. Section of placentomal arcade zone from a heifer examined at PIW 8. Note extensive inflammatory infiltrate separating chorioallantois (short arrow) from maternal septa (long arrow). HE. Bar = 100 μm.

Figure 2. Section of placentome from a heifer examined PIW 8. Note chorionic villus surrounded by inflammatory cells and necrotic cell debris. Trophoblastic epithelial cells are enlarged and granular in appearance due to numerous intracellular bacteria (arrows). HE. Bar = 50 μm.
component of the inflammatory infiltrate. SRB51 immunoreactive macrophages were most prominent adjacent to the tips of the maternal septa and were rarely seen adjacent to the trophoblast layer (Figure 4). Neutrophils were seen in the tunicae intima and muscularis of arterioles of the chorioallantois with fewer located in the stroma of the chorioallantois. Endothelial cells of chorioallantoic arterioles were rounded with some containing clear, smoothly contoured cytoplasmic vacuoles. Bacteria immunoreactive for SRB51 were not seen within maternal septal epithelial cells of the placentome. Tips of maternal septa contained areas of lymphoplasmacytic infiltration with mild to moderate fibrosis. Small ulcerations of maternal septa were seen with loss of maternal epithelial cells, infiltrates of moderate numbers of neutrophils and small numbers of bacteria which were immunoreactive for SRB51 antigen. Intercotyledonary placenta had similar placentitis to that seen in placentomes with inflammatory infiltrate which separated maternal and fetal tissues.

Foci of endometritis subjacent to areas of placentitis contained infiltrates of numerous lymphocytes, plasma cells, macrophages and neutrophils. These infiltrates were most intense in the strata compactum and spongiosum but also extended deep to surround uterine submucosal glands. Immunostaining of T-lymphocytes in the endometrial inflammation revealed most to be of the CD4 type, with few present of the CD8 or γ/δ (CD4- CD8-) subtype (Figures 5a, 5b). Infiltrates of neutrophils and fibrin were in submucosal glands of the uterus (Figure 6). Low numbers of submucosal gland epithelial cells, neutrophils and macrophages with cytoplasmic staining for SRB51 antigen were seen within and surrounding submucosal glands.

Mammary glands had multifocal interstitial lymphoplasmacytic infiltrates and intraluminal infiltrates of large numbers of neutrophils within some alveoli and intralobular ductules. Supramammary lymph nodes had infiltrates of large numbers of macrophages and neutrophils in medullary sinuses which extended superficially to the deep cortex. Cortical regions contained prominent follicles with germinal centers.
Figure 3. Placentome from full term heifer, chorionic villus. Note extensive intracellular staining for SRB51 antigen. Avidin-biotin-alkaline phosphatase method, Gill's hematoxylin counterstain. Bar = 35 μm.

Figure 4. Placentome from full term heifer, maternal septa (arrow). Note extensive inflammatory cells occupying space between chorioallantoic membrane and maternal septa which stain positive for SRB51 antigen. Avidin-biotin-alkaline phosphatase method, Gill's hematoxylin counterstain. Bar = 50 μm.

Figure 5. Section of uterus from heifer at PIW 8. Periglandular lymphocytic infiltrate is composed primarily of CD4+ T-cells (a), with rare γ/δ T-cells (arrow)(b). Avidin-biotin-alkaline phosphatase method, Gill's hematoxylin counterstain. Bar = 35 μm.
Figure 6. Section of uterus from full term heifer. Note periglandular inflammation and intraluminal neutrophils. HE. Bar = 50 μm.
Maternal spleens had large numbers of macrophages with abundant cytoplasm which separated white pulp areas and obliterated red pulp. These cells stained strongly for the macrophage marker CD68. Areas of lymphoid follicle formation in white pulp were normal.

Calves examined at PIW 8 contained no significant microscopic lesions. In spleens, red pulp was well developed, periarteriolar lymphoid sheaths were small, and germinal centers were absent.

**Full term** - Placentae from 4/5 full term cows had placentitis resembling that seen at PIW 8. However, placentitis was more widespread and extended deeper into the placentome involving not only the arcade zone but primary chorionic villi and maternal epithelium and stroma. Bacterial colonies, immunoreactive for SRB51 antigen, were seen among the inflammatory infiltrate separating trophoblast from maternal septa. Immunostaining for SRB51 antigen showed results similar to those seen at PIW 8 with abundant staining of trophoblast epithelial cell cytoplasm. Similar microscopic lesions were observed in other organs as those seen in cows examined at PIW 8. As with the calves examined at PIW 8, full term calves showed no splenic germinal center formation and small periarteriolar lymphoid sheaths. No other gross or microscopic lesions were seen in full term calves.

Extensive necrosis was seen in placentomes from heifer # 9 which delivered a premature weak calf. Necrosis of chorioallantois and trophoblastic layers extended deep to the base of the placentome. Aggregates of basophilic coccobacilli between trophoblastic and maternal septal epithelium were immunoreactive for SRB51 antigen. Endometritis characterized by infiltrates of large numbers of lymphocytes, plasma cells and neutrophils was present in the superficial layers of the uterus as well as surrounding submucosal uterine glands. Multifocal small erosions and ulcerations of uterine mucosa with numerous neutrophils were also seen.
Mammary tissue from heifer #9 had alveoli containing moderate numbers of intraluminal neutrophils. Low numbers of lymphocytes were seen within the interstitium surrounding such alveoli. Supramammary lymph node changes in heifer #9 were consistent with those observed in cattle examined at PIW 8.

Lung from the calf from heifer #9 had very mild multifocal interstitial thickening due to infiltrates of moderate numbers of macrophages and neutrophils. These areas were often found but not limited to peribronchiolar regions. Increased numbers of cells immunoreactive for CD68 were seen within the interstitium. Bronchi and bronchioles were normal with no intraluminal or intraepithelial inflammation.

Placentomes from noninfected, normal cows did not contain areas of necrosis or inflammation. In some sections, small areas of hemorrhage were present within the arcade zone. Foci of hemorrhage were not associated with necrosis of trophoblastic epithelial cells or with infiltrates of inflammatory cells.

Discussion-

This study shows that SRB51 has a tropism for the bovine placental trophoblast and induces placentitis which can result in premature birth. Abortion is defined as the premature expulsion from the uterus of the products of conception, of the embryo, or of a nonviable fetus. Although the calf from heifer #9 was premature and weak, the judgement of potential viability is subjective. To avoid confusion we have classified this delivery as a premature, weak calf and not an abortion. The intravenous inoculation of heifers in the sixth month of gestation represents a stringent test of virulence for any brucellosis vaccine candidate. It is unlikely that SRB51 would be given intravenously in field situations. However, despite this stringent test, lack of abortion shows that SRB51 is less virulent than other brucellosis vaccine strains, including S19, when administered intravenously. This decreased virulence in pregnant cattle contributes to the attractiveness of SRB51 as a vaccine for the
prevention of bovine brucellosis. Moreover, lack of antibodies which are detected by brucellosis surveillance tests, e.g. standard tube test, allows differentiation of SRB51-vaccinated animals from naturally infected animals.\textsuperscript{42} Such differentiation is not possible following S19 vaccination.

SRB51-induced placental and uterine lesions in our cattle are similar to those reported in cattle and other species infected with virulent \textit{B. abortus},\textsuperscript{27,33} and are consistent with the role of the trophoblast epithelium as the primary site of infection.\textsuperscript{5,39} Placental inflammation and trophoblast epithelial cell necrosis could allow passage of bacteria into the allantoic sac. Such a mechanism could explain the presence of SRB51 in allantoic fluid of 40\% of our heifers while the amniotic fluid was infected in only 10\%. The amniotic membrane is not in intimate association with maternal tissues making extension of infection from the trophoblast layer less likely. The disseminated infection of many of the fetuses as well as the chorioallantoic vasculitis suggest that hematogenous spread of infection also plays a role in fetal infection.

T-lymphocytes of the CD4 type are known to be important in infections with intracellular bacteria.\textsuperscript{19} The activation of CD4\(^+\) T-cells and resultant secretion of IFN-\(\gamma\) have been shown to be important in the protection of mice infected with other intracellular pathogens such as \textit{Listeria monocytogenes} and \textit{Mycobacteria} spp.\textsuperscript{19} The presence of numerous CD4\(^+\) T-cells in the endometritis seen in the present study is consistent with a role for CD4\(^+\) T-cells in the response to SRB51. Although IFN-\(\gamma\) levels were not measured in our cattle, IFN-\(\gamma\) has been shown to be important in the control of intracellular growth of SRB51 in murine macrophages\textsuperscript{18} as well as the infection of murine spleens with S2308.\textsuperscript{43}

\(\gamma/\delta\) T-cells do not appear to play a major role in SRB51-induced endometritis. The rare identification of \(\gamma/\delta\)-T-cells in our study differs from the proposed mechanism of \(\gamma/\delta\)-T-cells in protection against intracellular pathogens in other species.\textsuperscript{7} In mice, \(\gamma/\delta\) T-cells colonize various tissues, including lung, tongue, skin, intestine, uterus, vagina and placenta.\textsuperscript{7}
In *M. tuberculosis*-infected mice deficient in γ/δ T-cell receptors, pulmonary infection is disseminated and not confined in granulomata as it is in genetically normal control mice or mice deficient in only the α/β T-cell receptor. This suggests a role for γ/δ T-cell in the containment of *M. tuberculosis* infection. A similar protective role has been seen in mice infected with *L. monocytogenes*.

Extrapolation of results regarding lymphocyte subsets in other species to cattle may not be appropriate. In contrast to mice, γ/δ T-cells comprise a significantly higher proportion of peripheral blood mononuclear cells in ruminants. Although the precise role and significance of γ/δ T-cells in ruminants is not clear, one study has shown that numbers of γ/δ T-cells are not altered in draining lymph nodes following subcutaneous inoculation of SRB51. It may be possible that γ/δ T-cells are not major effector cells in SRB51-induced inflammation; however, in the present study, the lack of periodic analysis of γ/δ T-cell involvement over the course of infection does not rule out the early involvement and/or possible destruction of γ/δ T-cells in SRB51-induced endometritis.

The incidence of fetal lung lesions in *Brucella*-infected, aborted fetuses or premature weak calves is highly variable, and is characterized as bronchitis or bronchopneumonia with predominantly mononuclear infiltrates. Interstitial lesions similar to those seen here have been reported.

Persistence of SRB51 infection to the termination of our study at 14 weeks differs from previous studies with SRB51 in which SRB51 was cleared by 6-weeks after inoculation in subcutaneously inoculated heifers. Prolonged persistence may be due to differences in inoculation route, dosage or pregnancy status. Route of inoculation has been shown to influence the outcome of infection. Subcutaneous inoculation with *B. abortus* strain 45 is innocuous while intravenous inoculation results in abortion. Additionally, our inoculum dose, 10^10 CFU, is slightly higher than the dose of 5 to 7 x 10^9 used in previous vaccination studies. The pregnancy status of cattle at inoculation also plays an important role in the
development of infection as other *Brucella abortus* strains have been shown to have a
tropism for the placental trophoblast.\(^5,39\)

In contrast to the persistence in tissue, lack of persistence of SRB51 in blood samples
in the current study is possibly due to increased phagocytosis and killing of rough strains as
compared to smooth strains.\(^12,15,34\) Previous studies have isolated virulent smooth *B. abortus*
from pregnant cows 52 - 62 days following intravenous inoculation.\(^12\) SRB51 is more
sensitive to the bactericidal effects of complement when compared to smooth strains.\(^10\)
These differences have been attributed to the lack of O-side chain on SRB51.\(^10,13\)

Weak or negative serologic responses to SRB51 by perinatal calves are consistent
with previous findings in SRB51 vaccinated cattle.\(^9\) Morphologic evidence for this lack of
response was evident in the present study as splenic germinal center formation was absent in
all calves examined. Although anatomically the components of the fetal immune system may
be present by day 175 of gestation,\(^45\) fetal changes responsible for immunologic maturity
may not occur at the same time for all antigens.\(^31\) In a previous study, bovine fetuses
experimentally infected with the smooth S2308 had increased levels of both IgM and IgG.\(^14\)
Being a rough derivative of S2308, it is possible that the rough nature of SRB51 may
contribute to decreased immunogenicity in fetuses.

References -

1. Ackermann MR, DeBey BM, Stabel TJ, Gold JH, Register KB, Meehan JT: Distribution
   of anti-CD68 (EBM11) immunoreactivity in formalin fixed, paraffin-embedded bovine


CHAPTER 3. SAFETY AND IMMUNOGENICITY OF THE VACCINE *BRUCELLA ABORTUS* STRAIN RB51 IN PREGNANT CATTLE

A paper submitted to the *American Journal of Veterinary Research*
(In press, accepted for publication 11/19/96)

Mitchell V. Palmer DVM, Steven C. Olsen DVM, PhD, and Norman F. Cheville DVM, PhD

**Objective**- Determine the safety and immunogenicity of *Brucella abortus* strain RB51 as a vaccine in pregnant cattle.

**Animals**- Twelve Polled Hereford heifers obtained from a brucellosis-free herd and bred on site at 16 months of age with a brucellosis-free bull.

**Procedure** - Pregnant heifers were vaccinated SC at 6 months’ gestation with $10^9$ CFU *Brucella abortus* strain RB51 (n=5), $3 \times 10^8$ CFU *B abortus* strain 19 (n=5), or sterile pyrogen free saline (n=2). Periodic blood samples were collected for serology and lymphocyte blastogenesis assays. At full gestation, heifers were euthanatized and maternal and calf tissues and fluid specimens were collected for bacteriologic culture, histologic analysis, and lymphocyte blastogenesis using various antigenic stimuli ($\gamma$-irradiated strain RB51, $\gamma$-irradiated strain 19, strain RB51 LPS or strain 2308 LPS).
Results- None of the strain RB51- or strain 19-vaccinates aborted or had gross or microscopic lesions at necropsy consistent with brucellosis. Two of five strain RB51-vaccinates had strain RB51 in superficial cervical lymph nodes draining the site of vaccination. No other maternal or calf tissues contained strain RB51. Maternal PBMC from strain RB51-vaccinates and strain 19-vaccinates showed proliferative responses to both γ-irradiated strain RB51 and strain 19 which were greater than nonvaccinated controls. In contrast, maternal superficial cervical lymph node cells from strain 19-vaccinates, but not strain RB51-vaccinates, demonstrated proliferative responses to γ-irradiated strain RB51 or strain 19 bacteria that were greater than that seen in nonvaccinated controls. PBMC from calves of strain RB51- and strain 19-vaccinates showed proliferative responses greater than nonvaccinated controls to irradiated strain RB51, strain RB51 LPS, and strain 2308 LPS. None of the heifers vaccinated with strain RB51 developed antibodies detected by the standard tube agglutination test, but all developed antibodies to strain RB51 that reacted in a dot ELISA test using irradiated strain RB51 as antigen.

Conclusions- Pregnant cattle can be safely vaccinated with $10^9$ CFU strain RB51 without subsequent abortion or placentitis. Furthermore, strain RB51 is immunogenic in pregnant cattle and induces humoral and cell mediated responses.
Clinical relevance- Safety and immunogenicity of *B. abortus* strain RB51 in pregnant cattle, with the absence of antibodies that interfere with serologic diagnosis of field infections, make it a desirable vaccine to be used in situations in which whole herd vaccination is necessary.

Brucellosis is an economically important abortifacient disease of cattle caused by the bacterium *Brucella abortus*. Vaccination of heifers with the vaccine *Brucella abortus* strain 19 (S19) has been integral to the decreased prevalence of bovine brucellosis in the United States since the inception of the brucellosis eradication effort in 1940. Although effective as a vaccine, S19 has several limitations including the ability to induce abortion in pregnant cattle.

Another limitation which continues to interfere with brucellosis eradication efforts is the production of antibodies following S19 vaccination which are indistinguishable on brucellosis surveillance tests from those produced by natural infection with field strains.

Limitations of the current brucellosis serologic tests makes the distinction of naturally infected animals from S19-vaccinates problematic.

*Brucella abortus* strain RB51 (SRB51), a rough mutant of the virulent *Brucella abortus* strain 2308, has recently been approved in the United States for use as a brucellosis vaccine for calves. SRB51 has been shown to be protective in cattle and does not produce antibodies which cross react on serologic surveillance tests, enabling the distinction between vaccinated and naturally infected animals. The possibility of placental and fetal infection with SRB51 was established by the experimental IV inoculation of pregnant cattle with very high numbers of SRB51, however, abortion was not observed. The question of
placental tropism when SRB51 is given in lower numbers and by the recommended route, i.e., SC inoculation, has not been established.

This study was designed to determine the safety and immunogenicity of SRB51 in pregnant cattle when the vaccine is administered by the recommended route, using a dosage which has been used in previous field investigations. Both humoral and cell mediated immune responses were analyzed as a measure of immune responsiveness.

Materials and Methods
Polled Hereford heifers used in the experiment were obtained from a nonvaccinated, brucellosis-free herd. Heifers were 16 months old at breeding. Prior to shipment and again prior to vaccination, all cattle were serologically negative for brucellosis by the standard tube agglutination test (STAT). Upon arrival, and throughout the experiment, cattle were kept on pasture. Breeding was by natural service using a Polled Hereford bull which was serologically negative for brucellosis as determined by STAT.

At six months' gestation, animals were randomly divided into 3 groups and injected with 9.4 x 10⁹ CFU SRB51 (n=5), 3 x 10⁷ CFU S19 (n=5), or 2 ml sterile pyrogen free saline, (n=2). All animals were injected SC in the right prescapular region. The dosage of SRB51 used previously in adult vaccination studies is 10⁹ CFU, while the recommended adult dose of strain 19 is 3-10 x 10⁸ CFU.
Body temperatures were recorded daily for the first four days after vaccination and animals were observed twice daily for the duration of the experiment for adverse reactions or clinical signs.

Necropsy- All cattle were euthanatized at full gestation by IV administration of sodium pentobarbital. Samples of maternal uterus, placentome, intercotyledonary placenta, mammary gland, supramammary lymph node, liver, spleen, and calf liver, spleen, lung and bronchial lymph node were obtained for bacteriologic culture and histologic analysis. Immunohistochemical staining for SRB51 antigen was done on tissues from SRB51-infected heifers and calves. Maternal blood, milk, vaginal swab specimens, allantoic and amniotic fluids and calf blood, cerebrospinal fluid, abomasal contents and rectal swab specimens, were also collected for bacterial culture.

Histologic and Immunocytochemical analyses- Tissue specimens were placed in neutral buffered 10% formalin. Tissues were processed by routine paraffin embedment techniques, cut 4 to 6 μm thick and stained with H&E. Unstained sections were used for immunohistochemical staining of SRB51 antigen using an avidin-biotin-alkaline phosphatase complex staining technique as described and a commercially available kit. Polyclonal anti-SRB51 antibodies were prepared by hyperimmunization of rabbits with whole cell, γ-irradiated SRB51.

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*Sleeaway, Fort Dodge Co., Fort Dodge, IA.

*Histomark, Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD.
Anti-SRB51 antiserum was used at a dilution of 1:1000 based on previous work using sections of SRB51-infected murine spleens or bovine lymph nodes. Control slides were stained with serum from nonimmunized, clinically normal rabbits. Sections of SRB51-infected BALB/c mouse spleen and liver were used as positive control slides.

**Bacteriologic analysis** - *B abortus* strain RB51 and strain 19 were used in this study. SRB51 vaccine was obtained from a commercial vaccine supplier and diluted appropriately using the supplied diluent. Strain 19 vaccine was obtained and prepared as directed for the vaccination of adult animals.

Tissue and fluid samples to be tested for SRB51 or S19 were processed as described using tryptose agar (TSA) and TSA with 5% bovine serum, cyclohexamide (30 μg/ml), bacitracin (7.5 U/ml), polymyxin B sulfate (1.8 U/ml) and ethylviolet (1:300,000).* Brucella* colonies were identified by colony morphology and growth characteristics.

**Collection and handling of blood for culture** - At time periods corresponding to postvaccination day (PVD) 1-4 and postvaccination week (PVW) 1-12, 100 ml of blood was collected aseptically for culture and processed as described. Calf blood was processed similarly at necropsy.

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*Colorado Serum Co., Denver, CO.

*National Veterinary Services Laboratory, Ames, IA.*
Serologic analysis - Blood was collected by venipuncture from each heifer prior to vaccination and at PVW 1-12, and from calves at necropsy. Maternal and calf blood was collected into sterile 10-ml tubes, allowed to clot for 6-24 hrs at 4°C, and centrifuged. Serum was divided into 2 ml aliquots, frozen, and stored at -70°C. The STAT, and a previously described dot ELISA for antibodies to SRB51 were used to evaluate antibody responses.

Lymphocyte proliferation assay - Assays were done on peripheral blood mononuclear cells (PBMC) collected prior to vaccination (PVD 0), on PVW 2, 4, 6, 8, 10 and 12, and from calves at necropsy. Fifty milliliters of blood was collected in 5 ml acid citrate buffer as anticoagulant. Following centrifugation, plasma was decanted and cells were placed on a Ficoll gradient (s.g. 1.083 g/dl) for separation. PBMC were processed and resuspended to a concentration of 10^7 viable cells/ml in RPMI 1640 medium. A 50 µl aliquot (5 x 10^5 cells) of the PBMC suspension was added in duplicate to separate flat-bottom wells of a 96-well microtiter plate containing 100 µl of various concentrations of γ-irradiated B abortus SRB51 (10^9 to 10^6 bacteria/well), S19 (10^9 to 10^6 bacteria/well), SRB51 LPS (20 to .02 µg/well), or S2308 LPS (2.5 to .025 µg/well) in a solution of RPMI 1640 medium containing L-glutamine, 25mM Hepes, 10% fetal bovine serum, 100U/ml penicillin, 100µg/ml streptomycin and 5 x 10^-5 M 2-mercaptoethanol. Lymphocytes were collected for blastogenesis assays at necropsy from maternal sites (superficial cervical lymph nodes, supramammary lymph nodes), and calf sites (parotid lymph nodes, mesenteric lymph nodes and spleen) and prepared as described. A 50 µl aliquot (5 x 10^5 cells) of lymph node (LN) cell suspension was added in duplicate to separate flat-bottomed wells of a 96-well microtiter plate as described. Lymph node and PBMC cell cultures were incubated for 7

Histopaque 1083, Sigma Diagnostics, St. Louis, MO.
days at 37°C in 5% CO₂, pulsed with 1.0 μCi per well of [³H]-thymidine for 18 hours, 
harvested on to glass filters and counted for radioactivity in a liquid scintillation counter. 
Cell proliferation results were expressed as the mean counts per minute (CPM) ± the 
standard error of the mean.

**Statistical analysis** - Differences in responses between treatments were compared by a 
repeated measures, general linear models procedure using the SAS system. For statistical 
comparisons, [³H] thymidine incorporation and antibody titers were also evaluated as the 
logarithm of the CPM or logarithm of the antibody titer. Means for individual treatments 
were separated by use of a least significant difference procedure (P < 0.05).

**Results**

**Clinical Signs** - All animals remained clinically normal throughout the experiment. Febrile 
responses were not detected in any animal during the first 4 days after vaccination. 
Abortions or premature births did not occur. Evidence of arthritis or anaphylactic shock was 
not seen in animals in either vaccine group.

**Postmortem examination** - Gross or microscopic lesions were not seen in cattle vaccinated 
with either SRB51 or SI9. Similarly, lesions were not seen in calves from vaccinated cattle 
in either group. Immunohistochemical staining of tissue sections from SRB51-vaccinates 
revealed no SRB51 antigen.

**Bacteriology results** - Strain RB51 was isolated from superficial cervical lymph nodes on 
the side of initial vaccination in 2/5 cases. No other maternal tissues, including placentomes

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[SAS Institute Inc., Cary, NC.]
or intercotyledonary placenta contained SRB51 or S19. Similarly, no maternal blood samples taken at any time point from either vaccine group contained SRB51 or S19. No tissues or fluid specimens examined from calves from either group contained SRB51 or S19.

**Serology** - All SRB51-vaccinates remained serologically negative by STAT throughout the experiment (Fig 1). S19 vaccinates showed peak STAT titers at PVD 14 which steadily declined to the termination of the study. Both SRB51- and S19-vaccinates showed dot ELISA titers to γ-irradiated SRB51 (Fig 2). However, peak dot ELISA titers, were greater (P<0.05) in SRB51-vaccinates at PVD 42, 56 and 70. Nonvaccinated control heifers and calves from all groups showed no titers by either STAT or dot ELISA (data not shown).

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**Figure 1** - Serum antibody responses of heifers vaccinated during pregnancy with *Brucella abortus* SRB51 or S19 as measured by the standard tube agglutination test. Results are expressed as mean titer ±S.E.
Figure 2- Serum antibody responses of heifers vaccinated during pregnancy with *Brucella abortus* SRB51 or S19 as measured by a dot ELISA test for antibodies to SRB51. Results are expressed as mean titer ± S.E. Titers of SRB51-vaccinates at 6, 8 and 10 weeks after vaccination are significantly (P<0.05) greater than titers of S19-vaccinates.

Maternal cell mediated response - Blastogenesis assays of maternal PBMC revealed a greater (P<0.05) proliferative response in both vaccine groups than in nonvaccinated controls when PBMC were incubated with either γ-irradiated-SRB51 or S19 (Fig 3). Although proliferative responses to γ-irradiated S19 were greater (P<0.05) in S19-vaccinates than in SRB51-vaccinates on PVD 28 and 70, proliferative responses to γ-irradiated SRB51 were
significantly different between S19 and SRB51-vaccinates at PVD 56 only. Similarly, differences were not seen (P>0.05) between vaccinated and nonvaccinated cattle when maternal PBMC were incubated with LPS derived from SRB51 or S2308 (data not shown).

Response of maternal superficial cervical node cells obtained at necropsy from SRB51-vaccinates did not differ (P>0.05) from that seen in nonvaccinated controls to any of the antigens tested (Fig 4). However, superficial cervical lymph node cells from S19-vaccinates showed greater (P<0.05) proliferative responses to γ-irradiated SRB51, and γ-irradiated S19 than that seen in nonvaccinated controls and SRB51-vaccinates. Cells from maternal supramammary lymph node of SRB51- or S19-vaccinates did not show proliferative responses that were significantly different (P>0.05) from controls (data not shown).

**Calf cell mediated response** - PBMC from calves of SRB51-vaccinates showed greater (P<0.05) proliferation than nonvaccinated controls in response to γ-irradiated SRB51, RB51 LPS and 2308 LPS (Fig 5). PBMC from calves of S19-vaccinates also showed significantly higher (P<0.05) responses to γ-irradiated SRB51, SRB51 LPS and S2308 LPS, as well as γ-irradiated S19 than did nonvaccinated controls (Fig 5). PBMC from calves of S19-vaccinates had greater responses (P<0.05) to γ-irradiated SRB51, γ-irradiated S19, and SRB51 LPS than did PBMC from calves of cows vaccinated with SRB51. Cells from calf parotid lymph node, calf mesenteric lymph node and calf spleen of SRB51- or S19-vaccinates did not show proliferative responses that were significantly different (P>0.05) from controls (data not shown).
Figure 3- Proliferation of PBMC from SRB51-vaccinated, S19-vaccinated, or saline-inoculated pregnant heifers to A) $10^8$ γ-irradiated SRB51 or B) $10^8$ γ-irradiated S19. PBMC were incubated for 7 days with killed bacteria prior to being pulsed for 18 hrs. with $[^3]$H-thymidine. Results are presented as mean ± S.E. cpm of duplicate cultures; PVD = postvaccination day.
Figure 4- Proliferation of maternal superficial cervical lymph node cells from SRB51- vaccinated, S19- vaccinated, or saline-inoculated pregnant heifers to: A- $10^9 \gamma$-irradiated SRB51, B- $10^9 \gamma$-irradiated S19, C- 2.5 $\mu$g SRB51 LPS, or D- 3.1 $\mu$g S2308 LPS. Lymph node cells were incubated for 7 days with antigen and then pulsed for 18 hrs. with $[^3H]$- thymidine. Results are expressed as mean ± S.E. cpm of duplicate cultures. Means with different superscripts differ significantly ($P<0.05$).
Figure 5- Proliferation of calf PBMC from SRB51-vaccinated, S19- vaccinated, or saline-inoculated pregnant heifers. See figure 4 for legend.
Discussion

Results of this study support the use of SRB51 in pregnant cattle by showing that pregnant cattle can be safely vaccinated with SRB51 when inoculated SC using a dose of $10^9$ CFU SRB51. Specifically, vaccination of pregnant cattle with SRB51 did not result in generalized infection of maternal or calf tissues but did induce cell mediated and humoral immune responses. However, small animal numbers decrease the likelihood of detecting rare vaccine-induced abortions. Likewise, no S19-induced abortions were seen in the present study. Although S19-induced abortions do occur, a large study involving the SC vaccination of $>10,000$ pregnant cattle with S19, showed abortions occur in $<1\%$.  

Lymphocyte blastogenesis results of maternal PBMC in this study indicate that pregnant cattle develop a cell mediated immune (CMI) response to SRB51 by PVD 28. Heifer calves have been shown to develop a CMI response to SRB51 and S2308 protein fractions 10-12 wks after vaccination with a slightly higher dose of SRB51. Blastogenic responses to S2308 and SRB51 proteins were similar even though SRB51 fractions do not contain the O-side chain of LPS. Our studies further confirm that the LPS of S2308 is not blastogenic for PBMC or superficial cervical lymph node cells from SRB51-vaccinated cattle. The blastogenic and humoral responses of S19-vaccinates to γ-irradiated SRB51 was not unexpected as the outer membrane proteins of Brucella species have been shown to be remarkably similar. Results of this study are also similar to data obtained from another study in which S19-vaccinates developed humoral responses to irradiated SRB51. These studies suggest that S19 and SRB51 share common epitopes to which immune responses are directed.

The development of a CMI response in the PBMC of calves from SRB51- or S19-vaccinates suggests exposure of the calf to vaccine antigens although SRB51 or S19 were not isolated from calf tissues or placental membranes. It cannot be excluded that fetal infection occurred following vaccination, with bacteria cleared by immune responses prior to
parturition. Fetal immune responses to Brucella antigens are possible as the anatomic components of the fetal immune system are present by day 175 of gestation. Although pregnant heifers were vaccinated at 180 days of gestation, lack of CMI response by other calf lymphoid tissues and the lack of a calf humoral response suggest that the calf immune system may not be functionally mature in relation to response to Brucella antigens.

A previous study using IV inoculation of pregnant cattle with SRB51 showed generalized fetal infection with SRB51 while only one of ten fetuses developed a humoral response. Another previous study with bovine fetuses experimentally inoculated with S2308 showed increased levels of both IgG and IgM. Differences in fetal response to S2308 and SRB51 may be due to the lack of the O-side chain of the lipopolysaccharide of SRB51 or to differences in the amount of antigen to which the fetus was exposed.

The results of this study further indicate that route of vaccination and dosage have significant effects on the outcome of vaccination with SRB51. Intravenous inoculation of 10^{10} CFU SRB51 to cattle at 6 months of gestation results in placentitis and fetal infection. Conversely, lack of placentitis as well as absence of localization of SRB51 in other tissues, i.e. spleen, liver, etc., in the present study, suggest that systemic spread and colonization of SRB51 do not occur following SC vaccination with 10^9 CFU SRB51. Route of inoculation has also been demonstrated to be critical with other brucellosis vaccines such as strain 45 or S19 which consistently cause abortion when administered IV but cause few or no abortions when administered SC.

Persistence of SRB51 in the present study in superficial cervical lymph nodes lymph nodes to 12 weeks after vaccination differs from previous findings. In calves vaccinated SC with 10^{10} CFU SRB51 in the superficial cervical region, SRB51 was not isolated from lymph node biopsies beyond 6 weeks after vaccination. Persistence in pregnant cattle may be related to age or pregnancy status at vaccination which may influence immune function. Moreover, clearance of S19 from superficial cervical lymph nodes prior to the clearance of
SRB51 in the present study differs from that seen elsewhere. However, the adult dose of S19 used in the current study is roughly 30-fold less than that used previously.

In agreement with previous findings, our SRB51-vaccinates did not develop antibody titers which were detected by STAT, thereby, facilitating the differentiation of SRB51-vaccinates and naturally infected animals. Antibodies detected in the STAT are directed to the O-side chain; therefore, lack of STAT response in SRB51-vaccinates is thought to be due to the lack of O-side chain on SRB51.

Lymphocyte blastogenesis and serologic reactions in the present study suggest an active immune response in pregnant animals without generalized infection of maternal reproductive or calf tissues. However, vaccination with SRB51 during pregnancy followed by challenge with a virulent strain of Brucella would be necessary to prove protective immune responses. Additionally, field tests with large numbers of pregnant cattle would also be needed to determine the true prevalence of SRB51-induced abortion. In outbreak situations it has been the policy of the USDA to “whole herd vaccinate”, vaccinating both female adults and calves. The current findings suggest that vaccination of pregnant cattle with SRB51 would provide a safe means to enhance resistance to Brucella infection.

References


Abstract

TNF-α is a key cytokine in inflammatory processes and mediates many of the clinical signs associated with endotoxemia due to gram negative bacterial infection. In order to determine the influence of brucellosis vaccination on TNF-α levels in pregnant cattle and the possible role of the placenta in TNF-α production, pregnant cattle were vaccinated IV with Brucella abortus strain RB51 (n=10), SC with B. abortus strain RB51 (n=5), or SC with B. abortus strain 19 (n=5); controls received pyrogen free saline SC (n=2). Radioimmunoassays showed no elevations in TNF-α levels in serum or plasma from IV or SC vaccinated cattle that differed from controls (P>0.05). Similarly, TNF-α levels in amniotic and allantoic fluids from SC vaccinated cattle were not different from controls (P>0.05). Immunohistochemistry for TNF-α revealed increased immunoreactivity within trophoblastic epithelial cells in SC and IV vaccinated cattle. Immunoreactivity was most extensive in IV vaccinated cattle that developed vaccine induced placentitis. These studies indicate that SC vaccination for the prevention of brucellosis using recommended adult dosages does not result in elevations of
TNF-α in plasma, serum or placental fluids; however, vaccination of pregnant cattle does stimulate trophoblastic epithelial cells to express TNF-α even when placentitis is not histologically evident.

1. Introduction

Tumor necrosis factor-alpha (TNF-α) is a cytokine capable of altering immune and pathophysiologic responses. TNF-α is expressed in a variety of cell types; the most quantitatively important being the macrophage (Myers et al., 1995; Tracey et al., 1992). However, TNF-α is also produced by lymphocytes, mast cells, NK cells, neutrophils, B-cells, keratinocytes, microglial cells, smooth muscle cell and various tumor cells (Tracey et al., 1992). Various cell types also carry receptors for TNF-α (Le et al., 1987; Dayer et al., 1985). Consequently, the biological effects of TNF-α are numerous including: inhibition of lipoprotein lipase (Beutler et al., 1985), pyretogenesis (Dinarello et al., 1986; Le et al., 1987), activation of neutrophils (Tsujimoto et al., 1986; Klebanoff et al., 1986), T-cell activation (Le et al., 1987), osteoclast activation (Bertolini et al., 1986), induction of endothelial cell procoagulant activity (Nawroth et al., 1986; Nawroth et al., 1986), mitogenic action on fibroblasts (Vilcek et al., 1986; Sugarman et al., 1985), induction of oncogenes, and induction of synthesis of acute phase proteins, prostaglandins (Dinarello et al., 1986; Dayer et al., 1985), collagenases (Dayer et al., 1985), granulocyte/macrophage-colony stimulating factor, IL-1 and TNF-α itself (Dinarello et al., 1986; Nawroth et al., 1986).
TNF-\( \alpha \) levels are increased in response to a variety of stimuli, the most potent of which is endotoxin (Adams et al., 1990; Kenison et al., 1991) (Mannel et al., 1987), a lipopolysaccharide (LPS) in the cell wall of gram-negative bacteria. Endotoxin exposure results in numerous clinical signs, many of which are mediated through the production of TNF-\( \alpha \), including fever, anorexia, depression, hypotension, metabolic acidosis, transient hyperglycemia followed by hypoglycemia, gastrointestinal infarction, hemorrhage of the adrenal glands and pancreas, acute nephrosis, and interstitial pneumonitis (Mannel et al., 1987). Endotoxin exposure can also have significant effects on reproductive performance in cattle. Endotoxin has been shown to be luteolytic (Benyo et al., 1992), causing shortened interestrous intervals. Infusion of *Salmonella typhimurium* endotoxin in pregnant cows induces abortion within 4 to 10 days of infusion (Foley et al., 1993). Endotoxin infusion is followed by a rapid increase in maternal but not fetal TNF-\( \alpha \) (Foley et al., 1993).

Bovine brucellosis is an economically important abortifacient disease caused by the gram-negative bacterium *Brucella abortus*. Since 1940, an integral part of brucellosis prevention and eradication in the United States has been the vaccination of female calves with the live vaccine *Brucella abortus* strain 19 (S19). Although effective in decreasing the incidence of brucellosis, S19 has several disadvantages including the induction of abortion in some pregnant cattle (Nicoletti, 1977). A new vaccine, *Brucella abortus* strain RB51 (SRB51), has recently been approved in the United States as an official calfhood vaccine. A study in pregnant cattle showed that IV injection of SRB51 results in placental infection, placentitis and fetal infection; although, abortion was not seen (Palmer et al., 1996). Further
study showed SC vaccination of SRB51 did not result in placental infection, placentitis or fetal infection (Palmer et al., (in press)).

This work was part of a vaccine safety study involving vaccination of pregnant cattle with SRB51. Clinical signs and pathology associated with reproduction of all groups have been reported elsewhere (Palmer et al., 1996; Palmer et al., (in press)). Briefly, one heifer vaccinated IV with SRB51 delivered a weak calf one month prior to the predicted calving date. No other premature births or abortions were noted in either the IV or SC vaccinated groups (Palmer et al., 1996; Palmer et al., (in press)). Eight of ten IV vaccinated cattle and 0/10 SC vaccinated cattle developed placentitis which is described in detail elsewhere (Palmer et al., 1996; Palmer et al., (in press)).

In the IV vaccinated group, 80% of cattle were febrile during the initial 24 to 48 hrs. after vaccination. All but two animals had normal body temperatures by 48 hrs. after vaccination and the remaining two were normal by 96 hrs. after vaccination. Cattle vaccinated SC with SRB51 or S19 showed no febrile response and did not differ from controls (Palmer et al., 1996; Palmer et al., (in press)).

The current investigation was designed to determine the changes in TNF-α levels following vaccination with SRB51 or S19 as a measure of the toxicity of vaccine-associated LPS. Additionally, this investigation was designed to determine placental tissue for cellular sites of TNF-α production.
2. Materials and Methods-

Polled Hereford heifers used in this experiment were obtained from a nonvaccinated, brucellosis-free herd. Heifers were 16-27 months old at breeding. All cattle were serologically negative for brucellosis by standard tube agglutination (Alton et al., 1988). Upon arrival, and throughout the experiment, cattle were kept on pasture. Breeding was by natural service using a Polled Hereford bull which was serologically negative for brucellosis by standard tube agglutination.

**IV vaccination** - At 6 months of gestation heifers were injected IV in the right jugular vein with $10^{10}$ CFU SRB51 suspended in 10 ml sterile saline (0.85% NaCl). At postvaccination week 8 (PVW), 5 heifers were randomly selected, euthanatized, and examined. The remaining cattle were euthanatized at term (n=3) or following premature delivery (n=1).

**SC vaccination** - At six months of gestation animals were randomly divided into 3 groups and injected SC in the right prescapular region with $9.4 \times 10^8$ CFU SRB51, (n=5), $3 \times 10^8$ CFU S19, (n=5), or 2 ml sterile pyrogen free saline, (n=2). The exact inocula dose reported above was determined retrospectively. The dosage of SRB51 used in adult vaccination studies is $10^9$ CFU (Palmer et al., 1995), while the recommended adult dosages of
S19 is 3-10 x 10^8 CFU (United States Department of Agriculture et al., 1986). All animals were euthanatized at full term of pregnancy.

In both IV and SC vaccinated cattle, body temperatures were recorded daily for the first 4 days following vaccination and animals were observed twice daily during the experiment for clinical signs of abortion, illness or other adverse reactions.

**Radioimmunoassay** - Blood was collected 3 times prior to vaccination on days -7, -5 and on the day of vaccination (day 0) and on postvaccination days (PVD) 1, 2, 3, 4, 7, 14, 21, 28, 35, 42, 49, 56, 63. Blood was collected from the calves at necropsy. Amniotic and allantoic fluids were collected at necropsy from cattle vaccinated SC with SRB51 or S19. Blood and placental fluids from SC vaccinated cattle were immediately placed in sterile tubes with EDTA, and centrifuged. Samples were filtered through a 0.22 μm low protein binding filter and aliquotted in 2 ml vials and frozen at -70 C. Similarly, serum was collected from IV vaccinated cattle in sterile tubes without anticoagulant and allowed to clot for 4 to 6 hrs at 4 C and centrifuged. Serum was removed, filtered as above, and aliquotted in 2 ml vials and frozen at -70 C. Plasma, serum, and placental fluid TNF-α levels were measured using a double-antibody radioimmunoassay (RIA) as described (Kenison et al., 1990).

**Immunohistochemistry** - Placental samples were placed in neutral 10% buffered formalin and processed by routine paraffin embedment techniques, cut 4-6 μm thick and left unstained. Staining for bovine TNF-α was done as described (Cirelli et al., 1995), with
modifications for deparaffinization of paraffin sections. An avidin-biotin-alkaline phosphatase complex staining technique was used as described (Palmer et al., 1996; Meador et al., 1986). Anti-bovine TNF-α antibody was prepared as described (Kenison et al., 1990), and used at a dilution of 1:500 - 1:1000. Blocking with 1% normal bovine serum for 15 min. prior to the application of the anti-TNF-α antibody was done to reduce background staining. Negative control slides included histologic sections stained with serum from clinically normal rabbits and slides stained with omission of the primary antibody. Macrophages within the individual tissue sections were highly immunoreactive for bovine TNF-α and were used as an internal positive control.

**Statistical Analysis** - Differences in TNF-α levels between groups were compared by a repeated measures, general linear models procedure using the SAS system (SAS Institute, Cary, NC). Differences between means were considered significant at a level of P<0.05. Prevaccination means on days -7, -5 and 0 were combined within groups to yield one prevaccination TNF-α value per group.

3. Results -

*Plasma TNF-α levels* - Tumor necrosis factor-α levels in plasma of cattle vaccinated SC are shown (Figure 1). There were not significant differences between cattle vaccinated SC with SRB51 or S19 or given saline (P>0.05). However, in these three groups a significant rise (P<0.05) was seen in TNF-α levels over the course of the experiment which
peaked at 42-56 days after vaccination. Similarly, levels of TNF-α in serum of IV vaccinated cattle did not show significant elevations as compared to controls. Levels of TNF-α in amniotic fluid and allantoic fluid from SC vaccinated cattle were either undetectable or not elevated above that seen in control cattle (Figure 2). Calf plasma or serum did not contain significantly elevated TNF-α as compared to controls (Figure 3).

Figure 1. Plasma TNF-α levels in cattle vaccinated SC with $10^9$ CFU SRB51 (n=5) or $3\times10^8$ CFU S19 (n=5), IV with $10^{10}$ CFU SRB51 (n=10), or given saline (n=2). TNF-α levels were determined by radioimmunoassay and represent mean ± standard error. * Significantly greater in SC vaccinates than values at 0-14 days after vaccination (P<0.05).
Figure 2. TNF-α levels in amniotic (A), and allantoic (B) fluids from cattle vaccinated SC with $10^9$ CFU SRB51 (n=5), $3 \times 10^8$ CFU S19 (n=5) or sterile saline (n=2). Values are expressed as mean ± standard error.
**Immunohistochemistry Results** - Placental sections from SRB51 IV vaccinated cattle with placentitis showed staining of large regions of the trophoblast layer. Strongly immunoreactive trophoblastic epithelial cells contained diffuse cytoplasmic staining for TNF-α (Figure 4). In contrast, placental sections from SC vaccinated cattle (S19 and SRB51), which did not have placentitis, showed a punctate staining of trophoblastic epithelial cells with few cells having diffuse cytoplasmic staining (Figure 5). Immunoreactive punctate foci within trophoblastic epithelial cells were multiple, round, 1-3 μm in diameter and strongly immunoreactive for TNF-α. Staining was seen in multiple groups of adjacent cells bounded by groups of cells without TNF-α immunoreactivity. Macrophages which stained strongly for TNF-α were present within the chorion, endometrium, myometrium and within the submucosal region of the uterus surrounding glands and vessels of both IV and SC vaccinated cattle (Figure 6).

Saline-inoculated control cattle showed immunoreactivity for TNF-α in low numbers of macrophages within the myometrium and subserosa. Macrophages between the outer longitudinal muscle layer and serosa, and macrophages adjacent to submucosal glands or vessels, stained strongly for TNF-α (Figure 7). Low numbers of individual trophoblastic epithelial cells were also immunoreactive for bovine TNF-α with moderate diffuse staining of the entire cytoplasm; however, the remainder of the trophoblast layer lacked immunoreactivity for TNF-α (Figure 8).
Figure 3. TNF-α levels in plasma of calves from dams vaccinated SC with $10^9$ CFU SRB51 (n=5), $3 \times 10^8$ CFU S19 (n=5), IV with $10^{10}$ CFU SRB51, or sterile saline (n=2). Values are expressed as mean ± standard error.

4. Discussion

These studies indicate that bovine trophoblastic epithelial cells can produce TNF-α and that TNF-α is produced by more trophoblastic epithelial cells during SRB51-induced placentitis than in controls or vaccinated cows without placentitis. The diffuse cytoplasmic TNF-α immunoreactivity seen in cases of placentitis may reflect increased expression of TNF-α. Intense punctate immunoreactivity for TNF-α seen in SC vaccinated cows also suggests increased expression over that seen in controls.
Figure 4. Section of placenta from heifer vaccinated IV at 6 months gestation with $10^{10}$ CFU B. abortus strain RB51 and examined 8 weeks later. Note diffuse cytoplasmic immunoreactivity for TNF-α in trophoblastic epithelial cells. Alkaline phosphatase method, Gill’s hematoxylin counterstain. Bar = 30 µm.

Figure 5. Section of placenta from heifer at full term which was vaccinated SC at 6 months gestation with $10^9$ CFU B. abortus strain RB51. Note punctate cytoplasmic immunoreactivity for TNF-α in trophoblastic epithelial cells. Alkaline phosphatase method, Gill’s hematoxylin counterstain. Bar = 30 µm.

Figure 6. Section of placenta from heifer at full term which was vaccinated SC at 6 months gestation with $10^9$ CFU B. abortus strain RB51. Note immunoreactivity for TNF-α in macrophages surrounding chorioallantoic arteriole. Alkaline phosphatase method, Gill’s hematoxylin counterstain. Bar = 50 µm.

Figure 7. Section of placenta from control heifer at full term which was injected SC with saline at 6 months gestation. Note lack of immunoreactivity for TNF-α in trophoblast layer. Alkaline phosphatase method, Gill’s hematoxylin counterstain. Bar = 50 µm.

Figure 8. Section of uterus from control heifer at full term which was injected SC with saline at 6 months gestation. Note immunoreactivity for TNF-α in macrophages in subserosa. Alkaline phosphatase method, Gill’s hematoxylin counterstain. Bar = 30 µm.
However, *Brucella* were not recovered from SC vaccinated cattle and histologic signs of inflammation were not seen. In mice, endotoxin treatment results in the appearance of TNF-α in secretory granules of granulocytes, monocytes and neutrophils in all tissues examined (Schmauder-Chock et al., 1994). The punctate TNF-α immunoreactivity in trophoblastic cells of SC vaccinated cattle may represent secretory granules containing TNF-α.

The dose of endotoxin associated with our vaccinations may have been insufficient to induce changes seen in cattle injected with endotoxin of other bacterial species. An experimental study in pregnant cattle with endotoxemia showed elevations in maternal plasma TNF-α following injection of *Salmonella* endotoxin; however, fetal plasma and amniotic and allantoic fluid TNF-α levels were not elevated above controls (Foley et al., 1993). The endotoxin of *Brucella* has been shown to be 10,000 fold less potent than that of *Escherichia coli* in eliciting fever in rabbits (Goldstein et al., 1992), and 1,000 fold less potent than *Salmonella* endotoxin in eliciting lysozyme release in neutrophils (Rasool et al., 1992). It should be noted that radioimmunoassay measures TNF-α regardless of bioactivity. Factors which inhibit TNF-α bioactivity, such as soluble TNF-α receptors, may interfere with bioactivity but not immunoreactivity of secreted TNF-α (Myers et al., 1995).

It may be possible that SC vaccination induced changes within the placenta which were significant enough to increase TNF-α expression, but not significant enough to result in chemotaxis of inflammatory cells and disruption of cells or tissue. Immunoreactivity of TNF-α in bovine trophoblastic epithelial cells in cases of placentitis has not been demonstrated previously but is consistent with studies in women with preterm labor due to
infection. Elevated TNF-α has been measured in the amniotic fluid of women in preterm labor (Romero et al., 1989; Romero et al., 1989) (Laham et al., 1994) (Halgunset et al., 1994) with clinical as well as subclinical intrauterine infection. Furthermore, TNF-α production has been documented in human cytotrophoblasts and decidual cells (Chen et al., 1991; Stallmach et al., 1995). In response to TNF-α and/or IL-1, several cell types, including amnion and decidua synthesize prostaglandins (Dayer et al., 1985) (Riley et al., 1984) (Romero et al., 1989). Similarly, 10 μg of recombinant human TNF-α causes abortion in mice (Parant, 1987), while in a model of malaria-induced abortion, as little as 1.5 μg will induce abortion in mice infected with Plasmodium vinckei (Clark et al., 1988). Therefore, it is hypothesized that bacterial products, such as LPS, stimulate maternal and/or fetal cells to produce cytokines, such as TNF-α, which in turn increase prostaglandin synthesis by intrauterine tissues resulting in the onset of labor (Romero et al., 1989). In cultured bovine luteal cells, TNF-α induces a dose dependent increase in luteal PGF$_2$α and has an inhibitory effect on lutenizing hormone-induced progesterone production (Benyo et al., 1992). Both increased PGF$_2$α and decreased progesterone levels are changes consistent with the onset of parturition in cattle.

The reason is unknown for the increase in plasma TNF-α seen in SC vaccinated and control cattle which occurred 42-56 days after vaccination. This time period corresponds to approximately 7.5 to 8 months’ gestation. Mononuclear cells from periparturient cattle have been shown to produce significantly more TNF-α than cows in mid to late lactation (Sordillo et al., 1995). However, all cattle in our study were housed together during the experiment:
therefore, the possibility of subclinical disease affecting all cattle which resulted in elevations of plasma TNF-α cannot be ruled out.

Reasons for lack of postvaccination fever in our SC vaccinated cattle may include vaccine dose and age at vaccination. A previous report with S19 vaccination described fever and anorexia of 3-5 days duration in dairy and beef calves (Dale et al., 1957). However, the animals used were calves and the dose of S19 vaccine was unspecified.

This study shows that SC vaccination with SRB51 or S19 and IV vaccination with SRB51 results in increased TNF-α immunoreactivity in histologic sections of placenta with and without placentitis. Although neither IV vaccination with SRB51 nor SC vaccination with SRB51 or S19 resulted in elevations in plasma TNF-α, the increased immunoreactivity of TNF-α indicates the potential exists for a role in bovine abortion for TNF-α similar to that suspected in humans.

References


CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

These studies show the vaccine *Brucella abortus* strain RB51 (SRB51) does have a tropism for the bovine placenta, similar to other strains of *B. abortus*; however, even with moderate placentitis and disseminated fetal infection, abortion was not seen. Placentitis could only be induced by higher than recommended dosages and IV inoculation. Subsequent studies showed that pregnant cattle can be safely vaccinated SC with $10^9$ CFU SRB51 which induced both humoral and cell mediated immune responses without placentitis or fetal infection.

When infected herds have been identified in the United States, action taken by the USDA may involve vaccination of the whole herd, including pregnant animals. The results presented here support the vaccination of pregnant cattle with SRB51. The protective value of SRB51 vaccination in pregnant cattle; however, can only be suggested from the data presented. Studies involving vaccination of pregnant cattle followed by challenge with the virulent strain 2308 would be required to determine true efficacy.

These results further suggest that SRB51 induced abortion is a rare event in cattle. Vaccination of 10,000 head of pregnant cattle with S19 resulted in $<1\%$ abortion. The number of cattle used in the present studies was small and the true prevalence of SRB51-induced abortion will require the vaccination of large numbers of pregnant cattle. Field studies with SRB51 have vaccinated $>800$ pregnant cattle in various stages of gestation with 1 SRB51-induced abortion.
The disseminated infection of full term calves following IV vaccination without immune response by the calf suggests that calf infection may occur very late in gestation prior to calving or calves may not be functionally capable of mounting a response to SRB51. The presence of SRB51 infection in calves as early as 8 weeks after infection and 4 weeks prior to calving discounts the possibility of calf infection immediately prior to calving. In contrast, calves infected in utero with the virulent strain 2308 developed increased IgG and IgM. Differences between responses reported in S2308 infected calves and those seen here may be due to the rough nature of SRB51. Smooth *Brucella* spp. survive in macrophages which are important antigen presenting cells as well as effector cells of the immune response.

Humoral and cell mediated responses in pregnant cattle are similar to those seen in nonpregnant cattle. Lack of humoral responses detected by routine brucellosis surveillance tests, such as the card test or standard tube agglutination test, allow the differentiation of SRB51 vaccinated animals from naturally infected animals. Such differentiation is not possible with S19 vaccinated animals and should accelerate the brucellosis eradication effort.

Adverse effects due to LPS induced TNF-α elevations may be of minimal concern under the parameters described in these studies. Lack of significant increases in circulating TNF-α levels following IV or SC vaccination with SRB51 and SC vaccination with S19 may be due to the decreased toxicity of *Brucella* LPS as compared to other gram negative pathogens. However, immunohistochemical detection of TNF-α in trophoblastic epithelial
cells of vaccinated cattle but not control cattle suggests that vaccination did result in increased TNF-α expression by trophoblastic epithelial cells. The proposed role of placental TNF-α in women with premature labor,10,11 and abortion in mice,28 is a key one. Our findings suggest that vaccination does have an effect on bovine placental TNF-α; however, this role remains poorly defined and deserves further investigation.

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