Pathogenesis of placentitis in the goat (Capra hircus) inoculated with Brucella abortus

Timothy Dean Anderson

Iowa State University

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PATHOGENESIS OF PLACENTITIS IN THE GOAT (CAPRA HIRCUS) INOCULATED WITH BRUCELLA ABORTUS

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Pathogenesis of placentitis in the goat (Capra hircus) inoculated with Brucella abortus

by

Timothy Dean Anderson

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Approved:

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Iowa State University
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1985
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GENERAL INTRODUCTION

Bovine brucellosis is caused by a bacterium, *Brucella abortus*, and is of major significance due to economic losses caused by abortions. In 1982, 121 out of 160 countries reporting brucellosis considered bovine brucellosis, caused by *B. abortus*, to be more important than porcine brucellosis caused by *B. suis*, caprine brucellosis caused by *B. melitensis*, ovine brucellosis caused by *B. ovis*, and canine brucellosis caused by *B. canis*. In the USA in 1972, the direct loss of meat (as a result of abortions, infertility, and weight losses) in infected cattle herds has been estimated at 15% of the expected herd total. Milk production may decrease 20% per infected cow. In the USA alone, federally funded control and eradication programs cost $90 million in 1982, up from $30 million in 1975. In addition, brucellosis has great public health significance and is considered an occupational hazard for veterinarians, farmers, and packing plant workers who come in contact with infected animals or infected placentae.

Brucellosis in the pregnant cow is characterized by placentitis, intracellular replication of *B. abortus* in trophoblasts, large numbers of *B. abortus* in placentae, fetal death, and abortion. The pregnant cow is generally infected by ingestion of brucellae from an aborted fetus or placenta, or contaminated uterine discharges from infected herdmates. After infection, bacteremia is followed by localization of the bacteria in spleen and lymph nodes, mammary gland, and pregnant uterus of the female. Infection of the pregnant uterus
leads to placentitis, fetal death and abortion, or the delivery of weak calves.\textsuperscript{35,64}

The affinity (tropism) of \textit{B. abortus} for trophoblasts and its subsequent intracellular replication probably contributes greatly to placental localization, placentitis, and abortion. Mechanisms of placental localization and trophoblast tropism in ruminant brucellosis are poorly understood.

The objectives of this study were i) to evaluate a caprine model for study of ruminant brucellosis, ii) characterize the ultrastructural lesions associated with brucellar placentitis and, iii) determine mechanisms whereby trophoblasts may facilitate placental localization and bacterial replication.

This dissertation is presented in the alternate format and consists of three manuscripts submitted to refereed scientific journals. The format used is that of Veterinary Pathology. The review of the literature precedes the first manuscript. The first and second manuscripts have been submitted for publication to Veterinary Pathology. The third manuscript has been submitted to The American Journal of Pathology. A general summary and discussion follows the last manuscript. A list of references appears at the end of each manuscript. Literature cited in the introduction, literature review, and general summary and discussion appears at the end of the dissertation.

The Iowa State University Committee on the Use of Animals in Research reviewed this project and concluded that the animal subjects were treated humanely in all procedures involved in this research.
The Ph.D. candidate, Timothy Dean Anderson, was the principal investigator for each study.
LITERATURE REVIEW

Description of the organism: Taxonomically, the genus Brucella comprises 6 species, which differ in host specificity, epidemiology, pathogenicity to man and animals, and metabolic behavior. *B. abortus* causes contagious abortion in cattle and undulant fever in man, its main reservoir hosts are domestic cattle and water buffalo. In endemic areas, sheep, goats, pigs, dogs, foxes, jackels, and hyenas may serve as alternate hosts. *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* have as their chief reservoir hosts, goats, swine, sheep, dogs, and the desert wood rat respectively. Each species can be distinguished by their oxidative utilization of certain amino acids and carbohydrates as well as their sensitivity to bacteriophages. *B. abortus* isolates can be divided into different biotypes on the basis of CO₂ requirements, H₂S production, growth on cultural media containing dyes, and their reaction to monospecific antisera. Determination of biotype is useful in epidemiological investigations.¹⁸,⁸⁸

*B. abortus* is a Gram-negative short rod measuring 0.5 to 0.7 μm by 0.6 to 1.5 μm. The rods are usually arranged singly, although short chains may form in liquid cultures. Because it is a facultative intracellular bacterium, *B. abortus* is frequently found in clumps (within cells) in smears made from uterine exudates. It is an aerobic, non-sporeforming, non-motile bacterium which requires 5 to 10 percent CO₂ for primary isolation.

*B. abortus* grows slowly *in vitro* and initially produces small non-hemolytic colonies on tryptose agar, albimi agar, and trypticase-soy
agar enriched with blood or serum. The colonies may be of the smooth or rough type, are barely visible after 2 days growth and reach maximum size only after 5 to 7 days. Various solid media with a beef, veal, or potato base supplemented with 5 to 10 percent serum or blood are suitable for growing *B. abortus*.18,30,77,88

**Historical background:** Brucellosis was first described in man in 186144,93 but the association of brucellosis with a bacterium was made in 1887 by Sir David Bruce,13 a British physician stationed in Malta. His major task was to care for soldiers ill with "Malta fever", the name given to a disease in man characterized by recurring fever and associated with the Malta islands. Bruce succeeded in culturing a "micrococcus" from the spleens of four people with fatal cases of Malta fever. Shortly thereafter, he successfully transmitted the disease to monkeys, designated the disease as Mediterranean fever and the etiological agent as *Micrococcus melitensis*, and presented a brief clinical discussion of the disease.13-15

The human disease, undulant fever (brucellosis), was not associated with animals until after Bruce reported on an epidemiological study commissioned by the Royal Society of Great Britian.33 In preparation for a large-scale study of undulant fever, the commission evaluated the Malta island's goat population as a possible source of experimental animals. The commission eventually determined that, of the island's 20,000 goats, 40 percent had positive agglutination reactions for *M. melitensis*, and about 2,000 secreted milk containing viable organisms.23 The incidence of undulant fever declined remarkably after
the use of all goat's milk and its products were absolutely prohibited in governmental establishments.

The first description of brucellosis in cattle and its association with a bacterium was by Bang, a Danish veterinarian in Copenhagen, who established *Bacillus abortus* as the cause in 1897. However, according to Fleming, an English veterinarian of the nineteenth century, contagious abortion in cattle had plagued farmers for centuries. He cites an instance in the Roman Empire: "The Tarentian War was succeeded by a most desolating pestilence, invading both cities and suburbs, and carrying off chiefly women and cattle. In 278 B.C. it was known as the Abortus epidemicus, and was particularly fatal to pregnant females and cows at Rome."25

In 1895, Professor Bang purchased a cow that was aborting and removed the calf and placental membranes. Careful study of the maternal and fetal structures revealed no abnormality in the uterus. Between the uterine wall and the placental membranes, however, there was "a copious, odorless, gelatinous exudate, which on microscopic examination was found to be packed with intracellular microorganisms." Bang was able to grow the microbe at two optimum concentrations of oxygen, one just below the content of atmospheric air, the other close to 90 percent. Nocard, a professor in France, had earlier reviewed the subject of contagious abortion in cattle in 1886 and had concluded that abortions were due to the presence of microorganisms between the fetal membranes and the uterus. He was correct, but he did not isolate the causative organism.31
Bang performed further studies on aborted placentae and succeeded repeatedly in isolating the same bacillus. He was also able to produce the disease in healthy pregnant heifers and ewes. He incriminated the bull as being partly responsible for spreading the disease and recommended prophylactic measures.4

At this point, two species of Brucella and their associated diseases had been described by Bruce and Bang but the relationship of the two microorganisms remained unrecognized for many years. Whereas Bruce had designated his organism as a micrococcus reproducing readily on the surface of culture media, Bang had described his organism as a bacillus, the growth of which did not occur under atmospheric conditions, but in relation to certain concentrations of oxygen.31

MacNeal and Kerr at the University of Illinois were the first to report the existence of B. abortus in the United States in 1909.41 Alice Evans, a bacteriologist with the United States Bureau of Animal Industry, showed in 1918 the close relationship between B. abortus and M. melitensis and implicated milk from infected cows as a possible source of infection for human beings.24 Meyer and Shaw confirmed Evan's findings and suggested the genus Brucella as the name of both organisms.46 Theobold Smith later published the first description of the preferential location of B. abortus in chorioallantoic trophoblasts in 1919.78

The majority of research on brucellosis since the 1920s has concentrated on two major objectives, the development and standardization of diagnostic methods for detecting the disease, and the immunization of susceptible animals against infection.
Diagnostic methods: Although protection against infection by *B. abortus* is largely a result of cell-mediated immunity,\textsuperscript{17} most diagnostic tests developed to date rely on detecting a response in the humoral immune system.\textsuperscript{82} Bacterial culture and positive identification of *B. abortus* is the most reliable diagnostic test.\textsuperscript{58,59}

Standard serologic tests currently used include buffered plate, rivanol, card, and tube agglutination and complement fixation. Passive hemagglutination, indirect hemolysis, counter-electrophoresis, enzyme- and radioimmunoassay tests are being evaluated in efforts to increase sensitivity and specificity of current tests. Increased effort is also being directed to the development of in vitro assays for cell-mediated immunity. Most workers have employed lymphocyte blastogenesis assays, however, serious problems exist with specificity and interpretation in these procedures.\textsuperscript{92}

Immunogens: As early as 1906, Bang reported that protection against brucellosis in cattle was attained following the injection of living cultures of brucellae, but no protection was acquired with killed organisms.\textsuperscript{5} An attenuated strain of *B. abortus* (Strain 19) was first isolated by Buck in 1923 from bovine milk, and has since been utilized as an immunogen. The culture was originally virulent, however, the virulence of the strain became attenuated after accidentally remaining on an agar slant at room temperature for a year.\textsuperscript{27}

While the ideal vaccine for brucellosis does not exist, the smooth Strain 19 of *B. abortus* is considered to be superior to other vaccines for protection against *B. abortus* infection.\textsuperscript{59} Strain 19 rarely produces permanent infection in cattle vaccinated at calfhood. There
has been no proven change in antigenicity and little change in virulence.\textsuperscript{59,82}

A major difference exists between field strains of \textit{B. abortus} and Strain 19 in their utilization of erythritol. Field strains of \textit{B. abortus} utilize erythritol in preference to glucose, however, strain 19 is inhibited by erythritol\textsuperscript{12,38,47} due to lack of an NAD-dependent D-erythrulose 1-phosphate dehydrogenase essential for erythritol catabolism.\textsuperscript{79,80} This may explain the preference of fully virulent \textit{B. abortus} for the bovine erythritol-containing pregnant uterus and the failure of Strain 19 to colonize the uterus and produce abortion in the majority of pregnant vaccinated animals.

Several problems exist with the use of live modified Strain 19. These include post-vaccinal titers, persistence of Strain 19 in vaccinated animals, reversion to erythritol catabolism and full virulence, and incomplete efficacy of protection.\textsuperscript{19,58,59}

\textbf{Treatment:} A satisfactory treatment for brucellosis in domestic animals has not been developed.\textsuperscript{59} \textit{B. abortus} is a facultative intracellular pathogen that resides within host cells. It is largely inaccessible to currently available chemotherapeutic agents and the humoral immune system. Consequently, brucellosis is a chronic disease often refractory to treatment and diagnosis.\textsuperscript{8,26,30,77}

\textbf{Epidemiology:} Nearly all bovine brucellosis is caused by ingestion and subsequent infection by one of the biotypes of \textit{B. abortus}. Natural or artificial infections usually persist indefinitely.\textsuperscript{42} The source is usually by association with uterine discharges or aborted placentae of an infected cow. Eighty-five percent of infections are from biotype 1.
In the United States, biotypes 2 and 4 are also found. Abortions occur during the first and possibly second pregnancies after the initial infection, however, subsequent pregnancies are usually normal.\(^8,26,35,58,59\)

A higher prevalence and probability of infection exists in cattle in large herds (more than 250 head) with a high cattle density. This higher prevalence occurs because of the large exposure potential following an abortion. In addition, large herds acquire their replacement cattle from a large variety of outside sources.\(^58\)

Sexually immature cattle are usually considered resistant to infection with \textit{B. abortus}. Susceptibility increases with sexual development and pregnancy. However, calves may acquire infections \textit{in utero} or by ingestion of contaminated milk. A small percentage of heifer calves which are infected in early life and are negative to serologic tests will abort or have an infected calf during the first pregnancy. These calves with latent infections often infect the herd they join in later life.\(^8,55,58,59,68\)

Bulls can be infected orally and, after infection, brucellae may localize in the testes, epididymis, seminal vesicles, and ampullae of the ductus deferens. Excretion of brucellae is common during the acute disease but eventually ceases and male infertility is not a normal sequel. Transmission of \textit{B. abortus} to susceptible cows by natural service has not been demonstrated in controlled experiments.\(^67,70\)

The incubation period of brucellosis varies considerably and is affected by several factors such as gestation, exposure dose, age, vaccination, and other unknown host-resistance influences. The
incubation period is inversely proportional to the stage of fetal
development at the time of exposure. Incubation periods of 53 to 251
days have been reported.\textsuperscript{89}

It is not unusual for antibodies to \textit{B. abortus} to develop only
after abortion. In one study of 2300 brucellosis-related abortions,
13.3\% of the dams were serologically negative and the infection was
diagnosed by bacteriological results alone.\textsuperscript{61,89}

The variable incubation period and the difficulties of diagnosing
infection until after abortion and transmission have occurred are among
the most serious problems in the epidemiology of brucellosis.

\textbf{Anatomy of the ruminant placenta:} Ruminant placentae are
cotyledonary, villous, and adeciduate. Cotyledonary placentae contain
tufts of chorionic villi, the cotyledons, which interdigitate with
well-vascularized stroma of endometrium, the caruncles. Cow, sheep, and
goat placentae contain 60-100 cotyledons which develop only in those
parts of the placenta which overlay uterine caruncles. Cotyledons are
separated by less specialized areas of smooth chorion. Adeciduate
placentae have a clearcut separation of fetal from maternal components
whereas deciduate placentae carry with them a substantial amount of
maternal tissue when they are delivered after parturition.\textsuperscript{60,69,83}

The exact nature of the ruminant placental barrier is
controversial. The barrier has been termed both syrdenesmochorial and
epitheliochorial because of confusion over the origin of syncytial cells
that cover maternal septae in placentomes.\textsuperscript{7,84,99}

The terms syrdenesmochorial and epitheliochorial were used in
Grosser's placental classification scheme in 1909.\textsuperscript{29,69} Grosser's
histologic classification was based on the number of layers of tissue which appeared to separate fetal from maternal bloodstreams. In epitheliochorial placentae, there are six layers of tissue: 1) endothelium of fetal capillaries, 2) fetal connective tissue, 3) fetal chorionic epithelium, 4) maternal uterine epithelium, 5) maternal connective tissue, and 6) maternal endothelium. Syndesmochorial placentae do not contain maternal epithelium in the barrier and thus are comprised of only 5 layers. Grosser originally classified ruminant placentae as syndesmochorial. Lawn, Bjorkman and others have demonstrated that ruminant placentae are epitheliolchorial in nature, however, Wooding has recently presented evidence that ovine placentae are syndesmochorial.

Placental units of cotyledons and caruncles are termed placentomes, there are two basic types. In the placentome of the cow, the endometrial elevation has roughly the shape of a mushroom with a covering sheath of chorionic villi that project into its substance. The second type is present in the sheep and goat, in these species the endometrium rises above the surface of the uterine wall in the form of a cup into which the chorionic villi fit. The placentomes of other ruminants constitute variations on these basic types.

Within the placentome, the epitheliochorial organization is apparent. Stroma of endometrium containing maternal capillaries, connective tissue, and syncytial epithelium interdigitate with chorionic villi comprised of fetal trophoblasts, connective tissue, and placental capillaries.
The basic fetal component of the placenta is the trophoblast, which differentiates from a single layer of cells surrounding the ovum. Trophoblasts are in intimate contact with uterine epithelium, and cover intercotyledonary areas of the chorion and cotyledonary chorionic villi. The outstanding morphologic characteristic of the trophoblast is its border of microvilli, which interdigitate with similar structures on maternal cells.\(^\text{10,20,39,83,84}\)

Ruminant placentae contain two distinct types of trophoblasts, columnar cytotrophoblasts and round binucleated giant cells. The cytotrophoblast serves as an epithelial covering of placental membranes. It functions in placental attachment, histotropic and hemotropic nutrition of the fetus, steroid and protein hormone secretion, and as a source of binucleate cells.\(^\text{2,10,21,32,83,84,96,100}\)

Binucleate cells are formed from uninucleate cytotrophoblasts by a failure of cytokinesis after normal nuclear division. They constitute approximately one-fifth of the total trophoblasts in ruminant placentae and are interspersed between cytotrophoblasts.\(^\text{100,101}\) They are usually separated from the fetal-maternal interface and the trophoblast basement membrane by cytotrophoblast cytoplasm.\(^\text{84}\) Placental lactogen is a prominent component of binucleate cells and is released into maternal tissues after binucleate cell migration across the microvillous border.\(^\text{97,98,100,101}\) Autoradiographic studies in ovine placentae have demonstrated that syncytial epithelium bordering maternal septae in placentomes is partially formed by fusion of the migrated binucleate cells and a partial loss of the uterine epithelium.\(^\text{97,99,100}\)
The ovine and caprine placentome contain erythrophagocytic cytotrophoblasts which lie between the bases of chorionic villi at the placentomal hilus. Extravasted maternal blood, which escapes from capillaries within tips of the maternal septae, is responsible for the characteristic pigmentation of the placentomal hilus. This maternal blood is phagocytosed and broken down by the erythrophagocytic trophoblasts; probably as a significant source of fetal iron. Hematomas are present at the tips of maternal septae in the bovine placentome, however, erythrophagocytic bovine trophoblasts has yet to be described.

Between placentomes, in intercotyledonary zones, an avillous chorion lies loosely opposed to endometrial epithelium. Vascular, absorptive areole composed of chorioallantoic trophoblasts are opposite endometrial glands. Both cytotrophoblasts and binucleate cells cover the chorioallantoic membrane.

In late pregnancy, trophoblasts in the intercotyledonary areas contain large numbers of dense, heterogenous, and crystalline inclusions (the Stabchen of Bonnet). The significance, composition, or function of these crystalline inclusions is unknown.

**Pathogenesis:** Following oral infection with *B. abortus*, the bacteria first localize in regional lymph nodes of the port of entry. Spread from here is chiefly hematogenous and intermittent bacteremia may occur for several months or cease entirely. In chronic infections, bacteremia may recur irregularly for 2 years in 5 to 10% of animals. In the bacteremic female, localization is largely restricted to the spleen, mammary glands, mammary lymph nodes, and pregnant uterus.
B. abortus selectively localizes in and replicates to large numbers in pregnant bovine uteri. In a study of five naturally infected pregnant cows, it was found that brucellae infection was confined to the fetal cotyledons, fluids, and chorion which contained respectively 60-85%, 1-25%, and 2-8% of the total organisms (3-14 x 10^{13}) found in the mother and fetus. Numbers of B. abortus in fetal cotyledons may reach 10^{11} to 10^{13} organisms per gram of tissue.

Localization in the pregnant bovine uterus has been attributed to its content of the four-carbon sugar, erythritol. Erythritol is present in the placentae and fetal fluids of those animals susceptible to acute brucellar placentitis, such as the cow, ewe, goat, deer, and sow. Erythritol is not present in the placentae of man, rat, rabbit, or guinea pig; species not susceptible to acute brucellar placentitis. Although the exact cellular site of erythritol synthesis is unknown, placental perfusion experiments in the ewe have demonstrated that the placenta is the site of production. B. abortus utilizes erythritol as an energy source in preference to glucose and the bacterium's growth is stimulated by microgram quantities of erythritol. Energy metabolism and erythritol catabolism are tightly linked in Brucella as erythritol is an electron donor in the Brucella electron transport system. Unmetabolizable analogues of erythritol will inhibit B. abortus replication and decrease the severity of disease.

Lesions: B. abortus has a special affinity for the ruminant placenta where it produces extensive lesions. In all descriptions of brucellar placental lesions, since the first in 1886, the investigators
report an abundant yellowish exudate which contains necrotic debris and numerous brucellae\textsuperscript{31,35,51-53,64,81} in the intercotyledonary area between the endometrium and chorioallantoic membrane.

Nocard, a French veterinarian, first described the lesions of contagious abortion in 1886. He recognized the infectious nature of the disease and that it was caused by a bacterium, however, his attempts to isolate a causative bacterium failed. He examined several cows which had recently aborted and found "between the uterus and the foetal envelopes a considerable quantity of yellowish, flocculent, purulent material." He concluded that contagious abortion was not a disease of the uterine mucosa, but rather, a bacterial disease of the fetus and "its envelopes."\textsuperscript{81}

Bang, in 1897, isolated a bacterium associated with contagious abortion which he called \textit{Bacillus abortus}. He necropsied several cows with "the well-known premonitory signs of abortion" and found between the uterine wall and the foetal envelopes "an abundant, odourless, exudate—a dirty yellow, somewhat thin, pultaceous material, of a slimy, somewhat lumpy character." His examination of this exudate demonstrated the presence of a small bacterium, usually present in dense clumps within distended cells. The cell nucleus and a portion of the cell cytoplasm could still be discerned peripheral to the mass of intracellular bacteria. Another constant feature of placentitis which Bang reported was "the oedematous condition of the fine subchorial connective tissue."\textsuperscript{4}

Bang produced the disease in pregnant heifers and ewes by intravaginal and intravenous administration of the bacterium which he
isolated from field cases of contagious abortion. Similar placental lesions were present in each case of experimentally produced disease. 4

Theobold Smith, in 1919, described the characteristic localization of *Bacillus abortus* in trophoblasts covering the chorioallantoic membrane. He described chorioallantoic trophoblasts as a "layer of cells which faces the epithelial covering of the uterine mucosa and is in intimate contact with it, covers the intercotyledonary areas of the chorion, and is continuous with the epithelium of the villous areas of the cotyledons which dip into the depressions of the maternal caruncles." He further stated that in contagious abortion "these cells, either individually or in series, are densely filled with minute bacilli." Smith theorized that the bacteria enter the uterochorionic space by way of the blood vessels in the uterine wall and "are rubbed into the substance of the cell by the pressure exerted by the uterine wall on the chorion." He believed that the bacteria found the intracellular residence in chorionic epithelial cells a favorable medium for multiplication and a protection against phagocytosis. The other types of epithelium, such as those of the uterine mucosa and of the amnion, were not found to contain bacilli. Smith considered this as the earliest stage of the disease "by which the organism gains by rapid unchecked multiplication, a considerable advantage over the host." 78

Since the original description of *B. abortus* by Bang in 1897, a mass of literature has accumulated concerning the bacteriology, pathology, and immunology of ruminant brucellosis. The first thorough description of the pathogenesis and lesions of bovine brucellosis were made by Payne in 1959. 64 Payne inoculated 11 cows with *B. abortus* in an
experiment designed to "elucidate the mechanism whereby *B. abortus* enters the uterus of the pregnant cow and induces changes which lead to fetal death and abortion." The cows were necropsied at weekly intervals and most organs studied by techniques of bacteriology and pathology.

Payne concluded that, after conjunctival infection, brucellae spread to regional lymph nodes of the head. Brucellae then spread hematogenously to lymphoid tissue elsewhere, such as the spleen and iliac and supramammary lymph nodes. An acute lymphadenitis characterized by lymphoid hyperplasia and plasma cell infiltration was present. After localizing in lymphoid organs, Payne believed that blood-borne organisms infect the gravid uterus by way of the endometrium and uterine glands.

The earliest uterine lesion reported was inflammation in the periglandular connective tissue of the endometrium. Uterine inflammation was mild even though exudate within the uterine lumen contained large numbers of brucellae. Purulent exudate formed within uterine glands and was excreted into the uterine lumen (intercotyledonary space). He believed this to be the origin of the heavily-infected uterine exudate so characteristic of the disease. Severe ulcerative endometritis followed glandular inflammation.

Large numbers of *B. abortus* were isolated from the uterine lumen in the space between the uterine mucosa and the chorioallantoic membrane (intercotyledonary space). The chorioallantoic membrane was separated from the uterus and placentome by a thick layer of pale eosinophilic exudate composed of necrotic debris and dead cells filled with masses of
bacteria. Payne stated that *B. abortus* multiplied to enormous numbers within chorionic trophoblasts.

In many cases where the uterine lumen was heavily infected, positive cultures were obtained from only one or two cotyledons. Payne felt that cotyledons slowly became involved by spread of the intercotyledonary exudate along the chorioallantoic membrane. This concurs with the observations of others, in that, the primary site of intrauterine bacterial replication was in the chorioallantoic trophoblasts and intercotyledonary space (uterine lumen).

At or near abortion, large numbers of bacteria and inflammation were present in connective tissue of the chorionic (cotyledonary) villus. He felt that the brucellae arrived there hematogenously, via the fetal blood stream, after ulceration of chorioallantoic membranes.

Mollello reported lesions similar to Payne's descriptions when *B. abortus, B. melitensis,* or *B. ovis* were inoculated into pregnant sheep. Consistent lesions were extensive placental edema, peri- to interplacental exudates, and ulcerated chorioallantoic membranes. The chorioallantoic exudate contained necrotic cellular debris, bacteria, macrophages and neutrophils, and desquamated epithelial cells containing bacteria. Changes in late stages of placentitis were necrosis of cotyledonary villi and chorioallantoic membranes. Large numbers of bacteria were present in connective tissue of inflamed or necrotic villi.

Mollello concluded that after maternal bacteremia in sheep, brucellae are phagocytosed by placental trophoblasts; they then replicate in adjacent chorioallantoic trophoblasts; and are subsequently
released in massive numbers into the uterine lumen after trophoblast necrosis. He believed that brucellae gained access to the fetal circulation through sub-epithelial capillaries beneath ulcerated chorioallantoic membranes. He emphasized the marked affinity of brucellae for chorionic epithelium and the relative immunity of maternal uterine tissues to brucellae infection.

**Brucellosis in goats:** The goat is the natural host for *B. melitensis* and thus nearly all caprine brucellosis studies utilize *B. melitensis*-infected goats. The goat was first proposed as suitable for studies regarding the pathogenesis of bovine brucellosis caused by *B. abortus* in 1939. At that time, Doyle produced abortion in 36 out of 48 pregnant goats conjunctivally inoculated with *B. abortus*. *B. abortus* could be isolated from milk or lymphoid tissues of inoculated goats for up to 386 days. There was no marked change in the pathogenicity of two bovine strains of *B. abortus* serially passaged through goats.

**Bacterial placentitis in ruminants:** The list of bacterial infections known to produce sporadic abortion in ruminants is long. Any bacteremia occurring during pregnancy carries with it the risk of bacterial colonization of the fetal chorion. However, there are bacteria with tropism for the placenta that produce characteristic placental lesions and which are commonly associated with ruminant abortions. These common abortifacients will be examined first, followed by a discussion of other bacteria which rarely produce abortions or characteristic placentitis in ruminants.
Bacteria which commonly cause placentitis and abortion in ruminants include *Campylobacter fetus* var. *venerealis*, *Campylobacter fetus* var. *intestinalis*, *Listeria monocytogenes*, *Chlamydia psittaci*, *Coxiella burnetti*, and *Leptospira* spp.

*Campylobacter fetus* var. *venerealis* causes a venereal disease in cattle. The organism is carried by the bull and transmitted at coitus. Early embryonic death is the common manifestation, however, abortions can occur about the fourth to sixth month of gestation. Placental lesions resemble those in brucellosis but are less severe. The intercotyledonary placenta is edematous and leathery with a diffuse histiocytic infiltration. Cotyledons contain yellow necrotic foci and large numbers of polymorphonuclear leukocytes among denuded villi and in the villous stroma. Thrombosed vessels are present in the chorion and chorionic villi. *Campylobacter fetus* var. *venerealis* also parasitizes the chorionic trophoblastic epithelium, however, to a lesser degree than observed with *Brucella* species.\(^{35,73}\)

*Campylobacter fetus* var. *intestinalis* causes genital infection in sheep characterized by abortions, premature births, and the birth of weak lambs. It occasionally causes bovine abortion but is much less important in cattle than *Campylobacter fetus* var. *venerealis*. *Campylobacter fetus* var. *intestinalis* causes primarily intestinal infection; transmission is by ingestion, and uterine localization follows bacteremia in non-immune sheep.\(^{35}\) Placental lesions and bacterial localization resemble those present in brucellosis. Eighty to ninety percent of the total organisms present in the ewe are present in the cotyledons, allantoic fluid and chorion.\(^{40}\)
In ovine campylobacteriosis, hematomas and trophoblasts at the placentomal hilus are the first placental sites involved, these sites contain large numbers of bacteria. Chorioallantoic membranes are edematous and infiltrated with leucocytes. Campylobacter colonies distend the cytoplasm of chorionic trophoblasts and endothelial cells and fill capillary lumens. Vasculitis and thrombi are present in caruncular septae. Multifocal necrotizing hepatitis is present in aborted fetuses.34

Infections caused by *Listeria monocytogenes* in ruminants are usually encephalitic, however, localization in pregnant uteri and subsequent abortions are not uncommon. Abortions occur in cattle, sheep, and goats during the last trimester and rarely occur with the encephalitic form of the disease. Like in the encephalitic disease, route of infection is usually oral. Abortions may be sporadic or involve up to 50% of the herd. In sheep, hematomas and chorionic epithelium at the placentomal hilus contain large numbers of bacteria. In all ruminants, chorioallantoic membranes are ulcerated and covered by exudate containing bacteria, necrotic debris, and desquamated trophoblasts. Small numbers of chorionic trophoblasts contain bacteria. Necrotic tips of villi are covered by a purulent exudate containing large numbers of bacteria. Multifocal necrotizing hepatitis is present in aborted fetuses.28,35,54,62,85,92

*Campylobacter fetus* var. *intestinalis* and *Listeria monocytogenes*, like *B. abortus* selectively localize and proliferate in ruminant placentae. They, however, show no growth enhancement when exposed to
Chlamydia psittaci causes abortion in sheep and goats. The disease is known as enzootic abortion of ewes, and is manifested as abortion late in gestation or premature lambings. Placental lesions in sheep and goats resemble closely those present in brucellosis. The first site of chlamydial localization is the trophoblastic epithelium lining hematomas in the placentome. The chorioallantoic membrane is edematous or thick and leathery, and infiltrated with leucocytes. As with brucellosis, large numbers of organisms are present within chorioallantoic trophoblasts. Chorioallantoic necrosis and ulceration and widespread placental vasculitis are prominent features of chlamydial placentitis. Histologic lesions in the fetus are mild and nonspecific.\textsuperscript{35,86,87}

The role of Chlamydia psittaci in causing bovine abortions is unknown. Despite previous reports, it is not currently believed to be the etiological agent of epizootic bovine abortion (foothills abortion). The organisms readily produce placentitis in experimental animals, and numerous investigators have isolated chlamydia from aborted fetuses, but a definite role for Chlamydia psittaci in bovine abortions has yet to be proven.\textsuperscript{35}

Coxiella burnetti causes abortions in sheep and goats. Persistent infection follows inhalation of the organism in contaminated dust. Abortion occurs late in gestation, usually after initial exposure, but may be uncommon in endemically infected flocks. The chorioallantoic membrane is thickened and leathery, with copious exudate covering the intercotyledonary areas. Large numbers of organisms distend the
cytoplasm of infected chorioallantoic trophoblasts. An acute diffuse suppurative placentitis is largely confined to the intercotyledonary areas. Vasculitis is not a prominent feature as it is with chlamydial placentitis. Histologic lesions in aborted fetuses are mild and nonspecific.43,63,94

*Leptospira interrogans* serovars *hardjo* and *pomona* are the most common causes of bovine leptospiral abortion. Most abortions occur in the last third of gestation after leptospiremenia and leptospiral localization in placentae. The placentas may be edematous, but leucocyte infiltration is absent or mild in spite of large numbers of leptospires within chorionic trophoblasts. Caruncles may contain increased interstitial connective tissue. Interstitial nephritis is present in affected fetuses.35,56,57

Other bacterial pathogens can produce abortion but are not generally associated with herd outbreaks of reproductive failure. Salmonellosis is an important cause of abortion in certain geographic areas. *Salmonella dublin* and *Salmonella typhimurium* are the most frequent isolates involved. The majority of *Salmonella* induced abortions are not associated with disease in the dam. Affected placentae are edematous and the intercotyledonary area is covered by a yellow exudate in which organisms are abundant. The placentitis is not histologically distinctive, and the fetus contains few lesions.35

*Hemophilus somnus* may produce abortions in cattle unassociated with the encephalitic form of the disease. Experimentally, abortions have been produced by amniotic and intravaginal bacterial inoculation. Placentitis is characterized by chorioallantoic necrosis, necrotizing
vasculitis, thrombi, and numerous bacteria within trophoblasts. A necrotizing metritis and vaginitis are present in the dam. Mild, nonspecific lesions are present in aborted fetuses.35,48,49

Other organisms that rarely produce bacteremias in adult animals may produce placentitis and sporadic abortions. These organisms include Corynebacterium pyogenes, E. coli, Bacillus spp., Pasteurella spp., Staphylococcus spp., Yersinia pseudotuberculosis and Streptococcus spp. There are no distinctive characteristics of these abortions and neither the placentitis or fetal lesion is specific. In order to confirm abortion due to these organisms, bacteria should be demonstrable in large numbers in smears from the placenta, there should be evidence of placentitis, and bacteria should be present in fetal abomasal contents.35,36

Thus, a large number of bacteria can colonize the ruminant chorion, infect the fetus, and cause abortion. The ability of a bacterium to produce abortions depends primarily on its ability to survive intracellularly and produce a bacteremia during pregnancy. A majority of the bacteria which are capable of producing epidemic abortion outbreaks are facultative or obligate intracellular pathogens which can survive and replicate in trophoblasts after hematogenous placental infection. The basis of preferential bacterial replication in trophoblasts is unknown for all of these placental pathogens.
PATHOGENESIS OF PLACENTITIS IN THE GOAT INOCULATED WITH BRUCELLA ABORTUS.

I. GROSS AND HISTOLOGIC LESIONS
PATHOGENESIS OF PLACENTITIS IN THE GOAT INOCULATED
WITH BRUCELLA ABORTUS.

I. GROSS AND HISTOLOGIC LESIONS

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ABSTRACT

Pregnant goats were inoculated intravenously or in uterine arteries with Brucella abortus. Tissues from the uterus and placentae were examined at various post-inoculation intervals to study mechanisms of placental infection. Placentitis was present by 5 days post-inoculation and abortions occurred within 11 days. B. abortus was identified in placentae by light microscopy and immunoperoxidase techniques. Brucellae were first seen in erythrophagocytic trophoblasts of the placentome. Subsequently, high numbers of brucellae were seen in periplacentomal chorioallantoic trophoblasts. Trophoblast necrosis, chorioallantoic ulceration, and large numbers of brucellae in chorionic villi were present in later stages of infection. These results suggest that entry and replication of brucellae in trophoblasts precede placentome and fetal infection and that trophoblasts are the source of brucellae for these tissues. Experimental caprine brucellosis closely resembles bovine and ovine brucellosis and it provides a model to study the intracellular development of B. abortus in trophoblasts.
Introduction

Brucella abortus has a marked predilection for the ruminant placenta. In acute infections of pregnant cows, up to 85% of the bacteria are in cotyledons, placental membranes, and allantoic fluid. Numbers of B. abortus in placental tissues can reach $10^{10}$ organisms/ml of allantoic fluid or $10^{11}$ to $10^{13}$ per gram of fetal cotyledons. In 1919, Theobald Smith described the characteristic intracellular localization of B. abortus in ruminant chorionic trophoblasts. The marked affinity (tropism) for and replication of B. abortus in trophoblasts contributes significantly to placentitis.

Placental lesions and the pathogenesis of brucellosis have been characterized in cattle and sheep. In the pregnant cow, B. abortus localizes initially in lymph nodes, infects the gravid uterus during bacteremia and multiplies to enormous numbers in chorionic trophoblast cells. Replication of brucellae in chorionic trophoblasts also occurs in ovine brucellosis caused by B. ovis, B. melitensis, and B. abortus. In all instances, fetal bacteremia occurs after replication of brucellae in trophoblasts, trophoblast necrosis, and chorioallantoic ulceration. Subsequently, fetal viscera and placental cotyledons become heavily infected with brucellae. Placentitis and abortions also occur in caprine brucellosis due to B. abortus.

Mechanisms of placental localization, trophoblast tropism and abortion in ruminant brucellosis are poorly understood. The objective of this study was to evaluate a caprine model for study of ruminant brucellosis and to characterize the lesions associated with placentitis.
We were particularly interested in determining how trophoblasts contribute to the entry, localization, and replication of brucellae in placentae.
MATERIALS AND METHODS

Experimental design: Twenty 2-to-3-year-old pregnant alpine crossbred goats in their last third of gestation were inoculated with varying amounts of \textit{Brucella abortus} (strain 2308 supplied by Dr. B. Deyoe, National Animal Disease Center, Ames, IA 50010) in jugular veins, uterine arteries, or the uterine lumen (Tables 1 & 2). Goats were maintained in isolation until necropsy. Four pregnant goats were not inoculated and served as controls. All goats lacked serologic titers to \textit{B. abortus} (card and tube agglutination test) and no clinical evidence of brucellosis was apparent in the herd history.

Inoculation procedures: Jugular vein inoculations were done by injecting varying amounts of \textit{B. abortus} into either the right or left jugular vein (Table 1). Uterine artery inoculations were done by ventral laparotomy on goats anesthetized with 2 to 4 mg/kg ketamine hydrochloride (Ketaset, Bristol Laboratories, Syracuse, NY 13201) and 0.1 mg/kg xylazine (Rompun, Haver-Lockhart, Shawnee, KS 66201). Gravid uteri were removed from the abdomen, the middle uterine arteries exposed and varying amounts of \textit{B. abortus} (Table 1) suspended in phosphate buffered saline injected into one or both arteries. One goat was inoculated into the uterine lumen with $10^6$ \textit{B. abortus} after an unsuccessful attempt at inoculating the uterine artery. At first, random dosages were selected to evaluate the effect of dose upon lesion development. Later, most goats received $10^9$ brucellae/ml of diluent (Tables 1 & 2). All goats appeared clinically normal after inoculations and were observed twice daily until necropsy.
Necropsy and tissue collection: Goats were necropsied from 2 to 23 days post-inoculation at randomly selected times. At necropsy, gravid uteri were exposed by ventral laparatomies on anesthetized, heparinized goats. After sodium pentobarbital overdose uteri were fixed by intraarterial perfusion into the middle uterine arteries of 1.25% glutaraldehyde and 1.0% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. Uterine veins were severed and perfusion was started by flushing erythrocytes with Hanks' balanced salt solution, pH 7.4 containing 0.1% procaine followed by the aldehyde fixative. Uteri were perfused with 3 to 5 liters of fixative over a period of approximately 5 to 10 minutes. The uteri were then removed from vaginal attachments, and placental tissues were collected for light and electron microscopy.

Uteri became firm and hard within 2 to 4 minutes after fixative perfusion was started. Uteri of goats which had aborted were not perfused due to uterine edema and reduced size of uterine arteries. Specimens for bacteriology were collected from placentae before fixative perfusion and from the remainder of maternal and fetal tissues following uterine perfusion.

Preparation of tissues: Multiple 1 cm thick tissue sections of uterus, placentome, fetal lung and liver, and several sections of peri- and interplacentomal chorioallantoic membrane were randomly collected from each goat and fixed for 24 hours in 10% neutral buffered formalin. Tissues were dehydrated in graded alcohols, cleared, infiltrated and embedded in paraffin, cut at 6 μm, and stained with hematoxylin and eosin (H & E), and Brown-Brenn modified Gram stain for histologic examination.
Bacteriology: Maternal tissues were collected from uteri, inguinal and peripheral lymph nodes, spleen, mammary gland, and supramammary lymph node. Fetal spleen, lung, liver, kidney, peritoneal fluid and stomach contents were also collected. Bacteria were cultured by direct smear of sliced tissue surfaces or fluid samples onto tryptose agar containing 5% bovine serum, ethyl violet (1:800,000), cyclohexamide (30 μg/ml), bacitracin (7.5 units/ml), and polymixin B (1.8 units/ml). Numbers of B. abortus were quantitatively determined on selected samples of placentome and allantoic fluid. For quantitative determinations, portions of placentome were homogenized in physiologic saline. This homogenate and the allantoic fluid were serially diluted prior to plating on agar. B. abortus colonies were identified by colony morphology, growth characteristics and agglutination by Brucella abortus-specific antisera.

Immunoperoxidase: Localization of Brucella abortus in selected placental tissues was performed by immunoperoxidase techniques utilizing biotinylated secondary antibody and an avidin-biotin-peroxidase complex using techniques previously described. Primary antibody was bovine anti-Brucella abortus IgG precipitated from milk whey of a B. abortus infected cow (Judith M. Patterson, National Animal Disease Center, Ames, IA 50010). Biotinylated anti-goat IgG (immunologically reacts with bovine IgG) and the avidin-biotin-peroxidase complex were commercially available (Vectastain™ ABC kit, Vector Laboratories, Inc., Burlingame, CA 94010).
RESULTS

Clinical observations: Abortion occurred in 6 of 20 goats from 11 to 18 days after inoculation; no other evidence of clinical disease was detected. Aborted fetuses were often passed with no maternal straining or preparturient signs. Two of 10 goats inoculated by the jugular vein aborted on post-inoculation days 11 and 15. Four of 10 goats inoculated via the uterine artery had aborted, or were in the process of aborting, at the time of euthanasia on post-inoculation days 12, 14 and 18 (Table 1). No clinical signs were observed in the remaining 14 infected animals.

Gross lesions: Placental lesions were present in 5 of 10 jugular-inoculated goats and 10 of 10 goats inoculated in the uterine arteries or lumen (Tables 1 and 2). Diffuse inflammation of the chorioallantois was the most prominent placental lesion and was characterized by thick, tenacious, yellow exudate covering the trophoblastic side of a diffusely edematous and thickened chorioallantoic membrane (Figs. 1,2,3). The single goat inoculated in the uterine lumen had focal exudate on the chorioallantoic membrane at the site of inoculation.

In those goats with mild placentitis, lesions were limited to the periplacentomal chorioallantoic membrane. Severe placentitis was characterized by periplacentomal chorioallantoic exudate and diffuse interplacentomal exudate and/or edema (Figs. 1,2,3). Four of the 15 goats with placentitis had mild lesions (defined previously). The remaining 11 goats, including those which had aborted, had severe
Lesions. Lesions in placentomal cross-sections were present in two goats with severe placentitis (Figs. 4,5).

Diffuse suppurative metritis, characterized by layers of green-tinged, hemorrhagic exudate covering caruncles and endometrium was present in goats which had aborted. Placentae of these goats were expelled within 12 hours following abortion. Hemorrhagic and suppurative vaginal discharge was observed for several days after abortion and was generally present at necropsy.

Dead fetuses were present in 4 of 16 goats necropsied before or at the time of abortion. All aborted fetuses, from the 4 goats necropsied after abortion, were dead when expelled. Lesions in dead fetuses were cherry-red subcutaneous edema and a fibrinous peritonitis with fibrin sheets covering abdominal viscera.

Lesions were not observed in respiratory, cardiac, gastrointestinal, mammary, or urinary systems of inoculated and control goats or in the four placentae of control goats.

**Histological lesions:** Brucellae were first present in the hilus of the placentome. Bacterial colonies were present in hematomas between tips of maternal septa and erythrophagocytic trophoblasts at the base of chorionic villi (Figs. 6,7). Hematomas contained bacterial colonies, neutrophils, and necrotic cellular debris. Erythrophagocytic trophoblasts contained intracellular bacteria adjacent to phagocytosed erythrocytes (Fig. 8) and were continuous with brucellae-filled chorioallantoic trophoblasts.

Immunoperoxidase localization of *Brucella abortus* in placentae demonstrated brucellae in placentomal hematomas and erythrophagocytic
trophoblasts (Fig. 6) concurrent with chorionic trophoblast infection. This was always present before brucellae or inflammation were observed in placentomal parenchyma.

The most prevalent placental lesions were present in chorioallantoic membranes and were characterized by diffuse filling of chorioallantoic trophoblasts with intracellular brucellae (Fig. 9). In later stages many trophoblasts were necrotic and the chorioallantoic membranes were ulcerated and covered with exudate (Fig. 10). Chorioallantoic ulceration was often adjacent to brucellae-filled trophoblasts. The cytoplasm of bacteria-infected trophoblasts contained large numbers of brucellae which surrounded cell nuclei. Intracellular bacteria were observed only in trophoblastic epithelium (Fig. 9). The exudate covering ulcerated chorioallantoic membranes consisted of desquamated, brucellae-filled trophoblasts, necrotic cellular debris, free bacteria, macrophages, and neutrophils (Fig. 10). Lesions of chorioallantoic membranes were prominent before bacteria or inflammation were present in placentomal parenchyma.

When chorioallantoic lesions were severe, prominent lesions were present in placentomes (Figs. 4,5). Bacterial colonies were prevalent in connective tissue of chorionic villi: either rimmed by intact trophoblastic epithelium or surrounded by neutrophils and necrotic cell debris (Fig. 5). Intraepithelial brucellae were not observed in placentomal trophoblasts or maternal syncytial epithelium. The syncytial epithelium lining maternal septa was often necrotic.

Goats with severe placentitis had a mild suppurative endometritis with exudate in endometrial glands and lymphoid cells in endometrial
lamina propria. Endometrial epithelium was intact. No brucellae were present in endometrial epithelium or lamina propria. Necrosis of caruncles, endometrial ulceration, severe suppurative metritis, and numerous surface bacterial colonies were present in uteri after abortion.

Suppurative bronchopneumonia, fibrinous perihepatitis and perivascular hepatitis were present in dead fetuses. Perivascular infiltrates in livers consisted of macrophages and lymphocytes.

**Bacteriology:** *B. abortus* was isolated from all goats inoculated with doses of at least $10^6$ *B. abortus/ml* of inocula (Table 3). No placental lesions were present and no bacteria were isolated from the single goat inoculated intravenously with $10^5$ *B. abortus/ml*. The number of tissues culture positive for *B. abortus* was higher in the maternal and fetal tissues of those animals inoculated via the uterine artery. The spleens, lymph nodes, and placentomes consistently contained *B. abortus*. Fetal tissues often contained brucellae even when allantoic fluid did not. Average placentome concentration of *B. abortus* in 5 placentomes was $2.2 \times 10^8$ organisms/gm. Two samples of allantoic fluid contained $2 \times 10^3$ and $5 \times 10^9$ *B. abortus/ml*. Brucellae were not isolated from control goats.
Fig 1—Placenta of a goat inoculated in the middle uterine arteries with $10^9$ B. abortus and necropsied on day 12 post-inoculation. Severe placentitis is present and characterized by diffuse interplacentomal (black arrows) and periplacentomal (white arrows) exudate. Placentomes appear normal. Bar = 2 cm.

Fig 2—Placenta of a goat inoculated in the middle uterine arteries with $10^9$ B. abortus and necropsied on day 12 post inoculation. Cross-section of placentome (P) is normal, however, thick and tenacious exudate is present on the trophoblastic side of the chorioallantoic membrane (arrow). Uterine wall (U), chorioallantoic membrane (CA). Bar = 1 cm.
Fig 3—Diffuse chorioallantoic edema obscuring view of placentome in a goat inoculated intravenously with $10^9$ B. abortus and necropsied on post-inoculation day 19. Whitish periplacentomal exudate is prominent (arrows). Bar = 1 cm.

Fig 4—Placentome from a goat inoculated in a middle uterine artery with $10^9$ B. abortus and euthanized while aborting on post-inoculation day 14. Multiple foci of abscessation and hemorrhage are present in placental cross-sections. Uterine wall (U), placentome (P), chorioallantoic membrane (CA). Bar = 1 cm.
Fig 5—Multifocal abscessation and bacterial colonies in placentome of Fig 4. a) Colonies of B. abortus (Br) are in connective tissue of chorionic villi and rimmed by intact trophoblastic epithelium (Tr). Exudate (arrows) separates maternal septa (S) and capillaries (C) from bacteria-filled chorionic villus. Bar = 30 um b) Bacteria-filled phagocytes (arrow) and necrotic cell debris separating maternal septa (S) from trophoblasts (Tr). Brown-Brenn Gram stain. Bar = 20 um.

Fig 6—Immunoperoxidase localization of B. abortus, placentome from a goat inoculated intravenously with $10^9$ B. abortus and necropsied on post-inoculation day 23. a) Bovine anti-B. abortus IgG used as primary antibody. Labeling of B. abortus in hematomas at base of chorionic villi (arrow) and in exudate covered chorioallantoic membrane (CA-arrow). Endometrial glands (E) and placentome (P) are normal and no bacteria are present in connective tissue of fetal chorionic villi (small arrows). Placentomal parenchyma is free of brucellae. b) Same placentome, normal bovine serum used in place of primary antibody. No peroxidase reaction product is visible. Hematoxylin. Bar = 0.5 cm.
Fig 7—Immunoperoxidase localization of *B. abortus* in placentome from Fig 6. Brown peroxidase reaction product signifies location of brucellae cells. a) Intracellular brucellae are present in chorioallantoic trophoblasts (bottom) but not uterine endometrium, glands, or stroma (top). Magnification = 100x. b) Brucellae are clustered in perinuclear cytoplasm of chorioallantoic trophoblasts. Magnification = 400x.
Fig 8—Hematoma from placentome in Fig. 6. Bacterial colonies (Br) are surrounded by maternal erythrocytes (RBC) and adjacent to erythrophagocytic trophoblasts (Tr). Intracellular B. abortus (arrow) are adjacent to phagocytosed erythrocytes. Brown-Brenn Gram stain. Bar = 15 um.

Fig 9—Chorioallantoic membrane from a goat inoculated intravenously with $10^9$ B. abortus and necropsied on post-inoculation day 23. Chorionic trophoblasts filled with intracellular brucellae (small arrows) are adjacent to inflamed uterine epithelium. One brucellae-filled chorionic trophoblast has nearly sloughed into the uterine lumen (arrow). H & E stain. Bar = 40 um.

Fig 10—Chorioallantoic membrane from Fig. 9. Necrosis of brucella-filled trophoblasts with ulceration of chorioallantoic membrane (arrows) is adjacent to intact brucella-filled chorionic trophoblasts (Tr). Overlying exudate contains brucella-filled trophoblasts (Tr), free brucellae, neutrophils (N) and necrotic cell debris. H & E stain. Bar = 30 um.
Table 1. Inoculation dosages, clinical signs, and necropsy observations of goats infected intravenously with *Brucella abortus* arranged by time of necropsy

<table>
<thead>
<tr>
<th>Goat ID</th>
<th>Inoculation dose</th>
<th>Necropsy PID(^a)</th>
<th>Clinical signs</th>
<th>Degree of placentitis(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 ml (10^9)</td>
<td>2</td>
<td>ketosis and death</td>
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</tr>
<tr>
<td>2</td>
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<td>none</td>
</tr>
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<td>9</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>4</td>
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</tr>
<tr>
<td>6</td>
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<td>aborted PID 15</td>
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</tr>
<tr>
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<td>none</td>
</tr>
<tr>
<td>8</td>
<td>1 ml (10^9)</td>
<td>19</td>
<td>aborted PID 11</td>
<td>severe</td>
</tr>
<tr>
<td>9</td>
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<td>19</td>
<td>none</td>
<td>severe</td>
</tr>
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<td>1 ml (10^9)</td>
<td>23</td>
<td>none</td>
<td>severe</td>
</tr>
</tbody>
</table>

\(^a\)PID = Post-inoculation days.

\(^b\)Criteria for degree of placentitis defined in gross lesion results.
<table>
<thead>
<tr>
<th>Goat ID</th>
<th>Inoculation dose</th>
<th>Necropsy PID(^a)</th>
<th>Clinical signs</th>
<th>Degree of placentitis(^b)</th>
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<tbody>
<tr>
<td>11</td>
<td>1 ml (10^9)</td>
<td>5</td>
<td>none</td>
<td>mild</td>
</tr>
<tr>
<td>12</td>
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<td>6</td>
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<td>1 ml (10^9)</td>
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<td>aborting PID 12</td>
<td>severe</td>
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</tr>
<tr>
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<td>1 ml (10^9)</td>
<td>14</td>
<td>aborting PID 14</td>
<td>severe</td>
</tr>
<tr>
<td>18</td>
<td>1 ml (10^9)</td>
<td>15</td>
<td>none</td>
<td>severe</td>
</tr>
<tr>
<td>19</td>
<td>1 ml (10^9)</td>
<td>18</td>
<td>aborted PID 18</td>
<td>severe</td>
</tr>
<tr>
<td>20</td>
<td>1 ml (10^9)</td>
<td>19</td>
<td>aborted PID 18</td>
<td>severe</td>
</tr>
</tbody>
</table>

\(^a\)PID = Post-inoculation days.

\(^b\)Criteria for degree of placentitis defined in gross lesion results.

\(^c\)Goat was inoculated directly into uterine lumen.
Table 3. Tissues of inoculated goats from which *Brucella abortus* was isolated

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Jugular vein</th>
<th>Uterine artery or lumen&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>9/10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10/10</td>
</tr>
<tr>
<td>Lymph nodes&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Placenta</td>
<td>5/7</td>
<td>10/10</td>
</tr>
<tr>
<td>Allantoic fluid</td>
<td>4/10</td>
<td>5/10</td>
</tr>
<tr>
<td>Fetal tissues&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6/10</td>
<td>9/9</td>
</tr>
</tbody>
</table>

<sup>a</sup>One goat was inoculated into the uterine lumen. *B. abortus* was cultured from all maternal and fetal tissues.

<sup>b</sup>Data are expressed as number of goats from which *B. abortus* was isolated/number of goats cultured.

<sup>c</sup>Lymph nodes included parotid, mandibular, retropharyngeal, prescapular, internal iliac, and supramammary.

<sup>d</sup>Fetal tissues included spleen, lung, liver, kidney, stomach contents, and peritoneal fluid.
DISCUSSION

Erythrophagocytic and chorioallantoic trophoblasts play a dominant role in the entry and spread of *B. abortus* in caprine placental tissues. We believe that initial infection of placentomal erythrophagocytic trophoblasts with subsequent spread of brucellae to adjacent chorioallantoic trophoblasts accounts for periplacentomal placentitis which is present in acute brucellosis.\textsuperscript{10,13-16} Infection of chorioallantoic trophoblasts and massive intracellular replication of brucellae preceded placentomal and fetal infection. Thus, trophoblasts were likely the source of brucellae for these tissues.

Erythrophagocytic trophoblasts may serve as the initial entry site of brucellae into placenta. In addition, these trophoblasts are continuous with chorioallantoic epithelium and may serve as an initial source of infection to chorioallantoic trophoblasts. Immunoperoxidase labeling of brucellae in placentae demonstrated brucellae in placentomal hematomas and erythrophagocytic trophoblasts concurrently with chorioallantoic trophoblast infection. Extravasated maternal blood, which escapes from capillaries and larger blood vessels within the tips of maternal septa, is responsible for the characteristic pigmentation of the central depression of the placentome in small domestic ruminants.\textsuperscript{5} The trophoblasts in this region are actively engaged in the uptake and subsequent breakdown of maternal erythrocytes, probably as an important source of iron for the fetus. During bacteremia, brucellae may also be released from the maternal blood stream and be phagocytosed by erythrophagocytic trophoblasts with maternal erythrocytes. This proposed mechanism requires further study in the cow.
Intracellular replication in chorioallantoic trophoblasts was responsible for the accumulation of large numbers of brucellae in placentae. Trophoblasts may enhance growth of \textit{B. abortus} because of erythritol content, hormone synthesis, or by other undefined mechanisms. Ruminant placentae produce erythritol and progesterone. Both erythritol and progesterone enhance \textit{in vitro} growth of \textit{B. abortus}.

Necrosis of trophoblasts released large numbers of organisms into the uterine lumen. Chorionic villi of placentomes and fetal viscera became heavily infected after hematogenous dissemination of brucellae from uterine lumens through ulcerated chorionic membranes. In bovine brucellosis, brucellae are first located in chorionic trophoblasts and the uterine lumen (intercotyledonary space). In later stages, large numbers of brucellae are hematogenously disseminated to chorionic villi and fetal tissues from the uterine lumen. Payne suggested that bacteremic brucellae first localize in uterine lamina propria and then spread to the uterine lumen and chorionic trophoblasts. A similar sequence of tissue infection occurred in our goats, however, we believe that brucellae initially infected placentomal erythrophagocytic trophoblasts directly from the vascular system. Chorioallantoic trophoblasts were infected by extension from the placentome. Endometrial stroma or epithelium did not appear to be important in the sequence of events in placental infection. Thus, we believe trophoblasts to be the primary cell type involved in the entry, localization, and replication of brucellae in placentae.
Our observations on the sequence of events in caprine brucellar placentitis concur with those of Molello's for ovine brucellosis. He reported that after maternal bacteremia in sheep, brucellae are phagocytosed by placentomal erythrophagocytic trophoblasts, replicate in adjacent chorionic trophoblasts, and are released in massive numbers into the uterine lumen after trophoblast necrosis. As in our goats, brucellae present in the uterine lumen are disseminated hematogenously to placentomal chorionic villi and fetal viscera through ulcerated chorionic membranes.

Experimental caprine brucellosis closely resembles natural and experimental bovine and ovine brucellosis, i.e., intracellular replication of \( B. \) \textit{abortus} in chorioallantoic trophoblasts, peri- and interplacentomal exudate, high numbers of \( B. \) \textit{abortus} in placental tissues, and abortion. The caprine placenta is anatomically similar to bovine and ovine placentae, all are cotyledonary villous, epitheliolchorial, and nondeciduate. Ultrastructural examination of placentae from experimentally infected goats may allow further definition of cellular mechanisms by which brucellae infect placentae, replicate in chorioallantoic trophoblasts, and cause abortion.
LITERATURE CITED


6 Doyle TM: *Brucella abortus* infection of goats. J Comp Path and Ther 52:89, 1939


PATHOGENESIS OF PLACENTITIS IN THE GOAT INOCULATED WITH BRUCELLA ABORTUS.

II. ULTRASTRUCTURAL STUDIES
PATHOGENESIS OF PLACENTITIS IN THE GOAT INOCULATED WITH BRUCELLA ABORTUS.

II. ULTRASTRUCTURAL STUDIES

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ABSTRACT

Pregnant goats were inoculated intravenously or in uterine arteries with *Brucella abortus* and tissues from the uterus and placenta were examined by electron microscopy. Identification of *B. abortus* in placentae was with antibody-coated colloidal gold. Brucellae were first seen in phagosomes of erythroagocytic trophoblasts and in the rough endoplasmic reticulum of chorioallantoic trophoblasts. Subsequently, trophoblast necrosis and ulceration of chorioallantoic membranes were present. Coincidently, brucellae were present in the lumen of placental capillaries. In late stages of infection, placental vasculitis was present and placentomal trophoblasts were separated from maternal syncytial epithelium. In lesions with vasculitis, large numbers of brucellae were in connective tissue of chorionic villi. Within the placentome, trophoblasts that lined chorionic villi contained no intracellular bacteria and were separated from *B. abortus* by intact basement membranes. These results suggest that bacteremic brucellae are endocytosed by erythroagocytic trophoblasts and that brucellae replicate in the rough endoplasmic reticulum of chorioallantoic trophoblasts. Replication of brucellae in trophoblastic rough endoplasmic reticulum is unique; we believe that *B. abortus* may utilize endoplasmic reticulum for glycosylation of bacterial membrane proteins or that *B. abortus* catabolizes trophoblast secretory proteins.
INTRODUCTION

Brucellosis in pregnant ruminants is characterized by placentitis, intracellular replication of Brucella abortus in trophoblasts, large numbers of B. abortus in placental tissues, fetal death and abortion. Preferential localization (tropism) of B. abortus in bovine chorioallantoic trophoblasts was first reported in 1919 and later described in cattle, sheep, and goats. Gross and histologic lesions of brucellar placentitis are well described. Localization of bacteria in chorioallantoic trophoblasts is important in the pathogenesis of many reproductive diseases of the ruminant. For example, Coxiella burnetti and Chlamydia psittaci fill chorioallantoic trophoblasts of aborted placenta in sheep and goats. Listeria monocytogenes, Brucella melitensis, Brucella ovis, and Campylobacter fetus var. intestinalis initially infect placentomal erythrophagocytic trophoblasts of the sheep with subsequent spread to chorioallantoic trophoblasts. In addition, Campylobacter fetus var. venerealis infects chorioallantoic trophoblasts of the cow. Mechanisms of placental infection and trophoblast tropism in these diseases are poorly understood.

B. abortus is tropic for both caprine and bovine placentae and placentas of both species have similar responses to infection by this bacterium. We have used the caprine placenta to characterize the ultrastructural lesions in brucellar placentitis and to elucidate some of the mechanisms of placental infection. We were particularly interested in: 1) how brucellae enter placentae, 2) how trophoblasts
facilitate placental localization and bacterial replication, and 3) the mechanisms of abortion.
MATERIALS AND METHODS

Experimental design: Twenty 2 to 3 year-old pregnant crossbred goats in their last trimester of gestation were inoculated with varying doses of *B. abortus* (strain 2308, supplied by Dr. B. Deyoe, National Animal Disease Center, Ames, IA) in jugular veins or surgically in middle uterine arteries as previously described. Four pregnant goats were not inoculated and served as controls. All goats were considered *B. abortus*-free based on herd history and negative serologic titers to *B. abortus* (card and tube agglutination test).

Necropsy procedure and tissue preparation: Goats were necropsied at randomly selected times from 2-23 days post-inoculation. Gravid uteri of anesthetized and heparinized goats were surgically-exposed for perfusion fixation of placentae. After barbiturate overdose, placentomes were fixed for electron microscopy by perfusion of middle uterine arteries with 1.0% glutaraldehyde and 1.25% paraformaldehyde in 0.1M cacodylate buffer for 5 to 10 minutes. Samples of fixed placentome and chorioallantoic membrane were randomly selected from perfused uteri and minced into 1 mm³ blocks for immersion fixation in the glutaraldehyde-paraformaldehyde mixture at 4°C. After 2 hours of fixation tissue samples were rinsed in cacodylate buffer, stained en bloc in 1% osmium tetroxide, dehydrated in alcohols, cleared in propylene oxide, and embedded in epoxy resin.

Electron microscopy: All sections of placenta were cut at 1 um, stained with toluidine blue, and examined by light microscopy. Areas from these sections were randomly selected and ultrathin sections
of these areas were cut, stained with uranyl acetate and lead citrate, and examined with a Philips 410 transmission electron microscope. The number of placental sections examined from goats at each post-inoculation interval are depicted in Table 1.

**Immunocytochemistry:** Immunogold labeling of *B. abortus* was performed on thin sections of resin-embedded placental tissue mounted on nickel grids using post-embedding gold labeling techniques as described by Bendayan. The immunogold probe consisted of primary anti-*B. abortus* antibody coupled to 20 nm colloidal gold. Colloidal gold was prepared by reduction of aqueous tetrachloroauric acid with ascorbic acid and coupled directly to bovine anti-*B. abortus* IgG (supplied by Judith M. Patterson, National Animal Disease Center, Ames, IA). Placental sections were overlaid with non-antibody coated colloidal gold solutions to assess non-specific binding of the immunogold. Resin-embedded sections of other randomly selected bacteria such as *Salmonella choleraesuis, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella ozaenae* were used as controls to insure specificity of the immunogold.

Clinical signs and gross and histologic placental lesions present in these goats have been previously reported.
RESULTS

Placentitis was present in 18 of the 20 inoculated goats. Abortion occurred in 6 of the 20 goats from 11 to 18 days post-inoculation. Tissue changes of uterine artery-inoculated goats are described in time sequences; lesions in the goats inoculated intravenously were identical but occurred at later times.

Chorioallantoic membrane: At five days post-inoculation chorioallantoic trophoblasts contained numerous brucellae within dilated cisternae of the rough endoplasmic reticulum and perinuclear envelope. Bacteria occupied the perinuclear cell cytoplasm and displaced cell organelles (Figs. 1, 2). Ribosomes lined the cytoplasmic side of both the normal and brucellae-containing rough endoplasmic reticulum. Cisternae contained cross-sections of one or more bacteria and dividing bacteria. At first, only periplacental chorioallantoic trophoblasts were infected. By 12 days post-inoculation, most interplacental chorioallantoic trophoblasts were also infected.

In infected trophoblasts, overt evidence of cell degeneration (cell swelling, proteolysis, and vacuolation) was minimal to absent. When chorioallantoic epithelium was intact, no lesions were seen in basement membranes, fetal capillaries, or subepithelial mesenchymal tissue.

At eight days post-inoculation necrosis of trophoblasts, ulcers of the chorioallantoic membrane and subepithelial inflammation were present (Fig 3). Exudate overlying ulcerated membranes contained cell debris, fibrin, necrotic brucellae-filled trophoblasts, and free brucellae. When chorioallantoic membranes were ulcerated, subepithelial and
Chorioallantoic ulceration was the predominant lesion in 4 goats which were killed while aborting or after abortion on post-inoculation days 12, 14, 18, and 19. However, areas of brucellae-filled trophoblasts covering non-ulcerated membranes were present in all aborted placentae. Brucellae in trophoblasts were always within the rough endoplasmic reticulum.

**Placentome:** At 5 days post-inoculation, large numbers of brucellae were present in placentomal hematomas at the tips of maternal septa, on microvillous surfaces of erythrophagocytic trophoblasts, and in phagosomes of erythrophagocytic trophoblasts with maternal erythrocytes. (Fig. 5). Brucellae-filled erythrophagocytic trophoblasts at the edges of placentomes were continuous with brucellae-filled chorioallantoic trophoblasts. Infection of erythrophagocytic trophoblasts was present concurrent with chorioallantoic trophoblast infection, however, before brucellae were present in connective tissue of chorionic villi.

At 8 days post-inoculation, endothelium of maternal and fetal capillaries in placentomes was swollen and neutrophils had infiltrated into maternal septae (Fig. 6). A few bacteria were present in capillary lumens and in perivascular connective tissue of chorionic villi. The microvilli of fetal and maternal epithelium were still intact and in close apposition, however, swollen mitochondria and cisternal dilatation were present in maternal syncytial cells (Fig. 6). Erythrophagocytic trophoblasts were necrotic and numerous brucellae and neutrophils were in placentomal hematomas. Exudate covering erythrophagocytic
trophoblasts was continuous with exudate in the uterine lumen which covered ulcerated chorioallantoic membranes.

At 12 days post-inoculation, severe vasculitis and separation of maternal from fetal epithelium at the microvillous border were present (Figs. 7-12). In lesions with vasculitis, numerous brucellae were in connective tissue of chorionic villi (Figs. 8-12). Brucellae were not present in placentomal trophoblasts or maternal syncytial cells. Capillaries of maternal septa and fetal chorionic villi contained swollen endothelium, intraluminal and perivascular leucocytes, platelets, fibrin, and brucellae (Figs. 7,8). Basement membranes of capillaries in chorionic villi were separated from endothelium by fibrin deposits, neutrophils, and platelets (Fig. 8).

Six goats were aborting on or had aborted by post-inoculation days 11, 12, 14, 15, and 18. Placentae from 4 of these goats were examined by electron microscopy. Vasculitis, separation of maternal from fetal epithelium, and brucellae-filled chorionic villi were present in placentomes from these placentae. Cisternal dilatation, swollen mitochondria with fragmented cristae, and cytoplasmic clearing were present in both maternal syncytial cells and placentomal trophoblasts (Figs. 7,10-12). Placentomal trophoblasts were separated from maternal syncytial epithelium at the microvillous border. Microvesicles bulged from degenerate microvilli (Fig 11). Syncytial epithelium was necrotic and detached from maternal septa (Fig. 7). Endothelium lining capillaries in chorionic villi was necrotic or absent and capillary lumens contained cell debris and brucellae (Fig. 10). Fetal phagocytes in chorionic villi which contained brucellae were necrotic (Fig. 12).
**Bacterial cells:** Brucellae in placental tissues were characterized by an undulant outer membrane, a periplasmic space, and a plasma membrane encircling bacterial cytoplasm. Granular aggregations of ribosomes were randomly dispersed throughout the cytoplasm. No mesosomes or other membranous structures were seen. Bacterial cross-sectional diameter ranged from 0.3 to 1 micron. The largest bacterial cross-sectional length was 1.5 microns. Immunogold particles attached to outer membranes and occasionally overlaid bacterial cytoplasm. No gold labeling with the anti-*B. abortus* immunogold was present on the other selected Gram-negative bacteria. No gold labeling of brucellae was present in placental sections overlaid with non-antibody coated colloidal gold.
Fig. 1—Chorioallantoic membrane from a goat inoculated intravenously with \textit{B. abortus}. Chorionic trophoblasts (CT) contain numerous brucellae (large arrow) which occupy membrane-bound cisternae of perinuclear cytoplasm. One trophoblast (small arrow) has nearly sloughed into the uterine lumen (UL). Fetal capillaries (FC) and subepithelial connective tissue appear normal. Bar = 7 \textmu m.
Fig. 2a & b—Cytoplasm of a chorioallantoic trophoblast similar to those in Fig. 2. *B. abortus* (Br) is present in distended rough endoplasmic reticulum (RER). A section of normal RER (RER-arrows) leads directly into a brucella-containing and distended section. Ribosomes line the cytoplasmic side of all brucellae-containing cisternae. Trophoblast nucleus (N). a) Unlabeled *B. abortus* in RER. Bar = 0.5 um. b) similar cell as in Fig. 2a, however *B. abortus* is labeled with immunogold.

Bar = 0.5 um.

Fig. 3—Chorioallantoic membrane from a goat inoculated in the uterine arteries with *B. abortus*. Cell debris of necrotic trophoblasts and brucellae (Br) are in uterine lumen (UL) and above remaining trophoblast basement membrane (TBM). The basement membrane of subepithelial capillaries (CEM) remains. Capillary lumens (CL) contain brucellae, platelets, fibrin, and necrotic endothelium. Bar = 7 um.

Fig. 4—Fetal capillary (Fc) in placentome of a goat inoculated in uterine arteries with *B. abortus*. Bacteremic *B. abortus* (Br-arrow), fetal erythrocyte (RBC), placentomal trophoblast (PT), and maternal syncytiun (MS). Bar = 1 um.
Fig. 5—Placentome from a goat inoculated intravenously with *B. abortus*. 
a) Erythrophagocytic trophoblast (ET) contains brucellae (Br-arrows) in membrane-bound cisternae. Maternal erythrocytes (RBC) and brucellae emeshed in surface microvilli. Mitochondria (M). Bar = 2 um. 
b) Similar cell as in a. Intracytoplasmic membrane-bound brucellae (Br) labeled with immunogold adjacent to membrane-bound maternal erythrocyte. Mitochondria (M). Bar = 1 um.
Fig. 6—Placentome of a goat inoculated intravenously with B. abortus. Capillary in maternal septa contains neutrophil (N) and swollen endothelium (ME). A neutrophil and macrophage (M) are present in perivascular connective tissue. Maternal syncytial epithelium (MS) contains swollen mitochondria and dilated cisternae but is still attached to placentomal trophoblasts (PT) at microvillous border. Fetal capillary (FC) in chorionic villus connective tissue. Bar = 4 um.
Fig. 7—Placentome of a goat inoculated intravenously with *B. abortus* and in late stages of placentitis. Maternal capillaries contain disrupted endothelium (ME), neutrophils (N), mononuclear leucocytes (M), platelets (P) and fibrin (arrows). Maternal syncytial epithelium (MS) is necrotic and separated from placentomal trophoblasts (PT). Fetal connective tissue (Fct) of chorionic villi. Bar = 4 um.

Fig. 8—Same placentome as in Fig. 7. Fetal capillary in chorionic villus connective tissue contains intraluminal and perivascular platelets, fibrin, and cell debris. Fetal endothelium (FE) is swollen and a fetal phagocyte (FP) is present in perivascular tissue. Capillary basement membrane is separated from endothelium by cell debris and fibrin accumulation. Basement membrane (BM-open arrow) of placentomal trophoblasts. *B. abortus* (Br-arrows) present in surrounding fetal connective tissue (Fct). Bar = 2 um.

Fig. 9—Perivascular fetal connective tissue (Fct) in chorionic villus of a placentome at an earlier stage of infection than in Fig. 8. Immunogold labeled *B. abortus* adjacent to collagen fibers (C). Basement membrane (BM) and endothelial cell (FE) of fetal capillary appear normal. Bar = 0.5 um.
Fig. 10—Placentome of a goat inoculated intravenously with *B. abortus* and killed at the time of abortion. Placentomal trophoblasts (PT) separating from maternal syncytium (MS) at degenerate microvillous border. Fetal microvilli (white arrows) have detached from maternal microvilli (large black arrows). Numerous brucellae (small black arrows) and necrotic fetal phagocytes are in fetal connective tissue (Fct) of chorionic villus. Basement membrane (BM) of distended fetal capillary; endothelium is absent and lumen contains brucellae.

Bar = 4 μm.
Fig. 11—a) Placentome pictured in Fig. 10. Brucellae (Br-arrows) in fetal connective tissue beneath placentomal trophoblast (PT). Separation of trophoblast from maternal syncytiun (MS) at microvillous border (large arrows). Mitochondria (M). Bar = 1 um. b) Enlargement of Fig. 11a. Microvesicles (small arrow) protrude from degenerate microvilli. Bar = 1 um.

Fig. 12—Placentome of a goat inoculated intravenously with B. abortus. Fetal connective tissue (Fct) of chorionic villus filled with brucellae (Br-arrows) and degenerate fetal phagocytes (FP). Placentomal trophoblasts (PT) are degenerate but do not contain intracellular bacteria and lay on an intact basement membrane. Fetal capillaries (C) contain necrotic endothelium and cell debris. Bar = 3 um.
Fig. 13—Diagram of caprine placentome which summarizes the pathogenesis of placental and fetal infection as determined in this study. 1) Brucellae were first seen in phagosomes of erythrophagocytic trophoblasts (ET). 2) Subsequently, brucellae replicated in the rough endoplasmic reticulum of chorioallantoic trophoblasts (CAT). 3) After trophoblast infection, necrosis of chorioallantoic trophoblasts, spewing of brucellae into the uterine lumen (UL), and ulceration of the chorioallantoic membrane occurred. 4) Coincidently, intravascular brucellae, present in placental capillaries, spread to chorionic villi (CV) and fetal viscera. No brucellae were present in placentomal trophoblasts (PT), maternal septae (MS), syncytial epithelium (SE), or endometrium. Erythrophagocytic, placentomal, and chorioallantoic trophoblasts form the continuous epithelial sheet which covers chorionic villi of the cotyledon and the chorioallantoic membrane. They are differentiated here due to differences in anatomic location, function, and their role in brucellar placentitis.
ALLANTOIC CAVITY

COTYLEDON

ALLANTOIC EPITHELIUM

CHORIOALLANTOIC MEM.

UTERINE LUMEN

ENDOMETRIUM
Table 1. Number of placental sections examined from *B. abortus*-inoculated goats and non-inoculated controls\(^a\)

<table>
<thead>
<tr>
<th>Days PI(^b)</th>
<th>Goat ID(^c)</th>
<th>Light microscopy(^d)</th>
<th>Electron microscopy(^e)</th>
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<td>4</td>
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<td>3</td>
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<tr>
<td>13</td>
<td>4, 5, 15</td>
<td>48</td>
<td>13</td>
</tr>
<tr>
<td>14</td>
<td>16, 17(^f)</td>
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<td>15</td>
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</tr>
<tr>
<td>18</td>
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<tr>
<td>19</td>
<td>7, 9, 20(^f)</td>
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<td><strong>Total</strong></td>
<td><strong>16</strong></td>
<td><strong>260</strong></td>
<td><strong>59</strong></td>
</tr>
<tr>
<td><strong>Control goats</strong></td>
<td><strong>4</strong></td>
<td><strong>34</strong></td>
<td><strong>9</strong></td>
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</table>

\(^a\) Tissues from 4 inoculated goats were not examined by electron microscopy due to autolysis of aborted placentae (goats 6,8) or lack of lesions at the light microscopic level (goats 1,12).

\(^b\) PI = post-inoculation.

\(^c\) Goat ID corresponds to previously reported studies.\(^3\)

\(^d\) 1 \(\mu m\) sections were stained with toluidine blue and examined by light microscopy.

\(^e\) Ultrathin sections were labeled with colloidal gold and examined by transmission electron microscopy.

\(^f\) Goats were killed while showing clinical signs of abortion (goats 14,17) or after abortion (goats 19,20).
DISCUSSION

This study indicates that during bacteremia Brucella abortus first enters and replicates within erythroagocytic trophoblasts (Fig. 13). We believe that brucellae next replicate in the rough endoplasmic reticulum of chorioallantoic trophoblasts. Chorionic villi and fetal viscera are infected hematogonously after trophoblast necrosis and ulceration of chorioallantoic membranes have occurred. It is likely that brucellae in placentomal chorionic villi caused vasculitis and separation of trophoblasts from maternal syncytial epithelium.

Epithelial cell penetration and intracellular replication have been reported for many bacterial pathogens and are referred to as the "epithelial phase" of facultative intracellular parasitism. Intracellular replication has been reported primarily for bacteria which enter and replicate in intestinal epithelium. For example, Listeria monocytogenes, Chlamydia psittaci, Shigella flexneri, and Shigella dysenteriae cause disease only after entering intestinal epithelium, replicating intracellularly, and causing enterocyte degeneration. In addition, Coxiella burnetti, Chlamydia psittaci, Listeria monocytogenes, Brucella melitensis, Brucella ovis, Campylobacter fetus var. intestinalis, and Campylobacter fetus var. veneralis also infect and likely replicate in ruminant chorioallantoic trophoblasts. The contribution of enterocytes or trophoblasts to replication of bacteria is unknown.

Erythroagocytic trophoblasts of small ruminants phagocytose maternal erythrocytes which leak from tips of maternal septa.
hemoglobin likely serves as a significant source of iron for the developing fetus. Although it is probable that brucellae were passively phagocytosed by trophoblasts with maternal erythrocytes, we could not rule out active entry of the trophoblast by brucellae. Infection of erythrophagocytic trophoblasts allowed spread of brucella to periplacentomal chorioallantoic trophoblasts. This may account for the predominant periplacentomal chorioallantoic exudate observed in early lesions of brucellosis.\textsuperscript{20,28-30} This method of placental entry requires evaluation in cattle. The bovine placentome contains hematomas at the tips of maternal septae in late gestation, however, a distinct erythrophagocytic area has not been described.\textsuperscript{48}

Chorionic trophoblasts could be infected by: 1) cell-to-cell lateral transfer of bacteria from infected trophoblasts or 2) by rupture of infected trophoblasts, spewing of bacteria into the uterine lumen, and endocytosis of luminal bacteria by adjacent trophoblasts. Chorioallantoic trophoblastic epithelium is adjacent to and continuous with erythrophagocytic trophoblastic epithelium and thus both mechanisms were plausible in our study. Our observations suggest that cell-to-cell lateral transfer is possible as large numbers of brucellae often were within chorioallantoic trophoblasts without bacteria in the uterine lumen or on the trophoblast surface. Cell-to-cell transfer through the lateral plasmalemma has been demonstrated for \textit{Shigella flexneri}\textsuperscript{34} and has been suggested for \textit{Listeria monocytogenes}\textsuperscript{38} and \textit{Escherichia coli}.\textsuperscript{44} However, in support of the second mechanism, trophoblasts are endocytic\textsuperscript{9} and ample opportunity for endocytosis of luminal brucellae by trophoblasts exists. Large numbers of brucellae would be released into
the uterine lumen when necrotic trophoblasts rupture. As with enterocyte infection by Chlamydia psittaci, trophoblasts may be infected by endocytic uptake of free brucellae, brucellae-filled necrotic cells, or brucellae-containing cytoplasmic fragments.

Brucellae clearly replicate within trophoblasts. Infected trophoblasts had massive numbers of intracellular bacteria, without corresponding numbers in the uterine lumen or on trophoblast surfaces. A requirement for epithelial entry and replication may exist in the pathogenesis of placentitis caused by B. abortus, as it does in the pathogenesis of enteritis caused by Shigella dysenteriae. Trophoblasts appear to provide a suitable environment for marked brucella replication and may contain factors which enhance brucella growth. Ruminant placentae contain high concentrations of erythritol, although the exact site of synthesis is unknown. In addition, trophoblasts synthesize progesterone throughout gestation. Both erythritol and progesterone enhance in vitro growth of B. abortus.

Replication of brucellae in the rough endoplasmic reticulum of epithelial cells is a unique mechanism of intracellular parasitism. Only Legionella pneumophilia has a similar intracellular location, but it inhabits the rough endoplasmic reticulum of macrophages. Other protozoan and bacterial intracellular pathogens are either free in the cytoplasm or within membrane-bound phagosomes. The rough endoplasmic reticulum synthesizes two types of proteins; those destined for secretion and those that become an integral part of new cell membranes. Future studies should determine if B. abortus utilizes the proteins
synthesized in trophoblastic rough endoplasmic reticulum for bacterial metabolism or as part of the glycoproteins in bacterial membranes.

Intracellular replication in chorioallantoic trophoblasts caused trophoblast necrosis and chorioallantoic ulceration. Large numbers of brucellae in chorioallantoic membranes was the source of bacteria for placentomes and fetal tissues. Similar events occur in bovine disease caused by Chlamydia psittaci, an obligate intracellular pathogen. Chlamydia occurs only after chlamydial uptake by enterocytes, intracellular chlamydia replication, enterocyte necrosis and basement membrane degeneration. Likewise, in brucellosis, the proximity of placental capillaries to an ulcerated chorioallantoic surface allowed access of brucella-filled exudate to the fetal circulation with hematogenous spread of bacteria to chorionic villi and fetal tissues.

The numerous brucellae in chorionic connective tissue may be due to failure of fetal phagocytes to destroy brucellae and subsequent bacterial replication. Degenerative fetal phagocytes containing intact brucellae were prevalent in chorionic villi; it is likely that they die or are killed after phagocytosis of brucellae. In disease caused by Salmonella typhimurium, Chlamydia psittaci, and Listeria monocytogenes, large numbers of intact organisms are present in phagocytes of lamina propria after intraepithelial bacterial replication and enterocyte necrosis. Our observations concur with those of others, in that non-activated macrophages may not arrest the multiplication of facultative intracellular pathogens, such as brucella, listeria, and salmonella, at the early stages of infection.
Furthermore, *B. abortus* may inhibit bacteriocidal mechanisms of ruminant neutrophils.\(^{39}\)

Vasculitis in maternal and fetal tissues and separation of fetal trophoblasts from maternal syncytial epithelium may be due to endotoxin released from the large numbers of brucellae in chorionic villi. Endothelial damage and desquamation, platelet aggregation, neutrophil infiltration and fibrin deposition occur in vascular lesions associated with enterobacterial endotoxin.\(^7,^{16,26,33}\) The endotoxin of *B. abortus* shares many biologic properties with enterobacterial endotoxin, including complement-activation, mouse lethality and abortifacient properties.\(^{32}\) Either the ischemia which accompanies vasculitis, or a direct effect of endotoxin, may be responsible for degeneration in placentomal trophoblasts and maternal syncytial epithelium. Thus, once brucellae enter placentae and replicate to such large numbers, endotoxin may have the final role in causing abortion.

Mechanisms of abortion in brucellosis may include either: 1) impairment of placental circulation and oxygenation with subsequent fetal death,\(^{36}\) 2) fetal infection and stress causing elevated cortisol levels,\(^{22,35}\) or 3) a combination of 1) and 2). Our observations support a combination of these mechanisms. Elevated fetal cortisol associated with fetal stress or normal hormonal fluctuations is likely involved in abortion, premature deliveries, and normal initiation of parturition.\(^{37}\) Infusion of cortisol into the ovine fetus results in a separation of trophoblasts from maternal syncytial epithelium at their microvillous border,\(^{18}\) similar to the separation site present in our brucella-infected goats. However, this type of separation was present
concurrent with severe, widespread placental vasculitis. The exact mechanism of abortion was not clear in our study, however, maternal and placental vasculitis was prominent and we believe that it contributed greatly to placentomal lesions, separation of maternal from fetal epithelium, fetal death and abortion.


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ULTRASTRUCTURAL MORPHOMETRIC ANALYSIS OF
BRUCELLA ABORTUS-INFECTED TROPHOBLASTS IN
EXPERIMENTAL BRUCELLAR PLACENTITIS
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BRUCELLA ABORTUS-INFECTED TROPHOBLASTS IN
EXPERIMENTAL BRUCELLAR PLACENTITIS

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ABSTRACT

Trophoblasts in normal and Brucella abortus-infected placentae were characterized by ultrastructural morphometric analysis. Identification of B. abortus in placentae was with antibody-coated colloidal gold. Uninucleate trophoblasts had large numbers of brucellae in cisternae of rough endoplasmic reticulum; binucleate trophoblasts did not contain bacteria. Volume and surface density of normal rough endoplasmic reticulum which did not contain brucellae were: control trophoblasts (normal placentae), 2.8% and 0.30 μm²; non-infected trophoblasts from infected placentae, 3.3% and 0.34 μm²; and infected trophoblasts, 0.48% and 0.06 μm². Volume and surface density of trophoblast rough endoplasmic reticulum which contained brucellae were 27.4% and 0.50 μm². This six-fold reduction of normal rough endoplasmic reticulum in infected trophoblasts and the corresponding hypertrophy of brucellae-filled rough endoplasmic reticulum suggests that B. abortus used trophoblastic rough endoplasmic reticulum for bacterial replication. We believe that B. abortus may use trophoblastic rough endoplasmic reticulum for synthesis and glycosylation of bacterial membrane proteins and that this use of trophoblast enzymes enhances bacterial replication in trophoblasts.
Brucella abortus has a marked affinity for chorioallantoic trophoblasts in the pregnant cow and other ruminants. Marked bacterial replication occurs in trophoblasts and placentae. Placentae contain up $10^{13}$ organisms per gram which accounts for the high infectivity of aborted bovine placentae for animals and man. In human brucellosis, B. abortus also infects the placenta and will cause abortion.

During bacteremia B. abortus is endocytosed by erythrophagocytic cytotrophoblasts in the placentome. The bacteria then replicate in the rough endoplasmic reticulum of periplacental chorioallantoic trophoblasts; this is an uncommon site of intracellular bacterial parasitism. After host cell death, brucellae are shed into surrounding tissue and the cycle of cell infection, intracellular replication, and cell death continues. Legionella pneumophila has a similar intracellular location, however, it replicates in rough endoplasmic reticulum of monocytes and macrophages.

We believe that B. abortus uses trophoblastic rough endoplasmic reticulum for synthesis and glycolysis of bacterial membrane proteins and that bacterial replication is enhanced in trophoblasts by this mechanism. This study was designed to quantitatively analyze morphologic changes in B. abortus infected chorioallantoic trophoblasts. We therefore wished to establish the precise structural relationship of intracellular brucellae with different cytoplasmic organelles.
MATERIALS AND METHODS

**Tissue preparation:** Placentae from 7 goats with experimentally-induced brucellar placentitis and 3 non-inoculated control goats were fixed by perfusion of middle uterine arteries with 1.0% glutaraldehyde and 1.25% paraformaldehyde in 0.1M cacodylate buffer as previously described.\(^5,6\) Samples of chorioallantoic membrane were randomly selected from at least 12 periplacental locations and cut into 1 mm\(^2\) squares for immersion fixation in the glutaraldehyde-paraformaldehyde mixture at 4°C. After 2 hours of fixation tissue samples were rinsed in cacodylate buffer, stained en bloc in 1% osmium tetroxide, dehydrated in alcohols, cleared in propylene oxide, and embedded in epoxy resin. Embedding was done in rectangular rubber molds with the tissue blocks being oriented so that sections would be cut perpendicularly to the epithelial surface.

Sections of chorioallantoic membrane were cut at 1 um, stained with toluidine blue, and examined by light microscopy. Only blocks which demonstrated an epithelial layer and an epithelium-connective tissue junction, and which thus had been cut to include the full epithelial thickness, were used in this investigation. Several blocks with advanced lesions were discarded due to ulceration of the chorioallantoic membrane. Ultrathin sections of appropriate blocks were cut, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope.

**Experimental design:** Animals were placed into one of three groups. Animals in group one were four females which were inoculated with
B. abortus and in which chorioallantoic trophoblasts which contained brucellae were selected for electron microscopic morphometry. Animals in group two were three females which were inoculated with B. abortus, and had placentitis, however, chorioallantoic trophoblasts which did not contain brucellae were selected for morphometry. Animals in group three were three normal non-inoculated females. Animals in groups two and three provided samples of normal non-infected chorioallantoic trophoblasts in inoculated and non-inoculated animals respectively.

Morphometry and stereology: All morphometric procedures followed previously reported methods. Three blocks of chorioallantoic membrane were obtained from each animal and one section was prepared from each block. Electron microscopic morphometry was conducted on each section at two levels of magnification.

At the first level, four micrographs were recorded from each section of random, but not overlapping, fields of trophoblast epithelium at a primary magnification of 1700 x. Point counting morphometry was conducted on micrographs printed at 4420x using a lattice test grid with 49 points. Relative volume densities ($V_v$) were expressed as a percentage of the epithelial sheet covering chorioallantoic membranes. This was represented as the ratio of points falling on nuclei, trophoblast cytoplasm, and brucellae-filled cisternae respectively, to total epithelial points (defined as the sum of total points falling on trophoblast cytoplasm, nuclei, and brucellae-filled cisternae) multiplied by 100. The mean number of brucellae per trophoblast cross-section was determined from eight cells in each section (24 cells per animal).
At the second level of magnification, eight micrographs were recorded of trophoblast cytoplasm from each section at a magnification of 6900x. Alternate areas of basal and luminal cytoplasm of every fifth trophoblast were photographed. These were photographically enlarged to 17,940x and point counting morphometry was conducted with a double lattice coherent test grid with 42 coarse points and 672 fine points. Stereologic parameters were estimated only on intact, non-degenerate trophoblasts which were still basally attached to the chorioallantoic basement membrane and laterally attached to neighboring trophoblasts. Binucleate giant cells were not included in cytoplasmic morphometry at the second level of magnification.

Relative volume densities \( (V_v) \) obtained at this second level were expressed as a percentage of trophoblast cytoplasm. This percentage was represented as the ratio of points falling on rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi apparatus, and brucellae-filled cisternae respectively, to total cytoplasmic points (defined as the sum of total points on rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi apparatus, brucellae-filled cisternae, the remainder of cytoplasmic organelles and cytoplasmic matrix) multiplied by 100. Twenty-four cells from each animal (96 total cells in group 1 and 72 total cells in groups 2 and 3) were analyzed at this level.

Relative surface densities \( (S_v) \) of the rough endoplasmic reticulum and brucellae-filled cisternae were expressed as square microns of cisternal surface membrane per cubic micron of trophoblast cytoplasm.
Surface densities were determined using the equation:

\[ S_v = \frac{I}{P_{\text{cyt}} \times d} \]

where \( I \) is the number of intersections of horizontal test lines with the outer surfaces of the rough endoplasmic reticulum and brucellae-filled cisternae and \( P_{\text{cyt}} \) (total cytoplasmic points) \( \times d \) (distance between points) is the length of the horizontal test lines overlying trophoblast cytoplasm.

Numerical density (\( N_v \)) of brucellae per unit volume of trophoblast cytoplasm was estimated by counting bacterial profiles per area of cytoplasm covered by the grid overlay and applying the following formula:

\[ N_v = \frac{K}{B} \cdot \frac{N_a^{3/2}}{V_v^{1/2}} \]

where \( N_a \) = the number of bacterial profiles per square micron of trophoblast cytoplasm, \( V_v \) = volume density of brucellae-filled cisternae, and \( K \) and \( B \) are coefficients which adjust for relative size and shape of the particle being counted. \( K \) was arbitrarily assigned a value of 1.05. \( B \) relates particle volume to its mean profile area. For a cylindrical brucellae cell with an axial ratio (length/diameter) of 3:1, \( K = 2.25 \).

The distance between test points and total area covered by the test grid was measured by semiautomatic computerized morphometry (Bioquant...
At a magnification of 17,940, d was equal to 0.41 microns and the point counting grid overlay an area of 111.3 cubic microns.

Statistics: All means were calculated on an individual animal basis. These data were subjected to an analysis of variance with individual degrees of freedom used to compare group 1 with groups 2 and 3 and group 2 with group 3.

Immunocytochemistry: Immunogold labeling of B. abortus was performed on thin sections of resin-embedded placental tissue mounted on nickel grids using post-embedding gold labeling techniques as described previously. The immunogold probe consisted of primary anti-B. abortus bovine IgG coupled to 20 nm colloidal gold.

Clinical signs and gross, histologic, and ultrastructural placental lesions present in these goats have been previously reported.
RESULTS

Rough endoplasmic reticulum (membranes, ribosomes, cisternae, and intracisternal brucellae) occupied 27.4% of trophoblast cytoplasm (Figs. 1, 2, 5, Table 1). This rough endoplasmic reticulum contained single or multiple bacteria and was continuous with normal rough endoplasmic reticulum (Fig. 6). Outer membranes of brucellae-filled cisternae were continuous with the outer nuclear membrane (Fig. 7). In heavily infected cells, multiple brucellae were within both the lumen of cytoplasmic rough endoplasmic reticulum and the perinuclear space (Fig. 7). Ribosomes lined the cytoplasmic side of the outer nuclear membrane when the nuclear envelope was distended by brucellae.

Brucellae-filled trophoblasts were necrotic and present in the uterine lumen when brucellae-filled cisternae occupied more than 36.4% of trophoblast cytoplasm (Figs. 3, 4). Volume densities of brucellae-filled cisternae in non-degenerate trophoblasts ranged from 23.8% to 36.4% (Table 1).

Trophoblasts with volume densities less than 36% had intracytoplasmic brucellae in the rough endoplasmic reticulum. In trophoblasts with volume densities greater than 36%, brucellae were always within membrane-bound cisternae, however, these cisternae were not always lined with cytoplasmic ribosomes. Brucellae-containing cisternae in these heavily infected cells contained multiple pleomorphic bacteria, and an osmiophilic, granular material which surrounded bacterial cells. Bacterial pleomorphism was characterized by variation in cell diameter from 0.5 to 1.5 μm and excessive folding of outer
membranes. Membrane whorls, similar in morphology to bacterial outer membranes, were also present in these large brucellae-containing cisternae.

Cell degeneration in brucellae-containing cytotrophoblasts was characterized by cytoplasmic vacuolization and clearing, dissolution of the nucleolus, chromatin clumping, and disappearance of all cell organelles. Necrotic trophoblasts were either free in the uterine lumen or had ruptured and remained attached to the basement membrane. Bacteria occupied nearly 100% of the cytoplasm in necrotic, sloughed trophoblasts (Fig. 4). Free bacteria, cell debris, erythrocytes, and necrotic trophoblasts were present in the uterine lumen.

There was no significant difference in cellular composition, morphology, or stereologic parameters (Table 3) between chorioallantoic membranes from groups 2 and 3 (inoculated and control groups without brucellae in trophoblasts). These two groups will be considered as normal and their stereologic parameters will be averaged and discussed together.

Rough endoplasmic reticulum occupied 3.0% of perinuclear cytoplasm and had a surface area of 0.32 \( \text{um}^2 \) in normal trophoblasts. In brucellae-infected cells the normal rough endoplasmic reticulum, not containing bacteria, occupied 0.48% of the cytoplasm and had a surface density of 0.06 \( \text{um}^2 \) (Table 2). This normal appearing rough endoplasmic reticulum in infected cells was directly continuous with brucellae-filled rough endoplasmic reticulum. Surface area of brucellae-filled rough endoplasmic reticulum was 0.50 \( \text{um}^2 \) (Table 1).
This difference in content of normal rough endoplasmic reticulum, which did not contain bacteria, was significant ($P = .01$).

No significant difference in the average volume density of smooth endoplasmic reticulum or Golgi apparatus was present among the three groups. However, in one brucellae-infected animal, hypertrophy of the smooth rough endoplasmic reticulum was present. It occupied 1.9% of trophoblast cytoplasm and was adjacent to brucellae-filled cisternae (Fig. 9 and Table 2).

Each cytotrophoblast cross-section contained an average of 161.2 bacterial profiles. Numeric density of brucellae in trophoblasts was 0.60 bacterial cells/$\mu m^3$ trophoblast cytoplasm (Table 1). Brucellae in trophoblasts were characterized by an undulant outer membrane, a periplasmic space, and a plasma membrane encircling bacterial cytoplasm. Granular aggregations of ribosomes were randomly dispersed throughout the cytoplasm. Several bacterial profiles compatible with dividing bacteria were present. No mesosomes or other membranous structures were seen. Bacterial cross-sectional diameter ranged from 0.3 to 1 micron. Immunogold particles attached to outer membranes and occasionally overlaid bacterial cytoplasm.

As determined at the low magnification, brucellae occupied an average of 36 + 9.0% of the entire trophoblastic epithelial sheet. There was no significant difference between the three groups in nuclear to cytoplasmic ratios.

Binucleate trophoblasts (giant cells) were not infected by brucellae even when adjacent to heavily infected cytотrophoblasts (Fig. 8). Binucleate trophoblasts were present in the chorioallantoic
membranes of all three groups. They were separated from the basement membrane and uterine lumen by cytotrophoblast cytoplasm and usually contained multiple, circular, membrane-bound secretion granules but never contained intracellular brucellae.

Dense, homogenous, square and rectangular inclusions were present in the same cisternae which contained brucellae (Fig. 9). Inclusions were present in the majority of normal and brucellae-infected cytotrophoblasts and in some binucleate giant cells from all three groups. When trophoblasts had only a few inclusions, they were located within the rough endoplasmic reticulum. When the majority of cytoplasm contained inclusions, they were in membrane-bound cisternae unlined by ribosomes.
Fig. 1—Chorioallantoic membrane from a goat inoculated with \textit{B. abortus}. Chorioallantoic trophoblasts contain numerous intracellular brucellae in perinuclear cytoplasm. UL = uterine lumen, C = placental capillary containing fetal erythrocytes. Bar = 6 \mu m.
Fig. 2—Chorioallantoic trophoblast infected with *B. abortus*. Numerous brucellae (arrow) in perinuclear cytoplasm but no cell degeneration is present. Microvillous surface facing uterine lumen (UL). Bar = 4 μm.

Fig. 3—Chorioallantoic trophoblast in which the cytoplasm is nearly filled with brucellae. It has separated from neighboring trophoblasts and will be sloughed into the uterine lumen (UL). The nucleus is indented by numerous brucellae distending the perinuclear envelope (arrows). Bar = 4 μm.

Fig. 4—Necrotic chorioallantoic trophoblast present in the uterine lumen. The cytoplasm is completely filled with brucellae but the microvillous surface (MV-arrows), basal surface (BS), and nucleus (N) are still identifiable. Bar = 4 μm.

Fig. 5—Trophoblast similar to Fig. 3. The perinuclear envelope contains numerous immunogold-labeled brucellae which indent the inner nuclear membrane. The outer and inner membrane are still apposed at three locations (arrows) and the lumen of the perinuclear envelope is continuous with the lumen of brucella-filled rough endoplasmic reticulum (open arrow). Bar = 1 μm.
Fig. 6—a) Normal trophoblast in a non-inoculated goat. Rough endoplasmic reticulum (arrows) is present in perinuclear cell cytoplasm. Bar = 1 um. b) Brucellae-infected trophoblast. The perinuclear cytoplasm contains brucellae-filled rough endoplasmic reticulum (arrows), however, no normal rough endoplasmic reticulum is present. Bar = 1 um.

Fig. 7—Rough endoplasmic reticulum of a brucellae-infected trophoblast which contains numerous brucellae (Br) and which is continuous with normal rough endoplasmic reticulum (arrows). All brucellae-containing cisternae are lined on their cytoplasmic face with ribosomes. Bar = 1 um.
Fig. 8—Chorioallantoic membrane from a goat inoculated with *B. abortus*. Two binucleate trophoblasts (Bn) in different stages of maturation are surrounded by the cytoplasm of brucellae-infected uninucleate trophoblasts. Brucellae fill cytoplasmic cisternae of uninucleate trophoblasts (Br-arrows) but are not present in binucleate trophoblasts. One binucleate cell contains numerous secretion granules. Bar = 7 μm.

Fig. 9—Chorioallantoic trophoblast which contains immunogold-labeled brucellae (Br) and dense, homogenous inclusions in distended cisternae of the rough endoplasmic reticulum. Ribosomes line the cytoplasmic face of all brucellae-containing cisternae. The smooth endoplasmic reticulum (SER) was hypertrophied in the trophoblasts of this animal. Bar = 1 μm.
Table 1. Volume ($V_v$), surface ($S_v$), and numeric ($N_v$) density of brucellae-filled cisternae and brucellae in trophoblasts from four infected animals (group 1)

<table>
<thead>
<tr>
<th>Animal</th>
<th>$V_v$</th>
<th>$S_v$</th>
<th>$N_v$</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>23.8</td>
<td>0.53</td>
<td>0.58</td>
</tr>
<tr>
<td>2</td>
<td>22.4</td>
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<td>0.50</td>
</tr>
<tr>
<td>3</td>
<td>36.4</td>
<td>0.65</td>
<td>0.87</td>
</tr>
<tr>
<td>4</td>
<td>26.9</td>
<td>0.31</td>
<td>0.44</td>
</tr>
<tr>
<td>Mean</td>
<td>27.4</td>
<td>0.50</td>
<td>0.60</td>
</tr>
<tr>
<td>SEM(^a)</td>
<td>3.2</td>
<td>0.07</td>
<td>0.09</td>
</tr>
</tbody>
</table>

\(^a\)Standard error of the mean.
Table 2. Volume and surface densities of normal rough endoplasmic reticulum, not containing bacteria, ($V_{\text{vrer}}$ and $S_{\text{vrer}}$), and volume densities of smooth endoplasmic reticulum ($V_{\text{vser}}$) and Golgi apparatus ($V_{\text{vgolgi}}$) in brucellae-infected and normal trophoblasts

<table>
<thead>
<tr>
<th>Group #</th>
<th>1</th>
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<th>3</th>
<th>1</th>
<th>2</th>
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<tr>
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<td>0.60</td>
</tr>
<tr>
<td>4</td>
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<td></td>
<td></td>
<td></td>
<td>1.90</td>
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Mean 0.48* 3.30 2.80 0.60 0.49 0.62
SEMa 0.11 0.50 0.13 0.44 0.01 0.04

<table>
<thead>
<tr>
<th>V_{\text{vgolgi}}</th>
<th>S_{\text{vrer}}</th>
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<tr>
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<tr>
<td>Animal #</td>
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</tr>
<tr>
<td>1</td>
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<td>3</td>
<td>0.06</td>
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<tr>
<td>4</td>
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</tbody>
</table>

Mean 0.05 0.07 0.04 0.06* 0.34 0.30
SEMa 0.005 0.04 0.005 0.02 0.08 0.03

Group 1 = brucellae infected trophoblasts.
Group 2 = non-infected trophoblasts in inoculated goats (normal).
Group 3 = normal trophoblasts in non-inoculated goats.
*Significant difference from other means among groups ($P < 0.01$).
aStandard error of the mean.
Table 3. Statistical analysis of volume and surface.
Comparisons were between group means and results are reported as a significant (S) or non-significant (NS) F ratio.

<table>
<thead>
<tr>
<th>Group comparison</th>
<th>$V_{vrer}$</th>
<th>$V_{vser}$</th>
<th>$V_{vgolgi}$</th>
<th>$S_{vrer}$</th>
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<tbody>
<tr>
<td>1 vs 2 and 3</td>
<td>S</td>
<td>NS</td>
<td>NS</td>
<td>S</td>
</tr>
<tr>
<td>2 vs 3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Significant difference from other means among groups (P < 0.01).

Group 1 = brucellae infected trophoblasts.

Group 2 = non-infected trophoblasts in inoculated goats (normal).

Group 3 = normal trophoblasts in non-inoculated goats.
DISCUSSION

These studies establish that Brucella abortus replicates in trophoblastic rough endoplasmic reticulum. A six-fold reduction of normal rough endoplasmic reticulum, which did not contain bacteria, and the corresponding hypertrophy of brucellae-filled rough endoplasmic reticulum was associated with intracellular bacterial replication and necrosis of trophoblasts. We believe that B. abortus uses trophoblastic rough endoplasmic reticulum for synthesis and glycosylation of bacterial membrane proteins and that this organelle is largely responsible for the marked placental tropism of B. abortus.

Five morphologic criteria were useful in verifying that brucellae-filled cisternae were trophoblastic rough endoplasmic reticulum: 1) brucellae-filled cisternae were lined on their cytoplasmic face by ribosomes, 2) cisternae were continuous with normal rough endoplasmic reticulum which did not contain brucellae, 3) outer membranes of brucellae-filled cisternae were continuous with outer nuclear membranes, 4) lumena of brucellae-filled cisternae were continuous with, and brucellae were present in, the perinuclear envelope, and 5) quantities of normal rough endoplasmic reticulum were markedly reduced in brucellae-infected trophoblasts.

B. abortus may utilize trophoblastic rough endoplasmic reticulum for synthesis of bacterial-encoded membrane or cytosolic proteins. Co-translational translocation of both bacterial and eukaryotic secretory proteins occurs across mammalian endoplasmic reticulum by similar mechanisms. The signal sequence on bacterial proteins
recognizes and can attach to signal recognition particle, a peripheral
membrane complex in mammalian rough endoplasmic reticulum which binds
protein synthesizing polysomes to the endoplasmic reticulum membrane.\textsuperscript{1,32} Thus, bacterial proteins encoded for by \textit{B. abortus} DNA
could be correctly translocated across and processed by trophoblastic
rough endoplasmic reticulum. Cytochemistry, utilizing DNA
hybridization, may prove useful to determine the location and function
of bacterial DNA in infected trophoblasts.

\textbf{Cell walls of \textit{Brucella} spp.} contain carbohydrates and trophoblastic
rough endoplasmic reticulum may be involved in the glycosylation of
bacterial membrane proteins. Glycosylation is a major biosynthetic
function of the rough endoplasmic reticulum and most proteins
sequestered in its cisternae are glycoproteins. Glycosyl transferases
in the rough endoplasmic reticulum membrane add N-acetyl glucosamine,
glucose, and mannose to synthesized proteins, these sugars are present
in brucellae cell walls.\textsuperscript{1,14}

Localization in rough endoplasmic reticulum and catabolism of
mammalian secretory proteins may provide a basis for the enhanced
intracellular replication observed with \textit{B. abortus} in trophoblasts and
\textit{Legionella pneumophila} in macrophages. Most strains of \textit{Brucella} spp.
require complex media containing several amino acids, thiamin,
nicotinamide and magnesium ions. Only some laboratory adapted strains
can grow on media containing ammonium salt as the sole nitrogen
source.\textsuperscript{14} \textit{Legionella pneumophila}, which inhabits rough endoplasmic
reticulum of macrophages, also requires amino acids for growth; they are
the primary source of carbon and energy for the legionellae.\textsuperscript{21}
The production of steroid hormones by trophoblasts may influence intracellular growth of *B. abortus*. Progesterone enhances *in vitro* growth of *B. abortus*, is synthesized by smooth endoplasmic reticulum, and is secreted in large quantities by ruminant trophoblasts in late gestation.\(^1,3,20,30\) Brucellae were not present in smooth endoplasmic reticulum, however, quantities of this organelle did increase markedly in the trophoblasts of one infected animal. Because this hypertrophy was not seen in all animals, we could not morphologically substantiate the role of steroid hormones, like progesterone, in growth enhancement of *B. abortus* *in vivo*. Ruminant trophoblasts also produce polypeptide hormones.\(^3,20\) Chorionic gonadotropin is produced in low amounts by trophoblasts throughout ruminant gestation. Its effects on the growth of *B. abortus* *in vitro* have not been evaluated. Placental lactogen is produced by binucleate trophoblasts (giant cells)\(^47,48\) but in this study brucellae did not infect binucleate trophoblasts. It is not likely that trophoblast polypeptide hormones enhance growth of brucellae.

Lack of changes in content of Golgi apparatus suggests that increased trophoblastic content of brucellae-filled rough endoplasmic reticulum is not associated with increased modification and sorting of glycoproteins destined for secretion or incorporation into cellular organelles.\(^1,13\) This, in turn, suggests that *B. abortus* utilizes the majority of newly synthesized proteins in the hypertrophied rough endoplasmic reticulum and there is no need for an increased level of post-translational protein modification.

The role of the perinuclear envelope in growth of brucellae is uncertain but it is likely that brucellae are present in the perinuclear
envelope only after excessive bacterial replication and overflow of brucellae from the lumen of the rough endoplasmic reticulum. This is because bacteria within the perinuclear envelope were observed most frequently in degenerate cells in which the cytoplasm was completely filled with brucellae. In addition, the degree of similarity between the rough endoplasmic reticulum and the nuclear membranes in terms of composition, organization, and functional activity are limited.\textsuperscript{1,37} Thus, the perinuclear envelope is probably of no functional significance to brucellae replication.

The morphology of normal ruminant trophoblasts has been described and our results agree with those of others.\textsuperscript{7,8,15,28,35,43,47,48} The connection between the four carbon alcohol erythritol and rough endoplasmic reticulum in trophoblasts is uncertain. Localization and replication of \textit{B. abortus} in the pregnant ruminant uterus has been attributed to its content of erythritol.\textsuperscript{27,34,39,40,46} Microgram quantities of erythritol enhance growth of \textit{B. abortus in vitro}.\textsuperscript{4,30,39,40,44} Erythritol is utilized by \textit{B. abortus} in preference to glucose and functions as an electron donor in the \textit{Brucella} electron transport system.\textsuperscript{42} Placental perfusion studies in the ovine have shown that erythritol is synthesized by ruminant placentae, but the exact cellular site of synthesis is unknown.\textsuperscript{9}

We believe that chorioallantoic trophoblasts are infected by endocytosis of free brucellae, or cytoplasmic fragments containing brucellae, which have been released after rupture of adjacent necrotic trophoblasts. After initial infection of placental erythrophagocytic trophoblasts, placental infection spreads from trophoblast to
trophoblast, on the chorioallantoic membrane, in a periplacentomal pattern. Trophoblasts are phagocytic cells and chorioallantoic trophoblasts are especially phagocytic as they absorb "uterine milk" secreted by endometrium. In recently infected trophoblasts, which contained few brucellae, bacteria were found near the microvillous surface in membrane-bound structures compatible with phagosomes. No ribosomes lined these brucella-containing phagosomes. This finding was a rarity and most brucellae in cells with few bacteria were within perinuclear rough endoplasmic reticulum.

The dense, osmiophilic inclusions present in the rough endoplasmic reticulum of trophoblasts are present in normal late-gestation ruminant placentae. Their composition, significance, and function are unknown. They may be crystalized protein aggregates from an over production or under utilization of normal trophoblast proteins in late gestation. Because they were present in normal trophoblasts from inoculated and non-inoculated goats, the location of inclusions in the same cisternae as brucellae is probably not significant.

B. abortus produces placentitis and Legionella pneumophila produces pneumonia, however, they share many similarities. They are both facultative intracellular pathogens which inhibit phagolysosome fusion and survive within phagocytic cells. B. abortus inhabits the rough endoplasmic reticulum of trophoblasts and Legionella pneumophila inhabits the rough endoplasmic reticulum of monocytes and macrophages in vivo and in vitro in several species. It appears that both organisms utilize rough endoplasmic reticulum for bacterial replication.
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GENERAL DISCUSSION AND SUMMARY

Although several studies have characterized the gross and microscopic lesions associated with ruminant brucellar placentitis, ultrastructural studies of the placental lesions have never been reported. In this study, a caprine model of ruminant brucellar placentitis was developed and used to characterize the histologic and ultrastructural changes of brucellae-infected placentae.

Results of the first study indicate that experimental caprine brucellosis closely resembles bovine and ovine brucellosis and that it provides a model to study the intracellular development of \textit{B. abortus} in trophoblasts. Brucellae were first seen in placentomal erythrophagocytic trophoblasts and periplacentomal chorioallantoic trophoblasts. Endocytosis of organisms during bacteremia by erythrophagocytic trophoblasts may be an important mechanism of placental infection for many placental pathogens. Intracellular replication of brucellae in chorioallantoic trophoblasts was the source of bacteria for the placentome and fetus.

Ultrastructurally, brucellae were first seen in phagosomes of erythrophagocytic trophoblasts and in the rough endoplasmic reticulum of chorioallantoic trophoblasts. This indicates that maternal bacteremic brucellae enter placentae by utilizing the normal process of trophoblastic erythrophagocytosis. Marked bacterial replication occurred in the rough endoplasmic reticulum of chorioallantoic trophoblasts. Binucleate trophoblasts were not infected even when adjacent to heavily infected chorioallantoic trophoblasts. This
indicates that the rough endoplasmic reticulum of chorioallantoic trophoblasts is selectively parasitized by \textit{B. abortus} and that it enhances bacterial replication. Large numbers of brucellae in placentae were associated with widespread placental vasculitis. Vasculitis and subsequent fetal hypoxia may be important mechanisms of abortion in ruminant brucellosis.

Rough endoplasmic reticulum (not containing brucellae) is markedly reduced in brucellae-infected trophoblasts. Quantitative stereology was used to show this marked reduction in normal rough endoplasmic reticulum associated with hypertrophy of brucellae-containing rough endoplasmic reticulum. This suggests that \textit{B. abortus} uses trophoblastic rough endoplasmic reticulum for replication. In addition, five morphologic criteria were used to establish that brucella-filled cisternae were indeed trophoblastic rough endoplasmic reticulum.

Intracellular replication of \textit{B. abortus} in ruminant trophoblasts has been recognized since the studies of Bang in 1897.\textsuperscript{5,64,78} This intracellular replication is associated with a unique tropism for trophoblastic rough endoplasmic reticulum, an organelle specialized for protein synthesis and glycosylation. In an infected animal, with bacteremia, \textit{B. abortus} is present in every organ; however, only in the placenta does the bacterium replicate to excessive numbers. This growth enhancement of \textit{B. abortus} in placentae may be due to its utilization of trophoblastic rough endoplasmic reticulum for synthesis and glycosylation of bacterial membrane proteins.

The studies in this dissertation have resulted in a better understanding of the pathogenesis of placentitis and intratrophoblastic
replication of *B. abortus* in ruminant brucellosis. Since this was primarily a morphologic study in a model system, several important questions remain to be evaluated. Is the ultrastructure of *B. abortus*-infected bovine trophoblasts similar to those in our study? What enzymes or mechanisms of biosynthesis does *B. abortus* use in trophoblastic rough endoplasmic reticulum to enhance bacterial growth? Is erythritol located in the trophoblast, and if so, what are its biochemical connections with the rough endoplasmic reticulum. Will strain 19 *B. abortus*, which is inhibited by erythritol,\textsuperscript{12,18,47} also replicate in trophoblastic rough endoplasmic reticulum. If so, a previously unrecognized substance or biosynthetic mechanism, other than placental erythritol content, may be responsible for the placental tropism of *Brucella* spp.


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