A histological and ultrastructural study of experimental *Brucella abortus* infection in embryonated chicken eggs

by

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INTRODUCTION

Brucella abortus, a facultative intracellular bacterium, produces a chronic infection which in pregnant cattle is manifested by abortion.[34] B. abortus preferentially localizes in chorioallantoic trophoblasts.[5,61,89,103,127] Recently Anderson et al., reported that B. abortus first enters the erythrophagocytic trophoblasts of caprine placentome and then replicates in rough endoplasmic reticulum of periplacentomal chorioallantoic trophoblasts.[3-5] This localization of B. abortus in trophoblasts is unique; other intracellular bacterial and protozoan parasites are usually located inside phagosomes or free in host cell cytoplasm.[26,94] Only Legionella pneumophila has been described inside the rough endoplasmic reticulum in monocytes and macrophages.[8,50,58,68] Mechanisms of B. abortus entry into the erythrophagocytic trophoblast and its transport to the rough endoplasmic reticulum of the chorioallantoic trophoblast are still unknown.

Brucellae can infect embryonated eggs by all classical inoculation methods. Since the first report of B. abortus growth in embryonated eggs in 1937[54], chick embryos have been used in B. abortus research. B. abortus multiplies intracellularly in almost all cells derived from the three primary germ layers[21,63] but has a greater affinity for cells of mesodermal origin, especially endothelial cells.[21]

To determine the suitability of embryonated chicken eggs as a useful in ovo model to study early cellular events of B. abortus

infection in embryonic membranes, a histopathological and ultrastructural study of B. abortus infection was done. We were especially interested in the organs and cells preferentially parasitized by the organism and the exact location of B. abortus in its target cells.

LITERATURE REVIEW

Bovine Brucellosis

Brucellosis is a highly contagious disease caused by bacteria of the genus Brucella. In cattle the disease is due to B. abortus and is characterized by abortion.[61]

Historical background

The first description of brucellosis as a disease entity was by Marston, an Assistant Surgeon in the British Army, who in 1860 wrote on the "Mediterranean gastric remittent fever", also called "Malta Fever", a human disease characterized by recurring fever. The disease had been endemic for centuries in the Mediterranean area, as suggested by Hippocrate's description, in 450 B.C., of a febrile illness compatible with brucellosis. The association of the disease with a bacterium was made by Sir David Bruce, a British Physician on assignment in a military hospital on Malta. Stimulated by the recent discoveries in the field of bacteriology, Bruce was determined to seek the cause of the fever. In 1886, he isolated from spleen at autopsy, and later from patient's blood and spleen, a micrococcus that reproduced the disease in monkeys. In a brief clinical discussion published in 1887, he called the disease "Mediterranean fever" and its etiological agent "Micrococcus melitensis".[37,82,131]

The source of the organism was not discovered until 1904, when, in preparation to a large scale epidemiological study on Malta fever, the goat population in Malta was evaluated as a possible source of experimental animals. It was found that 40% of the island's goats had positive agglutination reactions against M. melitensis and that the organism could be cultured from the milk of apparently healthy animals. The consumption of raw goat's milk was forbidden and the incidence of Malta fever declined rapidly.[131]

Abortion in domestic livestock has probably plagued herdsmans since animals were first domesticated.[82] According to a British veterinarian of the nineteenth century, the disease was known in the roman empire as "Abortus epidemicus" and was particularly fatal to "pregnant females and cows" in Rome.[131] The first modern investigation of epizootic abortion in cattle was by Nocard, a French veterinary bacteriologist, who, in 1886, concluded that abortions were due to the presence of microorganisms between the fetal membranes and the uterus.[100] Nocard, however, failed in his attempts to isolate the causative organism, probably because he was not in possession of an appropriate nutritive medium.[7] A decade later, in Copenhagen, Bang was able to grow the organism, using a serum-enriched medium maintained at an optimal oxygen concentration. In subsequent studies Bang repeatedly succeeded in isolating the same organism and reproduced the disease in pregnant heifers and ewes.[7] He called the etiological agent Bacillus abortus and the disease became known worldwide as "Bang's disease".

Despite the similitude of the diseases they produce in animals, the relationship between Bruce's Micrococcus melitensis and Bang's Bacillus abortus remained unrecognized for many years. Bruce had described his organism as a micrococcus growing under normal aerobic conditions, whereas Bang's organism was a bacillus the growth of which did not occur under atmospheric oxygen concentrations.[36,37] In 1918, the American bacteriologist Alice Evans correctly identified M. melitensis as a bacillus and recognized the common identity of the two bacteria.[36] She also implicated cow's milk as an important source of brucellosis in human beings.[131] A few years later, Meyer and Shaw, confirmed Evans' findings and proposed the name "Brucella" for the new genus.[85]

The organism

The genus Brucella, comprises six bacterial species and forms a discrete homogeneous group not closely related to any other genus. The three principal species were originally differentiated on the basis of their major animal sources: goats for B. melitensis, cattle for B. abortus and swine for B. suis. The three, more recently described, minor species, B. ovis, B. canis and B. neotomae have as their natural reservoir hosts, sheep, dogs and the desert wood rat, respectively. This speciation has since been supported by cultural, metabolic and antigenic differences. These differences, however, are slight and quantitative, rather than qualitative, and the number of biotypes in each species is large. Distinction between species and, within each

species, between different biotypes, is based on their utilization of certain amino acids and carbohydrates, their sensitivity to bacteriophages, CO_2 requirements, H_2S production, growth on cultural media containing dyes and their reaction to monospecific sera.[28,49,56,117,128]

B. abortus is a Gram-negative, non-motile, non-spore forming, aerobic coccobacillus measuring 0.5-0.7 µm by 0.6-1.5 µm. In vitro growth is slow, especially on initial cultivation. The colonies, barely visible after 2 days, reach maximum size after 5 to 7 days. Nutritional requirements of brucellae are complex, B. abortus will grow on tryptose agar, albimi agar and trypticase-soy agar supplemented with serum or blood. All strains of B. ovis and most strains in some biotypes of B. abortus, require 5 to 10% CO_2 for primary isolation.[28,49,56,117,128]

As a rule, freshly isolated B. abortus form smooth (S) colonies on solid media. However, when grown serially on laboratory media the smooth isolates tend to be replaced by intermediate (I), mucoid (M) and rough (R) forms. The colonial variants differ in virulence, antigenic properties and in phage susceptibility. They can be differentiated on the basis of their precipitate characteristics in 1:1000 acriflavine.[28,128]

Epidemiology

Brucellosis in livestock is produced by 4 of the 6 recognized species of the genus Brucella. In cattle nearly all infections are

caused by B. abortus, with 85% of the isolates belonging to biotype 1. Biotypes 2 and 4 are also found in the United States.[98] The disease is widespread in cattle and of major economical importance in most countries of the world. The true incidence of bovine brucellosis is difficult to estimate, as it varies considerably between countries, regions and herds.[14,49,61,98,99]

In most countries, cattle are the primary reservoir of B. abortus, and the usual source of infection is aborted fetuses and contaminated uterine discharges, where the organism achieves its greatest concentration. B. abortus infection occurs also in sheep, goats, horses, dogs and birds and has been found in several species of free-living animals, including bison, elk, moose, reindeer, fox and hare. Although, substantiated evidence of spread between feral animals and cattle is lacking in Europe and North America, in Africa, game animals are involved in the epizootiology of bovine brucellosis.[14,49,98,99]

B. abortus is susceptible to heat, desiccation and standard disinfectants, but survives under freezing conditions or in cool areas protected from direct sunlight. In temperate climates, infective organism can survive on grass for 100 days in the winter and 30 days in summer. Consequently, the possibility of indirect transmission by insects, pets, wild animals, fodder and inanimate objects exists.[14,49,98]

The susceptibility of cattle to infection by B. abortus increases with sexual maturation and becomes maximal during pregnancy. Sexually

immature animals are usually considered resistant. Most calves fed contaminated milk or calves which are infected *in utero* clear the organism in a few weeks. In a small proportion of heifer calves, however, organisms remain latent until shortly before or after the animals first parturition.[14,49,98,106,109]

The incubation period of bovine brucellosis varies considerably and is influenced by several factors such as gestation, age, exposure dose, vaccination and other unknown factors. Depending on the stage of gestation, incubation periods of 53 to 251 days have been reported. The shortest incubation periods are usually observed in late pregnancy.[98,109]

The spread of brucellosis from one herd to another is usually the result of the introduction of an infected animal into a non-infected susceptible herd. The higher prevalence of brucellosis in large size herds (more than 250 cows) can be related to several factors. First, the increase in herd size is usually accompanied with an increase in cattle density resulting in higher exposure potential, especially following abortion. Also, large sized herds are often maintained by introduction of replacement cattle from outside sources, increasing the risk of acquiring the disease. Finally, once infected, the time required to eliminate brucellosis is significantly greater in large size herds.[14,98,99]

From the viewpoint of human health, bovine brucellosis is an important disease because *B. abortus* can cause undulant fever in man. Human infection, however, is usually accidental and humans are almost

always end-hosts of the organism. Most cases in humans are occupationally related and occur in farmers, meat-packing plant workers and veterinarians. The drinking of unpasteurized infected milk and milk products can also be a source of human infection.[14,117,128]

Pathogenesis

The usual route of infection of cattle is alimentary, but infection can also occur by conjunctival, vaginal and intramammary routes, and through scarified skin.[14,61] Coital infection and transmission of B. abortus by artificial insemination have been reported.[10,81] Following exposure, the organisms are rapidly ingested by polymorphonuclear neutrophils (PMN) and carried to the regional lymph node where bacterial replication occurs.[56,61,104,134]

Spread of the organism is chiefly hematogenous. The duration of the bacteremia, which may persist for several months, depends on the susceptibility of the host. In immune animals the bacteria are probably quickly destroyed by the phagocytic cells. However, in susceptible animals the organism can resist intracellular killing by phagocytes and persist for weeks or months within macrophages. In these chronic infections, bacteremia becomes intermittent and recurs irregularly for at least 2 years in 5 to 10% of the animals.[49,56,61,117]

The localization of B. abortus in bacteremic females is largely restricted to the spleen, mammary gland, supramammary lymph nodes and pregnant uterus.[61,103] In the male, the organism localizes in lymphoid

tissues, testis and accessory sexual glands.[10,61,77] In a natural infection of pregnant cows, Smith and al.[124] found 3-14x10^{13} organisms found in the mother and fetus; the brucellae were confined to the fetal cotyledons, fluids and chorion, which contained 60-85%, 1-25% and 2-8% of the organisms, respectively. It is believed that bacteremic brucellae are endocytosed by erythrophagocytic trophoblasts and then replicate within chorioallantoic trophoblasts.[4,5,89] Placentomal chorionic villi and fetal viscera are infected hematogenously following trophoblast necrosis and ulceration of chorioallantoic membranes.[4,89]

The special affinity of B. abortus for the pregnant bovine uterus has been attributed to uterine content of erythritol.[70,104,125] This four-carbon polyhydric alcohol ($HOCH_2$-CHOH-CHOH-CH_2OH), stimulates growth of B. abortus in vitro and is present in the placenta and fetal fluids of animals (cattle, sheep, goats, swine and deer) prone to brucellar placentitis.[70,112] It is, however, not detected in the placenta of species which are less susceptible to acute placentitis after brucella infection (human, rat, rabbit and guinea-pig).[70] In males, erythritol is found in the seminal vesicles of the bull, ram, buck and boar but not in human seminal vesicles.[70] Erythritol is used by B. abortus as an energy source in preference to glucose and the bacterium's growth is inhibited by unmetabolizable analogues.[2,122,129]

The survival of B. abortus within PMNs and non-activated macrophages is believed to be due to the production by the organism of a cell wall component that interferes with bactericidal mechanisms of phagocytes.[123,128,134] Recent studies indicate that B. abortus

releases two components that inhibit the myeloperoxidase-H_2O_2-halide antibacterial system of bovine PMNs.[23,24] These two components, identified as 5'-guanosine monophosphate and adenine, were shown to act by preferential inhibition of primary granule release.[11,23]

Placental lesions

B. abortus has a special affinity for the ruminant placenta where it produces extensive lesions.[61] This was first recognized by Nocard, who in 1886 reported the presence, between the uterus and the fetal envelopes, of a considerable quantity of yellowish, flocculent, purulent material.[100] Bang, who isolated the causative bacterium in 1897, observed its intracellular localization in infected placentae.[7] In 1919, Theobald Smith identified the infected cells as the trophoblastic cells covering the chorioallantoic membrane.[127]

Grossly, the placental lesions caused by B. abortus are characteristic but not pathognomonic.[61] Typically, the placenta is edematous, the fetal membranes and umbilical cords being saturated with a clear edema fluid. A thick, tenacious, yellow exudate is present between the endometrium and the intercotyledonary chorion.[5,61,89,103] The lesions vary greatly in severity, depending on the course of the infection and the susceptibility of the host.[5,61] In animals with mild placentitis the lesions are limited to the periplacentomal chorioallantoic membranes.[5] In more severe cases, the cotyledons may become necrotic and an abundant exudate is diffusely present in interplacentomal areas. These lesions are usually not uniformly

distributed, as some cotyledons may appear normal while others are extensively necrotic.[5,61]

Histologically, a diffuse filling of the chorioallantoic trophoblasts with intracellular brucellae is the first and most prevalent placental lesion.[5] Many infected cells slough into the uterine cavity and consequently the chorioallantoic membrane becomes ulcerated and covered with exudate. The exudate covering the ulcerated membranes consists of desquamated brucella-filled trophoblasts, necrotic cellular debris, free bacteria, and inflammatory cells. In severe placentitis, the edematous placental stroma becomes necrotic and contains increased numbers of inflammatory cells and free bacterial colonies. The maternal portions of the placentome are usually not much involved except in the placental arcades where maternal and fetal villi are intimately apposed. Necrosis of caruncles, endometrial ulcerations and severe suppurative metritis are the usual uterine lesions observed after abortion.[5,61,89,103]

In a recent ultrastructural study of experimental B. abortus placentitis in goats, it was reported that in chorioallantoic trophoblasts, intracellular bacteria were located within the cisternae of the rough endoplasmic reticulum.[3,4]

Diagnosis

The clinical manifestations of brucellosis are not pathognomonic, rendering laboratory assistance absolutely necessary for the diagnosis of the disease.[14,49,61] The available techniques can be classified in

bacteriological, serological, allergic and cellular methods.[98,132] Bacterial culture and positive identification of B. abortus is the most reliable diagnostic test.[14,98]

In the absence of a positive culture, a presumptive diagnosis is usually made based on the presence of antibodies in serum, milk, whey, vaginal mucus or seminal plasma. Many countries use the standard tube agglutination method, or buffered antigen plate agglutination tests, for the screening of cattle populations, and the complement fixation test as a confirmatory serological method. The Rose Bengal test (card test) is simple and fast and can be used at farm or market. The Rivanol and mercaptoethanol tests are useful if IgG and IgM titers need to be differentiated. The inexpensive "milk ring test" is frequently used for the surveillance of dairy herds. Passive hemagglutination, indirect hemolysis, counter-electrophoresis, enzyme- and radioimmunoassay tests are being evaluated in an effort to increase sensitivity and specificity of current tests.[14,98,132]

Protection against infection by B. abortus is largely the result of cell-mediated immunity. For this reason, skin tests and lymphocyte stimulation tests have been recommended as supplemental procedures for the detection of incubative infections in cattle of unknown status.[132]

Control and prevention

Maximum control and prevention of bovine brucellosis is achieved when the three following procedures are combined: 1) vaccination,

2) test and isolation or slaughter of seropositive cattle, and 3) improved management practices that reduce exposure potential.[98] Despite a considerable research effort, the ideal vaccine for bovine brucellosis has yet to be developed.

Strain 19 of B. abortus was isolated in the United States by Buck in 1930 from bovine milk. The strain lost its virulence after remaining on an agar slant at room temperature for a year.[131] The seed culture of strain 19, dispensed by the United States Department of Agriculture (USDA) after 1956, does not grow on medium containing erythritol.[19,71] This inhibition seems to be due to a lack in the NAD-dependant D-erythrulose 1-phosphate dehydrogenase, an enzyme essential for erythritol catabolism.[129,130]

The smooth attenuated strain 19 is considered to be superior to other vaccines for protection against B. abortus infection. When inoculated subcutaneously in female calves, 3 to 10 months old, the live modified strain 19 induces effective immunity for four to five pregnancies. In cattle, calfhood vaccination rarely produces permanent infection. The vaccination of adult cattle is controversial, as it may result in abortion and permanent infection. The persistence of post-vaccinal serological titers, which makes the differentiation of vaccinated animals from naturally infected ones difficult, is the major problem with the use of this live attenuated vaccine.[14,49,98]

The control of brucellosis should emphasize populations more than individuals. Classically, control programs are based on the detection and elimination from the herd of infected cattle followed by the

vaccination of the remaining animals. General principles of hygiene are imposed to prevent spread and reintroduction of infection.[82,98]

As the control and prevention of bovine brucellosis is far more complex than testing cattle and slaughter of reactors, a good knowledge of the disease and a close cooperation of livestock owners is essential for the success of any eradication program.

Use of Chicken Embryonated Eggs in Brucellosis Research

Introduction

It is well established that chicken embryos provide a convenient and inexpensive means by which microorganisms can be studied. Chicken embryos have been widely used for the isolation and propagation of a number of viruses and intracellular bacteria. Despite tremendous developments of cell culture techniques, embryonated eggs are still the optimal system for the diagnosis of certain viral (e.g.: bluetongue, Newcastle, influenza, avian infectious bronchitis) and bacterial (e.g.: legionnaire's disease, Tizzer's disease, chlamydiosis, rickettsiosis) diseases.

Embryonated chicken eggs, protected by shell, membranes and albumen, are usually a germ-free environment[15] that provides living cells in various developmental stages. Avian embryos are immunologically immature and therefore receptive to many microorganisms.

Historical background

The first report on the use of chicken embryos for the study of infectious agents was published in 1906 by Constantin Levaditi,[79] a Rumanian bacteriologist, who worked with spirochetes.[20,53] However, according to Levaditi himself, the first use for this purpose of chicken embryos was by Borrel, a French bacteriologist. Borrel reproduced spirochetale septicemia in chicken embryos by inoculation of blood from Spirillum gallinarum (Borrelia gallinarum)-infected hens.[79]

In 1911, at the Rockefeller Institute for Medical Research in New York, Rous and Murphy inoculated embryonated eggs with tissue suspensions and cell-free filtrates of the newly described transmissible chicken sarcoma (Rous sarcoma).[116] The development of tumoral growths at the site of inoculation injury on the chorioallantoic membrane (CAM), represented the first use of chicken embryo not only for the experimental study of a virus, but also for the transplantation of graft tissue. For almost two decades, with the exception of a few experiments with avian pest and vaccinia viruses, the method was only used for tissue grafting.[12,29,53,95]

Major credit is given to Goodpasture and his collaborators for first emphasizing in 1931[55] the tremendous possibilities in the use of embryonated eggs for the study of infection and immunity.[12,29,80] Working first with fowl-pox, vaccinia and herpes simplex viruses,[55,140] they rapidly extended their technique to the study of pathogenic bacteria, including B. abortus.[54] The success of their studies relied probably on the inoculation method they used. Their adaptation of

Clark's technique[27] to gain access to the CAM, offered a more reliable and reproducible inoculation site than the blind intraembryonic injection used by previous workers.

It was reported that the histology of the lesions induced by many microorganisms in chicken embryos was similar to that of lesions observed in the natural host.[20,54] Bordetella pertussis, for example, proliferates on the ciliated columnar epithelium of the chick embryo trachea and bronchi mimicking the classical lesions of whooping cough in humans.[46] Similarly, following CAM inoculation, some strains of Haemophilus influenzae, invade the meninges and cerebrum of the chicken embryo, as frequently occurs in children.[45]

The advantages of the method was also emphasized for the isolation and production of pure viral and bacterial cultures and for the study of sera and other therapeutic agents for the control and treatment of infectious diseases.[52,53,106] Rapidly, embryonated eggs became widely used for the preparation of viral vaccines on a commercial scale.[12,29]

During the same period, other investigators started using the technique for the cultivation of rickettsiae, fungi, protozoa and even nematodes.[12,29,80,90]

Factors influencing chicken embryos' susceptibility to Brucella infection

It is well known, that the route of inoculation, the age of the embryo when inoculated and the inoculation dose are important factors affecting the embryo's susceptibility to a given microorganism.[12,20]

Route of inoculation Among factors determining the susceptibility of the chicken embryo to a given virus, the route of inoculation is reported as the most important.[12] Unlike viruses, bacterial infection seems to be less dependent on the site of inoculation.[74] Reports in the literature indicate that the chicken embryo is susceptible to B. abortus by all routes of inoculation, although no mention of intravenous inoculation was found. In a comparative study, Kamal observed that after inoculation of the yolk sac, allantois, chorioallantois, amnion and embryo, all embryonic tissues and fluids were infected.[62] The relation between the survival time of the embryo and the route of inoculation is difficult to establish as most investigators, including Kamal, used variable inoculation doses and embryos of different ages. For example, sixteen day-old embryos are more resistant to allantoic inoculation of Vibrio cholerae, than to intravenous inoculation.[41]

Age at inoculation As the chicken embryo develops, its resistance to bacterial infection increases.[15,20,41] When 2 million B. abortus cells were injected into the yolk sac of 4, 6, 8, 11 and 14 day-old embryos, the average embryo survival time was found to be 2.6, 4, 5.2, 7 and 11 days, respectively. The oldest embryos survived until after hatching, but died during the first week post hatching.[62] Kamal attributed this difference to the rapid increase in the volume of the embryo rather than to a true increase in resistance to bacterial infections.[62]

Resistance to bacterial infections with increasing age of the embryo can be related to several factors, since a number of anatomical, physiological and metabolic changes are occurring during the embryonic period.[41,102,113,114] Older embryos, for example, are less susceptible to endotoxin inoculation.[40,41,126] Experiments with enterobacterial endotoxins demonstrated a 10,000-fold decrease in susceptibility, between the eleventh and fifteenth day of incubation.[40] However, the cessation of the mesonephric function, rather than the increase in endotoxin resistance, is believed to cause the decreased susceptibility of older embryos to allantoic inoculation of Vibrio cholerae.[41] With the formation of the metanephros, the mesonephric duct is obliterated, isolating the intra-allantoic vibrios from the mesonephros and thence from the embryonic circulation.[41] Other factors intervening in the resistance of older embryos, include the increase in efficacy of the mononuclear phagocytic system and the appearance of non-specific serum factors (e.g., complement, lysozyme and transferrin).[15,66,67,136]

Inoculation dose Studies with several bacterial species indicate that the concentration of the inoculum influences greatly the outcome of the infection. With B. abortus, the greater the inoculum concentration is, the shorter is the time which elapse between inoculation and death of the embryo.[34,57,62,119,120] However, age of the embryo at the time of inoculation is important, as older embryos may survive infection and hatch normally, regardless of the dose.[34] Following yolk sac inoculation of a field strain of B. abortus into 6 day-old embryos, a 3 days increase in the average survival time was

observed when the inoculation dose was reduced from $20x10^6$ to 4 organisms.[61] Experiments with Listeria monocytogenes showed that large doses placed on the CAM, kill the embryo rapidly without producing any lesions. When a smaller inoculum is used, the infection remains localized and the embryo survives.[101]

Role of the strain and of the colony morphology Chicken embryos were frequently used for the analysis and comparison of different bacterial strains' virulence factors. Several investigators reported that the smooth avirulent strain 19 of B. abortus behaves, in the chicken embryo, as a fully virulent strain.[34,63,84] Reports on the behavior of non-smooth strains are controversial. De Ropp claimed that rough avirulent strains are incapable of producing fatal infection, even when inoculated in large numbers.[34] Kamal, on the other hand, using mucoid and rough forms of a virulent smooth strain, did not find any decrease in virulence. In ovo reversion to the smooth form was not observed, indicating that rough and mucoid strains were fully virulent for the chicken embryo.[63] Selective effects of the chicken embryo, favoring the establishment of virulent smooth strains of B. abortus and B. suis in eggs inoculated with mixtures of smooth and non-smooth forms, have been reported.[17]

Repeated egg-to-egg transfers reportedly increase the virulence of B. abortus for chicken embryos[57,63] and mice.[63] In an attempt to adapt a freshly isolated smooth strain to chicken embryos, Hall and Spink serially passed it once weekly via the allantoic sac. After seven passages the LD_{50} for 11-day embryos declined from 57,000 to 43

organisms.[57] Metzger and Stokes, however, reported that eleven egg-to-egg transfers of two strains of B. abortus, failed to induce any change in virulence.[84]

Histological studies

In their original comparative study on bacterial infections in embryonated chicken eggs, Ernest Goodpasture and Katherine Anderson, included B. abortus. They reported that, when inoculated onto the CAM, the organism induces little inflammatory reaction and replicates locally within ectodermal and mesodermal cells. They described the infected mesodermal cells as large mononuclear cells and fibroblasts and compared the intracellular localization within ectodermal cells to the chorionic epithelium infection, observed by Smith[127] in bovine placenta.[54]

A few years later, Buddingh and Womack, compared the infections of chicken embryos by B. suis, B. abortus and B. melitensis. The three species of Brucella were shown to behave differently when inoculated on the CAM of 12 days old embryos. B. suis and B. abortus invaded the embryonic tissues and replicated within the spleen, liver, kidney and myocardium. In addition to chorioallantoic ectoderm and liver parenchyme, both organisms had a marked affinity for cells of mesodermal derivation. Vascular endothelium invasion was especially marked after B. suis infection: B. abortus most commonly invaded mononuclear cells and fibroblasts. B. melitensis limited its intracellular growth to the chorioallantoic ectodermal epithelium and

was not observed in embryonic organs, although it was recovered from cardiac blood.[21]

Following yolk sac inoculation of 10 day-old embryos, Kamal observed a similar distribution of B. abortus in most embryonic tissues. However, unlike Buddingh and Womack, he reported the infection of the renal tubular epithelium and of the allantoic endoderm. In the yolk sac wall, he found B. abortus within endothelial cells and mononuclear leukocytes but never in other mesodermal cells or within the vitelline epithelium.[63]

Use of chicken embryos as a culture medium

The value of embryonated chicken eggs as a potential differential medium for the isolation of B. abortus was first recognized in 1939.[83] The isolation of B. abortus from infected milk, by means of an artificial medium, was often compromised by the overgrowth of other organisms, primarily Streptococcus agalactiae. From the observation that 10 day-old embryos are able to survive the CAM inoculation of 50,000 S. agalactiae organisms, it was proposed to use this method to detect the presence of B. abortus in milk samples. Eleven embryonated eggs were inoculated with brucella-infected bovine milk contaminated with S. agalactiae; B. abortus was recovered from ten infected embryos.[83]

Chicken embryos were rapidly recognized as a practical, economical, and reliable medium to isolate brucellae from blood of infected humans and animals.[25,47,118] Different studies reported that

yolk sac inoculation of 5 or 6 day-old embryos was the optimal method. It was shown to be superior to artificial media and guinea-pig inoculation.[30] Chicken embryos were found to be a convenient model to monitor the relative effect of various therapeutic agents upon the course of well-established Brucella infections.[57,119,120]

MATERIALS AND METHODS

White Leghorn chick embryos were obtained from the National Animal Disease Center (NADC) inbred line. The eggs delivered two days before inoculation were kept on arrival and throughout the experiments in a humid chamber at 37°C.

The study was divided into three experiments. In experiment I, 30, 6 day-old embryos were inoculated via the yolk sac (YS). In experiment II, 30, 12 day-old eggs were inoculated on the chorio-allantoic membrane (CAM). Samples from these two experiments were examined by light and electron microscopy. In experiment III, 25, 15 and 25, 10 day-old embryos were inoculated using the YS, CAM and intravenous (IV) routes, respectively. These last embryos were processed for histologic examination only. All eggs were inoculated with varying amounts of B. abortus (Strain 19 supplied by Dr. B. Deyoe, NADC, Ames IA) using standard techniques.[13,42] To avoid deposition of brucellae on the CAM, YS inoculation of 10 day-old embryos was done through the albumen. Bacteria were suspended in 0.2 ml saline solution for YS and CAM inoculations and in 0.1 ml for IV injection. Saline solution inoculated eggs were controls (Tables 1 and 2).

Eggs were candled twice daily. Eggs dying within the first 24 hours were discarded. Beginning on post-inoculation day 2, living and dead embryos were sampled at regular intervals until the last viable embryo was collected (Tables 1 and 2).

Table 1. Inoculation dosages and sampling protocol of embryos inoculated at different ages and by different routes with B. abortus

Route	Age (days)	Dosage (CFU/egg)	Group	Time of sampling[a]							Av. time of death (hrs)
				PID 2		PID 3		PID 4		PID 5	
				48h	60h	72h	84h	96h	108h	120h	
Yolk Sac	6	$2.1x10^3$	Infected	3[b]	7(6)	8(5)	7(4)				70.4
			Control	1	1	1	2				
Chorio-allantoic Membrane	12	$1.9x10^4$	Infected	3	0	3	0	11(8)	2(2)	4(2)	102.0
			Control	1	0	1	0	1	0	3	

[a]Post-inoculation day (PID).

[b]Number of embryos sampled (number of dead). The original plan to sample 3 embryos at 12 hour intervals was changed because of early death in the infected groups.

Table 2. Sampling protocol of embryos[a] inoculated by different routes with 1.5×10^3 CFU of B. abortus

Route	Group	Time of Sampling[b]							Av. time of death (hrs)
		PID 2		PID 3		PID 4		PID 5	
		48h	60h	72h	84h	96h	108h	120h	
Yolk Sac	Infected	2[c]	0	2	0	2	8(8)	0	111.3
	Control	1	0	1	0	1	0	1	
Chorio-allantoic Membrane	Infected	2	0	2	0	2	3(3)	1	108.0
	Control	1	0	1	0	1	0	1	
Intra-venous	Viable	2	8(7)	10(10)					67.1
	Control	1	1	2					

[a] 10 day-old when inoculated.

[b] Post-inoculation day.

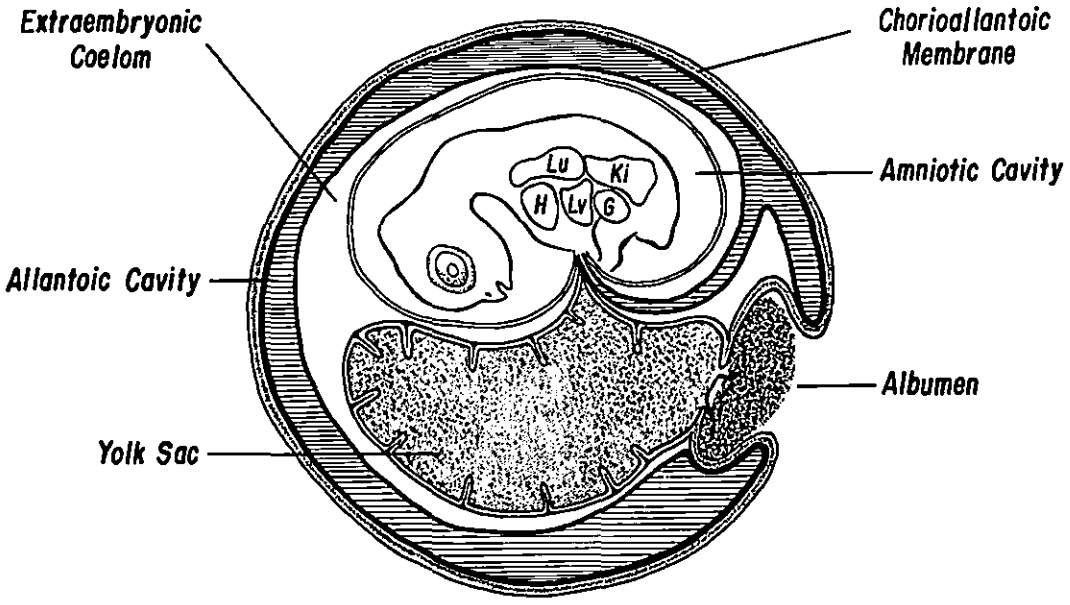
[c] Number of embryos sampled (number dead). The original plan to sample 2 embryos at each time was changed because of early death in the infected group.

In experiments I and III, each egg was opened at the large pole and the embryo exposed by cutting chorioallantoic and amniotic membranes (Figure 1). Large sections of the yolk membrane were dissected in ovo and transferred to a fixative-filled petri dish where they were further dissected into smaller strips. The embryo was removed, cut sagittally in half and both parts immersed in ice-cold fixative. Samples of amniotic and chorioallantoic membranes were taken. Large embryos in experiment II were dissected before fixation. Samples included yolk and amniotic membranes, liver, spleen, kidney, lung and heart. Chorioallantoic membranes were fixed in ovo with cold fixative. After 15 to 30 minutes, 0.3cm x 2cm strips of the membrane were detached from the shell and transferred to fresh fixative.

In experiment I, samples from viable inoculated and control embryos were fixed overnight in a mixture of 3% glutaraldehyde and 2% paraformaldehyde in a 0.1 M sodium cacodylate buffer, pH 7.2 with 5% sucrose and 0.025% anhydrous calcium chloride added.[87] For experiment II, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 was used. In addition, in both experiments, a few samples were fixed for 1.5 hour in a 4% solution of osmium tetroxide in water. After aldehyde or osmium fixation, samples were washed and stored in 0.1 M sodium cacodylate buffer at $4^{o}C$. Dead embryos in experiments I and II and all embryos in experiment III were fixed in 10% neutral buffered formalin.

Sections from all sampled tissues, including one half of the embryo in experiments I and II, were processed for histologic examination. They were dehydrated in graded alcohols, cleared,

Figure 1. Diagram of a chicken embryo and its extraembryonic membranes in the ninth day of incubation. The shell and shell membranes are not shown. (After B. M. Patten[102])


Extraembryonic Coelom
Chorioallantoic Membrane
Lu
Ki
H
Lv
G
Amniotic Cavity
Allantoic Cavity
Albumen
Yolk Sac

infiltrated and embedded in paraffin, cut at 6 μm and stained with hematoxylin and eosin (HE) and Brown-Hopps modified Gram stain.[121]

Samples for electron microscopy were postfixed in osmium tetroxide, infiltrated and embedded in epoxy resin and sectioned at 70 to 90 nm and examined with a Philips 410 electron microscope.

Yolk, allantoic fluid or amniotic fluid were sampled with a sterile cotton swab which was then streaked on a plate of tryptose agar containing 5% bovine serum. After 48 to 72 hours incubation, B. abortus colonies were identified by colony morphology, growth characteristics and agglutination by strain 19 B. abortus antisera.

Immunoperoxidase labeling, involved a biotinylated secondary antibody and an avidin-biotin-peroxidase complex.[60] Primary antibody was rabbit anti-B. abortus (St.19) IgG (Dr. B. Deyoe, NADC, Ames IA). Biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complex from a commercial source were used (Vectasin TM ABC kit, Vector Laboratories, Inc., Burlingame CA).

Immunogold labeling was done on resin-embedded thin sections mounted on nickel grids using techniques described by M. Bendayan.[9] The immunogold probe (20 nm) was prepared by reduction of aqueous tetrachloroauric acid with ascorbic acid[115] and coupled directly to rabbit anti-B. abortus cell surface proteins IgG (Dr. L. Tabatabai, NADC, Ames IA).[32,51]

RESULTS

Pure culture of B. abortus was isolated from all inoculated eggs. Death occurred between 2 and 5 days post-inoculation depending on age of the embryo and route of inoculation (Tables 1 and 2). Because postmortem changes occur rapidly in eggs incubated at 37°C, descriptions of lesions were based on observations made in embryos sampled before death.

Experiment I: Yolk Sac Inoculation of 6 Day-old Embryos

The average time of death after inoculation by this route was 70.4 hours (Table 1). Infected embryos were hyperemic (Figure 2), but no other gross change was seen.

Light microscopy

B. abortus was first detected in the area vasculosa of the yolk membrane (Table 3). In 48- and 60-hour samples, small numbers of bacteria were within individual endodermal cells; these infected cells were distributed randomly throughout the area vasculosa. Below the endoderm, numerous B. abortus were present; they were on both sides of the exocoelom, in mesenchymal cells of the yolk and allantoic mesoderm, and at the distal end of the area vasculosa in the chorionic somatopleur (Figure 3).

Table 3. Tissue distribution of B. abortus after yolk-sac injection in 6 day-old embryos. Data are expressed as number of tissues in which B. abortus was identified by immunoperoxidase/number of tissues examined

Time After Inoculation (hrs)	Tissues with B. abortus Antigens				
	Yolk Membrane	Stalk	Peritoneal epithelium	Glomeruli	Organs invasion
48	2/3	2/3	0/3	0/3	0/3
60	7/7	6/6	6/7	2/6	0/7
72	8/8	7/7	6/8	5/6	0/8
84	7/7	6/6	6/6	6/6	5/7

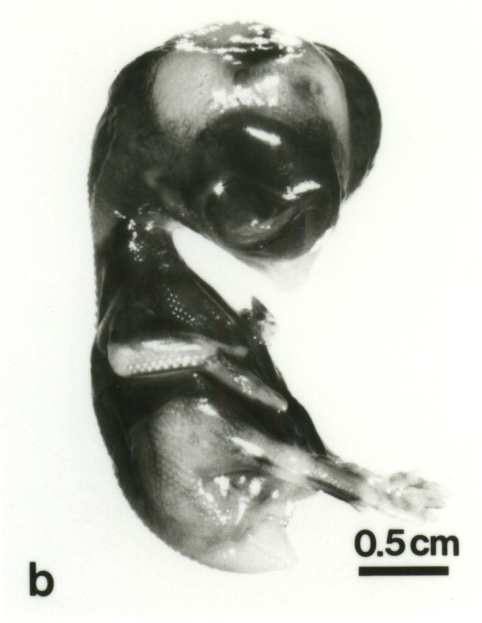
Figure 2. Nine day-old embryos sampled 3 days after yolk sac inoculation.

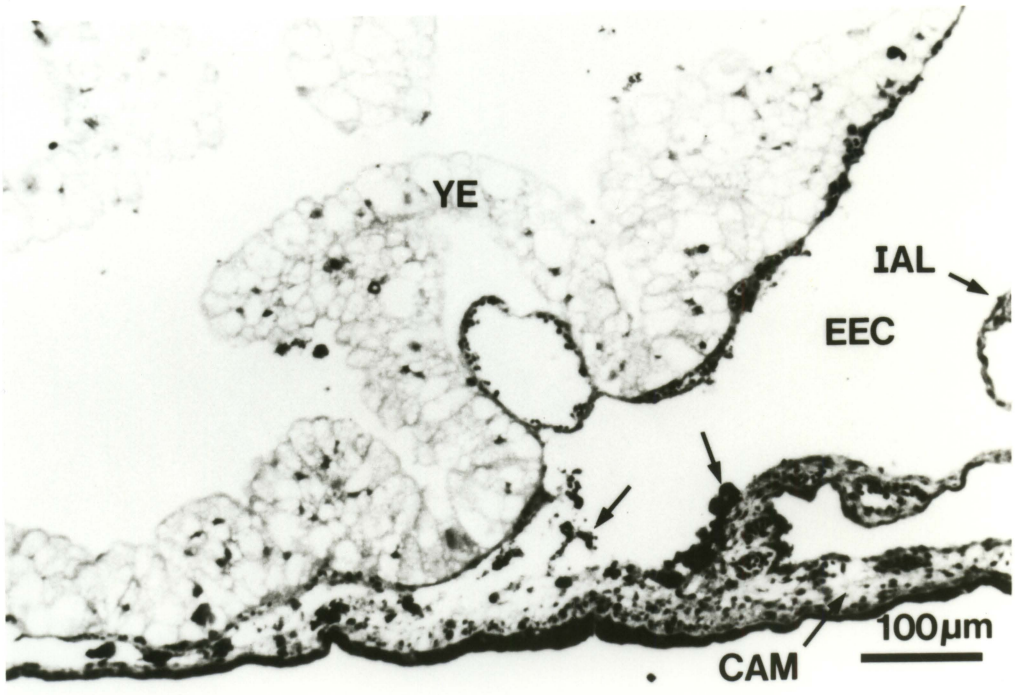
a. control embryo.

b. hyperemia, embryo dead at time of sampling

Figure 3. Yolk sac distal end, 60 hours after yolk sac inoculation. Extra- and intracellular B. abortus (arrows) within the extraembryonic coelom (EEC). [Chorioallantoic membrane (CAM), inner allantoic limb (IAL), yolk endoderm (YE)]. (Immunoperoxidase-hematoxylin)


a
0.5cm


b
0.5cm


YE
IAL
EEC
CAM
100µm

In 60-hour and later samples, the yolk, allantoic and chorionic mesoderm were infiltrated with large round brucella-filled mesenchymal cells. These cells were particularly abundant below the chorionic epithelium at the growing end of the allantoic sac where they were in large clusters (Figure 5). Large extracellular colonies of B. abortus were also present in the mesoderm, between infected mesenchymal cells, and within the exocoelomic cavity (Figure 4).

In 72-hour samples, the organism was diffusely present in the vitelline epithelium. The cytoplasm of infected endodermal cells contained large numbers of B. abortus surrounding intracellular vacuoles and yolk granules. Bacterial colonies were more abundant at the vitelline and vascular poles of the cells.

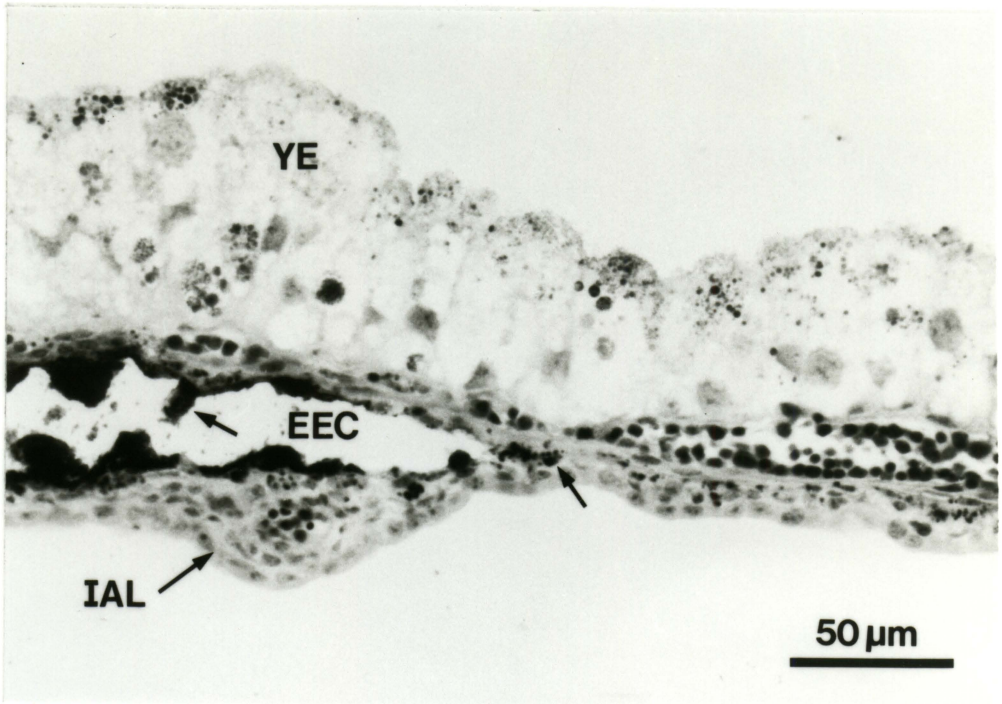
In sections made below the umbilicus across the yolk and allantoic stalks, B. abortus were in mesothelial cells lining the extraembryonic somatopleur and splanchnopleur.

Intraembryonic localization of B. abortus was first seen at 60 hours post-inoculation in dead embryos and at 72 hours in viable embryos (Table 3). The number of brucellae increased with time, however even at the last sampling period only few organisms were found in intraembryonic tissues. Extracellular bacteremic B. abortus were present in most blood vessels.

The peritoneal epithelium and the glomeruli of the mesonephros were the first and most prevalent embryonic localization of B. abortus (Figure 6). A diffuse filling of mesothelial cells with intracellular B. abortus was present in different segments of the peritoneal

Figure 4. Yolk sac wall, 60 hours after yolk sac inoculation. Extra- and intracellular B. abortus (arrows) within the extraembryonic coelom (EEC). [Inner allantoic limb (IAL), yolk endoderm (YE)]. (Immunoperoxidase-hematoxylin)

Figure 5. Yolk sac wall distal end, 60 hours after yolk sac inoculation. Brucella-filled mesenchymal cells (arrows) between the chorionic epithelium (CE) and the yolk endoderm (YE). (Immunoperoxidase-hematoxylin)


YE
EEC
IAL
50 µm

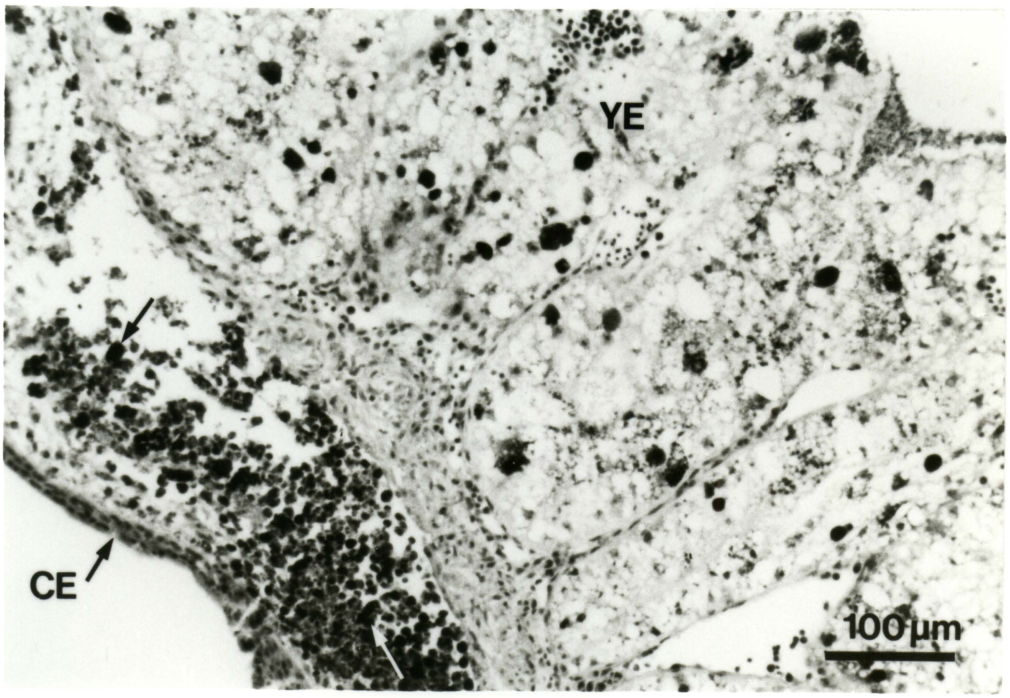

YE
CE
100 µm

Figure 6. Embryo sagittal section, 84 hours after yolk sac inoculation. B. abortus present within kidney (Ki) glomeruli (large arrows) and serosal surfaces (arrows) but not present within liver (Liv), spleen (Sp), gizzard (Gi) and lungs (Lg).

a. Rabbit anti-B. abortus IgG used as primary antibody, labeling of B. abortus.

b. Normal rabbit serum used in place of primary antibody, no peroxidase reaction product visible.

(Immunoperoxidase-hematoxylin)

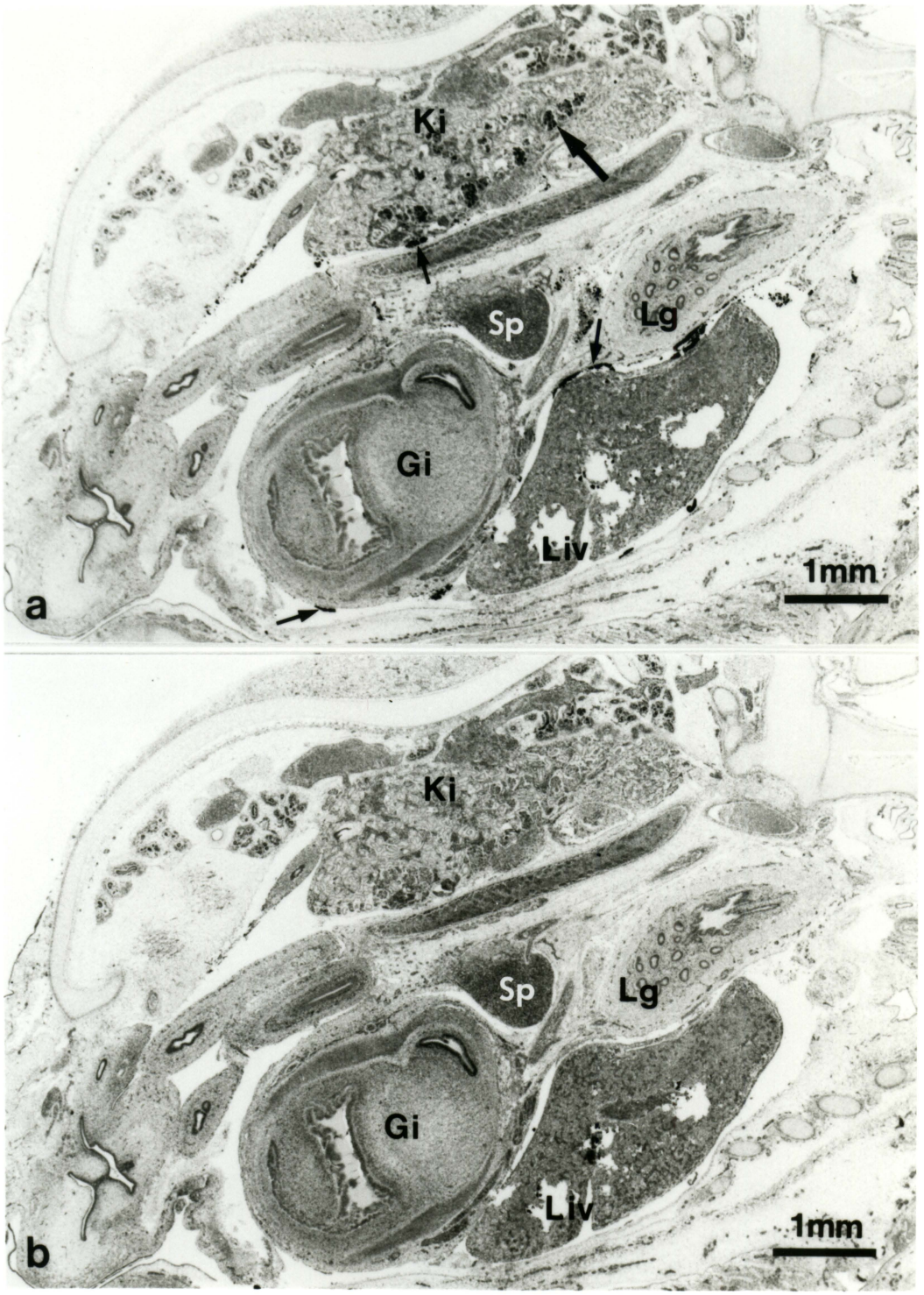
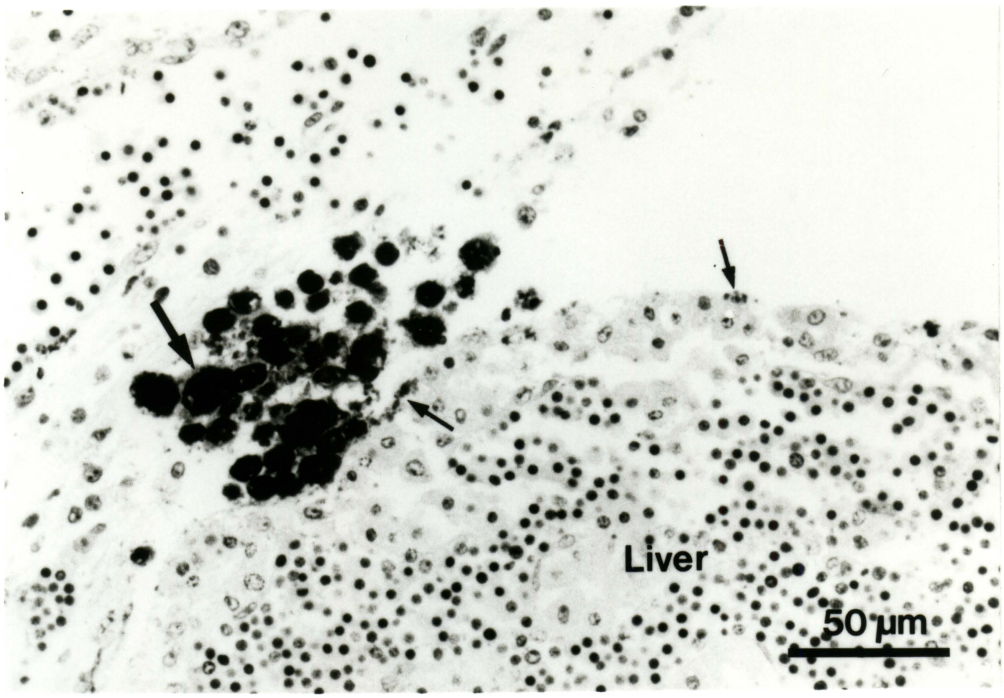
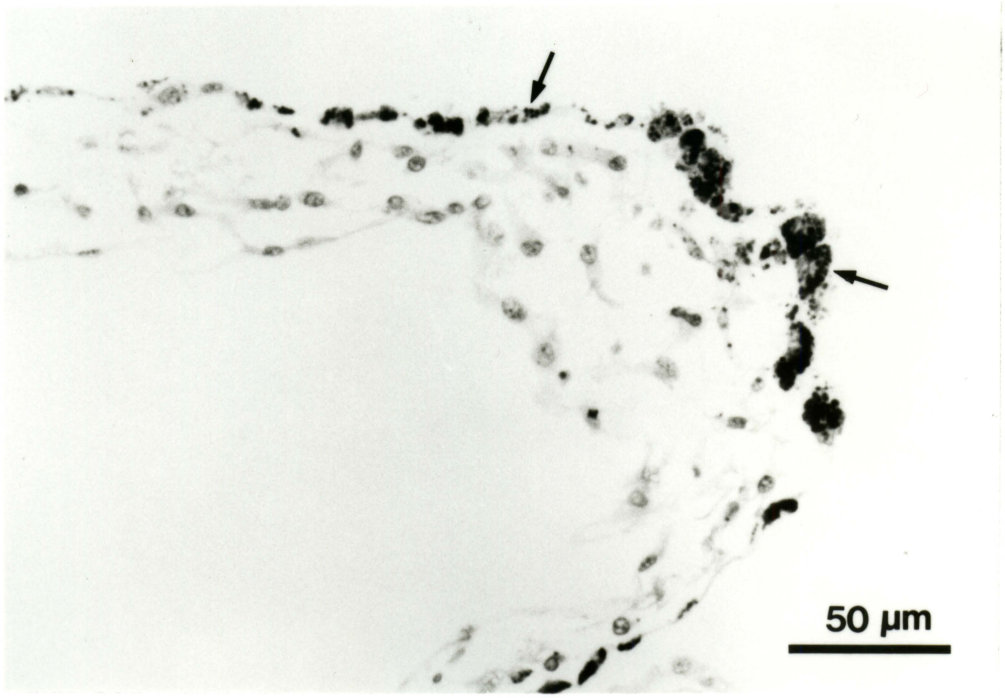

Ki
Sp
Lg
Gi
Liv
1mm
a
Ki
Sp
Lg
Gi
Liv
1mm
b

Figure 7. Liver, 84 hours after yolk sac inoculation. B. abortus within mesothelial cells of the peritoneal serosa (small arrows). Brucella-filled mesothelial cells have sloughed (large arrows) into the peritoneal cavity. (Immunoperoxidase-hematoxylin)

Figure 8. Pericardium, 84 hours after yolk sac inoculation. B. abortus within mesothelial cells (arrows). (Immunoperoxidase-hematoxylin)


Liver
50 µm


50 µm

epithelium (Figures 7 and 8). The extent and localization of the serosal infection were highly variable. Parietal and visceral serosa in the inferior part of the abdominal cavity, around the region where the yolk-stalk enters the body, were frequently involved. Infected mesothelial cells were swollen. In later stages clusters of large round brucella-filled desquamated mesothelial cells were occasionally present in the peritoneal cavity (Figure 7). Individual brucellae were often found in mesenchymal cells underneath infected mesothelia.

In glomeruli of the mesonephros, intracellular B. abortus were present in capillaries and mesangium. The organism was rarely found outside the glomeruli, except for brucella-filled necrotic cells in the lumen of some tubules.

At 84 hours post-inoculation, B. abortus were diffusely distributed in most tissues and organs. The bacteria were mainly intracellular within mesenchymal cells. However extracellular organisms were present inside blood vessels or in the interstitium surrounding infected cells. In liver, isolated bacteria were also free in intercellular spaces. Bacteria were seen in the heart and in fibroblasts lining cartilaginous formations at 84 hours.

Electron microscopy

In the yolk sac endodermal cells, B. abortus were present, throughout the cytoplasm between the intracellular yolk drops (Figures 9 and 10). The brucellae were located within the cisternae of the rough endoplasmic reticulum. The limiting membranes of the

Figure 9. Yolk endoderm, 72 hours after yolk sac inoculation. B. abortus are located within cisternae of rough endoplasmic reticulum (large arrows) between intracellular yolk drops (YD). Free brucellae (small arrows) are present in the yolk (Y). Insets. B. abortus within bristle-coated pit and phagosome. (Glutaraldehyde fixation)

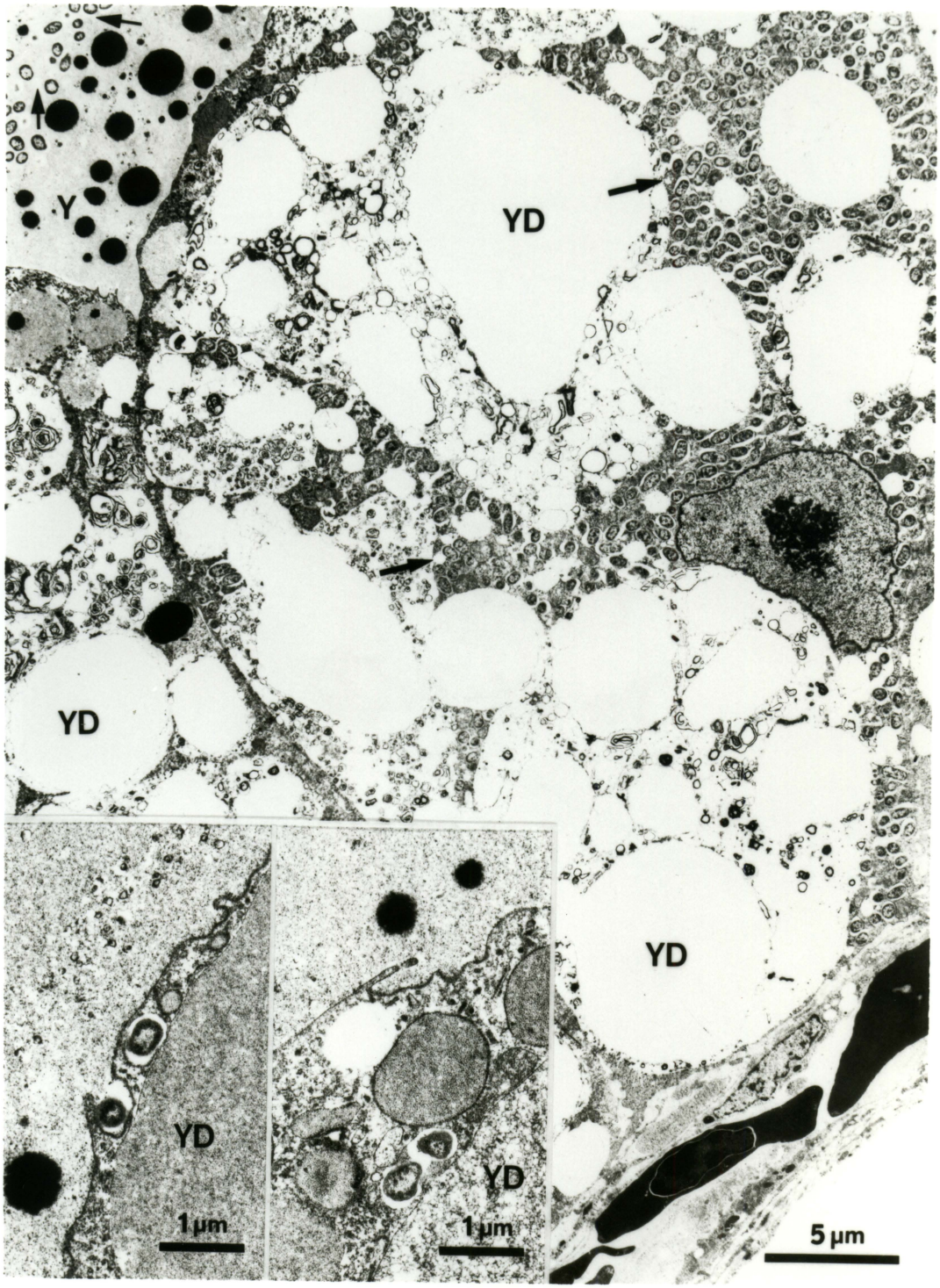

Y
YD
YD
YD
YD
1 µm
YD
1 µm
5 µm

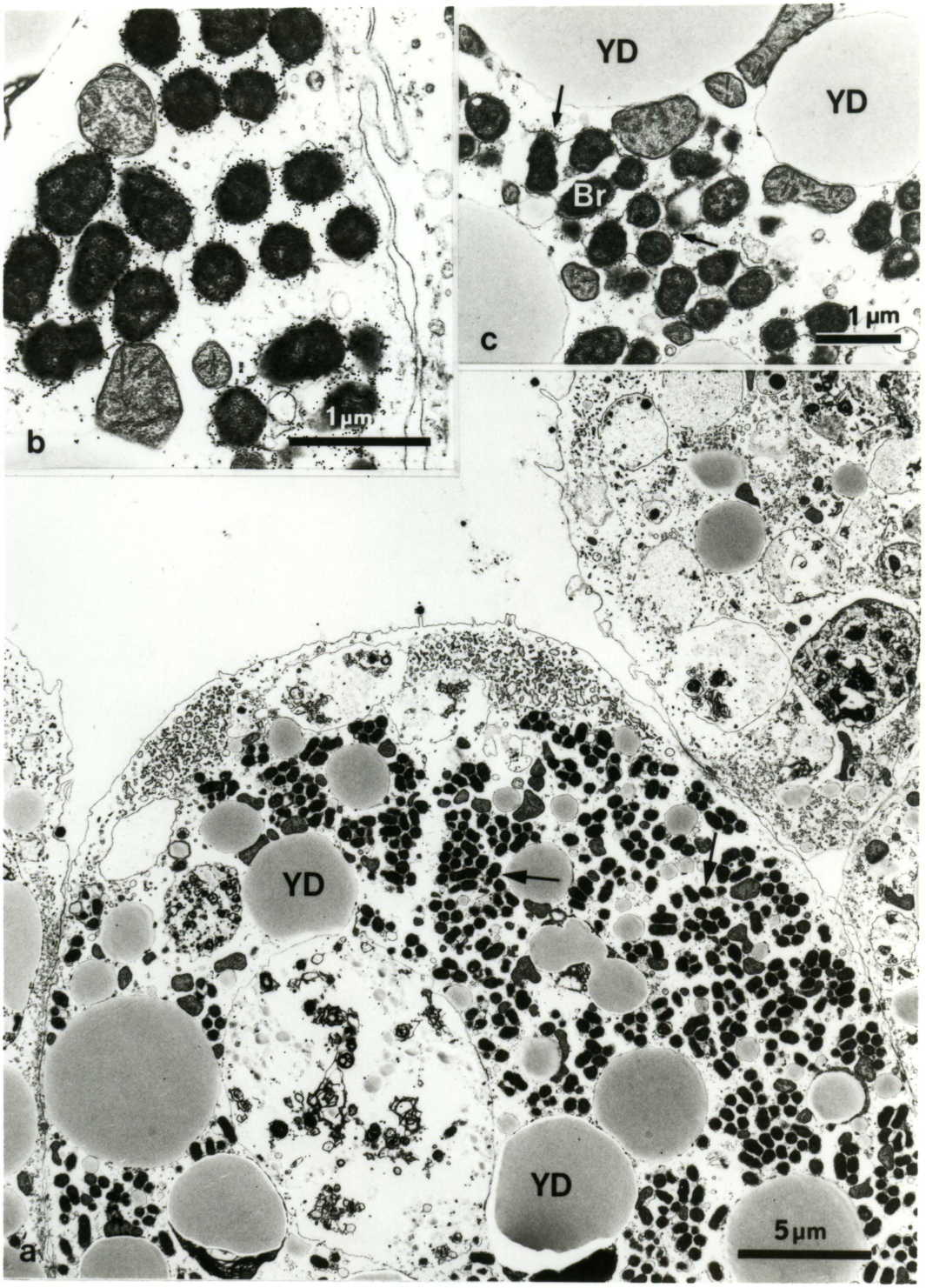
Figure 10. Yolk endoderm, 72 hrs after yolk sac inoculation.

a. B. abortus in cytoplasm (arrows) between intracellular yolk drops (YD).

b. Immunogold labeling of B. abortus.

c. B. abortus (Br) is located within cisternae of rough endoplasmic reticulum between intracellular yolk drops (YD). Cytoplasmic face of brucellae-filled cisternae are lined by ribosomes (arrows).

(Osmium fixation)


YD
YD
Br
1 µm
c
b
1µm
YD
YD
5µm
a

brucella-filled cisternae were folded around the organisms and discontinuously lined by ribosomes (Figure 10). In one out of ten infected endodermal cells, B. abortus were observed inside pleomorphic intracellular yolk drops.

Bristle-coated pits were abundant along the vitelline surface of the endodermal cells. Extracellular dense yolk granules, entrapped by apical microvilli, were seen in association with these coated regions. Individual brucellae in contact with apical plasma membranes, in bristle-coated pits or inside phagosomes between the vitelline surface and apical vacuoles, were seen in two of ten infected cells (Figure 9). Some of these phagosomes also contained yolk material.

Below the endoderm, B. abortus were within pleomorphic, undifferentiated, mesenchymal cells (Figure 11a). In moderately infected cells, the organisms were located within dilated cisternae of the rough endoplasmic reticulum (Figures 11b and 11c), while in heavily infected ones, the brucellae were inside membrane-bound vacuoles. Intracytoplasmic lipid droplets, commonly found in mesenchymal cells, were more abundant within severely infected cells. Extracellular bacterial colonies were abundant between the infected cells (Figure 11a). Extracellular brucellae were also present in the lumen of vitelline blood vessels.

Inside mesothelial cells, B. abortus were within cisternae of the rough endoplasmic reticulum (Figure 12). This was seen along the extraembryonic somatopleur and splanchnopleur at the level of the yolk stalk as well as within parietal and visceral serosal epithelia. There

Figure 11. Yolk-sac wall mesoderm, 72 hours after yolk sac inoculation.

a. Severely infected mesenchymal cells contain B. abortus within membrane-bound vacuoles (large arrow) and distended cisternae of rough endoplasmic reticulum (small arrow). Extracellular brucellae (Br) are abundant.

b. Moderately infected mesenchymal cell, a section of normal rough endoplasmic reticulum (RER-arrows) leads directly into a section containing brucellae (Br).

c. Similar cell as in Fig 4b., B. abortus (Br) are labeled with immunogold.

(Glutaraldehyde-paraformaldehyde fixation)

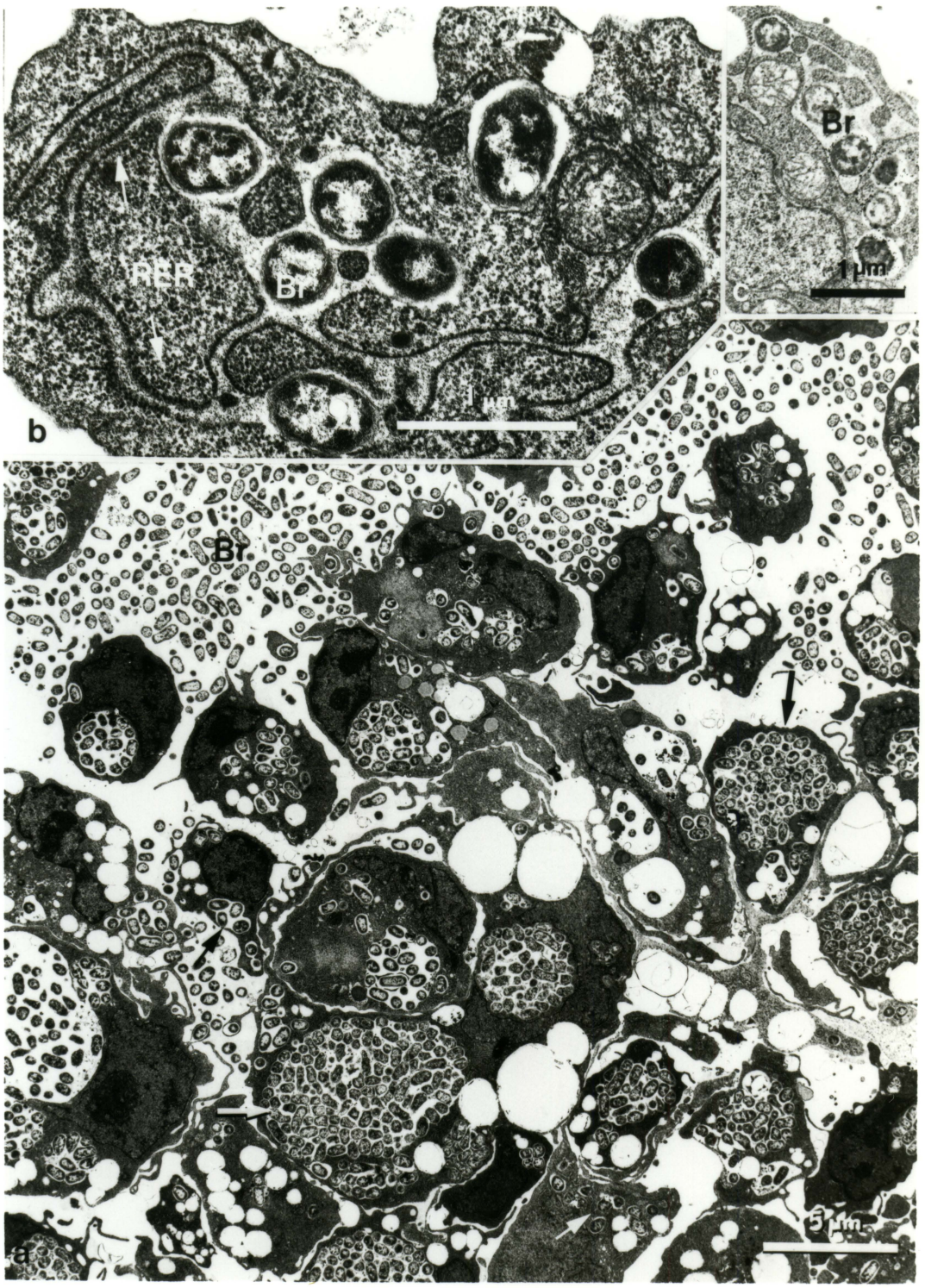
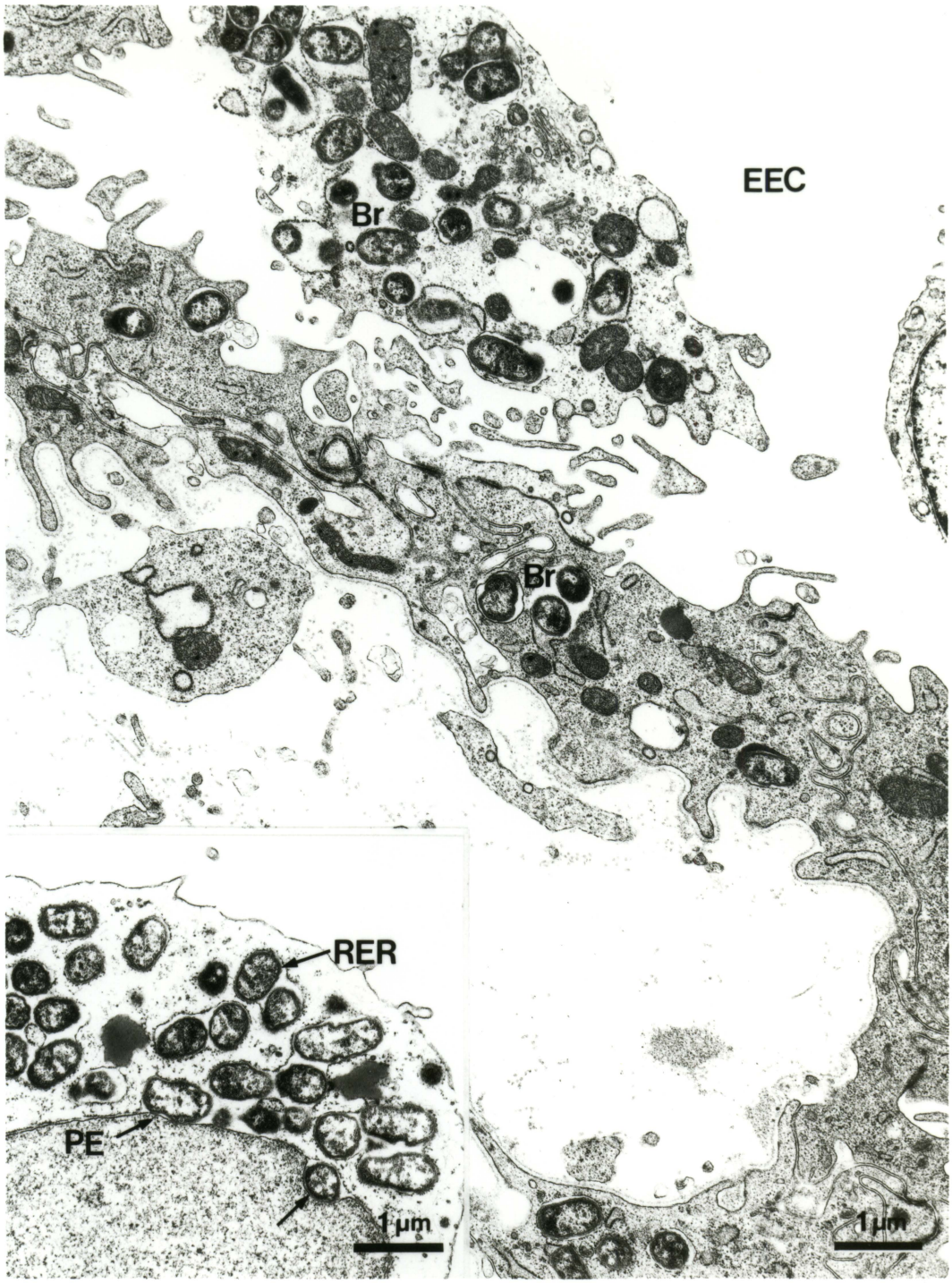

RER
Br
1 µm
b
Br
1 µm
a

Figure 12. Mesothelium of the allantoic stalk, 72 hours after yolk sac inoculation. B. abortus (Br) present within cisternae of rough endoplasmic reticulum. One degenerated mesothelial cell has sloughed into the extraembryonic coelom (EEC). (Glutaraldehyde fixation). The inset shows a mesothelial cell. Ribosome-lined membrane of brucellae-filled rough endoplasmic reticulum (RER) is continuous with the outer membrane of the perinuclear envelope (PE). B. abortus within perinuclear envelope (arrow). (Osmium fixation)


EEC
Br
Br
RER
PE
1 µm
1 µm

was no evidence of cell swelling, proteolysis or vacuolation. However, more severely infected cells had signs of degeneration and some had sloughed into the extraembryonic coelom or abdominal cavity (Figure 12).

In the mesonephros, B. abortus were in glomeruli. Bacteria were located inside membrane-bound vacuoles within endothelial and mesangial cells. Occasionally extracellular organisms were present between endothelium and basement membrane supporting podocytes of the visceral epithelium. Monocytes containing a large brucellae-filled vacuole were found in the same location.

Experiment II: Chorioallantoic Inoculation of 12 Day-old Embryos

Deaths occurred on day 4 or 5 after inoculation (Table 1). In inoculated eggs, the CAM was diffusely thickened and edematous with an increase in opacity at the site of inoculation. Grossly, inoculated embryos were normal except for areas of hemorrhage at the top of the skull of dead embryos (Figure 13). Liver and spleen sampled on day 4 and 5 were grossly enlarged (Table 4) and had a mottled tan and red surface (Figure 13). A slightly opaque chorioallantoic membrane at the site of inoculation was the only lesion found in control eggs.

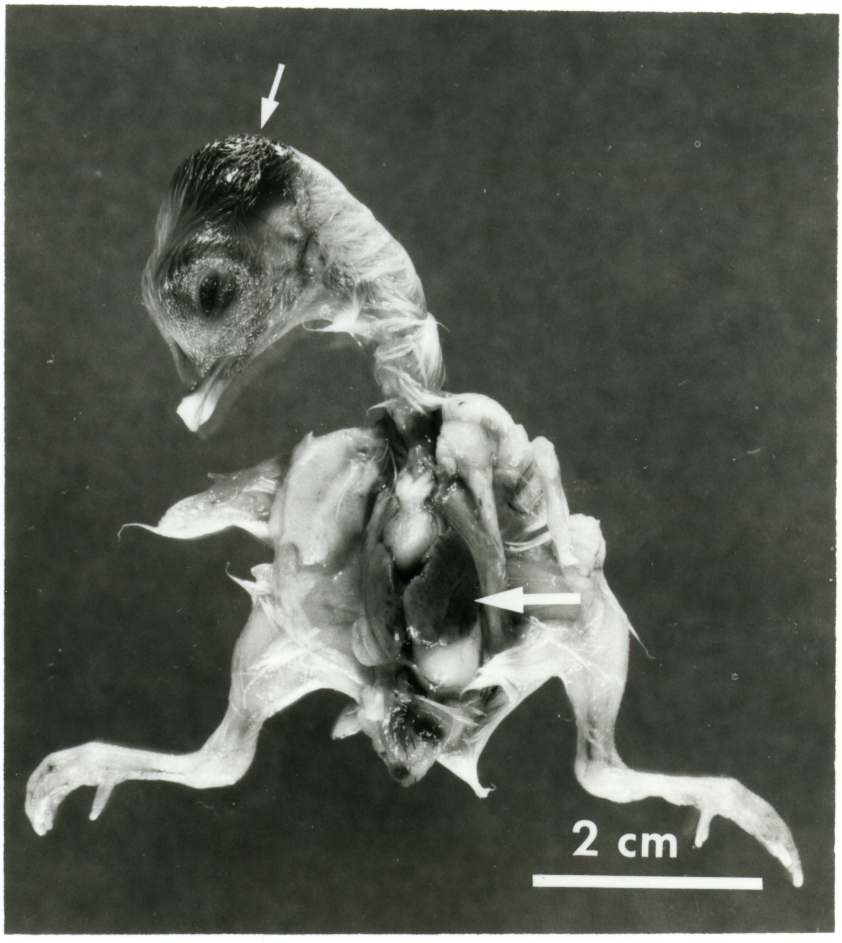
Light microscopy

Microscopically, on day 2 and 3 post-inoculation, moderate lesions were present at the inoculation site in inoculated embryos. They were

Table 4. Relative weights of liver and spleen of embryos dead after chorioallantoic membrane inoculation at 12 days with 1.9×10^4 CFU *B. abortus*

Time after inoculation (Hours)	Number of embryos	Liver (% Body Weight)	Spleen (%)
96	8	4.58	0.24
108	2	3.86	0.16
132	2	5.47	0.28
132 (control)	2	2.90	0.07

Figure 13. Fourteen day-old embryo that died 4 days after chorioallantoic membrane inoculation. Hemorrhagic area at the top of the skull (small arrow) and mottled liver (large arrow)


2 cm

characterized by diffuse squamous metaplasia of the chorionic epithelium, cellular debris and hematomas at its surface, and a sparse infiltration of heterophils in the mesodermal layers. A few B. abortus were seen inside necrotic debris.

In later stages, severe vasculitis, characterized by necrotic vascular walls with infiltrations of heterophils, was present throughout the chorioallantoic membrane. Abundant cellular exudate was diffusely present in the mesoderm. At first heterophils largely outnumbered mononuclear phagocytes, however the latter dominated the cellular exudate in later samples.

Numerous bacilli were present throughout the chorionic epithelium and in the mesoderm. Large clumps of bacteria were seen inside phagocytic cells trapped in the intraepithelial capillaries (Figure 14a). At the site of inoculation, where normal ectoderm was replaced by stratified squamous epithelium, colonies of B. abortus were found in the cellular debris and hematomas covering the membrane (Figure 14b). No organisms were present in intact ectodermal cells.

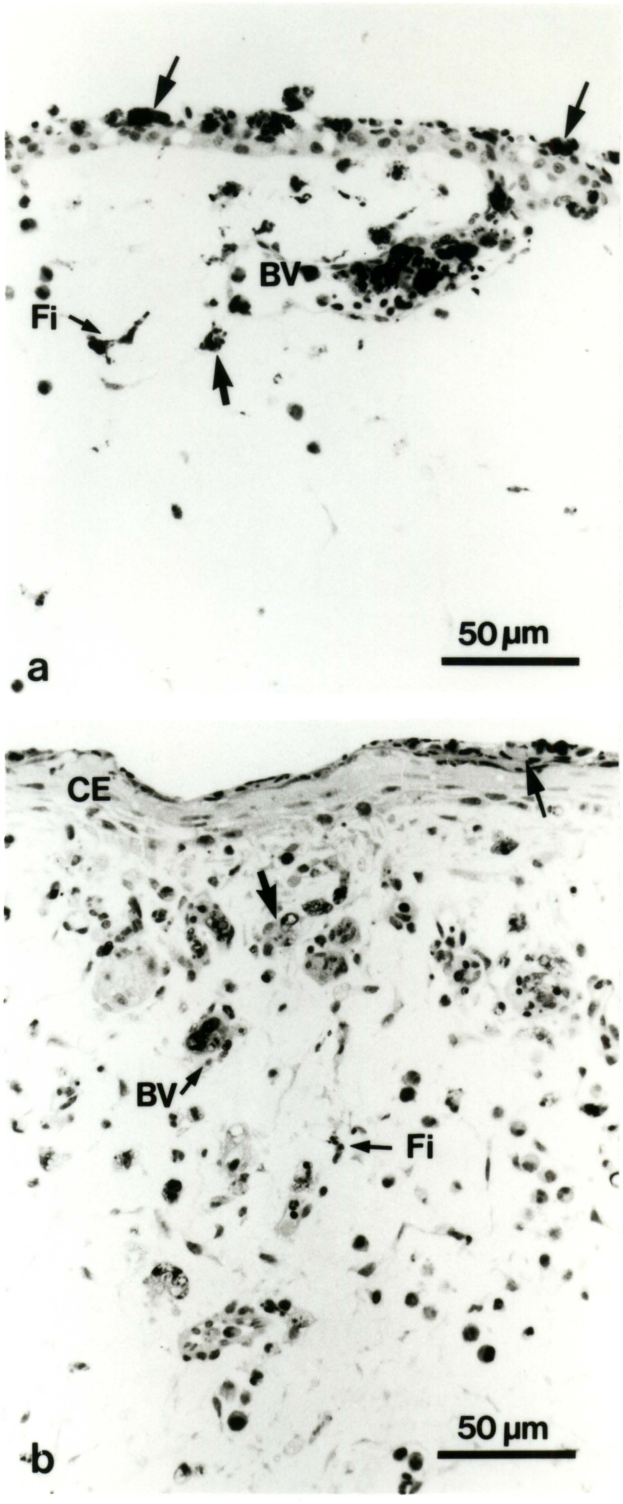
In the mesoderm bacteria were present in endothelial cells lining the capillary plexus directly underneath the ectoderm and in inflammatory cells surrounding infected vessels. Large foamy macrophages filled with B. abortus were abundant inside and around the mesodermal vessels. In severely affected areas, the bacteria were also present inside fibroblasts (Figure 14). Bacteria were seen in all blood vessels and in hemorrhagic foci surrounding necrotic vessels.

Figure 14. Chorioallantoic membrane, 5 days after chorioallantoic membrane inoculation.

a. Chorioallantoic membrane away from the inoculation site. Brucellae-filled phagocytes within chorionic epithelium capillaries (small arrows), mesodermic blood vessels (BV) and mesoderm (large arrow). B. abortus also present within fibroblasts (Fi).

b. Chorioallantoic membrane at site of inoculation. Squamous metaplasia of the chorionic epithelium (CE) and absence of intraepithelial capillaries. B. abortus within necrotic debris (small arrow) and mesodermic phagocytes (large arrow). Cellular and vascular reaction in the mesoderm is more severe than in Figure 14a.

(Immunoperoxidase-hematoxylin)


BV
Fi
50 µm
a
CE
BV
Fi
50 µm
b

Endodermal cells lining the allantoic cavity remained intact and free of B. abortus.

Dissemination of the infection to the embryo was first found at 3 days post-inoculation (Table 5). All embryonic tissues were invaded with both meso- and metanephros, liver, spleen and heart being most prominently infected.

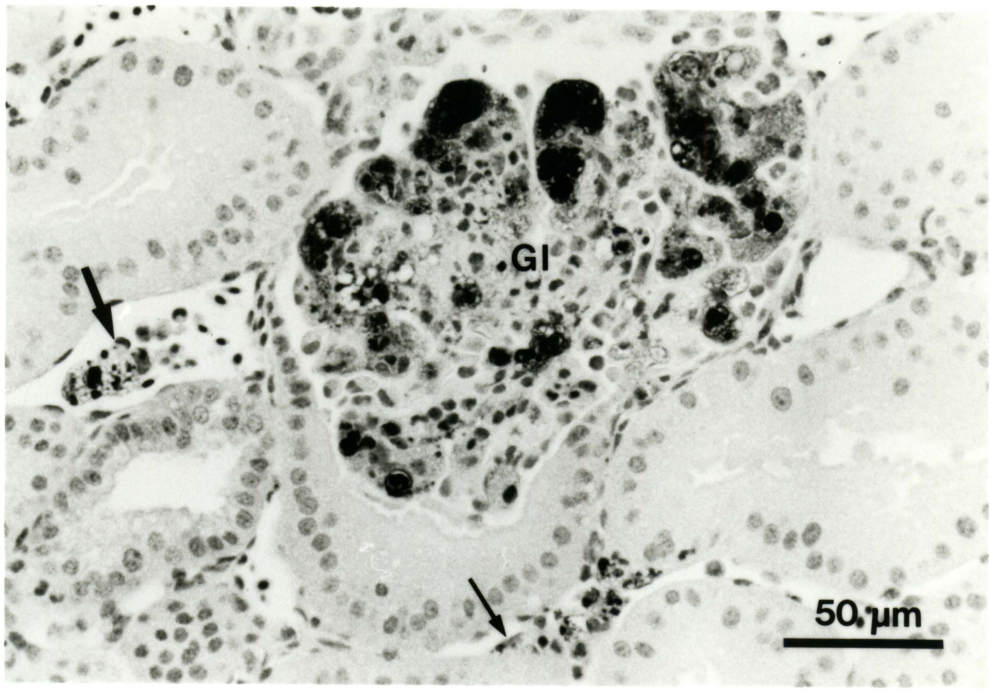
B. abortus were present in capillary endothelium, and surrounding mesenchymal cells, and in mononuclear cells throughout the liver, spleen, heart and renal glomeruli (Figures 15, 16, 17 and 18). Later, most B. abortus were present inside large foamy macrophages similar to the ones seen in the chorioallantoic membrane. These macrophages were mainly intravascular although in the liver, brucella-filled macrophages were also seen between the sinusoidal capillaries and hepatocytes (Figure 16). Free bacteria were also found in the intercellular space separating hepatocytes. Extension of the infection to hepatic parenchymal cells was seen in the two viable embryos collected 5 days after inoculation (Figure 16). In dead embryos, scattered hepatocytes filled with brucellae were found throughout the liver. Multifocal accumulations of brucella-filled foamy macrophages in areas depleted of proliferating granulopoetic cells were present in severely infected spleens (Figure 17). In the kidneys, the normal architecture of the glomeruli was masked by the phagocytic cell infiltration (Figure 15). Outside the glomeruli, these cells were also abundant in the interstitium and individual B. abortus were present in the cytoplasm of

Table 5. Tissue distribution of B. abortus after chorioallantoic membrane (CAM) inoculation in 12 day-old embryos. Data are expressed as the number of tissues in which B. abortus was identified by immunoperoxidase/number of tissue examined

Time After Inoculation (day)	Tissues with B. abortus Antigen						
	CAM						
	A	B	Liver	Spleen	Kidney	Heart	Yolk Memb.
2	3/3	0/3	0/3	0/3	0/3	0/3	0/3
3	3/3	1/3	1/3	1/3	1/3	1/3	1/3
4	3/3	3/3	3/3	3/3	3/3	3/3	3/3
5	2/2	2/2	2/2	2/2	2/2	2/2	2/2

Figure 15. Kidney, 5 days after chorioallantoic membrane inoculation. Severely infected glomerulus (Gl). B. abortus also present within interstitial phagocytes (large arrow) and peritubular mesenchymal cells (small arrow). (Immunoperoxidase-hematoxylin)

Figure 16. Liver, 5 days after chorioallantoic membrane inoculation. B. abortus within fixed and circulating macrophages (arrows) and scattered hepatocytes (He). (Immunoperoxidase-hematoxylin)


Gl
50 µm

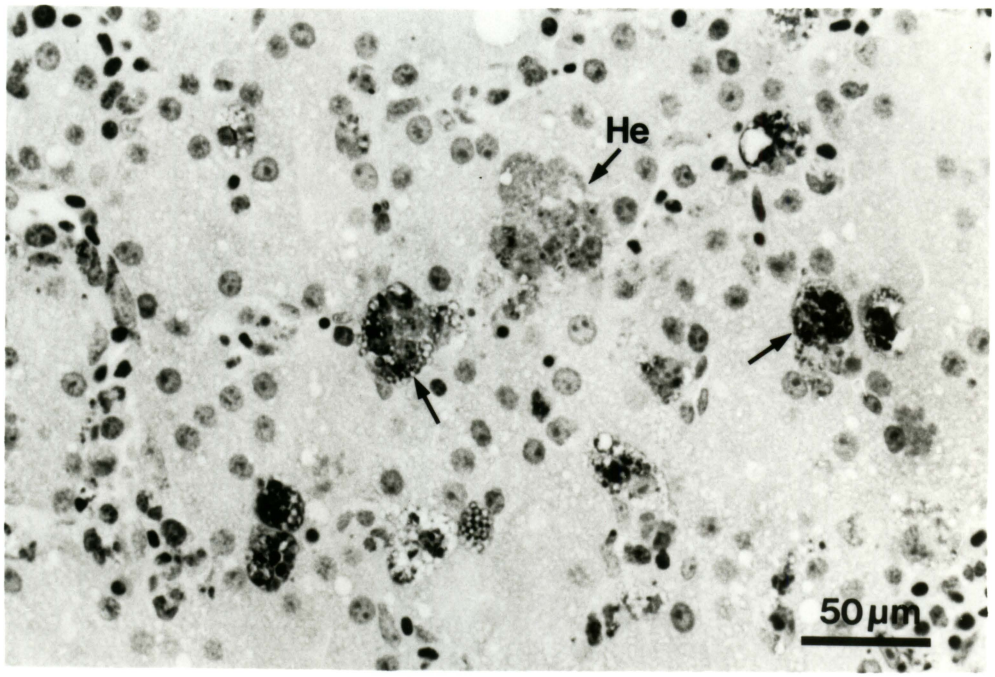
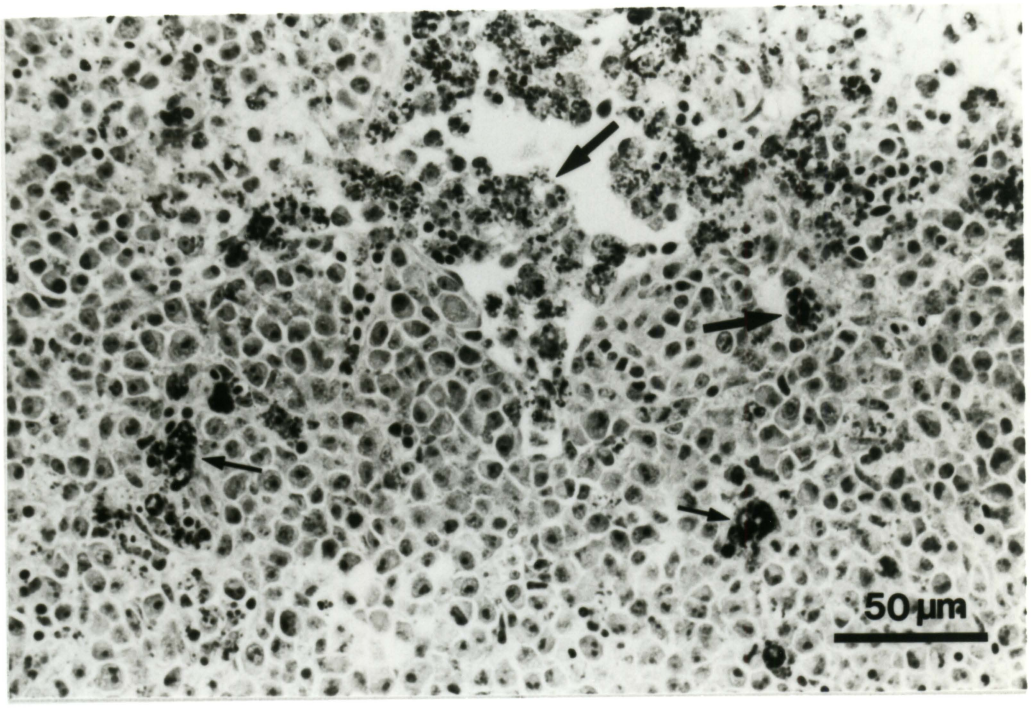
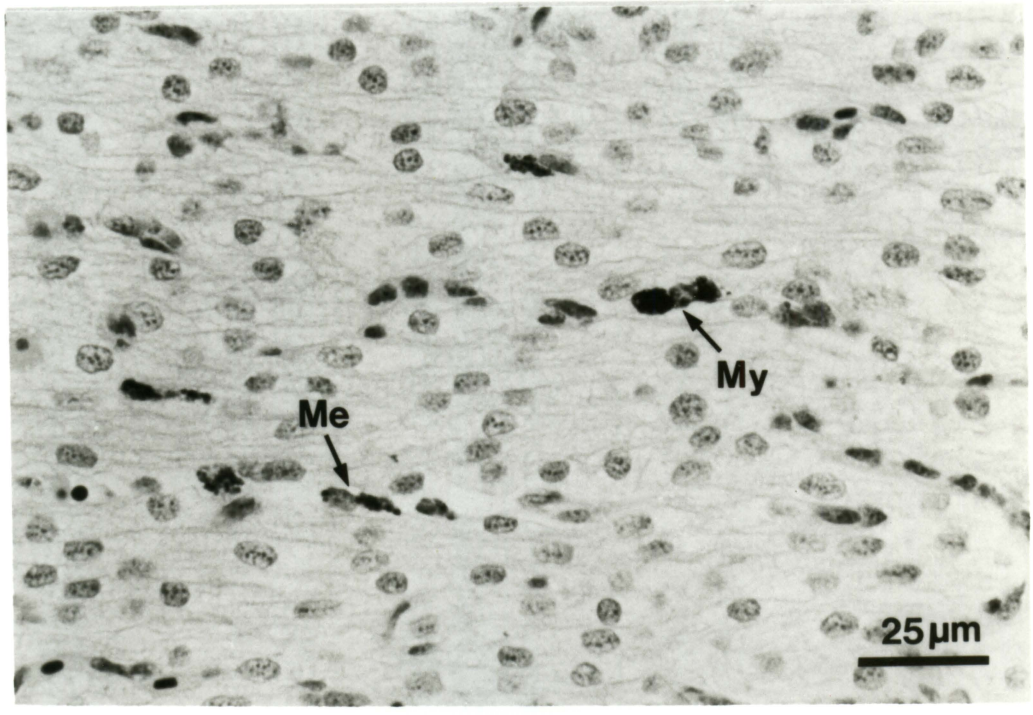

He
50 µm

Figure 17. Spleen, 5 days after chorioallantoic membrane inoculation. B. abortus within fixed (small arrows) and circulating macrophages (large arrows). (Immunoperoxidase-hematoxylin)

Figure 18. Myocardium, 5 days after chorioallantoic membrane inoculation. B. abortus within mesenchymal (Me) and myocardial cells (My). (Immunoperoxidase-hematoxylin)


50µm


My
Me
25µm

mesenchymal cells lining the nephrons. In the heart B. abortus were also found within scattered myocardial cells (Figure 18).

In the yolk membrane, B. abortus were within the subendodermal endothelium by 3 days post-inoculation. At 4 days post-inoculation, brucella-filled foamy phagocytic cells were seen in most vessels and hematopoetic islets. The organism was also seen in perivascular mesenchymal cells. By 5 days post-inoculation, B. abortus were found inside endodermal cells and bacterial colonies were present in the yolk.

Electron microscopy

In control eggs, at the site of inoculation, degenerative and inflammatory changes were present in the chorionic and mesodermal layers of the chorioallantoic membranes. Most of the chorionic epithelium was composed of variable numbers of squamous cell layers and was lacking the normally extensive superficial vascular network. Intracytoplasmic bands of tonofilaments in electron-dense flattened ectodermal cells were considered as evidence of squamous metaplasia. The chorionic surface was covered with cellular debris. Scattered necrotic and degenerative ectodermal cells and a slight heterophil infiltrate of the underlying mesoderm were present.

In brucella-infected embryos more severe changes were found at the site of inoculation. At 2 and 3 days post-inoculation, in these areas, small clusters of B. abortus were present throughout the necrotic debris covering the chorion. In addition to the squamous metaplasia

and the absence of superficial blood sinuses, there were cell degeneration (cell swelling, vacuolation, proteolysis) and necrosis (dark homogeneous cell) in the chorionic epithelium (Figure 19a). Intraepithelial infiltrations of leukocytes, mainly heterophils, were present in severely affected areas.

By 3 days post-inoculation, heterophil infiltrates in the mesoderm, directly underneath the chorionic epithelium were prominent. Leukocytes (monocytes and heterophils) were also frequently found within the lumen of blood vessels at the ectodermo-mesodermal junction. Some of the phagocytic cells contained small clusters of B. abortus inside membrane-bound vacuoles.

At 4 days post-inoculation, severe vasculitis and disruption of sub-ectodermal blood vessels were present. The mesodermal connective tissue contained with extravasated red blood cells, heterophils and necrotic cells. Several endothelial cells had pseudopodia which joined with similar projections from neighboring cells to form vascular channels. Small fibrin deposits were frequent in blood vessels. Numerous B. abortus were distributed randomly throughout the lesion. Groups of bacteria were seen within mesenchymal cells, inside large membrane-bound vacuoles filled with a lipid-rich substance morphologically similar to blood plasma. The organisms were also present within heterophils, endothelial cells or free in the lumen of blood vessels (Figures 20a and 20b).

Thrombi were frequent in deeper layers of the mesoderm. Vascular lumens were plugged with heterophils, necrotic phagocytes, red blood

Figure 19. Chorionic epithelium, 3 days (a) and 5 days (b) after chorioallantoic inoculation.

a. Membrane at site of inoculation. B. abortus within necrotic debris (small arrow). Heterophils are present in the mesoderm (large arrows).

b. Chorionic epithelium away from the site of inoculation. B. abortus (arrows) is present within a lipid-laden phagocyte located in an intraepithelial capillary.

(Glutaraldehyde fixation)

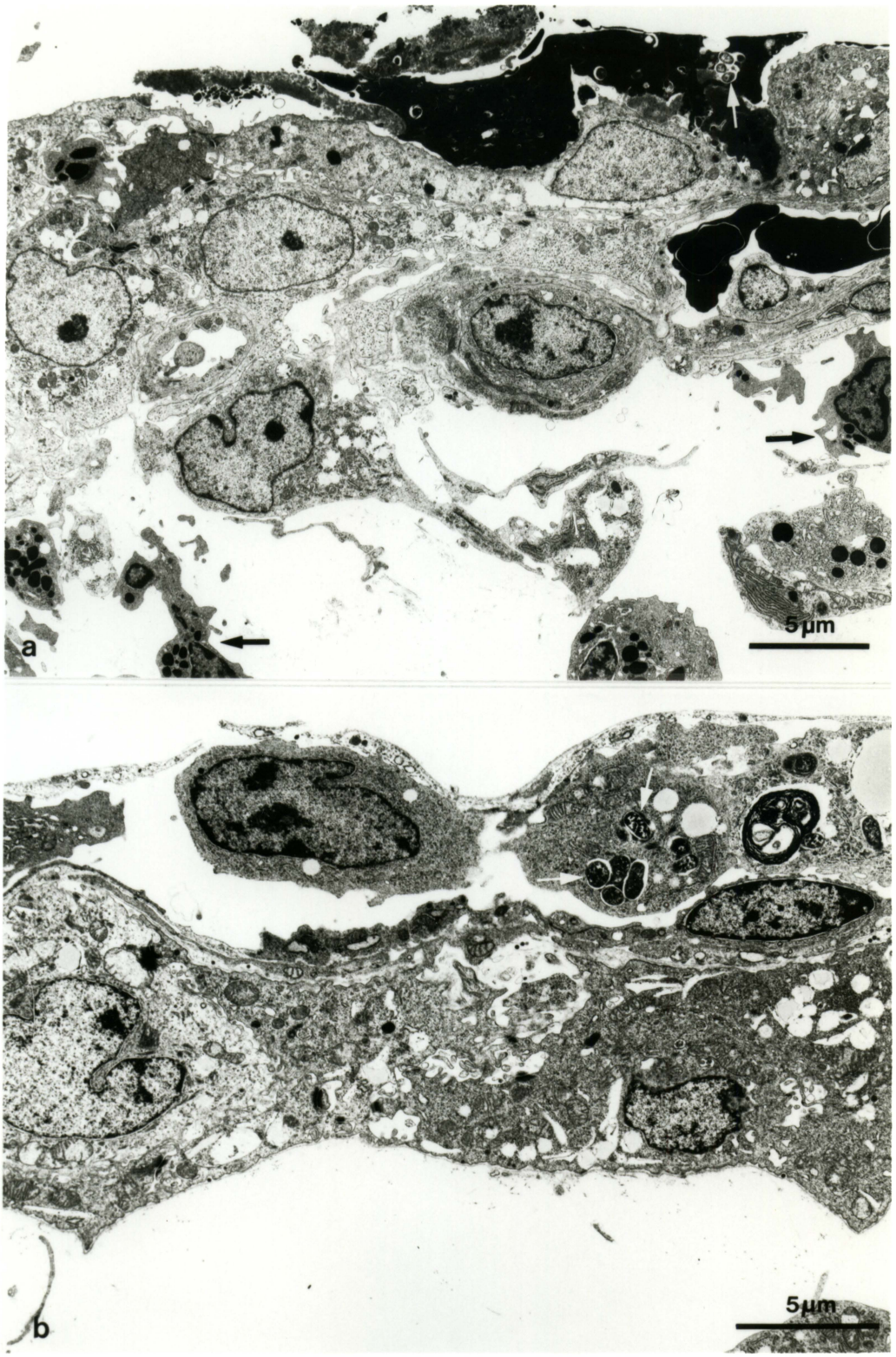

a
5µm
b
5µm

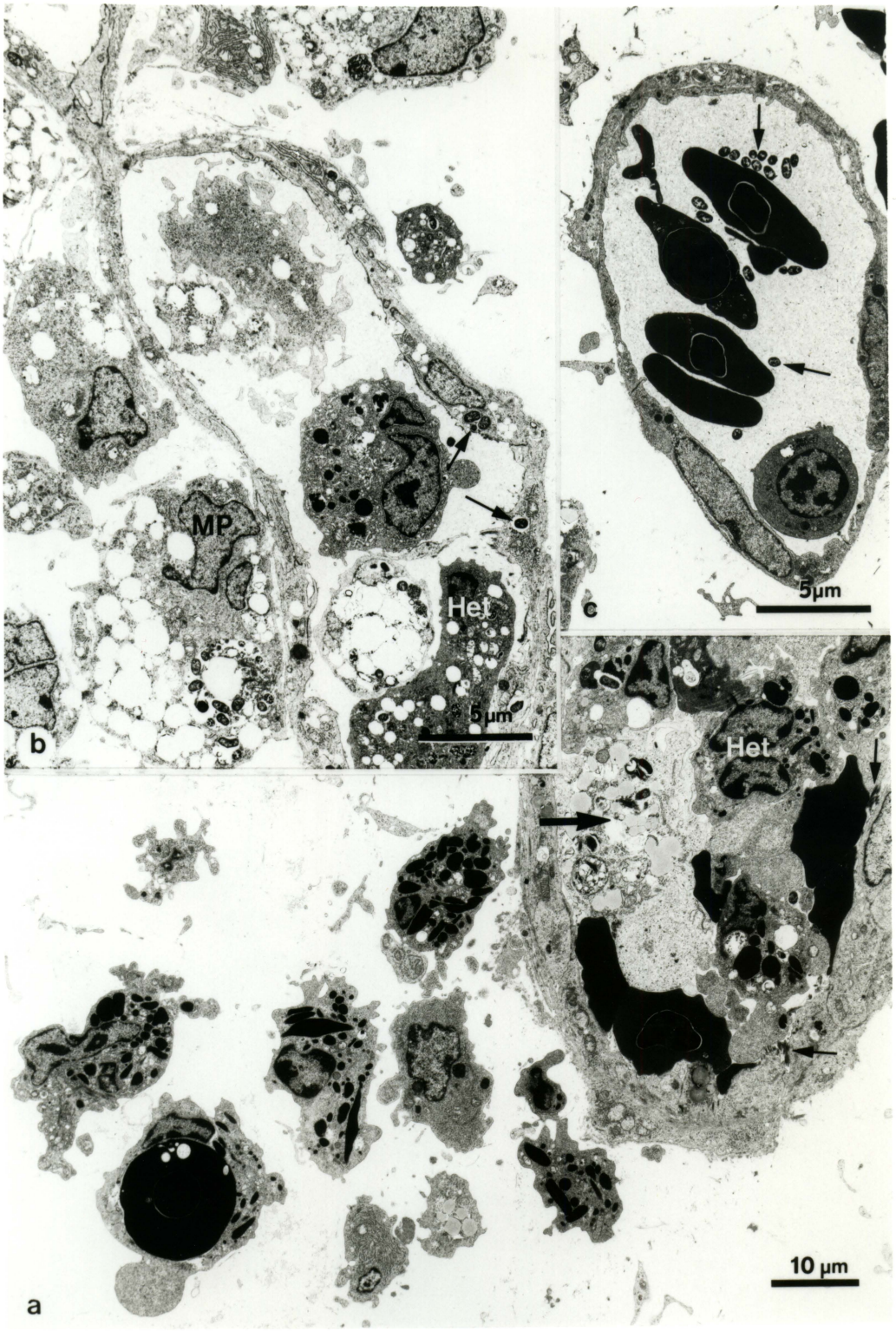
Figure 20. Mesoderm of the chorioallantoic membrane, 3 days (a) and 5 days (b,c) after chorioallantoic membrane inoculation.

a. Thrombosed vessel, the lumen is plugged with heterophils (Het), necrotic phagocytes (large arrow) and fibrin (small arrows). Heterophils are abundant around the vessel.

b. B. abortus within circulating and tissular lipid-laden mononuclear phagocytes (MP) and heterophils (Het). Isolated brucellae are found within endothelial cells (arrows).

c. Extracellular B. abortus within a mesodermic blood vessel (arrows).

(Glutaraldehyde fixation)


MP
Het
5µm
b
c
5µm
Het
10 µm
a

cells and small amounts of fibrin (Figure 20a). Extra- and intracellular bacteria were present in the thrombi. Outside areas of vasculitis and thrombosed vessels, mesodermal infection with B. abortus was sparce. In perivascular areas, a few organisms were seen in heterophils or within the rough endoplasmic reticulum of fibroblasts.

Lesions and numbers of organisms were greater at 5 days post-inoculation. Large vacuolated pleomorphic mononuclear phagocytic cells dominated the cellular infiltrate (Figure 20b). Some of these cells had a polyhedral to stellate shape, the others were round with numerous cytoplasmic projections. Their cytoplasm contained numerous spherical lipid droplets ranging from 1 to 2 μm in diameter. The remainder of the cytoplasm was occupied by an irregularly outlined eccentric nucleus, mitochondria, golgi and short segments of rough endoplasmic reticulum. Most of these cells harbored clusters of B. abortus within vacuoles which also contained electron dense amorphous materials, lipids and cell debris. The round cells had a more perivascular distribution and were frequently found in the lumen of blood vessels (Figure 20b).

With the exception of an intact chorionic epithelium and a later onset, similar changes were present throughout the chorioallantoic membrane outside the inoculation site (Figure 19b).

At 3 days post-inoculation, free bacteremic B. abortus were found in the blood vessels of different organs (Figure 20c). Intracellular localization of the organism was present in the liver and in the kidney glomeruli. In the kidneys, the lumen of several glomerular capillaries

was obliterated by large lipid-laden phagocytic cells containing small clusters of bacteria. These cells, similar to the phagocytic cells seen in the chorioallantoic membrane, were either inside the capillary lumen or between the endothelium and the basement membrane supporting the podocytes. In half the samples, individual organisms were found inside endothelial cells or free below the visceral epithelium basement membrane. In later samples, the infection had increased in severity and had spread to other areas of the kidney. Infected phagocytic cells were more numerous and contained larger numbers of bacteria. Brucellae-filled phagocytes plugged most of the glomerular capillaries (Figure 21) and infiltrated the mesangium. Mesangial and mesenchymal interstitial cells contained the organisms within rough endoplasmic reticulum cisternae. Fibrin deposits and circulating infected or intact leukocytes were in less affected capillaries. Outside glomeruli, the organisms were only found in the interstitium. The largest number of B. abortus were within lipid-loaded macrophages located around and inside blood and lymphatics capillaries (Figure 22). Organisms were also present, although in smaller numbers, within the cisternae of the rough endoplasmic reticulum of lymphatic endothelial cells (Figure 22) and fibroblasts (Figure 23). Tubular or glomerular epithelial cells remained free of organisms and were normal morphologically. In one sample, however, leukocytes containing a few brucellae inside phagolysosomes were seen within the tubular epithelium. Degenerated lipid laden infected cells were present within the lumen of a few tubules.

Figure 21. Kidney glomerulus, 5 days after chorioallantoic membrane inoculation. The normal architecture is masked by mononuclear phagocytes laden with B. abortus (Br) and lipid globules (L). Podocytes (Po) and other epithelial cells are free of brucellae. (Glutaraldehyde fixation)

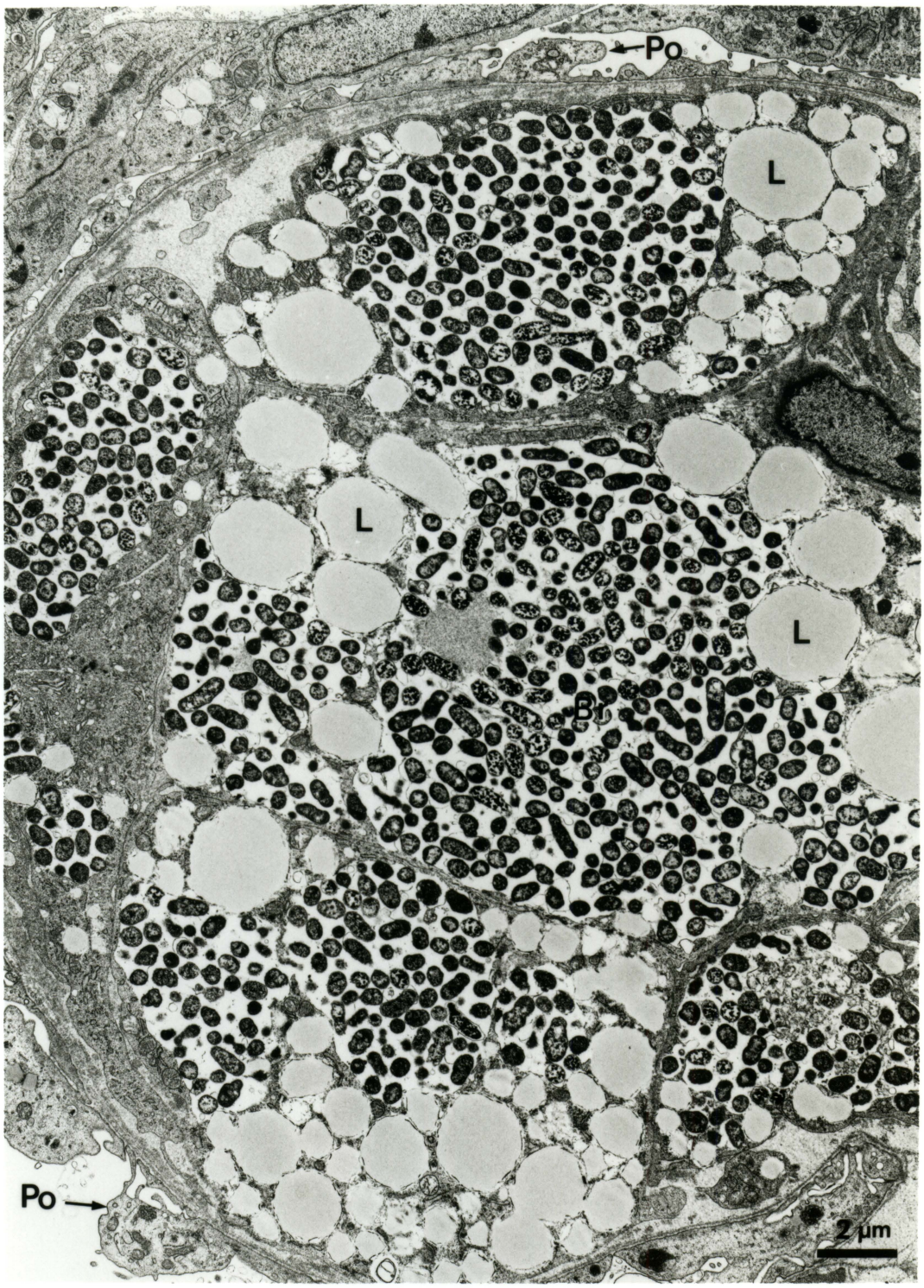

Po
L
L
L
B
Po
2 µm

Figure 22. Kidney, 5 days after chorioallantoic membrane inoculation. B. abortus within a mononuclear phagocytic cell (MC) located in the peritubular interstitium. B. abortus is also present within the rough endoplasmic reticulum of a lymphatic endothelial cell (arrows) and, as shown in the inset, the brucellae-filled cisterna is continuous with the perinuclear envelope. (Glutaraldehyde fixation)

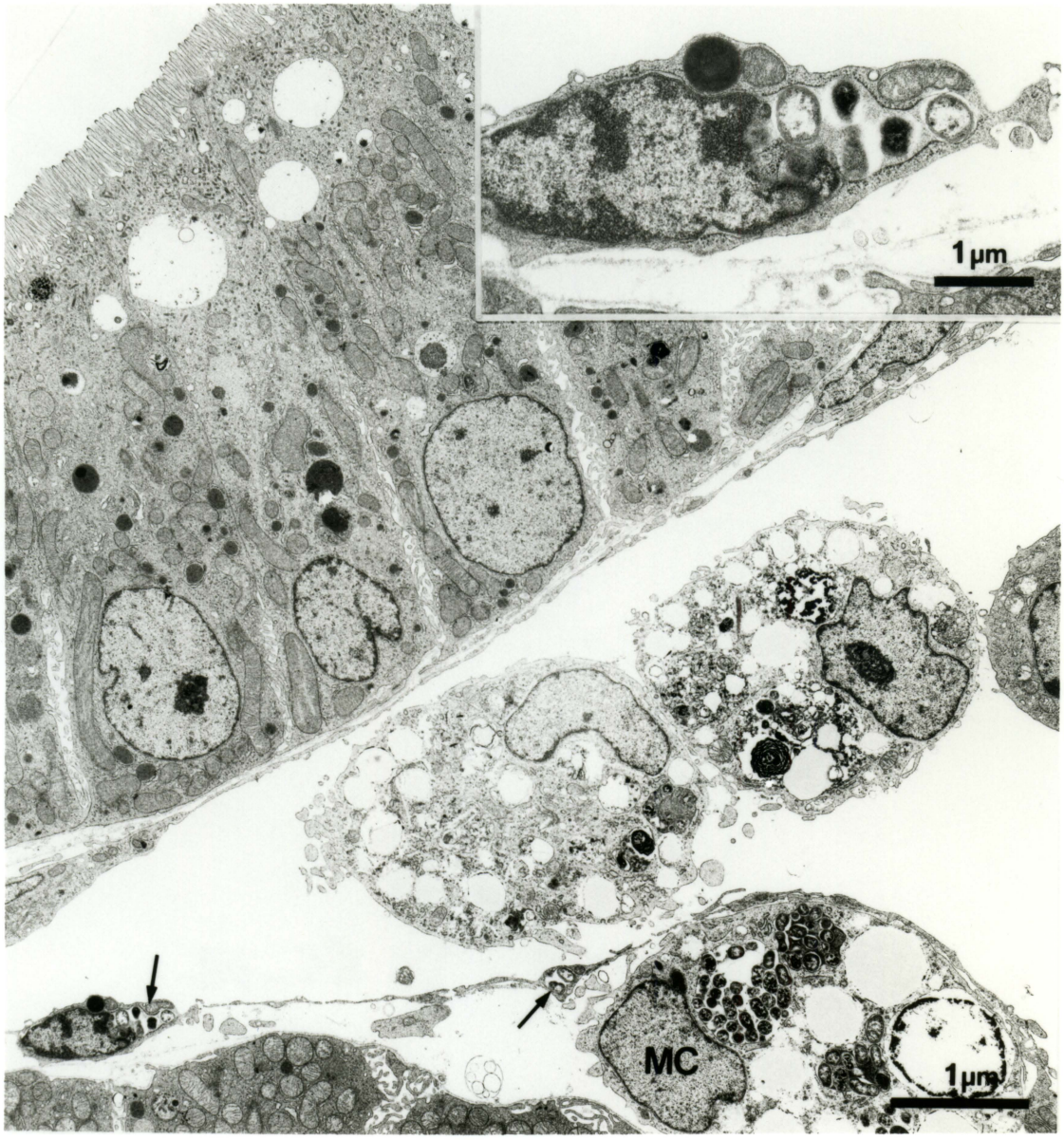
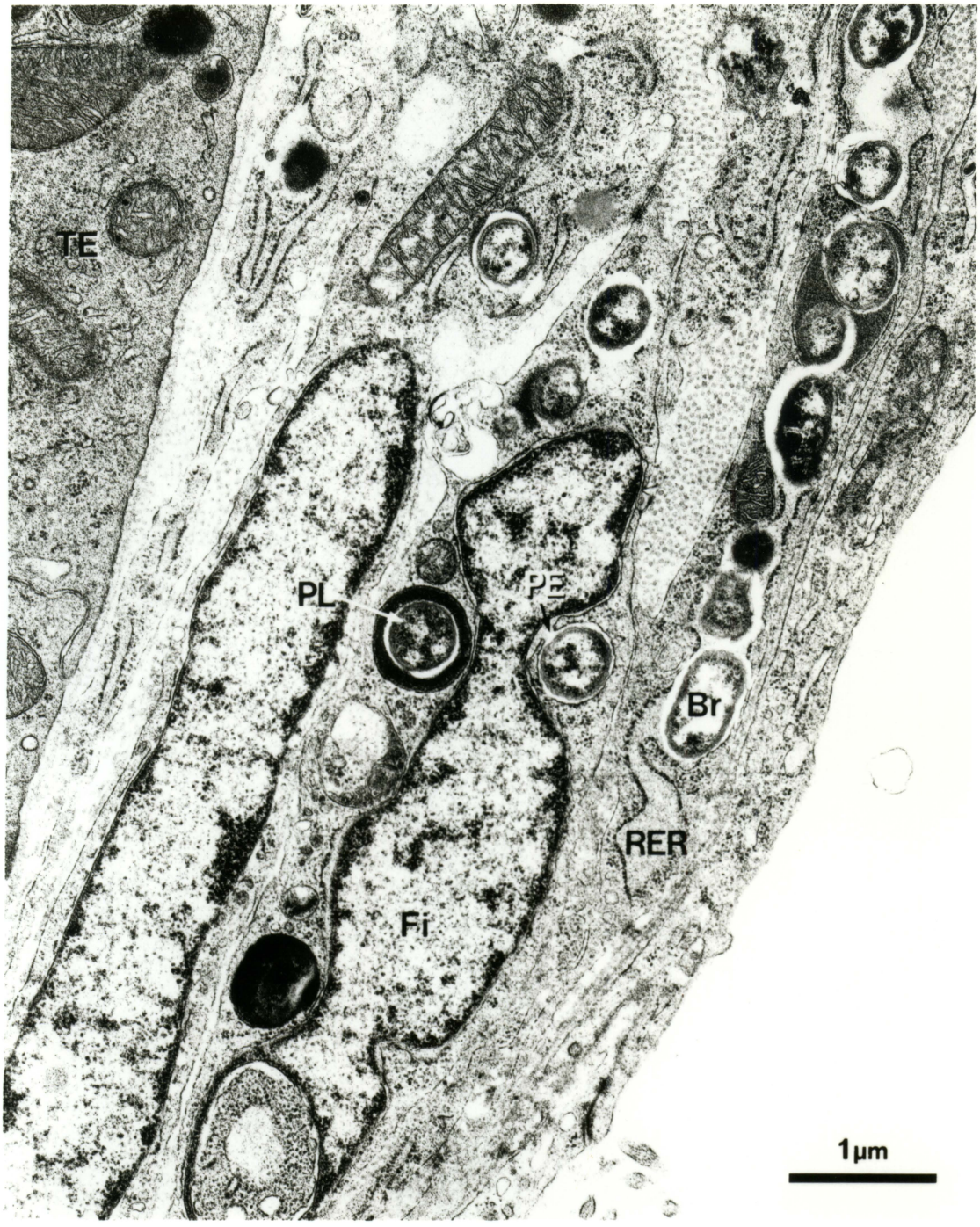

1 µm
MC
1 µm

Figure 23. Kidney, 5 days after chorioallantoic membrane inoculation. In fibroblasts (Fi) surrounding renal tubules, B. abortus (Br) are located within cisternae of rough endoplasmic reticulum (RER), perinuclear envelope (PE) and phagolysosome (PL). B. abortus are never found within tubular epithelium (TE). (Glutaraldehyde fixation)


TE
PL
PE
Br
RER
Fi
1µm

Samples taken on post-inoculation day 3 and thereafter, had intra- and extracellular B. abortus throughout the liver, either in blood or in the perivascular granulopoietic areas and spaces of Disse. Intracellular organisms were only within large lipid-laden phagocytes (Kupffer cells), in endothelial cells and within stellate mesenchymal cells forming the perivascular reticulum (Figure 24a). Individual extracellular bacteria were frequently present between hepatocytes. At 5 days post-inoculation, the liver was heavily infected, mainly with intracellular bacteria. Brucella-infected phagocytic cells still predominated, however the organisms were also abundant within perivascular mesenchymal cells and, in two embryos, hepatocytes (Figure 24b). In the hepatocytes and reticular cells, B. abortus were located within dilated cisternae of the rough endoplasmic reticulum (Figures 24c and 25). Other than the heavy cellular infiltrate, which plugged the lumen of most sinusoidal capillaries and sometimes distorted the normal liver architecture, signs of hepato-cellular degeneration were absent.

B. abortus localization in other tissues followed a similar pattern. Most bacteria were intracellular within lipid-laden phagocytic cells and inside the rough endoplasmic reticulum of interstitial mesenchymal cells. In the spleen organisms were also found within circulating granulocytes (Figures 26 and 27). However, cells of the granulopoietic islets were never infected. In the heart, the organisms were within the cisternae of the sarcoplasmic reticulum in the center of several myocardial cells (Figure 28). The outer

Figure 24. Liver, 4 days (a) and 5 days (b,c) after chorioallantoic membrane inoculation.

a. B. abortus are found within lipid-laden mononuclear cells (MC) and free (arrow) between hepatocytes.

b. Immunogold labeled B. abortus are present in rough endoplasmic reticulum of hepatocytes (He) surrounding a biliary canaliculus (BC).

c. B. abortus (Br) within dilated cisternae of hepatocytes rough endoplasmic reticulum. Biliary canaliculus (BC).

(Glutaraldehyde fixation)

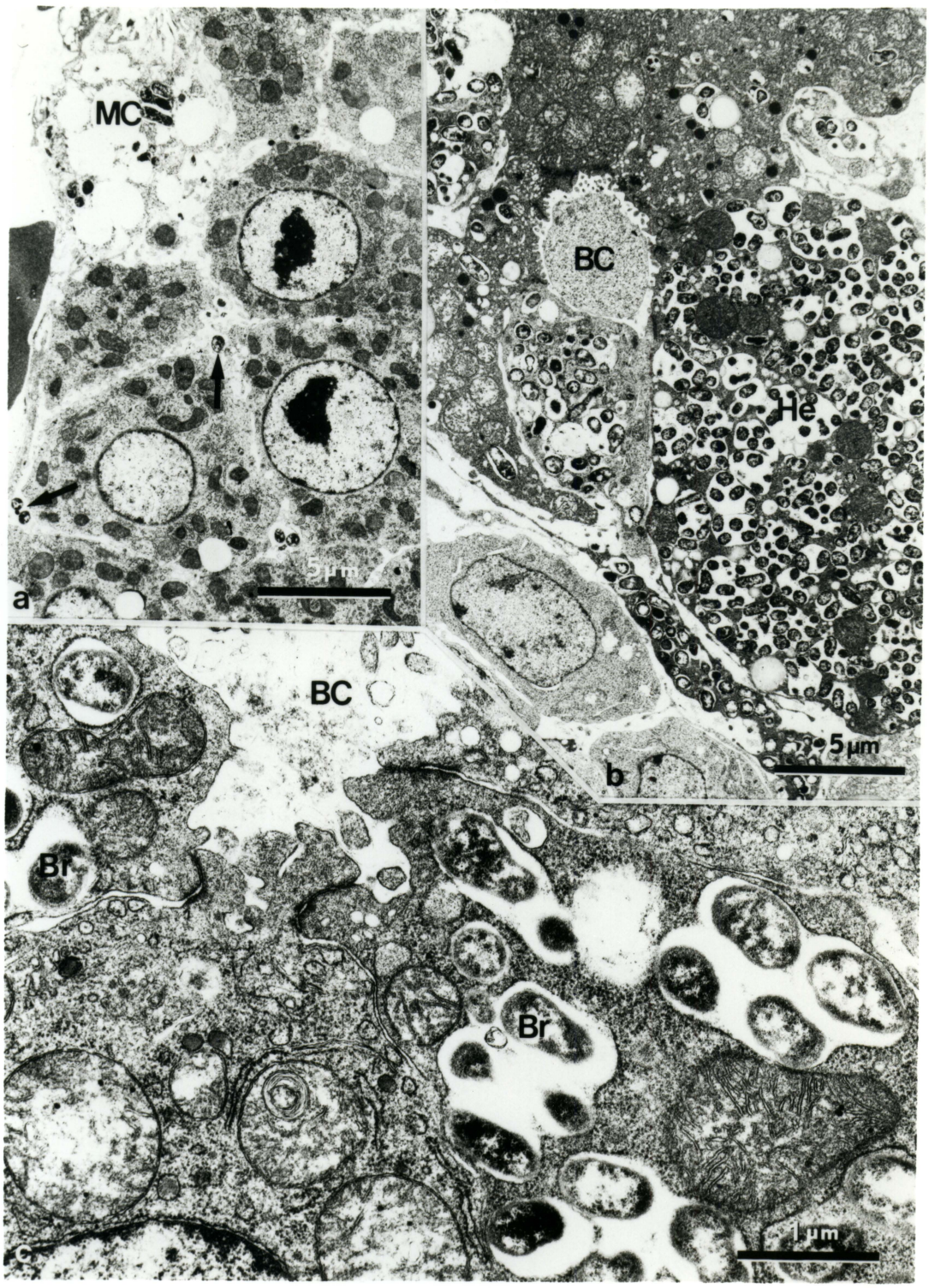

MC
5µm
a
BC
He
5µm
b
BC
Br
Br
1µm

Figure 25. Liver, 5 days after chorioallantoic membrane inoculation. B. abortus are present within a perivascular mononuclear phagocyte (MC) and in the rough endoplasmic reticulum of an interstitial mesenchymal cell (arrow). Circulating granulocytes (Gr) are present in the hepatic sinusoids (HS). (Glutaraldehyde fixation)

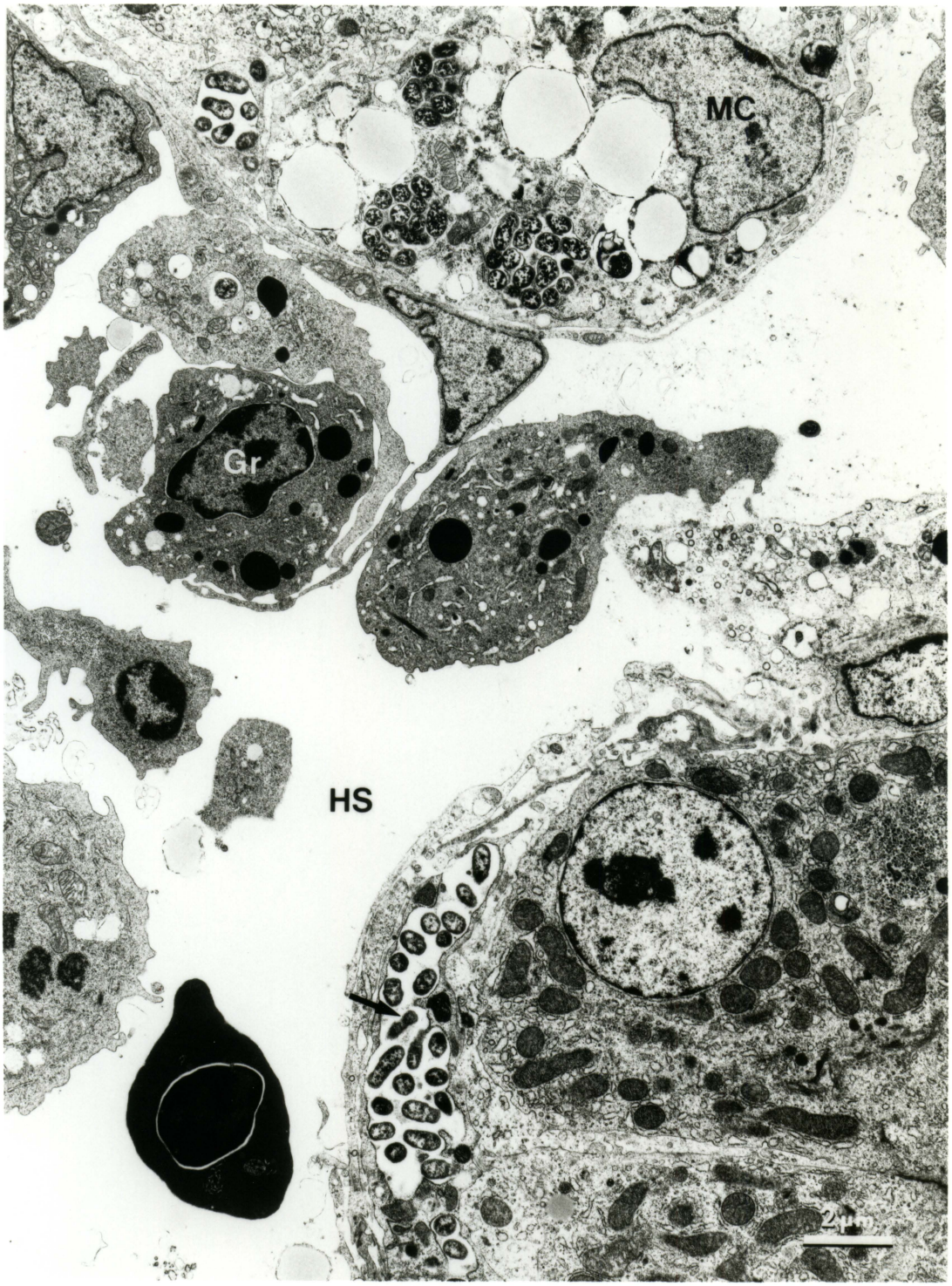

MC
Gr
HS
2μm

Figure 26. Spleen, 5 days after chorioallantoic membrane inoculation. B. abortus are found within phagolysosomes in fixed mononuclear cells (MC) and circulating granulocytes (Gr). The inset shows numerous B. abortus within cisternae of rough endoplasmic reticulum of a splenic reticular cell (RC). (Glutaraldehyde fixation)

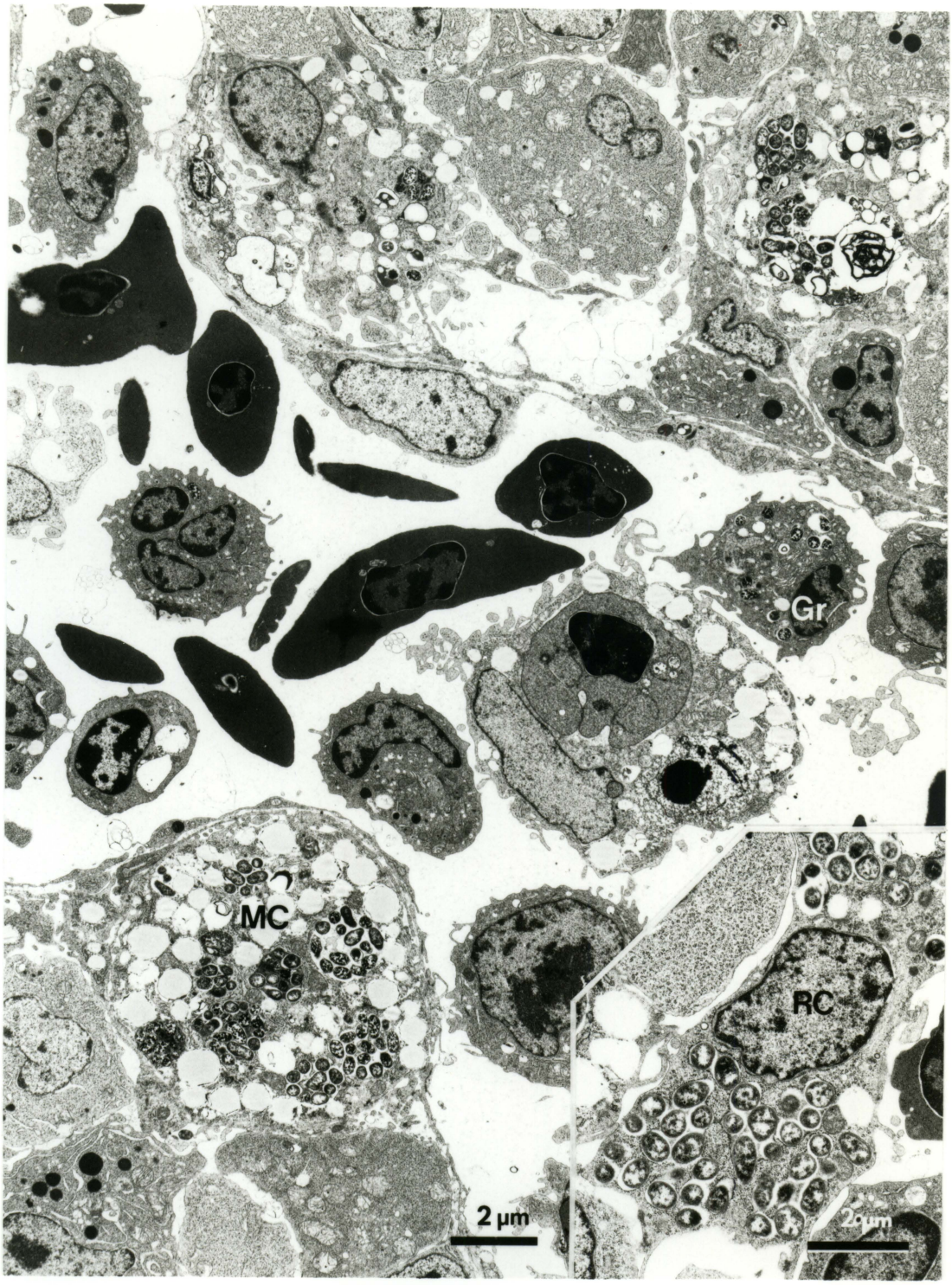

Gr
MC
RC
2 µm
2µm

Figure 27. Spleen, 5 days after chorioallantoic membrane inoculation. Splenic reticular cell (RC) contains numerous B. abortus within cisternae of rough endoplasmic reticulum (large arrow). Clusters of B. abortus are within phagolysosomes in intraluminal (Mo) and tissue mononuclear cells (MC). Isolated brucellae are frequently seen within endothelial cells (small arrow). (Glutaraldehyde fixation)

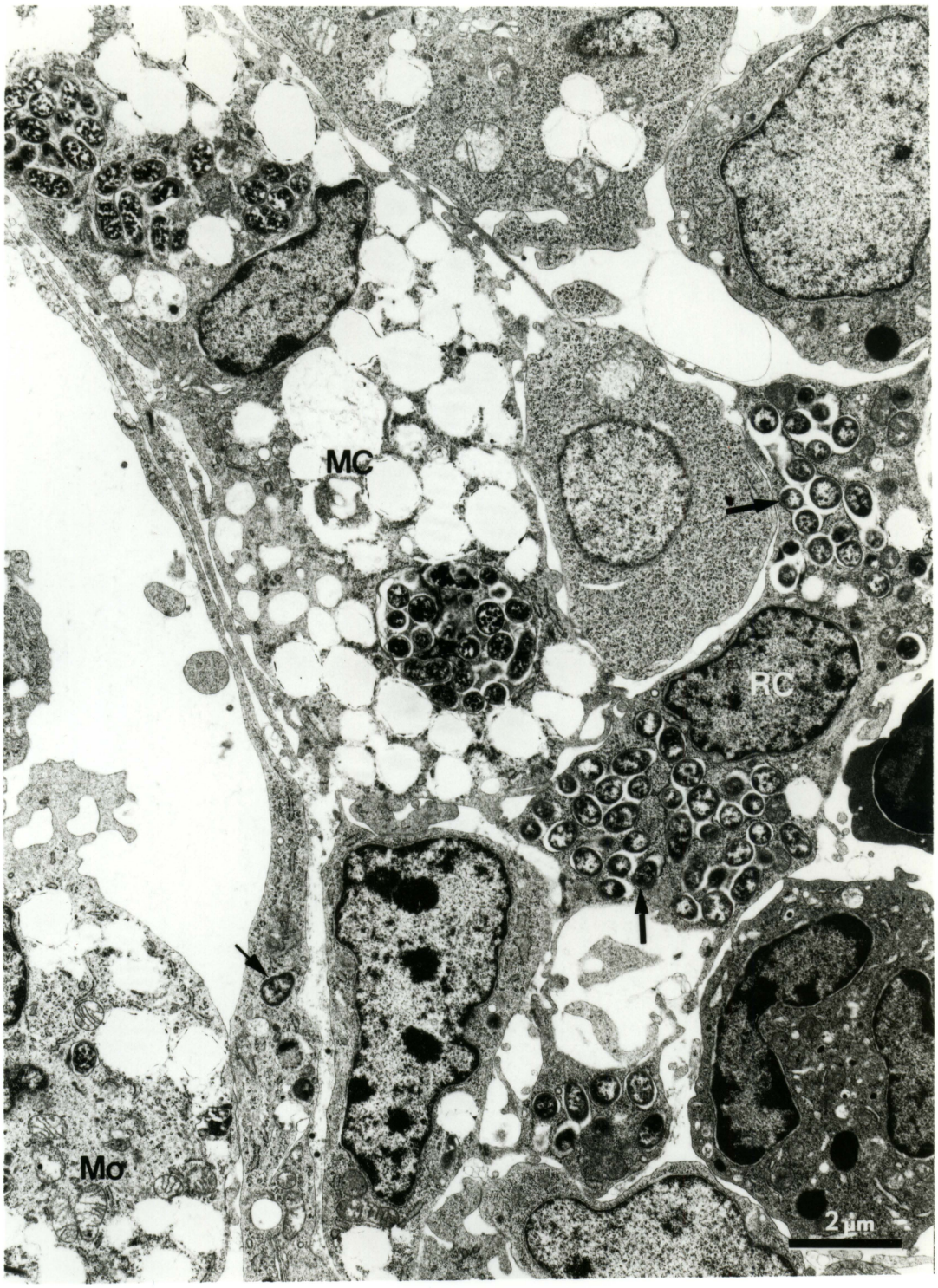
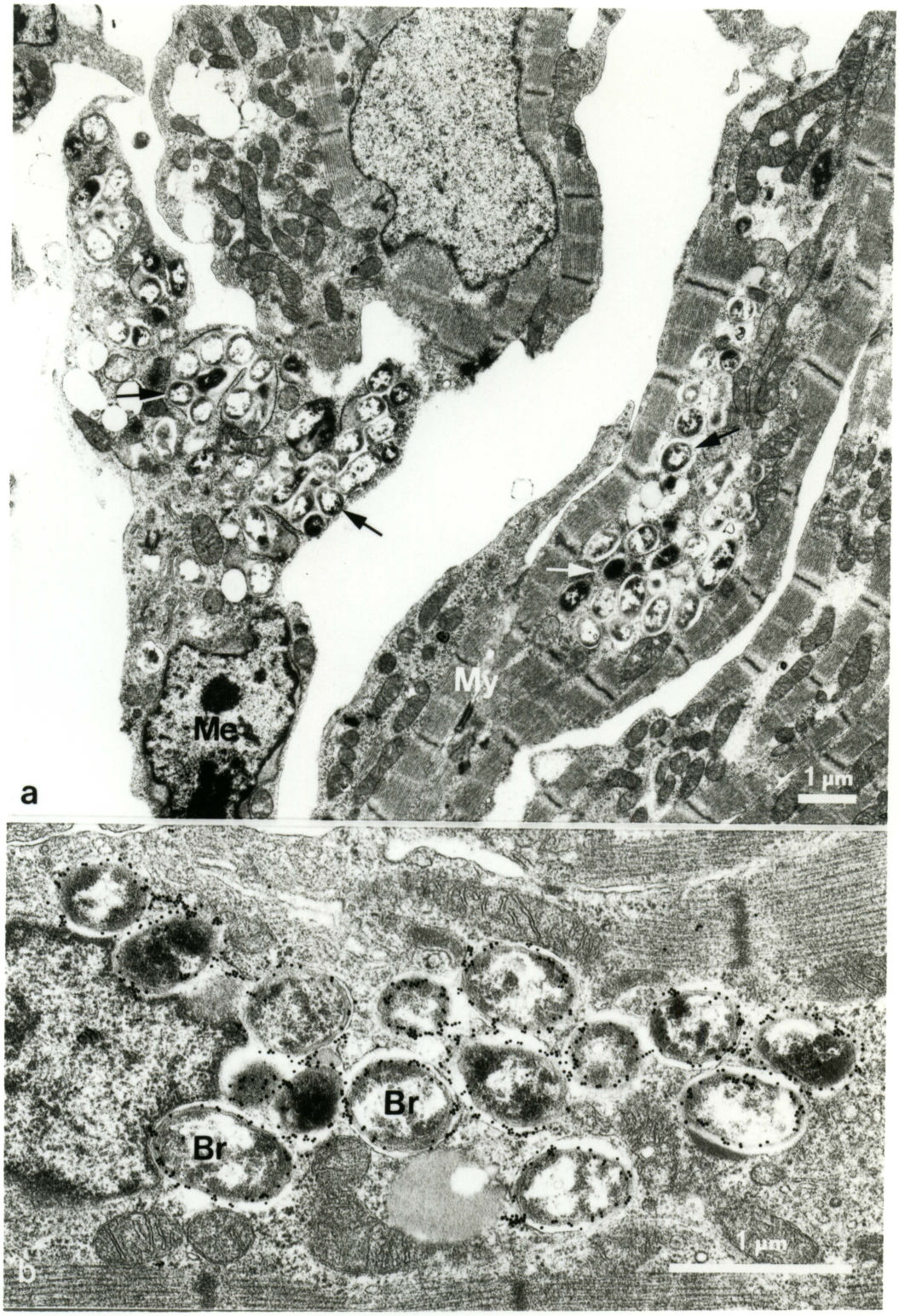

MC
RC
Mσ
2 µm

Figure 28. Myocardium, 5 days after chorioallantoic membrane inoculation.

a. B. abortus (arrows) are located within a myocardial cell (My) and within the rough endoplasmic reticulum of an interstitial mesenchymal cell (Me).

b. Immunogold labeled brucellae (Br) in perinuclear cytoplasmic cisternae of a myocyte.

(Glutaraldehyde fixation)


Me
My
1 µm
a
Br
Br
1 µm
b

membrane of the brucellae-filled cisternae was not lined by ribosomes, but was continuous with the outer membrane of the perinuclear envelope (Figure 28).

Experiment III: Yolk sac, Chorioallantoic and Intravenous Inoculations of 10 day-old Embryos

The progression of the infection after yolk sac injection in 10 day-old embryos differed from that in 6 day-old embryos. The filling of vitelline endodermal cells with intracellular B. abortus was more diffuse with almost all cells throughout the membrane infected. Organisms were not found at the vascular side of the endoderm before 3 or 4 days post-inoculation. At this time, intracellular brucellae were present within the vitelline endothelium and within cells of the serosal and subendodermal mesoderm. Extracellular bacteria and brucellae-filled foamy phagocytic cells were found within the vitelline blood vessels and hematopoietic islets. No infected cells or free bacteria were ever found in the extraembryonic coelom. Progression of the infection to the other embryonic tissues was present at 4 days post-inoculation and followed a similar pattern as in embryos inoculated on the chorioallantoic membrane.

Gross and histopathological findings in embryos inoculated on the chorioallantoic membrane at 10 days of age were indistinguishable from those in embryos inoculated at 12 days by the same route.

Average time of death after intravenous inoculation was 67 hours. Only 3 embryos were alive when sampled. At 48 hours post-inoculation, intravascular brucellae were found throughout all embryonic tissues, with the kidney glomeruli being the most heavily infected. The organisms were within endothelial cells or free in the blood. In later samples the number of brucellae were markedly increased. All tissues were infiltrated with numerous intra- and extracellular organisms. Localization of bacteria was similar to that seen in dead embryos sampled after chorio-allantoic inoculations at 10 or 12 days of age or yolk sac injection at 10 days. However, inflammatory cell infiltrates, mainly in the chorioallantoic membrane, were less prominent.

DISCUSSION

This study indicates that bacteremic B. abortus spread throughout all avian embryonic tissues and localized mostly within cells of mesodermal derivation. Invasion of internal organs, after CAM inoculation, was not seen in two previous studies,[54,75] although in one the organism was recovered from cardiac blood.[75]

B. abortus is selective for cells of mesodermal derivation, especially mononuclear phagocytes, fibroblasts and endothelial cells.[21] In our study, B. abortus was never seen within cells of ectodermal and, other than vitelline epithelium, of endodermal origin. This differs markedly from most previous studies which found B. abortus within chorionic ectodermal cells.[21,54,63,75] B. abortus proliferation within cells of the three germ layers, including kidney tubular epithelium and chorionic endoderm was described following yolk sac inoculation of 10 day-old embryos.[63] We did not find bacteria in these two locations; however, numerous organisms were present within yolk endoderm and kidney glomeruli which were reported as remaining free of bacteria.[63]

Mononuclear phagocytic cells of the reticulo-endothelial system played a major role in the multiplication and spread of B. abortus in embryonic tissues. Phagocytic activity of this system begins early in chick embryonic life[97] and different embryonic tissues have various phagocytic patterns throughout chick development.[15,66,69,86] The mesodermal layer of the yolk sac wall, the areolar connective tissue of the mesenteries and body wall and the kidney glomeruli play a dominant

role in uptake of colloidal materials in 3 to 9 days old embryos.[69] Later, their importance diminishes as liver and spleen become the principal phagocytic sites.[15,66,69,86] We found a similar distribution with B. abortus, e.g., embryos inoculated at 6 days had brucellae-filled macrophages chiefly in the yolk sac wall mesoderm and kidney glomeruli, while embryos injected at 10 and 12 days had infected macrophages mainly within kidney glomerular, hepatic and splenic capillaries.

The numerous brucellae present within cells of the mononuclear phagocytic system indicates the failure of embryonic phagocytes to destroy B. abortus and to inhibit its intracellular replication. Degenerative phagocytes containing intact brucellae were found in all infected tissues. Depending on the bacterial species, marked differences exist in the bactericidal capacity of chick embryonic phagocytes.[135] After chorioallantoic inoculation in 12-15 day chicken embryos, Staphylococcus aureus is rapidly phagocytized and destroyed by both polymorphonuclear and mononuclear leukocytes.[54] Despite rapid uptake, macrophages of chicken embryos are unable to destroy a rough strain of Escherichia coli until late in chicken embryonic life.[67]

Morphological evidence indicates that the mechanism of intracellular replication of B. abortus differs from that of other intracellular bacteria. We found B. abortus within the rough endoplasmic reticulum of mesenchymal, mesothelial, yolk endodermal and hepatic parenchymal cells. Similar localization has been reported within caprine chorioallantoic trophoblasts.[3,4] Legionella pneumophila

has a similar intracellular localization within macrophages and monocytes of several species.[8,50,58,68] However, in vitro studies with human blood monocytes[58] and the amoeba Naegleria fowleri,[96] suggest that L. pneumophila actually inhabits ribosome-studded cisternae. Phagocytized legionellae are first enclosed in vacuoles which are encircled by mitochondria and subsequently lined by ribosomes. In chicken embryonic cells, as in caprine trophoblasts,[3,4] brucellae-filled cisternae were continuous with normal rough endoplasmic reticulum and with perinuclear envelopes. Also, B. abortus were occasionally present within cisternae of the perinuclear envelope. We consider these morphological criteria as evidence of B. abortus localization within rough endoplasmic reticulum cisternae rather than within ribosome-lined phagosomes.

Penetration and replication of B. abortus within vitelline endodermal cells is probably the initial event following yolk sac inoculation of the chick embryo. Entry or replication of bacteria within epithelium, the "epithelial phase" of intracellular bacterial parasitism,[108] has been reported primarily for organisms entering the intestinal epithelium, e.g., Listeria monocytogenes,[108] Chlamydia psittaci[35] and Shigella dysenteriae.[48] Accordingly, it has been suggested that penetration and intracellular replication of B. abortus within ruminant's chorioallantoic trophoblasts represent an "epithelial phase" of brucellosis.[4] Similar events occurred in chick embryos inoculated via the yolk sac.

For entry into cells, B. abortus depends more upon the normal high phagocytic activity of the host cell than on an active bacteria-induced mechanism of endocytosis. This idea is suggested by the observation that intracellular brucellae are mostly found within cells which principal function involves phagocytosis, e.g., cells of the mononuclear phagocytic system and, in pregnant ruminants, erythrophagocytic and chorioallantoic trophoblasts.[4,5,16,22,31,89,127] Our observations in chick embryos support this idea. Yolk endodermal cells are also phagocytic.[88] Endothelial, mesothelial and undifferentiated mesenchymal cells, especially in younger embryos, are involved in the uptake of particulate material injected systemically.[86,110] However, presence of B. abortus within fibroblasts, hepatocytes and myocardial cells may represent bacteria-induced endocytosis.

Yolk endodermal cells remained intact despite massive intracellular replication of B. abortus and I believe that bacteria are released into the subendodermal mesoderm by exocytosis at the vascular pole of the host cell. Intracellular bacteria may leave the host cells in different ways.[94,108] For example, infected cells may be destroyed by the extensive bacterial proliferation or intact host cells may continuously shed infectious progeny.[94] In ruminant brucellosis, intracellular replication of B. abortus causes trophoblast necrosis allowing access of brucellae-filled exudate to the fetal circulation.[4] Similar events were observed in most infected cells of the chick embryo.

To gain access to the rough endoplasmic reticulum, B. abortus probably depends on a specific receptor mediated vesicular transport. The transport of macromolecules and particles between various organelles of eucaryotic cells involves the sequential formation and fusion of membrane-bounded vesicles.[1,93] Membrane receptors are believed to play a major role in the regulation of intracellular traffic.[1,93] In some yolk endodermal cells membrane-bound structures compatible with phagosomes, were found below the vitelline surface. These brucellae-containing phagosomes were also described in recently infected tropholasts.[3] When latex spheres, a particulate tracer, are injected beneath the endoderm of chicken embryo's yolk sac, they are transported via bristle-coated pits and vesicles into pleomorphic intracellular yolk drops.[88] This suggests that brucellae-containing vesicles bear different membrane signals in order to transfer B. abortus to the rough endolplasmic reticulum rather than to intracellular yolk drops. Because B. abortus are found within the rough endoplasmic reticulum of cells of different origin and function, I believe that the recognition signal is bacteria-induced.

Localization within the rough endoplasmic reticulum may provide B. abortus a basis to escape intracellular digestion and a favorable environment enhancing bacterial growth. Inhibition of phagolysosome fusion is one of the mechanisms used by intracellular parasites, including B. abortus[44,134] and Legionella pneumophila,[59] to resist intraleukocytic killing.[33] In absorbing epithelia, macromolecules can be transported unaltered across cells, bypassing lysosomes and thereby

avoiding intralysosomal digestion.[38] Antibodies and serum proteins follow a similar intracellular pathway in yolk endodermal cells of the rabbit[137] and guinea-pig[72,73] and in primate trophoblasts.[139] Rough endoplasmic reticulum may contain factors which enhance the replication of B. abortus. However, in chicken embryos the most extensive replication of B. abortus occurred within mononuclear phagocytes inside membrane-bound vacuoles not lined by ribosomes. Multiple factors are probably involved in the intracellular enhancement of B. abortus replication. Intracellular replication of B. abortus within phagocytic cells is documented,[18,43,76,111,134] ultrastructural studies indicate that it takes place within phagosomes.[65,111,134]

As the chicken embryo develops, survival time following B. abortus inoculation increases. Between the sixth and tenth day of incubation, a two day increase in average survival time was seen in yolk sac inoculated embryos. Unlike Kamal,[62] I believe that this increase in resistance is age-related rather than the result of rapid increase in embryonic volume. Immediately after hatching, newborn chicks become resistant to experimental inoculation with B. abortus and are able to clear the organism in a few weeks.[39,131] As suggested by studies with Salmonella gallinarum, improved bactericidal capabilities of embryonic phagocytes may explain this increase in resistance.[67] The appearance as embryonic development proceeds, of non-specific serum factors like complement,[136] lysozyme,[15] and transferrin[64,138] may participate in these improved bactericidal capabilities. The high lipidemia, existing during embryonic and perinatal life,[114] may have blockading effects on

the reticulo-endothelial system.[15] The age related increased cellular resistance of the chicken embryo to certain viral infections has been related to the higher responsivness of older embryonic cells to interferon.[91,92] Endotoxin, including brucella endotoxins, are excellent interferon inducers[133,141,142] and gamma-interferon plays a major role in phagocytic cells activation.[107]

In addition to the increased efficacy of the mononuclear phagocytic system, susceptibility to endotoxin may be involved in the age-related resistance of chicken embryos to bacterial infections. During embryonic development, chicken embryo susceptibility to the lethal effect of endotoxin changes from exquisite sensitivity to marked refractoriness.[40,126] With enterobacterial endotoxins a 10,000-fold increase in resistance has been demonstrated between the eleventh and fifteenth day of incubation.[40] Aqueous ether extracts of B. abortus strain 19 are lethal for 10 days embryos.[6] However, in comparison with E. coli endotoxins, B. abortus endotoxins are relatively innocuous for chicken embryos.[78]

In chicken embryos, dissemination of B. abortus infection occurs mainly via the bloodstream. Bacteremic brucellae, free or within degenerate phagocytes, were seen in all inoculated embryos. Intracellular replication of B. abortus and B. suis within endothelial cells leading to the dissemination of the infection by way of the blood circulation has been reported in chicken embryos.[21] Our observations suggest that transendothelial transport is possible, as isolated

B. abortus were seen within and on both sides of endothelial cells. However, intraendothelial replication of B. abortus was never seen. Migration of brucella-filled phagocytes probably played an important role in the transfer of the organism between embryonic tissues and blood stream, as peri- and intravascular phagocytes were abundant in infected areas.

In chicken embryos inoculated via the yolk sac on the sixth day of incubation, in addition to bacteremia, spreading of B. abortus along serosal surfaces is important in the dissemination of the infection. As suggested in caprine chorionic trophoblasts infection,[3,4] chicken embryo serosa could be infected by phagocytosis of free brucellae or cytoplasmic fragments containing brucellae, which have been released into the coelomic cavity after rupture of adjacent necrotic mesothelial cells. In the yolk stalk region, the mesothelial lining of the extraembryonic coelomic cavity is continuous with the intraembryonic serosal surfaces.[102,113] Necrosis of infected mesenchymal cells and phagocytes of the yolk sac wall probably initiated the coelomic infection. However, the possibility that the embryonic serosal infection resulted from the bacteremic dissemination of the organism cannot be excluded. Furthermore, cell-to-cell lateral transfer of B. abortus within the mesothelial epithelium is plausible, as organisms were frequently found within intact mesothelial cells without bacteria in the surrounding coelom.

SUMMARY AND CONCLUSIONS

Embryonated chicken eggs were inoculated with B. abortus (strain 19) to determine their suitability as a model of study for brucellosis cytopathology. The study was divided into three experiments. First, 6 day-old embryos were inoculated via the yolk sac. In the second experiment 12 day-old embryos were inoculated on the chorioallantoic membrane. In the third experiment, 10 day-old embryos were inoculated using the yolk sac, chorioallantoic and intravenous routes. Saline inoculated eggs served as control. Beginning on post-inoculation day 2, living and dead embryos were sampled at regular intervals until the last viable embryo was collected. Samples for light and electron microscopy included yolk, amniotic and chorioallantoic membranes, yolk stalk, liver, spleen, kidney, lung and heart. Only samples from embryos still viable when collected were processed for electron microscopy. B. abortus was labeled by avidin-biotin immunoperoxidase and immunogold techniques. All eggs were examined bacteriologically.

B. abortus grew in all inoculated eggs. Death occurred within 2 and 5 days post-inoculation, depending on age of the embryo and route of inoculation. The shortest survival times were observed in embryos inoculated in the yolk sac when 6 day-old and in embryos infected intravenously on the 10th day of incubation. Organ distribution and degree of bacterial replication varied with age of the embryo at time of inoculation. In the youngest embryos, B. abortus localized preferentially in the endoderm and mesoderm of the yolk sac wall, in

the extra- and intraembryonic serosal epithelia and in the glomeruli of the mesonephros. In older embryos, inoculated on the 10th or 12th day of incubation, B. abortus spread to all tissues. The kidney glomeruli, liver, spleen and heart were the most severely infected organs. Intracellular B. abortus were present within the rough endoplasmic reticulum of mesenchymal, mesothelial, yolk endodermal and hepatic cells. In other target cells, including mononuclear phagocytes, endothelial cells and granulocytes, bacteria were located within membrane-bound vacuoles.

The results of this study indicate that, in chicken embryos, B. abortus localizes preferentially within cells of mesodermal derivation and within the rough endoplasmic reticulum of some of its target cells. The mononuclear phagocytic system plays a major role in the multiplication and spread of B. abortus in chicken embryos. I believe that embryonated chicken eggs provide a useful in vivo model to study the early cellular events of brucella infection in embryonic membranes. Because its involvement is somewhat similar to the role of the trophoblastic epithelium in the absorption and transfer of nutrients to the embryonic circulation, the yolk endoderm infection is of particular interest.

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