Penetration and intracellular growth of Brucella spp in Vero cells

Philippe Gabriel Detilleux

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Penetration and intracellular growth of Brucella spp. in Vero cells

Detilleux, Philippe Gabriel, Ph.D.
Iowa State University, 1989
Penetration and intracellular growth of Brucella spp. in Vero cells

by

Philippe Gabriel Detilleux

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

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For the Graduate College

Iowa State University
Ames, Iowa

1989
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GENERAL INTRODUCTION

Bacteria of the genus *Brucella* are intracellular pathogens of man and animals, causing zoonoses of worldwide importance. The genus *Brucella*, comprises six species with different major animal sources, i.e., *B. melitensis* in goats, *B. abortus* in cattle, *B. suis* in swine, *B. canis* in dogs, *B. ovis* in sheep and *B. neotomae* in desert wood rat (24, 55, 122). Brucellae produce chronic infections with persistent or recurrent bacteremia (55, 121). In pregnant susceptible animals, the organism has a marked affinity for the placenta, where it replicates within trophoblasts causing placentitis and abortion (5, 55, 121). Except for *B. neotomae*, each species of *Brucella* is capable of intratrophoblastic replication in its natural host (19, 20, 55, 81, 110).

In ruminant trophoblasts, *B. abortus* replicates within cisternae of the rough endoplasmic reticulum (RER) (3, 4, 75). Similar intracellular localization has been seen in chicken embryo mesenchymal, yolk endodermal and hepatic cells (25). Replication within RER is a unique mechanism of intracellular bacterial parasitism. Usually, intracellular bacteria inhabit phagosomes, e.g., *Salmonella typhimurium* (61) and *Yersinia enterocolitica* (30), or are free in host-cell cytoplasm, e.g., *Shigella flexneri* (41) and *Listeria monocytogenes* (36). Mechanisms of *B. abortus* entry into trophoblasts and its transport to the RER are unknown.

The ability of *B. abortus* to enter, survive, and replicate within
cells plays a key role in bovine brucellosis (55, 121). Establishment of infection requires penetration through conjunctival or oro-pharyngeal mucosae (10, 55, 121), probably by migration through lympho-epithelial cells (1). Persistent chronic infection results from the resistance of \textit{B. abortus} to microbicidal activity of host phagocytic cells (17, 55, 107, 121). This results in intermittent bacteremia, during which brucellae localize in the genital tract, mammary glands, and other tissues (10, 55, 121). Brucella placentitis is initiated by the entry and intracellular replication of \textit{B. abortus} within trophoblasts (4, 5).

In vitro cell cultures have contributed greatly in progress made toward the understanding of the molecular mechanisms of cellular penetration and survival (30, 105). Cell cultures provide relatively uniform populations of cells that can be infected under defined conditions, allowing for selective modifications of either agent or host-cells.

The objectives of this study were: (i) to develop and characterize an in vitro system to study the entry and intracellular growth of \textit{B. abortus} in non-phagocytic cells, (ii) investigate the role of the host-cell and of the bacterium in the internalization and transfer of \textit{B. abortus} to the RER, and (iii) characterize ultrastructurally the entry and intracellular localization of various species of \textit{Brucella} in non-phagocytic cells.

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

The Ph.D. candidate, Philippe G. Detilleux, was the principal investigator for each study.
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 (55).

**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 Hippocrates' 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*" (28, 76, 115).

The source of the organism was not discovered until 1904, when, in
preparation for 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 \textit{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 (115).

Abortion in domestic livestock has probably plagued herdsmen since animals were first domesticated (76). 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 (115). 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 (84). 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 \textit{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 (27, 28). In 1918, the American bacteriologist Alice Evans correctly identified M. melitensis as a bacillus and recognized the common identity of the two bacteria (27). She also implicated cow's milk as an important source of brucellosis in human beings (115). A few years later, Meyer and Shaw, confirmed Evans' findings and proposed the name "Brucella" for the new genus (77).

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, i.e., B. melitensis in goats, B. abortus in cattle, and B. suis in swine (24, 55). Cross-infections however do occur as most species of mammals are susceptible to Brucella (55). Because it survives almost exclusively in animals, Brucella has the tendency to acquire different phenotypes when infecting new hosts (24, 55). This led to the description of three minor species, i.e., B. ovis in sheep, B. canis in dogs and B. neotomae in desert wood rat, and of numerous variants such as B. suis biovar 2 that infect hares and B. suis biovar 4, naturally pathogenic for reindeer (24).
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 biovars in each species is large. Distinction between species and between different biovars, within each species, is based on their utilization of certain amino acids and carbohydrates, their sensitivity to bacteriophages, CO₂ requirements, H₂S production, growth on cultural media containing dyes and their reaction to monospecific sera (24, 44, 98, 112, 122).

*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, albimí agar or trypticase-soy agar supplemented with serum or blood. All strains of *B. ovis* and most strains of *B. abortus*, require 5 to 10% CO₂ for primary isolation (24, 44, 99, 112, 122).

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 (24, 112).
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 biovar 1. Biovars 2 and 4 are also found in the United States (82). 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 (10, 55, 82, 83, 122).

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 (10, 82, 83, 122).

*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 organisms 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 (10,
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 (10, 82, 90, 93, 122).

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 (82, 93).

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 sized 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 sized herds (10, 82, 83).
From the viewpoint of human health, bovine brucellosis is an
important disease because \textit{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 (10, 98, 112).

\textbf{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 (10, 55). Coital infection and transmission of
\textit{B. abortus} by artificial insemination have been reported (8, 71).
Following translocation through mucosal epithelium, the organisms are
rapidly ingested by phagocytic cells and carried to the regional lymph
nodes where bacterial replication occurs (44, 55, 121). Recently, it
was shown that when inoculated into calf ileal loops, \textit{B. abortus} are
transported across the lymphoepithelial cells (M cells) covering the
Peyer's patches and are ingested by macrophages and polymorphonuclear
neutrophils (PMN) in the lamina propria (1).

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 (44, 55, 98, 122).

The localization of \textit{B. abortus} in bacteremic females is largely restricted to the spleen, mammary gland, supramammary lymph nodes and pregnant uterus (55, 87). In the male, the organism localizes in lymphoid tissues, testis and accessory sexual glands (7, 55, 66). In a natural infection of pregnant cows, Smith et al. (108) found large numbers of organisms 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, 80). Placentomal chorionic villi and fetal viscera are infected hematogenously following trophoblast necrosis and ulceration of chorioallantoic membranes (4, 80).

The special affinity of \textit{B. abortus} for the pregnant bovine uterus has been attributed to uterine content of erythritol (57, 88, 109). This four-carbon polyhydric alcohol (\textit{HOCH}_2-\textit{CHOH-CHOH-CH}_2\textit{OH}) stimulates growth of \textit{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 (57, 96). In males, erythritol is found in the seminal vesicles of the bull, ram, buck and boar (57). Erythritol is used by \textit{B. abortus} as an energy source in preference to glucose and the bacterium's growth is inhibited by unmetabolizable analogues (2, 106,
113). This requirement for erythritol, however, is not absolute; strain 19 of *B. abortus*, which in vitro is inhibited by erythritol, is fully capable of causing placentitis when inoculated in pregnant goats (T. D. Anderson, N. F. Cheville, and P. G. Detilleux, Abstr. Ann. Meet. Am. Coll. Vet. Pathol. 1986, p. 164). Similarly, rat, rabbit and guinea-pig placentae, which do not contain erythritol (57), are susceptible to *Brucella* infection (11).

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 (107, 112, 121). Recent studies indicate that *B. abortus* releases two components that inhibit the myeloperoxidase-H$_2$O$_2$-halide antibacterial system of bovine PMNs (17, 18). These two components, identified as 5'-guanosine monophosphate and adenine, were shown to act by preferential inhibition of primary granule release (9, 17).

**Placental lesions**

*B. abortus* has a special affinity for the ruminant placenta where it produces extensive lesions (55). 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 (84). 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 (110).
Grossly, the placental lesions caused by *B. abortus* are characteristic but not pathognomonic (55). 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, 55, 80, 87). The lesions vary greatly in severity, depending on the course of the infection and the susceptibility of the host (5, 55). 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, 55).

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, 55, 80, 87).

In a recent ultrastructural study of experimental \textit{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). The same intracellular localization is seen in bovine trophoblasts (75) and in chicken embryo mesenchymal, yolk endodermal and hepatic cells (25).

**Diagnosis**

The clinical manifestations of brucellosis are not pathognomonic, rendering laboratory assistance absolutely essential for the diagnosis of the disease (10, 55, 122). The available techniques can be classified in bacteriological, serological, allergic and cellular methods (82, 116). Bacterial culture and positive identification of \textit{B. abortus} is the most reliable diagnostic test (10, 82).

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-immunoelectrophoresis, enzyme- and radioimmunoassay tests are being evaluated in an effort to increase sensitivity and specificity of current tests (10, 82, 116).

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 (116).

**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 (82). 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 (115). The seed culture of strain 19, dispensed by the United States Department of Agriculture (USDA) after 1956, does not grow on medium containing erythritol (13, 58). This inhibition seems to be due to a lack in the NAD-dependant D-erythulose 1-phosphate dehydrogenase, an enzyme essential for erythritol catabolism (113, 114).
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, vaccination of calves rarely results in a 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 (10, 82, 122).

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 (76, 82).

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 close cooperation of livestock owners is essential for the success of any eradication program.
Comparison of the Strategies Used by Enteroinvasive Bacteria to Invade Cultured Animal Cells

**Introduction**

*Shigella*, *Salmonella* and *Yersinia* are genera of the Family Enterobacteriaceae which contain species capable of invading intestinal epithelial cells. Despite shared invasive abilities, the pathology of infections caused by *Shigella* species differs markedly from that found in infections with *Salmonella* and *Yersinia* species. *Shigella* species, leave the enterocytes but remain localized within the colonic mucosa, rarely spreading beyond the lamina propria (65, 118, 119). Intracellular bacterial growth, followed by cellular destruction and infection of adjacent cells, induce a strong inflammatory reaction which results in acute ulcerative colitis (65, 118). In contrast, *Salmonella* and *Yersinia* species seem to be transported across the intestinal epithelium, without harming the epithelial cells (117, 124). These bacteria replicate within macrophages in the lamina propria and gut-associated lymphoid tissues where they initiate an inflammatory reaction. The resulting enterocolitis is frequently associated with mesenteric lymphadenitis and in severe cases, bacteremia and septicemia.

These differences in pathology are correlated with the different mechanisms by which these enteroinvasive bacteria establish infection; either they replicate within the intestinal epithelial cells, as exemplified by *Shigella* spp., or they pass through the intestinal epithelium in order to establish systemic infection, as seen with
Salmonella and Yersinia spp. Both strategies are used by B. abortus in bovine brucellosis, i.e., B. abortus translocates across mucosal epithelium as a prelude for establishing systemic infection (1) and it replicates within trophoblasts causing placentitis and abortion (4, 5). Consequently, the bacterial mechanisms and genes necessary for the invasion of cultured animal cells by Shigella, Salmonella and Yersinia species, will be discussed here for comparative purposes.

Invasion of a host-cell by a bacterium can be usefully divided into four steps: (i) initial interaction, (ii) internalization and entry, (iii) intracellular survival and replication and (iv) escape from the host-cell.

Initial interaction

Before internalization a bacterium must come in contact with the host-cell, and subsequently adhere to its surface (52, 54). Stable bonds between the bacterium and the host-cell are required prior to and during internalization (54).

Although the initial contact of a bacterium with a cellular surface may be random event (even when motility is involved), it may also be chemotactically directed (52). Chemotaxis and motility contribute to the entry of Salmonella spp. into eukaryotic cells (69, 123, 126). Flagellar motility and chemotaxis are required for the invasion of HeLa cells by S. typhi; motility mutants created by transposon (Tn5) mutagenesis, are unable to enter HeLa cells (69). S. typhimurium are chemotactically attracted to HeLa cells mildly damaged by exposure to
low pH (123). Apparently, damaged HeLa cells release a diffusible
attractant, tentatively identified as glycine, that increases the
frequency of collisions between S. typhimurium and the epithelial cell
surface (123). In vivo, the chemotactic response of Salmonella to dying
cells of the intestinal villus tips may facilitate the establishment of
S. typhimurium infection. In vitro, this chemotactic dependency of S.
typhimurium for adherence can be overcome by centrifugation of the
bacteria onto the monolayer (123). Since Shigella spp. are non-motile
and Yersinia spp. are not actively motile at 37°C, chemotactic activity
is probably not involved in the adhesiveness and invasiveness of these
organisms (32, 79).

Adherence of a bacterium to an eukaryotic cell is profoundly
affected by the physicochemical properties of both bacterial and
cellular surfaces (6, 52). In an aqueous environment, bacteria and the
host-cells are surrounded by positive ions which are attracted to the
fixed negative charges on their respective surfaces. According to the
Derjaguin-Landau and Verwey-Overbeek (DLVO) theory, forces of attraction
\( V_A \) and forces of repulsion \( V_R \) generated between two surfaces of like
charge are additive, with states of mutual attraction occurring at two
distances of separation when \( V_A > V_R \) (6, 52, 54). At long distances of
separation (10-100 nm from the plasma membrane) the weak attractive
forces are those of the secondary minimum, whereas at shorter distances
(less than 1 nm) the stronger forces of attraction of the primary
minimum become apparent. Between the primary and secondary minima, the
interaction of the ionic surroundings interpose an energy barrier that
causes mutual repulsion between the two surfaces and $V_A < V_R$.

Reversible bacterial attachment to an eukaryotic cell occurs at the secondary minimum (52, 54). Although it is a weak non-specific adherence, reversible attachment is a prerequisite for the strong irreversible attachment that is essential for bacterial internalization. Continuous fluid shear produced by rocking the culture medium, prevented the reversible attachment of S. typhimurium to HeLa cells and greatly reduced irreversible bacterial attachment (54). Therefore, it was inferred that the period of reversible attachment provides time for bacterial adhesins to bind to the host-cell surface (54).

Adhesins are bacterial structures or molecules that mediate adhesion (6). The adhesins that mediate the strong irreversible attachment that hold enteroinvasive bacteria to the surface of their host-cells are as yet not well characterized (52). The nature of the adhesins responsible for adherence of Salmonella spp. to surface of epithelial cells is unclear. While some authors suggest a role for mannose-sensitive type 1 fimbriae (120), others report that non-fimbrial mannose-resistant hemagglutinins are involved (53, 74). Unlike Salmonella and Shigella species, Y. pseudotuberculosis are adherent at 4°C and this adherence is significantly reduced by addition of proteases (15, 49). This suggests that the adhesion of Yersinia spp. is mediated by bacterial outer membrane protein(s) that are continuously expressed. Since it is not possible to genetically separate the attachment and invasion phenotypes of cloned Yersinia invasion determinants (79), adhesiveness of Yersinia may be mediated by the same protein responsible
for invasiveness.

Increased hydrophobicity of the bacterial surface facilitates the establishment of irreversible adherence (43). By displacing water from interacting surfaces, a hydrophobic interface reduces the energy barrier responsible for mutual repulsion. Correlation between bacterial hydrophobicity and the degree to which they adhere to and invade cultured cells has been demonstrated for *Salmonella* spp. (59, 60) and *Yersinia* spp. (102, 103). Hydrophobicity alone, however, is not sufficient to confer adhesiveness and invasiveness to a bacterium. In a comparative study, Schiemann et al. (102) reported that although it was the most hydrophobic of the *Yersinia* spp. tested, the non-pathogenic *Y. kristensenii* exhibited negligible adherence to cultured animal cells. Bacterial hydrophobicity is probably only an accessory factor that promotes closer apposition of the bacteria with the epithelial cell, allowing specific bacterial structures to establish adherence and induce invasion (102).

**Bacterial internalization**

Although attachment is a prerequisite for bacterial uptake, tight adherence to surfaces of eukaryotic cells does not necessarily result in internalization. While the introduction of a recombinant plasmid encoding *E. coli* adhesin AFA-I (64) rendered strains of *Sh. flexneri* highly adherent to HeLa cells, it did not modify the invasive or non-invasive phenotype of each *Shigella* strain (21).

Studies of phagocytosis using scanning and transmission electron
microscopy, established that, after the bacterium has established intimate contact with an eukaryotic cell, protoplasmic projections extrude from the surface of the cell and encircle the bacterium; the organism is then internalized (12, 15, 37, 43, 61, 78, 100, 128). Bacterial internalization is an energy-requiring process (41, 61, 62). The integrity of the plasma membrane is maintained throughout the invasion process. This was demonstrated for Sh. dysenteriae 1 by prelabeling HeLa cells with \[^3\text{H}\] uridine; bacterial invasion did not cause leakage of labeled cytoplasmic RNA (39). If uptake of bacteria resembles phagocytosis morphologically, it may also involve receptor clustering as seen in receptor mediated endocytosis. Clathrin coated-pits have been described in association with penetrating salmonellae (33), shigellae (37) and yersiniae (78).

The role of cytoskeletal microfilaments of the host cell during bacterial entry has been studied indirectly with inhibitors of microfilament function and directly by fluorescence microscopy. Cytochalasins, which block phagocytosis by inhibiting actin microfilament polymerization, prevent invasion of cultured cells by Yersinia (31), Salmonella (31, 62), and Shigella species (31, 41). In contrast, microtubule-acting drugs, e.g., colchicine, vincristine and vinblastine, have no effect on bacterial uptake by host-cells (31, 41). Using NBD-phallicidin, a fluorescent marker for filamentous actin, Clerc and Sansonetti (22) demonstrated that actin polymerizes on the cytoplasmic side of the plasma membrane in areas where HeLa cells interact with invasive Sh. flexneri. Myosin accumulated at the same
sites, as revealed by indirect immunofluorescence using an anti-myosin monoclonal antibody (22). An adherent but non-invasive transformant of the same invasive Sh. flexneri strain did not induce any modification of cellular microfilaments, suggesting that bacterial uptake is triggered by specific signal(s) from the bacterium (22).

The role of Salmonella in the invasion process was analyzed by killing the bacterium with heat or ultraviolet radiation, by incubating it at low temperature or by treating it with chloramphenicol, an inhibitor of bacterial protein synthesis, or rifampicin, an inhibitor of bacterial RNA synthesis (33, 34, 60). These treatments greatly reduced the association of Salmonella with eukaryotic cells, indicating that the adherence to and invasion of host-cells by Salmonella is an active process that requires bacterial protein and RNA synthesis (34, 60). In contrast, DNA replication is not required since treatment with nalixidic acid, an inhibitor of bacterial DNA synthesis, had no effect on these processes (34). Recently, Finlay et al. (34) reported that, upon contact with cultured epithelial cells, S. typhimurium and S. cholerae-suis synthesize several new polypeptides required for adherence and invasion. The synthesis of these bacterial proteins was apparently induced by specific glycoprotein(s) at the surface of the host-cells, i.e., it was not induced by all cell lines tested nor by host-cells pretreated with trypsin or neuraminidase (34).

Conflicting evidence exists regarding the requirements of bacterial viability for the internalization of Yersinia (79). While, Y. enterocolitica inactivated by ultraviolet radiation or formalin is
internalized into cultured cells, heat-inactivated organisms are not (89, 111, 127). Loss of invasiveness by heat-treatment is probably due to the thermolability of invasin, a bacterial surface protein required by *Y. enterocolitica* and *Y. pseudotuberculosis* for adherence and invasion. Inhibition of bacterial RNA or protein synthesis were reported to decrease the levels of *Y. enterocolitica* invasion of HeLa cells (67), an observation that was not confirmed by others (32). The reason for these discrepancies is probably related to differences in experimental techniques used to distinguish attached versus internalized bacteria. Intracellular *Yersinia* were quantified by microscopic observation of Giemsa-stained monolayers, a method which makes distinction between intracellular bacteria and those adherent to the surface subjective. Nevertheless, in a recent review article, Finlay and Falkow conclude that, unlike invasion by *Salmonella*, de novo bacterial biosynthesis is not required for *Yersinia* invasion (32). They suggest that bacterial components required for *Yersinia* invasion are constitutively expressed at the surface of the organism.

In a first report on the role of shigellae in the induction of endocytic activity in cultured cells, Hale and coworkers (38) concluded that *Shigella* spp. must be metabolically active to enter into host-cells; bacteria treated with ultraviolet radiation, kanamycin or mild heating did not invade Henle 407 cells (38). When they later repeated these experiments using centrifugation to enhance the contact between inactivated *Sh. dysenteriae* 1 and HeLa cells, they found that lethal doses of ultraviolet or kanamycin do not immediately destroy the ability
of shigellae to invade host-cells (43). They speculated that Shigella express heat-labile surface structures, required for invasion, which are degraded by endogenous proteases in non-viable bacteria (43).

**Intracellular survival and replication**

Immediately after internalization Salmonella, Yersinia and Shigella organisms are located within phagosomes. Later, however, the intracellular behavior of the members of each genus varies greatly.

Although *Salmonella* spp. are reported to remain membrane-bound during intracellular replication, adequate ultrastructural evidence is lacking (30, 128). Using polarized epithelial MDCK cells grown on permeable supports, Finlay et al. (33) demonstrated that, when added to the apical surface of the cells, *S. choleraesuis* and *S. typhimurium* can be transported across an intact epithelial monolayer. Transmission electron microscopy revealed that salmonellae passed through but not between the MDCK cells, in a process resembling transcytosis. This bacterial transcytosis was inefficient when *S. choleraesuis* were added to the basolateral surface of the MDCK cells. Non-invasive *E. coli* failed to penetrate through the monolayers even when added with the salmonellae. Intracellular growth was not required for transcytosis, as a Tn5 insertion mutant with decreased rates of intracellular replication passed through the monolayer at rates similar to those of wild-type *S. choleraesuis* (35).

Yersinia spp. remain within membrane-bound vacuoles throughout the infectious cycle in non-phagocytic cells (12, 30, 125). However,
destruction of the vacuolar membrane and localization within the host-cell cytoplasm has been suggested for *Y. pseudotuberculosis* (68). Conflicting reports exist regarding the intracellular replication of *Y. enterococolitica* and *Y. pseudotuberculosis* in vitro (79). The number of intracellular yersiniae remained constant or decreased slowly over time in HEp-2 and HeLa cells, in some studies (12, 26, 105); in others, intracellular replication occurred in HeLa cells and human fibroblasts (12, 16, 68). Taken collectively, *Yersinia* spp. probably multiply within cultured animal cells but growth is slow and can be more easily detected in certain cell lines (fibroblasts) than in others (HeLa cells) (79). The transport of *Yersinia* spp. across epithelial cells, as reported for *S. choleraesuis* in MDCK cells, has not been adequately studied.

*Shigella* spp., in contrast to *Salmonella* and *Yersinia*, escape from the phagosome and enter the host-cell cytoplasm (21, 37). Soon after bacterial entry (within 15 minutes), a plasmid-encoded contact hemolysin is produced that lyses the membrane of the endocytic vacuole (21). Once free in the cytoplasm, *Shigella* spp. inhibit host protein synthesis through the action of shiga toxin and multiply rapidly (21, 39). Release from the phagosome, but not inhibition of host-cell protein synthesis, is a prerequisite for the multiplication of *Sh. flexneri* within HeLa cells; *Shigella* intracytoplasmic replication is strongly correlated with its ability to produce hemolysin but not with the induction of shiga toxin (100). Similar intracytoplasmic replication occurs with *Listeria monocytogenes*, which produce listerialysin, a
secreted hemolysin that is required to lyse the phagosome (36, 63, 92). *L. monocytogenes* mutants unable to produce listeriolysin cannot replicate intracellularly (92).

**Escape from the host-cell**

Escape of *Yersinia* and *Salmonella* from non-phagocytic cells has not been studied. It is known that after pulsing infected monolayers with antibiotics, the number of extracellular bacteria increases with time. This bacterial release could result from host-cell death and lysis due to intracellular bacterial replication or could result from the fusion of bacteria-containing vacuoles with the host-cell membrane in an exocytic-like process. The observation that *Salmonella* spp. can be transported across intact MDCK monolayers suggests that bacterial exocytosis occurs.

*Sh. flexneri* is capable to escape from an infected host cell before lysis, and invade a contiguous cell without being exposed to the extracellular environment (85). This was demonstrated by incubating infected HeLa cell monolayers in the presence of an agarose overlay containing tissue culture medium and gentamycin, which eliminated extracellular growth (85). This process resulted in the formation of plaques similar to those produced by cytopathic viruses (85). Dispersion of intracellular shigellae throughout the host-cell cytoplasm is required prior to plaque formation in HeLa cell monolayers (70, 86). The intracellular spread of *Sh. flexneri* is independent of bacterial motility, but requires host-cell microfilament function as it is
inhibited by cytochalasin D (86).

**Genetics of bacterial invasion**

Two approaches are commonly used to identify the genes required for the invasion of epithelial cells by enteroinvasive organisms: (i) analysis of transposon insertion mutants and (ii) cloning of essential DNA regions into an avirulent non-invasive *E. coli* strain (usually strain K-12) (29). *Shigella*, *Salmonella*, and *Yersinia* spp. differ markedly in the nature and the complexity of the genetic information required for the expression of the invasive phenotype.

Most genes required for *Shigella* spp. invasion are encoded on a 120 to 140 megadaltons virulence plasmid; transfer of this plasmid to a non-invasive *E. coli* strain confers the ability to penetrate into HeLa cells (99). Anucleate minicells isolated from *Sh. flexneri* can invade HeLa cells, providing additional evidence that chromosomal genes are not absolutely required for invasion (42). Chromosomal genes, however, play a role in the regulation of the expression of invasiveness, e.g., the thermoregulation of the invasive phenotype is under the control of the chromosomal *virR* gene (72, 73).

Three regions involved in invasiveness have been identified on the virulence plasmids of *Shigella* spp. (21). The first region contains *virF*, a positive regulator gene which controls the plasmid-encoded expression of invasive phenotype as well as the production of the contact hemolysin required for phagosomal lysis and intracellular replication (56). The second region contains the *virC* gene, which
encodes a 140 kd protein that mediates the spread of intracellular shigellae to neighboring cells (70, 86). The \textit{virG} gene is apparently regulated by the \textit{virF} gene and by a chromosomal locus designated \textit{kcpA}, because it is necessary for the induction of keratoconjunctivitis (Sereny test) (86). \textit{Sh. flexneri} strains which bear transposon \textit{Tn5} mutation in the \textit{virG} plasmid gene (70) or in the \textit{kcpA} chromosomal locus (86) enter and replicate within HeLa cells, but remain localized and fail to invade adjacent cells. The third region contains several loci also implicated in the invasion process (101).

The genetics of \textit{Salmonella} invasion are not as well defined as that of \textit{Shigella} invasion. Highly pathogenic \textit{Salmonella} species (with the exception of \textit{S. typhi}) harbor a plasmid which is essential for virulence. In contrast to \textit{Shigella}, this extrachromosomal DNA is not needed by \textit{Salmonella} spp. to enter epithelial cells. Recently, Finlay et al. described six classes of transposon (\textit{TnphoA}) mutants of \textit{S. choleraesuis} which are unable to penetrate through MDCK cells (35). All mutants were unable to adhere to MDCK cells, and yet none were in the genes encoding type 1 pili or mannose-resistant hemagglutinin. Two of the six classes of mutations, caused defects in the core or O-side-chain lipopolysaccharide molecules, suggesting that, in contrast to previous reports (59), smooth LPS is required for the invasion of cultured cells. One class of mutants was unable to synthetize the invasion proteins, whose expression is normally induced upon contact with epithelial cells (34).

The genes required by \textit{Yersinia} spp. for the invasion of epithelial
cells are apparently located on the Yersinia chromosome rather than on the virulence-associated plasmid, because strains lacking the plasmid remain invasive (46, 91, 97, 126). Recently, a single chromosomal gene that is sufficient to convert the innocuous E. coli K-12 strain into an organism capable of invading cultured animal cells, was cloned from Y. pseudotuberculosis (49). This gene, designated inv, encodes a 108,000 daltons surface protein, called invasin, that promotes adherence to and invasion of epithelial cells by Y. pseudotuberculosis (51). Miller and Falkow (78) found that Y. enterocolitica chromosome harbor two loci that can confer the invasive phenotype to the K-12 strain of E. coli. One locus is similar to the inv gene of Y. pseudotuberculosis, while the other, called ail for attachment-invasion locus, is not homologous to either inv genes. The ail gene confers a target tissue specificity on the bacterial host different from that conferred by the inv genes (78). While the inv gene directs a high level of attachment and invasion to most cell types tested, E. coli carrying the ail gene are internalized far better by CHO cells than by other cell lines (78, 79).
PENETRATION AND INTRACELLULAR GROWTH OF BRUCELLA ABORTUS
IN NON-PHAGOCYTIC CELLS IN VITRO
Penetration and Intracellular Growth of *Brucella abortus*

in Non-Phagocytic Cells in Vitro

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Running Head: Infection of non-phagocytic cells by *B. abortus*

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ABSTRACT

*Brucella abortus* penetrates and replicates within the rough endoplasmic reticulum (RER) of non-phagocytic cells. In this study, Vero cells were infected with *B. abortus* to investigate bacterial translocation to the RER. Studies using double fluorescence staining procedures and quantitative bacteriological culture of disrupted host-cells, showed that various *B. abortus* strains replicate within Vero cells, including smooth virulent (2308S, 544), smooth attenuated (19) and rough (45/20, 2308R) strains. Rough brucellae, were more adherent and invaded a greater number of Vero cells. Intracellular replication occurred in a larger percentage of cells with smooth virulent (2308S, 544) than with smooth attenuated (19) or rough strain (45/20, 2308R). Differences in adhesiveness and invasiveness were correlated to hydrophobicity of the organism, as measured by hydrocarbon adherence.

*B. abortus* 2308S infected other established cell lines (MDBK, ROS, Pk-15 and Jeg-3) but failed to invade MDCK cells. Ultrastructurally, intracellular smooth (2308S) and rough (45/20) brucellae were consistently within cisternae of the RER and nuclear envelope. The results suggest that transfer to the RER is the limiting step in the infection of non-phagocytic cells by *B. abortus*. 
**INTRODUCTION**

*Brucella abortus* is an intracellular pathogen that causes chronic infections with persistent or recurrent bacteremia in man and several species of ruminants. The organism has a marked predilection for the gravid uterus of cattle; an important step in the pathogenesis of *Brucella* placentitis is the invasion of trophoblastic epithelium. Studies in a caprine model (1), indicate that bacteremic *B. abortus* enter and replicate within erythrophagocytic trophoblasts and then colonize the chorioallantoic trophoblastic epithelium. Marked intracellular replication of *B. abortus* kills trophoblasts and organisms are shed into surrounding tissues and fluids. The cycle of cell invasion, intracellular replication and cell death continues, leading to overt placentitis and abortion (1).

In trophoblasts, *B. abortus* replicates within cisternae of the rough endoplasmic reticulum (RER) (1). The same intracellular localization is seen in bovine trophoblasts (25) and in chicken embryo mesenchymal, yolk endodermal and hepatic cells (6). Replication within RER is a unique mechanism of intracellular bacterial parasitism. Usually, intracellular bacteria inhabit phagosomes, e.g., *Salmonella typhimurium* (22) and *Yersinia enterocolitica* (7), or are free in host cell cytoplasm, e.g., *Shigella flexneri* (35).

Most studies on the interactions of *B. abortus* with host cells deal with monocytes and polymorphonuclear neutrophils. In vitro growth of *B. abortus* within non-phagocytic cells has been reported, e.g. virulent
strains of *B. abortus* replicate within chick embryo fibroblasts (17), hamster-kidney cells (13), HeLa cells (37) and primary or subcultures of different bovine adult and fetal cells (5, 29, 30). Rough *B. abortus* and the attenuated smooth strain 19 fail to replicate in chicken embryo fibroblasts in vitro (17).

This study was designed to evaluate the internalization and intracellular growth of *B. abortus* within non-phagocytic cells in vitro. A double fluorescence staining procedure was used to discriminate between extra- and intracellular organisms and to determine the effects of various experimental conditions on monolayer infection. Replication of *B. abortus* in Vero cells was shown to occur within the RER.

(This research was reported in part at the 69th Annual Meeting of the Conference of Research Workers in Animal Diseases, abstract No. 40, Chicago, Ill., 1988.)
MATERIALS AND METHODS

Bacterial Strains and Cultures

Five strains of *B. abortus* were used: Strain 2308S is a CO₂-independent virulent smooth strain; strain 544, the international type strain for *B. abortus*, is a CO₂-dependent virulent smooth strain; strain 19 is an attenuated smooth strain used worldwide as live vaccine; strain 45/20 is a rough strain used as killed-cell vaccine; and strain 2308R is a stable rough strain derived from 2308S.

*B. abortus* was grown on potato infusion agar slants for 48 h at 37°C. Cells were harvested by gentle washing with sterile 0.85% NaCl and standardized turbidimetrically to a concentration of $1 \times 10^{11}$ cells/ml. This suspension was then diluted in cell culture medium supplemented with 10% fetal calf serum to $1.0 \times 10^9$ CFU/ml.

Tissue Culture Cells

African green monkey kidney fibroblasts (Vero), and Madin-Darby bovine kidney (MDBK), Madin-Darby canine kidney (MDCK) and porcine kidney (PK-15) epithelial cells were obtained from the National Veterinary Services Laboratories (NVSL, Ames, Iowa). Human choriocarcinoma (Jeg-3, ATCC-HTB36) cells were purchased from American Type Culture Collection, Washington, DC. Rat osteosarcoma (ROS) cells were a gift from Dr T. Reinhard (NADC, Ames, IA). Cells were routinely
grown in Eagle minimal essential medium (MEM; GIBCO Laboratories, Grand Island, NY) supplemented with L-glutamine (2 mM), fetal calf serum (FCS, 10%), and penicillin-streptomycin. For monolayer infection, 35-mm plastic tissue culture dishes (Becton Dickinson Labware, Oxnard, CA), each containing a 22 by 22 mm glass coverslip, were seeded with 3 ml of a suspension of 150,000 to 200,000 cells/ml. After 5 h incubation (24 h for Jeg-3 cells), subconfluent monolayers were washed 3 times with MEM at 37°C and were incubated overnight in 1 ml of MEM supplemented with 0.25% FCS and 2 mM glutamine but without antibiotics.

Monolayer Infection

After overnight incubation, medium was aspirated from the culture dishes and 2 ml of bacterial suspension was added. The culture dishes were centrifuged for 20 minutes at 550g at room temperature and placed in a humidified incubator with an atmosphere of 5% CO2 at 37°C. At intervals, coverslips were removed from the petri dishes, washed in 2 changes of PBS at 37°C, placed in six-well tissue culture plates (Costar, Cambridge, MA) and further incubated in MEM supplemented with 0.25% FCS and gentamycin (50 µg/ml) (Gentocin; Stering Corporation, Kenilworth, NJ) at bactericidal level to kill extracellular brucellae. Medium was replaced at 24 hours. The inoculation period was defined as the period between the exposure of monolayers to B. abortus and the introduction of gentamycin in the culture medium. Experiments were carried out in triplicate or duplicate and were repeated at least twice for each
The polycation diethylaminoethyl-dextran (DEAE-D) increases the infectivity of *Chlamydia* in cell culture, presumably by neutralizing mutually repulsive electrostatic forces (12). To investigate the effect of DEAE-D on the infectivity of *B. abortus* for Vero cells, monolayers were incubated for 1 h prior to inoculation in a 20 µg/ml suspension of DEAE-D (Pharmacia, Uppsala, Sweden) in MEM. Coverslips were then washed twice in sterile PBS at 37°C and exposed for different times to *B. abortus* strain 2308S.

**Double Fluorescence Staining**

At intervals, coverslips were washed in PBS, fixed for 5 min in methanol at 4°C, dehydrated for 5 min in acetone at 4°C and air dried. Brucellae were labeled by indirect immuno-fluorescence using goat or rabbit anti-*B. abortus* 2308S antiserum (smooth organisms) and rabbit anti-*B. canis* antiserum (rough brucellae) as primary antibodies (all antisera provided by Dr. B. L. Deyoe). Secondary antibody was either goat anti-rabbit or rabbit anti-goat FITC-conjugated antiserum (NVSL, Ames, IA). After washing in PBS containing 3% bovine serum albumin (PBS-BSA), coverslips were incubated for 1 h in a 1/500 dilution of the primary antiserum. Following washes in PBS-BSA, coverslips were incubated for 1 h in a 1/50 dilution of appropriate secondary antiserum. Following immunolabeling, coverslips were washed in PBS and treated for 5 min with propidium iodide (25 µg/ml in PBS) (Sigma Chemical Co., St.
Propidium iodide fluoresces bright red when intercalated in double stranded nucleic acid and exposed to ultraviolet light. Following staining, coverslips were washed three times in PBS, rinsed in distilled water and mounted on glass slides with a 90% solution of glycerin in PBS (pH 8.5). Specimens were examined by epifluorescence microscopy with either a blue (excitation at 490 nm and emission at 515 nm) or green (excitation at 545 nm and emission at 590 nm) filter.

For discrimination between intracellular and extracellular bacteria, the primary antibody was applied onto coverslips for 30 min at 4°C prior to fixation. Antibodies do not penetrate through the plasma membrane of unfixed cells (10, 20), consequently only adhering extracellular bacteria reacted with the primary antibody. After washing, monolayers were fixed and stained as described above, starting after the primary antibody labeling. Since propidium iodide was applied after solubilization of the cells by methanol-acetone fixation, both bacterial and host cell DNA were stained.

**Enumeration of Brucellae**

The number of intracellular viable *B. abortus* was determined at 4, 8, 16, 24, 36 and 48 h post-inoculation (PI). Except for the 4 h samples, gentamycin was introduced in all samples 8 h after inoculation. The 4 h and 8 h samples were treated with gentamycin for 1 h before bacterial counts. After exposure to gentamycin, infected monolayers on
coverslips were washed in PBS and incubated for 10 to 15 minutes in 2 ml of a 0.1% solution of deoxycholate in distilled water. This procedure disrupted the host cells without affecting the viability of brucellae. Samples of the lysate were serially diluted in PBS for quantitation of CFU of *B. abortus* on tryptose agar plates containing 5% bovine serum. *Brucella* colonies were identified by colonial morphology and growth characteristics (40).

**Enumeration of Infected Cells**

For each of 3 coverslips stained by double fluorescence, the number of cells per mm$^2$ was estimated, at a total magnification of 400x, by counting cell nuclei in 5 fields located along a diagonal across the coverslip (15 to 25 cell nuclei/field). The surface area was defined by an eyepiece reticle. The number of infected cells per mm$^2$ was then estimated, at a total magnification of 100x, by counting infected cells in 25 systematically ordered fields (1 to 20 infected cells/field). Results were expressed as percentage of infected cells.

In selected experiments, the percentage of Vero cells containing 0, 1, 2, 3 to 5, 6 to 10, and more than 10 intracellular brucellae was estimated by examining 200 randomly selected Vero cells in each of 2 or 3 coverslips.
Hydrocarbon Adherence Assay

Cell surface hydrophobicity of *B. abortus* (strains 2308S, 19, 2308R and 45/20) was measured by determining the degree to which they associate with hydrocarbons (p-Xylene, n-Heptane, iso-Octane), using a modification of the hydrocarbon adherence method of Rosenberg et al. (33). Standardized bacterial suspensions in 0.85% saline were adjusted to an optical density of 0.20 at 600 nm (approximately $2 \times 10^9$ CFU/ml). Each bacterial suspension was divided into thirty-six 4 ml samples to which various volumes (50, 100, 150, or 200 μl) of hydrocarbon were added. Three tubes without hydrocarbon were controls. After agitation for 30 seconds, tubes were allowed to stand for 15 min to permit phase separation and optical density of the aqueous phase was determined spectrophotometrically at 600 nm. Results were expressed as percentage of the optical density of control tubes.

Transmission Electron Microscopy

To validate the model we needed to ascertain that intracellular replication of *B. abortus* occurs within the rough endoplasmic reticulum (RER) of Vero cells. Vero cells were grown on microporous membranes in 12-mm Millicell-HA inserts (Millipor, Bedford, MA) placed in a 24-well tissue culture plate (Costar, Cambridge, MA). Following overnight incubation in antibiotic-free MEM, 1 ml of *B. abortus* inoculum was centrifuged onto inserts placed in 35-mm petri dishes, as described
above for the coverslips. After 8 h incubation, the inserts were rinsed in PBS, placed in a 24-well plate and incubated for 40 h in MEM containing 50 µg/ml gentamycin. Inserts were then washed in cold PBS and fixed for 1 h by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C. Following fixation, membranes were removed from the inserts and stored in 0.1 M sodium cacodylate buffer at 4°C. Membranes were postfixed in osmium tetroxide, infiltrated and embedded in epoxy resin, sectioned at 70 to 90 nm and examined with a Philips 410 electron microscope.
RESULTS

Microscopic Observations

Using the double fluorescence labeling technique, with the blue filter, brucellae had an intense green yellow fluorescence while eukaryotic cell nuclei were red (Fig. 1A-D). When the primary antibody was applied before fixation, yellow-green fluorescence was restricted to extracellular organisms, while intracellular bacteria stained red (Fig. 1E-G). With a green filter, both bacteria and cell nuclei were stained with an intense red fluorescence.

Intracellular localization of *B. abortus* 2308S in Vero cells was evident at 2 h PI. At 4 h PI, 30 to 40% of Vero cells contained one or more bacteria and at the end of the inoculation period, 8 h PI, 40 to 50% of Vero cells were infected (Figs. 1A and 2). Intracellular brucellae were isolated rods throughout the cytoplasm (Figs. 1A and B). The number of cells containing at least one organism did not increase significantly after 8 h, but in a small proportion of these cells, the number of intracellular bacteria increased sharply between 24 and 48 h (Figs. 1B and C). At 48 h PI, 1 to 10% of the cells were filled with bacteria (Fig. 1C) (referred to hereafter as infected cells). Most infected cells were in clusters of 2 to 6 cells. Brucellae replication occurred throughout the cytoplasm except in the nuclear area. Infected cells in different phases of the mitotic cycle were frequent (Fig. 1C). At 72 h, some heavily infected cells had ruptured and organisms were
Figs. 1 A-G. Light micrographs of Vero cells infected with smooth (2308S) and rough (2308R) *B. abortus*. (A) 2308S, 8 h PI: Vero cells with few intracellular brucellae. (B) 2308S, 24 h PI: Slight increase in the number of intracellular brucellae. (C) 2308S, 48 h PI: Vero cells with cytoplasm full of *B. abortus*. Note the presence of intracellular brucellae in mitotic cells (arrows). (D) 2308S, 72 h PI: Vero cell rupture due to excessive intracellular growth of *B. abortus*. (E) 2308R, 4 h PI: Greater adhesiveness of rough organisms (compare to 1G). (F) 2308R, 4 h PI: Clumps of internalized rough *B. abortus* (arrow). (G) 2308S, 4 h PI: Note brucellae adherent to areas of the coverslip not covered by Vero cells (arrowheads) (F and G). (A-E and G) Bar = 20 μm. (F) Bar = 10 μm.
Fig. 2. Interactions of *B. abortus* 2308S with Vero cells after 4, 8 and 24 h of incubation. Data are grouped according to the number of intracellular bacteria per cell, estimated for 200 Vero cells. Each bar represent the mean of 2 samples.
Number of B. abortus / Vero Cell

% of Cells with Intracellular Brucellae

0 1 2 3-5 6-10 >10

0 1 2 3-5 6-10 >10

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

24 hours 8 hour 4 hours
scattered over the field (Fig. 1D).

The most accurate determination of the percentage of infected cells was obtained with coverslips sampled at 48 h PI. At that sampling time, the number of infected cells was easily quantitated by scanning the coverslips at low magnification. At later sampling times, because increasing numbers of infected cells became disrupted, the estimation of the percentage of infected cells was less accurate. Therefore, in all subsequent experiments where the number of infected cells was determined, coverslips were sampled 48 h PI.

Interaction of four *B. abortus* strains (544, 19, 2308R and 45/20) with Vero cells was compared to that of strain 2308S. All smooth and rough strains invaded and grew in Vero cells and at 48 h PI, infected cells had similar morphology. There was no difference between strains 2308S and 544, two virulent smooth strains (Table 1). While larger numbers of *B. abortus* strain 19 adhered to Vero cells, invasion was less efficient (Fig. 3) and resulted in a lower percentage of infected cells (Table 1).

Rough brucellae were markedly more adherent and invasive than smooth (Fig. 3). At 4 h PI, 75% of Vero cells had 6 or more associated (extra- and intracellular) rough brucellae. With smooth organisms, less than 10% of Vero cells had 6 or more associated bacteria. Some Vero cells infected with rough brucellae (especially strain 45/20) were covered by extracellular bacteria (Fig. 1E). In 4 to 24 h PI samples, the percentage of Vero cells containing intracellular brucellae was higher with rough organisms (Fig. 3). Intracellular rough brucellae
Table 1. Relative infectivity of different *B. abortus* strains in comparison to strain 2308S in Vero cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>2308S</td>
<td>Smooth</td>
<td>100(^a)</td>
<td></td>
</tr>
<tr>
<td>544</td>
<td>Smooth</td>
<td>90 (2)(^b)</td>
<td>60 - 126</td>
</tr>
<tr>
<td>Str 19</td>
<td>Smooth</td>
<td>15 (13)</td>
<td>6 - 31</td>
</tr>
<tr>
<td>2308R</td>
<td>Rough</td>
<td>11 (2)</td>
<td>9 - 13</td>
</tr>
<tr>
<td>45/20</td>
<td>Rough</td>
<td>19 (2)</td>
<td>18 - 20</td>
</tr>
</tbody>
</table>

\(^a\)Results are expressed as percentage of strain 2308S (mean of 2 or 3 samples).

\(^b\)Number between parentheses indicates the number of determinations.
Figs. 3 A-B. Interactions of 4 strains of *B. abortus* with Vero cells after 4 h of incubation. Data are grouped according to the number of associated (A) or intracellular (B) bacteria per cells, estimated for 200 Vero cell. Each bar represent the mean of 3 samples.
were frequently seen as clumps of 5 or more bacteria (Fig. 1F); this was not seen with smooth organisms. Despite the higher adherence and invasiveness of rough strains, the percentage of infected cells, 48 h PI, was less than 20% that of smooth 2308S (Table 1).

With all strains, extracellular adherent brucellae were more abundant at the periphery of the cell (Fig. 1E). In addition, especially with rough strains, numerous brucellae adhered to areas of the coverslips not covered by Vero cells (Figs. 1E and G).

Infection of Different Cell Lines

Microscopic analysis showed that B. abortus 2308S had the capacity to infect 5 of the 6 cell lines tested (Table 2). Production of progesterone (± 35 ng/ml/24h) by Jeg-3 cells was confirmed by radioimmunoassay (Dr. Janice M. Miller, NADC, Ames). The appearance of infected cells was similar to that described for Vero cells. Infection of MDCK cells was never observed. B. abortus infected a similar percentage of MDBK cells as of Vero cells. However, because of their tendency to form multilayered cellular nests rather than uniform monolayers, MDBK cells were unsuitable for accurate determination of the percentage of infected cells. When incubated with primary epithelial and fibroblastic cell cultures isolated from the placenta of a 3-month pregnant cow, B. abortus infected less than 1% of the cells (data not shown). Because of these results all further work was carried out in Vero cells.
Table 2. Infectivity of *B. abortus* 2308S for different cell lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Tissue of origin</th>
<th>Type</th>
<th>Percentage of infected cells (% ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>African Green Monkey Kidney</td>
<td>fibroblast</td>
<td>1.13 ± 0.25*</td>
</tr>
<tr>
<td>ROS</td>
<td>Rat Osteosarcoma</td>
<td>osteoblast</td>
<td>0.41 ± 0.05</td>
</tr>
<tr>
<td>PK-15</td>
<td>Porcine Kidney</td>
<td>epithelial</td>
<td>0.48 ± 0.07</td>
</tr>
<tr>
<td>Jeg-3</td>
<td>Human Choriocarcinoma</td>
<td>epithelial</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin-Darby Bovine Kidney</td>
<td>epithelial</td>
<td>positive^b</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
<td>epithelial</td>
<td>none</td>
</tr>
</tbody>
</table>

^aResults are expressed as the percentage of infected Vero cells estimated microscopically 48 h after inoculation (mean of 3 determinations ± standard error).

^bMDBK cells were too numerous to allow accurate counts.
Reproducibility of the Model

Variations in the percentage of infected cells on different coverslips in one experiment was ± 15%. While variation between different experiments was much greater, the relative effect of different treatments was constant. Variation between different experiments was probably related to variations in the condition of Vero cells.

Kinetics of B. abortus Intracellular Growth

The number of viable brucellae in Vero cell lysates, was determined at various times after infection (Fig. 4). While initial period of decrease in bacterial numbers (after 8 h PI) corresponded probably to the killing of extracellular cell-associated bacteria by gentamycin, the increase (after 16 h PI) reflected intracellular growth. For all strains the maximal rate of intracellular replication was observed between 24 and 36 h PI. Strain 19 and 2308S had similar intracellular growth patterns, but at all sampling times the number of viable brucellae in cellular lysates was about 10-fold lower with strain 19. There was no difference in growth pattern between the 2 rough strains (2308R and 45/20), which differed slightly from that of smooth organisms. With rough strains, the initial number of viable brucellae was more than 10-fold higher than that of 2308S, and the following decrease lasted longer. In a separate experiment, strain 544 had the same growth pattern as smooth 2308S (data not shown).
Fig. 4. Infection of Vero cells with $2 \times 10^9$ CFU of *B. abortus*: 2308S (O), strain 19 (▲), 2308R (●) and 45/20 (△). Results are expressed as the mean $\log_{10}$ viable *B. abortus* per sample (mean of 2 or 3 determinations).
Effects of Inoculation Period Length and of DEAE-D

To establish the optimal length of the inoculation period, coverslips were inoculated for 2, 4, 6, 8, 10, or 12 h prior to the introduction of gentamycin and sampled 48 h PI. The percentage of infected cells increased with increasing length of the inoculation period, reaching a plateau after 8 h (Fig. 5). An inoculation period of 24 h did not result in a significantly larger percentage of infected cells (data not shown).

For all inoculation periods longer than 2 h, pretreatment of the monolayers with DEAE-D resulted in a higher percentage of infected cells (Fig. 5). DEAE-treated monolayers infected with smooth B. abortus were microscopically similar to monolayers exposed to rough brucellae, i.e., numerous bacteria adhered to Vero cells and coverslips surfaces.

Effects of Inoculum Concentration and of Centrifugation

In experiments in which dilutions of cultures of strains 19 and 2308S were incubated with Vero cells, the percentage of infected cells increased with increasing bacterial concentration (Fig. 6). Infected cells could not be found when the inoculum contained less than 2 x 10^7 CFU with strain 19 and 2 x 10^5 CFU with strain 2308S. On average there were approximately 1.7 x 10^5 Vero cells per coverslips. The minimal multiplicity of infection (MOI; ratio of number of bacteria to number of Vero cells at time of inoculation) was 120 for strain 19 and 1.2 for
Fig. 5. Infection of Vero cells by *E. abortus* 2308S as a function of the length of the inoculation period. Some monolayers were pretreated with DEAE-dextran for 1 h before inoculation (●). Results are expressed as the percentage of infected Vero cells estimated microscopically 48 h after inoculation (mean of 2 determinations ± standard error).
Fig. 6. Infection of Vero cells by *B. abortus* strains 2308S (○) and 19 (●) as a function of the inoculum concentration. Results are expressed as the percentage of infected Vero cells estimated microscopically 48 h after inoculation (mean of 3 determinations ± standard error).
% of Infected Vero Cells

- 2308S
- Strain 19

Inoculum Concentration (CFU/ml)

$10^6$ $10^7$ $10^8$ $10^9$ $10^{10}$ $10^{11}$
2308S. For all further work an inoculum of $2 \times 10^9$ CFU was used, with a MOI of $1.1.2 \times 10^4$ brucellae/Vero cell. Centrifugation of the inoculum onto monolayers doubled the number of infected cells (data not shown).

Hydrophobicity of Various *B. abortus* Strains

Marked differences in adhesiveness were correlated to differences in hydrophobic characteristics. The relative hydrophobicity of 2 smooth (2308S, 19) and 2 rough (2308R, 45/20) *B. abortus* strains was determined by assessing their affinities for 3 hydrocarbons in aqueous-hydrocarbon biphasic systems (33). More rough than smooth brucellae partitioned in the hydrocarbon phase indicating that rough strains have a higher hydrophobicity (Fig. 7). While among rough strains, 45/20 exhibited the highest hydrophobicity, there were no differences between the two smooth strains.

Electron Microscopy

Two days after inoculation, most infected cells contained large numbers of bacteria (Fig. 8A). Both smooth and rough strains of *B. abortus* were within cisternae of the rough endoplasmic reticulum (Fig. 8B and C). The limiting membranes of brucellae-filled cisternae were discontinuously lined by ribosomes. In the most heavily infected cells, brucellae were also in perinuclear spaces (Fig. 8D). Evidence of
Fig. 7. Relative affinity of various *B. abortus* strains towards hydrocarbons expressed as percentage of the initial absorbance (600 nm) of the aqueous suspension as a function of hydrocarbon volume (mean of 3 independent determinations). Symbols: 2308S (O—O); Str. 19 (△—△); 2308R (●—●); and 45/20 (△—△).
Figs. 8 A-D. Electron micrographs of Vero cells infected with *B. abortus* strains 2308S (A, B and D) or 45/20 (C) at 48 h postinoculation. Vero cells containing numerous *B. abortus* are attached to the microporous membrane by cytoplasmic processes (A, arrows). Brucellae are located within cisternae of the rough endoplasmic reticulum (B and C) and within perinuclear envelope (D). *Brucella*-containing cisternae are discontinuously lined by ribosomes (B and C, open arrows) and are continuous with normal rough endoplasmic reticulum (C, arrow). Bar = 1 μm.
cellular degeneration (cell swelling and vacuolation) was minimal, although some heavily infected cells were necrotic, with increased cytoplasmic electron-density and the loss of structural detail. Clusters of 2 to 4 brucellae were also found within phagolysosomes, especially in less infected cells that had no organisms in the RER. While half of these brucellae were morphologically intact the other half was degraded. Myelin figures were frequently associated with intact and degraded brucellae in phagolysosomes.
DISCUSSION

Using a double fluorescence staining procedure and quantitative bacteriological culture, we were able to show that *B. abortus* invades and grows in non-phagocytic Vero cells. In vitro growth of *B. abortus* has been reported in HeLa cells (37) and in primary cultures of hamster kidney cells (13), chicken embryo fibroblasts (17), and different adult and fetal bovine cells (5, 29, 30). All *B. abortus* strains tested entered and replicated within Vero cells. In contrast, Holland and Pickett (17) reported that in chicken embryo fibroblasts cultures, non-smooth variants and the attenuated strain 19 were either destroyed or failed to replicate intracellularly. This discrepancy could be explained by the low multiplicity of infection (MOI) used by these authors. We found that with strain 19, no infection could be detected when Vero cells were exposed to less than 120 organisms per cell. In ovo, *B. abortus* strain 19 replicates within chicken embryo fibroblasts (6).

Electron microscopic analysis showed that *B. abortus* replicated within rough endoplasmic reticulum (RER) of Vero cells. As in trophoblasts of experimentally infected goats (1) and cows (25), intracellular brucellae were within cisternae which (i) were lined by ribosomes on their cytoplasmic faces, (ii) were continuous to normal RER, (iii) had outer membranes continuous with outer nuclear membranes, and (iv) had lumens continuous with the perinuclear envelope, which also contained brucellae. Among intracellular bacteria, only *Legionella*
pneumophila has been described in a similar intracellular location; it inhabits ribosome-lined cisternae in phagocytic (9, 18) and non-phagocytic (28) cells. However, continuity between these cisternae and RER or perinuclear envelope has not been reported. In addition, in vitro studies with blood monocytes (9) indicate that internalized legionellae are enclosed in vacuoles which are later surrounded by ribosomes, suggesting that L. pneumophila inhabits ribosome-studded phagosomes rather than RER cisternae.

Transfer to the RER may be required for unrestricted growth of B. abortus within non-phagocytic cells. Depending on the strain, B. abortus was internalized by 30 to 50% of the Vero cells. However, when estimated 2 days post-inoculation (PI), the maximal percentage of infected cells was 10%. Ultrastructurally, in cells with abundant intracellular organisms, B. abortus was always located within RER cisternae, while in cells containing few isolated brucellae, these were within phagosomes or phagolysosomes. This suggests that transfer to the RER, not internalization, is the limiting step for replication of B. abortus in Vero cells, and that brucellae that fail to enter the RER are eventually destroyed or eliminated by Vero cells. Differences in virulence between smooth and rough strains may be correlated to differences in their ability to gain access to RER.

Failure of rough B. abortus strains to replicate within some infected Vero cells, may also result from a higher susceptibility of rough strains to intracellular killing. Although rough attenuated brucellae invaded a higher proportion of Vero cells, the percentage of
infected cells, two days PI, was lower than with smooth virulent bacteria. Lack of O polysaccharide side chain in rough B. abortus is correlated to their higher susceptibility to killing by bovine serum (4). Similarly, components of cell wall LPS are important for the intracellular survival and replication of brucellae in phagocytic cells (11, 31, 32). In vitro, cell walls of smooth brucellae are more resistant to digestion by hydrolytic enzymes (23). However, studies with extracts of neutrophil and macrophage granules, indicate that both smooth and rough brucellae are resistant to lysosomal enzymes (32). Greater susceptibility of rough strains to intracellular killing by phagocytic cells, seems to depend on the myeloperoxidase-H$_2$O$_2$-halide system (32), which is probably not active in non-phagocytic cells.

The double fluorescence labeling procedure provides a useful tool to discriminate microscopically between intra- and extracellular organisms. Differentiation between the two locations of bacteria is the most difficult problem in microscopic assessment of cell culture monolayer penetration. Different methods have been proposed to overcome this problem, e.g., optical sectioning by light microscopy (26), fluorescence quenching technique (15), combined differential interference (Normarski) and ultraviolet light microscopy (3), combined immuno-fluorescence and bright field light microscopy (10, 20), and double immuno-fluorescence microscopy (16). These methods are cumbersome and slow, as for each determination they require the observation of either the same field under two different conditions (3, 15, 16, 26), or of two differently processed samples (10, 20).
addition, some of these methods (15) necessitate the observation of unfixed material, a hazardous situation when working with human pathogens like *B. abortus*. Our procedure allows identification of both intra- and extracellular organisms within the same microscopical field. By reducing the number of samples, it provides an easier, safer, faster and more accurate procedure.

Despite a high multiplicity of infection (MOI; ± 1.1 x 10⁴ brucellae/cell), *B. abortus* infected only a small percentage of cells in a monolayer. Similar observation is reported for *B. melitensis* infection of BHK21 cells (hamster kidney fibroblasts); the percentage of infected cells never increased above 20%, even with a MOI of 1,000 bacteria/cells and an inoculation period of 18 hours (34). This suggests that monolayers are heterogeneous in terms of susceptibility and/or that *B. abortus* are heterogeneous in terms of invasiveness. In favor of the first alternative is the observation that, when incubated with Vero monolayers *S. typhimurium* and *Sh. flexneri* invade less than 1% of the cells (2), while with Hela (8) and Henle 407 (10) monolayers more than 50% of the cells are infected. In our study however, a low percentage of infected cells was observed with 5 different cell lines. This may indicate that only a fraction of the *Brucella* inoculum express the invasive phenotype.

Differences in adherence and invasiveness between smooth and rough strains of *B. abortus* are correlated to differences in physico-chemical properties between the 2 types of brucellae. Adherence to cell surface is required for internalization by phagocytic cells, and this adherence
is profoundly affected by electrostatic charge and hydrophobicity of both host cells and bacteria (19, 21, 36). Correlation between bacterial hydrophobicity and the degree to which they associate with cells in culture has been demonstrated for S. typhimurium (21) and Y. enterocolitica (36). Loss of polysaccharide side chains increases the hydrophobicity of rough bacteria; this was reported for organisms like S. typhimurium (24) and E. coli (33) and is confirmed here for B. abortus, using the hydrocarbon adherence method (33). Centrifugation and DEAE-dextran treatment increased the percentage of infected Vero cells, indicating that B. abortus attachment is also, at least in part, charge-dependent. The positively charged DEAE-dextran and centrifugation enhances bacterial attachment by reducing electrostatic repulsion between negatively charged bacteria and host cells (12, 39).

Invasion of non-phagocytic cells by smooth B. abortus may be correlated to their virulence in vivo. The attenuated vaccine strain 19 infected a smaller percentage of Vero cells than the virulent strains 2308S and 544. This difference was probably not the result of a lower ability to enter RER or of decreased intracellular survival, as suggested for rough B. abortus, since with strain 19, fewer organisms entered Vero cells. Decreased virulence of strain 19 has been attributed to its inability to metabolize erythritol (38). Although we have not tested our Vero cell cultures for the production of erythritol, we feel it unlikely that differences in erythritol metabolism are responsible for the observed difference in invasiveness.

Cellular invasion by B. abortus is not restricted by the tissue or
species of origin of the eucaryotic cell. *B. abortus* 2308S was not more invasive in cells of bovine (MDBK) or trophoblastic (Jeg-3) origin. *B. abortus* tropism for ruminant placentae because of its content of progesterone has been suggested (1). Progesterone, which in vitro enhances *B. abortus* growth (27), is secreted by bovine trophoblasts in late gestation (14). Despite production of high levels of progesterone, Jeg 3 cells were not more susceptible to *B. abortus* invasion. Failure of *B. abortus* to invade MDCK cells is not due to a lack of susceptibility of these cells to bacterial invasion, e.g., *Shigella* sp., *Salmonella* sp. and *Yersinia* sp. have been shown to invade MDCK cells (7). However, while the 4 species of *Shigella* tested were able to invade Hep-2 (human larynx epithelia) and CHO (chinese ovary) cells, only one, *Sh. flexneri*, invaded MDCK cells, indicating that MDCK cells may be less receptive to bacterial invasion.
LITERATURE CITED


INTERNALIZATION AND INTRACELLULAR GROWTH OF BRUCELLA ABORTUS IN VERO CELLS: EFFECTS OF ENDOCYTIC AND METABOLIC INHIBITORS
Internalization and Intracellular growth of *Brucella abortus* in Vero cells: Effects of Endocytic and Metabolic Inhibitors

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Running Head: Vero cells infection by *B. abortus*

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ABSTRACT

Uptake, transfer to rough endoplasmic reticulum (RER), and intracellular growth of *B. abortus* was studied in Vero cells treated with endocytic and metabolic inhibitors. Infection of Vero cells was suppressed when inhibitors of energy metabolism (iodoacetate, dinitrophenol), receptor-mediated endocytosis (monodansylcadaverine, amantadine, methylamine), or endosomal acidification (chloroquine, ammonium chloride, monensin) were added to the inoculum. No inhibition was observed when these drugs were added after the inoculation period. Infection of Vero cells by *B. abortus* was inhibited by dibutyryl-cyclic adenosine monophosphate (cAMP) and *Vibrio cholerae* enterotoxin but was stimulated by dibutyryl-cyclic guanosine monophosphate (cGMP) and *Escherichia coli* STa enterotoxin. Uptake of *B. abortus* by Vero cells was not prevented by colchicine, but was abolished by cytochalasin B. Uptake of heat-killed *B. abortus* and non-invasive *E. coli* was similar to that of viable brucellae. Intracellular growth of *B. abortus* was not affected by cycloheximide. These results suggest that (i) *B. abortus* is internalized by a receptor-mediated phagocytic process, (ii) transfer of *B. abortus* from phagosomes to RER requires endosomal acidification and (iii) replication of *B. abortus* within RER does not depend on protein synthesis by the host cell.
Bacteria of the genus *Brucella* have been shown to localize and replicate within the rough endoplasmic reticulum (RER) of non-phagocytic host cells (2, 10, 11, 12, 30). In pregnant ruminants, where it causes placentitis and abortion, *Brucella abortus* invades placental trophoblasts (2, 30). The process in trophoblasts involves penetration, transfer to cisternae of the RER, and intracisternal growth. This site of replication is in contrast to the intraphagosomal growth of *B. abortus* in macrophages. We have suggested that transfer to RER is the rate limiting step in *B. abortus* infection of Vero cells (11). We believe that internalized *B. abortus* are able to induce this transport to RER cisternae, where unrestricted intracellular replication takes place.

Phagocytosis is a particle-activated, microfilament-dependent variant of endocytosis whereby eucaryotic cells take up particulate materials (26, 31). Intracellular bacteria enter cells with low phagocytic capacity by "parasite-specified endocytosis". Attachment to host-cell surface receptors is generally assumed to be a prerequisite for bacterial invasion (25). As bacteria are internalized, protons are pumped into the phagosome causing it to become acidic (31, 40). This decrease in pH is a critical step in most endocytic pathways, since it determines the subsequent sorting and fate of incoming receptors and ligands (31). Endosomal acidification is required by several enveloped viruses to escape the endosome before lysosomal fusion (21, 31, 42). A
recent study however, indicates that endosome acidification is not required for invasion and intracellular replication of *Salmonella cholerae-suis*, *Shigella flexneri* and *Yersinia enterocolitica* in cultured cells (15).

Each step of the endocytic pathway can be altered by chemicals. Phagocytosis is inhibited by drugs that block actin polymerization, and, as microfilament contraction requires energy, by inhibitors of glycolysis or oxidative phosphorylation (6, 20, 26, 36). Phagocytosis can also be modulated by altering intracellular cyclic nucleotide levels (20, 43, 41). Inhibitors of receptor-mediated endocytosis prevent the clustering of cell surface receptors, or interfere with receptor recycling (7, 14, 31, 39). Endosomal acidification can be inhibited by lysosomotropic weak bases and cationic ionophores (31, 40). In their unprotonated form, lysosomotropic agents enter acidic vesicles where they become protonated and too polar to escape; accumulation of these weak bases neutralizes the acid pH of the vesicle (8, 31). Cationic ionophores permit exchange of protons for potassium ions, effectively blocking the establishment of a proton gradient across the acidic vesicle membrane (40).

This study was designed to investigate the role of the host cell in uptake and transfer to the RER of *B. abortus*. Drugs that affect different steps of the endocytic pathway were tested for their effects on the infection of Vero cells by *B. abortus*. The role of *B. abortus* in inducing its own uptake and the dependency of *B. abortus* on host-cellular protein synthesis for intracellular growth, were also assessed.
MATERIALS AND METHODS

Microorganisms

*B. abortus* strains 2308S (virulent) and 19 (attenuated) (11), were used in this study. Non-invasive *Escherichia coli*, isolated from liver of turkeys with colisepticemia, were used as control: Strain AV02 (02:K-:H6) is inconsistently piliated and avirulent for turkey poult's, strain V078 (078:K80:H9) expresses type I pili and is highly virulent (3, 13). *E. coli* were grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI), washed twice in 0.85% NaCl and standardized turbometrically to a concentration of $1 \times 10^{10}$ CFU/ml.

For monolayer infection, a standardized bacterial suspension was diluted, to a final concentration of $1.0 \times 10^9$ CFU/ml, in Eagle minimum essential medium (MEM; GIBCO Laboratories, Grand Island, NY) supplemented with L-glutamine (2 mM) and 10% fetal calf serum (FCS).

Monolayer Infection

Vero cells (African green monkey kidney fibroblasts), obtained from the National Veterinary Services Laboratories (NVSL; Ames, Iowa), were used as host-cells. Cell culture methods and infection procedure were as described (11). Briefly, subconfluent monolayers on coverslips, were inoculated with 2 ml of the bacterial suspension. The inoculum was centrifuged onto monolayers for 20 minutes at 550g. After 8 h
incubation in an humidified incubator with an atmosphere of 5% CO₂ at 37°C, the coverslips were washed in phosphate buffered saline (PBS; pH 7.0) and further incubated in fresh MEM supplemented with 0.25% FCS and 50 μg/ml gentamycin (Gentocin; Sterling Corporation, Kenilworth, NJ), to kill extracellular bacteria. Medium was replaced at 24 hours. The inoculation period was defined as the period between addition of the bacterial suspension and introduction of gentamycin in culture medium.

In experiments where coverslips were sampled 4, 8 and/or 24 h after exposure to bacterial suspension, the 4 and 8 h samples were incubated in presence of gentamycin for 1 h before immunolabeling while the 24 h samples were treated as described above.

All experiments were carried out in triplicate and were repeated at least twice for each experimental treatment.

Heat Inactivation of Bacteria

Several 2 ml samples of bacterial suspension in 0.85% NaCl containing 1 x 10¹⁰ CFU/ml were placed in 20 ml test tubes and incubated for 1 hour in a 65°C water bath. After heat treatment, bacterial suspensions were cooled and diluted to a final concentration of 1 x 10⁹ CFU/ml in MEM supplemented with 10% FCS and 2 mM L-glutamine; loss of viability was confirmed by culture.
Coverslips, sampled 48 hours after inoculation were stained (11) by indirect immuno-fluorescence using as primary antibody, a rabbit anti-\( \text{E. abortus} \) 2308S antiserum, and as secondary antibody, a goat anti-rabbit FITC-conjugated antiserum (NVSL, Ames, IA). After immunolabeling, coverslips were treated for 5 min with a 25 \( \mu \text{g/ml} \) solution of Propidium iodide (Sigma Chemical Co., St. Louis, MO) and examined by epifluorescence microscopy.

In selected experiments, the procedure to discriminate between extra-and intracellular bacteria was used, e.g., the primary antibody was applied for 30 min at \( 4^\circ \text{C} \) prior to methanol-acetone fixation (11). Rabbit anti-\( \text{E. coli} \) 02 and 078 antisera were used as primary antibodies for \( \text{E. coli} \) labeling.

**Enumeration of Infected Cells**

For each of 2 or 3 coverslips, the number of cells per \( \text{mm}^2 \) was estimated, at a total magnification of 400x, by counting cell nuclei in 5 fields located along a diagonal across the coverslip. The surface of the area was defined by a reticle in one eye piece of the microscope. The number of infected cells per \( \text{mm}^2 \) was then estimated, at a total magnification of 100x, by counting the number of infected cell in 25 fields arranged in 5 rows and 5 columns. Results were expressed as percentage of infected cells.
In selected experiments, the percentage of Vero cells containing 0, 1, 2, 3 to 5, 6 to 10 and more than 10 intracellular bacteria was estimated by examining, at a magnification of 1,000x, a total of 200 cells on each of 2 or 3 coverslips.

Chemicals

The following chemicals were purchased from Sigma Chemical Co. (St Louis, MO): Amantadine hydrochloride, cholera toxin (from *Vibrio cholerae*), diphosphate salt of chloroquine, cytochalasin B, cycloheximide, sodium salt of N\(^6\),2'-0-dibutyryl adenosine 3':5'-cyclic monophosphate (dibutyryl-cAMP), sodium salt of N\(^2\),2'-0-dibutyryl guanosine 3':5'-Cyclic monophosphate (dibutyryl-cGMP), monodansylcadaverine, 2,4-dinitrophenol, *Escherichia coli* heat-stable enterotoxin (STa), iodoacetic acid and methylamine hydrochloride. Sodium salt of monensin was obtained from Calbiochem (La Jolla, CA). Colchicine was from Nutritional Biochemical Corporation (Cleveland, OH) and ammonium chloride from Mallinckrodt Inc. (Paris, KY).

Treatment of Vero Cells with Metabolic Inhibitors

Chemicals were dissolved in PBS; except for amantadine hydrochloride dissolved in Tris (pH 7.6), colchicine and cytochalasin B dissolved in dimethyl sulfoxide (DMSO; Matheson Coleman & Bell, Norwood, OH) and monensin dissolved in ethanol. Dinitrophenol and
monodansylcadaverine were solubilized using an ultrasonic cleaner (L&R Manufacturing Company, Kearny, NJ) to expedite solubilization of the crystals. After solubilization and first dilution in PBS or Tris, all solutions were forced through a 0.22 μm filter (Millipor, Bedford, MA) to achieve sterility and appropriate dilutions were prepared in MEM. For each chemical, fresh solutions were prepared the day before an experiment and were kept overnight at 4°C.

Unless otherwise specified, 50, 100 or 200 μl of a metabolic inhibitor solution was added to the monolayer concomitant with the 2 ml Brucella inoculum. Experimental controls included addition to the bacterial inoculum of equivalent amounts of fresh MEM or MEM with appropriate dilutions of DMSO or ethanol.

The effect on B. abortus viability of the highest concentration of each chemical used in the present study was estimated. At the end of the inoculation period (8 h after inoculation), the number of viable brucellae in the supernatant of treated monolayers was compared to that in controls, using standard plate counting procedures.
RESULTS

Effects of Chemicals on*B. abortus* Viability

Of 15 chemicals tested, only methylamine decreased the number of viable brucellae by more than 25% (Table 1). At its highest concentration (100 mM), methylamine had a bacteriostatic effect on *B. abortus*, e.g., the number of organisms in the inoculum did not increase during the 8 h inoculation period. At lower concentrations, methylamine had no effect on *B. abortus* viability (data not shown).

Effects of Microfilament and Microtubule Inhibitors on Infection of Vero Cells

Cytochalasin B totally inhibited the infection of Vero cells by *B. abortus* (Fig. 1B). Colchicine decreased the number of infected Vero cells (Table 2) and induced polymicronuclei in many infected and non-infected cells (Fig. 1C).

Effects of Metabolic and Endocytic Inhibitors on Infection of Vero Cells

When added to the inoculum, inhibitors of host-cell energy metabolism (Table 3), of receptor mediated endocytosis (Table 4), and of endosomal acidification (Table 5), suppressed the infection of Vero cell
Table 1. Effects of different chemicals on viability of *B. abortus* 2308S in culture medium placed on Vero cells

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Log\textsubscript{10} CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>9.32</td>
</tr>
<tr>
<td>Colchicine</td>
<td>2.0 (\mu g/ml)</td>
<td>9.34 (+4) (c)</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>2.5 (\mu g/ml)</td>
<td>9.28 (-8)</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>0.02 mM</td>
<td>9.30 (-4)</td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>0.50 mM</td>
<td>9.24 (-16)</td>
</tr>
<tr>
<td>Monodansylcadaverine</td>
<td>0.5 mM</td>
<td>9.32 (-16)</td>
</tr>
<tr>
<td>Amantadine</td>
<td>2.5 mM</td>
<td>9.20 (-23)</td>
</tr>
<tr>
<td>Methylamine</td>
<td>100 mM</td>
<td>9.03 (-48)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>100 (\mu M)</td>
<td>9.32 (+1)</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>50 mM</td>
<td>9.21 (-21)</td>
</tr>
<tr>
<td>Monensin</td>
<td>12 (\mu M)</td>
<td>9.23 (-18)</td>
</tr>
<tr>
<td>Dibutyryl-cAMP</td>
<td>1.0 mM</td>
<td>9.41 (+23)</td>
</tr>
<tr>
<td>-cGMP</td>
<td>1.0 mM</td>
<td>9.28 (-9)</td>
</tr>
<tr>
<td><em>E. coli</em> STa enterotoxin</td>
<td>100 (\mu g/ml)</td>
<td>9.31 (0)</td>
</tr>
<tr>
<td><em>V. cholerae</em> enterotoxin</td>
<td>10 (\mu g/ml)</td>
<td>9.33 (0)</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>2.5 (\mu g/ml)</td>
<td>9.24 (-17)</td>
</tr>
</tbody>
</table>

\(a\) The number of viable *B. abortus* in the culture medium 8 h after inoculation was estimated by plate counting (mean of 2 plates).

\(b\) Highest concentration used in this study.

\(c\) Percentage change in viable count compared to chemical-free control.
Figs. 1 A-D. Light micrographs of Vero cells infected for 8h with *B. abortus* 2308S in the presence of various metabolic inhibitors. Monolayers were stained, 48 h postinoculation by indirect immunofluorescence for *B. abortus* and counterstained with propidium iodide. (A) Positive control monolayer showing 7 infected cells. (B) Colchicine (2 μg/ml): Several infected cells have polynuclear nuclei (arrows). (C) Cytochalasin B (2.5 μg/ml): Lack of infected cells, but presence of scattered extra and intracellular bacterial debris. (D) Cycloheximide (2.5 μg/ml) during and after inoculation: Decreased number of Vero cells/mm² and presence of numerous infected cells (arrows). Bar = 40 μm.
Table 2. Effects of inhibitors of microfilament (cytochalasin B) and microtubule (colchicine) on infection of Vero cells by *B. abortus* strains 19 and 2308S

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Percentage of infected cells (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ug/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain 19 (attenuated)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0.68 ± 0.20</td>
<td>--</td>
</tr>
<tr>
<td>colchicine</td>
<td>2</td>
<td>0.36 ± 0.07</td>
<td>53</td>
</tr>
<tr>
<td>cytochalasin B</td>
<td>2.5</td>
<td>0.00 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>Strain 2308S (virulent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>3.24 ± 0.54</td>
<td>--</td>
</tr>
<tr>
<td>colchicine</td>
<td>2</td>
<td>2.13 ± 0.09</td>
<td>66</td>
</tr>
<tr>
<td>cytochalasin B</td>
<td>2.5</td>
<td>0.05 ± 0.02</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>All treatments were concomitant with exposure to *B. abortus* for 8 h. Data are expressed as the percentage of infected Vero cells ± standard error.
Table 3. Effects of inhibitors\textsuperscript{a} of energy metabolism on infection of Vero cells by \textit{B. abortus} 2308S

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Percentage of infected cells (%)\textsuperscript{b}</th>
<th>Percent of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodoacetate</td>
<td>0</td>
<td>2.29 ± 0.46</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>1.72 ± 0.23</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.18 ± 0.13</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.43 ± 0.21</td>
<td>19</td>
</tr>
<tr>
<td>2,4-dinitrophenol</td>
<td>0</td>
<td>2.29 ± 0.46</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.44 ± 0.20</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.17 ± 0.21</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.60 ± 0.05</td>
<td>26</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Inhibitors of glycolysis (iodoacetate) and oxidative phosphorylation (dinitrophenol).

\textsuperscript{b}All treatments were concomitant with exposure to \textit{B. abortus} for 8 h. Data are expressed as the percentage of infected Vero cells ± standard error.
Table 4. Effects of inhibitors of receptor-mediated endocytosis on infection of Vero cells by *B. abortus* 2308S

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Percentage of infected cells (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>monodansyl-cadaverine</td>
<td>0</td>
<td>1.02 ± 0.14</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>1.03 ± 0.20</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.63 ± 0.09</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.02 ± 0.02</td>
<td>2</td>
</tr>
<tr>
<td>amantadine</td>
<td>0</td>
<td>3.78 ± 0.53</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2.59 ± 0.07</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.31 ± 0.09</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.03 ± 0.01</td>
<td>1</td>
</tr>
<tr>
<td>methylamine</td>
<td>0</td>
<td>1.94 ± 0.46</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.75 ± 0.24</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.23 ± 0.06</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.10 ± 0.02</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>All treatments were concomitant with exposure to *B. abortus* for 8 h. Data are expressed as the percentage of infected Vero cells ± standard error.
Table 5. Effects of inhibitors of endosomal acidification on infection of Vero cells by *B. abortus* 2308S

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Percentage of infected cells (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chloroquine</td>
<td>0 µM</td>
<td>3.78 ± 0.53</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>3.15 ± 0.15</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>0.81 ± 0.03</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>0.14 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>ammonium chloride</td>
<td>0 mM</td>
<td>1.13 ± 0.25</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>1.38 ± 0.03</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>25 mM</td>
<td>0.53 ± 0.04</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>0.13 ± 0.04</td>
<td>12</td>
</tr>
<tr>
<td>monensin</td>
<td>0 µM</td>
<td>1.13 ± 0.25</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>0.55 ± 0.10</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>2 µM</td>
<td>0.42 ± 0.11</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>3 µM</td>
<td>0.14 ± 0.02</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>6 µM</td>
<td>0.06 ± 0.01</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup>All treatments were concomitant with exposure to *B. abortus* for 8 h. Data are expressed as the percentage of infected Vero cells ± standard error.
by \textit{B. abortus}, in a dose-dependent fashion. Among the inhibitors of receptor-mediated endocytosis, methylamine was much less effective than both monodansylcadaverine and amantadine.

To determine if the effect of these inhibitors was restricted to the internalization and transfer to the RER phases of \textit{B. abortus} invasion of Vero cells, concentrations of drugs giving maximum inhibition (Tables 3-5) were added to monolayers at various times after the inoculation period. Results from one representative drug from each group of inhibitors are presented in Fig. 2. With all drugs, maximal inhibition was observed when treatment was concomitant with inoculation. When added for 6 h starting at the end of the inoculation period, while the inhibitory effect of energy metabolism and receptor-mediated endocytosis inhibitors was significantly reduced, maximal inhibition was still achieved by inhibitors of endosomal acidification, i.e., chloroquine. When added for 10 h, starting 6 h after the end of the inoculation period, the inhibitory effect of all drugs was almost completely abolished.

\textbf{Effects of Cyclic Nucleotides on Infection of Vero Cells}

Dibutyryl-cAMP and dibutyryl-cGMP are cAMP and cGMP analogs which traverse intact cell membranes. Treatment of Vero cells with dibutyryl-cAMP reduced the percentage of \textit{B. abortus} infected cells in a dose-dependent manner (Fig. 3). In contrast, at concentrations in the range of 0.1 to 1 \textmu M, dibutyryl-cGMP induced approximately a 30% increase in
Fig. 2. Effect of various metabolic inhibitors on Vero cell infection by *B. abortus* 2308S. Dinitrophenol (0.5 mM), monodansyl-cadaverine (0.5 mM) and chloroquine (0.1 mM) were added: For 8 h during the inoculation period (■); for 6 h starting at the end of the inoculation period (□); or for 10 h, starting 6 h after the end of the inoculation period (▲). Data are expressed as percentage of control.
Fig. 3. Effect of dibutyryl-cAMP (○) and dibutyryl-cGMP (●) on Vero cell infection by B. abortus 2308S. Treatment were concomitant with exposure to B. abortus for 8 h. Data are expressed as the percentage of infected cells estimated microscopically 48 h after inoculation (mean of 2 determinations ± standard error).
Control Concentration of Cylic Nucleotide (M)

% of Infected Vero Cells

Concentration of Cylic Nucleotide (M)
the number of infected cells, while at 0.01 μM and above 10 μM it had no effect (Fig. 3).

These results were confirmed using the enterotoxin of *Vibrio cholerae* (choleragen) (24), which induces cAMP production in mammalian cells, and the heat-stable enterotoxin (STa) of *Escherichia coli*, which increases cGMP formation in mammalian enterocytes (18, 37). When the inoculum contained as little as 0.1 μg/ml of choleragen, a significant inhibition of infection by *B. abortus* was observed (Fig. 4). Addition of small amounts of STa toxin to the *B. abortus* inoculum, resulted in a 30% larger percentage of infected Vero cells (Fig. 4). The effect of STa toxin was maximal at concentrations of 0.1 and 1 μg/ml. In two preliminary experiments, a 50 and 70% increase in the percentage of infected cells was observed with 0.1 μg/ml STa toxin (data not shown). STa toxin had no effect on the number and morphology of Vero cells, at any of the concentrations used.

Effects of Protein Synthesis Inhibition on Infection of Vero Cells

When Vero cells were incubated overnight in the presence of cycloheximide (2.5 μg/ml), their ability to internalize *B. abortus* was reduced (Table 6). When cycloheximide was added during or after the inoculation period and maintained in the culture medium throughout the subsequent incubation, the percentage of infected cells was not significantly reduced (Table 6). Following cycloheximide treatment, Vero cells had reduced cytoplasmic and nuclear volumes and infected
Fig. 4. Effect of *Vibrio cholerae* enterotoxin (O) and *Escherichia coli* STa enterotoxin (●) on Vero cell infection by *B. abortus* 2308S. Treatments were concomitant with exposure to *B. abortus* for 8 h. Data are expressed as the percentage of infected cells estimated microscopically 48 h after inoculation (mean of 2 determinations ± standard error).
Table 6. Effects of cycloheximide (2.5 µg/ml) on infection of Vero cells by *B. abortus* strains 19 and 2308S

<table>
<thead>
<tr>
<th>Period of exposure</th>
<th>Strain 19 (attenuated)</th>
<th>Strain 2308 (virulent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- before inoculation</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30 %&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>- during and after inoculation</td>
<td>72 %</td>
<td>81 %</td>
</tr>
<tr>
<td>- after inoculation</td>
<td>61 %</td>
<td>76 %</td>
</tr>
</tbody>
</table>

<sup>a</sup>Not done.  
<sup>b</sup>Percentage of control.
Relative Efficiency of Uptake of Viable and Heat-Killed *B. abortus* by Vero Cells

To determine if metabolic activity on the part of infecting brucellae is a prerequisite for entry into host-cells, parallel samples were exposed to viable and heat-killed brucellae. From a previous study (5), we know that following heating for 1 hour at 65°C, *B. abortus* remains ultrastructurally intact.

At 4 h post inoculation, there was no difference in intracellular numbers of viable and heat-killed brucellae (Fig. 5A). In monolayers exposed to viable brucellae, the number of infected cells and the average number of bacteria per cell increased with time (Fig. 5A). In samples exposed to heat-killed *B. abortus*, those parameters remained constant throughout the experiment (Fig. 5B).

Relative Efficiency of Uptake of *E. coli* and *B. abortus* by Vero Cells

To determine if uptake of *B. abortus* by Vero cells is a bacterium-induced process (versus non-specific uptake of adherent particles), the uptake of heat-killed *B. abortus* 2308S was compared to that of two strains of non-invasive *E. coli*.

There was no significant differences in the number of associated (extra- and intracellular) as well as internalized bacteria between *B.*
Figs. 5 A-B. Interactions of viable (A) and heat-killed (B) *B. abortus* 2308S with Vero cells after 4, 8 and 24 h of incubation. The 4 and 8 h samples were treated for 1 hour with gentamycin before fixation and staining. For the 24 h samples, the inoculum was removed 8 h postinoculation and replaced by fresh medium containing gentamycin. Data are grouped according to the number of intracellular bacteria per cell, estimated for 200 Vero cells. Each bar represent the mean of 2 samples.
abortus and E. coli (Fig. 6). As pili might affect E. coli adherence to
Vero cells, piliation of both E. coli strains was checked by slide
agglutination using a type I pili specific rabbit antiserum. Both pre-
and post-inactivation samples of strain V078 were positive for type I
pili, while strain AV02 samples were negative.
Figs. 6 A-B. Interactions of heat-killed *R. abortus* 2308S and heat-killed *Escherichia coli* AV02 and V078 with Vero cells after 4 h of incubation. Data are grouped according to the number of associated (A) or intracellular (B) bacteria per cell, estimated for 200 Vero cells. Each bar represent the mean of 3 samples.
Results of this study suggest that *B. abortus* is internalized by a receptor-mediated phagocytic process but does not induce its own uptake. This study indicates that transfer of *B. abortus* to the RER requires endosome acidification.

*B. abortus* enters Vero cells by phagocytosis, e.g., by a microfilament-dependent and energy-requiring process that can be modulated by intracellular cAMP and cGMP levels. Similar observations were made for *Sh. flexneri* in a human intestinal epithelial cell line (20). Further evidence that bacterial penetration occurs through phagocytosis, is provided by the observation that invasive *Sh. flexneri* induce actin polymerization and myosin accumulation at the site of entry into host-cells (6). Ultrastructurally, ingestion of *B. abortus* by bovine ileal dome lymphoepithelial (M cells) cells, by yolk-endodermal cells and by Vero cells, has characteristics of phagocytosis (1, 10, 12).

Metabolic activity by *B. abortus* is not required for bacterial uptake by Vero cells. Uptake of heat-killed *B. abortus* and of non-invasive heat-killed *E. coli* strains by Vero cells was similar to that of viable brucellae. In contrast, salmonellae actively induce their internalization by synthesizing new bacterial proteins upon contact with target cells (16). In ligated ileal loops, *B. abortus* enters lymphoepithelial cells (M cells) but not enteroabsorptive cells (1), suggesting that *B. abortus* can only enter endocytically active cells.
We believe that *B. abortus* is passively internalized by its host-cells and that invasion of bovine trophoblasts is the consequence of their high phagocytic activity (2, 4, 35).

Endosome acidification may be required for *B. abortus* transfer to the RER. Expression of bacterial virulence genes is often influenced by environmental factors (32). Cholera toxin production by *Vibrio cholerae* is regulated by the *toxR* gene which encodes a transmembrane DNA-binding protein that may sense a variety of signals, including osmolarity, pH and the presence of certain amino acids (33, 34). Similarly, intracellular replication of *Coxiella burnetii* is triggered by phagolysosomal acidification (19). We suggest that endosomal acidification activates bacterial genes whose product(s) direct the transfer of *B. abortus* to RER.

Inhibitors of endosomal acidification could prevent the uptake of *B. abortus* rather than its transfer to the RER. By interfering with the uncoupling of receptor-ligand complexes, these drugs interfere with receptor recycling (8, 14, 31); consequently surface receptors remain internalized and unavailable for bacterial interaction (14). However, inhibition of endosomal acidification does not prevent the entry of *S. cholerae-suis*, *Sh. flexneri*, and *Y. enterocolitica* into epithelial cells (15). In addition, ultrastructural studies provide evidence that lysosomotropic agents have no effect on the binding and endocytosis of Semliki Forest and influenza viruses, but inhibit their release from endocytic vesicles (22, 42).

Studies with inhibitors of receptor-mediated endocytosis (RME)
suggest that receptor clustering on the surface of Vero cells is necessary for the uptake of *B. abortus*. Covalent coupling of dansylcadaverine to cellular membranes might account for its ability to interfere with RME (7). Fluorescence and electron microscopy provide evidence that amantadine, dansylcadaverine and methylamine, but not chloroquine, block $\alpha_2$-macroglobulin clustering on fibroblasts (29, 39). Similarly, uptake of *S. cholerae-suis* and *Y. enterocolitica* by epithelial cells, is inhibited by dansylcadaverine (14). However, the possibility that RME inhibitors decreased the percentage of *Brucella*-infected cells by interfering with endosomal acidification (8), can not be ruled out.

Microtubule integrity is not required for uptake of *B. abortus* by Vero cells, but may be necessary for its transfer to the RER. Many cells with polymicronuclei, contained intracellular brucellae indicating that despite microtubule disruption, colchicine failed to inhibit bacterial uptake. Microtubules play an important role in directing cytoplasmic vesicles involved in secretion (23, 38, 43) as well as in endocytosis and transcytosis (28, 43), i.e., microtubule-acting drugs interfere with the polarized delivery of influenza virus hemagglutinin to the apical surface of Madin-Darby canine kidney (MDCK) cells (38).

Infectivity of *B. abortus* for Vero cells can be modulated by interfering with intracellular cAMP and cGMP levels. In human leukocytes, phagocytosis and lysosomal enzyme release appear to be under bidirectional cyclic nucleotide control, with cAMP acting as an inhibitor and cGMP as an activator (43). Similar modulation by cyclic
nucleotides may govern *Chlamydia trachomatis* infectivity for HeLa cells (41) and invasion of Henle cells by *Sh. flexneri* (20). The STa-induced stimulation of *B. abortus* uptake by Vero cells was probably consecutive to guanylate cyclase activation, since similar increases were observed following cGMP treatment. However, STa-induced cGMP accumulation is considered tissue-specific (18, 37) and morphologically Vero cells are insensitive to STa enterotoxin (17, 27).

*B. abortus* does not require host-cell protein synthesis for intracellular replication, e.g., growth of *B. abortus* within Vero cells was not affected by cycloheximide treatment. Cycloheximide increases the infectivity of certain strains of *C. psittaci* (9), supposedly by increasing the pool of cellular nutrients available to the parasite (9, 21); a similar effect was not observed with *B. abortus*. In contrast, when added before inoculation, cycloheximide reduced the percentage of *Brucella*-infected cells. This decrease might have resulted from depletion in cellular factors necessary for bacterial uptake.


ENTRY AND INTRACELLULAR LOCALIZATION OF BRUCELLA SPP.
IN VERO CELLS: FLUORESCENCE AND ELECTRON MICROSCOPY
Entry and Intracellular localization of *Brucella* spp. in Vero Cells: Fluorescence and Electron Microscopy

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Running Head: *Brucella* spp. in Vero cells

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Vero cells were inoculated with the six species of *Brucella* and examined by fluorescence and electron microscopy. All *Brucella* spp. were internalized by Vero cells. Numbers of intracellular brucellae increased with time after inoculation except with *B. canis*. Intracellular brucellae were first seen within phagosomes and phagolysosomes. Subsequent localization and replication within cisternae of the rough endoplasmic reticulum were seen with all species of *Brucella*, except *B. canis*. Intracellular *B. canis* were restricted to phagolysosomes. These results suggest that phagocytized *Brucella* spp. are transferred: (i) to cisternae of the rough endoplasmic reticulum, where unrestricted bacterial replication takes place; or (ii) to phagolysosomes in which *Brucella* spp. fail to replicate. We believe that the various strains of *Brucella* spp. differ in their ability to induce their own transfer to the rough endoplasmic reticulum.
Bacteria of the genus Brucella are intracellular pathogens of man and animals, causing zoonoses of worldwide importance. The genus Brucella, comprises three principal species originally differentiated on the basis of their major animal sources, i.e., B. melitensis in goats, B. abortus in cattle, and B. suis in swine (7, 18). Cross-infections, however, do occur as most species of mammals are susceptible to Brucella (18). Because it survives almost exclusively in animals, Brucella has the tendency to acquire different phenotypes when infecting new hosts (7, 18). This led to the description of three minor species, i.e., B. ovis in sheep (3), B. canis in dogs (4) and B. neotomae in desert wood rats (36), and of numerous variants such as B. suis biovar 2 that infect hares (6) and B. suis biovar 4, naturally pathogenic for reindeer and caribou (24).

Brucellae produce chronic infections with persistent or recurrent bacteremia (18). In pregnant susceptible animals, where it causes placentitis and abortion, the organism replicates within placental trophoblasts (18). Except for B. neotomae, each species of Brucella is capable of intratrophoblastic replication in its natural host (4, 5, 18, 30, 35).

In ruminant trophoblasts, B. abortus replicates within cisternae of the rough endoplasmic reticulum (RER) (1, 22). Similar intracellular localization is seen in chicken embryo mesenchymal, yolk endodermal and hepatic cells (10). We observed that in Vero cells, B. abortus
replicates within less than 20% of the cells it invades; within these cells *B. abortus* is located within the RER (8). We suggested that transfer to the RER is the rate limiting step in *B. abortus* infection of Vero cells and that brucellae that fail to enter the RER are destroyed or eliminated (8).

In this morphological study we describe the penetration and intracellular localization of *Brucella* spp. in Vero cells. Our goals were to determine, (i) if localization within RER is a characteristic of other species of *Brucella*, (ii) how brucellae enter Vero cells, (iii) how brucellae are transferred to the RER and (iv) the fate of internalized brucellae that fail to replicate within Vero cells.
MATERIALS AND METHODS

Microorganisms

Eight strains representing the six species of *Brucella* were used in this study (Table 1). Except for *B. ovis*, cultures were grown on potato infusion agar slants for 48 h at 37°C. *B. ovis* was grown on tryptose agar slants containing 5% bovine serum for 72 h at 37°C in an atmosphere of 5% CO₂. Brucellae were harvested by gentle washing with sterile 0.85% NaCl and standardized turbidimetrically to a concentration of 1 x 10¹¹ cells/ml. For monolayer infection, bacterial suspension was diluted, to a final concentration of 1.0 x 10⁹ CFU/ml, in Eagle minimum essential medium (MEM; GIBCO Laboratories, Grand Island, NY) supplemented with L-glutamine (2 mM) and 10% fetal calf serum (FCS).

Monolayer Infection

Vero cells (African green monkey kidney fibroblasts), obtained from the National Veterinary Services Laboratories, Ames, Iowa, were used as host-cells. Cell culture methods and infection procedure were as described (8). Briefly, subconfluent monolayers on coverslips were inoculated with 2 ml of *Brucella* suspension. The inoculum was centrifuged onto monolayers for 20 minutes at 550 g. After 8 h incubation, in a humidified incubator with an atmosphere of 5% CO₂ at 37°C, the coverslips were washed in PBS and further incubated in fresh
Table 1. Relative infectivity of different species of *Brucella* in comparison to *B. abortus* strain 2308S in Vero cells.

<table>
<thead>
<tr>
<th>Brucella Species</th>
<th>Strain</th>
<th>Natural host</th>
<th>Type</th>
<th>Mean (%)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. Abortus</em></td>
<td>2308S</td>
<td>Cattle</td>
<td>Smooth</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45/20</td>
<td>Cattle&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rough</td>
<td>19 (2)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18 - 20</td>
</tr>
<tr>
<td><em>B. Melitensis</em></td>
<td>16M&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Goat</td>
<td>Smooth</td>
<td>32 (1)</td>
<td>30 - 32</td>
</tr>
<tr>
<td><em>B. Suis</em> biovar 1</td>
<td>1330&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Swine</td>
<td>Smooth</td>
<td>134 (2)</td>
<td>101 - 185</td>
</tr>
<tr>
<td>biovar 4</td>
<td>IAB-2579</td>
<td>Reindeer</td>
<td>Smooth</td>
<td>24 (2)</td>
<td>21 - 26</td>
</tr>
<tr>
<td><em>B. Neotomae</em></td>
<td>418</td>
<td>Desert Wood Rat</td>
<td>Smooth</td>
<td>28 (2)</td>
<td>27 - 30</td>
</tr>
<tr>
<td><em>B. Canis</em></td>
<td>RM6-66&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Dog</td>
<td>Rough</td>
<td>0 (1)</td>
<td>0</td>
</tr>
<tr>
<td><em>B. Ovis</em></td>
<td>692</td>
<td>Sheep</td>
<td>Rough</td>
<td>2 (2)</td>
<td>2 - 3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Results are expressed as percentage of strain 2308S (mean of 2 samples).

<sup>b</sup>Laboratory-derived from a smooth isolate

<sup>c</sup>Number in parentheses indicates the number of determinations.

<sup>d</sup>Type strain [Bergey's Manual (7)]
MEM supplemented with 0.25% FCS and 50 μg/ml gentamycin (Gentocin; Stering Corporation, Kenilworth, NJ), to kill extracellular bacteria. Medium was replaced at 24 hours. Four coverslips were sampled at 4, 8, 16, 24, 36 and 48 h postinoculation (PI); the 4 and 8 h coverslips were incubated in presence of gentamycin for 1 h before further processing. Two coverslips were washed in PBS and incubated for 10 to 15 minutes in 2 ml of a 0.1% solution of deoxycholate in distilled water to disrupt host cells. Samples of the Vero cell lysate were plated on tryptose agar containing 5% bovine serum. Brucella colonies were identified by colonial morphology and growth characteristics (38).

Double Fluorescence Staining

At each sampling time, 2 coverslips were stained (8) by indirect immuno-fluorescence using as primary antibodies, a rabbit anti-\textit{B. abortus} 2308S antiserum (smooth brucellae) or a rabbit anti-\textit{B. canis} antiserum (rough brucellae), and as secondary antibody, a goat anti-rabbit FITC-conjugated antiserum (NVSL, Ames, IA). To discriminate between extra- and intracellular bacteria, on coverslips sampled at 4, 8, 16 and 24 h PI, the primary antibody was applied for 30 min at 4°C before methanol-acetone fixation (8). After immunolabeling, all coverslips were treated for 5 min with a 25 μg/ml solution of propidium iodide (Sigma Chemical Co., St. Louis, MO) to stain DNA and examined by epifluorescence microscopy (blue filter, excitation at 490 nm and
emission at 515 nm).

Enumeration of Infected Cells

For each of the 2 coverslips sampled 48 h PI, the number of cells per mm$^2$ was estimated, at a total magnification of 400x, by counting cell nuclei in 5 fields located along a diagonal across the coverslip (15 to 25 cell nuclei/field). The surface of the area was defined by a reticle in one eye piece of the microscope. The number of infected cells per mm$^2$ was then estimated, at a total magnification of 100x, by counting the number of infected cell in 25 systematically selected fields, arranged in 5 rows and 5 columns (1 to 20 infected cells/field). Results were expressed as percentage of infected cells.

The percentage of Vero cells containing 0, 1, 2, 3 to 5, 6 to 10 and more than 10 intracellular bacteria was estimated by examining, at a magnification of 1,000x, a total of 200 cells in each of the 2 coverslips sampled at 4, 8, 16 and 24 h PI.

Electron Microscopy

Vero cells, grown on microporous membranes in 12-mm Millicell-HA inserts (Millipor, Bedford, MA), were exposed to 1 ml of the various suspensions of Brucella, as described for coverslips. Sterile MEM-inoculated inserts were controls. Two inserts were sampled at 8, 24 and
48 h PI, washed in cold PBS and fixed for 1 h by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C. Following fixation, microporous membranes were removed from inserts and stored in 0.1 M sodium cacodylate buffer at 4°C. Four 2 mm² pieces (sampled from the center of each membrane) were postfixed in 1% osmium tetroxide, dehydrated in alcohols, cleared in propylene oxide, and infiltrated and embedded in epoxy resin. Embedding was done in rectangular rubber molds with the membranes being oriented so that sections would be cut perpendicularly to the Vero cell monolayers. Sections cut at 1 μm and stained with toluidine blue were examined by light microscopy. One piece of membrane, which demonstrated an intact Vero cell layer and adherent or intracellular bacteria was selected. Ultrathin sections were cut at 70 to 90 nm, stained with uranyl acetate and lead citrate and examined with a Philips 410 transmission electron microscope.

Immunogold Labeling

Brucellae were labeled on ultrathin sections of resin-embedded Vero cell monolayers mounted on nickel grids using a post-embedding indirect immuno-gold labeling procedure. Primary antibodies were as described above for double fluorescence staining. Immunogold probe was a goat anti-rabbit antiserum coupled to 20 nm colloidal gold (Auroprobe™EM; Janssen Life Science Products, Olen, Belgium). Tris-buffered saline
(TBS), pH 8.0, containing 0.05% triton X-100, 0.5 M NaCl and 1% bovine serum albumin (BSA) was used as diluting and washing solutions. Following etching for 10 min in 10% H₂O₂, sections were rinsed in ultra-distilled water and exposed for 10 min to 1% BSA to block non-specific reactions. Excess BSA was blotted from sections and specimens were incubated for 2 hours with a 1/500 dilution of the primary antibody. After a 15 min rinse in TBS, sections were incubated for 2 h with a 1/100 dilution of the immunogold probe. Sections were washed for 15 minutes in ultra-distilled water and stained with uranyl acetate and lead citrate.
RESULTS

Pure cultures of Brucella were isolated from all inoculated Vero cell monolayers. The number of intracellular organisms increased from 100 to 1,000 folds over the 48 h period, except with B. ovis where it remained constant and with B. canis where it decreased slightly (data not shown).

Fluorescence Microscopy

Using the double fluorescence labeling technique, brucellae had an intense green yellow fluorescence while nuclei of eukaryotic cells were red (Figs. 1-4). When the primary antibody was applied before fixation, yellow-green fluorescence was restricted to extracellular organisms, while intracellular bacteria stained red (Figs. 1-4).

Smooth brucellae (B. abortus 2308S, B. melitensis, B. suis biovars 1 and 4, B. neotomae) invaded and grew in Vero cells. Vero cells infected with the various species of smooth Brucella had similar morphology (Figs. 1-3). Depending on the strain of Brucella, at 4 h post-inoculation (PI), 25 to 40% of Vero cells contained one or more bacteria and, at the end of the inoculation period (8 h PI), 40 to 50% of Vero cells had intracellular brucellae (Figs. 5-6). With all strains of Brucella, the number of cells containing at least one organism did not increase significantly after 8 h (Figs. 5-6), but in a small
Figs. 1 a-c. Vero cell monolayers, 24 (a), 36 (b) and 48 (c) hours after inoculation with smooth *B. abortus* (2308S). Monolayers were stained by double immunofluorescence. The procedure to discriminate between intracellular (red) and extracellular (yellow-green) brucellae was applied to the 24 h samples (a).

Figs. 2 a-c. Vero cell monolayers, 24 (a), 36 (b) and 48 (c) hours after inoculation with smooth *B. suis* biovar 1 (1330) (Same staining as Fig. 1). The appearance of *B. suis* infected Vero cells at 36 h PI (a) is similar to that of *B. abortus*-infected cells at 48 h PI (Fig. 1c).

Figs. 3 a-c. Vero cell monolayers, 24 (a), 36 (b) and 48 (c) hours after inoculation with smooth *B. melitensis* (16M) (Same staining as Fig. 1). Infected Vero cells contain fewer organisms than those infected with *B. abortus* (Fig. 1b-c) or *B. suis* (Fig. 1b-c).

Figs. 4 a-c. Vero cell monolayers, 24 (a), 36 (b) and 48 (c) hours after inoculation with rough *B. canis* (RM6-66). Monolayers were stained by double immunofluorescence using the procedure to discriminate between intracellular (red) and extracellular (yellow-green) organisms. Note the few intracellular *B. canis* (arrows) and the lack of intracellular replication. Large number of extracellular brucellae adhere to Vero cells and directly to coverslips (open arrows).
Fig. 5. Interactions of *R. suis* biovar 1 (1330) and *R. melitensis* (16M) with Vero cells after 4, 8 and 24 h of incubation. Data are grouped according to the number of intracellular bacteria per cell, estimated for 200 Vero cells. Each bar represent the mean of 2 samples.
Fig. 6. Interactions of *B. suis* biovar 4 (IAB 2579) and *B. canis* (RM6-66) with Vero cells after 4, 8 and 24 h of incubation. Data are grouped according to the number of intracellular bacteria per cell, estimated for 200 Vero cells. Each bar represent the mean of 2 samples.
The diagram shows the percentage of Vero cells with different numbers of intracellular Brucella bacteria for two different strains of Brucella: B. suis Biovar 4 (IAB2579) and B. canis (RM6-66).

- **4 Hours**
  - B. suis Biovar 4 (IAB2579)

- **8 Hours**
  - B. suis Biovar 4 (IAB2579)

- **24 Hours**
  - B. canis (RM6-66)

The x-axis represents the number of intracellular bacteria per Vero cell, ranging from 0 to >10. The y-axis shows the percentage of Vero cells with different bacterial counts.

The data indicates a higher percentage of Vero cells with intracellular bacteria at 24 hours compared to 4 and 8 hours for both strains.
proportion of these cells, the number of intracellular bacteria increased sharply between 24 and 48 h (Figs 1-3). At 36 h PI, the cytoplasm of Vero cells infected with *B. suis* biovar 1 was filled with organisms (Fig. 2B). Similar brucellae-filled Vero cells were observed at 48 h PI with *B. abortus* 2308S, *B. suis* biovar 4 and *B. neotomae* (Fig. 1C). In contrast, at both 36 and 48 h PI, Vero cells infected with *B. melitensis* contained less bacteria than those infected with other brucellae (Fig. 3B and C). At 48 h PI, the percentage of brucellae-filled Vero cells varied from 1 to 9%; remaining Vero cells were either free of organisms or contained scattered clumps of intracytoplasmic Brucella-antigen. The largest percentage of infected Vero cells was found with *B. suis* biovar 1, followed by *B. abortus* 2308S, *B. melitensis*, *B. suis* biovar 4 and *B. neotomae* (Table 1). Most infected cells were in clusters of 2 to 6 cells. Infected cells in different phases of the mitotic cycle were frequent.

Rough brucellae (*B. abortus* 45/20, *B. ovis*, *B. canis*) were markedly more adherent and, except for *B. canis*, more invasive than smooth brucellae. Numerous rough brucellae adhered to the surface of Vero cells and to areas of the coverslips not covered by Vero cells (Fig. 4). At 4 h PI, 75% of Vero cells had 6 or more associated (extra- and intracellular) rough brucellae. With rough *B. abortus* 45/20 and *B. ovis*, some Vero cells were covered by extracellular bacteria. In 4 to 24 h PI samples, the percentage of Vero cells containing intracellular brucellae was higher with *B. abortus* 45/20 and *B. ovis* than with
brucellae of the smooth type. In contrast, intracellular \textit{B. canis} were in a smaller percentage of Vero cells (Fig 6B). Intracellular rough brucellae were frequently in clumps of 5 or more bacteria. At 36 and 48 h PI, less than 0.5% of Vero cells had their cytoplasm filled with \textit{B. abortus} 45/20 and \textit{B. ovis} (Table 1). Similarly infected Vero cells were not seen with \textit{B. canis}.

**Electron Microscopy**

Intracellular brucellae were seen with all species of \textit{Brucella} at all sampling times. Immunogold labeling of smooth brucellae was achieved using as primary antibody, either anti-\textit{B. abortus} 2308S (smooth) or anti-\textit{B. canis} (rough) antisera. Attempts to label rough brucellae on thin sections were unsuccessful.

At the end of the inoculation period (8 h PI), brucellae were seen in contact with Vero cell membranes, in different stages of engulfment by Vero cell, and intracellularly (Figs. 7-12). Adherent organisms were more frequent with rough brucellae (Table 2). Most adherent brucellae were on the Vero cell surfaces facing the insert's microporous membrane (Figs. 7 and 11). Brucellae attached to the apical surface of Vero cells were only seen with rough strains (Figs. 8 and 10). Several Vero cells had cytoplasmic processes that partially surrounded individual brucellae (Figs. 7 and 10); cytoplasmic membranes directly adjacent to brucellae were frequently thicker and occasionally formed coated-pits.
Fig. 7. *B. abortus* (45/20) attached to the basal surface of a Vero cell and enclosed by cytoplasmic extensions. Coated-pit associated with partially surrounded *B. abortus* (arrow). Vero cell monolayer 8 h post-inoculation. Bar = 0.5 μm.

Fig. 8. *B. abortus* (45/20) attached to the apical surface of a Vero cell. Thickening of the plasma membrane adjacent to the *B. abortus*. Coated-pit (arrow). Vero cell monolayer 8 h post-inoculation. Bar = 0.25 μm.

Fig. 9. Cytoplasmic process of Vero cell extending into microporous membrane. *B. abortus* (2308S) within a phagosome. Note multivesicular bodies (arrows) and abundant microfilaments (arrow heads). Vero cell monolayer 8 h post-inoculation. Bar = 1 μm.

Fig. 10. *B. ovis* (692) encircled by Vero cell apical extensions. Vero cell monolayer 8 h post-inoculation. Bar = 1 μm.

Fig. 11. *B. suis* biovar 4 (IAB 2579) attached to the basal surface of a Vero cell. Vero cell monolayer 8 h post-inoculation. Bar = 0.5 μm.

Fig. 12. *B. ovis* (692) within phagolysosomes (arrows). Myelin figures associated with intra-phagolysosomal brucellae (open arrows). Vero cell monolayer 8 h post-inoculation. Bar = 1 μm.
Table 2. Ultrastructural localization of *Brucella* spp. in Vero cells

<table>
<thead>
<tr>
<th><em>Brucella</em> spp.</th>
<th>Adherent to cell surface</th>
<th>Phagosomes/Phagolysosomes</th>
<th>Rough endoplasmic reticulum</th>
<th>Perinuclear envelope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8h 24h 48h</td>
<td>8h 24h 48h</td>
<td>8h 24h 48h</td>
<td>8h 24h 48h</td>
</tr>
<tr>
<td><em>B. abortus</em> (23088)</td>
<td>1^a 0 0</td>
<td>2 2 2</td>
<td>0 1 2</td>
<td>0 0 2</td>
</tr>
<tr>
<td>(45/20)^b</td>
<td>1 - 0</td>
<td>1 - 1</td>
<td>0 - 1</td>
<td>0 - 0</td>
</tr>
<tr>
<td><em>B. melitensis</em> (16M)</td>
<td>0 0 0</td>
<td>1 2 2</td>
<td>0 0 2</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>B. suis</em> (1330)</td>
<td>1 0 0</td>
<td>2 2 2</td>
<td>0 1 2</td>
<td>0 0 2</td>
</tr>
<tr>
<td>(IAB 2579)</td>
<td>2 0 0</td>
<td>2 2 2</td>
<td>0 1 2</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>B. neotomae</em> (418)</td>
<td>1 0 0</td>
<td>2 2 2</td>
<td>0 1 2</td>
<td>0 0 1</td>
</tr>
<tr>
<td><em>B. canis</em> (RM6-66)</td>
<td>1 2 0</td>
<td>2 2 2</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td><em>B. ovis</em> (692)</td>
<td>2 1 0</td>
<td>2 2 2</td>
<td>0 0 1</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>

^aNumber of samples (out of 2) in which *Brucella* has been found in that localization.

^bOnly one sample examined and no sample at 24 h post-inoculation.
(Figs. 7-8). Intracellular brucellae were within phagosomes, most of which contained only one morphologically intact bacterium (Fig. 9). Some brucellae, however, especially with rough strains, were within phagolysosomes. These contained 1 to 15 intact organisms as well as electron dense material, membrane debris and myelin figures (Fig. 12). Autophagic vacuoles and multivesicular bodies were frequent within Vero cells of infected monolayers, especially in cells containing intracellular brucellae. These structures were rare in control monolayers.

At 24 h PI, phagosomes and phagolysosomes containing morphologically intact brucellae were seen with all species of Brucella (Figs. 13-15). Isolated organisms within ribosome-lined cisternae were in Vero cells inoculated with B. abortus 2308S, B. suis biovars 1 and 4 and with B. neotomae (Table 2 and Fig. 15). Brucellae attached to Vero cells luminal or basal surfaces were only observed with rough strains of Brucella (Fig. 13). Intra-phagosomal and phagolysosomal smooth brucellae were frequently surrounded by concentric membranous structures similar to myelin figures (Figs. 13-14); in immunogold labeled samples, these structures were lined by gold particles (Fig. 14).

At 48 h PI, both smooth and rough brucellae (except B. canis) were within cisternae of the rough endoplasmic reticulum (Figs. 16-22). The membranes of Brucellae-filled cisternae were discontinuously lined by ribosomes and were continuous with membranes of normal appearing RER (Figs. 18-20). In the most heavily infected cells, brucellae were also
Fig. 13. *B. canis* (RM6-66) attached to the apical surface of a Vero cell and within phagolysosomes. Myelin figures associated with intraphagolysosomal brucellae (arrows). Vero cell monolayer 24 h post-inoculation. Bar = 0.5 μm.

Fig. 14. Intraphagosomal *B. melitensis* (16M) surrounded by immunogold labeled membranous structures. Vero cell monolayer 24 h post-inoculation. Bar = 0.5 μm.

Fig. 15. Immunogold labeled *B. suis* biovar 1 (1330) within cisternae of the rough endoplasmic reticulum. Vero cell monolayer 24 h post-inoculation. Bar = 1 μm.
Fig. 16. Vero cell contains numerous *B. suis* biovar 1 (1330) within cisternae of the rough endoplasmic reticulum and within perinuclear envelope (arrow). Vero cell monolayer 48 h post-inoculation. Bar = 1 μm.

Fig. 17. Immunogold-labeled *B. abortus* (2308S) within cisternae of the rough endoplasmic reticulum (arrows) and within phagolysosomes (open arrows). Immunogold-labeled bacterial debris and membranes in phagolysosomes (arrowheads). Brucellae were labeled with anti *B. canis* antiserum (anti-rough brucellae). Vero cell monolayer 48 h post-inoculation. Bar = 1 μm.
Fig. 18. *B. neotomae* (418) within distended cisternae of rough endoplasmic reticulum. A section of normal rough endoplasmic reticulum leads directly (arrow) into brucella-containing cisternae. Vero cell monolayer 48 h post-inoculation. Bar = 0.5 μm.

Fig. 19. Immunogold-labeled *B. melitensis* (16M) within cisternae of rough endoplasmic reticulum. A section of normal rough endoplasmic reticulum leads directly (arrow) into brucella-containing cisternae. Vero cell monolayer 48 h post-inoculation. Bar = 0.5 μm.

Fig. 20. *B. ovis* (692) within cisternae of rough endoplasmic reticulum. Vero cell monolayer 48 h post-inoculation. A section of normal rough endoplasmic reticulum leads directly (arrow) into brucella-containing cisternae. Vero cell monolayer 48 h post-inoculation. Bar = 0.5 μm.

Fig. 21. Intact and degraded *B. ovis* (692) within phagolysosomes. Vero cell monolayer 48 h post-inoculation. Bar = 1 μm.

Fig. 22. Membrane-bound and intraphagolysosomal *B. abortus* (2308S) within a Vero cell in mitosis. Note chromosome (X), centriole (arrow) and spindle microtubules (arrowheads). Vero cell monolayer 48 h post-inoculation. Bar = 1 μm.
in perinuclear spaces (Fig. 16). Evidence of cellular degeneration (cell swelling and vacuolation) was minimal, although some heavily infected cells were necrotic, with increased cytoplasmic electron-density and loss of structural detail. Clusters of 2 to 10 brucellae were also found within phagolysosomes, especially with *B. canis* and other rough brucellae (Figs 21-22). While most smooth brucellae were morphologically intact, half the rough organisms appeared degraded (Figs. 17 and 21-22). Myelin figures were frequent in association with intact and degraded brucellae in phagolysosomes (Figs. 17 and 21-22). Membrane-bound brucellae within mitotic cells were seen with *B. abortus* 2308S, *B. suis* biovar 1, *B. neotomae* and *B. ovis* (Fig. 22).
DISCUSSION

This comparative study indicates that all species of Brucella, attached to the surface of Vero cells, are internalized by phagocytosis. We believe that Brucella-containing phagosomes can follow one of two routes: (i) they can fuse with cisternae of the rough endoplasmic reticulum (RER) transferring organisms to the lumen of this organelle, where unrestricted bacterial replication occurs, or (ii) they can fuse with lysosomes forming phagolysosomes in which brucellae fail to replicate and are eventually destroyed (Fig. 23).

Brucella spp. clearly replicated within cisternae of the RER but not within phagolysosomes, i.e., heavily infected Vero cells had massive numbers of Brucella within the RER without corresponding numbers in phagolysosomes. Localization within RER may provide a favorable environment that enhance Brucella growth. On primary isolation, most Brucella strains are fastidious organisms requiring complex media containing several amino acids (7). Although brucellae do not require active protein synthesis by the host cell for intracellular replication (9), within the RER they may catabolize nascent polypeptides and incorporate liberated amino acids into bacterial proteins.

Results from this study confirm our theory that transfer to the RER, not internalization, is the limiting step for replication of Brucella spp. in Vero cells. Results also indicate that differences in intracellular replication within Vero cells between the various species
Fig. 24. Diagram which summarizes the infection of a Vero cell by Brucella spp. as determined in this study. Brucellae adherent to the cytoplasmic membrane are internalized by phagocytosis (1). Some intraphagosomal brucellae (2) induce their transfer to cisternae of the rough endoplasmic reticulum (3) where unrestricted replication occurs (4). Following massive intracisternal replication, some brucellae are within perinuclear envelope (5). Other Brucella-containing phagosomes (6) fuse with lysosomes forming phagolysosomes (7) in which brucellae fail to replicate and are eventually destroyed (8).
of *Brucella* are correlated to differences in their ability to enter the RER. Intracisternal *B. suis* biovar 4 and *B. abortus* 2308S, which infected the largest percentage of Vero cells, were predominantly within cisternae of the RER. In contrast, intracellular *B. canis*, which failed to replicate in Vero cells, were within phagolysosomes.

Results from this study provide additional evidence for the close relationship between the different species of the genus *Brucella*. Intracellular localization within the RER was seen with all strains of *Brucella* spp. tested, except *B. canis*. Phenotypic differences between various species and biovars are quantitative rather than qualitative (7, 18) and there is a high degree of DNA homology (96 ± 5%) between the various species of *Brucella* (37).

As reported for *Brucella* infected-trophoblasts in vivo (1, 22), *Brucella* spp. were not highly cytopathic for Vero cells, i.e., evidences of Vero cell degeneration were minimal even in heavily infected cells. The presence of numerous brucellae within mitotic Vero cells is additional evidence that intracellular *Brucella* interfere minimally with Vero cell metabolism. Brucellae are likely released when infected Vero cells disintegrate because of excessive intracellular bacterial replication. In contrast, intracellular pathogens like *Listeria monocytogenes* (13) and *Shigella dysenteriae* 1 (16) cause rapid cytolysis of infected host cells. The lack of cytopathogenicity of *Brucella* may be responsible for the delayed onset of abortion following experimental inoculation of pregnant ruminants with *Brucella* spp. (1, 28-30) as
compared to L. monocytogenes (27).

Bacteria-induced receptor-mediated vesicular transport is probably involved in Brucella spp. transfer to the RER. We believe that this process is bacterium-induced because Brucella resides within the RER of different cell types (1, 8, 10, 22). Although smooth and rough brucellae differ in lipopolysaccharide structure (7), they possess similar outer membrane proteins (32), most of which are conserved throughout the genus (2, 11, 32). Since most Brucella spp., including rough variants, enter the RER, we suggest that the recognition signal is provided by a bacterial protein.

The concentric membranous structures (myelin figures) that surrounded intra- phagosomal and phagolysosomal brucellae were of bacterial origin, as demonstrated by immunogold labeling. Similar structures have been described in phagocytic and non-phagocytic cells infected with B. abortus (17, 19, 23). Although some of these structures might be derived from dead brucellae, others may have resulted from de novo membrane synthesis by Brucella. Microorganisms can produce new surface proteins in response to changes in their environment (12, 14, 25), i.e., within minutes of entry into macrophages, Toxoplasma gondii secrete a protein-rich intraphagosomal membrane network, that is believed to interfere with phagosomal microbicidal activity (34). Phagosomal acidification may have triggered the formation of these membranous structures by Brucella.

The presence of coated-pits, in association with brucellae attached
at the surface of Vero cells, may indicate that Brucella-uptake is receptor-mediated. This would confirm the observation that monodansylcadaverine, an inhibitor of receptor-mediated endocytosis, prevents the infection of Vero cells by B. abortus (9). Similarly, coated-pits are seen in association with adherent invasive Yersinia enterocolitica (26) and Shigella flexneri (15), but not with adherent non-invasive Escherichia coli (26). However, since coated-pits were frequent at the surface of Vero cells and because bacterial engulfment involves the internalization of large areas of the cell surface, we can not rule out that the association of coated-pits with attached brucellae was coincidental.

Marked adhesiveness of B. abortus 45/20, B. ovis and B. canis for Vero cell surfaces was probably the result of higher hydrophobicity due to the rough characteristics (8). Cultures of B. ovis and B. canis are always in the rough or mucoid phase, even on primary isolation from infected animals (7). Adherence to cell surfaces is profoundly affected by electrostatic charge and hydrophobicity of both host-cells and bacteria (20, 31, 33). Correlation between bacterial hydrophobicity and the degree to which they associate with cells in culture has been demonstrated for various bacterial pathogens (20, 33), including B. abortus (8). Loss of polysaccharide side chains increases the hydrophobicity of rough bacteria; this was reported for S. typhimurium (21) and E. coli (31) and was recently confirmed for B. abortus (8).

Failure to immuno-label rough brucellae on ultrathin sections
probably resulted from the loss of antigenic reactivity during sample processing, perhaps due to the lack of O polysaccharide side chains in rough organisms. Immunogold labeling of surfaces of smooth *B. abortus* in ultrathin sections was observed using polyclonal anti-*B. canis* antiserum (anti-rough brucellae) as primary antibody. This indicates that within the wall of smooth brucellae some of the antigenic determinants recognized by this antiserum are not destroyed.


Pathogenesis of Brucella abortus infection in the mammary gland and 
24. Meyer, M. E. 1966. Identification and virulence studies of 
Brucella strains isolated from eskimos and reindeer in Alaska, 
regulation and sensory transduction in the control of bacterial 
in Yersinia enterocolitica that can promote invasion of epithelial 
Placental lesions of sheep experimentally infected with Listeria 
Placental pathology. II. Placental lesions of sheep experimentally 
Placental pathology. III. Placental lesions of sheep 
24:915-921.


An in vitro system was developed and used to characterize the entry and intracellular replication of *B. abortus* and other species of *Brucella* in non-phagocytic cells. Although several studies have shown intracellular growth of *Brucella* spp. in non-phagocytic cells in vitro (23, 45, 47, 94, 95, 104), none provided strong quantitative data nor precise ultrastructural indications on the intracellular localization of *Brucella*.

Results of the first study established that *B. abortus* were internalized by Vero cells and replicated within the rough endoplasmic reticulum (RER). Comparative studies with smooth and rough strains of *B. abortus* suggested that transfer to the RER, but not invasion, is the limiting step in the infection of non-phagocytic cells.

Studies with endocytic and metabolic inhibitors indicated that *B. abortus* is internalized by receptor-mediated phagocytosis and that its transfer to the RER requires endosome acidification. The uptake of heat-killed *B. abortus* and non-invasive *Escherichia coli* by Vero cells was similar to that of viable *B. abortus*, suggesting that *B. abortus* does not specifically induce its own uptake. Replication of *B. abortus* within the RER, was also shown not to depend on protein synthesis by the host-cell.

Results from the third study demonstrated that replication within cisternae of the RER is a characteristic shared by most species of
Brucella. Ultrastructural findings suggested that after phagocytosis, Brucella-containing phagosomes follow one of two routes: (i) they fuse with cisternae of the RER, transferring organisms to the lumen of this organelle (where unrestricted replication occurs), or (ii) they fuse with lysosomes forming phagolysosomes (in which brucellae fail to replicate and are eventually destroyed).

The biology of B. abortus interactions with its host-cell differs markedly from that of other intracellular bacteria. Unlike entero-invasive bacteria, B. abortus does not induce its own uptake but seems to depend on the normal endocytic activity of its target cell for entry (30, 34, 49, 105). Localization of B. abortus within cisternae of the RER differs from that of all other intracellular bacteria including Legionella pneumophila which resides within ribosome-studded phagosomes (48). While inhibition of endosomal acidification does not affect the entry and intracellular replication of enteroinvasive bacteria (30, 31), it inhibited the infection of Vero cells by B. abortus, probably by interfering with their transfer to the RER.

Many questions, concerning the interactions of brucellae with non-phagocytic host-cells, remain to be answered and even more have resulted from the present work. For example: Would specific anti-Brucella antibodies and/or complement interfere with the internalization of B. abortus? Are internalized heat-killed brucellae transported to the cisternae of the RER? What is the nature of the receptors (at the surface of the host-cell and of the bacterium) that are involved in the
uptake of \textit{B. abortus}? What is the origin and the nature of the signal(s) that induce the fusion of \textit{Brucella}-containing phagosomes with cisternae of the RER?

A logical continuation of this work would be to search for the bacterial genes required for \textit{B. abortus} entry into trophoblasts and transfer to the RER. One approach could be to identify the smallest amount of \textit{B. abortus} DNA necessary to confer these abilities to an innocuous \textit{E. coli} strain. This approach was recently used to find the genes conferring invasiveness to \textit{Yersinia pseudotuberculosis} (49, 50, 51). The identification of these genes, would represent a major achievement in the understanding of the pathogenesis of bovine brucellosis. It would also represent an original approach in the search of important antigenic determinants to be used as potential diagnostic tools and/or vaccine.
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