

## Development of a Rickettsia Isolated from an Aborted Bovine Fetus

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**An obligate intracellular rickettsial organism isolated from an aborted bovine fetus was studied in bovine turbinate and mouse macrophage cell cultures with light and electron microscopy. Development of the organism was similar in both cell types. The organism replicated within cytoplasmic vacuoles in a developmental cycle that resembled that of both the ehrlichiae and chlamydiae. The inoculum contained only electron-dense forms, which infected cells within 2 h postinoculation by adhering to cell membranes at thickened areas that appeared to be coated pits and then being endocytosed. A striking feature occurred next as the organisms became surrounded by host cell mitochondria and, by light microscopy, appeared to have halos. During this intimate association with mitochondria, the electron-dense organisms changed into large reticulated forms that began to divide by binary fission. These large forms were often in direct contact with mitochondrial membranes. The organisms continued to divide by binary fission, and host cells contained large cytoplasmic inclusions of reticulated organisms. The reticulated organisms gradually changed into electron-dense forms that were released from degenerated host cells.**

An obligate intracellular parasite was recently isolated from tissues of a first-trimester aborted bovine fetus (3, 4). On the initial cell culture passage, cytopathic effect was observed about 2 to 3 days postinoculation (p.i.) in bovine turbinate (BT) cell culture inoculated with pooled spleen and liver homogenates. The organism was serially passaged numerous times and consistently induced cytopathic effect. It replicated rapidly to high titers, reaching peak titers exceeding  $10^8$  50% tissue culture infective doses per ml within 3 days. Replication was inhibited by tetracycline but not by penicillin and gentamycin. Light microscopy (LM) revealed organisms within cytoplasmic inclusions that ranged in size from 0.2 to 0.4  $\mu\text{m}$ . Antigenic cross-reactivity of the organism was demonstrated by indirect immunofluorescence with *Cowdria ruminantium* (Kiswani isolate) (3, 4), a causative agent of heartwater disease in Africa and more recently the Caribbean (19, 27). This cross-reactivity suggested an antigenic relatedness between the two organisms. A lack of serologic reactivity has been shown with a variety of *Rickettsia*, *Coxiella*, *Wolbachia*, *Anaplasma*, and *Chlamydia* spp. (4).

The purpose of this study was to describe development of this newly isolated organism in two types of cultured cells and to define morphologic features assisting its identification in tissues of infected animals.

### MATERIALS AND METHODS

**Agent.** The organism used for these studies, initially isolated from an aborted bovine fetus at Washington Animal Disease Diagnostic Laboratory and designated WSU 86-1044 (3, 4), is referred to herein as WSU/1044. The organism was cloned in BT cells by terminal dilution three times serially at passages 11 through 13 and used for these studies at passage 14. The inoculum for cell culture was BT cells inoculated with a multiplicity of infection (MOI) of 0.01, cultured for 72

h, frozen in aliquots at  $-70^{\circ}\text{C}$ , and titrated as described previously (4).

**Cell culture.** Two cell types were used for these studies: (i) BT cells (ATCC CRL 1390), the host cell for the original isolation, and (ii) P388D1 mouse macrophage cells (ATCC TIB 63). The latter have been used for propagation of several members of the tribe *Ehrlichieae* (2, 9). BT cells were grown in Eagle minimal essential medium with 10% fetal bovine serum in culture conditions and infectivity titrations described previously (4). P388D1 cells were grown in RPMI medium containing 5% fetal bovine serum. Antibiotics were not used in any cultures. Subconfluent monolayers were grown in 25-cm<sup>3</sup> flasks, inoculated with the organism, and subsequently fixed at various intervals for LM and electron microscopy.

**Sample collection, fixation, and electron microscopy.** At each collection time following inoculation, cell culture medium was removed from the cultures and replaced with either 2% glutaraldehyde in a 0.2 M sodium cacodylate buffer with 2% sucrose or the glutaraldehyde-paraformaldehyde fixative of Karnovsky (12). The cultures were allowed to fix for 10 min at room temperature, after which the cells were scraped and pelleted in microcentrifuge tubes. The cell pellets were postfixed in 2% osmium tetroxide in 0.2 M sodium cacodylate buffer, dehydrated in a graded series of ethanol, and embedded in Dow 732 epoxy resin, with propylene oxide as the transitional fluid. Thick sections (1  $\mu\text{m}$ ) were prepared with glass knives and were stained with Mallory's stain (20). A second set of thick sections was prepared with a Diatome diamond knife and a Sorvall MT 5000 ultramicrotome just prior to fine sectioning of the tissue blocks; these sections were photographed in an Olympus photomicroscope. Thin (silver-gold) sections were cut, collected on 300-mesh copper grids, and stained with uranyl acetate and lead citrate (28). The sections were examined and photographed in a JEOL 100 CX STEM electron microscope.

The study was done in three experiments. In experiment

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1, two groups of BT cells were inoculated: group A was infected with an MOI of 10 and fixed at 15-min intervals for 12 h to study the early phase of infection, and group B was infected at a low MOI (approximately  $10^{-4}$ ) and fixed at 8, 24, 32, 48, 56, 72, 96, and 104 h p.i. to study the later stages of the developmental cycle. Experiment 2 was the same as experiment 1, except that cells were also fixed with the fixative of Karnovsky to determine the effects of a more rapidly penetrating fixative. Experiment 3 was done to compare the appearance of the organism in two different cell lines, BT and P388D1 cells. Both cell lines were inoculated and fixed as described for experiment 1. A sample of the inoculum was also fixed and pelleted in a microcentrifuge tube. In each experiment, controls (uninfected cells) were fixed at each collection time.

## RESULTS

**LM.** A similar developmental cycle of the organism was seen in both the BT (Fig. 1) and mouse macrophage cells by LM. Most cells infected with the high MOI contained one to several inclusions by 12 h p.i. These cultures were used to study infection of cells and early development (Fig. 1B and C). Subsequent development was studied in cell cultures inoculated with the lower MOI ( $10^{-4}$ ) (Fig. 1D to F).

Densely staining organisms were seen in the inoculum (Fig. 1A). By 2 h p.i. (MOI of 10), these dense forms adhered to and entered host cells. Individual dense forms could be seen in the cytoplasm and often were surrounded by clear space forming a halo (Fig. 1B). The dense forms changed into less densely staining, large reticulated forms that occurred in small clusters (Fig. 1C). As the cycle progressed, the cell cytoplasm contained many large colonies of organisms that contained reticulated forms (Fig. 1D). These reticulated forms gradually changed into dense forms (Fig. 1E). At the end of the experiment, most colonies contained dense forms, many of which occurred extracellularly, presumably having been released from degenerated cells (Fig. 1F).

**Electron microscopy.** The development of the WSU/1044 organism was also similar with electron microscopy in both the BT cells (Fig. 2 and 3) and mouse macrophage P388D1 cells (Fig. 4). Electron-dense organisms with a dense central core (Fig. 2A), but not reticulated forms, were seen in the inoculum. At 2 h p.i., these dense forms adhered to the host cell membrane (Fig. 2B and 4A). The organism appeared to adhere at thickened areas of the cell membrane that were similar in morphology to coated pits (Fig. 2B and 4A). The organisms entered the cells by invagination of a thickened portion of the host cell membrane by endocytosis (Fig. 2B and 4A) and via phagocytosis by cell extensions (Fig. 2C). Once inside a cell, individual electron-dense forms were found only within membrane-bound cytoplasmic vacuoles (Fig. 2D). Vacuoles containing organisms were surrounded by host cell mitochondria; the space between the organism and mitochondria appeared as a halo by LM (Fig. 2E and 4B). The electron-dense forms then changed into large reticulated forms (Fig. 2E and F and 4B). The outer membrane of the large reticulated forms often appeared to be in direct contact with the outer mitochondrial membrane (Fig. 2F and 4C).

After changing into large reticulated forms, the organisms divided by binary fission, forming first small colonies and later large, morula-like inclusions of organisms in the cytoplasm (Fig. 3A and B and 4C). Following multiplication by binary fission, the organisms changed from reticulated into electron-dense forms (Fig. 3C) and mature inclusions contained predominantly electron-dense forms (Fig. 3D and 4D). Inclusions in different stages of maturity were often seen within the same culture, as illustrated in Fig. 3F, in which one adjacent inclusion contained predominantly reticulated forms and the other contained predominantly electron-dense organisms. In the low-MOI cultures fixed at 56 h p.i., electron-dense forms, presumably infectious particles, were seen outside host cells; at 72 h p.i., the cultures contained degenerating cells and dense forms were abundant extracellularly (Fig. 3F and 4D).

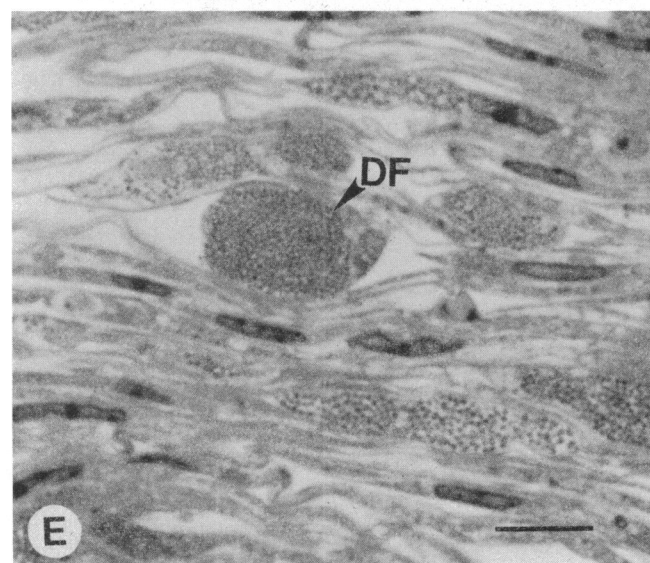
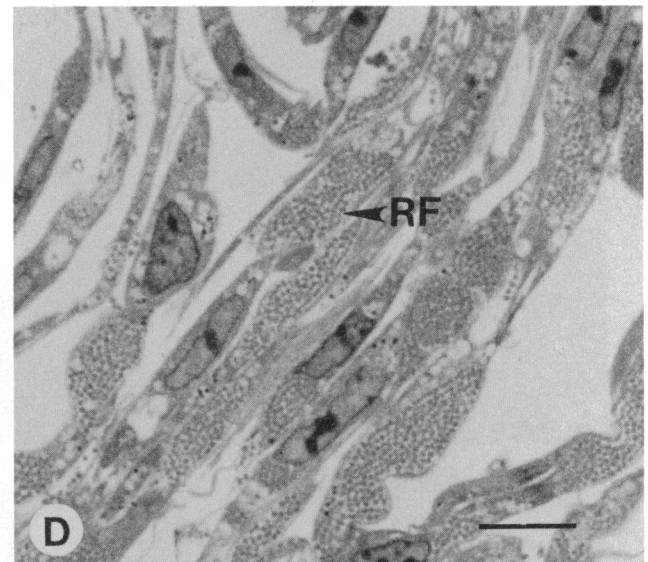
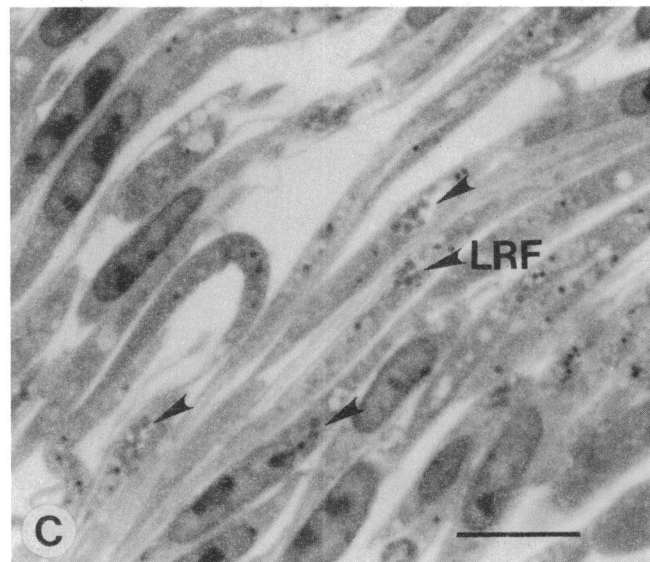
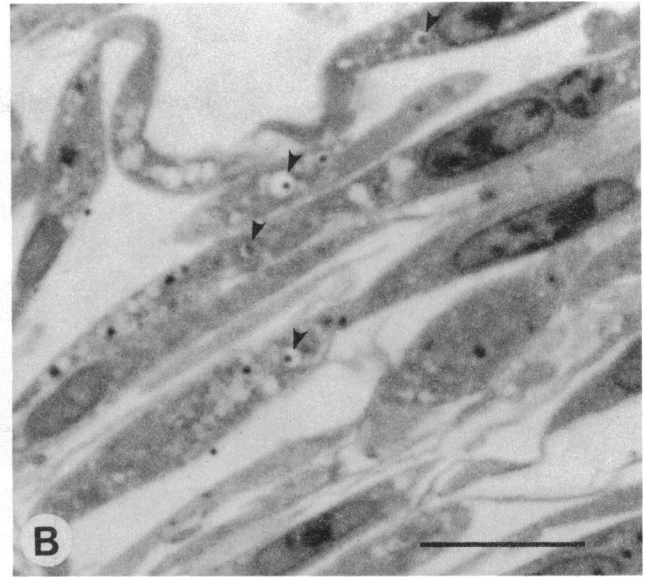
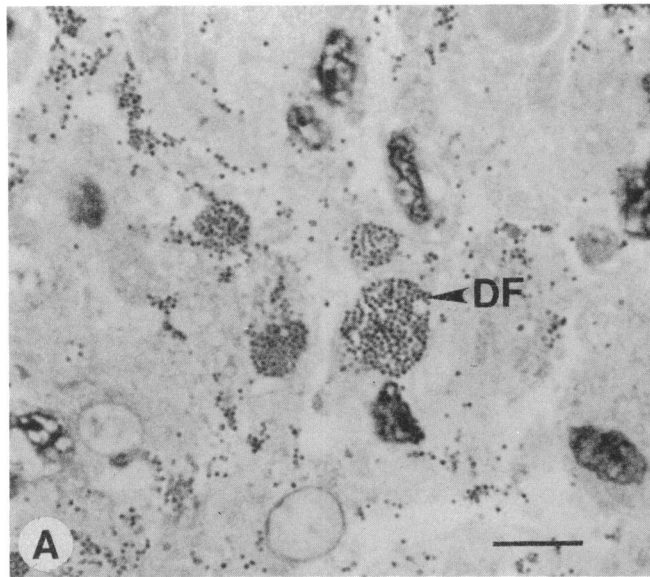
## DISCUSSION

The WSU/1044 organism proved to be a good subject for study with LM and electron microscopy because its rapid development was followed easily in 1- $\mu$ m plastic sections. By LM, individual organisms could be seen infecting host cells and later forming large colonies of electron-dense or reticulated forms.

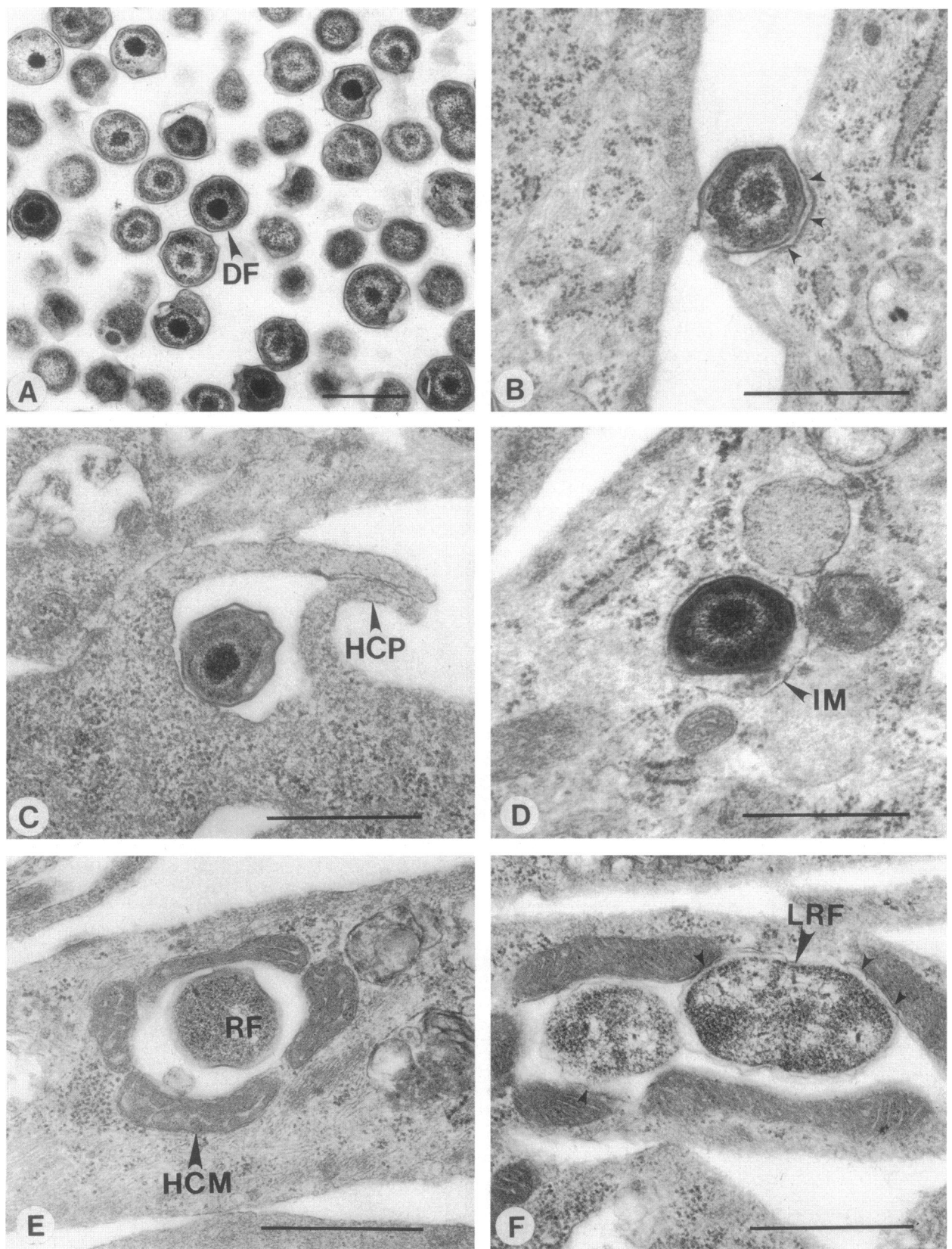
The taxonomical affiliation of this newly isolated organism has not yet been established. On the basis of its intracellular growth requirement and relative resistance to penicillin, it appeared to be a rickettsia, as broadly defined by Weiss and Moulder (30). The replication of the organism within cytoplasmic vacuoles of host cells and its antigenic cross-reactivity with *C. ruminantium* (3, 4) suggest a relatedness with the tribe *Ehrlichieae* (22). The ultrastructural studies reported herein demonstrated that WSU/1044 undergoes a developmental cycle involving electron-dense and reticulated forms. The electron-dense form appears to be the infective stage, as it occurred extracellularly both in the inoculum and in the samples collected late in the cycle, when the cells were degenerating. Also, the dense form was the one adhering to and entering cells during early infection. The dense form appeared to infect cells by adhering to coated pits in the cell membrane similar to that described for *Chlamydia* spp. (7, 8). Cell entry appeared to occur via endocytosis at coated pits and phagocytosis by host cell projections. After entry, electron-dense forms changed into reticulated forms by approximately 4 h p.i., after which they multiplied by binary fission within a membrane-bound vacuole. Following the period of active replication, reticulated forms changed into electron-dense organisms that were subsequently released from disintegrating cells. The cells examined for later stages were infected at a low MOI. The colonies seen at 48 h and beyond therefore probably represented secondary rather than primary growth cycles. Further studies are needed to document the specific time sequence of the developmental cycle of the organism.

A striking feature of development was the specific association of the organism with host cell mitochondria. Newly entered organisms became encircled by the mitochondria; the space between the organism and mitochondria appeared as a halo by LM. The outer membranes of the large reticu-

FIG. 1. Light micrographs of developmental stages of the WSU/1044 organism in BT cells. (A) Densely staining forms (DF) were the only organisms seen in the inoculum. (B) Organisms (arrowheads) that had recently entered cells were seen at 2 h p.i. and appeared to be surrounded by a halo. (C) Small clusters of large reticulated forms (LRF) were apparent. (D) These developed into large colonies of reticulated forms (RF) (48 h p.i.). (E) Reticulated forms changed into dense forms (DF) (56 h p.i.). (F) At the completion of the cycle, dense forms (DF) were seen outside degenerated host cells (72 h p.i.). (B and C) Cells infected at a high MOI; (D to F) cells infected at a low MOI. Bars, 5  $\mu$ m.







**FIG. 2.** Electron micrographs of the initial development of WSU/1044 in BT cells. (A) The inoculum contained dense forms (DF) of the organism with densely staining cores. (B and C) By 2 h p.i., dense forms entered cells via (B) endocytosis by attachment to cell membranes at coated pits (arrowheads) and (C) phagocytosis of organism by host cell projections (HCP). (D) Once in the cell, the organisms were within an inclusion membrane (IM). (E) There, they changed into reticulated forms (RF) and became surrounded by host cell mitochondria (HCM). (F) The organism changed to large reticulated forms (LRF) that often adhered to outer mitochondrial membranes (arrowheads). (B to F) Cells infected at a high MOI. Bars, 1  $\mu$ m.

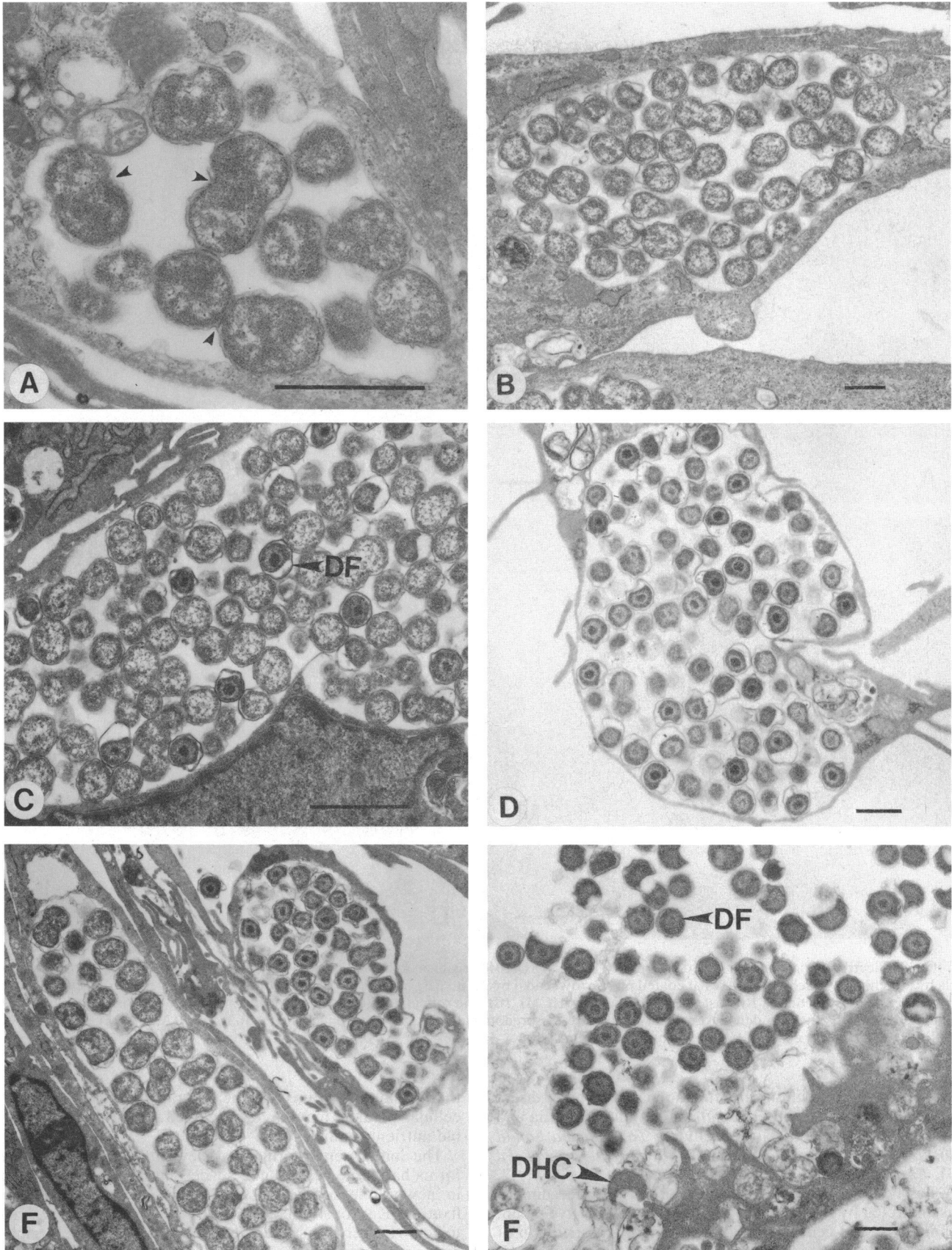


FIG. 3. Electron micrographs of later stages of development of the WSU/1044 organism in BT cells. Reticulated forms divided by binary fission (A, arrowheads), forming large colonies of reticulated organisms by 48 h p.i. (B). (C) The reticulated forms changed to dense forms (DF) as colonies matured (56 h p.i.). (D) Dense forms eventually were the predominant form in the inclusions. (E) Colonies varying widely in maturity were often seen adjacent to one another in the same cell. (F) Finally, dense forms (DF) were released from degenerated host cells (DHC) (72 h p.i.). Cells in all panels were infected at a low MOI. Bars, 1  $\mu$ m.

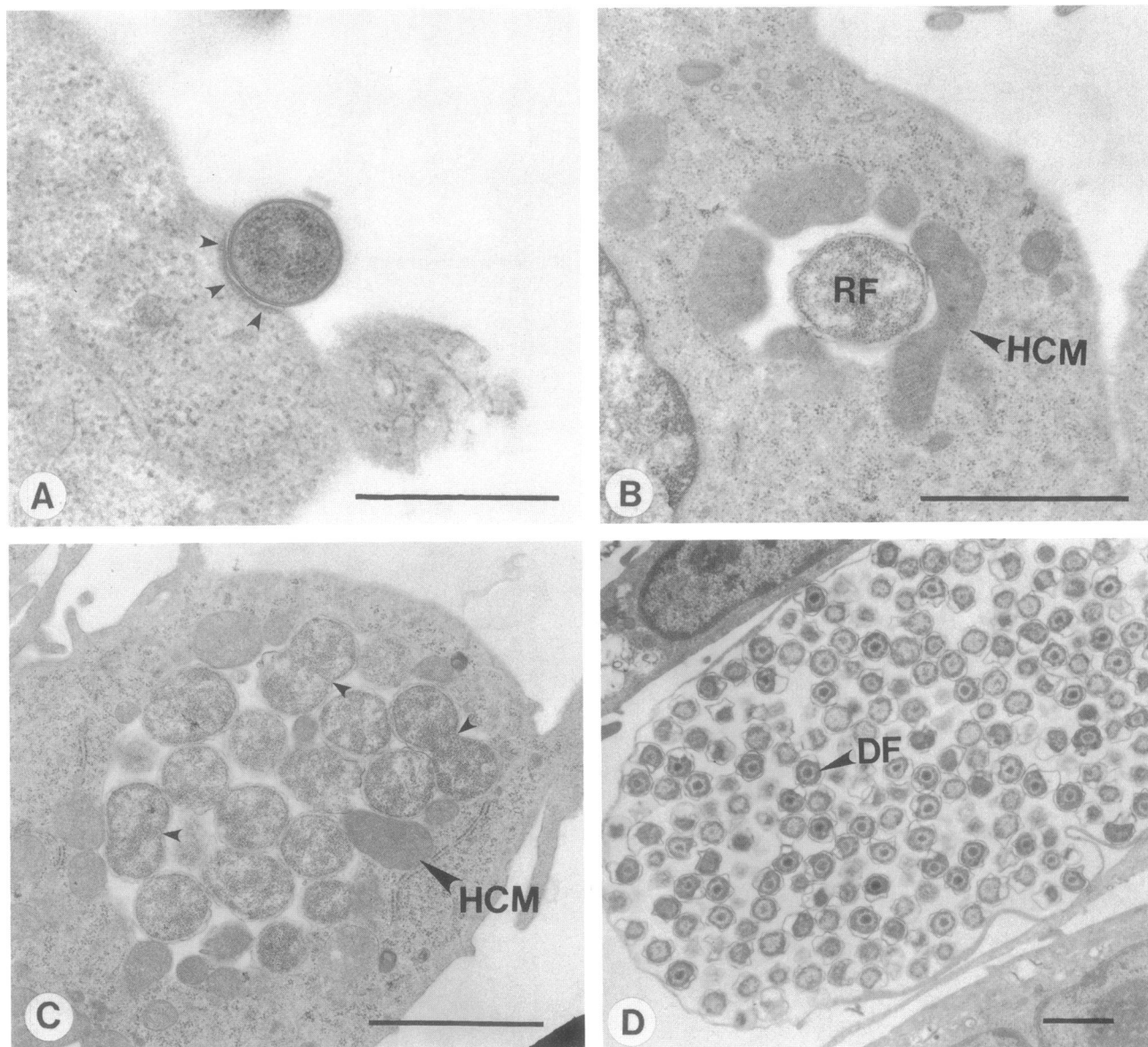


FIG. 4. Electron micrographs of the development of the WSU/1044 organism in P388D1 mouse macrophage cells. (A) Dense forms entered cells by attachment to cell membranes at coated pits (arrowheads) by 2 h p.i. (B) Once in the cell, the organisms changed into reticulated forms (RF) and became surrounded by host cell mitochondria (HCM). (C) The reticulated forms divided by binary fission (arrowheads). (D) The reticulated forms changed to dense forms (DF) and were the predominant form in mature inclusions (56 h p.i.). (A and B) Cells infected at a high MOI; (C and D) cells infected at a low MOI. Bars, 1  $\mu$ m.

lated forms and mitochondria often appeared to be in direct contact. An association between host cell mitochondria and other intracellular organisms, including *Toxoplasma gondii* (11), *Legionella pneumophila* (10), *Rickettsia prowazekii* (23), *Ehrlichia canis* (6), and *Chlamydia psittaci* (5, 16, 17, 24), has been described; however, none of these organisms was encircled by the mitochondria. Because *Chlamydia* spp. require host cell-generated ATP (17, 25), the association of WSU/1044 with mitochondria suggests that it too may require a source of exogenous ATP during the reproductive phase. The degree of mitochondrial association seen with WSU/1044, resulting in complete envelopment of the organism by mitochondria, appears unprecedented. It points to the uniqueness of this organism and raises speculation about

the possible mechanisms that this rapidly replicating intracellular parasite may have developed to satisfy its formidable nutrient requirements.

The initial dense forms that developed from reticulated forms had large periplasmic space that became less evident in newly released forms. We tested a faster-penetrating fixative (Karnovsky fixative [12]) in the second experiment to see whether this space is a fixation artifact. The periplasmic space appears to be a real feature, because it was evident only when the reticulated organisms were changing into electron-dense ones, and fixation with the faster-penetrating Karnovsky fixative did not eliminate this feature.

Several rickettsiae, including *Ehrlichia* (21, 26), *Cowdria* (18), *Anaplasma* (13), and *Chlamydia* (24, 25) spp., have



been shown to progress through phases characterized by electron-dense and reticulated forms. The morphologic features of the WSU/1044 developmental cycle, though not identical to those of any of the above organisms, possess similarities to each of them. In *Chlamydia* spp., the elementary body (electron-dense form) is the infectious entity that survives extracellular passage, whereas the reticulated form divides by binary fission with an intracellular inclusion to produce new progeny (24, 25). Morphologically similar forms have been shown for *Ehrlichia* and *Cowdria* spp. (14, 15, 21), but these organisms have not been studied as thoroughly in cell culture as the chlamydiae. The basic morphologic and developmental features of these three groups of organisms are similar. Their common attributes and their distinctness from the classical order *Rickettsiales* have prompted proposals to reclassify them into the same taxon (1). Although this organism multiplies by means of a developmental cycle that resembles that of the chlamydiae, an antigenic relation to the chlamydiae could not be demonstrated. However, it shares antigenic determinants with *C. ruminantium*, which is immunologically related to the genus *Ehrlichia*, of which one species, *E. risticii*, has recently been shown to be closely related to the genus *Rickettsia* (29). Taxonomic assignment will be deferred until more information is available.

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