Electron Microscopic Study on the Interaction Between Normal Guinea Pig Peritoneal Macrophages and Coxiella burnetii

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An electron microscopic study was conducted to explore the interaction between normal guinea pig peritoneal macrophages and phase I and II Coxiella burnetii previously treated with either normal or immune serum. A comparison was made on the efficiency of phagocytosis and subsequent killing of rickettsiae by macrophages. Both phases of rickettsiae previously treated with normal serum multiplied within phagosomes after phagocytosis with resultant destruction of macrophages. In contrast, suspending rickettsiae in immune serum rendered them more susceptible to phagocytosis and potentiated their destruction within macrophages.

The pathogenesis of Q fever and mechanisms of immunity are not well known. Although immune serum is not known to have a direct rickettsicidal action on Coxiella burnetii (1), it modifies the infection when passively transferred to nonimmune animals. Several groups of investigators have studied the action of antibody, phagocytic cells, and interaction of antibody-treated rickettsiae with phagocytic cells in vitro. Several of these studies have compared the two host-controlled variants of C. burnetii described by Stoker and Fiset (27), i.e., the naturally occurring phase I and phase II variants that appear on serial passage in the yolk sacs of embryonated hen eggs. Brezina and Kazár (6) and Wisseman et al. (30) found that polymorphonuclear leukocytes are capable of phagocytizing C. burnetii. The interaction between mouse and guinea pig peritoneal macrophages and C. burnetii was investigated by Downs (11) and Kazár et al. (16) by light microscopy. Downs found that phase II rickettsiae were more resistant to phagocytosis than phase I, whereas Kazár et al. concluded that phase II organisms were more susceptible than phase I rickettsiae. However, the fate of ingested rickettsiae was not resolved by these investigators.

In a comparison light microscopy study, phase I and II rickettsiae pretreated with normal serum multiplied and destroyed normal guinea pig peritoneal macrophages after phagocytosis, whereas antibody-treated phase I and II organisms were degraded by macrophages after ingestion (19).

Another rickettsial organism, Rickettsia mooseri (R. typhi), was shown by light microscopy to grow in human macrophages in culture when treated with normal serum (12). An electron microscopic study (4) with this system revealed that, after treatment with normal serum, phagocytized rickettsiae quickly escaped from the phagosome and entered the cytoplasm, where they multiplied. When immune serum-treated R. mooseri were ingested by macrophages, they remained in the phagosome and were destroyed.

Although the results of our previous light microscopic study on the interaction between normal and immune serum-treated rickettsiae and macrophages (19) are superficially similar to those described for R. mooseri (12), there is growing evidence that C. burnetii normally multiplies in a membrane-bound vacuole within the cytoplasm of its host cells (7, 13, 20). If this is the case also in macrophages, then the detailed relationship between phagocyte, C. burnetii, and antibodies might differ from that of R. mooseri. Therefore, the present study was undertaken to explore further the interaction of C. burnetii previously treated with either normal or immune serum and normal guinea pig peritoneal macrophages by means of transmission electron microscopy.

MATERIALS AND METHODS

Preparation of rickettsial stock suspensions. The third egg passage (EP-3) of the Henzerling strain of C. burnetii in phase I was obtained from the Na-
tional Drug Co., Philadelphia, Pa. The 88th egg passage (EP-88) of the phase II Nine-Mile strain was obtained from the stock culture collection of our laboratories. Chicken embryo cells were prepared and grown as a monolayer culture in roller bottles by the method of Kenyon and Pederson (18). The monolayer culture was then infected with 15 ml of a 10% rickettsial stock suspension (2 × 10^6 rickettsiae per ml) in Hanks balanced salt solution (HBSS). During the infection period the bottles were revolved at 0.4 rpm for 1 h at 35°C, the residual inoculum was removed, and 200 ml of Earle 199 medium (GIBCO, Grand Island, N.Y.) containing 2% fetal calf serum was added. The bottles were incubated on the roller apparatus (0.4 rpm) for 9 to 10 days at 35°C. Monolayers were frozen and thawed to release intracellular rickettsiae, the cellular debris was allowed to settle, and the rickettsiae in the supernatant fluid were pelleted by centrifugation at 8,400 × g for 2 h. Rickettsiae were resuspended in Earle 199 medium supplemented with 2% fetal calf serum and stored at −70°C. There were approximately 10^16 phase I or II rickettsiae/ml as determined by the method of Silberman and Fiset (26). Infectivity of the phase I and II rickettsial suspension was estimated to be 5 × 10^9/mi by intraperitoneal titration in outbred mice (Tac: SWiBR).

Sera. Outbred Hartley strain guinea pigs, weighing approximately 350 to 450 g and obtained from Buckberg Lab Animals, Tompkin Cove, N.Y., were immunized intraperitoneally with 0.2 ml of organism suspension containing approximately 10^6 formalin-killed phase I or II C. burnetii. Three additional inoculations were given at 2-week intervals. Serum complement-fixing antibody was determined 2 weeks after the last immunization. Serum complement-fixing titters from phase I-immunized guinea pigs were 1:128 to 1:256 against homologous antigen and 1:32 to 1:64 against heterologous antigen. Phase II-immunized guinea pigs usually had a titer of 1:256 to 1:512 against homologous antigen and 1:16 to 1:32 against phase I antigen. Negative CF titer sera were obtained from nonimmunized guinea pigs.

Preparation of peritoneal macrophages. Macrophages were collected 4 days after injection of guinea pigs with 25 ml of mineral oil (Marcol no. 90, Humble Oil and Refining Co., Houston, Tex.). The peritoneal exudate cells were harvested and processed by the method of David et al. (10). Approximately 5 × 10^9 macrophages were dispensed into petri dishes (60 by 15 mm; Falcon Plastics, Oxnard, Calif.) and incubated at 37°C for 2 h in a humid atmosphere of air containing 5% CO2. Nonadherent cells were removed by two washes in HBSS, 5 ml of fresh Earle 199 medium supplemented with 10% FCS was added, and the cells were incubated for an additional 18 h to 24 h.

Cell culture infection. Rickettsia (approximately 10^9/ml) of either phase I or II were incubated for 30 min at 37°C in medium containing either 10% normal or immune heat-inactivated guinea pig serum. Serum-treated rickettsiae were then added to macrophage cultures at a ratio of 100 rickettsiae/1 macrophage and incubated for 60 min at 37°C. The inoculum was removed by aspiration, and the macrophage cultures were washed three times with 5 ml of HBSS. Macrophages for immediate examination were processed at this time for transmission electron microscopy. Those held for longer periods were given fresh medium and incubated at 37°C. Infected cells were washed two times daily with HBSS, and fresh medium was added to minimize phagocytosis. No antibiotics were used in this study.

Electron microscopy. Infected macrophages for examination by transmission electron microscopy were washed three times with 5 ml of HBSS and fixed for 1 h at 4°C in 2% glutaraldehyde buffered to pH 7.4 with 0.1 M cacodylate buffer. The macrophages were then scraped off the petri dish with a rubber policeman and centrifuged at 250 × g for 10 min. The cell pellet was washed twice with 0.1 M cacodylate buffer, pH 7.4, containing 3.66% sucrose and then embedded in 2% Noble agar (Difco). After fixation in 1% osmium tetroxide the cells were dehydrated through an ethanol series, embedded in Epon 812, sectioned, and stained with uranyl acetate and lead citrate (23). For comparison, uninfected macrophages were processed simultaneously. All preparations were examined with a Hitachi HU-12 electron microscope operating at 75 kV.

Enzymatic analyses. Cells were harvested, washed in pH 7.3 phosphate-buffered saline, and resuspended to a concentration of 10^8 macrophages per ml. Triton X-100 was added to a concentration of 0.1%, and the cell suspension was sonicated treated with a Bronson Sonifier (model W 185D) for two 10-s periods at 70 W of power. The resulting homogenate was used directly for analysis of the following enzymatic activities. β-Glucuronidase (EC 3.2.1.31) was assayed in ml (final volume) of 0.15 M acetate buffer (pH 5.0) and 1 mM phenolphthalein β-glucuronidase (8). The reaction was stopped with 3 ml of glycine buffer (pH 10.7) and clarified by filtration, and the absorbancy was measured at 540 nm. Acid phosphatase (EC 3.1.3.2) was measured with p-nitrophenylphosphate (16 mM) as substrate and 0.12 M acetate buffer (pH 5.0). The 1-ml reaction system was stopped with 3 ml of 1.25 N NaOH, and the absorbancy was read at 420 nm (8). β-Acetylglucosaminidase (EC 3.2.1.30) and β-galactosidase (EC 3.2.1.23) were determined after the method of Beck and Tappel (5) using p-nitrophenyl-N-acetyl-β-D-glucosaminide and p-nitrophenyl-β-D-galactopyranoside as substrates, respectively. Lysozyme (EC 3.2.1.17) was determined by the lysoplate method of Osserman and Lawlor (22). Protein concentrations were determined by an automated Lowry procedure (21) using bovine serum albumin as standard.

RESULTS

Rickettsial phagocytosis. The percentages of macrophages containing ingested rickettsiae and subsequent fate of ingested organisms are based upon the mean of four replicates for each sample time. Approximately 100 to 200 cells were examined in each experiment. Examination of ultrathin sections revealed that phase I rickettsiae were more resistant to phagocytosis.
than phase II organisms. Four percent of the macrophages contained one to three phase I rickettsiae, whereas 55% of the macrophages contained five to ten phase II organisms when rickettsiae were pretreated with normal serum prior to interaction with macrophages. In contrast, pretreatment of rickettsiae with homologous immune serum enhanced the phagocytic uptake. Approximately 25% of the macrophages contained one to three phase I organisms; 90% of the macrophages had six to ten phase II organisms. Most rickettsiae were seen within well-defined phagosomes located near the periphery of the cell. Thick-walled rod-shaped rickettsiae (0.2 by 0.5 μm), containing either a filamentous or dense nucleoid mass in the central region, were usually seen within phagosomes (Fig. 1). All organisms were found within phagosomes, which suggests that they entered cells by phagocytosis rather than direct penetration into the cytoplasm. The morphological appearance of both phagocytized rickettsiae and infected macrophages remained relatively unaltered after the 60-min interaction period. The phagosomes of macrophages were relatively free of particulate material, except for the ingested rickettsiae.

Treatment of rickettsiae with homologous antibody enhanced the phagocytosis of both phases of organisms. Twenty-five percent of macrophages contained one to three antibody-sensitized phase I rickettsiae per cell after the 60-min interaction period, and 90% of macrophages contained six to ten antibody-sensitized phase II rickettsiae per cell.

Light microscopic studies on the macrophage-rickettsial interaction showed that phase I organisms previously treated with normal serum were phagocytized by 7% of the macrophages. An average of four rickettsiae was contained in each infected macrophage, based on counting 100 macrophages at random for each of four replicate experiments. In contrast, 80% of the macrophages contained an average of 20 phase II rickettsiae per infected cell. Treatment of rickettsiae with homologous immune serum enhanced the ingestion of rickettsiae by macrophages (19).

**Fate of ingested rickettsiae.** When macrophages ingested rickettsiae previously treated with normal serum, normally one to three phase I or five to ten phase II rickettsiae were within macrophage phagosomes after the 60-min interaction period. Phase I and II ingested rickettsiae multiplied freely in well-defined phagosomes for 2 to 3 days, resulting in a 5- to 10-fold increase in the number of rickettsiae per infected macrophage (Fig. 2). By day 3 after infection most of the macrophages were disrupted, with the ultimate liberation of rickettsiae. In one instance binary fission of rickettsiae separated by a cell wall was observed (Fig. 3).

An interesting morphological change of the rickettsiae was seen within infected macrophages after ingestion of both phases of orga-

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**Fig. 1.** Electron micrograph of rod-shaped rickettsiae within a macrophage phagosome. ×54,000; bar = 0.5 μm.
nisms treated with normal serum. Two forms were usually present. Rod-shaped forms were observed as well as round to oval organisms. The oval forms lacked thick walls, their nucleoid filaments were more dispersed, and they measured 0.4 by 0.8 μm (Fig. 4). These round forms were similar to those seen when macrophages ingested antibody-treated rickettsiae after the 60-min interaction period. The appearance of round forms occurred later than with the antibody-treated organisms. A few were seen at 24 h; many more were seen at 48 and 72 h. Some organisms were disrupted with the cytoplasm contracted from the cell wall, indicating death of the organism.

Macrophages that had ingested antibody-treated rickettsiae were studied 15, 30, 60, and 300 min and 1, 2, and 3 days after infection. After 15 min of infection, many rickettsiae were noted in various states of dissociation or degradation (Fig. 5). Both phases of antibody-treated rickettsiae were degraded. Rod-shaped forms remained fairly intact, but many round to oval forms showing alterations such as swelling or condensation of the cytoplasmic material were observed. In some instances triple-layered structures, representing cell wall fragments, remained after enzymatic digestion of the organism. Frequently, the internal contents of rickettsiae contracted away from the cell wall, or no

Fig. 2. Macrophage filled with multiplying rickettsiae within well-defined phagosomes 3 days after infection. ×10,800; bar = 1.0 μm.
Macrophages and C. burnetii infection

Rickettsial cytoplasm was seen. There was abundant cellular debris, including remnants of cell wall of rickettsiae and a very few intact and identifiable rickettsiae. Usually by 5 h postinfection, very few unaltered rickettsiae remained (Fig. 6). No intact organisms were observed 1 to 2 days after infection. Most of the macrophages at this time were essentially indistinguishable from controls. Results by light microscopy have substantiated the degradation of antibody-sensitized rickettsiae (19).

Lysosomal enzymatic activity. The specific activity of representative lysosomal enzymes (Table 1) demonstrates that the hydrolytic activities of oil-stimulated peritoneal macrophages were not significantly altered after culture for up to 2 days except for β-glucuronidase. Nonstimulated peritoneal cells, however, differed from oil-stimulated cells in that the former had slightly lower activities of β-galactosidase and lysozyme.

Experiments were conducted utilizing peritoneal macrophages from guinea pigs that were not stimulated with mineral oil. The phagocytic uptake and subsequent fate of ingested organisms were not significantly different from the results above.

Discussion

Phase I C. burnetii were more resistant to phagocytosis by normal guinea pig peritoneal macrophages than phase II organisms. Previ-
FIG. 4. Macrophage 3 days after infection containing both rod-shaped and round to oval forms. ×45,000; bar = 0.5 μm.

ously, resistance to phagocytosis by phase I organisms was described by Wisseman et al. (30) and Brezina and Kazár (6), who used light microscopy in interaction studies with polymorphonuclear leukocytes, and Kordová et al. (20), who used Earle cells. Our electron microscopic findings confirm our own light microscopic observations and those of Kazár et al. (16). We have no explanation for the findings of Downs (11), who found by light microscopy that phase II organisms were more resistant than phase I organisms. It appears that C. burnetii enters macrophages only by phagocytosis since all rickettsiae were found in the cytoplasm, surrounded by a limiting membrane. This finding is in agreement with other investigators (6, 7, 11).

Although some rickettsiae treated with normal serum were degraded after phagocytosis, most of these phase I and II rickettsiae multiplied unchecked after ingestion and ultimately destroyed the macrophages. Both phase I and II C. burnetii grew at about the same rate and were equally capable of growing within the macrophage. The finding of a rickettsia undergoing binary fission is in agreement with others on the mode of Coxiella reproduction (2, 13, 15). "Atypical" forms as described by Anacker et al. (2) or intermediate forms as described by Rosenberg and Kordová (24, 25) were not seen.

An interesting observation was that of large round to oval forms noted at various times after ingestion of phase I and II rickettsiae. These cells were observed initially 24 h after ingestion of normal serum-treated rickettsiae; they appeared earlier (60 min) and in greater numbers when treated with immune serum. Many of these larger forms appeared in various stages of degradation, and no intact rickettsiae were seen 1 day after macrophages ingested antibody-treated organisms. It appears that these larger cells were derived from the small, rod-shaped forms and were "swelling" prior to disruption. Although we cannot directly rule out the possibility that there is a complex cycle in the multiplication of C. burnetii, our data do not support it. Other investigators have observed two morphological types of phase I or II C. burnetii after purification by density gradient centrifugation (9, 28, 29). Wiebe et al. (29) speculated that the oval to round forms may be degenerate forms produced by host-mediated digestion or alteration by chemical treatment.
or that they may represent two stages in a complex developmental cycle. Wachter et al. (28) indicated that the larger forms could be derived from the rod-shaped rickettsiae by various stresses.

An important finding of this study is the role of immune serum in the uptake and subsequent fate of ingested rickettsiae. When phase I and II rickettsiae were treated with specific immune serum prior to interaction, both phases of rickettsiae were more avidly phagocytized by macrophages but, even more important, the immune serum potentiated the killing of ingested organisms. Enhancement of phagocytosis of antibody-treated C. burnetii by PMN was also observed by Brezina and Kazár (6).

Kazár et al. (15) reported that immune serum alone was not rickettsicidal, since phase I and II organisms treated with immune serum multiplied when subsequently inoculated into the yolk sac of chicken embryos. In our study the treatment of rickettsiae with immune serum potentiated the destruction of both phases of ingested organisms; the phenomenon occurred fairly rapidly. Many organisms were degraded during the 60-min interaction period; more were degraded by 15, 30, and 60 min after infection. Usually, by 5 h after infection very few intact organisms were seen. Rickettsiae had to be treated with immune serum prior to phago-

**Fig. 5.** Macrophage containing antibody-sensitized rickettsiae 60 min after infection. Round to oval as well as rod-shaped forms are seen. ×27,000; bar = 0.5 μm.
FIG. 6. Electron micrograph of antibody-sensitized rickettsiae 5 h after infection. Many organisms are in various stages of degradation. ×30,000; bar = 0.5 μm.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Oil stimulated</th>
<th>Nonstimulated</th>
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<tr>
<td></td>
<td>0&quot;</td>
<td>1</td>
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<tr>
<td>β-Glucuronidase</td>
<td>7.81 ± 0.25</td>
<td>8.09 ± 0.25</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>411 ± 25</td>
<td>421 ± 21</td>
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<tr>
<td>β-Acetylg glucosaminidase</td>
<td>1.07 ± 0.05</td>
<td>1.22 ± 0.05</td>
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<tr>
<td>β-Galactosidase</td>
<td>43.6 ± 2.9&quot;</td>
<td>49.0 ± 3.1&quot;</td>
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<tr>
<td>Lysozyme</td>
<td>29.2 ± 4.7&quot;</td>
<td>26.1 ± 2.4&quot;</td>
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*Expressed as nanomoles of substrate hydrolyzed/minute × milligrams of protein.
*Mean of six determinations ± standard error.
*Mean of two determinations.
*Days in culture
*P < 0.05 (Student's t test on day 0).
*Picomoles of substrate hydrolyzed/minute × milligram of protein.
*Micrograms of egg white lysozyme equivalents/milligram of protein.
cytosis for destruction by macrophages. Addition of the immune serum in the maintenance medium after ingestion of rickettsiae pre-treated with normal serum did not inhibit the growth of the organisms.

The ability of immune serum to opsonize rickettsiae for destruction has been studied in cultured cells by other investigators (7, 12, 15). Such studies suggest that the fate of antibody-sensitized rickettsiae was dependent upon the type of cell to which the organisms were exposed. Burton et al. (7) observed that rickettsiae were able to multiply in Earle cells, even after opsonization with immune serum. Kazár et al. (15) observed that both types of rickettsiae opsonized in immune serum reacted differently in chicken embryo cell cultures; the growth of phase II organisms was inhibited, and phase I rickettsiae grew unchecked.

Our results stress the importance of the host cell type on the subsequent fate of antibody-sensitized C. burnetii. Macrophages, which are considered phagocytic or scavenger cells, were consistently able to degrade ingested C. burnetii. The fate of antibody-sensitized R. prowazeki (31) or R. rickettsii and R. australis (17) with chicken embryo cells reemphasizes the importance of host cell type. Interaction studies utilizing antibody-sensitized intracellular parasites, such as R. mooseri (12) and Toxoplasma gondii (3), and normal macrophages have yielded results similar to ours.

The phagocytic uptake and subsequent fate of ingested organisms were essentially the same when rickettsiae were made to interact with macrophages from oil-stimulated or non-stimulated guinea pigs. There was no significant difference in the lysosomal hydrolase content of oil-stimulated macrophages when compared to nonstimulated macrophages. Neither was there any difference after culture, which would account for the difference in the fate of ingested rickettsiae.

Regardless of the low virulence of phase II rickettsiae (susceptibility to phagocytosis by macrophages), these organisms must still be considered virulent, since they were able to multiply within the macrophage and ultimately destroy the cell. Another parameter of virulence is their ability to infect laboratory animals.

Another consideration in immunity of C. burnetii is the role of immune serum. It appears that immune serum not only enhances phagocytosis of rickettsiae by macrophages but also potentiates the destruction of organisms within the phagocyte. Another potentially important area of the immune response is that of cell-mediated immunity. Our light microscopic study (19) and the lymphocyte transformation study of Jerrells et al. (14) indicate that cell-mediated immunity is involved in Q fever infection. We are currently studying this aspect of immunity.

Our results indicate that normal guinea pig peritoneal macrophages alone are not capable of killing phase I or II C. burnetii organisms. Immune serum acted as an opsonin but, more important, acted in concert with macrophages to potentiate destruction of ingested rickettsiae.

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LITERATURE CITED


