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Histologic, Immunofluorescence, and Electron Microscopic Study of Infectious Process in Mouse Lung after Intranasal Challenge with *Coxiella burnetii*

THEODOR KHAVKIN[1]* AND SIAMAK S. TABIBZADEH[2]

Interferon Sciences, Inc., New Brunswick, New Jersey 08901,[1] and City Hospital Center at Elmhurst, Elmhurst, New York 11373[2]

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A histologic, immunofluorescence, and electron microscopic study of the intracellular parasitism of *Coxiella burnetii* (the Q fever agent) in mouse lungs after intranasal challenge was undertaken. It was shown that this microorganism invades type I and, rarely, type II pneumocytes as well as pulmonary fibroblasts and histiocytes. The infectious process can be described as a focal intra-alveolar inflammation with the macrophages prevailing in the exudate. It is self-limited, with a complete resolution. The inflammation is associated with atelectases and with increased secretory activity by type II pneumocytes. Alveolar macrophages and granulocytes degrade *C. burnetii*. This degradation is followed by damage to and eventual disintegration of some macrophages and by damage to some bacterium-free pneumocytes and vascular endothelial cells in the vicinity of macrophages degrading organisms. The cell damage might be caused by lipopolysaccharide released from degraded organisms. The infectious process is also associated with the influx of T cells in the pneumonic foci, T-cell attachment to the macrophages degrading organisms, and fusion of some macrophages. These are considered a morphologic expression of cell-mediated immunity involved in the infectious process.

Q fever is an acute febrile infectious disease caused by the rickettsial organism *Coxiella burnetii*. The organism was originally considered pneumotropic, since Q fever is frequently manifested by influenzalike symptoms with pulmonary involvement (Q fever pneumonia) and since humans usually acquire the infection via aerosol inhalation. Hence, some early synonyms of Q fever were pneumorickettsiosis, pneumotyphus, and Balkan grippe (54). Subsequent observations have shown, however, that in human volunteers direct inhalation of *C. burnetii* results in the development of radiologically detectable pneumonia in only one-half of the cases (47). Furthermore, the rate of apparent pneumonia in reported Q fever outbreaks has varied from 4 to 90% of the cases (29). These observations have led to the reverse presumptions that *C. burnetii* is not well adapted for multiplication in the cells of the respiratory tract and that the lungs serve mainly as a portal of entry of the pathogen into the human body (48). The actual fate of *C. burnetii* in the respiratory tract and the pathogenesis of Q fever pneumonia are still obscure.

Contemporary notions about this disease are largely based on clinical and radiological data (29), since necropsy observations are scarce and are often complicated by concomitant nonspecific alterations (reviewed in reference 40). In both necropsy cases and experimental studies with laboratory animals, the pulmonary lesions have been characterized as either interstitial (13, 17, 38, 43) or intra-alveolar (1, 4, 14, 21, 30, 36, 37, 52) pneumonia with an exudate that is mainly mononuclear or as a combination of both interstitial and intra-alveolar lesions (45). No special attention has been paid to the alveolar interstitium. In experimental studies (4, 13, 43) and in studies of biopsies of human pulmonary tissue (31, 35) *C. burnetii* was detected in macrophages.

In our previous experiments, we also found coxiellae in macrophages as well as in cells of the peribronchial stroma (3, 4, 21). These experiments also showed that intranasal instillation of infected cells or animal tissues induced in mice a long-lasting infectious process. For 47 days after challenge coxiellae were detected in lungs both by passage in guinea pigs and yolk sacs and by immunofluorescence staining; on days 55 and 66, coxiellae were detected only by animal and yolk sac passages. As in other studies, however, we did not define the relationships of *C. burnetii* to cells of the respiratory parenchyma, the pneumocytes.

The aim of this work was a further morphologic study of experimental lesions produced by *C. burnetii* in mouse lungs, with emphasis on the interaction of the pathogen with cells of the respiratory parenchyma and pulmonary stroma. The mouse model of Q fever pneumonia applied in our previous studies and in this study appeared to be satisfactory for our goals, since there is a considerable similarity among mammalian species in overall lung composition and in the distribution and features of cells of the respiratory parenchyma (11, 46).

MATERIALS AND METHODS

Organism. The Louga strain of *C. burnetii* was used. It was isolated from a wild mouse (*Apodemus microtii*) in a guinea pig (2) and subjected to 20 yolk passages. Although it was serologically confirmed to be in phase 1, it rarely killed guinea pigs infected intraperitoneally. It multiplies extensively in cells of mouse spleen (4), chicken embryo yolk sac (24), guinea pig testicles (22), and MK2 cells of monkey kidney epithelium (23).

Challenge. Infected MK2 cells were chosen for inoculation. The choice was based on the following previous observations (3, 21). (i) Intranasal challenge of mice with a 5 to 10% suspension of an infected cell culture or animal tissue induced a pulmonary infectious process with organisms in lungs in numbers detectable with a microscope, without the death of animals, and, on autopsy, without visible lung alterations. (ii) Challenge with yolk sac cultures was com-

* Corresponding author.

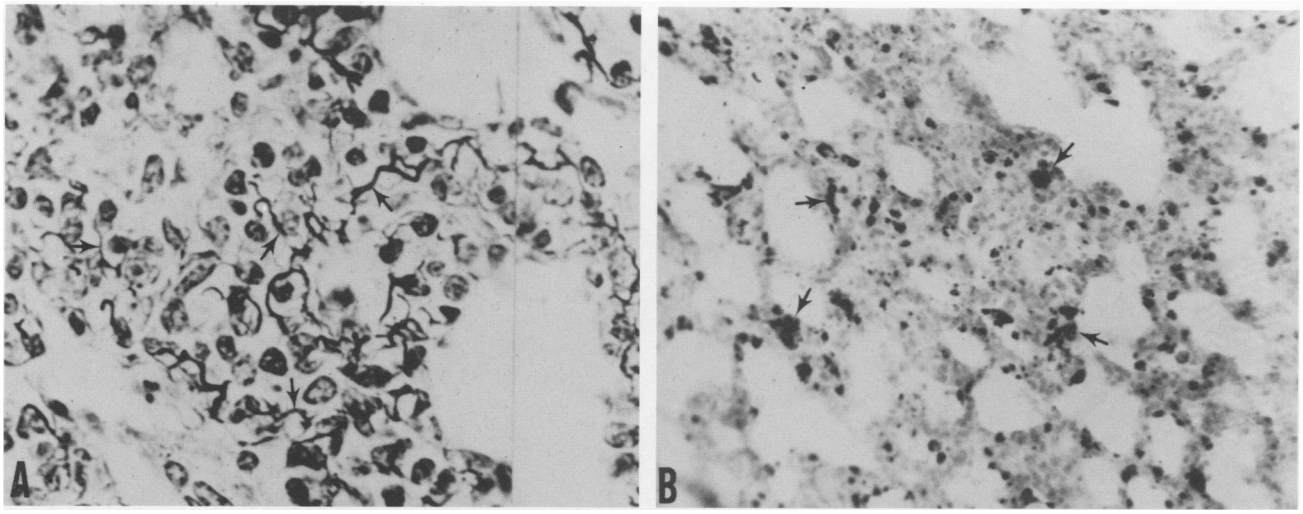

FIG. 1. Airless areas in lung parenchyma 3 (A) and 14 (B) days after challenge. (A) Argyrophilic fibrils (arrows) define walls of collapsed and semicollapsed alveoli. Paraffin section; silver impregnation; magnification, ×300. (B) Collections of granulocytes (arrows) in semicollapsed alveoli and single granulocytes within capillaries are defined by the myeloperoxidase reaction product. Frozen section; Sudan III–alpha-naphthol stain; magnification, ×200.

plicated by nonspecific pulmonary lesions and the death of some animals caused by admixture of the yolk sac material with the culture. (iii) Suspensions of noninfected cells or tissues, such as mouse spleen or lung and guinea pig spleen or testicles, elicited only leukocyte influx into the lungs which lasted 24 h; suspensions of MK2 cells were less contaminated by cellular debris than were cell suspensions prepared from animal tissues.

Sixty-two 2-week-old albino mice from a colony screened for the absence of adventitious lung lesions were inoculated intranasally with 0.1 ml of a 10% (vol/vol) suspension of homogenized 100%-infected MK2 cells. Upon intraperitoneal inoculation, this *C. burnetii* dose produced a week-long fever in guinea pigs and splenomegaly with multiplication of organisms in splenic cells in mice.

Material for investigations and staining procedures. At selected times (from 1 h to 23 days), smears and frozen and paraffin sections were prepared from lungs. For electron microscopy, lung samples taken from 15 mice 4, 5, and 13 days after inoculation were fixed in 1% OsO_4 in acetate-Veronal buffer and embedded in araldite. Smears and frozen and paraffin sections were stained with Giemsa stain, with the Sudan III–alpha-naphthol mixture for leukocyte myeloperoxidase (32), and with fluorescein-labeled rabbit anti-*Coxiella* immunoglobulin G produced at the Leningrad Pasteur Institute. At a working dilution of 1:32, this conjugate induces distinct specific fluorescence of coxiellae in both smears and paraffin sections (3, 4, 24). To demonstrate the reticular fibers, we also stained paraffin sections with periodate-leukofuchsine (periodic acid-Schiff method; 9) and impregnated them with silver by the Foot method (9). Ultrathin araldite sections were stained with uranyl acetate and lead citrate and examined with JEM7 and Hitachi H58 electron microscopes.

RESULTS

As in our previous studies (3, 21), the infection developed in mice without signs of sickness or death or, on autopsy, without macroscopically visible lung alterations.

Light microscopy. Scattered inflammatory foci with a predominantly granulocytic intra-alveolar exudate were found on day 1 after challenge. On day 2 granulocytes were for the most part replaced by macrophages. During the following 3 weeks, the major changes were pneumonic foci containing predominantly macrophages with an admixture of granulocytes and airless areas. The airless areas gave a coarse netlike appearance to the respiratory parenchyma suggestive of interstitial pneumonia. The periodic acid-Schiff reaction and silver impregnation that define the alveolar outlines (7) as well as staining for leukocyte myeloperoxidase demonstrated, however, that this appearance was actually due to collapsed alveoli rather than to inflammatory thickening of the alveolar septa (Fig. 1). From day 5 on there was an increase in the number of perivascular and peribronchial lymphocyte collections in comparison with those in lungs of unchallenged animals.

Microorganisms were identified by fluorescent antibody in smears from all the animals under study throughout the observation periods. In paraffin and semithin araldite sections, they were detectable only during week 1. Phagocytosis of coxiellae by macrophages and granulocytes was first noticed at 3 h; coxiellae were first noticed in parenchymal and stromal cells 1 day postchallenge. Instillation of noninfected MK2 cells elicited an immediate influx of granulocytes into alveolar ducts and some alveoli, without a serous exudate. Partially destroyed granulocytes were shifted into bronchioli 48 h after instillation; this shift was indicative of their elimination from the alveoli. There were no signs of inflammation in the lungs of control animals killed after day 3.

Electron microscopy. Circumscribed atelectases and pneumonic foci mostly with a macrophage-dominated exudate and enhanced secretory activity by type II pneumocytes were apparent on days 4 and 5 after challenge. Type II pneumocytes had numerous lamellate secretory bodies throughout. In some places the bodies were extruded into the alveoli (Fig. 2), indicating an enhanced secretory activity by these cells.

Seemingly unaltered coxiellae were found in type I and, rarely, type II pneumocytes as well as in histiocytes and fibroblasts of the peribronchial and perivascular stroma. Organisms represented mainly by large morphological vari-

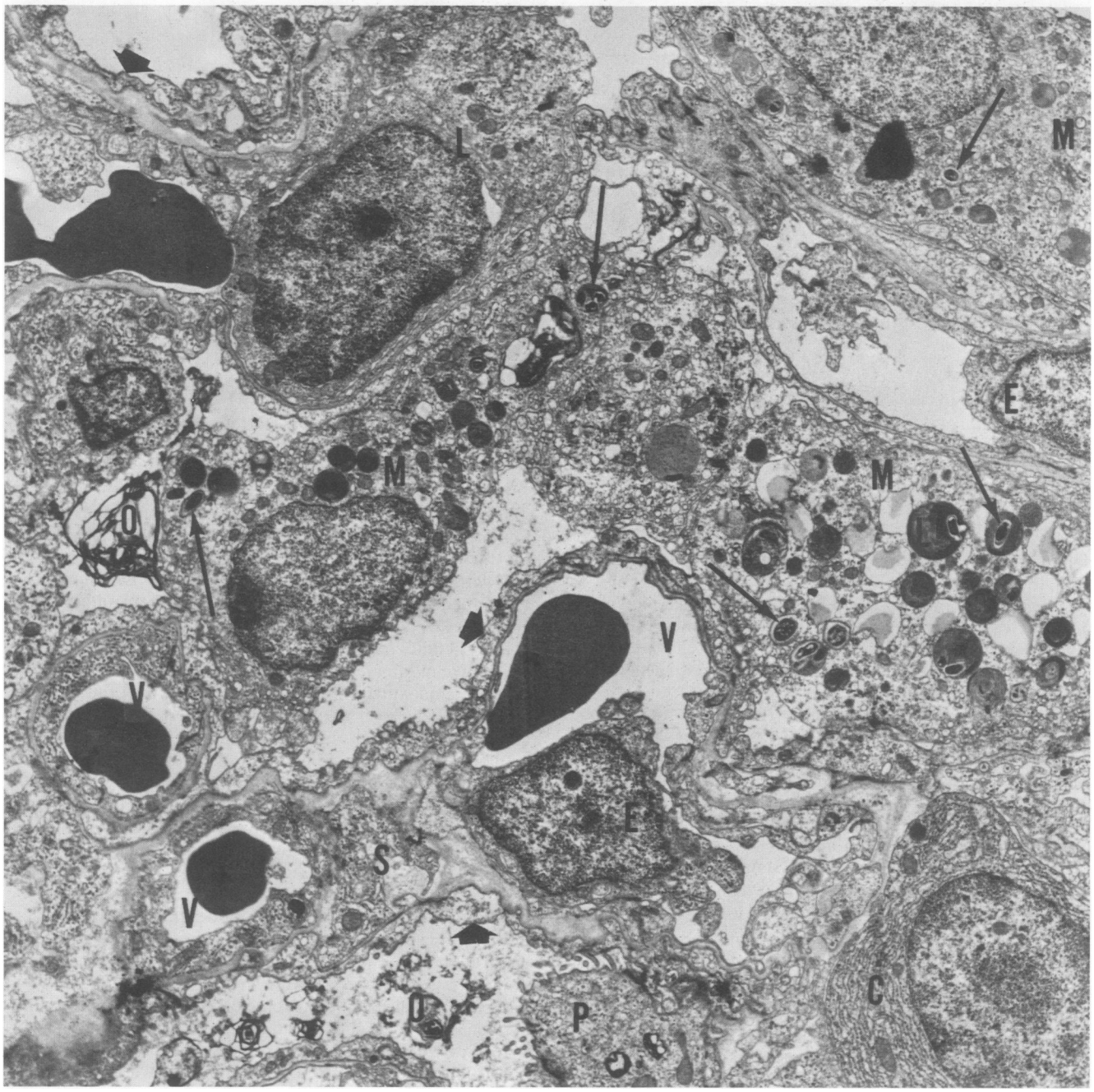

FIG. 2. Alveoli in a pneumonic area containing activated macrophages (M) and myelinlike osmiophilic bodies (O) of the alveolar surfactant. Some of these bodies may have been released from a type II pneumocyte (P). Arrows, Coxiellae in macrophage phagolysosomes; V, cross and oblique sections of the same capillary in the interalveolar septum (S); L, intravascular lymphocyte; E, nuclei of the capillary endothelial cells; arrowheads, type I pneumocytes; C, plasma cell in adjacent perivascular connective tissue. Day 4; magnification, ×2,400.

ants (5, 49) were confined within membrane-bound vacuoles that are typical of *Coxiella*-infected cells (5, 49), resulting in a protrusion of usually flattened type I pneumocytes into the alveolar lumen (Fig. 3A) and in a reduction in the number of organelles. Infected type II pneumocytes had reduced numbers of microvilli and secretory bodies (Fig. 3B). Heavily infected and damaged pneumocytes were covered by a serous exudate with an admixture of granulocytes (Fig. 3C). At the same time, there were undamaged infected pneumocytes of either type without an inflammatory response in their vicinity. These cells maintained their structural integrity and remained in intimate contact with the alveolar basal membrane (Fig. 3A and C), typical of pneumocytes (8, 11, 46).

We found no signs of an acute inflammatory response around infected stromal fibroblasts and histiocytes. The infected fibroblasts were recognized by their intimate contact with collagen and elastic fibers (Fig. 3D).

In alveolar macrophages, organisms were localized either in phagolysosomes or, rarely, in typical *C. burnetii*-bearing

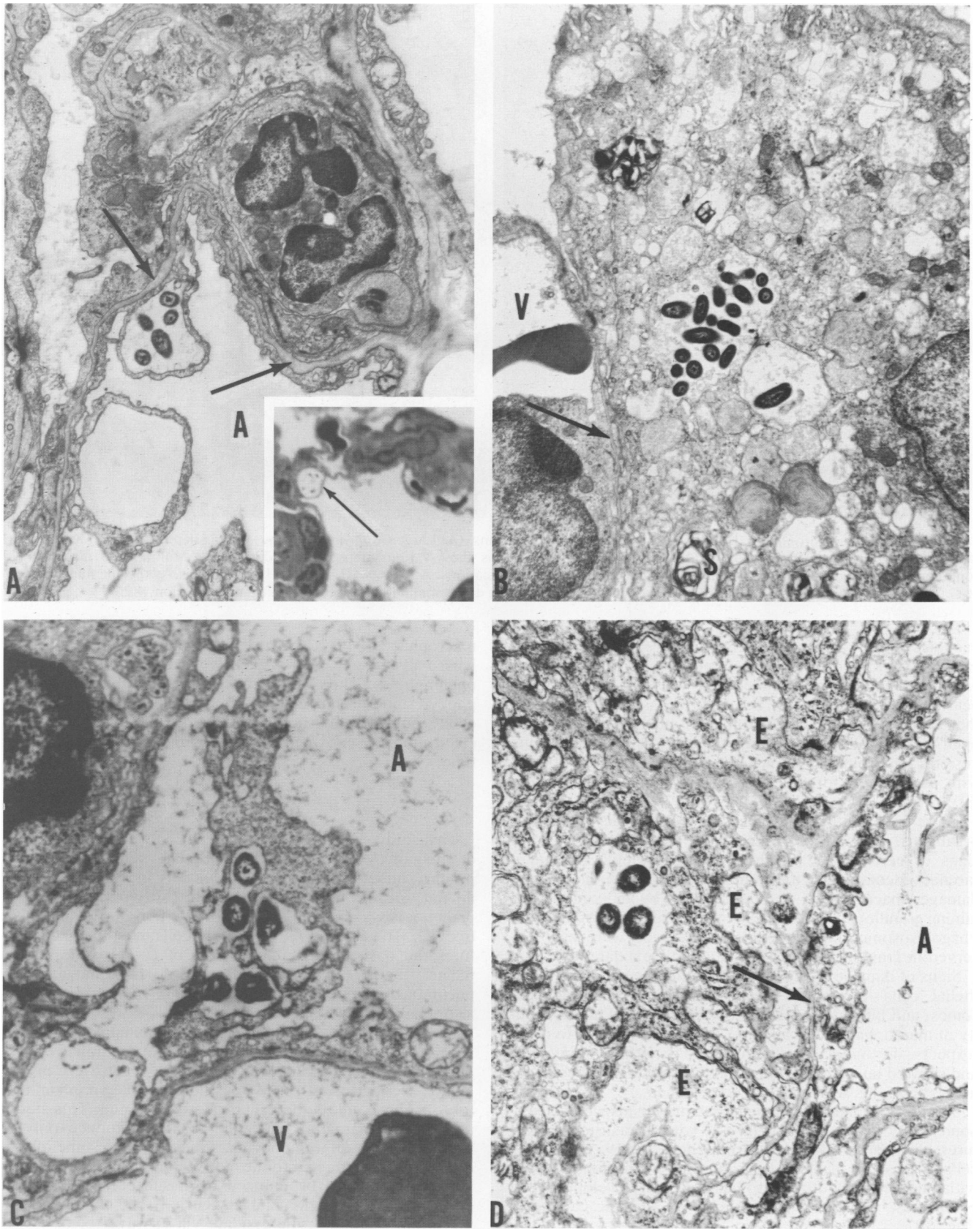

FIG. 3. *C. burnetii*-bearing cells of the respiratory parenchyma. (A) Type I pneumocyte with two vacuoles, one of which contains seemingly unaltered organisms. Arrows, Basal membrane separating an alveolus (A) from blood vessels. (B) Type II pneumocyte with several organism-containing vacuoles and myelinlike secretory granules (S). Arrow, Alveolar basal membrane; V, blood vessel. (C) Partially destroyed infected type I pneumocyte covered with serous exudate; V, adjacent capillary separated from an alveolus (A) by the basal membrane. (D) Pulmonary fibroblasts, one of which has an organism-containing vacuole. E, Cross sections of fibroblast-associated elastic fibers; arrow, basal membrane separating fibroblasts from type I pneumocytes and from an alveolus (A). Magnifications, ×5,200. Inset in panel A: infected pneumocyte (arrow) in an exudate-free alveolus. Semithick section; thionin stain; magnification, ×3,003.

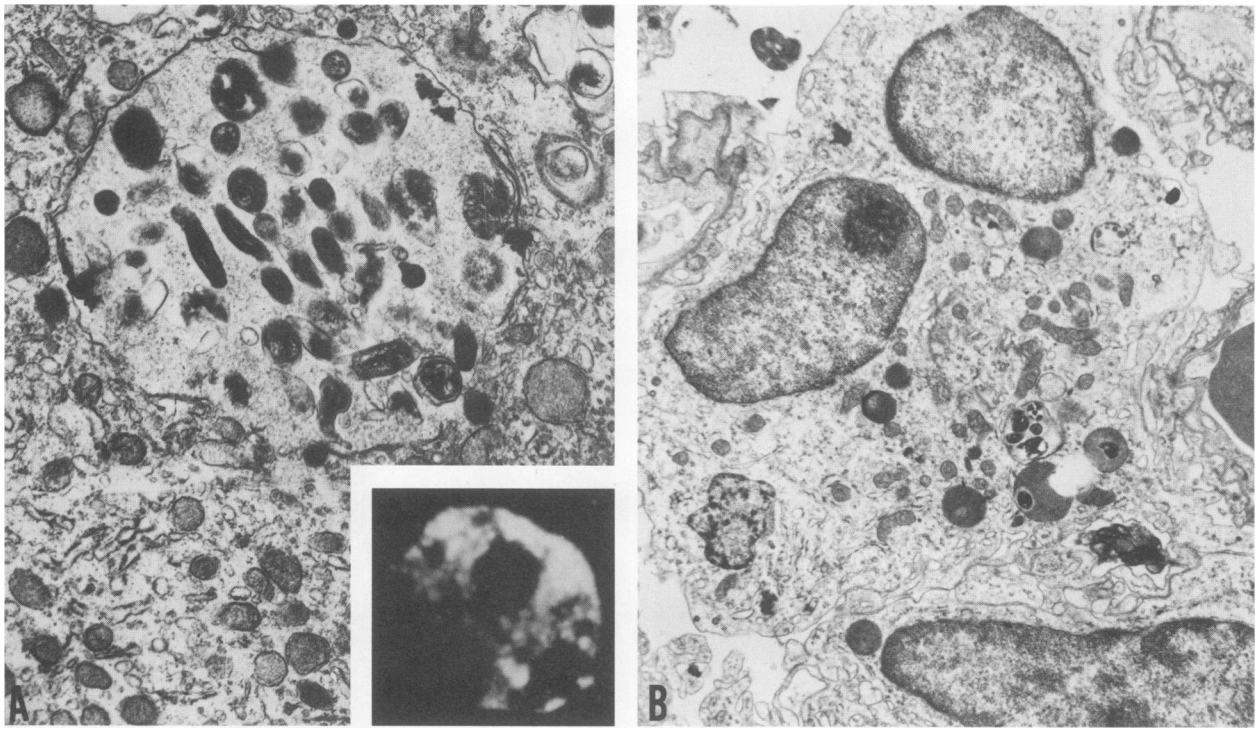

FIG. 4. Alveolar macrophages degrading intracellular microorganisms. (A) Large *C. burnetii*-bearing vacuole in an activated macrophage with numerous organelles and vesicles. Most intravacuolar organisms show various steps of degradation. (B) Multinuclear macrophage containing phagolysosomes with partially degraded organisms. Magnifications: A, ×9,600; B, ×2,400. Inset in panel A: specific fluorescence of *C. burnetii* antigen in a multinuclear alveolar macrophage. Imprint; direct immunofluorescence stain; magnification, ×200.

vacuoles. Most of these organisms showed signs of degradation: the loss of structural integrity and a patchy and diffuse osmiophilic appearance (Fig. 4A). The *C. burnetii*-bearing macrophages were activated cells, as evidenced by their euchromatic nuclei, prominent nucleoli, well-developed mitochondria and Golgi complexes, and numerous vesicles and phagolysosomes. Some macrophages fused with each other, forming bi- and multinucleate cells (Fig. 4B).

There were autophagosomes in macrophages degrading organisms. In some cells they were associated with other signs of cell damage, such as lipid accumulation and a rarefied edematous cytoplasmic matrix (Fig. 5A). Heavily damaged macrophages were surrounded by an exudate containing granulocytes and, occasionally, free microorganisms. Phagolysosomes with degraded organisms were also observed in granulocytes of the pneumonic exudate.

Signs of damage to organism-free pneumocytes and endothelial cells—a rarefied cytoplasmic matrix, autophagosomes, and blisterlike vesicles—were observed in the vicinity of macrophages degrading *C. burnetii* (Fig. 5B). In other respects, the alveolar wall maintained its structural integrity and showed no signs of interstitial inflammation. There were no erythrocytes within the alveolar lumen.

Many macrophages with degraded organisms were closely apposed by lymphocytes (Fig. 6A) with ultrastructural features of T cells (reviewed in references 15, 19, and 39). T cells with signs of activation, such as euchromatic nuclei with enlarged nucleoli, polysomes, and well-developed Golgi complexes, were also encountered in alveolar capillaries (Fig. 6B).

At 13 days after challenge, the following were typical changes: atelectases, circumscribed collections of activated macrophages with admixtures of granulocytes within collapsed alveoli, and an enhanced secretory activity by type II pneumocytes. There were also increased numbers of lymphocytes and plasma cells in the perivascular and peribronchial connective tissue. We were unable to observe by electron microscopy cells containing microorganisms at this time.

DISCUSSION

This study shows that the lungs not only serve as a portal of entry of *C. burnetii* into the body but are also an area of primary infectious focus. *C. burnetii* is indeed adapted to inhabit specific cells of the respiratory parenchyma, the pneumocytes, as well as pulmonary fibroblasts and histiocytes. The ability to invade these cells most likely constitutes the basis for pneumotropism of *C. burnetii*. It has recently been shown by Coalson et al. (10) that another pneumotropic pathogen, murine *Chlamydia trachomatis*, also inhabits type I pneumocytes.

C. burnetii shares its ability to reside in pneumocytes with *Rickettsia prowazekii* (26). Unlike the latter, however, *C. burnetii* is confined to specific vacuoles, which were previously described (5, 40), and does not provoke an inflammatory response unless the vacuoles are disrupted. This study also shows that after challenge, coxiellae induce only a transient influx of granulocytes, which degrade some of the organisms introduced into the mouse lungs. The granulocyte influx may in part be induced by tissue culture particles contaminating the rickettsial suspension. The elimination of the primary granulocytic exudate via airways seems to be followed by the inapparent multiplication of the pathogen in pneumocytes. Such an inapparent beginning of the infectious

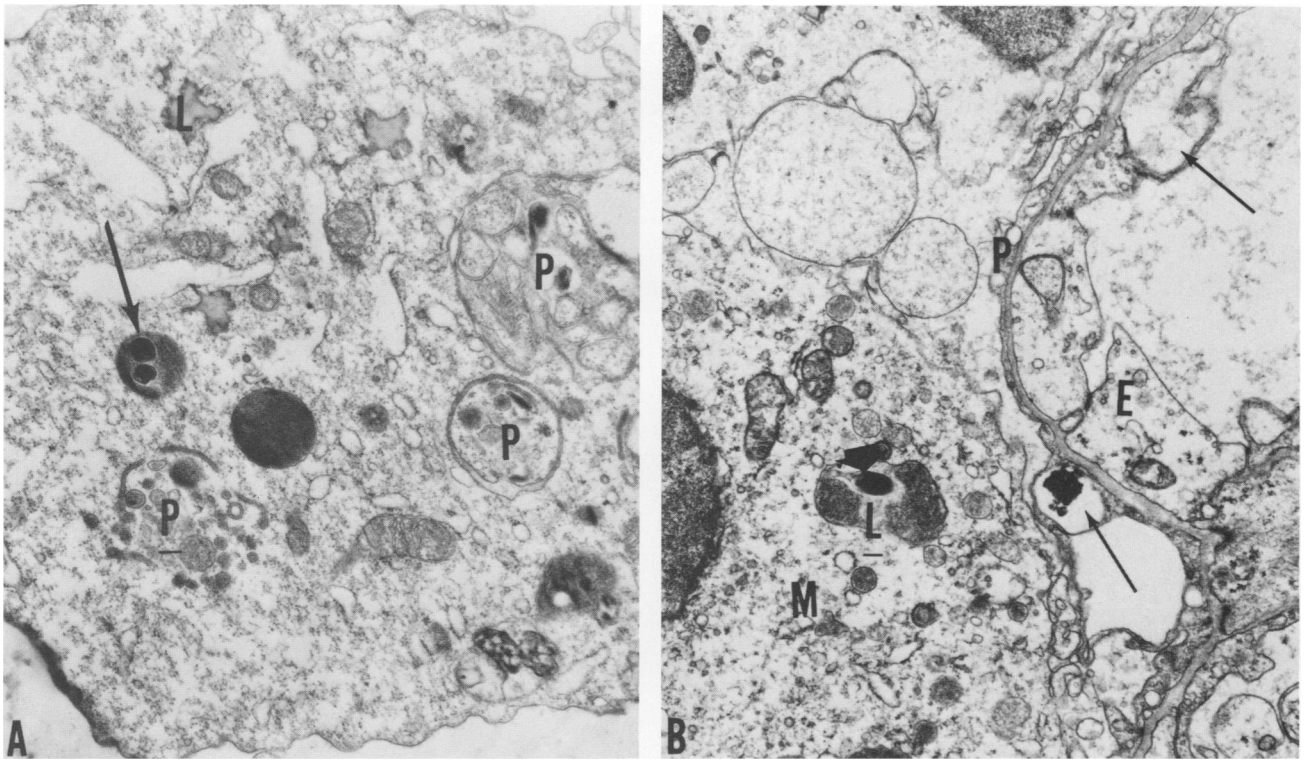

FIG. 5. Cell damage in a pneumonic area. (A) Highly damaged macrophage with autophagosomes (P) at various steps of formation. Lipid droplets (L) and widened profiles of the endoplasmic reticulum are also seen. Arrow, Lysosome with degraded organisms. (B) Damage of noninfected parenchymal cells in the vicinity of an alveolar macrophage (M) containing a phagolysosome (L) with a degraded organism (arrowhead). A vascular endothelial cell (E) and a type I pneumocyte (P) have blisterlike vesicles (arrows). Magnification: A, ×9,600; B, ×7,200.

process is consistent with published data that *C. burnetii* does not cause immediate damage to any of its host cells (5, 50) except for the gradual reduction of the cellular organelles because of the growing vacuole (4, 23).

Eventual destruction of infected pneumocytes is apparently associated with the development of focal intraalveolar, largely macrophage-dominated pneumonia. The pneumonia can be described as a self-limited pulmonary inflammation with complete resolution (18), owing to the effective destruction of the organisms by macrophages and granulocytes, and limited damage to the parenchymal cells. The cell damage is not accompanied by damage to the alveolar basal membrane. The membrane damage has been shown to be a major precondition for the development of interstitial pneumonia (12). Organisms which enter new pneumocytes may initiate new local infectious cycles which, however, eventually result in complete healing.

Extensive involvement of macrophages and T cells in the inflammatory process, T-cell attachment to *C. burnetii*-bearing macrophages, and macrophage fusion also suggest that effective control of the pulmonary infectious process is due to a mechanism of cell-mediated immunity. The pattern of *C. burnetii* degradation in alveolar macrophages resembles those that take place in peritoneal macrophages after the organisms have been opsonized by immunoglobulins or ingested by presensitized macrophages, as described by Kishimoto et al. (28). It is conceivable that the antigen released from destroyed *C. burnetii* at the macrophage surface promotes the attachment of sensitized T cells to the macrophages. The release of bacterial antigen on the surfaces of cells containing *R. prowazekii* has recently been described by Rollwagen et al. (42) as a possible mechanism of antigen presentation to the cells of the immune system. Some patterns of lymphocyte-macrophage adherence resemble those of conjugated cytolytic lymphocytes and target cells (6, 20). Still, the actual functional significance of lymphocyte attachment will be clarified in future experiments. As found by Mullbacher and Ada (34) in vivo, the same subpopulations of T lymphocytes may exert diverse, cytotoxic, helper, or suppressor activities towards infected cells.

The work presented here provides new information on some of the pathogenic features of *C. burnetii*, such as entry into the host cell and damage to cells and tissues. *C. burnetii* has been shown by Ariel et al. (4) and Hackstadt and Williams (16) to be a parasite of macrophage phagolysosomes that is resistant to lysosomal enzymes. The entry of *C. burnetii* into the cell has been interpreted as passive phagocytosis (5). This study shows that *C. burnetii* is also capable of inhabiting nonphagocytic cells such as pneumocytes and pulmonary fibroblasts in vivo, suggesting that *C. burnetii* shares with other rickettsiae (44, 51, 53) and some other intracellular pathogens (reviewed in reference 33) the ability to initiate entry.

The ability of *C. burnetii* to inhabit, without obvious damage, nonphagocytic cells with a long life span, such as pneumocytes and pulmonary fibroblasts, offers an explanation of the persistence of this organism in the lungs not only in experimental animals but also in humans, without apparent clinical or radiological manifestations (29, 48).

This study also shows that *C. burnetii* may damage phagocytic cells which degrade the organism and may cause

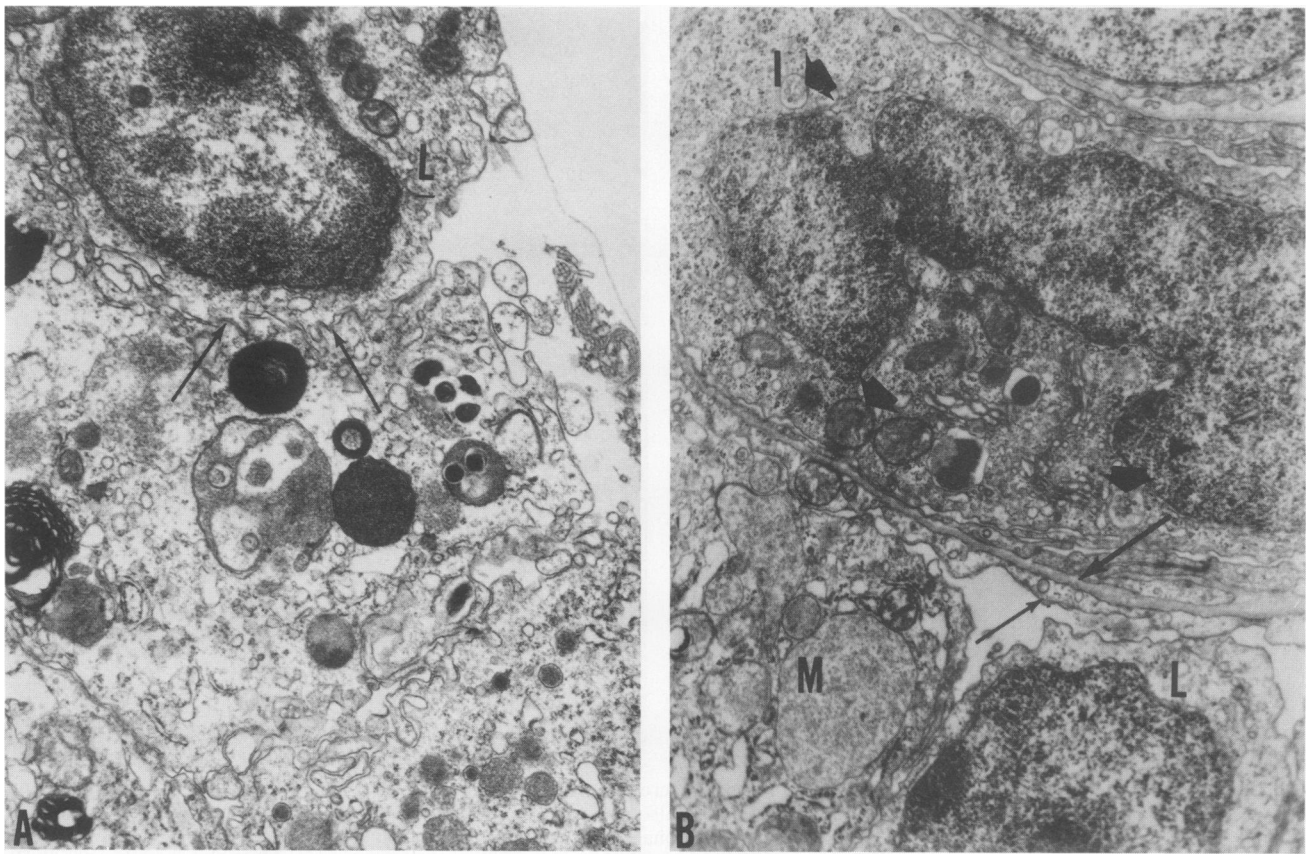

FIG. 6. Lymphocyte involvement in pneumonic foci. (A) A lymphocyte (L) with ultrastructural features of a T cell attached to a macrophage containing phagolysosomes with degraded organisms. Arrows, Interdigitating lymphocyte-macrophage contact area. (B) Intravascular lymphocyte (I) with a euchromatic reniform nucleus, an enlarged nucleolus, and well-developed Golgi complexes. Arrowheads, Membrane-bound microvesicular and dense bodies similar to those described as secretory granules in cytolytic (15, 21) and helper-suppressor (39) T cells in humans; large arrow, basal membrane separating a blood vessel from a type I pneumocyte (small arrow); L, intra-alveolar lymphocyte; M, intra-alveolar macrophage. Magnification, ×5,200.

indiredt damage to noninfected parenchymal cells in the vicinity of damaged phagocytes. It is conceivable that lipopolysaccharide released from degraded organisms is responsible for this cell damage. Unlike guinea pigs, mice have been shown to be resistant to the systemic toxic effect of lipopolysaccharide from *C. burnetii* (25). At the same time, this lipopolysaccharide may exert in mice a local damaging effect, as seen in our current observations. In infected macrophages local cytoplasmic alterations are subsequently sequestrated by autophagosomes. Similar autophagosome formation has previously been described in granulocytes that degrade *R. prowazekii* (27) and *R. tsutsugamushi* (41).

In this study we did not observe an acute interstitial inflammation in the vicinity of infected fibroblasts and histiocytes. It is conceivable that some transient focal inflammatory response occurs upon release of the organisms, but this has been overlooked. We had previously observed a transient inflammatory response around infected Kupffer cells in mouse and guinea pig livers. This was followed by complete resolution or by focal accumulations of lymphocytes and macrophages after the livers had been cleared of the organisms (22). In this study, similar cellular accumulations were found in the peribronchial and perivascular connective tissue as the only manifestation of interstitial involvement in the *C. burnetii*-induced pulmonary infectious process.

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