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Opsonin-Reversible Resistance of *Mycoplasma pneumoniae* to In Vitro Phagocytosis by Alveolar Macrophages

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Several species of mycoplasmas are responsible for respiratory disease in animals and man. As yet, little is known about the interaction of these pathogens with alveolar macrophages, one of the primary components of pulmonary resistance to infections. The present study was undertaken to develop an in vitro model to examine this organism-cell interaction, using a human pathogen, *Mycoplasma pneumoniae*, and normal guinea pig alveolar macrophages. During a 24-h incubation of *M. pneumoniae* with a monolayer of macrophages, mycoplasmas were found to attach directly to the surface of the cells without inducing significant phagocytosis. Ultrastructurally, the organisms appeared bound to the cell membrane by their characteristic attachment organelles. Only after the addition of specific anti-mycoplasma serum were cells able to engulf attached and surrounding organisms. These data suggest that the interaction of *M. pneumoniae* and alveolar macrophages is a potentially important aspect of disease pathogenesis, and immune factors which might alter this interaction merit further examination.

Alveolar macrophages have been studied extensively for their role in defense against a variety of microbial agents. The best available experimental data indicate that the bactericidal functions of the normal lung depend primarily on the phagocytic capacity of alveolar macrophages to kill, inactivate, or limit the growth of infecting organisms (16). This capacity is enhanced after immunization by either parenteral or respiratory routes, possibly through the development of opsonic antibody and/or cellular immunity (18).

In vitro studies have characterized several mechanisms which facilitate the ingestion and destruction of microbes by alveolar macrophages. Intracellular parasitic organisms such as *Listeria monocytogenes* are readily engulfed by normal alveolar macrophages (22) but are not killed optimally unless the macrophages have been activated by lymphokines from sensitized T-lymphocytes (24). Unopsonized gram-positive bacteria such as *Staphylococcus aureus* are also rapidly ingested (22), but intracellular killing of these organisms, even those opsonized with high-titer antiserum, is inefficient unless the organisms have been exposed to pulmonary surface lining material (21). Certain gram-negative bacteria such as *Pseudomonas aeruginosa* are poorly ingested by alveolar macrophages unless opsonized with antibody, but once ingested, whether opsonized or not, are rapidly destroyed (25).

These studies suggest that the factors important to altering macrophage-organism interaction are determined in part by characteristics of the invading pathogen. As yet, one important group of respiratory pathogens, mycoplasmas, have received limited attention regarding their interactions with alveolar macrophages. Since several species of mycoplasmas are known agents in respiratory infections of fowl, rodents, swine, cattle, and man, these studies are needed. This report deals with the in vitro interaction of *Mycoplasma pneumoniae*, a common human respiratory pathogen, and normal guinea pig alveolar macrophages. Using phase and electron microscopy and radioautography, we have observed the ability of *M. pneumoniae* to attach directly to the surface of the macrophages without inducing significant phagocytosis. After the addition to the system of specific rabbit anti-mycoplasma serum, organisms are rapidly ingested and degraded.

MATERIALS AND METHODS

Collection of macrophages. Young guinea pigs, weighing 300 to 500 g, were anesthetized by intraperitoneal injection of sodium pentobarbital and exsanguinated by intracardiac puncture. The trachea was exposed and cannulated with a 14-gauge Angiocath Teflon catheter (Deseret Pharmaceutical Co., Sandy, Utah), and the lungs were lavaged with 100 ml of heparinized (5 U/ml) 0.01 M phosphate-buffered saline at pH 7.2 in 10-ml aliquots. The cells were concentrated by centrifugation for 10 min at 200 × *g*,

washed with Hanks balanced salt solution, and resuspended in Eagle minimal essential medium (Flow Laboratories, Rockville, Md.) containing 292 mg of L-glutamine per liter, 100 U of aqueous penicillin G per ml, and 20% heat-inactivated fetal calf serum (Flow Laboratories). Cells (2×10^6) were placed on 22-mm square glass cover slips in Lux petri dishes (10 by 35 mm) (Microbiological Associates, Bethesda, Md.) and incubated overnight at 37 C in air with 5% CO_2. After removal of nonadherent cells, the resulting cell monolayers contained 98% macrophages and 2% eosinophilic leukocytes as judged by morphology and acid phosphatase staining. Cells were 99% viable as measured by trypan blue dye exclusion.

Organisms used to infect macrophages. Virulent *M. pneumoniae* strain M129B8 (eighth broth passage after isolation from a human host) was stored at −70 C after growth to log phase. Frozen cultures were thawed and inoculated 1:50 in PPLO broth (Difco Laboratories, Detroit, Mich.) containing 20% unheated horse serum, 10% yeast extract, 2% dextrose, 0.004% phenolphthalein, and 1,000 U of penicillin G per ml (Hayflick's medium, 17). After 4 days at 37 C, 100 ml of organisms was centrifuged at 14,000 × *g* for 30 min at 4 C, and resuspended in 20 ml of MEM containing 20% fetal calf serum, 20% PPLO broth, and 100 U of penicillin G per ml (MEM 20–20). This suspension was sonicated at 20 kHz for 15 s (Branson Sonifier, model LS75, Heat Systems Co., Great Neck, N.Y.), filtered through an 0.45-μm Millex filter (Millipore Corp., Bedford, Mass.), and incubated overnight at 37 C. Two milliliters of this suspension was then used to inoculate a Lux petri dish (10 by 35 mm) containing either a plain glass cover slip (25 by 25 mm) or a cover slip with an attached monolayer of alveolar macrophages. These dishes were further incubated for 18 to 24 h prior to examination.

Preparation of rabbit anti-mycoplasma serum. Log-phase cultures of *M. pneumoniae* strain Mac M were centrifuged at 14,000 × *g* for 30 min, washed once in phosphate-buffered saline, and resuspended in phosphate-buffered saline to a concentration of 10^8 colony-forming units per ml. Rabbits were inoculated subcutaneously twice at 14-day intervals with 1 ml of the organism suspension mixed with 1 ml of H37Ra complete adjuvant (Difco). Twenty-five days after the second inoculation, 1 ml of organism suspension was given intravenously. Rabbits were bled at 7 and 14 days after the intravenous inoculation. The serum was separated and frozen at −20 C. Before use, serum was heat inactivated at 56 C for 30 min. The mycoplasmacidal titer of the serum was >1:640 (6) and the growth inhibitory titer was 5.5 U (15).

Phase microscopy. After 18 to 24 h incubation with a suspension of *M. pneumoniae*, the cover slips containing glass-attached organisms or infected macrophage monolayers were rinsed in Hanks balanced salt solution and placed in cold 2.5% glutaraldehyde for 30 min. They were inverted over a drop of distilled water for examination through a Zeiss phase microscope at ×1,000 to 1,600 magnification. Where indicated, fresh medium containing either 1:100 rabbit anti-mycoplasma serum or 1:100 preimmune rabbit serum was added to the petri dish for varying time periods prior to fixation of the monolayers.

For time-lapse observations, the infected monolayers were rinsed in Hanks balanced salt solution, rimmed with paraffin-Vaseline, and inverted over a drop of fresh medium containing the test serum at 1:100 dilution. In some experiments, motion pictures were taken on Kodak Plus X negative film at 120 frames per min and a 0.35-s exposure time, using a ×40 or 100 phase contrast objective lens in a Zeiss microscope. The microscope stage was maintained at 37 C with a Sage curtain air incubator.

Electron microscopy. After fixation with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 15 min at 4 C infected or control cell monolayers were scraped with a rubber policeman, centrifuged at 100 × *g* for 5 min, and resuspended in 2.5% glutaraldehyde for 45 more min. The cells were pelleted, postfixed in 1% osmium tetroxide, and embedded in Epon. Thin sections were stained with lead citrate and uranyl acetate and examined in an AEI electron microscope at 60 kV using a 25-μm objective aperture.

Radioautography. For radioautographic studies, the mycoplasmas were radio-labeled with tritiated thymidine. [^{3}H]thymidine (10 μCi/ml) (Schwartz Mann, Orangeburg, N.Y.; specific activity, 50 Ci/mmol) was added to the sonicated, filtered suspension of *M. pneumoniae* and remained present in the media throughout the 18- to 24-h incubation of organisms with alveolar macrophages. Control studies showed that less than 2% of the monocytes incorporated radiolabel during this time period. After incubation of the organisms and macrophage monolayers, the cover slips were rinsed five times in Hanks balanced salt solution and transferred to new petri dishes containing fresh MEM 20–20 with 1:100 dilution of rabbit anti-mycoplasma serum or preimmune serum. After incubation for 10 to 60 min, monolayers were fixed in 2.5% glutaraldehyde for 30 min, rinsed in distilled water and air dried. Cover slips were mounted face up on glass slides, coated with undiluted NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.), stored in the dark for 4 days, and developed in Kodak D-19 for 5 min at 20 C. The developed radioautographs were stained for 30 min with filtered Giemsa stain prior to examination with conventional light microscopy.

RESULTS

Morphology of M. pneumoniae. When examined by phase microscopy, virulent *M. pneumoniae* cultured on glass cover slips in MEM 20–20 were morphologically identical to those cultured in Hayflick's medium as described by Bredt (3). Figure 1 illustrates representative stages in the growth cycle of organisms under the conditions used in these experiments. After sonic treatment and filtration, initial glass-adherent organisms appeared "dot-like" or as single filamentous structures, 1 to 5 μm long. Free-swimming mycoplasmas, with movement difficult to distinguish from Brownian motion, were seen to attach by a thickened end to the glass surface or to other organisms. After a

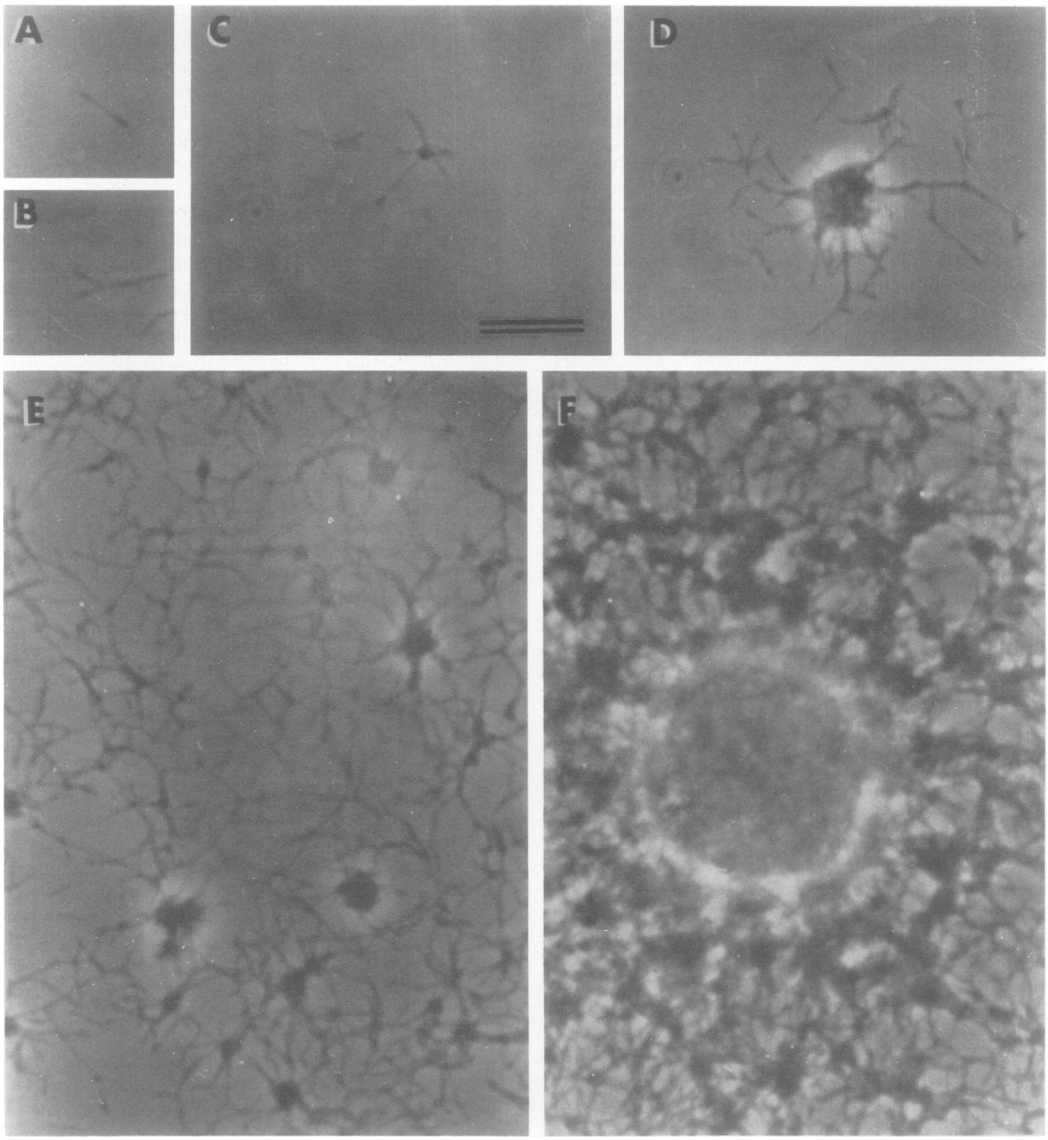

FIG. 1. *Phase-microscopy appearance of M. pneumoniae during different stages of growth onto a glass surface. (A) Single filamentous organisms found on the cover slip surface 4 h after inoculation. (B, C) Branching organisms which appear at 8 to 12 h. (D) Multiple branching of organisms around the nidus of a young colony; 12 to 24 h. (E) Latticework of branched organisms covering the glass surface; 24 to 36 h. (F) Dense colony found at 72 h. Bar = 5 μm.*

period of tail waving, the organisms attached full length to the cover slip where they demonstrated a slow, circling motion with the thickened end leading.

By 8 h, early branching structures were prevalent, resulting from incomplete binary fission or fusion of single organisms. After 24 to 36 h, there was a latticework of branched organisms covering the glass surface. Early colony forms appeared as irregular round densities within the latticework. Ultimately, growth led to a thick lawn of organisms connecting dense, amorphous colonies.

When examined with the electron microscope, *M. pneumoniae* utilized in these experiments appeared identical to those described by Biberfeld and Biberfeld (1). In a longitudinal plane of section, the organisms were dense, filamentous bodies 1 to 5 μm long and 0.1 to 0.3 μm wide (Fig. 2). They possessed a unique

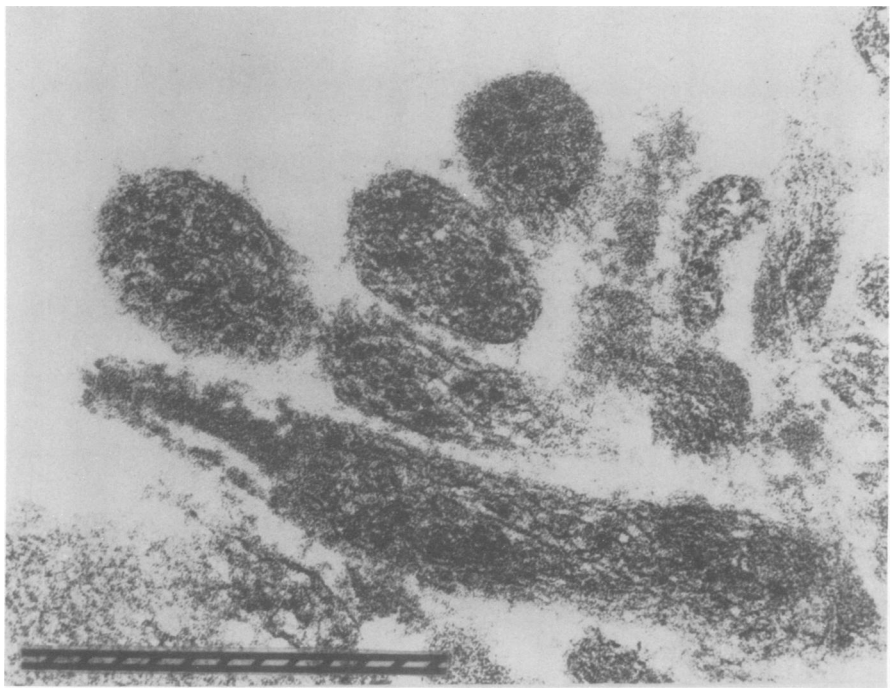

FIG. 2. *Electron photomicrograph of M. pneumoniae. In longitudinal sections, the organisms are filamentous and possess a unique tip structure. In cross-section they appear round or oblong. Bar = 1.0 μm.*

attachment organelle consisting of a dense central core surrounded by a lucent zone which was enveloped by an extension of the organism unit membrane. In cross section, the organisms appeared round or oval and possessed a dense staining granular cytoplasmic matrix.

Morphology of normal alveolar macrophages. Under the conditions of these experiments, uninfected alveolar macrophages at 48 h incubation were large, well-spread cells. Nuclear detail and cytoplasmic organelles such as mitochondria were readily viewed by phase microscopy. In the presence of 20% PPLO broth, cells formed numerous lipid droplets (Fig. 3A).

Infection of alveolar macrophages with M. pneumoniae. After 18 to 24 h incubation of a monolayer of alveolar macrophages with virulent *M. pneumoniae*, the monocytes, as viewed by phase microscopy, were surrounded by glass-adherent single or branching organisms (Fig. 3B). The macrophages had at least one and usually multiple organisms attached directly to the cell surface. The infected cells appeared more rounded than uninfected cells, nuclear detail was poorly defined, and there were few cytoplasmic vacuoles or lysosomal granules visible. Time-lapse studies revealed that ruffled membrane activity of the macrophages was minimal and that lamellopodia extending from these cells passed over adjacent glass-adherent mycoplasmas but retracted, leaving the organisms attached to the cover slip and undisturbed.

The exact number of mycoplasmas attaching directly to the cells was difficult to ascertain by phase microscopy. Organisms which attached to the cells in a perpendicular orientation at the focal plane of the cover slip surface were easily detected. However, motile organisms were observed to attach to the cell and soon affix themselves full length to the cell membrane, thereafter becoming impossible to distinguish from cell constituents. It could not be determined by phase microscopy whether such organisms were engulfed or remained extracellular.

When radioautographs of infected macrophages were examined, a lawn of silver grains activated by beta-emission from the [^{3}H]thymidine-labeled mycoplasmas was seen surrounding the cells (Fig. 4). Dense collections of grains were located contiguous to most cells in a cap-like pattern. These grains were more dense than those found attached to the cover slip, suggesting either a preferential attachment of mycoplasmas to cell membrane or an augmented growth of cell-associated organisms. By adjusting the focal plane, silver grains could also be seen over the body of the cells. Although

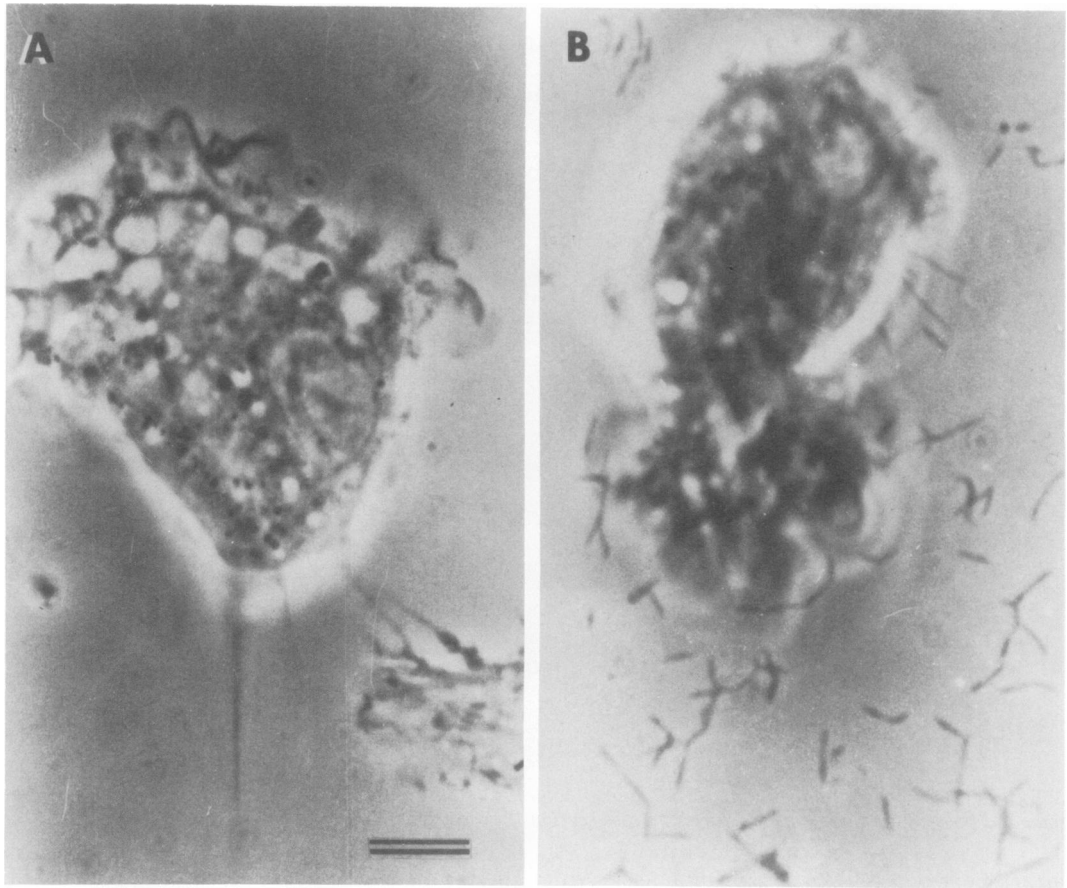

FIG. 3. *Phase microscopy appearance of normal guinea pig alveolar macrophages after 48 h in vitro. The uninfected cell (A) is well spread with readily visible nucleus, phase-dense cytoplasmic granules, ruffled membranes, and lipid vacuoles. The infected cell (B) is more rounded, and intracellular detail is vague. Mycoplasmas are seen surrounding the cell and attached directly to the cell membrane. Bar = 5 μm.*

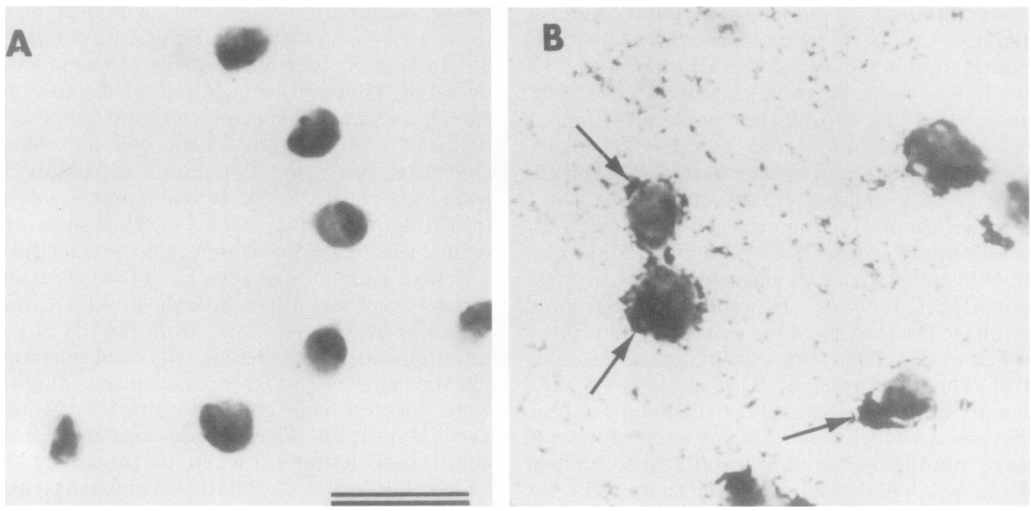

FIG. 4. *Radioautography of alveolar macrophages cultured for 24 h in vitro in medium containing [^3H]thymidine. The uninfected cells (A) demonstrate that the macrophages incorporate very little [^3H]thymidine, resulting in minimal background or cell-associated grains. In contrast, the infected cells (B) are surrounded by emulsion grains representing beta-emission from radiolabeled M. pneumoniae. Note the cap-like density of grains (arrows) contiguous to all cells. Giemsa stain over NTB-2 emulsion. Bar = 20 μm.*

accurate determination of their location was difficult, these grains were usually distributed over nuclear as well as cytoplasmic regions suggesting that they represented extracellular mycoplasmas.

To more accurately assess the location of cell-associated organisms, thin-section electron photomicrographs were examined. In general, organisms were seen clustered in close proximity to the external surface of the macrophages (Fig. 5); only rare cells containing intracellular mycoplasmas could be found. In the proper plane of section, whole filamentous organisms were seen attaching to the macrophage membrane by the tip structure unique to *M. pneumoniae*. Intracellular organelles of infected macrophages appeared no different than those of uninfected control cells.

Effect of antiserum on infected alveolar macrophages. Rabbit anti-mycoplasma serum led to rapid activation of infected macrophages. Immediately after the addition of 1:100 antiserum, these cells, as viewed in phase time-lapse studies, developed marked increase in ruffled membrane activity. Within 10 min, cells were spreading in random directions through extension of thin, semicircular lamellopodia. As these extending veils of cytoplasm passed over glass-attached mycoplasmas, the organisms remained transiently visible, appearing thickened and more phase dense. With centripetal retraction of the lamellopodium, mycoplasmas were removed from the cover slip surface and drawn into the cell where they were no longer visible (Fig. 6). On occasion, vacuoles which formed from the endocytosis of mycoplasmas could be clearly observed to fuse with lysosomal granules during their migration to a perinuclear location. Within 60 min, many cells were highly spread, vacuolated, and contained increased cytoplasmic, phase-dense granules. Organisms could no longer be found attached to these cells and the adjacent cover slip area was free of mycoplasmas. Organisms which were not within reach of the spreading cells remained motile, attached to the cover slip, and morphologically unchanged in the presence of antiserum.

Since intracellular mycoplasmas could not be visualized by phase microscopy, radioautographs were used to provide a more accurate measure of phagocytosis. This was accomplished by differential counts of infected macrophages which were categorized according to the distribution of grains relative to the cells (Fig. 7). Macrophages classified as type A cells showed clear evidence of phagocytosis as indicated by location of grains exclusively over the cytoplasmic region, lack of extracellular capping, and the presence of a zone cleared of silver grains surrounding the cell. Type B cells remained surrounded and capped by silver grains and therefore showed no evidence of significant phagocytosis. The phagocytic activity of some cells was indeterminant since they had neither intra- nor extracellular grain accumulations; these cells were classified as type C. The utilization of these criteria for quantitating the activity of infected macrophages in the presence of rabbit serum is indicated in Table 1. By 60 min after the addition of anti-mycoplasma serum, at least 50% of the cells had been activated to engulf attached and surrounding mycoplasmas; in contrast, less than 10% of cells were similarly activated during incubation with normal rabbit serum.

When electron photomicrographs of antiserum-activated macrophages were examined, sections containing intracellular organisms were easily found (Fig. 8). Within 10 min after the addition of antiserum, macrophages frequently contained one or more phagosomes with engulfed organisms. Mycoplasmas at this stage were morphologically intact suggesting no gross alterations had been caused by the opsonic antibody. By 60 min after the addition of antiserum, cells contained vacuoles packed with engulfed mycoplasmas. Since the generation time of *M. pneumoniae* is 6.5 h, this accumulation was most likely due to ongoing phagocytosis rather than organism replication. In some cells, organisms were found with peripheral chromatin clumping and reduction in density of the central matrix suggesting intracellular degradation of the mycoplasmas.

DISCUSSION

M. pneumoniae is a common human respiratory pathogen responsible for a significant incidence of upper respiratory illness, tracheobronchitis, and pneumonia in late childhood and adolescence (12). Studies of tracheal organ cultures (10) and infected human sputum samples (11) have revealed that this noninvasive extracellular parasite attaches to cilitated respiratory epithelium by a characteristic attachment organelle inducing cytopathic changes which result in cell death. Experimental animals infected with *M. pneumoniae* develop a nonfatal peribronchial pneumonitis which is histologically similar to human pathologic changes (7, 12). Upon recovery from a primary infection, these animals are rendered relatively resistant to reinfection with *M. pneumoniae*.

As yet, the mechanisms involved in host resistance to infection are only partially understood. Studies of human volunteers and mili-

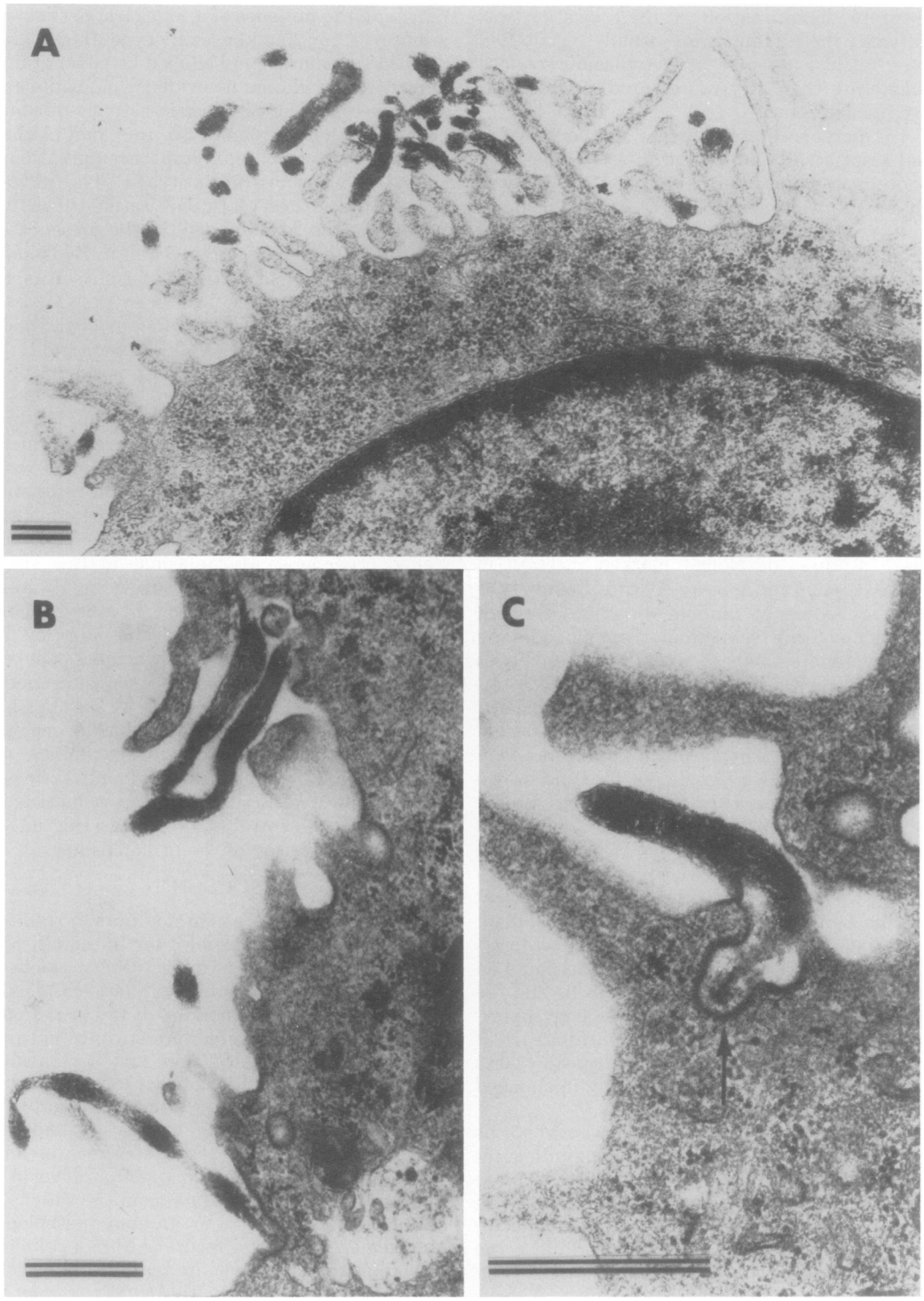

FIG. 5. *Electron photomicrographs of alveolar macrophages infected with M. pneumoniae. (A) Numerous organisms are seen adjacent to the cell membrane. Note the electron density and granularity of the M. pneumoniae cytoplasm contrasted with the microvillous projections of the macrophage. (B, C) Organisms sectioned longitudinally are oriented radially to the macrophage and appear bound to the cell membrane by their unique attachment organelle. Bars = 1.0 μm.*

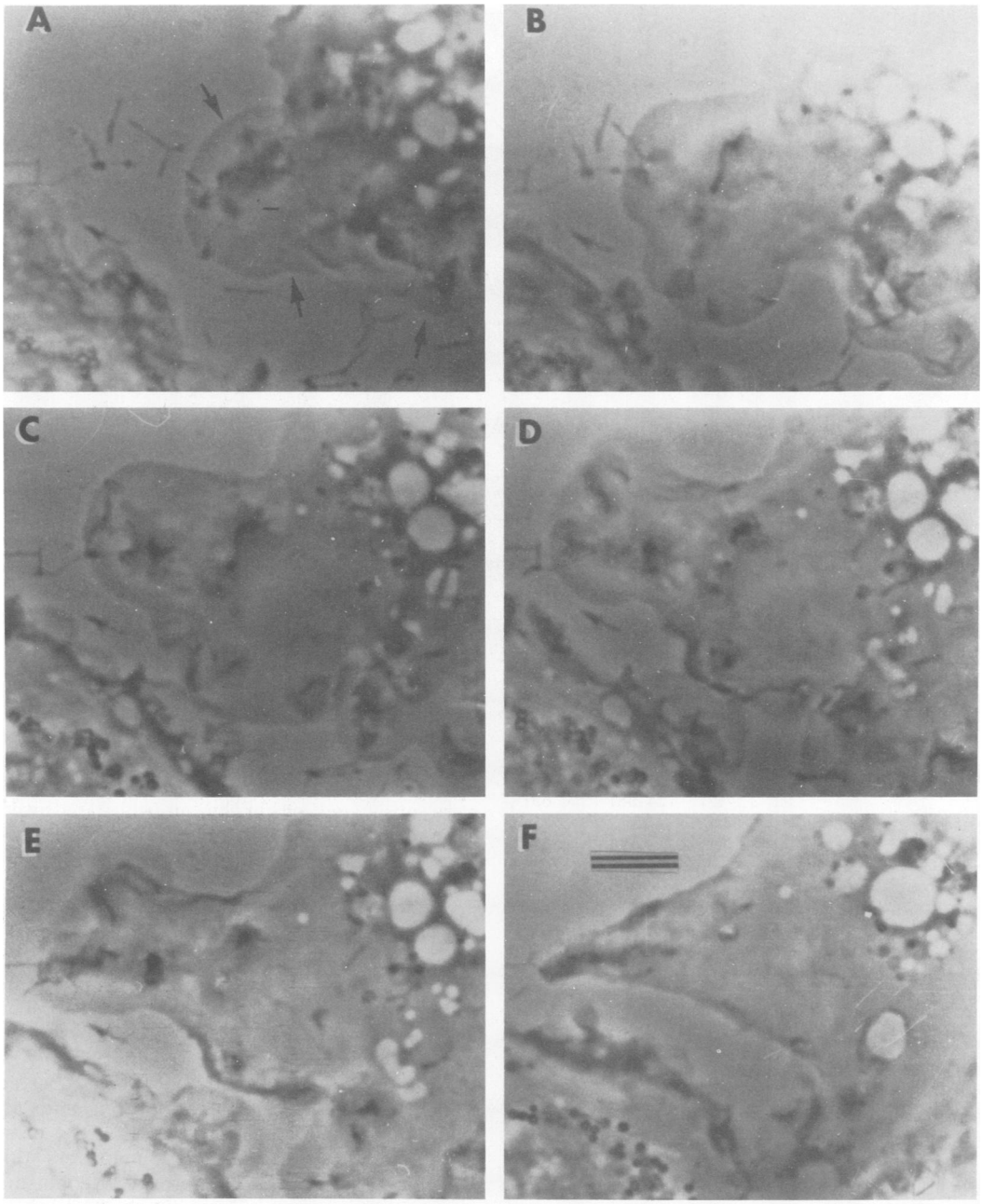

FIG. 6. *Phase-contrast time-lapse photomicrographs illustrating the phagocytosis of glass-adherent M. pneumoniae by an alveolar macrophage 30 min after the addition of anti-mycoplasma serum. (A–F) The same field photographed at 1-min intervals. Developing lamellopodia (A, arrows) approach clusters of organisms, pass over them (B, C, D), and retract (E, F) with disappearance of the mycoplasmas from the surface. Bar = 5 μm.*

tary recruits have linked the presence of serum antibodies (23) and/or secretory immunoglobulin (Ig) A (5) with lower rates of infection by *M. pneumoniae*. In experimental animal models, Fernald has shown that local pulmonary immunity rather than circulating antibody is crucial for protection to challenge infection (13).

The present report suggests that the interaction between *M. pneumoniae* and alveolar macrophages may play an important role in disease

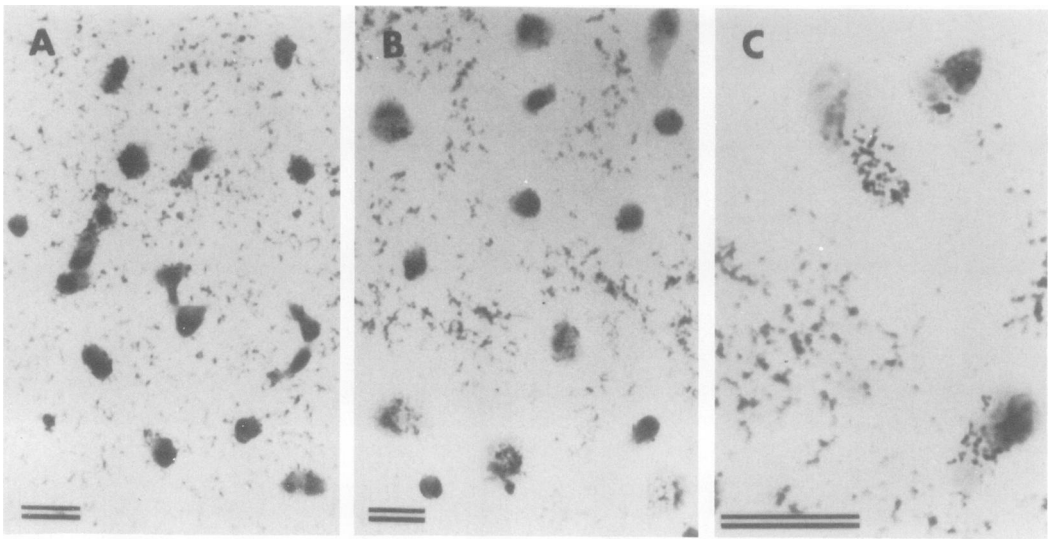

FIG. 7. *Radioautographs of M. pneumoniae-infected alveolar macrophages incubated for 60 min in the presence of preimmune serum (A) or anti-mycoplasma serum (B, C). Note the clear zones, lack of extracellular capping, and accumulation of intracytoplasmic grains within cells activated by antiserum. Bars = 20 μm.*

TABLE 1. *Phagocytic activity of alveolar macrophages infected with [^3H]thymidine-labeled M. pneumoniae*[a]

Serum	Cell type (%)[b]		
	A	B	C
Normal	7 (2–12)	67 (24–98)	26 (0–69)
Anti-mycoplasma	54 (51–61)	22 (3–45)	24 (4–41)

[a] From radioautographic preparations 60 min after addition of indicated serum.

[b] A, Clearly phagocytic; B, no evidence of phagocytosis; C, indeterminant. Results shown are mean of three experiments counting 200 cells each; range in parentheses.

pathogenesis and immunity. Under the in vitro conditions utilized in these studies, *M. pneumoniae* is able to surround and attach directly to normal alveolar macrophages without triggering phagocytosis to any significant extent. Attachment occurs primarily between the macrophage cell membrane and the mycoplasma unit membrane surrounding the unique tip structure of the organism. After the addition of rabbit anti-mycoplasma serum, alveolar macrophages were rapidly activated to a state of heightened phagocytosis, ingesting attached and surroundings organisms. Once ingested, organisms appeared to undergo degradation.

These findings are in apparent contrast with the studies of Zucker-Franklin et al. (27, 28), who demonstrated rapid ingestion of *M. pneumoniae* by human blood leukocytes with no apparent ability of the organism to resist phagocytosis. Although species and cell types may account for some of the differences between these two studies, it should be noted that unheated human plasma was contained in the incubation media of their experiments. It is possible that organisms in their experiments were opsonized either by alternate pathway components of complement or by specific antibody most likely present in pooled adult human serum (4).

Other workers have shown that several species of mycoplasmas possess anti-phagocytic mechanisms. Jones and Hirsch (19) have demonstrated extensive attachment of *M. pulmonis* to mouse peritoneal macrophages in vitro. The organisms were ingested only if killed, trypsinized, or opsonized by rabbit-specific anti-mycoplasma IgG (20). Cole and Ward have reported that *M. arthriditis*, *M. gallinarum*, and *M. pulmonis* show augmented growth in the presence of mouse macrophage monolayers (9). Specific hyperimmune rabbit anti-mycoplasma serum again lead to ingestion and killing of these organisms by the monocytes, but mouse antiserum to *M. arthriditis* did not induce engulfment of this organism. In fact, *M. arthriditis* in the presence of macrophages was protected from the growth inhibitory potential of the antiserum. Simberkoff and Elsbach (26) have shown that *M. arthriditis* and *M. hominis* induce membrane perturbations in human or rabbit polymorphonuclear leukocytes and resist

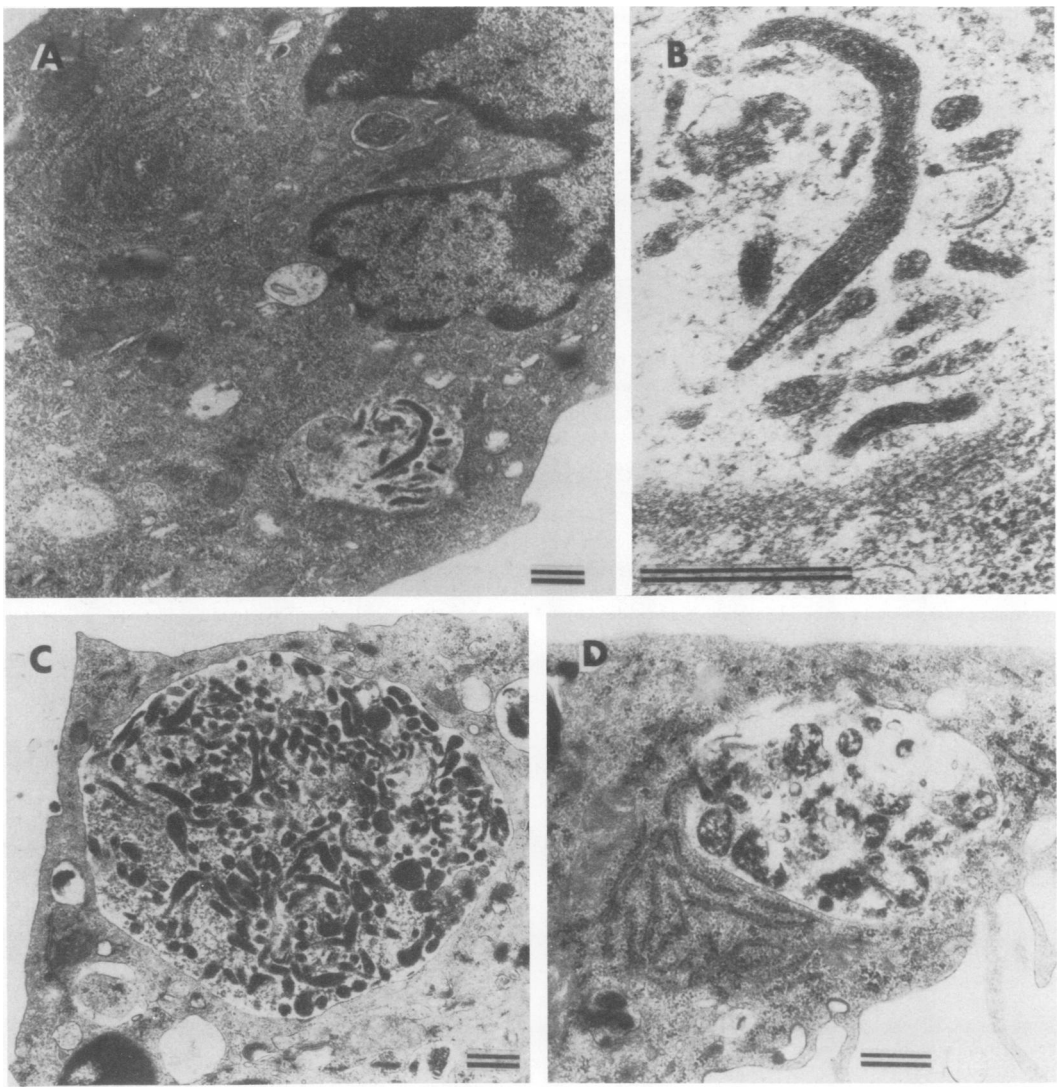

FIG. 8. *Electron photomicrographs of M. pneumoniae-infected alveolar macrophages after incubation with anti-mycoplasma serum. (A) Ten minutes after the addition of antiserum, mycoplasmas are present in phagosomal vacuoles. (B) Detail of phagosome in (A) indicating morphologic preservation of organisms (see Fig. 2). (C) Phagosome filled with organisms 60 min after addition of antiserum. (D) Mycoplasmas with clumped chromatin and clearing of the central matrix, suggesting intracellular degradation. Bars = 1.0 μm.*

phagocytosis by these cells even in the presence of specific antiserum. There is no evidence as yet to suggest that all species of mycoplasmas possess a common anti-phagocytic mechanism and little is known regarding the significance of such a mechanism to disease pathogenesis.

Studies by Cassel et al. (8) have suggested that impaired clearance of *M. pulmonis* by alveolar macrophages may be prominent in determining the extent of pulmonary disease in mice experimentally infected with this organism. In pathogen-free mice, clearance of *M. pulmonis* is inefficient, and high dose intranasal inoculation of this organism leads to an acute lower respiratory illness. Rats, on the other hand, show rapid and extensive clearance of *M. pulmonis* by alveolar macrophages and develop a more chronic upper respiratory infection.

Clearance of organisms by alveolar macrophages is clearly a primary host defense mechanism against bacterial pathogens (16). Whether this clearance depends on removal of ingested organisms through the pulmonary transport system or on inactivation of ingested organisms

within the phagocyte, the primary requirement of either mechanism is that the organism be engulfed. It is well known that specific antibody of the IgG class augments the rate and extent of in vitro phagocytosis of particulate antigens by macrophages. Reynolds and Thompson have recently recovered IgG opsonic antibody from the lower respiratory tract of rabbits after intranasal vaccination with killed pseudomonas (25). During natural and experimental *M. pneumoniae* infections, IgG, IgM, and IgA antibodies are found in the lower respiratory tract (2, 14). The importance of these immunoglobulins as specific opsonins has yet to be examined.

The techniques presented in this report will facilitate examination of pulmonary immunoglobulins as well as other immune parameters which might alter the interaction between *M. pneumoniae* and alveolar macrophages.

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