

THE INTERACTION IN VITRO OF MYCOPLASMA PULMONIS WITH MOUSE PERITONEAL MACROPHAGES AND L-CELLS*

BY THOMAS C. JONES, M.D. AND JAMES G. HIRSCH, M.D.

(From The Rockefeller University, New York 10021)

(Received for publication 23 September 1970)

Despite numerous studies in recent years, the relationship between mycoplasmas and their host cells remains uncertain. Some investigators have concluded that the mycoplasma organism is an intracellular parasite (1-5), whereas others have not found evidence to support intracellular survival of mycoplasmas (6-9). Few studies have examined the relationship of mycoplasmas to phagocytic cells (10-12). Observations on mycoplasma infection of mammalian cells in vitro or in vivo have been hampered by the fact that these microorganisms are usually small in size, pleomorphic, and lacking in distinguishing morphologic or staining properties. Most investigators have made such observations in stained sections (1-3) in which mycoplasmas may not be easy to identify and morphologic preservation is generally poor, or the studies have been made by electron microscopy (4, 5, 11-14) with technical limitations imposed by fixation procedures and examination of thin sections. Even under the electron microscope, mycoplasmas present no unique morphologic features, and it can be difficult to distinguish them from sections through pseudopods or from cell organelles.

The present work was designed to examine in detail the interaction between mycoplasmas and two mouse cell types: the fibroblast and the peritoneal macrophage. Evaluations were made by phase-contrast microscopy, time-lapse cinematography, electron microscopy, and radioautography. The interaction was observed in the presence and in the absence of anti-mycoplasma antibody, and the fate of intracellular organisms was determined.

Materials and Methods

Mouse Peritoneal Macrophage Cultures.—25 g NCS male mice were killed by cervical dislocation and inoculated intraperitoneally with 2 ml of heparinized phosphate-buffered saline at 37°C. The abdomen was kneaded gently and the peritoneal contents aspirated using a sterile Pasteur pipette. Material from five mice was used for each experiment. The suspension of peritoneal cells was centrifuged at 800 rpm for 5 min and the pellet was suspended in 8 ml of Eagle's minimal essential medium (MEM)¹ (Microbiological Associates, Bethesda, Md.) with

* Supported by Research Grant AI-01831 and AI-07012, U. S. Public Health Service.

¹ Abbreviations used in this paper: DPM, disintegrations per minute; HIFCS, heat-inactivated fetal calf serum; MEM, Eagle's minimal essential medium; PCA, perchloric acid.

20% heat-inactivated fetal calf serum (HIFCS) (Grand Island Biological Co., Grand Island, N. Y.). 0.5 ml of this suspension ($1-4 \times 10^6$ cells/ml; approximately half macrophages) was placed on 22 mm square cover slips in 35 mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.) and was incubated at 37°C in 5% CO₂-balanced air for 1 hr to allow the macrophages to adhere firmly to the glass. The medium was then aspirated, the cover slips were washed once with MEM, and finally overlaid with 2.5 ml of fresh MEM containing 20% HIFCS. The dishes were incubated stationary at 37°C in Ball Mason jars filled with 5% CO₂ and balanced air. Infection of macrophages was initiated after 24 hr of cultivation in vitro.

Mouse Fibroblasts.—L-cells (Microbiological Associates) were maintained in continuous culture in Eagle's MEM and 20% HIFCS. Monolayers were treated with 0.25% trypsin (Microbiological Associates) in MEM for 5 min at 37°C. The cell suspension was diluted 1:10 in fresh MEM with 20% HIFCS; the cell number was adjusted to $2-4 \times 10^6$ per ml and 2.5 ml were placed on 22 mm square cover slips in plastic dishes. After 2 hr the media was removed and infection with mycoplasmas initiated.

Mycoplasmas.—*Mycoplasma pulmonis* (WRAIR strain, kindly provided by Dr. Ruth G. Wittler of the Walter Reed Institute, Washington, D. C.), was passed every 3 or 4 days in heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 20% heat-inactivated horse serum (Microbiological Associates). The studies to be reported were conducted on organisms obtained from the 30th to 80th broth passage. 24–48 hr before macrophage infection 1 ml of the stock mycoplasma culture was added to 5 ml of heart infusion broth with 20% heat-inactivated horse serum and was incubated at 37°C. At the time of infection this subculture was diluted 1:100 into MEM containing 20% HIFCS and 10–30% heart infusion broth; the macrophage or fibroblast cultures were then placed in this medium.

Preparation of Anti-Mycoplasma Antibody.—48 hr subcultures of *Mycoplasma pulmonis* in heart infusion broth with 20% heat-inactivated horse serum were centrifuged at 21,000 rcf g for 30 min in a refrigerated centrifuge (Lourdes Instrument Corp., Old Bethpage, N. Y.). The pellet was washed once with phosphate-buffered saline, centrifuged again under the same conditions, suspended in distilled water, and lyophilized. The dry material was approximately 60% protein by Folin determination. Rabbits were inoculated at three injection sites, intrascapular and both thighs, with 10 mg total of lyophilized mycoplasmas suspended in distilled water and mixed with equal volumes of complete Freund's adjuvant (Difco Laboratories). 14 days later second inoculations of 10 mg of lyophilized mycoplasmas mixed with equal volumes of incomplete Freund's adjuvant were given. The rabbits were bled 9 or 14 days after the second inoculations. The serum was separated and frozen at –20°C. Before use serum was heat-inactivated at 56°C for 30 min. Agglutination titers done on heat-inactivated sera used in these experiments were 1:128 to 1:256. Preimmunization rabbit serum was used as a control.

Evaluation of Mycoplasma-Cell Association.

(a) *Phase-contrast microscopy:* The culture medium was aspirated and the cells were fixed in 2.5% glutaraldehyde (Fisher Scientific Co., Fairlawn, N. J.) in 0.1 M NaCacodylate buffer, pH 7.4, for 10 min at 4°C. The cover slips were then removed, inverted on a drop of distilled water, and rimmed with paraffin-vasoline. The cultures were examined for general morphology and for degree of mycoplasma infection by employing a Zeiss photomicroscope with 100× Neofluor phase objective. Preliminary study of the mycoplasma cultures fixed and observed under these conditions showed that the organisms were large (0.5–1.0 μ), round, phase-dense bodies. The mycoplasmas thus were readily seen under phase contrast, and were easily distinguished from round cellular structures; they were much larger and more phase dense than the macrophage or fibroblast lysosomal granules, and their phase density distinguished them from the transparent pinocytotic vesicles and vacuoles and from the refractile lipid droplets. It was necessary to examine the cells at various planes of focus since macrophages even when well spread were

thickened over the nuclear and centrosomal region, and the surface mycoplasmas were over these regions as well as the thinned pseudopodia. The percentage of macrophages or L-cells infected with mycoplasmas was determined, and the degree of infection was graded: 1+, less than ten mycoplasmas per cell; 2+, 10–50 mycoplasmas estimated per cell; 3+, more than 50 mycoplasmas estimated per cell.

(b) *Time-lapse cinematography*: Macrophages or L-cells were placed on number 1-1/2, 25 mm round cover slips, and were cultured and infected with mycoplasmas as described above. 20–24 hr after infection the cover slips were removed from the plastic dishes and placed in a Sykes-Moore chamber, filled with fresh culture medium, and maintained on the microscope stage at 37°C by an air curtain incubator (Sage Instruments, White Plains, N. Y.). Polyethylene tubes (i.d. 0.018) attached to 25 gauge needles provided inlet and outlet ports for introduction of medium containing antiserum. Motion pictures were taken on Kodak Plus-X negative film at speeds of 12, 40, or 120 frames per min and a 0.20 or 0.25 second frame exposure time, using a 40 X oil immersion phase-contrast objective in a Zeiss Standard WL microscope and Sage Model 505 cinemicrophotographic equipment.

(c) *Electron microscopy*: Medium was aspirated from the cultures and the cover slips were rinsed once with saline at room temperature. The cover slips were then flooded with 2.5% glutaraldehyde at room temperature, and after five min the cell sheet was scraped off with a plastic policeman. The suspended cells were transferred to a 3 ml conical tube and chilled in an ice bath. The cells were then fixed again in a mixture of glutaraldehyde and osmium, were exposed to uranyl acetate, and were embedded in agar and then in epon as described in detail previously (15). Thin sections stained with lead and uranyl solutions were examined in a Siemens Elmiskop I at 80 kv using a 50 μ objective aperture.

(d) *Radioautography and scintillation counting*: Macrophage or L-cell cultures infected with mycoplasma for 18–24 hr were exposed for 4 hr to new medium (MEM with 20% HIFCS) containing 1 μ Ci/ml of tritiated thymidine (New England Nuclear Corp., Boston, Mass.). For radioautography, cover slips were fixed in glutaraldehyde, washed, treated with 5% trichloroacetic acid for 1 hr at 4°C, washed and dried, coated with L-4 emulsion (Ilford Ltd., Essex, England), reacted for 6 days in the dark, developed for 2 min at 20°C, and stained for 4 min with methanol-giemsa, pH 5.75. Cover slips for scintillation counting, following exposure of the cultures to tritiated thymidine, were washed five times in MEM containing 10% heart infusion broth, incubated in MEM containing 20% HIFCS and 10% broth for 1 hr at 37°C, and the medium replaced with appropriate experimental medium. At intervals three replicate dishes were removed, and the media and cells were treated separately with perchloric acid (PCA) (0.25 N) (Allied Chemical, Morristown, N. J.) at 4°C overnight. After centrifugation at 1500 rpm for 10 min, the pellet was washed once with 0.25 N perchloric acid, then dissolved in formic acid. Perchloric acid-soluble and insoluble fractions were added to Bray's solution and counted for 4 min each in a scintillation counter (Nuclear-Chicago, Des Plaines, Ill.).

RESULTS

Preliminary Studies on Techniques for Observing Mycoplasmas and on Techniques for Establishing Infection of Macrophage and Fibroblast Cultures.—Several years ago it was noted in this laboratory that mycoplasma infection of long term fibroblast cultures could in some instances be detected readily by phase-contrast microscopic observation of living or of glutaraldehyde-fixed cells, the mycoplasmas appearing as phase-dense round bodies carpeting the cell surface. Preliminary studies were required to find a strain of mycoplasma suitable for our purposes, and to develop procedures for establishing infection in vitro of mammalian cells by these mycoplasmas.

The Walter Reed Army Institute for Research strain of *Mycoplasma pulmonis* could be maintained by passage in liquid culture to provide large numbers of dispersed organisms of fairly uniform size and shape. When examined by phase-contrast microscopy in the living state or after glutaraldehyde fixation, these mycoplasmas appeared as phase-dense round bodies, 0.5–1.0 μ in diameter.

TABLE I
Effect of Broth Concentration on the Extent of *Mycoplasma* Infection of Macrophages after 24 Hr of *in Vitro* Infection

Heart infusion broth (%)	Medium		Macrophages with mycoplasma (%)	Degree of macrophage infection
	HIFCS (%)	MEM (%)		
50	20	30	100	3+
30	20	50	90–100	2–3+
10	20	70	60–70	1–2+
5	20	75	10–20	1+
1	20	79	1–2	1+
0.5	20	79.5	0	0
0.1	20	79.9	0	0



FIG. 1. Growth of *Mycoplasma pulmonis* in MEM with 20% HIFCS, containing 10 or 30% heart infusion broth, with and without macrophages.

Their phase-contrast appearance was thus distinct from that of round organelles in the mouse macrophages and fibroblasts to be studied; the mycoplasmas were much larger and more phase dense than lysosomal granules, and their fairly uniform size and phase density made it easy to distinguish them from phase-lucent pinocytic vacuoles or vesicles, or from the refractile lipid droplets in the cells.

Mycoplasmas cultured for 24–48 hr in broth with 20% heat-inactivated horse serum and diluted 1:100 in appropriate final cell culture medium provided a

predictable degree of cell infection in 18–24 hr. Organisms taken from broth cultures at least 10 broth passages from agar-block passage exhibited less pleomorphism and clumping than those recently washed from agar medium.

The extent and reproducibility of cell infection by mycoplasmas was found to be influenced by a number of factors including composition of medium, cell type, and cell density. *Mycoplasma pulmonis* multiplied in MEM containing 20% HIFCS only if the medium also contained at least 5% heart infusion broth. Table I shows that the concentration of broth added to MEM with HIFCS also influenced the mycoplasma–macrophage association after 24 hr. Media containing 10% broth plus 20% HIFCS in MEM led to relatively light visible infection of 60–70% of the macrophages, whereas media containing 30

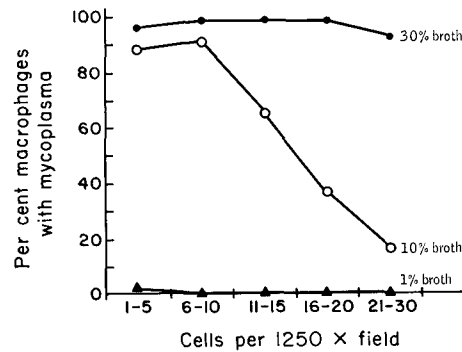


FIG. 2. Effect of macrophage cell density on mycoplasma–macrophage association.

or 50% broth led to heavy infection of all macrophages evidenced by a dense lawn of mycoplasma on their surfaces.

The number of colony-forming units of mycoplasma recoverable from the cultures after 24 hr was influenced also by the presence of macrophages (Fig. 1). As is seen in medium containing 10% heart infusion broth, the generation time of *M. pulmonis* grown without macrophages was 2 hr, whereas in the presence of macrophages it was 4 hr. However, this difference was not seen when 30 rather than 10% heart infusion broth was added to the medium. Heart infusion broth was not a necessary supplement when mycoplasma were grown in association with L-cells.

Macrophage cell density in stationary cultures also influenced the infection with mycoplasmas (Fig. 2). In culture medium containing 10% broth, the percentage of cells infected was inversely related to the cell density. In the presence of 30% broth nearly all cells were infected at all cell densities. In 1% broth little or no infection with mycoplasmas was seen at any cell density. A similar relationship of L-cell density to mycoplasma growth was not evident.

As a result of these observations the medium selected for these studies was Eagle's MEM with 20% HIFCS and varying concentrations of heart infusion broth determined by the desired degree of cell infection. Macrophages were used 24 hr after cultivation in vitro, and observation of infected macrophages

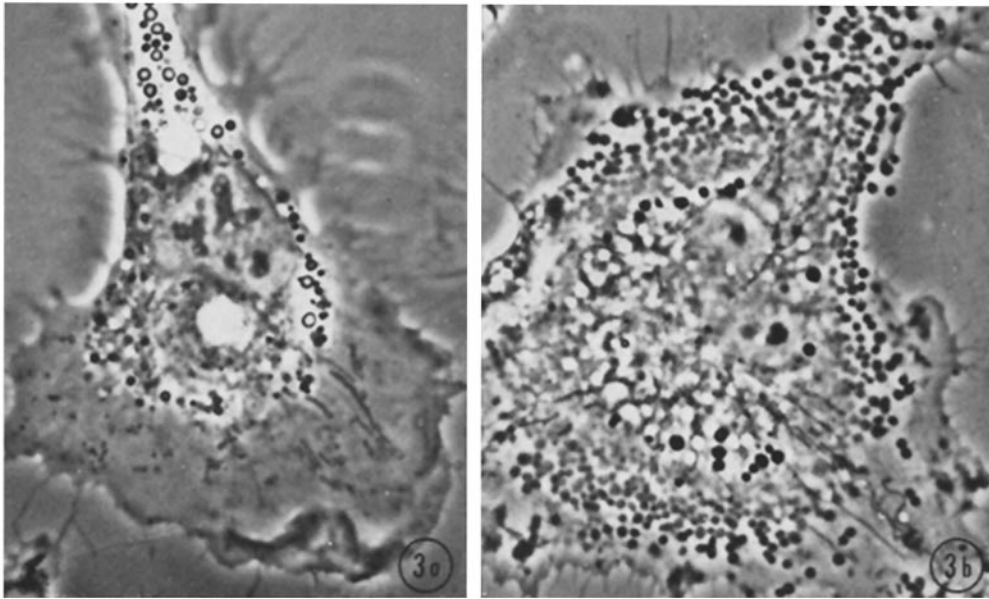


FIG. 3. Phase-microscopic appearance of mouse peritoneal macrophages cultured for 48 hr in vitro. The uninfected cell on the left (a) shows a reniform nucleus, perinuclear small vesicles, granules and lipid droplets, and a large veil of clear cytoplasm containing a few granules and elongated mitochondria. The cell on the right (b) had been infected for 24 hr with *Mycoplasma pulmonis*. The organisms appear as dark round bodies 0.5–1 μ in diameter blanketing the peripheral areas of the cell. The lawn of mycoplasmas also covered the central region of the cell, but these organisms are out of the plane of focus of this picture. Detailed features of the cell are masked by the microorganisms; the number of centrosomal clear vesicles is increased, but otherwise no differences from the control cell are apparent. Phase contrast; glutaraldehyde fixation; $\times 1600$.

was done by phase-contrast microscopy, using a 100 \times objective and examining cover slips showing five to fifteen macrophages per field. Fibroblasts were infected with mycoplasmas 2 hr after transfer using MEM with 20% HIFCS and 10% heart infusion broth.

Observations on Mycoplasma-Cell Interaction Using Phase-Contrast Microscopy.—Mouse peritoneal macrophages under the conditions of these experiments were large cells (30–70 μ) which increased in size during cultivation in

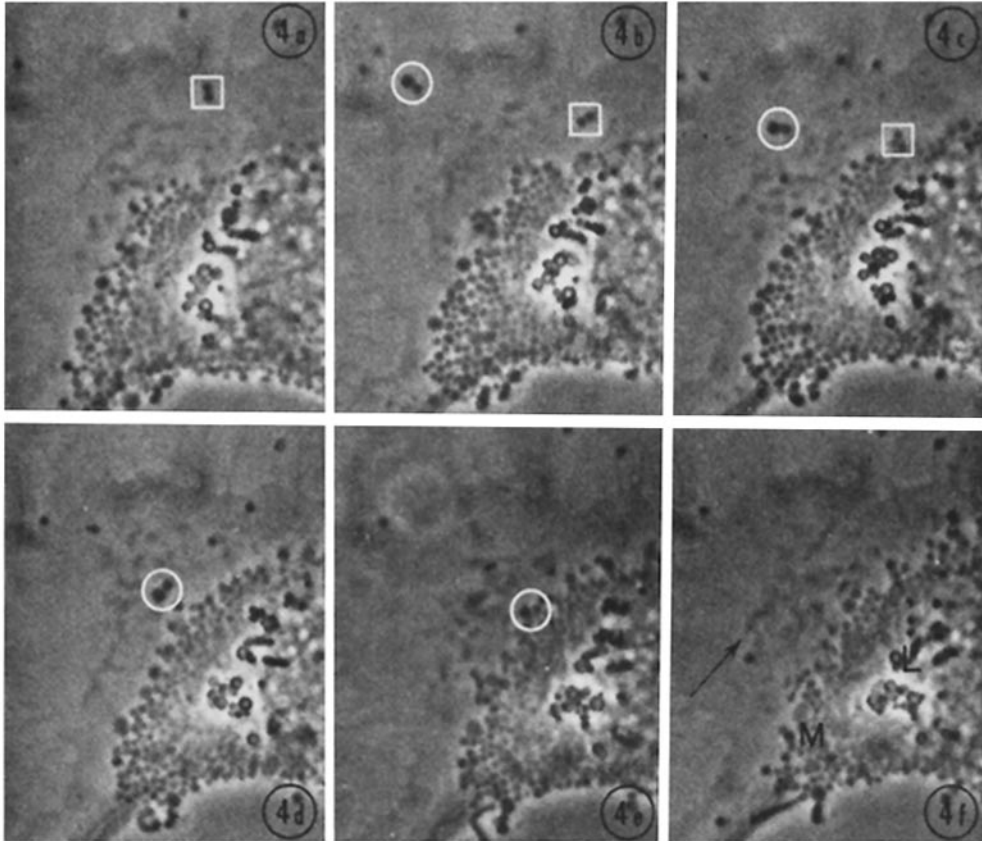


FIG. 4. Phase-contrast motion picture sequence of a living mouse peritoneal macrophage infected with *M. pulmonis*. The pictures presented are frames selected at intervals of 100 sec. The sequence depicts the translocation of two pairs of mycoplasmas from the cell periphery toward the central region. One of the pairs, outlined with a square, is seen first in frame *a*, and moves to join the mycoplasma lawn at the center of the cell in frames *b* and *c*. The second pair of mycoplasmas (circled) appears in frame *b* at the cell periphery, and in frames *c*, *d* and *e* is seen to move to the central region of the cell. The rate of translocation of these mycoplasmas was measured at $4 \mu/\text{min}$. Membrane ruffles (arrow), mycoplasmas (*m*), and lipid deposits (*l*), are labeled in frame (*f*). Approximately $\times 1200$.

vitro. They exhibited the characteristic morphologic features documented in previous studies from this laboratory (16). Culture in the presence of heart infusion broth led to somewhat enhanced membrane ruffling and pinocytic activity, and to the formation of numerous lipid droplets. (Fig. 3).

After addition of 1 to 10×10^5 *Mycoplasma pulmonis* to macrophages in

vitro only a few organisms were detectable during the ensuing 8 hr. These few organisms were usually located on the plasma membrane overlying the center of the cell. Membrane activity was marked over the surface of the cell,

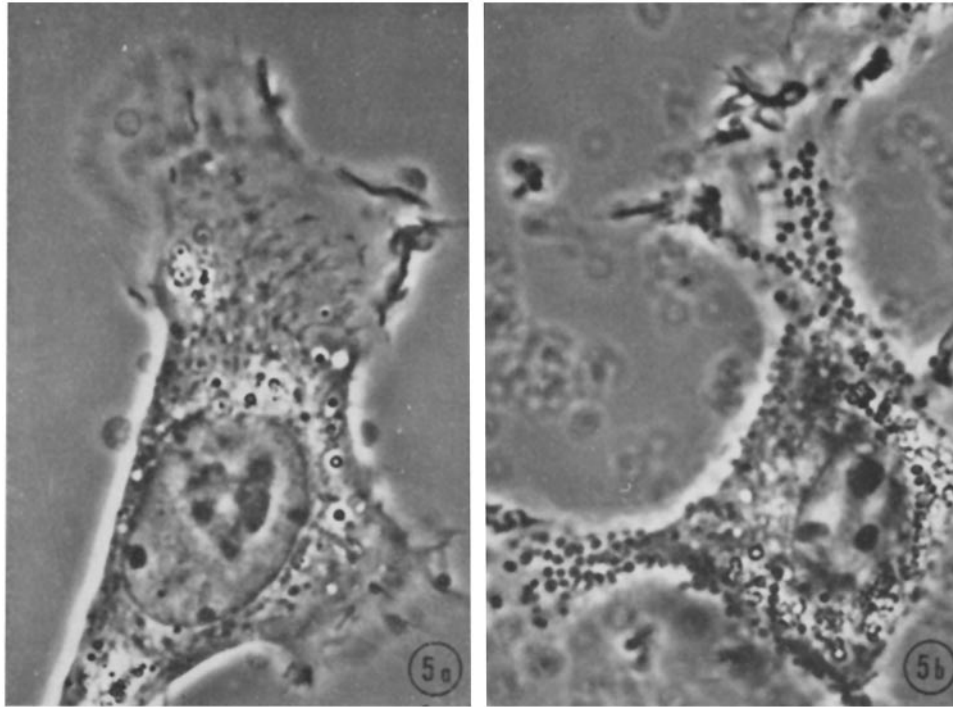


FIG. 5. Phase-microscopic appearance of mouse fibroblasts cultured for 24 hr in vitro. The cell on the left (*a*) is from an uninfected culture. It has a large oval nucleus with prominent nucleoli, perinuclear clear vesicles, granules and lipid deposits, and a few threadlike mitochondria in the clear peripheral cytoplasm. The cell on the right (*b*) is from a culture infected with *Mycoplasma pulmonis*. The pseudopods are covered with mycoplasmas which appear as dark round bodies 0.5–1.0 μ in diameter. The lawn of mycoplasmas also covered the central part of the cell, but these organisms are out of the focal plane of this picture. Mycoplasmas dispersed on the glass surface (out of focus in the illustration) are responsible for the numerous poorly defined circular shadows in the extracellular region. Phase contrast; glutaraldehyde fixation; $\times 1600$.

appearing as surface wrinkles or peripheral ruffles after glutaraldehyde fixation. The infected macrophages appeared identical to the cells in control cultures except for the presence of the organisms on the infected cells.

18–24 hr after infection hundreds of mycoplasmas were visible on the surface of each cell. At early time periods the organisms were seen on the surface overlying the central region of lightly infected cells; later with heavier infection

TABLE II
Titration of Opsonic Activity of Rabbit Anti-Mycoplasma Antibody

Reciprocal titer of antiserum	Macrophages* with mycoplasma (%)	Degree of macrophage infection
100	0	0
500	0	0
1000	0	0
2000	4	1+
4000	44	2-3+
Control	89	2-3+

* Approximately 90% of macrophages were infected at 2-3+ before the addition of antibody. 200 macrophages were examined in each specimen 4 hr after the addition of antibody or normal rabbit serum.

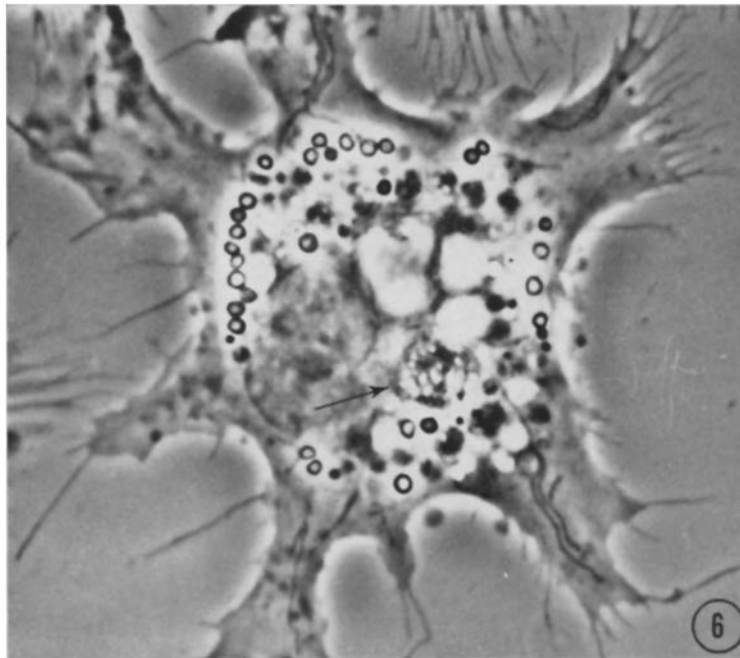
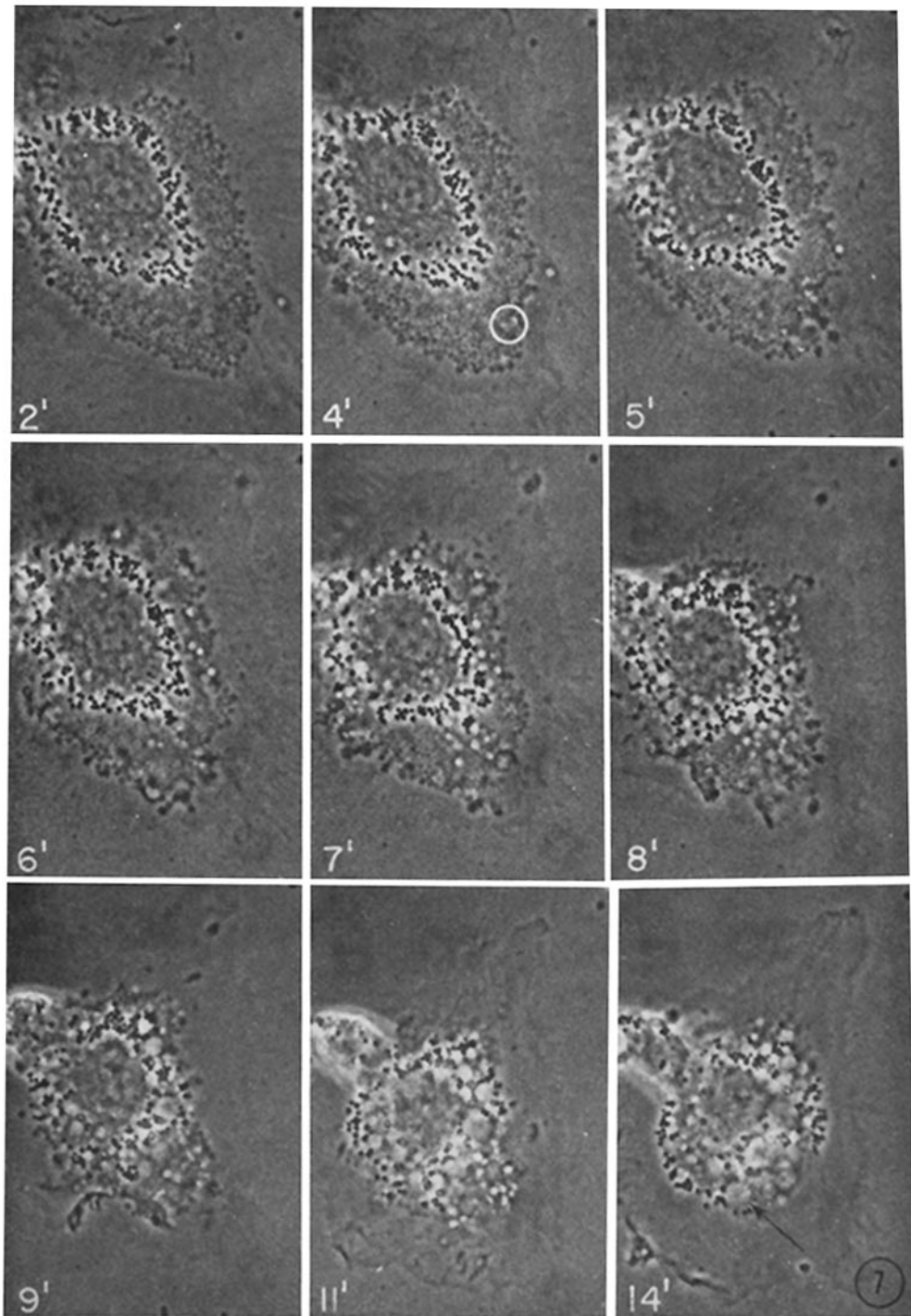


FIG. 6. This photomicrograph illustrates the effect of anti-mycoplasma antibody on mouse macrophages infected with mycoplasmas. Before the addition of antibody, the cells of this culture were well spread and covered with surface mycoplasmas (identical appearance to the cell illustrated in Fig. 3 b. 2 hr after the addition of antibody, striking changes occurred, as shown here. Cell pseudopods are retracted and surface mycoplasmas have disappeared completely. There are large central vacuoles some of which appear empty, whereas others contain deposits of dense material. One vacuole (arrow) contains a grapelike cluster of translucent small round bodies which may represent intracellular mycoplasmas. Phase contrast; glutaraldehyde fixation; $\times 1600$.



the surfaces of the cells were often completely covered by organisms. All mycoplasmas appeared to be on the cell surface, but it was not possible to rule out the presence of intracellular microorganisms under these conditions. Fig. 3 shows an infected macrophage (*b*) in comparison with an uninfected cell cultured under the same conditions (*a*). In the infected cell a lawn of round phase-dense bodies 0.5–1.0 μ in diameter is seen around the phase-lucent vacuoles and the nucleus. Mycoplasmas also covered the central portion of the cell shown in Fig. 3 *b*, but these organisms were not visible at the focal plane of this picture.

Observation of the infected cultures by time-lapse cinematography revealed a wave-like membrane motion of the macrophage surface directed centripetally. This inward sweeping action appeared to account for the collection of the mycoplasmas over the central part of the cell. Translocation of the mycoplasmas toward the cell center occurred at a rate of approximately 4 μ /min. Phagocytosis of the surface mycoplasmas was not seen. Mycoplasmas were seen as single organisms or in pairs, but occasionally in clumps or in chains. Fig. 4 is a sequence from phase-contrast cinematography showing the membrane motion and translocation of mycoplasmas toward the center.

Fig. 5 shows a comparison between control L-cells (*a*) and L-cells 24 hr after infection with *Mycoplasma pulmonis* (*b*). The cells were more heavily infected than similarly infected macrophage cultures and organisms were seen over the entire surface of the cell. The mycoplasmas were more numerous also on the glass surface than was the case in macrophage cultures. Mycoplasma infection had no discernible effect on L-cell functions such as membrane activity, pinocytotic vesicle formation, and cell division. However, in heavily infected specimens the L-cells began to round and detach from the cover slip after several days. Intracellular mycoplasmas were not seen.

The addition of rabbit anti-mycoplasma antibody caused prompt engulfment of the surface mycoplasmas in the macrophage cultures, but not in the cultures of infected L-cells. The opsonic titer of the antiserum as determined by morphologic examination of the infected cultures is shown in Table II. The rate of uptake of mycoplasmas was influenced by the density of macrophages and mycoplasmas as well as by the quantity of antiserum added; in general antiserum at 1:100 exhibited striking opsonic activity, whereas dilutions

FIG. 7. Phase-contrast motion picture series illustrating the phagocytosis of surface mycoplasmas by a macrophage after the addition of anti-mycoplasma antibody. At 2 min (2') the cell and the mycoplasmas appear unchanged from their appearance before the antibody addition. By 4 min, the mycoplasmas have disappeared from one area of the cell (circled). The following sequences illustrate progressive disappearance of the mycoplasmas, associated with local membrane activity and small clear vesicle formation. The cell contracts somewhat, and the perinuclear very dark bodies (lipid droplets) become dispersed and perhaps reduced in number. By 11 min mycoplasmas are no longer visible; at 14 min large vacuoles have formed, some of which contain clear small round bodies (arrow). Approximately $\times 1200$.

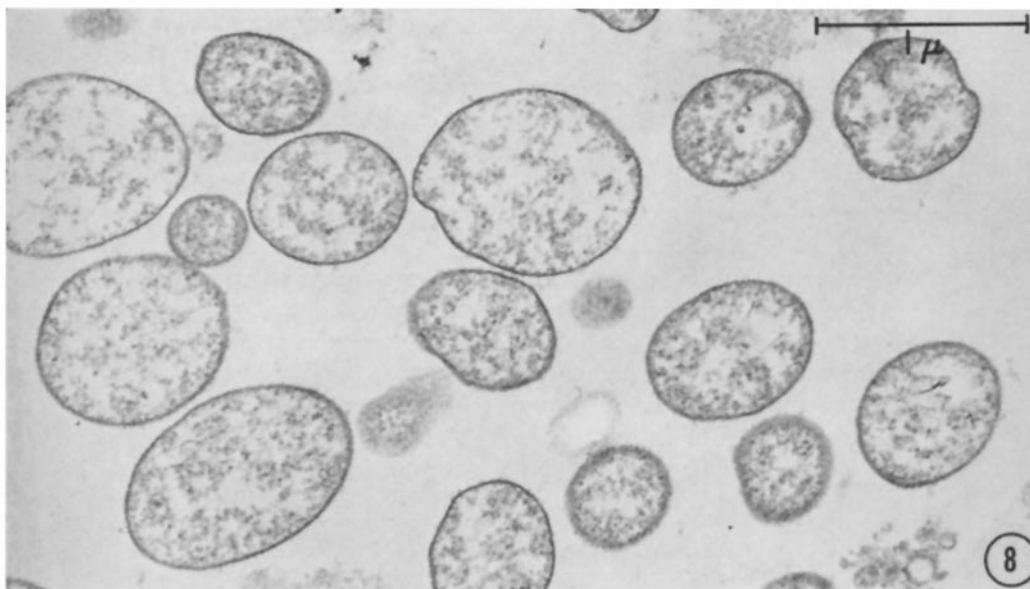
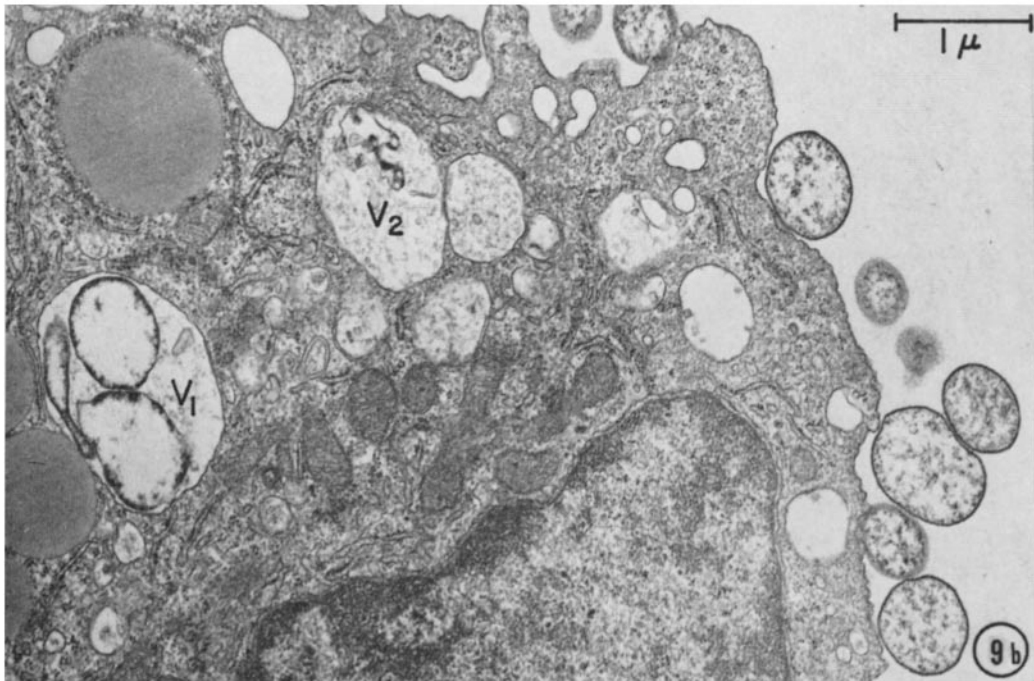
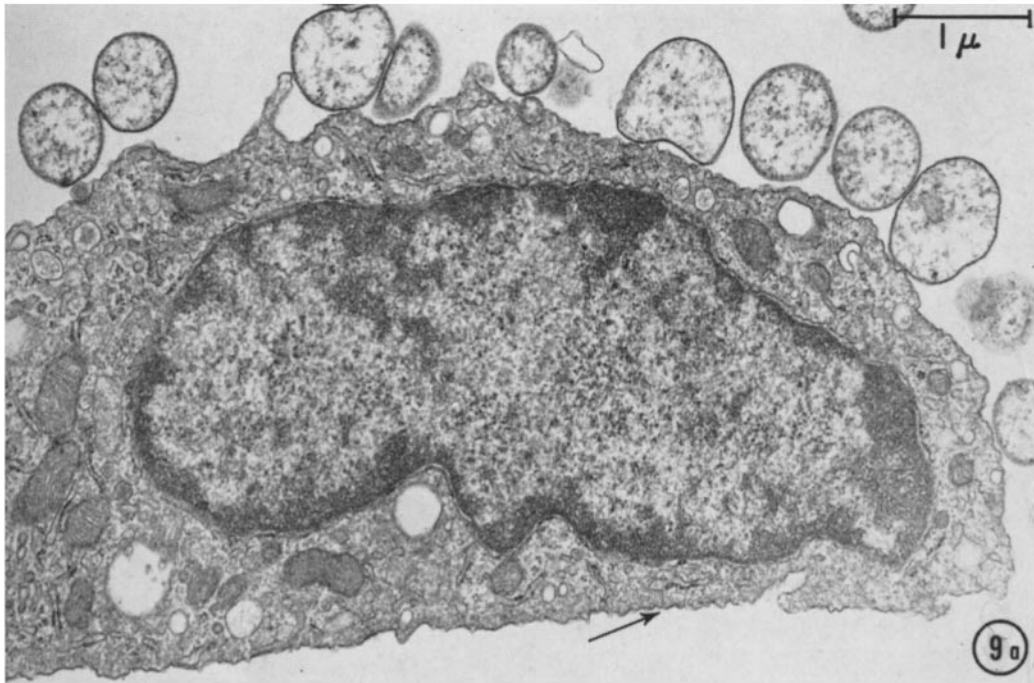


FIG. 8. Electron-microscopic appearance of *Mycoplasma pulmonis* from 24 hr broth culture. The organisms appear as round bodies, with central sections ranging from 0.5 to 1.0 μ in diameter. The limiting membrane is well defined. Contents include scattered ribosomal and amorphous densities, but no specific organelles. $\times 28,000$.

FIG. 9. Electron-microscopic appearance of mouse peritoneal macrophages 24 hr after infection with *M. pulmonis*. Section *a* shows a cell with many mycoplasmas attached to the upper surface. The macrophage surface which had been adherent to glass is flattened (arrow). The mycoplasmas are morphologically similar to those from broth culture. The membranes of the mycoplasma are separated from the cell plasma membrane by a distance of approximately 100 A. No unusual features are seen in the macrophage nucleus and cytoplasmic organelles visible in this section. No intracellular mycoplasmas are present. $\times 18,500$.

Section *b* shows another infected macrophage. Several mycoplasmas are seen attached to the cell surface on the right. Normal cytoplasmic structures are seen, including perinuclear mitochondria and vesicles, scattered endoplasmic reticulum and, at the far left, several large fat deposits. Also present in this section are phagocytic vacuoles (V_1 and V_2) which appear to contain amorphous material and partially degraded mycoplasmas (the large [0.8 μ] circular structures with clear content and peripheral dense material seen in V_1). $\times 18,500$.

higher than 1:2000 had little or no effect. Fig. 6 shows a macrophage 2 hr after the addition of specific antibody in a dilution of 1:100. The cell is somewhat retracted. Surface mycoplasmas are no longer present, but the cell shows several large phase-lucent vacuoles, some of which contain dense amorphous aggregates or lucent small round bodies. Time-lapse cinematography was utilized to document further the opsonization by specific antibody. Immediately after the addition of antibody a marked increase in surface membrane



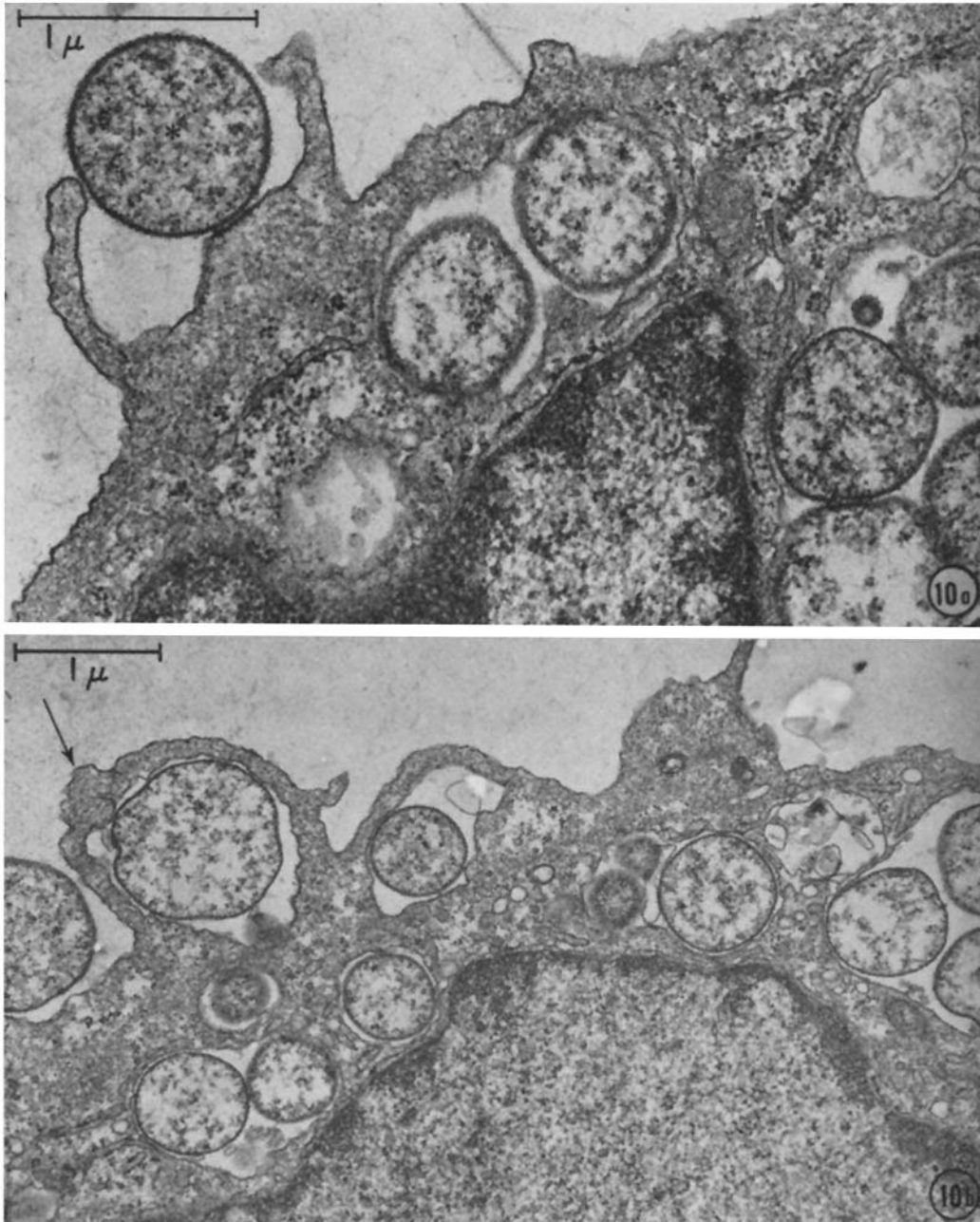


FIG. 10. Electron-microscopic appearance of mycoplasma-infected macrophages 5 min after the addition of anti-mycoplasma antibody. Many mycoplasmas are noted within phagosomes rather than at the cell surface. The mycoplasma remaining at the surface in Fig. 10 *a* (*) appears to be in the process of envelopment by pincers-like microprojections. In some instances these projections overlap and appear to have fused (Fig. 10 *b*, arrow). Most of the organisms within phagosomes appear morphologically normal. (*a*) $\times 33,000$; (*b*) $\times 20,000$.

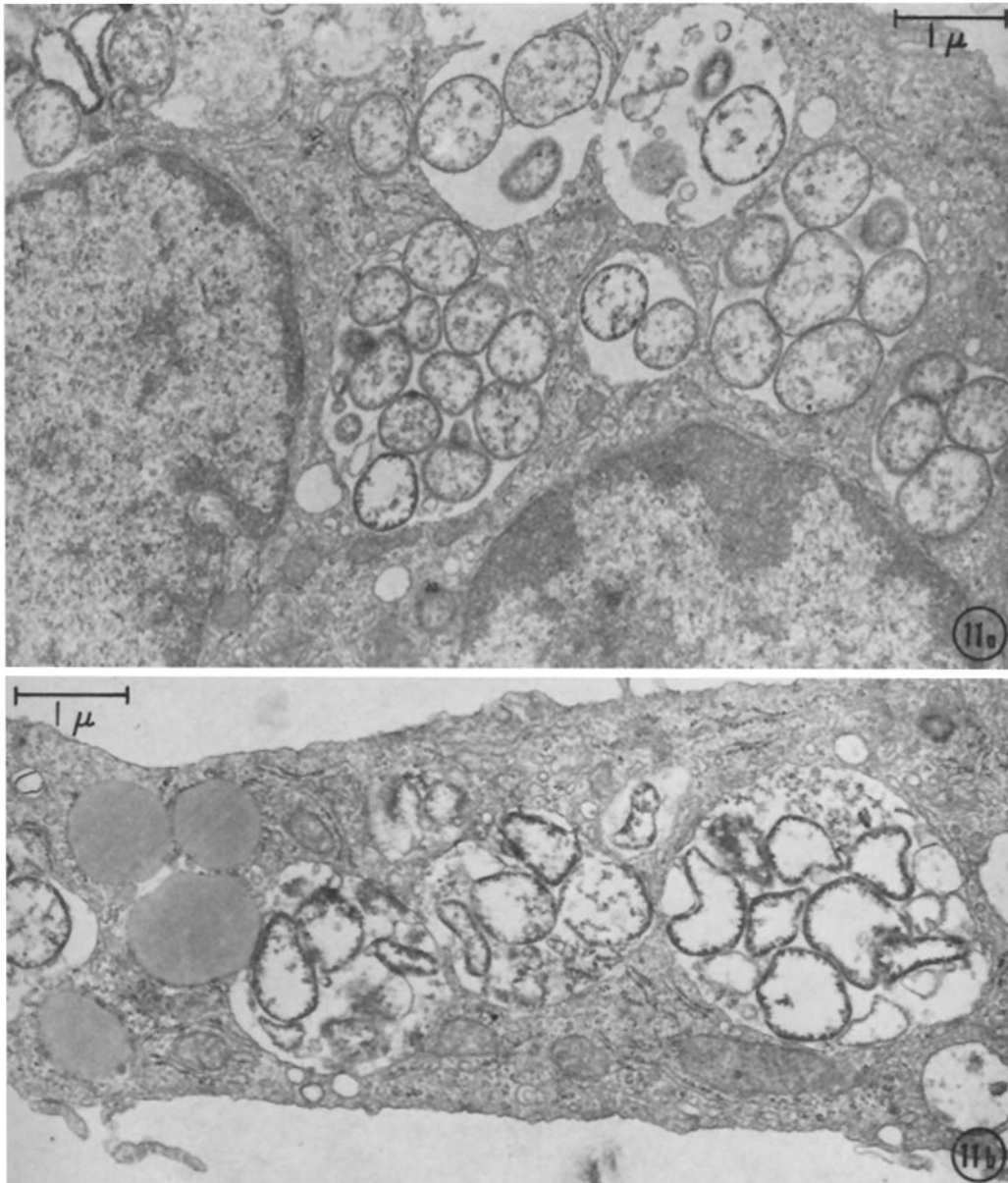


FIG. 11. Electron-microscopic appearance of mycoplasma-infected macrophages 2 hr after the addition of anti-mycoplasma antibody. Cell surface mycoplasmas are not seen. Large vacuoles, some of which appear to interconnect, contain numerous mycoplasmas. The microorganisms in 11 *a* appear for the most part normal, whereas those within vacuoles in the cell in 11 *b* show clearing of the central region and condensation of electron-opaque material at the periphery. These changes are interpreted as morphologic evidence of mycoplasma degradation. (*a*) $\times 17,500$; (*b*) $\times 17,500$.

ruffling was apparent. These ruffles surrounded the mycoplasmas and when they subsided the mycoplasmas had vanished. Removal of surface mycoplasmas was complete in 10 min even when the infection was heavy. During the 30 min

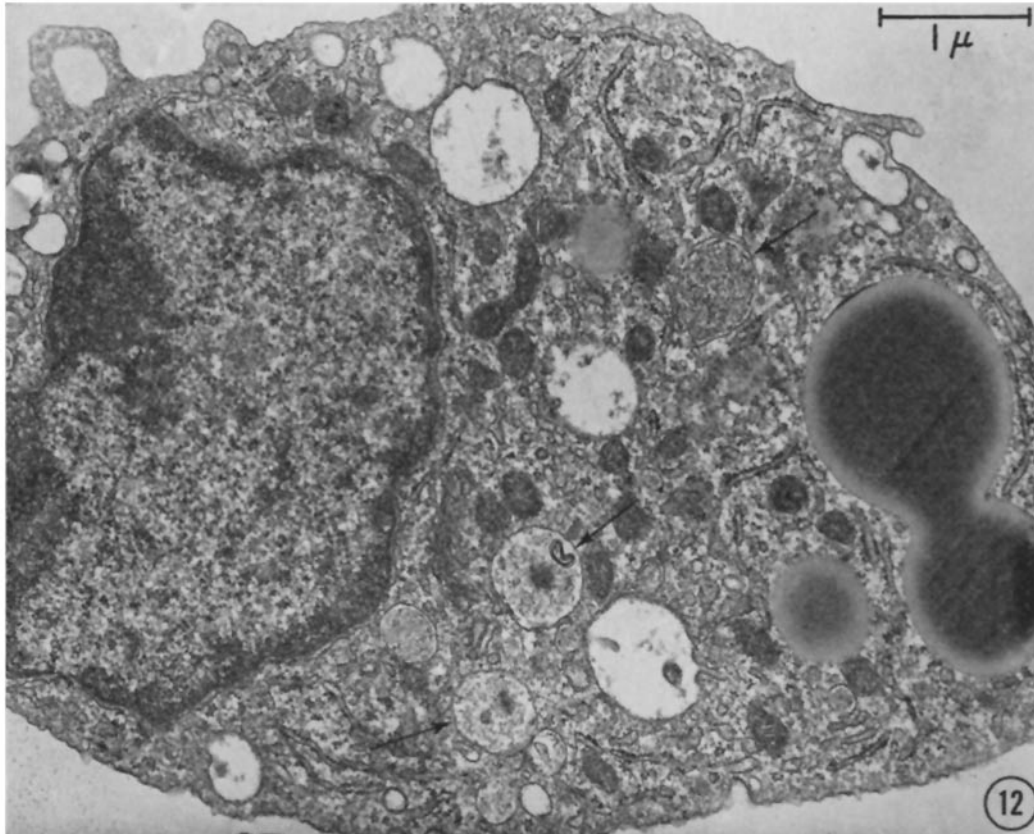


FIG. 12. Electron-microscopic appearance of an infected mouse peritoneal macrophage 24 hr after the addition of anti-mycoplasma antibody. Mycoplasmas cannot be detected. The cytoplasm contains the usual complement of mitochondria, clear vacuoles, endoplasmic reticulum, and lipid deposits. Present here but not seen in normal cells are several large vacuoles (arrows) filled with electron-opaque granular or lamellar debris; these probably are residual bodies resulting from the degradation of mycoplasmas. $\times 20,000$.

after this clearing of the surface, the mycoplasmas gradually became visible as pale spheres gathered in grape-like clusters inside of large phagocytic vacuoles in the centrosomal region of the macrophage. The sequence in Fig. 7 shows selected stages during this phagocytic process.

Time-lapse cinematography of mycoplasma-infected L-cells after addition of specific antibody showed no change in the surface mycoplasmas or in the cells.

Observations on Mycoplasma-Cell Interaction Using Electron Microscopy.—The ultrastructure of *Mycoplasma pulmonis* associated with macrophages or L-cells was not significantly different from that of organisms in 24 hr broth cultures. Fig. 8 shows organisms fixed in a broth culture. The mycoplasmas were somewhat pleomorphic, but usually appeared as round bodies 0.5–1.0 μ in diameter on thin section. The cytoplasm was generally electron lucent with scattered ribosomal and amorphous densities. No specific organelles were seen. The limiting membrane had a typical unit structure. Macrophages fixed 24 hr after mycoplasma infection are shown in Figs. 9 *a* and 9 *b*. The mycoplasmas were seen in close opposition to the plasma membrane; no special morphologic feature was evident at the attachment site. In some instances the mycoplasmas were situated in cup-like depressions of the cell surface. A clear space of approximately 100 A separated the unit membranes of the cell and the attached mycoplasma. In most sections of these infected macrophages no structures resembling intracellular mycoplasmas were seen. However, on occasions there were phagocytic vacuoles containing mycoplasmas which appeared to be partially degraded (Fig. 9 *b*).

Specimens fixed for electron microscopy 5 min or 2 hr after addition of rabbit anti-mycoplasma antibody showed a marked change in the macrophage-mycoplasma association. At 5 min (Fig. 10 *a, b*) most mycoplasmas were located in phagosomes rather than at the cell surface. These organisms appeared morphologically normal in most cases. Mycoplasmas remaining on the surface of the cell appeared to be in the process of envelopment by pincer-like microprojections. Fig. 11 shows infected macrophages fixed 2 hr after addition of anti-mycoplasma antibody. No organisms were seen on the cell surface. Large phagocytic vacuoles were present in these cells; in some cases (Fig. 11 *a*) the mycoplasmas in these vacuoles appeared for the most part normal, whereas in other cells (Fig. 11 *b*) the microorganisms were partially degraded as evidenced by clearing of the central region and condensation of electron-opaque material at the periphery. 24 hr after the addition of antibody to the infected macrophages, phagocytic vacuoles containing distinct mycoplasmas were no longer detectable (Fig. 12). The cytoplasm at this time contained large vacuoles some of which were filled with amorphous, electron-opaque material, probably representing residual bodies formed as the end stage of the digestion of engulfed mycoplasmas.

Electron micrographs of L-cells infected with mycoplasmas revealed organisms closely associated with the cell surface. Apparent indentation of the cell membrane and microprojections were common at sites of mycoplasma attachment (Fig. 13). Attachment sites showed no special morphologic fea-

tures, except for a clear space approximately 100 Å wide separating the unit membranes. Very rarely a degenerated mycoplasma was seen in an apparent phagocytic vacuole. L-cells fixed 2 hr after the addition of anti-mycoplasma antibody showed no change in comparison to control cultures. The organisms remained surface-associated. Intracellular organisms were not seen. There was no evidence of damage to or disappearance of mycoplasmas.

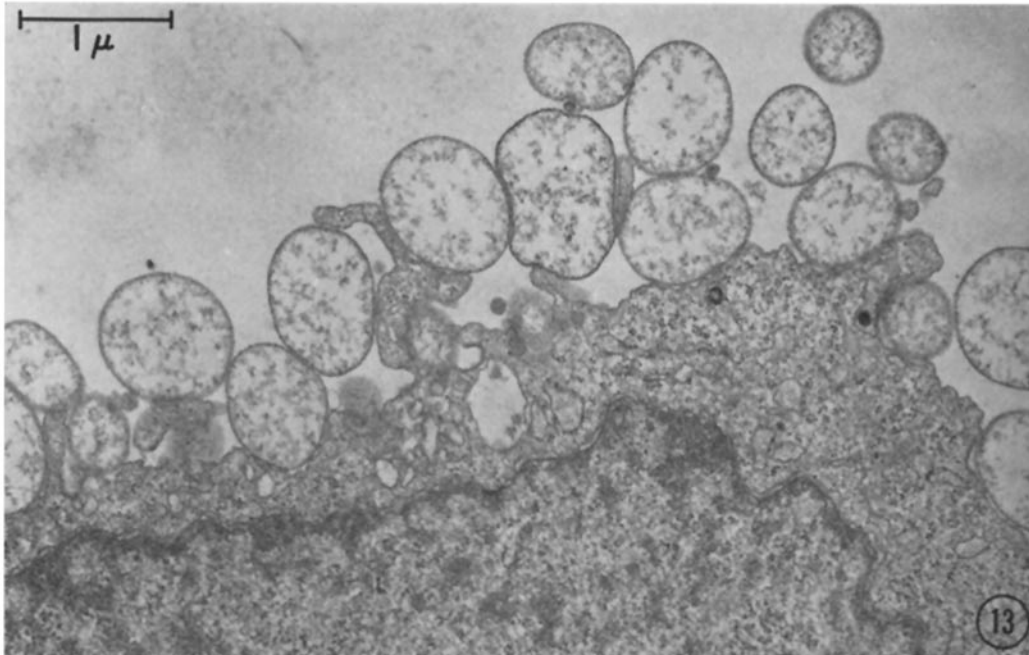


FIG. 13. Electron-microscopic appearance of a mouse fibroblast 24 hr after infection with *M. pulmonis*. Cell membrane indentations and microprojections are seen associated with mycoplasma attachment. The mycoplasmas are separated from the cell and from each other by an electron-lucent space of approximately 100 Å. No intracellular mycoplasmas are seen. $\times 20,000$.

These observations taken together indicate that mycoplasmas exist mainly on the membrane surface and occasionally within phagocytic vacuoles of mouse peritoneal macrophages. In the presence of anti-mycoplasma antibody all surface mycoplasmas are rapidly engulfed and appear to be degraded within the phagocytic vacuole. Mycoplasmas attach to the L-cell membrane surface and are not seen intracellularly in significant numbers whether or not specific antibody has been added.

Fate of Membrane-Associated Mycoplasmas after Exposure to Anti-Mycopl-

plasma Antibody.—It was of interest to determine whether heat-inactivated anti-mycoplasma antibody had killed the mycoplasmas on the cell surfaces as a prelude to their phagocytosis by the macrophage. Mycoplasmas on macrophages or L-cells were exposed to antibody in a dilution of 1:100 for 30 min, and the cover slips were washed twice with MEM containing 1% HIFCS. Fresh medium with 1 μ Ci/ml of tritiated thymidine was then added for 4 hr. Specimens were then fixed in glutaraldehyde and prepared for radioautography.

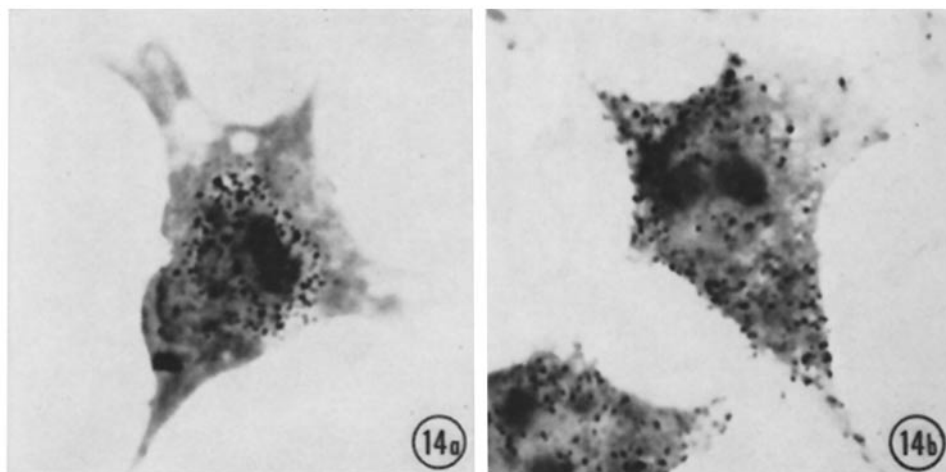


FIG. 14. Radioautograph of L-cells exposed to tritiated thymidine. (a) Shows an uninfected cell demonstrating grains limited to the central region, a finding consistent with incorporation of the labeled thymidine into cell-nuclear DNA. (b) Shows a cell infected with *M. pulmonis* for 20 hr and exposed to anti-mycoplasma antibody for 30 min before treatment with tritiated thymidine. Grains are distributed over the entire area of the L-cell, a finding consistent with incorporation of the thymidine into the DNA of mycoplasmas growing on the cell surface. Infected L-cells not treated with antibody showed this same pattern of grains after exposure to tritiated thymidine. $\times 1600$.

Fig. 14 shows an uninfected control L-cell with all the radiolabel over the nucleus (a), and an infected L-cell after exposure to anti-mycoplasma antibody with radiolabel distributed over the surface of the cell (b). L-cells infected with mycoplasmas appeared identical by this technique, whether or not they had been exposed to anti-mycoplasma antibody. Thus, antibody did not kill mycoplasmas on the surface of L-cells, as determined by the ability of the mycoplasmas subsequently to incorporate tritiated thymidine. Fig. 15 shows infected macrophages in cultures which were or were not exposed to anti-mycoplasma antibody as described above. Incorporation of tritiated thymidine occurred in both, although to a lesser extent in the cultures treated with anti-

body. In the infected cells grains were over the surface of the cell (Fig. 15 *a*), whereas after antibody exposure grains appear clumped intracellularly (Fig. 15 *b*). Therefore, as with the L-cell, incorporation of tritiated thymidine into mycoplasmas occurred after exposure to specific antibody. Whether the incorporation occurred before ingestion or after phagocytosis could not be determined from these experiments.

It was also important to establish whether or not mycoplasmas engulfed

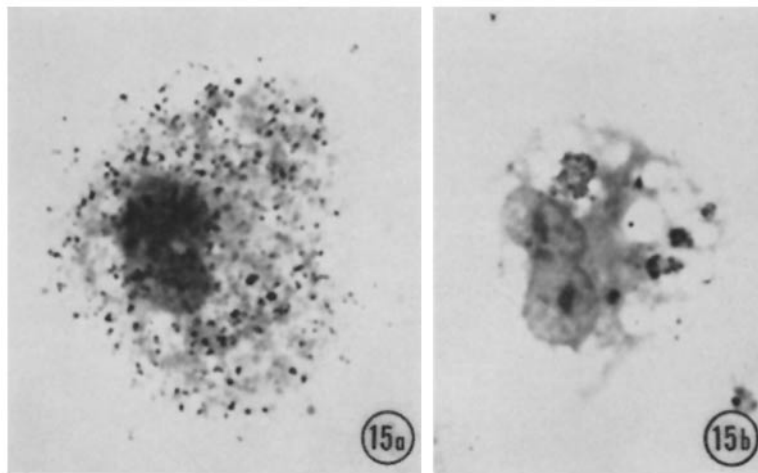


FIG. 15. Radioautographs of mycoplasma-infected mouse peritoneal macrophages treated with tritiated thymidine. Uninfected macrophages did not incorporate label into acid-insoluble constituents under these conditions. (*a*) Shows an infected macrophage with grains distributed widely over the outline of the cell. (*b*) Shows an infected cell which was exposed to anti-mycoplasma antibody for 30 min before the addition of the radiolabel. Grains are fewer in number and appear to be clumped within the vacuoles of the cell. $\times 1600$.

by macrophages survived intracellularly. The absence of organisms in cells 24 hr after antibody exposure indicated that they did not persist in recognizable forms for that period. To obtain further information on this point, mycoplasma-infected macrophage cultures were labeled with tritiated thymidine. The mycoplasmas incorporated the label into acid-insoluble DNA, whereas the macrophages did not synthesize DNA under these conditions. The conversion of DNA into acid-soluble material thus served as a sensitive marker of organism degradation (17). The effect of rabbit anti-mycoplasma antibody on the degradation of mycoplasma DNA was followed in cultures of the microorganisms alone as well as in the infected macrophage culture system.

Fig. 16 shows the per cent of total counts found in the perchloric acid-soluble fraction with time. In the infected macrophage cultures containing anti-

mycoplasma antiserum, less than 10% of the radiolabel remained in an acid-insoluble form after 3 hr. In infected macrophage cultures containing no antibody, and in control cultures of mycoplasmas without cells, 45% of the radiolabel remained in acid-insoluble form. These results indicated that engulfed mycoplasmas were rapidly degraded within the phagocytic vacuole.

DISCUSSION

The pleuropneumonia-like organisms now classified as *Mycoplasma pulmonis* was first isolated from lung lesions of rats by Klieneberger and Steabben in 1937 (18). The microorganism has since been isolated from the brain, lungs, liver, plasma cell tumor, and middle ear of mice (19). It has also been recovered

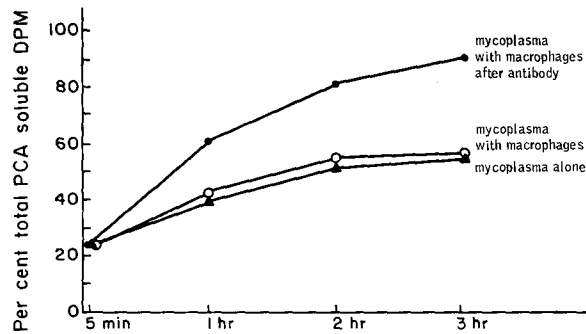


FIG. 16. Increase in acid-soluble radiolabel with time after the addition of anti-mycoplasma antibody to macrophages infected with mycoplasmas.

from various tissues of rats (19) and from the respiratory tract of rabbits (20), *M. pulmonis* has been demonstrated to cause infectious catarrh (pneumonia, otitis media, and rhinitis) (21), pneumonia (22), oophoritis and salpingitis (23), and arthritis (24, 25) in inoculated mice. The course of the pulmonary infection has been correlated with antibody response by Lemcke (26). All information to date indicates that this strain of mycoplasma is a natural pathogen for mice causing both acute illness and latent infection.

Under phase microscopy *M. pulmonis* in cell cultures appear as phase-dense spherical bodies 0.5–1 μ in diameter. They are larger and less pleomorphic than the microorganisms washed from agar. Freundt has proposed that large spherical forms may be correlated with organic acids in the growth medium produced by fermentation of glucose by the mycoplasmas (27). The phase appearance of mycoplasma is distinct from refractile lipid droplets and lucent pinocytic vacuoles, but it can be difficult to separate them from cytoplasmic granules within the cell except for the surface location of the mycoplasmas, their large size, and their widespread distribution. The definition of intra-

cellular mycoplasmas by phase microscopy is generally not adequate except at specific stages of engulfment when they appear as grape-like clusters (Fig. 6). In cells which are poorly spread, or cells too filled with lipid, good resolution of surface mycoplasmas is not possible. In spite of these limitations, phase-contrast microscopy is very useful for observations on mycoplasma-cell interactions. Phase contrast is clearly superior to stained preparations for determining whether organisms are on the cell surface or intracellular. It is our opinion that earlier observations on such stained specimens (1-3) led to the misinterpretation that surface organisms were intracellular.

The mycoplasmas shown in the electron photomicrographs are similar in ultrastructure to descriptions recorded by others (5, 9, 11-14). We found no significant difference in the ultrastructure of this strain of mycoplasma whether in broth medium or cell-associated. Attachment of mycoplasmas to the cell surface presents no striking morphological features. The unit membrane of the mycoplasma is usually clearly separated from the plasma membrane of the cell by approximately 100 Å. This space is relatively constant, and there is no evidence of fusion or junctional areas. Electron micrographs similar to those shown by others as evidence of intimate mycoplasma contact with the cell (28) or fusion (5) were seen occasionally in our specimens and were felt to be compatible with artifacts of sectioning rather than being morphologic evidence of a special attachment complex. We also have not observed mycoplasmas free within cytoplasmic ground substance as reported by others (5, 12, 13). As noted by Zucker-Franklin et al. (11), apparently intracellular mycoplasmas enclosed by membranes in some thin sections may in fact be extracellular, lying in invaginations of the cell surface. The studies reported here suggest that mycoplasmas may be seen intracellularly within phagocytic vacuoles under two conditions. First, in the absence of anti-mycoplasma antibody, degenerated forms of organisms are seen infrequently by electron microscopy within L-cells and macrophages. This may reflect a very slow rate of ingestion of normal mycoplasmas under these conditions, or it may reflect engulfment of the occasional mycoplasmas which are damaged. Second, in the presence of anti-mycoplasma antibody, surface organisms are phagocytized and then digested within lysosomes of macrophages, but not L-cells. Intracellular survival of the mycoplasma appears to be transient or nonexistent.

The use of radiolabeled thymidine to determine the fate of mycoplasmas after addition of antiserum was possible since mycoplasmas rapidly incorporate thymidine into DNA and macrophages do not. The radioautographs of infected L-cells and macrophages show that the mycoplasmas continue to incorporate thymidine after exposure to antibody for at least a few hours. One therefore cannot attribute the rapid opsonization of surface mycoplasmas to killing of the microorganism. However, once the organisms have been phagocytized by macrophages, the results show rapid movement of the radiolabel

from acid-insoluble to acid-soluble material, thus indicating degradation of the organisms within phagosomes. These results agree with those of Cohn (17) in following the fate of phagocytized bacteria. Thus, "latent" infection with mycoplasma is not likely due to persistence of the organisms within macrophages. Zucker-Franklin et al. (12) have suggested that mycoplasmas may remain viable for long periods within lymphocytes. We have not made any observations on the interaction of *M. pulmonis* with lymphocytes.

Early in these experiments it became clear that mycoplasma growth and cell association differed depending upon the cell type and the composition of medium. Mycoplasmas multiplied on L-cells in either MEM or Medium 199 plus 20% serum. In contrast, in macrophage cultures the mycoplasmas disappeared in these media. Addition of heart infusion broth at 5% or higher concentrations to these media allowed the organisms to thrive and to parasitize the surface of macrophages in culture. In mixed cultures of L-cells and macrophages in Medium 199, mycoplasmas grew well and covered the macrophage surfaces even when the media contained no added broth. These observations suggested that L-cells released into the medium a growth factor utilized by mycoplasma, and/or that Medium 199 contained a factor toxic to mycoplasma which was neutralized in the presence of L-cells but not macrophages. On the other hand in mixed L-cell and macrophage cultures in MEM and 20% serum, the mycoplasmas did not thrive and infect the cells; evidently in this situation a growth factor was sufficiently removed or toxic factor elaborated by the macrophages that mycoplasma did not grow on either cell. These observations obviously require further investigation, but they serve to emphasize the complexities of the mycoplasma-cell interactions in vitro, and probably in vivo as well.

The mechanism of association of mycoplasmas with the surface of cells is unclear. The Mycoplasmatales appear unique as cell-associated, extracellular organisms. There is no evidence to date documenting any metabolic requirement of mycoplasmas fulfilled by their intimate cell association (7). Sobeslavsky et al., showed that adsorption of *M. pneumoniae* colonies to epithelial cells and erythrocytes could be prevented by pretreatment of the cells with neuraminidase or influenza B virus, or pretreatment of the mycoplasma with neuraminic acid. Trypsin treatment of cells did not alter adsorption. A neuraminic acid receptor was therefore postulated as a mechanism of mycoplasma-cell association. These observations, however, were not applicable to other mycoplasma species studied, including *M. pulmonis*. Adsorption of *M. pneumoniae* was blocked by a specific antiserum, and this block was removed by absorption of the antiserum with the lipid fraction and the glycerophospholipid hapten of the lipid fraction of the organism. Antisera made against other mycoplasma species, however, did not block their adsorption (29).

Thomas (30) found that attachment of *M. gallisepticum* to erythrocytes, lymphocytes, and fibroblasts required ambient temperature and occurred after

the organisms were disrupted by freeze-thawing. Attachment was prevented when heat-killed or trypsin-treated organisms were used or when cells were treated with neuraminidase. His data suggested that attachment required a sialic acid cell determinant and a mycoplasma surface-protein component.

An immunologic mechanism of attachment of mycoplasma to macrophages, such as by macrophage cytophilic antibody of mouse, horse, or fetal calf serum origin, must be considered. LoBuglio et al. demonstrated the binding of red cells coated with immunoglobulin G to mononuclear cells (31). In this setting phagocytosis did not rapidly follow attachment. Free γ G inhibited attachment, and papain treatment released the red cells from the mononuclear cells. These experiments suggested that macrophages possess a γ G-binding site which does not trigger rapid erythrophagocytosis, but causes damaging changes to the erythrocyte coated with incomplete antibody. This appears an unlikely mechanism for attachment of mycoplasmas to the mouse peritoneal macrophage since even the small amount of free γ G present in the fetal calf serum would be expected competitively to inhibit attachment; partially surrounded or damaged mycoplasmas are not seen on the surface of the cells, and pretreatment of macrophages with trypsin or papain does not prevent attachment of mycoplasmas. The occurrence of attachment in spite of various combinations of cells and serum or broth products noted in our experiments makes attachment through cytophilic antibody to both the L-cell and macrophage unlikely. These results suggest that the attachment of this organism to mouse peritoneal macrophages and L-cells is probably a nonimmunologic process; the nature of the receptors remains unknown.

Rabinovitch described a procedure for examining separately the attachment and ingestion stages of phagocytosis using glutaraldehyde-treated erythrocytes (32). He demonstrated that attachment of a particle to the macrophage surface did not trigger ingestion unless proper temperature, serum, and divalent cations were also present. By carefully controlling these factors such that the rate of ingestion was limited, study of the two stages was possible (33). Others have used similar techniques, as well as metabolic inhibitors, to prevent engulfment in systems utilizing specific antibody (34-37) or serum containing natural antibody (38) to achieve attachment. Mycoplasmas provide a "natural" model of attachment in which ingestion does not occur, or occurs at a very slow rate, even when proper temperature, serum, and divalent cations are present. Engulfment is strikingly stimulated by antibody to the mycoplasmas. Further examination of this system should yield important information concerning the attachment and ingestion phases of the phagocytic process.

The marked opsonic effect of heat-inactivated rabbit anti-mycoplasma antiserum on mycoplasmas attached to the surface of mouse peritoneal macrophages has allowed microscopic documentation of the ingestion process. Under phase-contrast observation, addition of antiserum also results in marked stimulation of membrane activity, with ruffles surrounding the mycoplasmas;

when the ruffles subside, the organisms are no longer visible. At the electron microscopic level one observes, during the first minutes after exposure to specific antibody, the pincers-like microprojections of cell membrane and organelle-free cytoplasm enveloping the organisms. Subsequently, the coalescence of phagosomes and degradation of the ingested mycoplasmas can be seen. These changes are consistent with previous morphologic descriptions of macrophage function (see Review in reference 16) with the mycoplasma serving as a convenient marker.

The different effects of anti-mycoplasma antiserum on the mouse peritoneal macrophage compared to the L-cell underline the difference between the "professional" and "nonprofessional" phagocyte. The L-cell does not ingest the surface organisms after addition of anti-mycoplasma antiserum, in contrast to the peritoneal macrophage, which rapidly phagocytizes these organisms after addition of antiserum. These results agree with those of Rabinovitch, who demonstrated that L2 cells did not phagocytize glutaraldehyde-treated red cells after treatment with anti-erythrocyte serum (39), whereas macrophages did (33). The anti-erythrocyte serum in his model prevented attachment as well as ingestion, indicating that immunoglobulin receptors were not present on the L2 cells. Since attachment is already achieved in the mycoplasma-cell system, most likely at a nonimmunologic site, it is clear that anti-mycoplasma immunoglobulin acts as a "trigger" for ingestion by the macrophage but not by the L-cell.

The pathogenesis of mycoplasma infection is poorly understood. *Mycoplasma pulmonis* has been shown to grow on the surface of bronchial epithelial cells in vivo (10). Like *M. pneumoniae*, it produces hemolysis due to H₂O₂ (40), and a toxic property unrelated to hydrogen peroxide has also been demonstrated in living organisms (30). Separation of epithelial cells from each other has been shown in tracheal organ cultures infected with *M. pneumoniae* (41). In gnotobiotic mice inoculated with *M. pulmonis* extensive polymorphonuclear invasion of the bronchi is seen whereas in the peribronchial region a mononuclear response is evident (10). Attempts to demonstrate metabolism-inhibiting antibody in respiratory secretions of patients convalescing from *M. pneumoniae* infections have been unrevealing (42). In humans the presence of metabolism-inhibiting antibody (43, 44) and antibody measured by indirect immunofluorescence (45) has been correlated with resistance to *M. pneumoniae* infection. However, one-fourth of patients with serum metabolism-inhibiting antibody become reinfected (43, 44). Certainly, further information on both cellular and humoral responses to mycoplasma infection is required to understand properly the mechanisms of virulence and host resistance. The present observations on macrophages provide the first evidence that opsonic antibody must be considered as a mechanism of host resistance against mycoplasma infections.

There may be differences between the various types of macrophages in their

interaction with *M. pulmonis*. For example, preliminary observations on mouse alveolar macrophages cultured in vitro indicate that these cells exhibit moderately active ingestion of mycoplasmas in media containing no added anti-mycoplasma antibody. Differences between peritoneal and alveolar cells in their response to mycoplasma infection need further examination to determine whether they are due to the more efficient state of the alveolar macrophage (46), to exposure of alveolar cells to organisms antigenically unrelated to mycoplasma (47), or to local antibody or cellular responses resulting from prior mycoplasma infection. A study of germfree mice is planned to examine these questions.

Sites of mycoplasma localization and inflammation other than the lungs raise additional questions regarding pathogenesis. Nelson (23) demonstrated that *M. pulmonis* inoculated intraperitoneally localized and caused inflammation around the ovaries and the oviducts. Intraperitoneal inoculation of *M. pulmonis* also caused arthritis in mice but with less frequency than intravenous inoculation (23, 24). The pathogenesis of this arthritis requires further investigation. Thomas (30) has described the similarities of mycoplasma infections and "autoimmune diseases" and raised the interesting question whether mycoplasma-antibody-complement reactions adjacent to a cell surface could cause sufficient cell damage to induce a subsequent autoimmune reaction. These many unsolved problems call for additional investigation of the interactions between mycoplasmas and their host cells.

SUMMARY

Methods have been devised for establishing infection in vitro of mouse macrophages and fibroblasts with *Mycoplasma pulmonis*. The mycoplasmas attached to the cells and under appropriate cultural conditions grew into a lawn of microorganisms covering most of the cell surface. The mycoplasmas grew abundantly on fibroblasts cultured in minimal essential medium containing 20% fetal calf serum; supplementation of this medium with heart infusion broth was necessary to obtain similar growth on macrophages. The infection of these cells appeared to be essentially an extracellular process; only rarely were partially degraded mycoplasmas seen with phagocytic vacuoles.

The addition to heavily infected macrophage cultures of low concentrations of anti-mycoplasma antibody stimulated rapid, massive phagocytosis of the surface microorganisms. In sharp contrast, the same antiserum had no discernible effect on the mycoplasma-fibroblast relationship. The antibody effect in the macrophage system was apparently a direct opsonic one rather than an indirect result of microbial killing, since the mycoplasmas in macrophage or fibroblast cultures incorporated labelled thymidine into DNA after the addition of antiserum to the medium.

The phagocytic event and the subsequent fate of the mycoplasmas were studied in detail after the addition of antibody to the macrophage cultures.

Phase-contrast cinemicrophotography revealed membrane ruffles surrounding the surface mycoplasmas and disappearance from view of the organisms; 10–30 min later translucent grapelike clusters were seen in large phagocytic vacuoles. On electronmicroscopic study the surface mycoplasmas were surrounded by pincers-like projections of the macrophage. Numerous mycoplasmas were seen in phagocytic vacuoles; in the early minutes after the addition of antibody the intracellular mycoplasmas appeared normal, but within 2 hr they appeared partially degraded with a central electron-lucent area and electron-opaque deposits at the microbial cell margin. 24 hr after the addition of antiserum, digestion of the mycoplasmas was nearly complete; the cells appeared normal except for large residual bodies composed of amorphous moderately dense material and increased lipid deposits.

Degradation of mycoplasmas within macrophages was also studied using infected cultures in which the mycoplasmas, but not the macrophages, had incorporated tritiated thymidine into DNA. The appearance of large amounts of acid-soluble radiolabel after phagocytosis stimulated by antibody confirmed the degradation of the intracellular mycoplasmas.

REFERENCES

1. Hayflick, Leonard, and Warren R. Stinebring. 1960. Intracellular growth of pleuropneumonia-like organisms (PPLO). *In* Tissue Culture and In Ovo. *Ann. N. Y. Acad. Sci.* **79**:433.
2. Eaton, Monroe D., and Iolanda E. Low. 1967. Propagation of *Mycoplasma pneumoniae* and other fastidious strains of PPLO. *Ann. N. Y. Acad. Sci.* **143**:375.
3. Nelson, John B. 1960. The behavior of murine PPLO in HeLa cell cultures. *Ann. N. Y. Acad. Sci.* **79**:450.
4. Hummeler, Klaus, and Donald Armstrong. 1967. Observations on mycoplasma strains in tissue culture. *Ann. N. Y. Acad. Sci.* **143**:622.
5. Edwards, G. A., and J. Fogh. 1960. Fine structure of pleuropneumonia-like organisms in pure culture and in infected tissue culture cells. *J. Bacteriol.* **79**:267.
6. Pollock, M. E., and G. E. Kenny. 1963. Mammalian cell cultures contaminated with PPLO. III. Elimination of PPLO with specific antiserum. *Proc. Soc. Exp. Biol. Med.* **112**:176.
7. Hayflick, Leonard. 1969. Fundamental biology of the class Mollicutes, order Mycoplasmatales. *In* The Mycoplasmatales and the L-Phase of Bacteria. Leonard Hayflick, editor. Appleton-Century-Crofts. 15.
8. Thomas, Lewis. 1969. Relationship between mycoplasma and mammalian cells. *In* Cellular Recognition. R. T. Smith and R. A. Good, editors. Appleton-Century-Crofts. 139.
9. Hirth, R. S., M. E. Tourtellotte, and S. W. Nielson. 1970. Cytopathic effects and ultrastructure of *Mycoplasma agalactiae* var. *bovis* (Donetla strain). *Infect. Immun.* **2**:105.
10. Organick, A. B., K. A. Siegesmund, and I. I. Lutsky. 1966. Pneumonia due to mycoplasma in gnotobiotic mice. II. Localization of *Mycoplasma pulmonis* in the lungs of infected gnotobiotic mice by electron microscopy. *J. Bacteriol.* **92**:1164.

11. Zucker-Franklin, D., M. Davidson, and L. Thomas. 1966. The interaction of mycoplasma with mammalian cells. I. HeLa cells, neutrophils, and eosinophils. *J. Exp. Med.* **124**:521.
12. Zucker-Franklin, D., M. Davidson, and L. Thomas. 1966. The interaction of mycoplasma mammalian cells. II. Monocytes and lymphocytes. *J. Exp. Med.* **124**:533.
13. Hummeler, K. D., D. Armstrong, and N. Tomassini. 1965. Cytopathogenic mycoplasmas associated with two human tumors. II. Morphological aspects. *J. Bacteriol.* **90**:511.
14. Anderson, D. R., and R. A. Manaker. 1966. Electron microscopic studies of mycoplasma (PPLO strain 880) in artificial medium and in tissue culture. *J. Nat. Cancer Inst.* **36**:139.
15. Hirsch, James G., and Martha E. Fedorko. 1968. Ultrastructure of human leukocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "postfixation" in uranyl acetate. *J. Cell Biol.* **38**:615.
16. Cohn, Zanvil A. 1968. The structure and function of monocytes and macrophages. *In* Advances in Immunology. Academic Press, Inc., New York. 163.
17. Cohn, Zanvil A. 1963. The fate of bacteria within phagocytic cells. I. The degradation of isoptically labeled bacteria by polymorphonuclear leukocytes and macrophages. *J. Exp. Med.* **117**:27.
18. Klieneberger, E., and D. B. Steabben. 1937. On a pleuropneumonia-like organism in lung lesions of rats, with notes on the clinical and pathological features of the underlying condition. *J. Hyg.* **37**:143.
19. Tully, Joseph G. 1969. Murine mycoplasmas. *In* The Mycoplasmatales and the L-Phase of Bacteria. L. Hayflick, editor. Appleton-Century-Crofts. 571.
20. Deeb, Barbara J., and George E. Kenny. 1967. Characterization of *Mycoplasma pulmonis* variants isolated from rabbits. I. Identification and properties of isolates. *J. Bacteriol.* **93**:1416.
21. Nelson, J. B. 1967. Pathologic response of Swiss and Princeton mice to *M. pulmonis*. *Ann. N. Y. Acad. Sci.* **143**:778.
22. Lutsky, Irving I., and Avrum B. Organick. 1966. Pneumonia due to mycoplasma in gnotobiotic mice. I. Pathogenicity of *Mycoplasma pneumoniae*, *Mycoplasma salivarium* and *Mycoplasma pulmonis* for the lungs of conventional and gnotobiotic mice. *J. Bacteriol.* **92**:1154.
23. Nelson, J. B. 1954. The selective localization of murine pleuropneumonia organisms in the female genital tract on intraperitoneal injections in mice. *J. Exp. Med.* **106**:179.
24. Sabin, A. B. 1939. Experimental proliferative arthritis in mice produced by filterable, pleuropneumonia-like microorganisms. *Science (Washington)*. **89**:228.
25. Barden, Jerri A., and Joseph G. Tully. 1969. Experimental arthritis in mice with *Mycoplasma pulmonis*. *J. Bacteriol.* **100**:5.
26. Lemcke, R. M. 1961. Association of PPLO infection and antibody response in rats and mice. *J. Hyg.* **59**:401.
27. Freundt, E. A. 1969. Cellular morphology and mode of replication of the mycoplasmas. *In* The Mycoplasmatales and the L-Phase of Bacteria. L. Hayflick, editor. Appleton-Century-Crofts. 281.
28. Anderson, D. R. 1969. Ultrastructural studies of Mycoplasmas and the L-Phase of

- bacteria. *In* The Mycoplasmatales and the L-Phase of Bacteria. L. Hayflick, editor. Appleton-Century-Crofts. 365.
29. Sobeslavsky, O., B. Prescott, and R. M. Chanock. 1968. Adsorption of *Mycoplasma pneumoniae* to neuraminic acid receptors of various cells and possible role in virulence. *J. Bacteriol.* **96**:695.
 30. Thomas, Lewis. 1969. Mechanisms of pathogenesis in mycoplasma infection. *In* Harvey Lectures 1968-1969, Series 63. Academic Press, Inc., New York. 73.
 31. LoBuglio, A. F., R. S. Cotran, and J. H. Jandl. 1967. Red cells coated with immunoglobulin G; binding and sphering by mononuclear cells in man. *Science (Washington)*. **158**:1582.
 32. Rabinovitch, M. 1967. The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. *Exp. Cell Res.* **46**:19.
 33. Rabinovitch, M. 1967. The role of antibodies in the ingestion of aldehyde-treated erythrocytes attached to macrophages. *J. Immunol.* **99**:232.
 34. Uhr, J. W. 1965. Passive sensitization of lymphocytes and macrophages by antigen-antibody complexes. *Proc. Nat. Acad. Sci. U.S.A.* **54**:1599.
 35. Nelson, D. S. 1969. Endocytosis by macrophages. *In* Macrophages and Immunity. North-Holland Publishing Co., Amsterdam, The Netherlands. 94.
 36. Berken, A., and B. Benacerraf. 1966. Properties of antibodies cytophilic for macrophages. *J. Exp. Med.* **123**:119.
 37. Auzins, Deva, and D. Rowley. 1963. Factors involved in the adherence of *S. typhimurium* C5 and mouse peritoneal macrophages. *Aust. J. Exp. Biol. Med. Sci.* **41**:539.
 38. Brumfitt, W., A. A. Glynn, and A. Percival. 1965. Factors influencing the phagocytosis of *Escherichia coli*. *Brit. J. Exp. Pathol.* **46**:213.
 39. Rabinovitch, M. 1969. Uptake of aldehyde-treated erythrocytes by L2 cells; inhibition by anti-red cell antibody or by coating the erythrocytes with purified proteins. *Exp. Cell Res.* **54**:210.
 40. Cole, B. C., J. R. Ward, and E. H. Martin. 1968. Hemolysin and peroxide activity of mycoplasma species. *J. Bacteriol.* **95**:2022.
 41. Collier, A. M., W. A. Clyde, Jr., and F. W. Denny. 1970. Nature of respiratory epithelial infection by *Mycoplasma pneumoniae*. *Bacteriol. Proc.* 82.
 42. Smith, C. B., R. M. Chanock, W. T. Friedewald, and R. H. Alford. 1967. *Mycoplasma pneumoniae* infections in volunteers. *Ann. N. Y. Acad. Sci.* **143**:471.
 43. Chanock, R. M., H. H. Fox, W. D. James, R. R. Gutekunst, R. J. White, and L. B. Senterfit. 1967. Epidemiology of *M. pneumoniae* infection in military recruits. *Ann. N. Y. Acad. Sci.* **143**:484.
 44. Steinberg, P., S. L. Fuld, R. J. White, R. Gutekunst, R. M. Chanock, and L. B. Senterfit. 1969. Ecology of *Mycoplasma pneumoniae* infections in Marine recruits at Parris Island, South Carolina. *Amer. J. Epidemiol.* **89**:62.
 45. Taylor-Robinson, D., A. Shirai, O. Sobeslavsky, and R. M. Chanock. 1966. Serologic response to *Mycoplasma pneumoniae* infection. II. Significance of antibody measured by different techniques. *Amer. J. Epidemiol.* **84**:301.
 46. Oren, R., A. E. Farnham, K. Saito, E. Milofsky, and M. L. Karnovsky. 1963. Metabolic patterns in three types of phagocytic cells. *J. Cell Biol.* **17**:487.
 47. Blanden, R. V., G. B. Mackaness, and F. M. Collins. 1966. Mechanisms of acquired resistance in mouse typhoid. *J. Exp. Med.* **124**:585.