Studies on Attachment and Ingestion Phases of Phagocytosis of *Mycoplasma pulmonis* by Mouse Peritoneal Macrophages[1] (36165)

THOMAS C. JONES, SHIRLEY YEH, AND JAMES G. HIRSCH
The Rockefeller University, New York, New York 10021

We have described in a previous publication an *in vitro* system in which mycoplasmas attached and multiplied on the surface of mouse peritoneal macrophages (1). Addition of antimycoplasma antibody to the system led to prompt engulfment of the attached microrganisms. In this report, we present further observations on the requirements for attachment of mycoplasmas to the macrophage surface, and on conditions necessary for phagocytosis of the organisms.

Materials and Methods. Preparation of macrophage cultures using unstimulated mouse peritoneal cells followed techniques previously described (1). Five to 10×10^5 macrophages were allowed to adhere to cover slips in 35 mm plastic tissue culture dishes for 1 hr in minimum essential medium (MEM) containing 20% heat-inactivated fetal calf serum (HIFCS) at 37° in 5% CO_2–balanced air. This medium was then aspirated and the cells were overlayed with fresh MEM–20% HIFCS. The culture dishes were incubated for 24 or 48 hr at 37° in 5% CO_2–balanced air.

Mycoplasma pulmonis (WRAIR strain) was passed every 3 or 4 days in heart infusion broth containing 20% heat-inactivated horse serum (HIHS). Studies were conducted on organisms obtained from the 60th to 150th broth passage. Twenty-four to 48 hr before each experiment 1 ml of the stock mycoplasma culture was added to 5 ml of heart infusion broth–20% HIHS and incubated at 37°. Mycoplasmas from this culture were used in one of two ways. For studies of 24-hr infected macrophages the mycoplasma culture was diluted 1:100 in MEM containing 20% HIFCS–10% heart infusion broth. Macrophages were overlayed with this medium for 24 hr prior to each experiment. For studies of attachment or ingestion of mycoplasmas by 48 hr uninfected macrophages, mycoplasma stock cultures were diluted 1:100 in MEM containing 20% HIFCS and 30% heart infusion broth and placed in 60 mm plastic dishes each containing a 24 × 40 mm cover slip. After 24 hr, mycoplasmas were found in large numbers firmly attached to the cover slip. These mycoplasmas were then washed with MEM, treated with the experimental media, and the cover slips were scraped with a rubber policeman to obtain a suspension of mycoplasmas which then was used to overlay the macrophages.

Attachment and ingestion were evaluated on mycoplasmas, which had been treated in various ways and then incubated with the peritoneal macrophages for 2 hr. The preparations were washed twice with MEM and fixed in 2.5% glutaraldehyde in sodium cacocylate buffer (pH 7.4) for 10 min at 4°. In some experiments, such as when EDTA or only sucrose was in the suspending medium, the effects of these media on the macrophage necessitated shorter exposure periods. For these studies, macrophages were grown on 16 mm round cover slips; these cover slips were placed in an 18 mm glass flat-bottomed tube and the added mycoplasmas in suspension were brought rapidly into contact with the macrophage surface by centrifugation at 1000 rpm for 5 min. These slides were then washed and fixed in glutaraldehyde as described above. In some experiments the macrophages were treated with the various experimental media for 1 hr prior to the addition of mycoplasmas. For studies in the presence of glycolytic inhibitors, the macro-

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TABLE I. Phagocytosis of Mycoplasmas by Macrophages.

Additions to the macrophage cultures	Ingestion of mycoplasmas
Living mycoplasmas	0
Heat killed (120°) mycoplasmas	++
Frozen–thawed mycoplasmas	++
Glutaraldehyde (2.5%) killed mycoplasmas	+++
Glutaraldehyde (2.5%) killed mycoplasmas + 3×10^{-5} *M* iodoacetamide	+

phages were pretreated with inhibitor for 15 min prior to addition of the mycoplasmas; and the inhibitor was maintained during the remainder of the experiment. The ingestion phase of phagocytosis was also studied on macrophages infected for 24 hr with mycoplasmas. These were washed with MEM and the experimental media was added for 1 hr, followed by washing and fixation in 2.5% glutaraldehyde.

The percentage of macrophages with surface mycoplasmas was determined by examining 200 macrophages on duplicate samples. The most convenient method for estimating phagocytosis was to grade ingestion 0 to ++++ inversely related to the percentage of macrophages with surface organisms as detected by phase contrast microscopy. Grading was as follows for the results presented in Tables I, II, and III: $0 = > 95\%$ macrophages with surface mycoplasmas; $+ =$ 50–95%; $++ =$ 20–50%; $+++ =$ 5–20%; $++++ = < 5\%$. Disappearance of the surface mycoplasmas was correlated with appearance of intracellular organisms in vacuoles, and was blocked by addition to the medium of inhibitors of phagocytosis indicating that the disappearance from the surface was due to ingestion, not detachment, of the mycoplasmas.

The following preparations were used: lyophilized trypsin, 185 μg/ml; alpha-chymotrypsin, 50 μg/mg; trypsin-DFP (diisopropyl fluorophosphate; 0.58% trypsin); soybean trypsin inhibitor (1 mg inhibits 1.5 mg trypsin); chymotrypsinogen A; pepsin, 2830 μg/mg; all obtained from Worthington Biochemical Corporation, Freehold, NJ; phenylmethylsulfonylfluoride (PSF), neuraminidase (*Clostridium perfringens*, type IV), from Sigma Chemical Company, St. Louis, MO; iodoacetamide (2 × crystallized).

Antiserum against *M. pulmonis* was prepared in rabbits as previously described (1). Serum was heat inactivated at 56° for 30 min, precipitated with saturated ammonium sulfate, then fractionated by DEAE-cellulose column chromatography using established techniques (2). The material was dialyzed against acetate buffer (pH 4.0), treated with pepsin 2% by weight for 18 hr at 37°, and dialyzed against borate buffer (pH 8.0) for 20 hr (2, 3). The IgG and pepsin digest were concentrated by vacuum dialysis and checked for purity by placing on a G-100 Sephadex column. Protein concentrations were determined by the Lowry method. Fractions were maintained at —20° until use.

Results. Attachment of mycoplasmas to macrophages was examined by altering the suspending medium and the chemical or physical state of the mycoplasmas and the macrophages prior to mixing. The following alterations did not block attachment: low temperature (to 4°), increased tonicity (to 4 × isotonicity), absence of serum and broth, and the absence of divalent cations.

The following treatments of the mycoplasmas and/or the macrophages did not block attachment: treatment of macrophages or mycoplasmas with neuraminidase (up to 100 μg/ml), trypsin, chymotrypsin or lysozyme (up to 1 mg/ml); treatment of macrophages with glycolytic inhibitors; glutaraldehyde fixation of macrophages for 10 min at 4°; and treatment of mycoplasmas with glutaraldehyde, heat (120°), or freezing and thawing.

Attachment did not occur when both the mycoplasmas and the macrophages had been fixed in glutaraldehyde prior to mixing. At-

TABLE II. Stimulation of Ingestion by Treatment of Mycoplasma-Infected Macrophages with Proteases.

Additions to the mycoplasma-infected macrophage cultures	Ingestion of mycoplasmas
None	0
Trypsin, 100 μg/ml	+ + + +
20 μg/ml	+ + + +
4 μg/ml	+ + +
Trypsin, 20 μg/ml + soy bean inhibitor, 13 g/ml	+ +
26 g/ml	0
Trypsin-diisopropyl fluorophosphate, 8 μg/ml	0
Chymotrypsin, 100 μg/ml	+ + + +
20 μg/ml	+ +
4 μg/ml	0
Chymotrypsin, 100 μg/ml + phenylmethyl-sulfonylfluoride, 1×10^{-5} *M*	0
Chymotrypsinogen A, 100 μg/ml	0

tachment was also blocked when living organisms and cells were mixed in a 0.25 *M* sucrose medium rather than MEM.

Treatment of mycoplasmas attached to glutaraldehyde-fixed macrophages with trypsin resulted in detachment of the surface organisms; whereas trypsin treatment of mycoplasmas associated with living macrophages did not. These morphologic observations were confirmed using mycoplasmas labeled with tritiated thymidine, determining that radioactivity remained cell associated when living mycoplasma-infected cells were treated with trypsin; whereas the label moved into the suspending medium after trypsin treatment of glutaraldehyde-fixed-infected cells.

Phagocytosis of mycoplasmas occurred after adsorption to the macrophage surface if the mycoplasmas were altered by heating, freezing and thawing, or glutaraldehyde fixation as is shown in Table I. These studies were made by adding the mycoplasmas treated in these various ways to macrophage cultures, and assessing ingestion after 2 hr of incubation.

Table II shows the results of studies on the addition of the proteolytic enzymes to cultures of mycoplasma-infected macrophages. The degree of ingestion was estimated by following the disappearance of the surface lawn of mycoplasmas, and the concomitant appearance of intracellular microorganisms clustered in vacuoles. As shown, ingestion of mycoplasmas occurred with as little as 4 μg/ml of trypsin or 20 μg/ml of chymotrypsin. These effects were specifically inhibited by trypsin soybean inhibitor, diisopropyl fluorophosphate (DFP) or phenylmethylsul-

TABLE III. Effect of Antibody Fractions on Ingestion of *M. pulmonis* by Macrophages.

Antibody fractions added to mycoplasma-infected macrophage cultures	Ingestion of mycoplasmas
Rabbit antimycoplasma IgG, 100 μg/ml	+ + + +
10 μg/ml	+ +
1 μg/ml	0
Rabbit antimycoplasma $F(ab')_2$, 75 μg/ml	0
7.5 μg/ml	0
None	0

fonylfluoride. Figure 1 shows examples of macrophages infected with mycoplasmas 1 hr after the addition of 20 μg/ml of trypsin (Fig. 1b), or after addition of DFP-inactivated trypsin (Fig. 1a). No effect was seen with chymotrypsinogen A, a molecule similar to chymotrypsin but devoid of proteolytic activity. Other experiments showed that mycoplasmas pretreated with trypsin or chymotrypsin were rapidly ingested when added to macrophage cultures. In contrast, pretreatment of the macrophages prior to addition of untreated mycoplasmas did not result in ingestion. No reduction in the number of colony-forming units of mycoplasmas was observed after treatment with trypsin.

Neuraminidase (100 μg/ml), phospholipase-C (50 μg/ml), or lysozyme (1 mg/ml) did not induce ingestion or removal of surface mycoplasmas from macrophages.

A most effective trigger for ingestion of mycoplasmas by mouse peritoneal macrophages was the addition of antimycoplasma antiserum (1). It was of interest to characterize further the action of this antibody. Antimycoplasma IgG demonstrated marked opsonic activity at 10 μg/ml of antibody protein as shown in Table III. The $F(ab)'_2$ fragment of this antibody, prepared by pepsin digestion, agglutinated mycoplasmas but had no opsonic effect, even in concentrations up to 10 times higher than that in the intact IgG. Studies were also done on possible blocking of the opsonic action of antimycoplasma IgG by normal IgG. Blocking occurred only when the concentration of normal IgG was 10–50 times the concentration of IgG-containing antimycoplasma antibodies.

Discussion. Only recently has it been possible to study separately the two phases of the phagocytic process, namely attachment and ingestion (4). When red blood cells were used in such studies, it was necessary first to treat these cells in a special manner, opsonizing them with serum or fixing them with aldehyde, to promote their attachment to macrophages, and it was, furthermore, necessary to employ special conditions such as low temperature to prevent ingestion and thus permit observations on attachment alone. The mycoplasma infection of macrophages provides a useful natural model in which separate observations of attachment and of ingestion are easily made. The present work has surveyed several factors influencing the adherence of mycoplasmas to the macrophage surface, and has examined selected factors which trigger ingestion of these organisms.

Studies on the attachment of mycoplasmas to macrophages have revealed this process to have few if any identifiable physicochemical requirements; it has not been possible to discover the nature of the attractive force or bond responsible for the sticking. Attachment was not blocked by : (a) low temperature, (b) hypertonicity, (c) absence of divalent cations, (d) absence of serum, or (e) treatment of the mycoplasmas or of the macrophages with trypsin, chymotrypsin, neuraminidase, lysozyme, or glutaraldehyde. Attachment was prevented when both mycoplasmas and macrophages were glutaraldehyde fixed, or when a nonionic sucrose medium was employed. These results do eliminate attachment via some mechanisms previously found in other systems, such as sialic acid receptors (5), immune adherence (6), incomplete antibody (7), or calcium or magnesium ion bridging (8).

Three types of alterations in mycoplasmas, free or attached to the macrophage surface, were found to result in their ingestion: (i) killing the organisms in various ways, (ii) exposure to proteolytic enzymes, and (iii) addition of antimycoplasma IgG.

As reported previously (1), mycoplasmas grew on the surface of macrophages and resisted ingestion only if the medium contained broth and other growth factors. When infected cultures were transferred to deficient medium the attached mycoplasmas were engulfed during the subsequent 24 to 48 hr. Separate studies on mycoplasmas suspended in these deficient media established that they died under these conditions. As shown in the present work, killing the mycoplasmas in several other ways (glutaraldehyde fixation, heating at 120°, repeated freezing and thawing) also rendered them susceptible to ingestion by macrophages. The nature of the change in the surface of the organisms in these various situations is, of couse, not

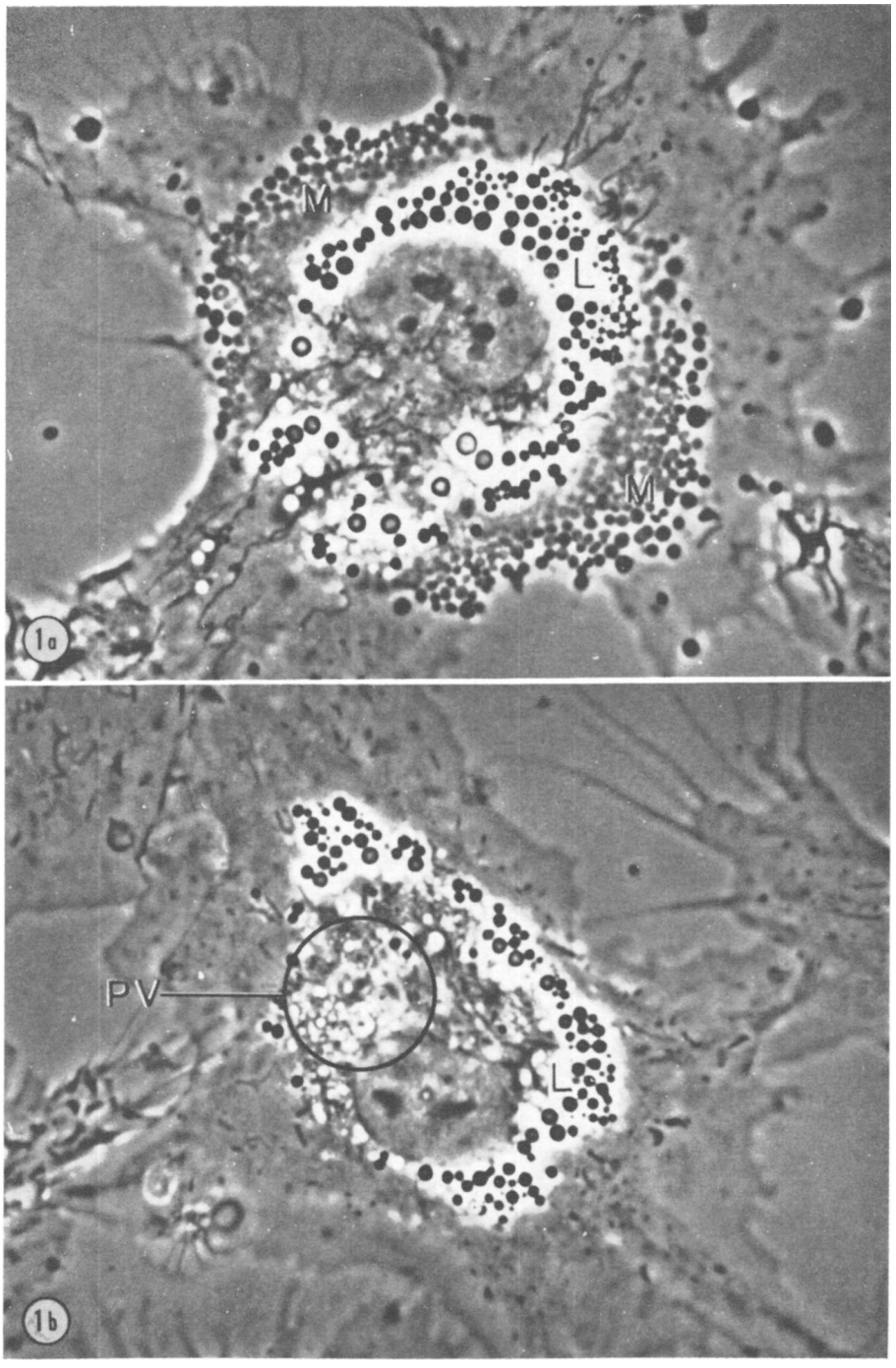

FIG. 1. Phase-contrast photomicrographs of mouse peritoneal macrophage 24 hr after infection with *M. pulmonis*: (a) after 1 hr of exposure to inactivated trypsin (trypsin-DFP, 8 μg/ml). This appearance was identical to that of controls not exposed to any trypsin preparation. The mycoplasmas (M) remain on the cell surface, appearing as phase dense spheres, as distinguished from the darker, refractile lipid bodies (L) which surround the nucleus and centrisomal region. The

known, and it may well be different from one case to another. It seems likely, however, that alteration in surface proteins may be a common feature of these several conditions lethal for the mycoplasmas, and that this alteration of surface protein renders the organisms susceptible to ingestion by macrophages by a mechanism independent of serum or antibody. Denaturation of surface protein has been suggested as a mechanism determining ingestion of effete erythrocytes (9). In our studies, viability of the organisms and alterations in their surface influenced ingestion but not attachment. Similar studies by others in different systems suggest an influence on attachment as well (10).

Stimulation of ingestion of mycoplasmas by proteases or by specific antibody is of particular significance since these agents did not kill the mycoplasmas *in vitro*. The proteases acted on attached or on free mycoplasmas to render them rapidly susceptible to ingestion. This action was shown to be a specific one dependent on proteolytic activity. It therefore can be concluded that this strain of mycoplasma, cultured under these conditions, produces a surface protein which blocks ingestion of the organism by mouse peritoneal macrophages. This antiphagocytic protein does not, however, block attachment of the mycoplasmas to the macrophage surface. This situation may be compared to that of the M protein of streptococci (11), a surface protein which renders the organism resistant to phagocytosis, probably in the main by interfering with the attachment phase of phagocytosis. The M protein is, of course, a primary determinant of virulence of the group A streptococci; no information is available on the possibility that the surface protein is also a virulence factor in *M. pulmonis*. Suffice it to say, *M. pulmonis* is a major rodent pathogen causing pneumonia (12) and arthritis (13), and mycoplasmas not known to cause disease in rodents such as *Mycoplasma hominis* and *Mycoplasma pneumoniae* are rapidly phagocytized by the mouse peritoneal macrophage.

We pointed out earlier the striking phagocytosis-promoting action of antimycoplasma antibody on mycoplasmas attached to macrophages (1). These results have now been extended to show that intact IgG molecules have this effect, but $F(ab')_2$ fragments do not. Thus the antibody probably is not acting merely by masking the mycoplasma antiphagocytic surface protein. The results suggest then that the intact immunoglobulin, and in particular the F_c portion of the molecule is playing a specific role in stimulating ingestion *per se*, over and above its recognized role in facilitating attachment between particle and phagocytic cell. The mechanism by which the IgG molecule stimulates the ingestion phase is unknown, but presumably it involves reaction with the F_c receptors on the macrophage surface (14). Spreading attachment between IgG molecules and these F_c receptor sites could act as a "zipper" leading to ingestion, although ultrastructural aspects of the ingestion process under these conditions does not entirely support this concept (1).

Summary. Studies have been made on factors which influence attachment of *Mycoplasma pulmonis* to the surface of mouse macrophages *in vitro*, and on factors which determine whether or not the attached microorganisms are ingested.

Attachment of mycoplasmas exhibited few specific requirements. Attachment occurred in media containing no broth, serum, or divalent cations, and it was not blocked by proteolytic enzymes, neuraminidase, lysozyme or by exposure of the mycoplasmas to glutaraldehyde, to heat, or to repeated freezing and thawing. Only glutaraldehyde fixation of both the macrophages and the mycoplasmas,

centrisomal region shows only a few phase lucent vacuoles. Mitochondria are seen to extend toward the cell periphery. (b) after 1 hr of exposure to trypsin, 20 μg/ml. Mycoplasmas are no longer seen on the cell surface. The centrisomal region contains numerous phase lucent phagocytic vacuoles (PV), some appearing as "grape-like" clusters. Refractile, phase dense lipid droplets (L) in the perinuclear area appear unchanged. The cell is somewhat contracted. These changes are consistent with recent ingestion of the surface mycoplasmas.

or use of a nonionic sucrose medium prevented attachment.

In enriched tissue culture medium, the attached mycoplasmas resisted ingestion by the macrophages. This resistance was lost if the mycoplasmas were damaged or killed in various ways. Mycoplasmas treated with proteolytic enzymes under conditions not lethal for the organisms became susceptible to ingestion by macrophages, suggesting the presence of an antiphagocytic surface protein. Mycoplasmas were also rendered susceptible to ingestion on exposure to nonlethal concentrations of antimycoplasma IgG antibody; $F(ab')_2$ fragments did not promote engulfment, suggesting that the F_c portion of the antibody molecule was important in the ingestion process, probably by reacting with F_c receptor sites on the macrophage.

1. Jones, T. C., and Hirsch, J. G., J. Exp. Med. **133**, 231 (1971).

2. Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D. H., "Methods in Immunology," 2nd ed., p. 193. Benjamin, New York (1970).

3. Nisonoff, A., Markus, G., and Wissler, F. C., Nature (London) **189**, 293 (1961).

4. Rabinovitch, M., *in* "Mononuclear Phagocytes", (R. Van Furth, ed.), p. 299. Blackwell, Oxford (1970).

5. Sobeslavsky, O., Prescott, B., and Chanock, R. M., J. Bacteriol. **96**, 695 (1968).

6. Nelson, D. S., "Macrophages and Immunity", p. 94. North-Holland, Amsterdam (1969).

7. Lo Buglio, A. F., Cotran, R. S., and Jandl, J. H., Science **158**, 1582 (1967).

8. Lay, W. H., and Nussenzweig, V., J. Immunol. **102**, 1172 (1969).

9. Rabinovitch, M., Proc. Soc. Exp. Biol. Med. **124**, 396 (1967).

10. Rabinovitch, M., Exp. Cell Res. **54**, 210 (1969).

11. Lancefield, R. C., J. Exp. Med. **78**, 465 (1943).

12. Organick, A. B., Siegesmund, K. A., and Lutsky, I. I., J. Bacteriol. **92**, 1164 (1966).

13. Barden, J. A., and Tully, J. G., J. Bacteriol. **100**, 5 (1969).

14. Abramson, N., Gelfand, E. W., Jandl, J. H., and Rosen, F. S., J. Exp. Med. **132**, 1207 (1970).

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