REVIEW ARTICLE

The phagocytosis of mycoplasmas

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Introduction

Mycoplasmas are recognised primarily as extra-cellular parasites or pathogens of mucosal surfaces, although recent evidence suggests that certain species may invade host cells.^{1–5} The role of mycoplasmas in disease is also being reassessed in the light of recent findings linking them with other infectious agents, including HIV,^{1,2,5–9} meningococci^{10,11} and chlamydiae.^{12,13}

Phagocytic cells, such as macrophages and polymorphonuclear leucocytes (PMNL), comprise the first line of defence against mycoplasmas invading the lung and genitourinary tract.¹⁴ Cells of the macrophage-monocyte lineage patrol the respiratory and genitourinary mucosae, accumulating at sites of mycoplasma infection.¹⁵ Not only do they carry out effector functions such as receptor-mediated phagocytosis,^{15,16} but they are also important accessory cells of the immune system involved in antigen presentation and the production of cytokines.¹⁷ After an inflammatory stimulus, PMNL (together with peripheral blood monocytes) are also recruited to the area by chemotaxis¹⁸ and large numbers amass in respiratory, synovial and cartilaginous tissues infected with mycoplasmas.^{19,20} Thus, at the onset of infection, these two cell types are the main mediators of immunity to mycoplasmas; indeed, the outcome of this interaction may determine the subsequent progression of disease.

Over the past 25 years, many of the reports concerning the interaction of mycoplasmas with phagocytes have appeared contradictory, and the mechanisms by which pathogenic mycoplasmas circumvent innate immune responses and go on to establish disease remain poorly understood. In this review, we summarise current knowledge concerning the phagocytosis of mycoplasmas, emphasising recent advances and highlighting areas where knowledge is incomplete, in an attempt to clarify the way forward for future studies. More comprehensive reviews detailing early studies can be found elsewhere.^{15,17,21}

Interaction between phagocytes and mycoplasmas *in vivo*

Innate responses to mycoplasma infection

Many reports describe the interaction of macrophages or PMNL, or both, with mycoplasmas *in vivo*.^{22–39} Much of the work has centred on the effects of *Mycoplasma pulmonis*, a mouse pathogen, on the cellular responses of mice and rats. In the early 1970s, Cassell *et al.*^{18,22} studied the clearance of *M. pulmonis* from the lower respiratory tract of Fischer rats and CD-1 strain mice. They found that rats were able to clear this mycoplasma from the lung relatively efficiently, with only limited infiltration of PMNL, and deduced that the organisms were taken up rapidly by alveolar macrophages. Mice, on the other hand, cleared mycoplasmas much more slowly and no association between mycoplasma antigen and macrophages was observed. Moreover, the accumulation of PMNL in the bronchi of infected mice was massive and much of the tissue destruction observed was attributed to PMNL hydrolytic activity.¹⁸ Lindsey and Cassell¹⁹ have also used immunofluorescence techniques to show the association of *M. pulmonis* antigen with PMNL in the alveoli of infected mice. Similar prominent alveolar infiltrations of macrophages and PMNL have been observed in mice injected with live, killed or membrane preparations of *M. pulmonis*, suggesting that mycoplasma antigen alone may be sufficient to attract phagocytes to foci of infection and to stimulate inflammatory responses.^{22,30} Recently, Ross *et al.*⁴⁰ have demonstrated that *M. pulmonis* membrane preparations possess a potent chemoattractant activity for peritoneal and alveolar macrophages from fresh rat serum. This is corroborated by Meier *et al.*,³³ who showed increased PMNL aggregation and activation (as measured by respiratory burst activity) in polyarthritic joint fluid from *M. arthritidis*-infected SPF-Lewis rats, and Komatsu,³⁴ who showed degranulation and specific chemotaxis of eosinophils towards *M. pneumoniae* in the lungs of guinea-pigs. In another study, Taylor and Taylor-Robinson²³ observed increased proliferation and dissemination of *M. pulmonis* and *M. pneumoniae* in T cell-depleted hamsters and mice, but a decreased

severity of disease (as measured by lesion score) when compared with control animals. This was attributed to the reduced accumulation of macrophages and PMNL, which were less activated and showed a reduced capacity to kill mycoplasmas and to induce inflammatory responses.

Many mycoplasma syndromes are characterised by the persistence of antigen in the lung parenchyma before the onset of antibody production, despite the presence of phagocytes. For example, *M. bovis* antigen has been shown to persist in the lung parenchyma of gnotobiotic calves for at least 4 weeks after infection,²⁹ and *M. pulmonis* strains have been shown to survive in the respiratory tract of mice, despite the accumulation of peritoneal macrophages.^{26, 27}

More recently, Trichopoulos and colleagues^{41, 42} have investigated the effect of *M. arthritidis* on the phagocytic activity of macrophages of rats and mice. By studying the kinetics of colloidal carbon clearance in the lungs, they have shown that phagocytic activity is significantly depressed 12 h post-infection, returns to normal at 24 h, then increases significantly by 7 days, and subsequently returns to control levels by the end of the fourth week.⁴² They suggest that the early depressive effect on phagocytic activity may be related to superantigen activity, with the production of suppressive cytokines, such as macrophage-de-activating factor. The later expansion of the macrophage population might result from the stimulation of auto-reactive clones of T and B lymphocytes. Presumably, increased macrophage activity may also be a factor in the development of the chronic arthritis found in mycoplasma-infected mice.

Factors that affect innate responses

The killing and clearance of *M. pulmonis* from the respiratory tract, in the absence of specific antibody, depend on the strain of mycoplasma,³¹ mouse²⁸ or rat³² studied; thus, differences in disease severity from host to host may relate to the effectiveness of non-specific phagocytic mechanisms in eliminating particular mycoplasma strains.³¹ Recently Lai *et al.*⁴³ studied the genetic control of resistance to *M. pulmonis* infection in mice, demonstrating that macrophages harvested from infected H-2b (C57BL/6) mice are much more effective at killing mycoplasmas than macrophages from infected CT (T2) mice. Activation of macrophage bactericidal function may help to explain genetic variation in resistance to *M. pulmonis* infection.

Different types of phagocytic cell also exhibit varying mycoplasmacidal activity. The clearance of *M. pulmonis* from the peritoneal cavity of SPF/CBA mice is enhanced by the presence of increasing numbers of peritoneal macrophages, but not PMNL.¹⁶ Brownlie *et al.*²⁵ demonstrated that PMNL-rich exudates, induced in the bovine mammary gland after challenge with *M. dispar* or ureaplasmas, were ineffective at eliminating these organisms, and suggested

that neutropenia following infection may facilitate mycoplasma growth and aggravate mastitis. Interestingly, an elevation in the number of mature and immature PMNL is found in the respiratory tract of neonates colonised with *Ureaplasma urealyticum*.⁴⁴

Natural killer (NK) cells may be the principal cells responsible for mycoplasmacidal activity in the respiratory tract of mice,³¹ a view supported by studies on mice with severe combined immunodeficiency⁴⁵ and BALB/c⁴⁶⁻⁴⁸ mice, infected with *M. pulmonis*; clearance of mycoplasmas is inhibited significantly by antibodies to interferon (IFN)- γ or NK cells. The mycoplasmacidal activity of NK cells is attributed mainly to their ability to produce IFN- γ , which activates macrophage bactericidal mechanisms.

Environmental factors, such as nitrogen dioxide (NO₂), may suppress non-specific immunity to mycoplasma infection and increase disease severity.^{39, 49} Exposure to NO₂ at doses > 5 ppm decreases intrapulmonary killing and exacerbates disease. Davis *et al.*³⁹ have shown that this effect results largely from NO₂-mediated damage to alveolar macrophages, leading to decreased viability and impaired bactericidal capability.

What do in-vivo studies tell us?

Clearly the outcome of the initial mycoplasma-phagocyte interaction plays an important role in determining the progression of infection and the severity of disease. Several factors appear to determine the capacity of a host to eliminate mycoplasmas by phagocytosis. First, phagocyte activation, induced by mycoplasma either directly or *via* cytokine production, is important in determining mycoplasmacidal activity. Second, the efficiency of phagocytosis varies with both the strain of mycoplasma and the individual host. Third, the nature and origin of a phagocyte influence its capacity to engulf and destroy mycoplasmas, probably as a result of differences in surface receptors or bactericidal mechanisms. Finally, environmental factors may modulate the innate immune system resulting in impaired phagocytic responses.

Interaction between phagocytes and mycoplasmas *in vitro*

The importance of opsonisation

Three basic types of interaction between phagocytes and mycoplasmas have been studied *in vitro*; phagocytosis of unopsonised mycoplasmas, phagocytosis of mycoplasmas exposed to non-specific serum opsonins (e.g., complement), and phagocytosis of mycoplasmas exposed to specific serum opsonins (e.g., IgG or IgA). Most mycoplasmas resist phagocytosis in the absence of opsonins (table). The capacity of phagocytes to recognise unopsonised bacterial surfaces is dependent on electrostatic charge, van der Waals attraction and hydrophobicity. In our view, the

Table. *In-vitro* studies of the interaction between mycoplasmas and phagocytic cells

Mycoplasma species	Phagocyte* type/source	Phagocytosis in opsonisation conditions†			Reference no.
		unopsonised	non-specifically opsonised‡	specifically opsonised‡	
<i>M. arthritis</i>	Human/rabbit PMNL	±	ND	+ [R]	52
	Mouse peritoneal Mφ	—	— [M] [GP]	+ [R]	53
	Rabbit peritoneal Mφ	—	— [M] — [R]	— [M]	53
<i>M. bovis</i>	Bovine PMNL	—	ND	ND	72
	Bovine PMNL	—	— [R]	— [B]	54
	Bovine Mφ	—	— [R]	+ [B]	54
	Mouse PMNL/Mφ	—	ND	+ [R] + [B]	16
<i>M. buccale</i>	Human PMNL	±	± [H]	± [R]	50
<i>M. dispar</i>	Bovine PMNL/Mφ	—	ND	+ [R] + [B]	16
	Bovine PMNL	—	— [R]	— [R] + [B]	54
	Mouse PMNL/Mφ	—	ND	+ [R] + [M]	27
<i>M. fermentans</i>	Human PMNL	±	± [H]	± [R]	50
<i>M. gallisepticum</i>	Human PMNL	+	+ [H] + [M]	ND	57
<i>M. hominis</i>	Human PMNL	±	± [H]	+ [C]	51
	Human/rabbit PMNL	±	± [H]	± [R]	50, 52
<i>M. hyorhinis</i>	Bovine PMNL	+	ND	ND	77
<i>M. neurolyticum</i>	Human PMNL	+	+ [H] + [M]	ND	57, 58
<i>M. orale</i>	Human PMNL	±	± [H]	± [R]	50
<i>M. pneumoniae</i>	Human PMNL	±	± [H]	+ [C] ± [R]	50, 51
		+	+ [H] + [M]	ND	57, 58
	Guinea pig alveolar & peritoneal Mφ	—	ND	+ [R]	63, 65, 76
		—	— [R]	ND	62
		—	+ [GP] + [R]	+ [R]	56
	Mouse peritoneal Mφ	—	ND	+ [R]	61
	P388D1 cell line	—	ND	± [R]	61
		—	ND	± [R]	61
<i>M. pulmonis</i>	Mouse alveolar & peritoneal Mφ	—	ND	+ [R]	61, 66, 67, 69
		—	ND	+ [R] — [M]	53, 71, 75
		—	ND	ND	26
	Mouse PMNL & Mφ	—	— [R] [M]	+ [R] + [M]	27
<i>M. salivarium</i>	Human PMNL & Mono	+	ND	+ [R]	55
	Human PMNL	±	± [H]	+ [C]	51
<i>Ureaplasma urealyticum</i>	Human PMNL	±	± [H]	± [R]	50
		±	± [H]	+ [C]	51
<i>Acholeplasma laidlawii</i>	Bovine PMNL	+	ND	ND	77
	Mouse peritoneal Mφ	+	ND	+ [R]	61
	P388D1 cell line	+	ND	+ [R]	61

* PMNL, polymorphonuclear leucocyte; Mφ, macrophage; Mono, peripheral blood monocyte.

† Phagocytosis: +, present; —, absent; ±, contradictory results (i.e., evidence for phagocytosis/phagocytosis-related phenomena, but an absence of intracellular killing of mycoplasmas by phagocytes); ND, assays not done.

‡ Serum source is indicated: [R] rabbit; [GP] guinea-pig; [B] bovine; [M] murine; [C] caprine; [H] human.

resistance of unopsonised mycoplasmas to phagocytosis is not particularly significant in pathogenesis as many species of bacteria avoid phagocytosis in the absence of opsonins, and unopsonised cells are essentially "artificial" and would rarely if ever be encountered by phagocytes *in vivo*.

The lack of published data on the interaction of non-specifically opsonised mycoplasmas with phagocytes is surprising as non-immune host defences are a key stage in resistance to infection. Non-specific opsonisation involves the deposition of complement breakdown products (C3b, C3bi, C3dg and C4b) on the mycoplasma cell surface, but other opsonins, such as C-reactive protein, fibronectin, α2-macroglobulin, lipoprotein surfactants, tuftsin and non-specific serum proteases may also be involved. For many mycoplasma species, viable count data suggest that non-specific opsonisation has little beneficial effect on phagocyte

bactericidal activity,^{27, 50–54} whereas studies with electronmicroscopy (EM), chemiluminescence (CL) and ¹⁴CO₂ production demonstrate increased phagocytosis and respiratory burst activity when mycoplasmas are treated with non-specific opsonins.^{50–52, 55–58} This may reflect phagocytosis of mycoplasmas which retain their viability. Surprisingly, little work has been performed on the phagocytosis of non-specifically opsonised cells of *M. pneumoniae*, the main mycoplasma pathogen in man (table). For pathogens such as *M. hominis*^{51, 52, 59} and *M. fermentans* strain incognitus,⁵⁹ data indicate survival following phagocytosis in the presence of non-specific opsonins; however, such mycoplasmas may be exceptional.⁶⁰

Most *in-vitro* studies have concentrated on specifically opsonised mycoplasmas (table). Opsonisation with specific antibody, either with or without complement is followed usually by rapid phagocytosis and

intracellular killing,^{16, 27, 50, 51, 61-65} although not always.^{50, 52-54} The increased efficiency of phagocytic uptake and killing results from the specific recognition of the Fc portion of mycoplasma-bound IgG or IgA by receptors on the phagocyte surface. Provided that it is of the right idiotype-isotype, antibody can act as an effective opsonin and agglutinin as well as directly neutralising mycoplasmas. Those cases in which antibodies have been ineffective in promoting phagocytosis probably reflect the fact that they were inappropriate or non-opsonic.

In-vitro methods for studying the mycoplasma-phagocyte interaction

Several approaches have been adopted in the search for a reliable in-vitro assay to assess the rate and extent of mycoplasma ingestion and killing by phagocytes, these include phase-contrast microscopy,^{56, 62, 66, 67} electronmicroscopy (EM)^{51, 55, 57, 58, 61-63, 66, 68-70} autoradiography,^{62, 66} uptake of radiolabelled mycoplasmas,^{63, 66} inhibition of ³H-uridine uptake by macrophages,⁶¹ stimulation of 1-[¹⁴C]-glucose metabolism by phagocytes,^{52, 71} immunofluorescence staining and flow cytometry,^{72, 73} spectrophotometry,^{69, 73} trypan blue exclusion,⁷⁴ enzyme-linked immunosorbent assay (ELISA),⁶³ titration of viable organisms,^{16, 26, 27, 50-55, 66, 75, 76} and luminol-dependent chemiluminescence (CL).^{50, 51, 72, 77-79}

Techniques such as microscopy, autoradiography, uptake of radiolabelled organisms, immunofluorescence and ELISA assess the topological relationship between mycoplasmas and phagocytes. Others, such as inhibition of ³H-uridine uptake, [¹⁴C]-glucose breakdown, spectrophotometric measurement of enzyme activity and luminol-dependent CL, analyse the metabolic response of phagocytes to mycoplasmas. Phagocyte viability can be measured by trypan blue exclusion assays, whereas viable counts determine extracellular and intracellular survival of mycoplasma.

Technical difficulties

In-vitro studies of the phagocytosis of mycoplasmas are hampered by factors such as bacterial aggregation,^{55, 75} the tendency of mycoplasmas to adhere spontaneously to phagocytes,⁶¹ the lack of distinguishing morphological or staining properties for mycoplasmas,⁶¹ the difficulty in differentiation of cell-adherent and ingested organisms,⁵⁰ mycoplasma susceptibility to lysis⁶¹ and discrepancies between bacterial cells grown *in vitro* or *in vivo*.²⁶

Ultrastructural studies (e.g., autoradiography and EM) reveal the intracellular or extracellular location of mycoplasmas. The presence of intact organisms in phagolysosomes does not, however, confirm their viability. A major problem in EM experiments is the difficulty of interpreting electronmicrographs because of the similarity between ingested mycoplasmas and

PMNL intracellular organelles. Gold-antibody labelling helps to resolve this; Lo *et al.*^{80, 81} successfully demonstrated *M. fermentans* strain incognitus in diseased tissues by immunochemical staining and EM.

CL and ¹⁴CO₂ production studies allow measurement of the phagocyte respiratory burst elicited by mycoplasmas, but this is not necessarily an indication that effective phagocytosis and intracellular killing has occurred. Both ¹⁴CO₂ production from glucose-1-[¹⁴C] and luminol-dependent CL emission indicate that *M. hominis* elicits a sizeable respiratory burst from PMNLs yet viable count studies show killing is relatively inefficient.⁵⁰⁻⁵²

Viable count studies are potentially of great value as they are the only direct means of measuring the mycoplasmacidal activity of phagocytes. Viable mycoplasma counts before and after low-speed centrifugation of the phagocyte-mycoplasma suspension enable the number of mycoplasmas associated with the phagocyte to be ascertained but do not differentiate between adherent and intracellular mycoplasmas. Parkinson and Carter⁵⁵ claim that viability studies are unsuitable for assessing the attachment and ingestion of mycoplasmas by phagocytes because of mycoplasma aggregation or disaggregation, transient attachment to the phagocyte and leucocytic sedimentation during the assay.

To count live, intracellular mycoplasmas, phagocytes must be lysed by a technique that does not affect mycoplasma viability. Ice-cold distilled water⁵⁰ and gentle sonication³⁵ have both been used successfully; sonication has the added benefit of dispersing mycoplasma aggregates, a source of error in viable-count assays.⁸²

Can in-vitro assays be related to in-vivo immune responses?

The applicability of in-vitro assays of phagocytosis in in-vivo cellular immune responses has been questioned.^{15, 26} Discrepancies in phagocytosis of in-vitro and in-vivo cultured mycoplasmas have been reported.^{27, 75} Standardisation of the mycoplasma culture is fundamental to attaining reproducible results. Culture age affects the outcome of the interaction with the phagocytic cell,⁵⁹ probably as a result of changes in mycoplasma affinity for PMNL binding sites and variations in production of metabolites toxic to PMNL during their life cycle. For example, attachment of encapsulated mycoplasmas to phagocytes is likely to vary with capsular size, which depends upon culture age^{83, 84} and growth rate.⁸⁵ Qualitative or quantitative changes may occur also in the expression of mycoplasma surface antigens *in vivo*. Alternatively, host material or non-opsonic antibody may become bound to mycoplasmas and subsequently inhibit the attachment of opsonic antibody or complement.²⁷

Howard and Taylor²⁶ demonstrated that in-vitro phagocytosis studies could not differentiate avirulent and virulent mycoplasma strains, as defined *in vivo*.

This probably reflects the dependence of virulence on a variety of factors, e.g., attachment, antibiotic resistance, production of toxic metabolites etc., rather than on one single determinant.⁸⁶ Krausse *et al.*^{50, 78} showed that poor stimulation of PMNL CL was associated with increased virulence of clinical isolates of *U. urealyticum* and a reference strain of *M. pneumoniae*. However, they could not correlate PMNL CL stimulation with virulence for *M. hominis*.⁵⁰

Studies of the phagocytosis of mycoplasmas attached to host cells rather than in suspension may resemble more closely the in-vivo relationship between phagocytes and mycoplasma.²¹ For other bacteria, CL responses of PMNL depend on whether organisms are adherent to glass or are in suspension.⁸⁷ A further problem of in-vitro studies is that PMNL from peripheral blood are not representative of the highly activated PMNL that marginate and migrate through tissues to foci of infection.⁸⁸

The method of opsonisation of mycoplasmas may affect the outcome of phagocytosis.⁷⁵ If mycoplasmas are treated with serum after attaching to phagocytes, some cells may be inaccessible to opsonins.⁷⁶ However, serum treatment before mixing with phagocytes results in aggregation, which affects attachment, ingestion and enumeration of mycoplasmas. Reports on other bacteria even suggest that pre-opsonisation may increase resistance to phagocytosis.⁸⁹ Variations in specific antibody titres and complement levels in serum may affect reproducibility. Contradictory data on the capacity of specific antisera to opsonise mycoplasmas effectively may have resulted from the use of non-homologous sera and cells,^{27, 53, 54, 61, 62, 71, 75} which can lead to erroneous conclusions about the role of cellular immune mechanisms in the natural host. Discrepancies between the opsonising potential of sera from different species may lie in the recognition of different mycoplasma antigens by the respective host immune systems. For example, antibody raised in different hosts may recognise different epitopes of the same *M. pneumoniae* P1 adhesin.⁸⁶ Specific immunoglobulin raised against different epitopes of mycoplasma antigens may also have varying opsonic capacity, and non-homologous anti-mycoplasma immunoglobulins may bind less efficiently to phagocyte Fc receptors due to differences in IgG sub-class distribution.^{22, 75}

Other methodological factors that affect the reproducibility of in-vitro assays include presence of medium components,^{51, 61, 64, 75} variation (or total disregard) in phagocyte-mycoplasma ratios and cell concentrations,^{51, 57, 58, 77, 90} incubation times of reaction mixtures,^{51, 56, 66} impurities of phagocyte preparations^{54, 55, 75} and opsonisation conditions.

Mycoplasma and phagocyte function (figure)

Attachment

Mycoplasmas attach to the surface membranes of competent phagocytes and often appear to prolifer-

ate.^{27, 50, 53, 55, 76} Jones and co-workers^{66, 67} found that *M. pulmonis* multiplied after attachment to the plasma membrane of peritoneal macrophages. Mycoplasmas may withdraw nutrients or gain protection from the action of complement and other serum components by this close association. Failure of IgG to enhance phagocytosis of *M. pulmonis* has been attributed to the inaccessibility of mycoplasmas within large accumulations of PMNL exudate, and to the concealment of mycoplasmas in the cytoplasmic processes and microvilli of infected cells.^{53, 76} The ability of certain mycoplasmas to bind host immunoglobulins non-immunologically through the expression of receptors for the Fc moiety^{91, 92} might also impair phagocytosis. EM shows an intimate association between the plasma membrane of the mycoplasma and that of the phagocyte,⁶⁶ with a gap of only c. 5–10 nm between the juxtapositioned membranes.⁹³ Radiolabelling, flow cytometry-immunofluorescence and ELISA studies have provided a wealth of information concerning the ability of mycoplasma cells or antigen to bind the surfaces of both professional and non-professional phagocytes.^{52, 61, 63, 66, 72} Unfortunately, relatively little is understood of the mechanisms of attachment or the mycoplasma surface moieties that determine the outcome of this interaction.⁶⁷

Hydrophobicity is important in determining the outcome of phagocytosis,⁹⁴ bacteria of high hydrophobicity tend to be engulfed more rapidly than those of low hydrophobicity. This phenomenon may be of particular importance to *Mycoplasma* spp. as their membranes contain large amounts of cholesterol, a highly hydrophobic molecule.⁹⁵ Furthermore, cholesterol is abundant in bronchial secretions⁹⁶ and is bound avidly by mycoplasmas;⁹⁷ the capacity of a mycoplasma to assimilate cholesterol from its environment may thus affect interaction with phagocytes.

Adherence of mycoplasmas to the cell surface probably involves lipoglycans,⁹⁸ which have been found in all species of *Acholeplasma*, *Anaeroplasma* and *Spiroplasma* studied, and in certain species of *Mycoplasma*. Lipoglycans exhibit both mitogenic and endotoxic properties, and they are thought to be similar in function to bacterial lipopolysaccharides.⁹⁸ At present, PMNL priming by lipoglycans has not been demonstrated; however, mycoplasma-derived high mol. wt material (MDHM) from *M. fermentans* has been shown to activate murine macrophages;⁹⁹ and MDHM, *M. arginini* TUH-14 membrane lipoproteins, as well as *M. pneumoniae* and *M. pulmonis* membranes are known to induce pro-inflammatory cytokines (i.e., TNF- α , IL-1 β , IL-6 and IFN- γ);^{100–107} thus, it seems likely that priming of PMNL or macrophages, or both, does take place.

M. pneumoniae and *M. genitalium* mediate attachment to epithelial cells *via* adhesin proteins,¹⁰⁸ and sialoglyco-conjugated proteins and lipids are important in the interaction of other mycoplasmas with

Figure. The stages of phagocytosis that might be circumvented by mycoplasmas. (a) Attachment to and phagocytosis of mycoplasmas may be inhibited by filamentous morphology, presence of antiphagocytic proteins or capsular material, binding of Fc portion of immunoglobulin, or localisation of mycoplasmas between cilia and cytoplasmic processes making them inaccessible to phagocytes. (b) Integrity of the phagosome membrane, phagocyte metabolism and activation of the respiratory burst may be affected by mycoplasma proteases, lipases and phospholipases and by oxygen radicals produced by mycoplasmas. (c) Phagosome-lysosome fusion may be impaired by the production of ammonia by mycoplasmas. (d) Mycoplasmas may survive phagocytosis and escape into the cytoplasm (mechanism unknown). (e) Mycoplasma survival following phagosome-lysosome fusion is unlikely.

eukaryotic cells.^{109,110} Glycosidases alter surface properties of mycoplasmas,⁹¹ but the effect of these enzymes on the attachment and phagocytosis of mycoplasmas has not been investigated.

Attachment of mycoplasmas to the phagocyte membrane may affect the phagocytosis of other bacteria. Phagocytosis and killing of *Escherichia coli* by PMNL are impaired by the presence of *M. hominis*,^{52,64} *M. arthritidis*,⁶⁴ *M. dispar*¹⁶ or *M. bovis*,¹⁶ adherence of *M. bovis* to bovine PMNL suppresses CL responses to zymosan and phorbol myristate acetate (PMA).⁷² The importance of mycoplasma viability in the suppression of phagocyte function is disputed, but this may reflect methodological differences.^{16,72} Adherence of mycoplasmas to the PMNL surface may result in interference with the exposed functional domain of the NADPH oxidase complex.⁷² Alternatively, mycoplasma attachment may prevent binding of other bacteria by the phagocyte, although adherence of *E. coli* to phagocytes is unaffected by pre-incubation of phagocytes with *M. bovis* at concentrations that inhibit bacterial killing.¹⁶ Flow cytometric studies may help to determine whether pre-incubation of PMNL with mycoplasmas interferes with the adherence of particulate targets or their subsequent ingestion, or both.⁷²

Phagocytosis

Bar-shavit *et al.*¹¹¹ found filaments of *E. coli* to be internalised less efficiently than coccoid bacteria by phagocytic cells, although this was overcome by opsonisation. Similar observations have been made

with mycoplasmas; exponential phase *M. mycoides* subsp. *capri* cells (predominantly filamentous), elicit reduced CL responses from PMNL when compared with younger or older cells (predominantly non-filamentous);⁵⁹ treatment of exponential-phase mycoplasmas with non-specific opsonins reverses these differences. Perhaps filamentous mycoplasmas have a non-homogeneous distribution of binding proteins that, in the absence of opsonins, prevents efficient interaction with the phagocyte surface and the circumferential interaction required for the engulfment stage.^{112,113}

Studies with trypsin suggest the presence of an anti-phagocytic surface protein on *M. pulmonis*.^{67,69} Following treatment with trypsin (100 µg/ml) the organism was more susceptible to phagocytosis by murine macrophages when observed under phase contrast microscopy^{67,114} or by scanning EM.⁶⁹

Many mycoplasmas possess a capsule, as determined by ruthenium red staining or EM,¹¹⁴ and several reports suggest their importance in resistance to phagocytosis. Capsules reduce mycoplasma cell surface hydrophobicity, increasing resistance to phagocytosis. Recent studies have demonstrated that bovine lung fibroblasts induce proliferation of the galactan that encapsulates *M. dispar*,^{115,116} this results in inhibition of the bactericidal and secretory functions of bovine alveolar macrophages.¹¹⁵ Both capsulate organisms and purified capsule preparations suppress tumour necrosis factor α (TNF- α) production, IL-1 production and glucose consumption by macrophages.¹¹⁵

The *M. mycoides* subsp. *mycoides* galactan capsule may also stimulate TNF- α , as large colony strains of this species induce TNF- α production in infected goats.¹¹⁵ Non-capsulate *M. mycoides* cells are more susceptible to non-specific opsonisation⁵⁹ probably as a result of decreased complement deposition. However, the capacity of capsulate *M. mycoides* strains to impair binding of opsonins to the cell surface does not appear to be shared by many other *Mycoplasma* species. Most mycoplasmas both bind antibody rapidly and activate complement by either the alternative^{56, 117, 118} or the classical¹¹⁸⁻¹²⁷ pathways, as well as interacting with the C1 component directly.^{51, 128}

PMNL membrane damage

Mycoplasma-mediated damage to the phagocyte membrane may occur in several ways. Proteases associated with the mycoplasma membrane¹²⁹ may damage phagocyte membrane integrity. The action of mycoplasma lipases or phospholipases^{130, 131} on the cell surface may reduce phagocytic function either by generating lipid hydrolysis products,⁷² such as sphingosines (potent inhibitors of NADPH oxidase activity¹³²), or by altering membrane fluidity,¹³³ one of several factors important in the activation of the PMNL respiratory burst.¹³³

Mycoplasma oxygen radical production, by damaging the phagocyte membrane, may act as a pathogenicity factor.¹³⁴⁻¹³⁸ Generation of these oxygen metabolites in close proximity to the PMNL surface may be important in allowing oxidation of PMNL membrane lipid components,¹³⁶ thereby affecting membrane fluidity and resulting in the formation of cytotoxic metabolites, such as malonyldialdehyde.¹³⁴ Membrane lipid peroxidation *via* arachidonic acid is also the first stage in a whole spectrum of PMNL metabolic pathways which result in the production of leukotrienes and prostaglandins, two important groups of cytokines involved in inflammatory and cellular immune responses. Mycoplasma radical production may disrupt these and other metabolic pathways, such as the glutathione redox cycle,^{136, 139} glutathione synthesis is coupled with respiratory burst activity in PMNL¹⁴⁰ and glutathione depletion as a result of mycoplasma oxidative attack might affect PMNL bactericidal capacity. Mycoplasma oxygen radical generation may result in premature inactivation of the PMNL NADPH oxidase, which is sensitive to oxidation by superoxide ion (O_2^-).¹⁴¹ Phorbol myristate acetate (PMA) by-passes receptor-mediated transmembrane signalling, acting directly on protein kinase C; the inhibition of PMA-induced CL by *M. bovis* implies that interference with NADPH oxidase activation must occur at the level of protein kinase C activation or later in the pathway.⁷²

M. fermentans strain incognitus has been shown to damage PMNL (and lymphocyte) membranes, as measured by the trypan blue method.⁷⁴ However, in this case, damage to the phagocyte membrane is

probably not attributable to autonomous production of oxygen radical, as *M. fermentans* strain incognitus induces only a relatively weak CL response from PMNL. In contrast, *M. fermentans* PG18 and *M. felis* elicit substantial PMNL CL responses, but only mildly affect PMNL membrane integrity.

Intracellular survival

Evidence for the survival of mycoplasmas within phagocytes is contradictory; this may reflect the fact that survival depends on the mechanisms by which mycoplasmas are phagocytosed. Antibody-mediated phagocytosis results almost invariably in ingestion and killing of *Mycoplasma* spp. by phagocytic cells.^{51, 63} In contrast, complement-mediated phagocytosis results in less effective killing of mycoplasmas.^{50-52, 59} In-vivo studies have demonstrated intracellular mycoplasma-like inclusions in mononuclear interstitial cells which stained positive for *M. fermentans* strain incognitus antigens.² Lo *et al.*¹⁴² identified *M. fermentans* strain incognitus in various cell and tissue types, including peripheral blood monocytes, by DNA hybridisation. The mechanism of entry to the PMNL cytoplasm is unknown, but may involve complement receptors; cellular uptake *via* CR3 avoids the triggering of some PMNL microbicidal mechanisms and is a strategy employed by a number of intracellular pathogens.¹⁴³

If mycoplasmas can exist intracellularly within phagocytes, how do they escape phagocyte bactericidal mechanisms? Resistance of mycoplasmas to oxygen radicals and chloramines generated by phagocytes during the respiratory burst is hard to explain, as these organisms are sensitive to the same oxygen radicals generated in cell-free systems.^{144, 145} The close proximity of mycoplasmas to the phagocyte membrane may be in some way protective.⁷⁶ Serum is an effective scavenger of H_2O_2 and binding of serum components, such as catalase, by mycoplasmas⁹⁷ might account for the resistance of serum-treated cells to phagocyte oxidative attack. Mycoplasmas themselves produce substantial amounts of active oxygen radicals and may thus possess efficient enzymic processes for their inactivation. However, reports of catalase and superoxide dismutase (SOD) activity in mycoplasmas are conflicting,^{68, 137, 146} and it seems unlikely they have sufficiently high levels of such enzymes to avoid damage by phagocyte oxygen radicals. Furthermore, levels of SOD and catalase production do not correlate with virulence or resistance to phagocytic killing for other bacteria.¹⁴⁷ Alternative mechanisms by which mycoplasmas may avoid phagocytic killing include inhibition of myeloperoxidase, and detoxification of oxidants *via* reducing agents secreted or exposed on the mycoplasma surface.

Resistance of mycoplasmas to non-oxidative microbicidal mechanisms of phagocytes is poorly understood. *M. pulmonis* elicits the release of hydrolytic enzymes from macrophages, suggesting that mycoplasmas are exposed to attack by serine proteases

and defensins.⁹⁰ Erb and Bredt⁷⁶ proposed that the high lipid content of mycoplasma membranes might protect against phagocyte intraphagosomal components but found no evidence for this. Lysosomal enzymes, such as lysozyme, would have little effect on mycoplasmas as they lack a cell envelope.⁵⁰ On the other hand, the lack of a cell wall predisposes mycoplasmas to direct attack by other lysosomal enzymes. The sensitivity of mycoplasmas to PMNL lysates and intracellular killing mechanisms of PMNL *in vitro* suggests that inherent resistance to non-oxidative killing is unlikely.^{52, 148, 149}

Ammonia may interfere with phagosome-lysosome fusion in PMNL^{150, 151} and its production by mycoplasmas has been proposed as a virulence factor.^{152, 153} Ammonia is formed by the hydrolysis of urea by ureaplasmas, and of L-arginine by many *Mycoplasma* spp. Furthermore, as these reactions are major or sole sources of energy generation in the species concerned, large quantities of substrate will be consumed during growth. Reduced availability of L-arginine can affect the metabolism of mammalian cells and, in particular, the biosynthesis¹⁵⁴ of nitric oxide. Macrophage-derived nitric oxide is involved in tumour cell cytostasis¹⁵⁵ and the killing or inhibition of growth of bacteria.¹⁵⁶ Thus, arginine-utilising mycoplasmas (e.g., *M. hominis* and *M. fermentans*) may disrupt phagocyte bactericidal capacity.

Conclusions

A number of conclusions can be drawn from the current extent of our knowledge of the phagocyte-mycoplasma interaction and its implications in the wider context of mycoplasma pathogenicity.

First, the presence of opsonins is important in determining the fate of the mycoplasma following interaction with the phagocyte. Opsonisation by specific antibody results almost invariably in the rapid phagocytosis and destruction of the mycoplasma. Further work is required to determine if this is a result of antibody neutralisation of mycoplasma anti-phagocytic mechanisms, increased aggregation of mycoplasmas, increased efficiency of Fc receptor-mediated phagocytosis, or a combination of factors. The outcome of complement-mediated phagocytosis is less clear; recent studies suggest that non-specifically opsonised cells are phagocytosed, but some may escape bactericidal mechanisms. The state of priming or activation of the phagocytic cell is likely to be important in determining the success of non-specific phagocytosis of mycoplasmas. Recent evidence suggests that the NADPH oxidase of activated cells produces enhanced amounts of H₂O₂ and superoxide ion.¹⁵⁷ In addition, the increased expression of membrane receptors and the enhanced efficiency of degranulation results in the more effective bacterial killing observed with activated phagocytes. The importance of priming on phagocyte function emphasises

the need to study the effect of *Mycoplasma* spp. on host cytokine production,^{102, 158} and in turn the effects of these cytokines on phagocyte function.^{115, 116}

Second, the type and origin of the phagocyte encountered by a mycoplasma is important in determining its subsequent survival. Both PMNL and mononuclear phagocytes can phagocytose mycoplasmas effectively in the presence of antibody, providing it is of the right idiotype-isotype. However, they may differ in their ability to kill mycoplasmas in the absence of antibody. Furthermore, NK cells, rather than PMNL or macrophages, have been reported to be responsible for mycoplasmacidal activity in the respiratory tract of mice.^{31, 46, 47} Future studies should investigate the role of different cell types in resistance to mycoplasma infection in the non-immune host.

Finally, the assumption that mycoplasmas are phagocytosed in the host only after an antibody response must be re-evaluated. Many investigators have disregarded the role of complement or other non-specific opsonins. Mycoplasma infection results in a massive increase in localised concentrations of complement components in bronchial secretions.¹⁵⁹ Increased vascular permeability during inflammation would be expected to elevate levels of complement further. In addition, a distinct functionally active leucocyte complement system exists on the surface of macrophages and lymphocytes,^{90, 160} and macrophages co-operate with PMNL in phagocytosis *in vitro* by providing opsonins.¹⁶¹ This evidence suggests strongly that mycoplasmas are exposed to complement, even at extracellular mucosal sites, in the non-immune host. Therefore, the use of unopsonised cells in in-vitro studies to represent the interaction of mycoplasmas with phagocytes in the non-immune host is inappropriate.

Recent studies highlight the susceptibility of patients with antibody deficiency to mycoplasma infection.^{51, 162} The emergence of mycoplasmas as opportunists in HIV infections,^{1, 5} where antibody levels are also depleted, is of particular note. The capacity of mycoplasmas to impair the phagocytosis of other bacteria *in vitro* emphasises their possible role as co-pathogens with chlamydiae, meningococci and HIV.

The ability of some pathogenic mycoplasmas to avoid killing in the presence of complement and PMNL is important to understanding the pathogenesis of mycoplasma infections. Enhanced PMNL oxidative activity in the presence of complement-opsonised mycoplasmas may result in alveolar damage¹⁶³ and may account for the necrotic nature of lesions associated with mycoplasma infection.²¹ Webster *et al.*⁵¹ even go so far as to suggest that phagocytosis of pathogenic mycoplasmas in the absence of antibody may be an important factor in the dissemination of mycoplasma disease.

The interaction between mycoplasmas, phagocytes and complement components, and their influence on recovery from mycoplasma disease are extremely

complex. Further study of the mechanisms involved in the mycoplasma-phagocyte interaction, and of the genetic basis for phagocytic competence against these bacteria, will enable us to understand more fully the

ways in which mycoplasmas cause disease in non-immune and immunocompromised hosts.

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