INTERACTION OF *MYCOPLASMA PULMONIS* WITH MOUSE PERITONEAL MACROPHAGES AND POLYMORPHONUCLEAR LEUCOCYTES

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MYCOPLASMA PULMONIS produces persistent respiratory disease and arthritis in mice (Barden and Tully, 1969; Lindsey and Cassell, 1973). A major histological feature of both diseases is the large number of polymorphonuclear (PMN) leucocytes and macrophages in the lesions at all stages (Lindsey and Cassell, 1973; Taylor, Taylor-Robinson and Keystone, 1978). Clearance of *M. pulmonis* from the alveoli coincides with the influx of phagocytic cells and development of specific antibody in respiratory secretions (Cassell, Lindsey and Baker, 1974). This observation and that of the presence of mycoplasmas within PMN leucocytes (Organick, Siegesmund and Lutsky, 1966) have led to the suggestion that immune phagocytosis may be important in resistance. However, after clearance from the alveoli, mycoplasmas remain localised on the bronchial epithelium where they persist despite the presence of phagocytic cells and specific antibody.

Resistance to *M. pulmonis*-induced arthritis and respiratory disease in mice can be transferred by convalescent serum (Cassell *et al.*, 1973; Taylor and Taylor-Robinson, 1976, 1977), and it has been suggested that the protective effect may be due, in part, to the ability of serum to promote phagocytosis of the mycoplasmas (Taylor and Taylor-Robinson, 1977). Resistance to *M. arthritidis*-induced arthritis in rats and mice can also be transferred to some extent by convalescent rat or mouse serum (Cole *et al.*, 1971; Cole and Ward, 1973*a*) but such sera did not promote in-vitro phagocytosis of *M. arthritidis* by rodent macrophages (Cole and Ward, 1973*b*). In contrast, specific antiserum produced in rabbits was very efficient at promoting phagocytosis of *M. arthritidis*. Although it is known that rabbit antiserum promotes the phagocytosis of *M. pulmonis* by mouse macrophages *in vitro* (Jones and Hirsch, 1971), the opsonic capacity of convalescent mouse serum for *M. pulmonis* has not been examined.

The following investigations were therefore undertaken to examine the ability of convalescent mouse serum to promote phagocytosis of *M. pulmonis* by mouse peritoneal macrophages and polymorphs *in vitro*. In addition, factors were examined that may contribute to the persistence of *M. pulmonis*, despite the presence of large numbers of phagocytic cells and antibody at the sites of infection.

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MATERIALS AND METHODS

Mycoplasma strains and culture media. *M. pulmonis* strain JB was the same as that described by Taylor, Taylor-Robinson and Slavin (1974). The medium used for culture of the mycoplasmas was similar to that described by Gourlay and Leach (1970), with the exception that 10% (v/v) horse serum inactivated at 56°C for 30 min. was used instead of foetal calf serum (FCS). Solid medium used for enumeration of mycoplasmas was the same as the liquid medium but contained 0.5% (w/v) agarose (Miles Lab. Inc., Elkhart, Ind. USA).

Collection and maintenance of cells. Medium 199 (Wellcome Research Laboratories, Beckenham, Kent) containing NaHCO_3 0.11% (w/v), ampicillin (Beecham Ltd) 0.1% (w/v) and FCS 10% (v/v) heated at 56°C for 30 min. was used to maintain cell cultures.

Macrophage cultures were prepared from male CBA mice obtained from the Specific-pathogen-free Unit at IRAD, Compton, Berks (MRC Laboratory Animal Centre's category-4 standard). Mice were killed with ether 72 h after intraperitoneal (i.p.) inoculation of 0.5 ml of 2% (w/v) sodium alginate (British Drug Houses) and the peritoneal cavity was washed with 5 ml of medium 199 containing heparin 10 units/ml at 4°C. Peritoneal washings from the mice were pooled and the cell count adjusted to 2×10^6 cells/ml with medium 199 + FCS; 2-ml amounts of the cell suspension were distributed to plastic petri-dishes (30 mm diameter). After incubation for 1 h at 37°C in 5% (v/v) CO_2 in air, the petri dishes were washed at least three times with 0.15M phosphate-buffered saline (PBS), pH 7.2, to remove non-adherent cells, and 2 ml of fresh medium 199 containing 10% FCS was added to each plate. The interaction of macrophages with *M. pulmonis* was examined after overnight incubation at 37°C. At this time more than 95% of cells were phagocytic as judged by their ability to ingest 1.0- μ latex beads.

Cultures of PMN leucocytes were prepared from CBA mice, killed 3 h after i.p. inoculation with alginate, as described above for macrophages, with the exception that peritoneal washings were collected at room temperature. The cell suspension was centrifuged at 100 g for 10 min. at room temperature and resuspended in medium 199 containing heparin to give 2×10^6 cells/ml. The interaction of PMN leucocytes with *M. pulmonis* was examined immediately.

As a control, mouse L-cells were used in tests for the interaction of *M. pulmonis* with non-phagocytic cells. The L-cells were also maintained in medium 199 + FCS.

Interaction of mycoplasmas with cells. Macrophage cultures and L-cells were washed twice with PBS and 4×10^6 c.f.u. of *M. pulmonis* in 1 ml were added to each plate. The cultures were incubated for 1 h at 37°C, washed twice with PBS to remove non-adherent mycoplasmas, and overlaid with 2 ml of medium 199 + FCS containing various mouse or rabbit sera. The cultures were incubated at 37°C and the numbers of mycoplasmas in the medium and the numbers associated with the cells were determined at 0, 3 or 4 and 24 h of incubation as described previously (Howard *et al.*, 1976).

The PMN-cell suspension containing approximately 2×10^8 cells was mixed with 2×10^8 c.f.u. of *M. pulmonis* and distributed to 30-mm plastic petri dishes in 2-ml amounts. After incubation for 30 min. at room temperature, the petri dishes were washed gently with PBS. The cells in Giemsa-stained preparations were > 75% PMN leucocytes. The PMN-cell cultures were overlaid with 2 ml of medium 199 + FCS containing various mouse or rabbit sera. The plates were incubated at 37°C in 5% CO_2 in air and examined after 0, 1½, and 3-h incubation for the numbers of mycoplasmas in the medium and the numbers associated with the cells as described above.

Cell-associated and supernatant counts at each time point were obtained from the geometric means of three cell cultures. All experiments were repeated at least once and because the results were essentially the same, the findings from only one of the duplicate experiments are reported.

Antisera. Rabbit antiserum was prepared as described by Howard and Gourlay (1972); the animal was hyperimmunised with *M. pulmonis* strain JB grown in rabbit digest broth containing 10% (v/v) heated normal rabbit serum. Mouse antisera to *M. pulmonis* were prepared from the pooled blood of 60 CBA mice that had been inoculated intravenously (i.v.) 6 weeks previously with 2×10^6 c.f.u. of *M. pulmonis* strain JB. The antibody titres to *M. pulmonis* in the various sera were determined by a modification of the single radial-haemolysis test (Howard, Collins and Gourlay, 1977) in which *M. pulmonis* was coupled to sheep red blood cells, by complement

TABLE I
Antibody to Mycoplasma pulmonis in rabbit and mouse sera

Sera	Area of SRH zone (mm ²)	CF titre	MI titre
Rabbit antiserum	125.7	NT	> 1024
Normal rabbit serum	0	NT	< 2
Convalescent mouse serum, batch 1	71.5	> 5120	< 2
Convalescent mouse serum, batch 2	51.0	2560	< 2
Normal mouse serum	0	< 10	< 2

SRH=Single radial haemolysis; CF=complement fixation; MI=metabolism inhibition; NT=not tested.

fixation (Taylor, Howard and Gourlay, 1977) and by metabolism inhibition (Taylor-Robinson *et al.*, 1966) and are shown in table I.

In the experiments described below, all sera were held at 56°C for 30 min. Rabbit sera were used at a final dilution of 1 in 500 and mouse sera at a final dilution of 1 in 50. Convalescent mouse serum batch 1 was used for all macrophage experiments and convalescent mouse serum batch 2 for all PMN-leucocyte experiments.

Cytochalasin B. The inhibitor of phagocytosis, cytochalasin B (ICI Pharmaceuticals, Macclesfield, Cheshire) was dissolved in dimethyl sulphoxide (Me₂SO) and added to medium 199 + FCS to give a final concentration of 10 µg/ml.

Preparation of M. pulmonis grown in vivo. Six to eight weeks after intranasal (i.n.) inoculation of mice with 5×10^5 c.f.u. of *M. pulmonis*, the animals were anaesthetised with sodium pentobarbitone (Sagatal, May and Baker Ltd), killed by exsanguination, and the lungs were irrigated with 1 ml of medium 199 + FCS (Taylor-Robinson *et al.*, 1972). Lung washings obtained in this way were pooled, centrifuged at 200 *g* for 10 min. to sediment cells and the supernatant was stored at -70°C. After thawing, the number of viable *M. pulmonis* in lung washings was determined by titration on solid media.

Statistical analysis of results. Because the number of in-vitro- and in-vivo-grown mycoplasmas attached to macrophages at 0 h was not the same, the data were adjusted to the 0-h values of the in-vitro-grown group. An analysis of variance was made on these adjusted data to compare differences in the numbers of in-vitro- and in-vivo-grown organisms killed by the macrophage cultures.

RESULTS

Effect of sera on the viability of M. pulmonis

Several dilutions of mouse and rabbit sera were examined for their effect on the viability of *M. pulmonis* suspended in medium 199 + FCS. Dilutions of sera that did not produce a decrease in mycoplasma viability were chosen for tests of the interaction of *M. pulmonis* with phagocytic cells. Thus, rabbit sera were used at a dilution of 1 in 500 and mouse sera at a dilution of 1 in 50. As shown in fig. 1, sera at these dilutions did not produce a significant decrease in numbers of mycoplasmas after 24-h incubation; in fact the numbers increased slightly except in cultures containing rabbit antiserum and convalescent mouse serum where they remained constant. Similar results were obtained when 1 in

FIG. 1.—Effect of rabbit and mouse sera on the survival of *M. pulmonis* in medium 199 + foetal calf serum. Medium contained, no additional sera (X), normal rabbit serum (Δ), rabbit anti-*M. pulmonis* serum (▲), normal mouse serum (○), convalescent mouse serum (●).

50 mouse sera and 1 in 500 rabbit sera were added to cultures of L-cells infected with *M. pulmonis*.

Interaction of M. pulmonis with peritoneal macrophages

There was no significant decrease in the numbers of mycoplasmas associated with cells or in the medium in macrophage cultures without added sera after 4-h incubation. After 24 h there was a marked increase in the numbers of mycoplasmas associated with the cells and in the culture medium (fig. 2). The numbers of *M. pulmonis* in macrophage cultures containing normal mouse or normal rabbit sera were not significantly different from those without sera after 4-h incubation. However, after 24 h there were more organisms in supernatant fluids from cultures containing normal mouse serum than in cultures without serum or with normal rabbit serum (fig. 2). In contrast, there was a marked decrease in the numbers of mycoplasmas in cultures containing convalescent mouse sera or rabbit antisera after 4-h incubation. However after 24 h, the number of mycoplasmas increased in macrophage cultures containing convalescent mouse sera, whereas the number of organisms in cultures containing rabbit antiserum continued to decrease (fig. 2).

Mycoplasmas inoculated into mice to produce convalescent mouse serum, and those used to infect macrophage cultures were grown in medium containing horse serum. Therefore, the killing of *M. pulmonis* by macrophages at 4 h may have been promoted by antibody directed against components of the medium and not the mycoplasma itself. To investigate this possibility mycoplasmas, grown in medium containing either 10% (v/v) normal CBA mouse

FIG. 2.—Effect of rabbit and mouse sera on the survival of *M. pulmonis* in cultures of mouse peritoneal macrophages. Symbols as in fig. 1; CA = cell-associated mycoplasmas; S = mycoplasmas in supernatant fluid.

serum or 10% (v/v) foetal calf serum, were used to infect macrophage cultures. The extent of antibody-mediated killing of mycoplasmas by macrophages was the same regardless of the source of serum used to supplement the mycoplasma medium, indicating that antibody to medium components was not involved.

Effect of cytochalasin B on the interaction of M. pulmonis with peritoneal macrophages. The effect of cytochalasin B on the decrease in numbers of *M. pulmonis* in macrophage cultures containing convalescent mouse serum was examined as described previously (Howard *et al.*, 1976). Neither Me₂SO nor cytochalasin B alone appeared to affect the viability of *M. pulmonis* and there was no significant killing of mycoplasmas in cultures containing normal mouse serum and cytochalasin B in Me₂SO. As seen previously there was a 76%

reduction in the numbers of organisms in cultures containing convalescent mouse sera after 3-h incubation, whereas there was a 58% reduction in similar cultures containing cytochalasin B in Me₂SO ($p < 0.01$, Student's *t*-test). However, the numbers of mycoplasmas in cytochalasin B-treated cultures were also significantly different from those to which normal mouse serum had been added ($p < 0.001$). Thus, cytochalasin B had not completely inhibited killing of mycoplasmas by macrophage cultures containing convalescent sera. The effect of cytochalasin B on the phagocytosis of yeast cells was also examined (Stewart, Lin and Adles, 1975). As determined by phase-contrast microscopy, 73% of mouse peritoneal macrophages had phagocytosed yeast cells after incubation for 30 min. at 37°C, whereas 46% of macrophages to which 10 µg/ml of cytochalasin B in Me₂SO had been added contained yeast cells.

Interaction of M. pulmonis with peritoneal macrophages obtained from infected mice. Simberkoff and Elsbach (1971) have suggested that mycoplasmas may interfere with phagocytosis; this could result in failure of eradication of organisms from sites of infection (Cassell *et al.*, 1978). To investigate this, we compared the ability of peritoneal macrophages obtained from previously infected mice to kill *M. pulmonis*, with that of macrophages obtained from uninfected animals. Mice were given i.v. inoculation of either 2×10^6 c.f.u. of *M. pulmonis* or 0.2 ml of mycoplasma medium and 6 weeks later alginate solution was inoculated intraperitoneally. Macrophages were obtained from the peritoneal cavity of these mice 72 h after inoculation with alginate. Cultures of these cells were inoculated with mycoplasmas and incubated in the presence of either normal or convalescent mouse serum. There were no significant differences in the inactivation of *M. pulmonis* by cells obtained from either group of animals.

Interaction of M. pulmonis grown in vivo with peritoneal macrophages. Cultures of peritoneal macrophages were infected with approx. 1×10^6 c.f.u. of either *M. pulmonis* grown *in vitro* or mycoplasmas grown *in vivo*. Three experiments were done with organisms obtained from three different groups of mice. In all three experiments, the attachment of in-vitro-grown organisms to macrophages was 6–7-fold greater than that of mycoplasmas grown *in vivo*. The results of one of these experiments is shown in fig. 3. As seen in previous experiments, after 3-h incubation there was a decrease in the numbers of organisms in macrophage cultures containing convalescent mouse sera. However, the decrease in numbers of in-vivo-grown organisms was not as great as that observed for *M. pulmonis* grown *in vitro*. A comparison of the antibody-dependent killing of in-vitro- and in-vivo-grown mycoplasmas by macrophages is shown in table II. There was a significant difference between the ability of macrophages to kill mycoplasmas that had been grown *in vitro* and those that had been grown *in vivo*. To determine whether this was due to differences in the numbers of mycoplasmas attached to the macrophages at the start of the experiment, cultures were inoculated with a relatively small number of in-vitro-grown organisms. Although there were only $10^{3.8}$ c.f.u. of *M. pulmonis* associated with macrophages at the start of the experiment there was an 84% decrease of viable organisms in cultures containing convalescent

FIG. 3.—Effect of mouse sera on the survival of *M. pulmonis* grown *in vitro* (a), and *in vivo* (b), in cultures of mouse peritoneal macrophages. Symbols as in fig. 1; CA = cell-associated mycoplasmas; S = mycoplasmas in supernatant fluid.

TABLE II

Comparison of the antibody-dependent killing of M. pulmonis grown in vitro and in vivo by macrophage cultures

Experiment no.	Decrease* in numbers of mycoplasmas grown		Standard error	p†
	<i>in vitro</i>	<i>in vivo</i>		
1	0.59	0.24	0.14	<0.05
2	0.42	0.03	0.04	<0.01
3	0.58	0.20	0.27	<0.05

* Log₁₀ c.f.u. in cultures containing normal mouse serum after 3-h incubation — log₁₀ c.f.u. in cultures containing convalescent serum after 3 h.

† Probability that the number of in-vitro-grown organisms killed is significantly different from the number of in-vivo-grown organisms killed.

FIG. 4.—Effect of rabbit and mouse sera on the survival of *M. pulmonis* in cultures of mouse peritoneal polymorphonuclear leucocytes. Symbols as in fig. 1; CA = cell-associated mycoplasmas; S = mycoplasmas in supernatant fluid.

mouse serum, which was approximately the same as that seen in cultures infected with much larger numbers of organisms.

*Interaction of *M. pulmonis* with peritoneal polymorphs*

There were no significant differences in the numbers of *M. pulmonis* in

cultures of polymorphs without any sera or with normal rabbit or normal mouse sera after 3-h incubation. In contrast, there was a marked reduction in the numbers of mycoplasmas in cultures to which convalescent mouse serum had been added (fig. 4). Similar results were also obtained when rabbit serum was added to polymorph cultures, although the decrease in numbers of mycoplasmas in cultures containing 1 in 500 rabbit anti-*M. pulmonis* serum was greater than that observed in cultures containing 1 in 50 convalescent mouse serum.

DISCUSSION

Mouse peritoneal macrophages and polymorphs cultured *in vitro* did not appear to kill *M. pulmonis* unless specific antibody, from either rabbit or mouse, was present. Cytochalasin B inhibits phagocytosis by polymorphs and macrophages (Davies, Estensen and Quie, 1971; Klaus, 1973) but does not affect macrophage cytotoxicity (Temple *et al.*, 1973). Because cytochalasin B caused only partial inhibition of the antibody-dependent killing of *M. pulmonis* by macrophages, it may be that mechanisms other than phagocytosis are involved. For example, Taylor-Robinson *et al.* (1978) have shown that *M. pulmonis* induces the release of hydrolytic enzymes from macrophages, which could be involved in the killing of *M. pulmonis*. However, we observed killing of mycoplasmas in macrophage cultures after 4-h incubation, which is several hours before hydrolytic enzymes were detected in culture fluids by Taylor-Robinson *et al.* (1978). Furthermore, because phagocytosis of baker's yeast by macrophages was also only partially inhibited by cytochalasin B, it is possible that under these experimental conditions cytochalasin B did not completely inhibit phagocytosis. During a 3-h incubation period, killing of *M. pulmonis* appeared to be more efficient in polymorph cultures than in macrophage cultures when both were supplemented with the same rabbit antiserum. A similar effect was observed with convalescent mouse serum even though the serum used to supplement polymorph cultures had an antibody titre lower than that used in macrophage experiments. Moreover, *M. dispar* appeared to be killed more efficiently in bovine polymorph cultures than in cultures of bovine macrophages, both supplemented with the same bovine antiserum (Howard *et al.*, 1976).

Although convalescent mouse serum diluted 1 in 50 was opsonic its ability to promote phagocytosis was not as great as that of rabbit antiserum diluted 1 in 500. Furthermore, killing of *M. pulmonis* in macrophage cultures supplemented with convalescent mouse serum occurred only up to 4 h of incubation whereas the numbers of viable organisms in cultures containing rabbit antiserum were still decreasing after 24 h. The relatively poor opsonic ability of convalescent mouse serum may be due to the presence of high levels of IgG1-specific antibody (Cassell *et al.*, 1974; G. Taylor, unpublished observations). This class of immunoglobulin, which neither promotes phagocytosis nor fixes complement, could block the binding of opsonic IgG2 antibody to the mycoplasma surface and thereby protect the organisms from the host's defence

mechanisms. Other factors may also contribute to persistence of mycoplasmas at sites of infection. For example, Simberkoff and Elsbach (1971) demonstrated that mycoplasma infection of polymorphs *in vitro* impaired their ability to phagocytose *Escherichia coli*. If mycoplasma infection impaired phagocytosis *in vivo*, this could contribute to persistence of mycoplasmas. However, in the presence of specific antibody there were no differences between the killing of *M. pulmonis* by macrophages obtained from persistently infected mice and from uninfected animals, although cells obtained from the site of infection may behave differently from those induced in the peritoneal cavity. In addition, clearance of *Staphylococcus aureus* from the respiratory tract of mice infected with *M. pulmonis* was the same as that from uninfected animals, suggesting that the pulmonary phagocytic defences are not seriously impaired by mycoplasma infection (Howard, Stott and Taylor 1978).

Organisms may persist if they are able to avoid the host's defences in some way. In an attempt to investigate this possibility we compared the antibody-dependent killing by macrophages of *M. pulmonis* obtained from the respiratory tract of persistently-infected animals with that of mycoplasmas grown *in vitro*. There were differences in the proportion of organisms attaching to the macrophages, in that more in-vitro-grown mycoplasmas were associated with the macrophages after the attachment phase, than mycoplasmas grown *in vivo*. This difference may have been due to the presence of opsonic antibody coating the in-vivo-grown mycoplasmas so that organisms were taken up and killed by the macrophages during the attachment phase, although continued killing of the mycoplasmas on further incubation was not observed. A more likely explanation may be that attachment sites on the mycoplasma were blocked by host material. In all three experiments the killing of mycoplasmas grown *in vivo* by macrophages was significantly less than that of organisms grown *in vitro*; this difference did not appear to depend on the numbers of mycoplasmas attached to the macrophages at the start of the experiment. Possible explanations for these differences are (1) that the uptake of both mycoplasma preparations by macrophages is the same, but the intracellular killing of in-vivo-grown organisms is less efficient as seen for in-vivo-grown *Neisseria gonorrhoeae* in human phagocytes (Witt *et al.*, 1976); (2) that there may be changes in the antigenic structure of mycoplasmas replicating *in vivo*, or (3) that host material or non-opsonic antibody or both may be bound to the mycoplasma and inhibit the attachment of opsonic antibody to the organism. There is some evidence that mycoplasmas can selectively "acquire" host antigens, and these may hinder their recognition by the host's defence mechanisms (Wise, Cassell and Acton, 1978).

These studies indicate that convalescent mouse serum, which is known to be effective in passively immunising against respiratory disease and arthritis, promotes the in-vitro phagocytosis of *M. pulmonis* by mouse peritoneal macrophages and polymorphs for up to 3–4 h. Specific antibody of the immunoglobulin class known to promote phagocytosis has been demonstrated in respiratory-tract secretions (Cassell *et al.* 1974; G. Taylor and C. J. Howard, unpublished). However, despite the presence of such antibody and the large

numbers of phagocytic cells at sites of infection, *M. pulmonis* persists for long periods. The increased resistance to macrophage killing of *M. pulmonis* isolated from persistently infected mice compared with that of organisms grown *in vitro*, and the relatively poor opsonic activity of convalescent mouse serum when compared with that of rabbit serum may contribute to the persistence of mycoplasmas at sites of infection.

SUMMARY

Mouse peritoneal macrophages and polymorphonuclear leucocytes were examined for their ability to kill *Mycoplasma pulmonis in vitro*. Killing of mycoplasmas was shown to require specific antibody, suggesting that antibody-dependent phagocytosis may be involved in resistance to infection. Convalescent mouse serum appeared to be less effective than rabbit antiserum in promoting the killing of mycoplasmas by phagocytic cells. Moreover, mycoplasmas recovered directly from the respiratory tract of infected mice were more resistant to killing by macrophage cultures than organisms grown *in vitro*. The possibility that these observations may contribute to an understanding of the persistent nature of *M. pulmonis* infections in mice is discussed.

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