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Interaction of Mycoplasmas and Phagocytes

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Aspects of the interaction of certain mycoplasmas with macrophages and neutrophils *in vivo* and *in vitro* have been studied using two systems, one involving *M. pulmonis* in mice and the other involving *M. bovis* with bovine leucocytes.

Studies with *M. pulmonis* indicated that the disappearance of viable organisms from the peritoneal cavity was not enhanced in SPF mice in which a peritoneal exudate rich in neutrophils had been induced. However, viable *M. pulmonis* organisms disappeared more rapidly from the peritoneal cavities with exudates containing increased numbers of macrophages.

Experiments *in vitro* studied the opsonic effect of bovine IgG isotypes for bovine neutrophils and alveolar macrophages. Both IgG1 and IgG2 promoted killing of *M. bovis* by alveolar macrophages but IgG2 was more effective than IgG1 at promoting mycoplasma killing by neutrophils.

Further studies *in vitro* indicated that certain bovine mycoplasma could inhibit killing of *Escherichia coli* by bovine neutrophils.

INTRODUCTION

In the normal non-inflamed respiratory tract, the alveolar macrophage is considered to be the predominant phagocytic cell. Therefore these cells might be expected to be involved in controlling mycoplasma infections at this site. After an inflammatory stimulus, neutrophils migrate into the area attracted by chemotaxis and these cells could also be a major component of the cellular response of the host before antibody synthesis has commenced. Antibody, produced as a result of vaccination or infection, may enhance phagocytosis, and a large number of reports exist describing the opsonic effect of antibody for mycoplasmas. Many of these *in vitro* studies have not used mycoplasmas, leucocytes, and antisera from the natural host which, it can be argued, is essential when mechanisms of defense and potential importance *in vivo* is being studied. For example, some mycoplasma infections do not result in an antibody response that is opsonic, e.g., *Mycoplasma arthritidis* in mice and rats, yet rabbit antisera is opsonic for this mycoplasma [1].

Another aspect of mycoplasma-phagocyte interaction that should be considered concerns the inefficient phagocytosis of mycoplasmas by certain leucocytes and a possible inhibitory effect of mycoplasmas. The inhibition of phagocytosis of *Escherichia coli* by neutrophils due to *M. arthritidis* has been reported previously [2,3].

Thus, three aspects of the interaction of mycoplasmas with phagocytic cells are important: *(1)* the interaction of mycoplasmas with macrophages and neutrophils in the absence of specific antibody; *(2)* the interaction of mycoplasmas with

macrophages and neutrophils in the presence of specific antibody; *(3)* the effect of mycoplasmas on the ability of phagocytic cells to function. These three aspects of mycoplasma-phagocyte interactions were investigated by *(1)* studying the effect of inducing exudates, rich in either neutrophils or macrophages, on the survival of *M. pulmonis* in the peritoneum of SPF mice; *(2)* examining the ability of purified bovine IgG1 and IgG2 to promote the killing of *M. bovis* by bovine peripheral blood neutrophils and alveolar macrophages; *(3)* examining the effect of infection of bovine neutrophils with mycoplasmas on the phagocytosis of *E. coli.*

MATERIALS AND METHODS

Peritoneal Clearance

Specific pathogen-free (SPF) CBA mice were used, and disappearance of viable mycoplasmas from the peritoneum was measured as described previously [4]. To induce a peritoneal exudate rich in neutrophils mice were injected intraperitoneally (ip) with 0.25 ml of 2 percent weight per volume sodium alginate and used three hours later. These animals and control mice were inoculated ip with 0.25 ml of a suspension of *M. pulmonis* strains JB or Negroni (5×10^6 CFU ml^{-1}) and the number of viable organisms was determined in peritoneal washings taken from groups of five mice over a three-hour period. To induce a peritoneal exudate rich in macrophages, mice were injected with 0.5 ml of sodium alginate and used three days later.

Mycoplasma Strains

The virulent JB and avirulent Negroni strains of *M. pulmonis* were used [4,5]. *M. bovis* strain Ab/1 and *M. dispar* strain Gri 226 were also used [6].

In Vitro *Phagocytosis*

The method for determining the effect of antibody, or Ig preparations, on the survival of viable mycoplasmas in the presence of neutrophils or macrophages cultured as monolayers in medium 199 and heated (56°C, 30 minutes) fetal calf serum (FCS) has been described [5,6] except neutrophils were isolated from bovine peripheral blood [7]. Briefly, *M. bovis* was added to leucocyte cultures to give about equivalent numbers of organisms and cells, unattached mycoplasmas were removed by washing and fresh medium, with and without dilutions of purified bovine IgG1 or IgG2, prepared according to Fey et al. [8], added. Numbers of viable organisms free in supernatants and cell-associated were determined in four replicates at time zero and after 1½ or 4 hours incubation, respectively, for the neutrophils and macrophages.

A second method used was to isolate neutrophils from bovine blood and suspend them in medium 199 with 5 percent heated FCS. Mycoplasmas and antibody dilutions were added to produce a final mixture containing about 2×10^6 neutrophils and 2×10^6 colony-forming units (CFU) of *M. bovis.* Triplicate samples were titrated at time zero and after rolling at 37°C.

To determine the effect of mycoplasmas on the killing of *E. coli* by bovine neutrophils 2×10^6 CFU *E. coli* were added per 2×10^6 neutrophils in medium 199 with FCS in triplicate. Under these conditions there was no decrease in CFU of *E. coli* after cultures had been incubated rolling at 37°C for two hours. Addition of 2 percent heated normal bovine serum promoted a decrease in viable count of $\geq$ 99 percent. Dilutions of washed suspensions of mycoplasmas (5 mg protein per ml) were added to the mixtures to determine their effect on the killing of *E. coli*

by bovine neutrophils. The effect of mycoplasmas on bacterial uptake was examined in a similar manner, but using *E. coli* labeled by growth in the presence of ^{35}S-methionine and washing neutrophils three times before measuring bound radioactivity.

RESULTS

Effect of Induction of an Exudate Rich in Neutrophils on the Survival of M. pulmonis *in the Peritoneal Cavity of Mice*

In normal mice the number of cells ml^{-1} peritoneal wash, and standard deviation, was $10^{4.9 \pm 0.23}$ and $\geq$ 95 percent were macrophages. In mice injected with alginate three hours previously, there were $10^{5.7 \pm 0.16}$ cells ml^{-1} and about 75 percent were neutrophils. As seen in Fig. 1, the disappearance of viable *M. pulmonis,* strains JB or Negroni, in mice in which a peritoneal exudate, consisting largely of neutrophils, had been induced was the same as that in control mice.

Effect of Induction of an Exudate Rich in Macrophages on the Survival of M. pulmonis *in the Peritoneal Cavity of Mice*

Three days after injection of mice with sodium alginate there were $10^{6.4 \pm 0.15}$ cells ml^{-1} peritoneal wash of which $\geq$ 90 percent were macrophages. The rate at which viable *M. pulmonis* strain JB disappeared from the peritoneal cavity of these mice was greater than the rate of disappearance from control mice (Fig. 2).

Effect of Antibody on Phagocytosis in vitro *by Bovine Phagocytes*

M. bovis and *M. dispar* have been found to survive or grow in cultures of bovine alveolar macrophages and bovine lacteal neutrophils. The addition of bovine serum containing specific antibodies promoted killing of mycoplasmas by both types of leucocyte [6].

The specific IgG1 and IgG2 antibody titers to *M. bovis* of fractions prepared from a bovine antiserum were $10^{3.9}$ and $10^{4.0}$, respectively, by radioimmunonassay. The IgG1 antibody titer of the IgG2 preparation and the IgG2 antibody titer of the IgG1

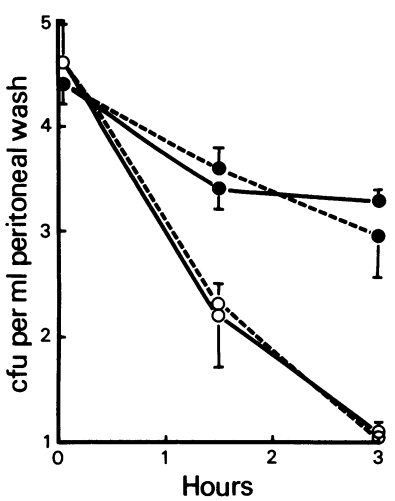

FIG. 1. Effect of neutrophil containing exudate on clearance of *M. pulmonis* strains from the peritoneal cavity of mice. ●——● strain JB in control mice; ●- - -● strain JB in mice injected with sodium alginate three hours previously; ○——○ strain Negroni in control mice; ○- - -○ strain Negroni in mice injected with sodium alginate. Groups of five mice used, mean and SE recorded.

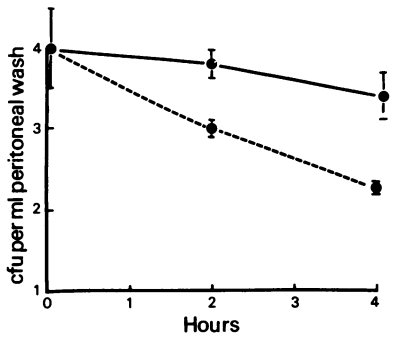

FIG. 2. Effect of a macrophage containing exudate on peritoneal clearance of *M. pulmonis* JB. ●——●, survival in control mice; ●- - -●, survival in mice injected with sodium alginate three days previously.

preparation were $\leq 10^1$. When added to *M. bovis*-infected alveolar macrophage cultures at dilutions of 1/50 and 1/100 the IgG1 antibody reduced the number of cell-associated *M. bovis* (± SD) by $10^{0.38 \pm 0.08}$ and $10^{0.47 \pm 0.09}$, respectively, and the IgG2 antibody reduced the number of *M. bovis* by $10^{0.38 \pm 0.06}$ and $10^{0.33 \pm 0.04}$, respectively, compared to control cultures with no added antibody.

With bovine peripheral blood neutrophils similarly cultured as monolayers on plastic, the addition of IgG2 antibody at dilutions of 1/50 and 1/100 reduced the number of CFU by $10^{0.80 \pm 0.05}$ and $10^{0.39 \pm 0.10}$, respectively. The addition of the same dilutions of IgG1 antibody caused no decrease in number of *M. bovis* compared to controls with no added antibody.

When neutrophils and *M. bovis* were incubated with rolling killing was not observed even when specific antibody was added.

Inhibition of Phagocytosis by Mycoplasmas

By adding suspensions of mycoplasmas to the mixtures of leucocytes and *E. coli,* it was shown that bacterial killing was inhibited by *M. bovis* and *M. dispar.* Neutrophils reduced the number of *E. coli* by $10^{2.2}$ CFU if 2 percent (final concentration) normal bovine serum was included in the medium. In the presence of *M. dispar* inhibition of bacterial killing was evident when the mycoplasma concentration was reduced to 12.5 μg per ml but not when present at only 2.5 μg per ml (Table 1).

TABLE 1
Effect of *M. dispar* on the Killing of *E. coli* by Neutrophils

Neutrophils[a] (2×10^6 ml^{-1})	Normal Bovine Serum (2%)	*M. dispar* (μg ml^{-1})	Decrease in CFU *E. coli* (10^n)
+	−	0	0.2
+	+	0	2.2
+	+	125	1.0
+	+	62.5	1.1
+	+	25	1.3
+	+	12.5	1.3
+	+	2.5	1.9
−	−	0	0
−	+	0	0

[a] For neutrophils and normal bovine serum + and − indicate their presence or absence at the indicated final concentration.

To examine the mechanism of inhibition of bacterial killing, the effect of prior incubation of mycoplasmas with normal bovine serum was determined. Killing of *E. coli* by neutrophils was unaffected by the absorption. Heat-inactivated *M. dispar* did not inhibit neutrophil-mediated killing as effectively as live organisms but mycoplasmas inactivated by freeze-thawing did. *M. bovis* also inhibited bacterial killing by neutrophils. However, this mycoplasma had no effect on the uptake of radiolabeled *E. coli;* thus the attachment step appeared unaffected and the inhibitory effect of mycoplasmas could be an ingestion and/or subsequent killing.

DISCUSSION

Studies by Cassell et al. [9] and Davis et al. [10] related the more rapid disappearance of *M. pulmonis* from the lungs of rats compared to mice with the more effective killing of the mycoplasma by rat alveolar macrophages. Also, Howard and Taylor [4] related the rate of disappearance of *M. pulmonis* strains, of differing virulence, from the respiratory tract of mice to rate of killing in the peritoneal cavity. This killing of the mycoplasmas was inhibited by silica and appeared to be dependent on peritoneal macrophages, and it was suggested that the ability to avoid phagocytosis was an attribute of virulent strains. These observations, together with the results of the effect of inducing a peritoneal exudate rich in macrophages, support the view that the interaction of mycoplasmas and macrophages in animals without antibody is of importance in determining the progression of infection. But, it should be noted that alginate may induce other changes beside inducing an exudate. In contrast, neutrophils did not appear to play a decisive role at this stage. However, the involvement of complement was not examined and this may promote neutrophil-mediated killing in the early stages of infection.

These observations in mice with *M. pulmonis* were similar to those reported by Brownlie et al. [11]; these workers observed that an exudate consisting largely of neutrophils, induced in one-quarter of the mammary gland of a cow by the injection of lipopolysaccharide from *Escherichia coli* 18 hours previously, did not protect the gland against infection with *M. dispar* or *Ureaplasma* sp. Such a treatment does afford some protection against staphylococcal and streptococcal infection.

It has been shown previously that killing of *M. bovis* and *M. dispar* by bovine alveolar macrophages and mammary neutrophils is promoted by bovine antibody [6]. Also, killing of *M. pulmonis* by murine peritoneal macrophages and neutrophils is promoted by murine antisera [5]. Not all immunoglobulin classes are equally effective at promoting effector cell activities, and appropriate receptors must be present on the leucocyte. Usually IgA is considered not to be opsonic; IgM has an effect in some situations, which may be enhanced by complement. IgG is regarded as the most efficient opsonin, but different subclasses have different activities. Murine IgG1, IgG2a, and IgG2b promoted phagocytosis of *M. pulmonis*-sensitized erythrocytes by murine peritoneal and alveolar macrophages, but IgG2a was probably the most effective molecule [12]. For phagocytosis to be effective *in vivo* the appropriate Ig isotype must be present at the appropriate site. In mice with *M. pulmonis* the isotype response varies with route of antigen presentation and time after exposure [13].

The comparative opsonic effects of IgG1 and IgG2 for mycoplasma are consistent with studies of receptors on bovine alveolar macrophages and neutrophils by rosetting [14] and phagocytosis and cytotoxicity studies with neutrophils [15,16]. For cattle IgG1 appears before IgG2, following exposure to an antigen, and IgG1 is the predominant Ig isotype in many secretions. Since IgG1 is selectively transferred into

colostrum it is the principal maternally derived antibody in calf sera. Therefore, although both IgG1 and IgG2 promoted killing of *M. bovis* by bovine alveolar macrophages, IgG1 may be the most important in the phagocytic defense system of the neonatal calf. IgG2 antibody was more effective than IgG1 at promoting phagocytosis of *M. bovis* by bovine neutrophils. Although IgM and IgG1 responses have developed at birth, IgG2 and IgA responses in cattle develop postpartum [17]; therefore, IgG2 antibody and neutrophils are probably more important as a phagocytic defense mechanism in older cattle.

The ability to inhibit bacterial killing by neutrophils appears to be a property of several mycoplasma species. This may be related to their ability to survive and grow on the phagocyte surface without being extensively ingested unless specific antibody is added [18] and could potentially be an important survival mechanism for the mycoplasma.

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