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Interaction of *Mycoplasma dispar* and *Mycoplasma agalactiae* subsp. *bovis* with Bovine Alveolar Macrophages and Bovine Lacteal Polymorphonuclear Leukocytes

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Mycoplasma dispar and *Mycoplasma agalactiae* subsp. *bovis* survived or grew in cultures of bovine lacteal polymorphonuclear leukocytes or bovine alveolar macrophages. In the presence of specific bovine antibody, macrophages and polymorphonuclear leukocytes appeared to kill both species of mycoplasma. Specific rabbit antisera also promoted the killing of these mycoplasmas by bovine macrophages but had no demonstrable activity for bovine polymorphonuclear leukocytes. It is suggested that phagocytosis of these mycoplasmas by bovine cells occurs only in the presence of specific antibody. The experiments also indicate that differences exist between bovine polymorphonuclear leukocytes and macrophages with regard to their receptor sites for immunoglobulins.

Mycoplasma dispar has been isolated from pneumonic calf lungs (9) and produces subclinical pneumonia gnotobiotic calves after endobronchial inoculation (12) and mastitis after inoculation of the mammary gland of cows (4). *Mycoplasma agalactiae* subsp. *bovis* has been isolated from severe outbreaks of mastitis (10) and from pneumonic calf lungs (21). This mycoplasma also produces mastitis in cows after intramammary inoculation (10) and pneumonia in gnotobiotic calves after endobronchial inoculation (unpublished observations). Macrophages and polymorphonuclear leukocytes (polymorphs) are the predominant cells in the bronchial exudate of these pneumonic lungs, and polymorphs are present in large numbers in infected milk. In addition there is a transient but enhanced polymorph infiltration in the milk of previously infected and immune quarters of the mammary gland after challenge with *M. dispar* (8).

Although accumulations of macrophages and polymorphs are common features of many mycoplasma infections, little is known of the interactions of these cells with mycoplasmas and the importance of such interactions in host defense mechanisms. Investigations have shown that *Mycoplasma pulmonis*, *Mycoplasma arthritidis*, and *Mycoplasma gallinarum* are phagocytosed by mouse peritoneal macrophages only in the presence of specific rabbit antiserum (5, 14). Similarly, the addition of specific rabbit antiserum was necessary for the phagocytosis of *M. pneumoniae* by guinea pig alveolar macrophages (17). In contrast the addition of either mouse or rat convalescent sera did not promote the phagocytosis of *M. arthritidis* by rodent macrophages (5).

There are also conflicting reports on the interaction of mycoplasmas with polymorphs. It has been reported (22) that *M. pneumoniae*, *Mycoplasma neurolyticum*, and *Mycoplasma gallisepticum* are ingested by peripheral blood leukocytes in the presence of autologous serum. On the other hand, Simberkoff and Elsbach (18) reported that human and rabbit neutrophils did not phagocytose *Mycoplasma hominis* and *M. arthritidis* even when specific rabbit antiserum was added.

The significance of these observations in relation to resistance to infection is not known, since the serum and/or cells were frequently obtained from animals that were not the usual host for the particular mycoplasma. Therefore, the following study was undertaken to investigate the interaction of bovine macrophages and polymorphs with *M. dispar* and *M. agalactiae* subsp. *bovis* in an allogeneic system, i.e., one in which the cells, antisera, and mycoplasma are obtained from the same animal species.

MATERIALS AND METHODS

Mycoplasma strains and media. *M. dispar* strain 462/2 has been described previously (11). *M. agalactiae* subsp. *bovis* strain Ab/1 was isolated from the lung of a calf that died during an outbreak of pneumonia (21) and was cloned by picking single colonies grown from filtered (450-nm membrane filters; Millipore Corp., Bedford, Mass.) suspensions of organisms three times (13). Both strains were grown in glucose-calf serum broth containing ampicillin (1).

Solid medium was similar to the broth but contained 0.5% agarose (Miles Laboratories, Inc., Elkhart, Ind.), and horse serum (Flow Laboratories, U.K.) was substituted for fetal calf serum.

Organisms for inoculation of cell cultures were grown in mycoplasma medium and sedimented by centrifugation. The pellet was washed in 0.15 M phosphate-buffered saline (PBS), pH 7.2, resuspended in PBS containing 5% fetal calf serum (Flow Laboratories), and stored at −70 C. The number of organisms was determined by titrations on solid media (see below) and expressed as colony-forming units (CFU) per milliliter.

Maintenance of cells. All cell cultures were maintained as monolayers in plastic petri dishes (30-mm diameter) with Medium 199 (Wellcome Research Laboratories, Beckenham, England) containing 0.11% $NaHCO_3$, 0.1% ampicillin (Beechams, G. B.), 25 units of mycostatin per ml (Squibb, G. B.), and 10% (vol/vol) heated (56 C, 30 min) fetal calf serum.

Alveolar macrophages were collected by washing out the lungs from 4- to 8-day-old calves with 1.5 liter of PBS. The cells were sedimented by centrifugation at 90 × *g* for 15 min at 5 C, washed with PBS, counted, and resuspended in maintenance medium containing heparin (10 units/ml) to give 2×10^6 cells/ml. Two-milliliter volumes of the cell suspension were distributed in petri dishes. After a 2-h incubation at 37 C in 5% CO_2 in air, the petri dishes were washed three times with PBS to remove nonadherent cells, and 2 ml of fresh maintenance medium was added to each plate. The cultures, which consisted of more than 90% mononuclear cells when Giemsa-stained petri dishes were examined, were further incubated at 37 C overnight.

Polymorphonuclear leukocytes were obtained from the bovine mammary gland 18 h after injection via the teat canal of 5 μg of *Escherichia coli* lipopolysaccharide (*E. coli* O55:B5, Difco Laboratories, Detroit, Mich.) in 5 ml of 0.15 M NaCl (3). Approximately 400 ml of milk was collected in a beaker containing 100 units of heparin. The milk cells were sedimented by centrifugation at 90 × *g* for 15 min at 20 C, washed with PBS containing 10 units of heparin per ml, counted, and resuspended in maintenance medium to give 2×10^6 cells/ml. Two-milliliter volumes of the polymorph suspension were distributed into petri dishes. The cells were allowed to settle at room temperature for 30 min (6) and then washed gently with PBS. More than 95% of the cells in Giemsa-stained preparations were polymorphonuclear leukocytes.

Interactions of mycoplasmas with cells. Macrophages, calf kidney (CK) cells or polymorphs, were washed with PBS containing 5% fetal calf serum. One milliliter of maintenance medium containing sufficient mycoplasmas to give a 1:1 mycoplasma-to-cell ratio was added to the cells. After a 60-min incubation at 37 C for macrophages and CK cells, or 20 C for the polymorphs, the cells were washed three times with PBS containing 5% fetal calf serum and 2 ml of maintenance medium containing various sera at final dilutions of 1:100 was added. At intervals after the addition of the sera the numbers of mycoplasmas present in the supernatants and the numbers associated with the cells were determined. A 0.5-ml volume of the supernatant fluid was removed for titration, and then the rest of the supernatant fluid was discarded. One milliliter of PBS was then added to each petri dish, and the attached cells were removed by means of a silicone bung. Single-cell suspensions were made by forcing the resuspended cells through a 25-gauge needle. Microscopic examination confirmed that the cells were not aggregated. Serial dilutions of the supernatants and the cell suspensions were made in PBS, and 25-μl volumes of each dilution were put onto solid mycoplasma medium. Plates were then incubated at 37 C in air for 14 days for *M. dispar* and 5 days for *M. agalactiae* subsp. *bovis*. All cell-associated and supernatant counts were performed on three samples, and all experiments were repeated at least once.

Antisera. Rabbit antisera to *M. dispar* strains 462/2 and F370 have been described elsewhere (11). Rabbit antisera to *M. agalactiae* subsp. *bovis* strain Ab/1 was prepared against organisms grown in rabbit serum and rabbit digest broth (11). The bovine sera to *M. dispar* were preinoculation and convalescent sera from two cows that developed mastitis following the intramammary inoculation of *M. dispar*. The bovine antisera to *M. agalactiae* subsp. *bovis* strain Ab/1 was obtained from a calf (no. 3) that had been inoculated intravenously with an ethanol-inactivated suspension of this mycoplasma and subsequently exposed to a calf infected endobronchially with *M. agalactiae* subsp. *bovis*.

The titers of the antisera to *M. dispar* 462/2 determined by the metabolism inhibition (19) and indirect hemagglutination tests (11) are shown in Table 1. The antibody titer to *M. agalactiae* subsp. *bovis* strain Ab/1 of the rabbit antiserum was ≥10,240 by the metabolism inhibition and 2,560 by the indirect hemagglutination test. The titer of the bovine antisera to strain Ab/1 was ≥10,240 by the indirect hemagglutination test. All sera were heated at 56 C for 30 min before use and used at a final dilution of 1:100.

RESULTS

Effect of sera on the survival of mycoplasmas in maintenance medium. To determine whether the antisera alone killed the mycoplasmas, *M. dispar* was added to maintenance medium containing the various sera listed in Table 1 and the number of organisms present in the media was determined at intervals up to 48 h. None of the sera caused a significant decrease in the number of organisms over an incubation period of 24 h. After 48 h only the convalescent bovine serum from cow no. 1 caused a slight decrease (0.6 $\log_{10}$) in the number of organisms.

The effect of antisera on the survival of *M. agalactiae* subsp. *bovis* in the absence of cells was also examined. In medium alone no change in the number of mycoplasmas was detected during an incubation period of 48 h. Addition of

TABLE 1. *Antibody titers to M. dispar 462/2 in rabbit and bovine sera tested by MI and IHA tests*[a]

Serum	Titer by MI to 462/2 as antigen	Titer by IHA to 462/2 as antigen
Preinoculation rabbit serum 1	<20	<20
Normal rabbit serum 2	<20	<20
Rabbit anti-*M. dispar* 462/21	640	5,120
Rabbit anti-*M. dispar* F370 2	1,280	10,240
Preinoculation bovine serum 1	<2	2
Preinoculation bovine serum 2	4	256
Convalescent bovine serum 1	16	1,024
Convalescent bovine serum 2	64	≥2,048

[a] Abbreviations: MI, Metabolism inhibition; IHA, indirect hemagglutination.

normal rabbit serum, rabbit antiserum, preinoculation bovine serum, or bovine antiserum to the maintenance medium did not affect the survival of this mycoplasma.

Effect of sera on the growth of *M. dispar* in cultures of CK cells. Having determined the effect of antiserum alone on the mycoplasmas, the effect of antiserum on the growth of mycoplasmas in cultures of nonphagocytic cells was examined. *M. dispar* multiplied in cultures of CK cells to which no other sera had been added (Fig. 1). When the sera listed in Table 1 were added to the culture medium, only bovine sera to *M. dispar* appeared to affect mycoplasma multiplication. In the presence of this bovine serum the number of *M. dispar* remained fairly constant.

Interaction of *M. dispar* with bovine alveolar macrophages. As seen with CK cultures *M. dispar* multiplied in macrophage cultures incubated in medium to which no other sera had been added (Fig. 2). Neither of the normal rabbit sera had any effect on the growth of the mycoplasmas. However, replication of mycoplasmas appeared to be inhibited in macrophage cultures treated with rabbit anti-*M. dispar* serum (Fig. 2). The effect of the rabbit antiserum appeared to be more pronounced on the number of organisms in the supernatants. The second rabbit serum to *M. dispar* (R2) also inhibited the increase in number of cell-associated mycoplasmas and caused a decrease in the number of organisms in the supernatants of the macrophage cultures. This effect of rabbit antisera and macrophages is in contrast to that of the same sera and CK cells noted above, where mycoplasma replication did not appear to be affected.

There were no differences in the number of mycoplasmas in macrophage cultures treated with preinoculation bovine serum (cow no. 1) and in cultures incubated in the absence of any added sera. However, the addition of convalescent serum from the same cow, which had an antibody titer of 1,024 by the indirect hemagglutination test, caused a marked decrease in the numbers of mycoplasmas associated with the macrophages and in the supernatants (Fig. 2). In contrast, this serum only inhibited the increase in numbers of mycoplasmas in CK cell cultures.

The addition of preinoculation bovine serum (cow no. 2), which had an antibody titer to *M. dispar* of 256 by the indirect hemagglutination test, to macrophage cultures resulted in a slight decrease in the number of mycoplasmas in the supernatants and inhibited multiplication of cell-associated mycoplasmas compared with cultures not treated with antibody. However,

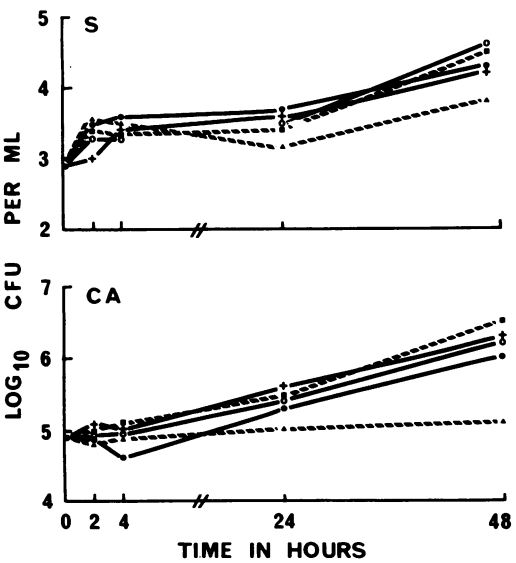

FIG. 1. *Effect of bovine and rabbit antisera on the survival of M. dispar strain 462/2 in cultures of bovine CK cells. Abbreviations: S, Number of colony-forming units per milliliter of supernatant; CA, number of colony-forming units per milliliter of cell suspension. Medium contained: (●) No additional sera; (+) normal rabbit serum (sample 1); (○) rabbit (1) anti-M. dispar 462/2 serum; (■) preinoculation bovine serum (cow no. 1); and (▲) bovine anti-M. dispar serum (cow no. 1). Mean values of three samples are recorded.*

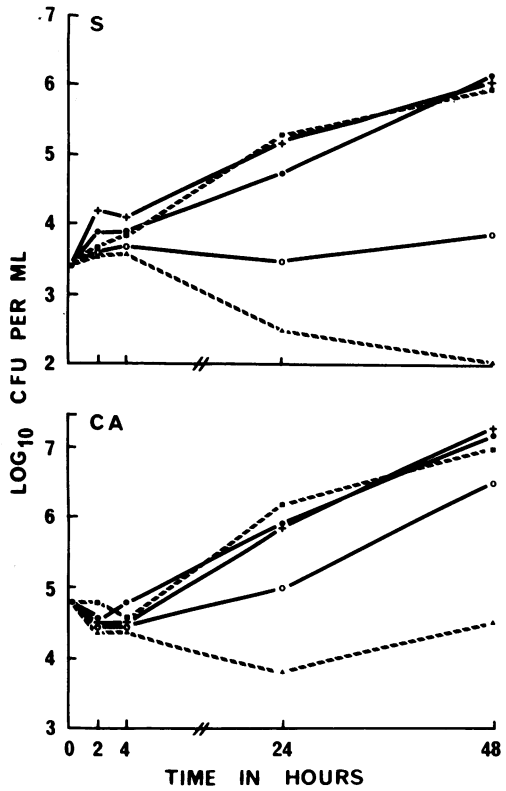

FIG. 2. *Effect of bovine and rabbit antisera on the survival of M. dispar strain 462/2 in cultures of bovine alveolar macrophages. Symbols as in Fig. 1.*

the addition of convalescent serum from the same cow resulted in a much more marked decrease in the number of mycoplasmas in the supernatants and associated with the cells.

Interaction of *M. dispar* with bovine lacteal polymorphs. There were no differences in the numbers of organisms in polymorph cultures incubated in the absence of any added sera or treated with normal rabbit serum, rabbit antiserum to *M. dispar*, or preinoculation bovine sera (Fig. 3). In contrast, when *M. dispar*-infected polymorph cultures were incubated in the presence of either of the two bovine antisera to this organism, a marked decrease in the number of mycoplasmas present in the supernatants and associated with the cells was observed.

Interaction of *M. agalactiae* subsp. *bovis* with bovine alveolar macrophages and bovine lacteal polymorphs. Rabbit and bovine sera to *M. agalactiae* subsp. *bovis* were also examined for their ability to promote the killing of strain Ab/1 by bovine macrophages and polymorphs. In the absence of serum *M. agalactiae* subsp. *bovis* survived in cultures of bovine alveolar macrophages without any apparent change in the number of organisms over the first 3 h of incubation. After 24 h considerable multiplication of the mycoplasmas had occurred. The addition of normal rabbit serum or preinoculation bovine serum (calf no. 3) did not affect the multiplication of *M. agalactiae* subsp. *bovis* (Fig. 4). In contrast there was a marked decrease in the number of mycoplasmas in macrophage cultures treated either with rabbit anti-*M. agalactiae* subsp. *bovis* serum or with bovine antiserum to that mycoplasma. By 48 h the number of mycoplasmas had increased more rapidly in the presence of bovine antibody than in the presence of rabbit antibody.

M. agalactiae subsp. *bovis* strain Ab/1 survived equally well in bovine polymorph cultures incubated in the absence of any added serum and in the presence of normal rabbit

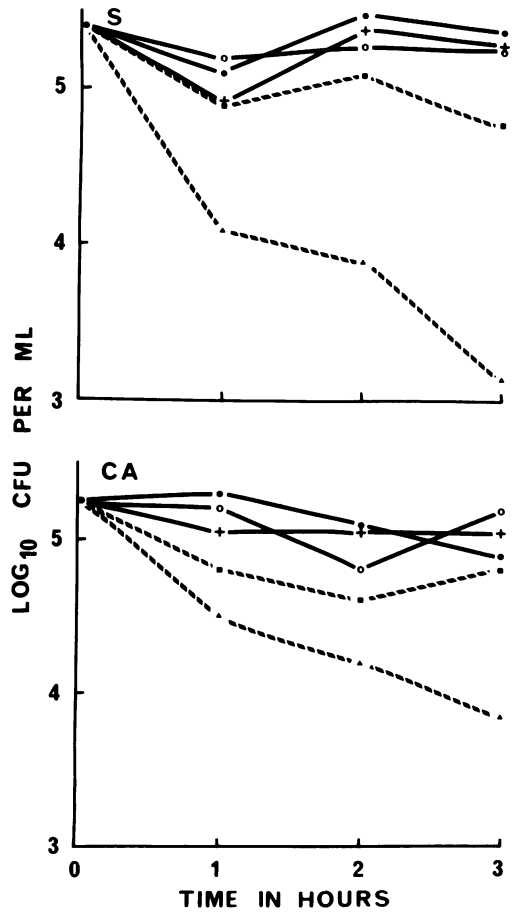

FIG. 3. *Effect of bovine and rabbit antisera on the survival of M. dispar strain 462/2 in cultures of bovine lacteal polymorphonuclear leukocytes. Symbols as in Fig. 1.*

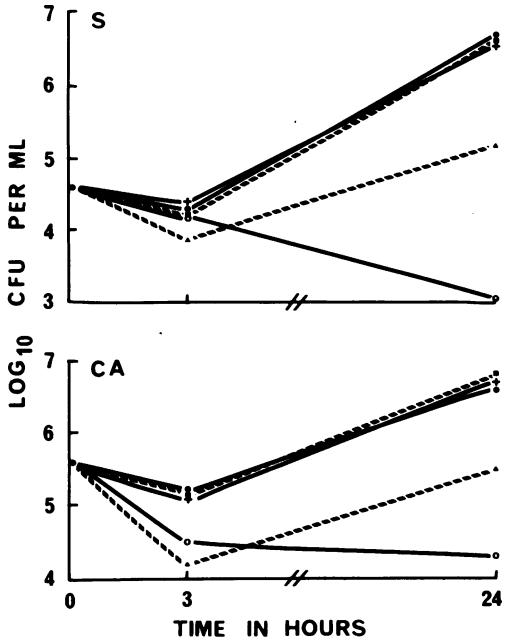

FIG. 4. *Effect of bovine and rabbit antisera on the survival of M. agalactiae subsp. bovis strain Ab/1 in cultures of bovine alveolar macrophages. Abbreviations: S, Number of colony-forming units per milliliter of supernatant; CA, number of colony-forming units per milliliter of cell suspension. Medium contained: (●) No additional sera; (+) normal rabbit serum (1); (○) rabbit anti-M. agalactiae subsp. bovis serum; (■) normal bovine serum (calf no. 1); and (▲) bovine anti-M. agalactiae subsp. bovis serum. Mean values of three samples are recorded.*

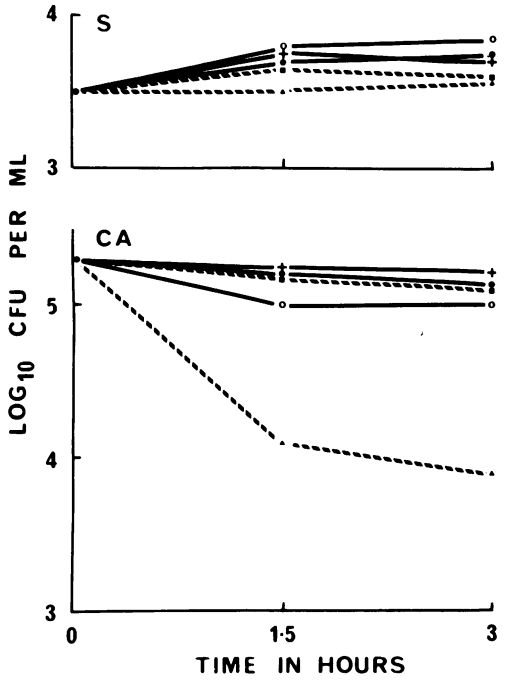

FIG. 5. *Effect of bovine and rabbit antisera on the survival of M. agalactiae subsp. bovis strain Ab/1 in cultures of bovine lacteal polymorphonuclear leukocytes. Symobls as in Fig. 4.*

serum, rabbit antibody, or preinoculation bovine serum (calf no. 3) (Fig. 5). The addition of bovine antiserum against *M. agalactiae* subsp. *bovis* to these polymorph cultures resulted in a marked decrease in the number of cell-associated mycoplasmas.

Effect of cytochalasin B on survival of *M. agalactiae* subsp. *bovis* in cultures of bovine polymorphs. Cytochalasin B (ICI Pharmaceuticals, Macclesfield, England) dissolved in dimethyl sulfoxide (Me_2SO) was added to medium containing *M. agalactiae* subsp. *bovis* to give 10 μg/ml as a final concentration of cytochalasin B and 1:100 as a final dilution of Me_2SO. As controls the mycoplasmas were suspended in medium or in medium containing Me_2SO at a final dilution of 1:100. At time zero $10^{5.6}$ CFU of strain Ab/1 per ml of medium were present. After 24 h of incubation at 37 C $10^{5.8}$ CFU were present in medium alone, $10^{5.7}$ CFU were present in medium containing Me_2SO, and $10^{5.7}$ CFU were present in medium containing Me_2SO and cytochalasin B. Thus, neither Me_2SO nor cytochalasin B appeared to have any effect on the viability of *M. agalactiae* subsp. *bovis*. The effect of cytochalasin B on the survival of this mycoplasma in bovine polymorph cultures incubated in the presence and absence of bovine antibody is shown in Fig. 6. These results showed that cytochalasin B reduced the killing of *M. agalactiae* subsp. *bovis* by bovine polymorphs in the presence of bovine antibody.

DISCUSSION

In the absence of specific antibody neither bovine alveolar macrophages nor bovine lacteal polymorphonuclear leukocytes appeared to kill either *M. dispar* or *M. agalactiae* subsp. *bovis*. However, the addition of bovine antibody against these mycoplasmas to cultures of macrophages or neutrophils resulted in a decrease in the number of viable organisms detected after a 3- or 24-h incubation. In the absence of cells the bovine antisera had little or no effect on the survival of these mycoplasmas. Besides this, the addition of bovine antiserum to CK cells infected with *M. dispar* only inhibited the increase in the number of organisms. These observations indicate that the bovine sera contain antibodies that promote phagocytosis and killing of *M. dispar* and *M. agalactiae* subsp.

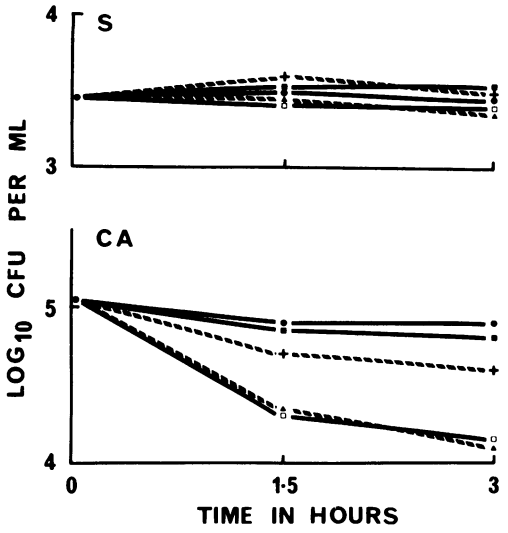

FIG. 6. *Effect of cytochalasin B on the killing of M. agalactiae subsp. bovis in cultures of bovine lacteal polymorphonuclear leukocytes in the presence of bovine antiserum. Abbreviations: S, Number of colony-forming units per milliliter of supernatant; CA, number of colony-forming units per milliliter of cell suspension. Medium contained: (●) No additional substances or serum; (▲) bovine anti-M. agalactiae subsp. bovis serum; (□) bovine anti-M. agalactiae subsp. bovis serum and dimethyl sulfoxide; (+) bovine anti-M. agalactiae subsp. bovis serum, dimethyl sulfoxide, and cytochalasin B; and (■) dimethyl sulfoxide and cytochalasin B. Mean values of three samples are recorded.*

bovis by both bovine alveolar macrophages and bovine lacteal polymorphonuclear leukocytes.

Cytochalasin B has been shown to inhibit the phagocytosis of opsonized bacteria by polymorphs (7) and of opsonized erythrocytes by macrophages (15). Macrophage cytotoxicity is not affected (20). The antibody-dependent killing of mycoplasmas by bovine polymorphs was found to be reduced in the presence of cytochalasin B. This observation supports the suggestion that the killing of mycoplasmas reported here involved phagocytosis.

The addition of hyperimmune rabbit serum to bovine macrophage cultures infected with *M. dispar* resulted in an inhibition of growth, or killing, of mycoplasmas, whereas it had no effect on the survival of *M. dispar* in medium alone or in cultures of CK cells. Surprisingly, rabbit antisera did not promote the killing of *M. dispar* by bovine polymorphs. This apparent discrepancy was also observed with *M. agalactiae* susp. *bovis*; i.e., rabbit antisera appeared to promote the phagocytosis of this mycoplasma by bovine macrophages but not by bovine polymorphs. An explanation for these observations may be that there is a deficiency of receptor sites on bovine polymorphs for certain rabbit immunoglobulins. If this were so, then it is also necessary to postulate differences in the immunoglobulin receptor sites on bovine macrophages and bovine polymorphs. It is worth noting that Patterson and Suszko (16) suggested that guinea pig and rabbit phagocytes did not have receptor sites for avian antibody, but avian phagocytes did have receptor sites for guinea pig antibody.

The finding that *M. dispar* attaches to the surface of bovine phagocytes, and is not subsequently phagocytosed in the absence of specific antibody, may account for the observation that, although injection of endotoxin into the mammary gland of normal cows induces a large increase in the number of polymorphs in the milk, inoculation of the mammary gland with *M. dispar* 18 h later results in a more severe infection in the endotoxin-treated quarter than in the untreated quarter injected with this mycoplasma (unpublished observations). This is in contrast to the finding of Blobel and Katsube (2), who reported that an induced leukocytosis resulted in enhanced resistance of the mammary gland to infection with *Staphylococcus aureus*. In addition, the rapid elimination of mycoplasmas from a quarter of the bovine udder previously infected with *M. dispar* is associated with a transient, but enhanced, infiltration with neutrophils (8). It seems reasonable to suggest that, on challenge of a previously infected quarter with *M. dispar*, either locally produced or serum antibody promotes rapid phagocytosis of *M. dispar* by the polymorphs, leading to an enhanced rate of elimination of organisms.

In conclusion, this investigation suggests that in the presence of specific antibody macrophages and polymorphs are capable of phagocytosing *M. dispar* and *M. agalactiae* subsp. *bovis* and may contribute to the defense mechanisms of cattle against these mycoplasmas.

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