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Interaction of *Mycoplasma dispar* with Bovine Alveolar Macrophages

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The capacity to avoid phagocytosis and the activation of bovine alveolar macrophages (BAM) by encapsulated *Mycoplasma dispar* or purified *M. dispar* capsule was investigated. Encapsulated and unencapsulated *M. dispar* were cocultured with BAM in the presence or absence of antisera prepared against unencapsulated *M. dispar* or purified capsule antiserum. Unopsonized mycoplasmas resisted phagocytosis, while only anti-capsule antibodies enhanced the phagocytosis of encapsulated mycoplasmas. BAM were cultured in the presence of purified *M. dispar* capsule or either live or heat-killed encapsulated or unencapsulated *M. dispar*. These BAM were then activated with *Escherichia coli* endotoxin or left without further activation. The supernatants of these cultures were assayed for tumor necrosis factor, interleukin 1, and glucose consumption as indicators of macrophage activation. Tumor necrosis factor and interleukin 1 were produced by BAM stimulated with unencapsulated *M. dispar* but not when encapsulated *M. dispar* or its purified capsule was used. Similarly, glucose consumption was increased in the presence of unencapsulated *M. dispar*, but not when BAM were cocultured with encapsulated *M. dispar* or purified capsule. When BAM were treated with purified capsule or encapsulated mycoplasmas, they could not be subsequently activated by endotoxin. These results indicate that encapsulated *M. dispar* or purified capsule exerts an inhibitory effect on the activity of BAM and prevents the activation of these cells.

Alveolar macrophages are central in the protection of the lower portions of the lungs, operating in many nonspecific modes of defense and augmented by specific immunologic mechanisms. Previous studies have shown that alveolar macrophages ingest, inactivate, and degrade inhaled microorganisms within 8 h of their entrance into alveolar regions (11). Such antimicrobial ability of macrophages is centered on their phagocytic capabilities and in the production of potent microbicidal and degradative substances within the macrophage.

It has been shown that macrophages have innate functional capacities that extend far beyond phagocytosis. They can act as secretory and regulatory cells, can initiate and prolong inflammatory processes, and can stimulate synthesis of extracellular matrix proteins (4, 13). Thus, they can adjust their response to their microenvironment and exert regulatory activities on other cells such as neutrophils and lymphocytes, mounting the defense system that protects the respiratory parenchyma from microbial invasion (4). However, if macrophage function is impaired because of pollutant or microbial exposure, the host-parasite balance is upset and disease ensues.

In the absence of specific antibodies, several mycoplasma species can colonize the surface of macrophages without stimulating phagocytosis (2, 5, 7, 17). In contrast, other mycoplasma species were capable of stimulating macrophage activity, inducing secretion of cytokines, expression of major histocompatibility complex molecules, and release of hydrolytic enzymes from these cells (6, 25, 28).

Experiments with *Mycoplasma dispar* have shown that this mycoplasma species can induce immunosuppression when injected in combination with other antigens, and it was postulated that the production of a soluble inhibitory factor(s) was responsible for the observed immunosuppression (10). The present studies examined the interaction of *M. dispar* with bovine alveolar macrophages (BAM) and found that *M. dispar* capsule exerts a suppressive effect. The bactericidal activity of macrophages as well as the secretory functions of these phagocytic cells were reduced by the presence of encapsulated *M. dispar* or polysaccharide capsule.

MATERIALS AND METHODS

Macrophages. BAM were obtained from 75- to 90-day-old calves from a respiratory mycoplasma-free herd. The cells were harvested as has been described previously (14), the proportion of macrophages was estimated by phagocytosis of latex beads (Poly Sciences), and viability of the cells was checked by Trypan blue exclusion.

The proportion of macrophages was enriched by using the selective adherence of macrophages to plastic surfaces, following the protocol described by Pennline (20). Briefly, cells were spun down (200 × *g* for 10 min at 4°C) and resuspended in Eagles's minimal essential medium (MEM) containing 15% fetal bovine serum (FBS; GIBCO BRL) with 62 μg of penicillin G per ml, 100 μg of kanamycin per ml, and 100 μg of streptomycin per ml and placed in petri dishes at a concentration of 3×10^5 cells per cm^2. The cells were incubated overnight at 37°C in a 4% CO_2 atmosphere. Adherent cells were then harvested as follows: after washing the monolayer to remove nonadherent cells, ice-cold modified Hank's solution (E-HBSS: 2.5 mM EDTA, 0.4 g of KCl per liter, 0.06 g of KH_2PO_4 per liter, 8.0 g of NaCl per liter, 0.35 g of $NaHCO_3$ per liter, 0.09 g of $Na_2HPO_4 \cdot 7H_2O$ per liter, penicillin G [62 μg/ml], kanamycin [100 μg/ml], streptomycin [100 μg/ml], and amphotericin B [50 μg/ml]) was added and allowed to stand at 4°C for 30 min. Cells were then washed twice with warm E-HBSS, spun down as described

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above, and resuspended in MEM–10% FBS. Viability and percentage of macrophages were measured as described above. Macrophages were then placed in 96- or 12-well cluster culture plates at a concentration of 3×10^5 cells per cm^2 and incubated at 37°C in a 5% CO_2 atmosphere. Absence of mycoplasma contamination was ascertained by culturing the BAM without antibiotics, followed by microscopic examination after staining with the fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride (Calbiochem).

Microorganisms. The SD-O strain of *M. dispar* was isolated from a pneumonic calf (29), was cloned twice, and was used at passage level 7. *M. dispar* was grown in Friis modified broth medium as previously described (18). Logarithmic-phase cultures were centrifuged for 10 min at 500 × *g* to remove debris, and the resulting supernatant was centrifuged at 34,000 × *g* for 30 min. The pellet was washed three times with 0.01 M phosphate-buffered saline solution (PBS), pH 7.2. Mycoplasma suspensions were stored after quick freezing at −70°C in 1-ml aliquots (30 mg of protein per ml). Protein concentrations were determined by the method of Lowry et al. (19) with alkali treatment prior to the assay to solubilize membranes (1). For induction of *M. dispar* capsule, we followed the methodology described by Almeida and Rosenbusch (3). Briefly, *M. dispar* cells were cocultured with bovine lung fibroblast monolayers for 23 h. Capsulated mycoplasmas were then harvested from the cell culture medium by differential centrifugation at 200 × *g* for 10 min at 4°C and washed three times by high-speed centrifugation at 23,000 × *g* with cold PBS supplemented with 1 mM Tris and 1 mM EDTA (E-PBS). For some experiments, aliquots of unencapsulated and encapsulated mycoplasmas were heat killed by placing them in boiling water for 3 min. For use as a marker, *Serratia marcescens* NIMA was grown on tryptose agar plates (Difco Laboratories, Detroit, Mich.) and incubated at room temperature for 36 h while *Staphylococcus aureus* (ATCC 10390) was grown in 7% bovine blood agar plates at 37°C overnight. The bacterial lawns were harvested and resuspended in lactose glutamate diluent (22) at a concentration of 6.0×10^9 and 6.5×10^8 CFU/ml, respectively, fractionated in 1-ml aliquots, and kept at −70°C until use.

Capsule purification. Capsule was extracted from the mycoplasmas by placing them in E-PBS (pH 8.0) at 37°C for 1 h. After centrifugation at 34,000 × *g* for 30 min, the supernatant was dialyzed for 72 h against the same buffer. Capsule material was then captured on immobilized *Ricinus communis* II lectin (Vector), washed in decreasing salt concentrations (2, 1.1, and 0.2 M NaCl in 1 mM Tris [pH 8.0] and 1 mM EDTA), eluted with 0.2 M lactose, and purified by size exclusion chromatography (Biogel P4; Bio-Rad) with E-PBS (pH 8.0) as a running buffer. Collected fractions were analyzed by thin-layer chromatography with thin-layer chromatography silica gel plates (E. Merck, Darmstadt, Germany) and a mixture of pyridine, ethanol, water, and *n*-butanol at a ratio of 4:1:1:1 as a solvent system. Presence of capsule was detected by staining the plates with a saturated solution of silver nitrate diluted 1:10 in a mixture of H_2O and acetone (1:1 ratio) and then by washing steps with 0.5 M NaOH in ethanol and then 6 M ammonium hydroxide solution. Fractions showing presence of capsule material were then analyzed by following a radioimmunoprecipitation protocol previously described (3). Positive fractions were then pooled, lyophilized, resuspended at 15 μg/ml in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–0.85 M NaCl–0.1 mM $CaCl_2$ buffer (pH 8.0), and kept in 500-μl aliquots at −70°C.

Mycoplasma phagocytosis assay. The phagocytosis protocol described by Davis et al. (8) was followed. Briefly, 12-well cluster plates containing 3×10^5 BAM per ml were treated with 3×10^6 color-changing units of encapsulated or unencapsulated *M. dispar* per ml reacted previously for 10 min at 37°C with or without rabbit anti-capsule or anti-*M. dispar* sera at a 1:100 final dilution. Plates were then centrifuged at 800 × *g* at 4°C for 20 min and then incubated for 15 min at 37°C to promote mycoplasma attachment. Cultures were washed five times with warm MEM–5% FBS to remove nonadherent mycoplasmas and incubated at 37°C in MEM with 10% FBS. At 4, 6, 12, and 18 h after the addition of mycoplasmas, macrophages of three wells per treatment were scraped from the plastic surface and centrifuged at 14,000 × *g* and 4°C for 10 min. The pellets were resuspended in MEM–10% FBS and sonicated on ice for 60 s (8-mm probe, 120 W; Braunsonic), and the lysate was serially diluted 10-fold and plated in duplicate modified Friis plates (18); agar plates were incubated at 37°C in a 5% CO_2 atmosphere for 5 to 7 days before colony counting. Concomitantly with the last sampling, macrophage viability was checked in nontreated control wells by Trypan blue exclusion.

Tumor necrosis factor (TNF) assay. BAM were treated with live or heat-inactivated encapsulated or unencapsulated *M. dispar* or treated with 7.5 μg of purified capsule per ml, 2 mg of *Escherichia coli* K-235 endotoxin (Sigma) per ml, or culture medium and incubated at 37°C in a 5% CO_2 atmosphere. Each treatment was conducted in triplicate wells, and 300-μl samples of the supernatants were taken at 4, 8, 12, and 24 h after treatment. Samples were then centrifuged at 14,000 × *g* for 10 min at 4°C and kept at −70°C until use. Concomitantly with the last sampling, BAM viability in nontreated control wells was checked by Trypan blue exclusion.

For the TNF assay, supernatants from the macrophages were assayed by using the TNF-sensitive L929 cell line. Briefly, L929 cells were grown in MEM with 10% horse serum (HyClone), resuspended in the same media, placed in 96-well culture plates at a concentration of 2.4×10^4 cells per well, and incubated at 37°C in a 5% CO_2 atmosphere. The medium was then changed to MEM with 10% horse serum and 3 μg of actinomycin D (Sigma) per ml, and after this, wells were inoculated with 100 μl of 1:10, 1:50, and 1:250 dilutions of macrophage supernatants and incubated overnight at 37°C in a 5% CO_2 atmosphere. After fixation with 10% formaldehyde, cells were washed three times with saline and stained with a 0.2% solution of crystal violet for 20 min. Plates were then washed to remove crystal violet and dried for 24 h at 37°C. One hundred microliters of a 1:1 mixture of PBS (pH 7.2) and 100% ethanol was added to each well and then the plate was read at 595 nm in an automated enzyme-linked immunosorbent assay reader (Bio-Tek Instruments).

IL-1 assay. BAM were treated with live or heat-killed encapsulated or unencapsulated *M. dispar* or with 7.5 μg of purified capsule per ml, *E. coli* K-235 endotoxin (2 μg/ml), or culture medium. Each treatment was conducted in triplicate wells (the experiments for TNF and interleukin 1 [IL-1] were done concurrently by using the same supernatants), and 300-μl samples of the supernatants were taken at 4, 8, 12, and 24 h after treatment. With the last sampling, macrophage viability was checked in nontreated control wells by Trypan blue exclusion. Samples were then centrifuged at 14,000 × *g*

for 10 min at 4°C and kept at −70°C until use. For IL-1 activity, supernatants were assayed by using the IL-1-dependent D10G4.1 cell line. Briefly, triplicate wells were inoculated with 1:5, 1:25, and 1:125 dilutions of macrophage supernatants in RPMI 1640 supplemented with 5% FCS and incubated at 37°C in a 5% CO_2 atmosphere for 48 h and then pulsed for 24 h with 50 μCi of [^{3}H]thymidine per ml. Cells were then harvested on glass microfiber filters (Whatman), which were allowed to dry. Scintillation vials containing individual glass microfiber filters were filled with 2.5 ml of scintillation cocktail (Ready-Solv Hp/b; Beckman), and counts per minute were determined in a scintillation counter (Packard Instruments).

Glucose consumption. Ten-microliter aliquots of supernatant from BAM treated with live or heat-killed encapsulated or unencapsulated *M. dispar* or 7.5 μg of purified capsule per ml or incubated with *E. coli* K-235 endotoxin (2 μg/ml) or culture medium were assayed for glucose consumption as an indication of BAM activation, following the protocol described by Ryan et al. (24). Briefly, 10 μl of each supernatant was mixed with 100 μl of a 5 mM NADP solution, 100 μl of a 20 mM ATP solution, and 5 ml of a hexokinase (250 U of hexokinase per ml) and glucose 6-phosphate dehydrogenase mixture (Sigma) in 0.1 M Tris buffer (pH 7.5). This mixture was added to a cuvette containing 700 μl of 0.1 M Tris-HCl (pH 7.5)–64 mM NaCl–3.5 mM $MgCl_2$–0.15 mM $CaCl_2$. Two minutes after the solutions were mixed, the optical density at 340 nm was read to determine NADPH production.

Assays with activated macrophages. To determine if capsulated *M. dispar* or purified capsule could inhibit the activation of BAM by endotoxin, BAM were treated with unencapsulated *M. dispar*, encapsulated *M. dispar*, or purified capsule, and after 4, 8, 12, and 24 h of incubation, supernatants were removed and the BAM were gently washed (three times) with warm MEM containing 15% FBS and 60 μg of Tylosin per ml. Macrophages were then incubated in MEM containing 15% FBS and Tylosin with the addition of *E. coli* K-235 endotoxin (2 μg/ml). After 12 h, aliquots were obtained and assayed for TNF and IL-1 production. For glucose consumption, BAM were incubated with unencapsulated or encapsulated *M. dispar* or purified capsule for 16 h. The BAM were then gently washed and incubated with MEM containing Tylosin and *E. coli* K-235 endotoxin as described above, and glucose consumption was determined as described above.

Statistical analysis. A Student's *t* test for unpaired samples was used to determine the statistical significance of the effect of encapsulated or unencapsulated *M. dispar* or of purified capsule on BAM.

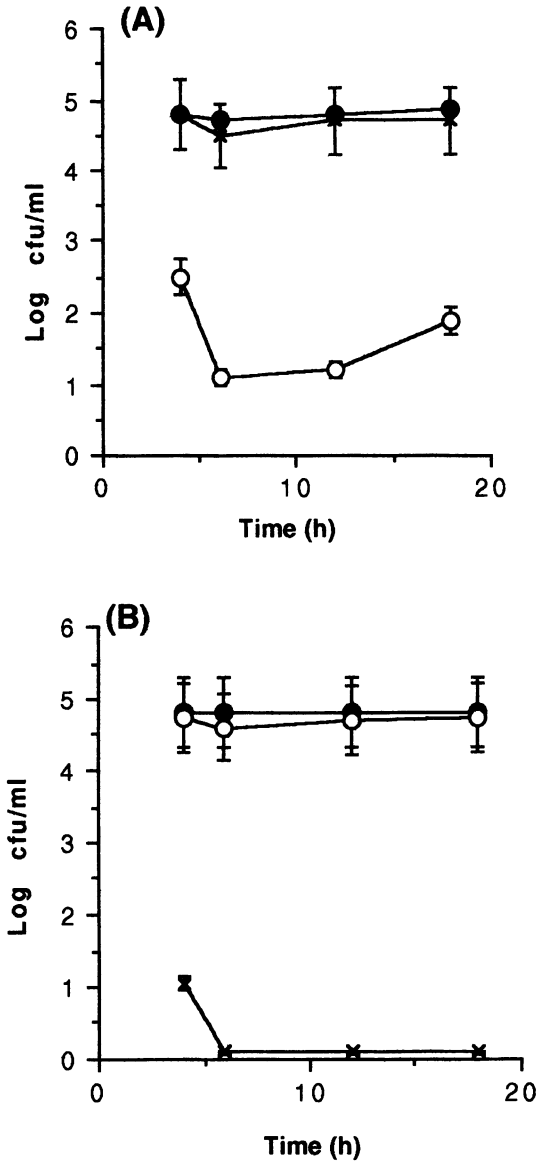

FIG. 1. Phagocytosis assay conducted with BAM and unencapsulated *M. dispar* (A) or encapsulated *M. dispar* (B) in the presence and absence of specific antiserum against *M. dispar* or purified capsule. Symbols: ●, *M. dispar* without antibodies; ○, *M. dispar* with anti-*M. dispar* antibodies; ×, *M. dispar* with anti-capsule antibodies. Means of triplicate wells with standard deviations of the means are recorded.

RESULTS

Mycoplasma phagocytosis. The results from the mycoplasma phagocytosis assay are shown in Fig. 1A and B. When unencapsulated or encapsulated *M. dispar* was cultured with BAM in the absence of antibodies specific for *M. dispar* surface antigens, no reduction of the titer of the microorganisms was observed. The need for specific antibodies to promote phagocytosis was evidenced by a decrease of 2 to 3 $\log_{10}$ units in the titer of the mycoplasma at all sampling times ($P < 0.05$). There was no decrease in BAM viability throughout the assay period.

TNF production. Unencapsulated *M. dispar* induced marked TNF production that presents patterns of induction over time similar to that observed in endotoxin-stimulated BAM (Fig. 2A). When BAM were treated with encapsulated *M. dispar*, TNF production was significantly less ($P < 0.05$) than that of BAM stimulated with endotoxin or unencapsulated *M. dispar*. Likewise, purified capsule also had an inhibitory effect on TNF production by BAM. When BAM were treated with encapsulated *M. dispar* or purified capsule, they became insensitive to activation by endotoxin (Fig. 2B). This inhibitory effect was expressed after all treatment times tested ($P < 0.05$). Addition of heat-killed unencapsulated *M. dispar* had the same effect as live unencapsulated *M. dispar*, while heat-killed capsulated *M. dispar* reacted like the live capsulated organisms (data not shown).

IL-1 production. BAM produced IL-1 upon stimulation with unencapsulated *M. dispar*, but the level reached was lower than that observed with endotoxin-stimulated BAM

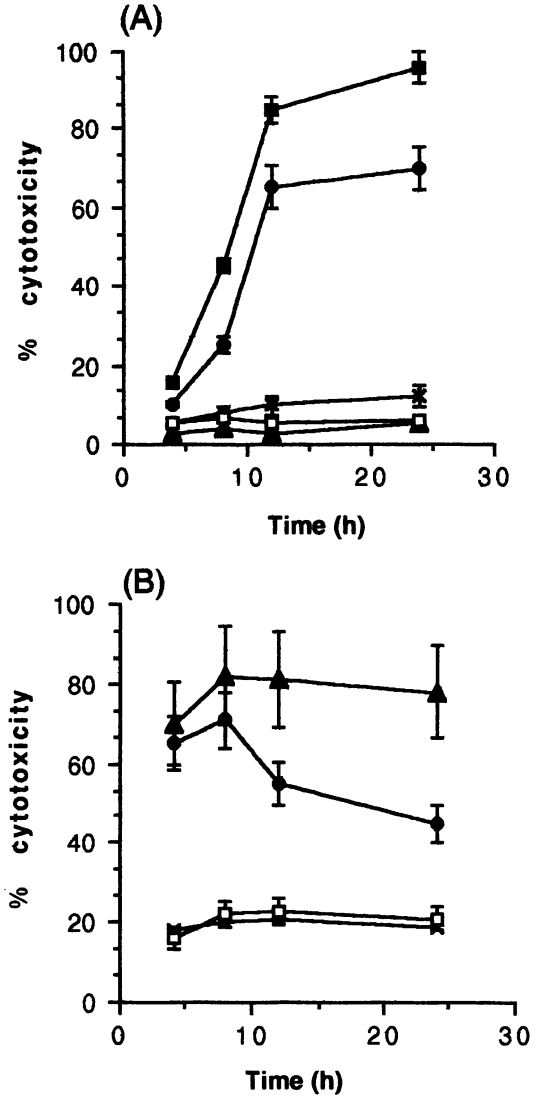

FIG. 2. Production of TNF activity by BAM treated with encapsulated and unencapsulated *M. dispar* and purified capsule in the absence of endotoxin stimulation (A) or after endotoxin stimulation (B). Symbols: ▲, untreated BAM; ■, BAM treated with endotoxin; □, purified capsule; ×, encapsulated *M. dispar*; ●, unencapsulated *M. dispar*. Means of triplicate wells with standard deviations of the means are recorded.

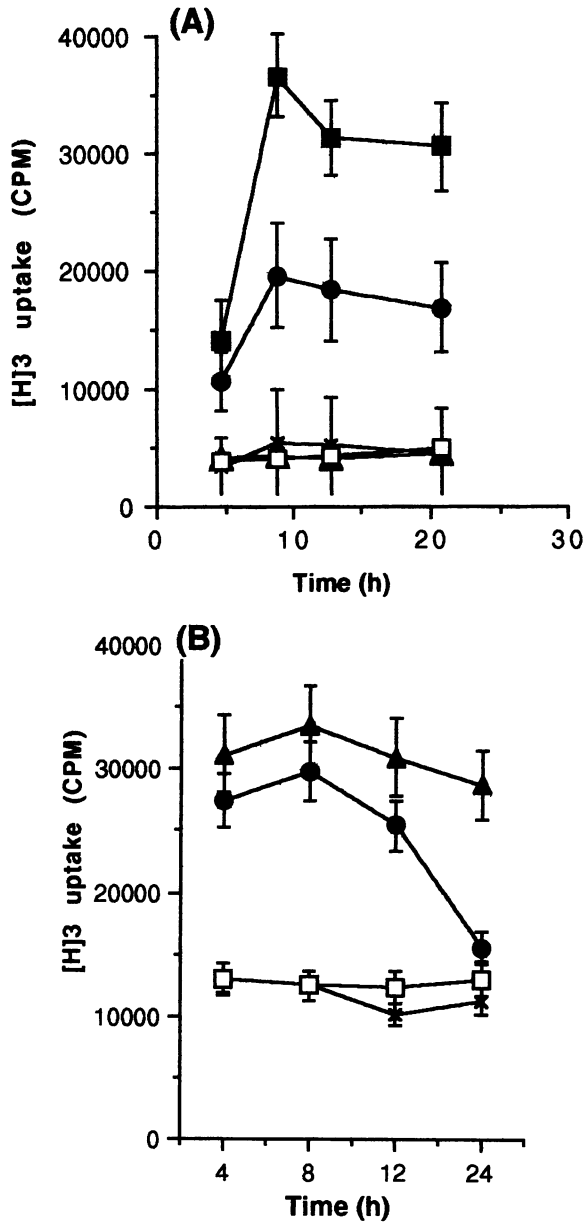

FIG. 3. Production of IL-1 by BAM treated with encapsulated and unencapsulated *M. dispar* and purified capsule in the absence of endotoxin stimulation (A) or after endotoxin stimulation (B). Symbols are as described in the legend to Fig. 2. Means of triplicate wells with standard deviations of the means are recorded.

(Fig. 3A). In contrast, the IL-1 production induced by encapsulated *M. dispar* or purified capsule was negligible and was no different from that detected with untreated macrophages. When heat-killed encapsulated or unencapsulated *M. dispar* was used, it was observed that IL-1 was produced and at levels similar to those of the corresponding live mycoplasma. In contrast, heat-killed encapsulated mycoplasmas induced higher levels of IL-1 than live encapsulated mycoplasmas (data not shown). Macrophages treated with encapsulated *M. dispar* or purified capsule became refractory to activation by endotoxin (Fig. 3B), while macrophages treated with unencapsulated *M. dispar* became refractory only after 24 h of treatment.

Glucose consumption. Glucose consumption was measured as an indicator of macrophage activation. In the presence of unencapsulated *M. dispar*, the rate of glucose consumption detected was similar to that observed in BAM treated with endotoxin (Fig. 4A). In the presence of encapsulated *M. dispar*, however, BAM showed only minimal consumption of glucose which became significantly lower than that observed in endotoxin-treated macrophages after 90 min ($P < 0.05$). Moreover, when purified capsule was used, glucose consumption was less than that observed with encapsulated *M. dispar* and significantly different ($P < 0.05$) from that of BAM stimulated with endotoxin or treated with unencapsulated *M. dispar*. Glucose consumption as a result of endotoxin activation provided results that were parallel to those seen with TNF production and IL-1 induction. When BAM were treated with purified capsule or encapsulated myco-

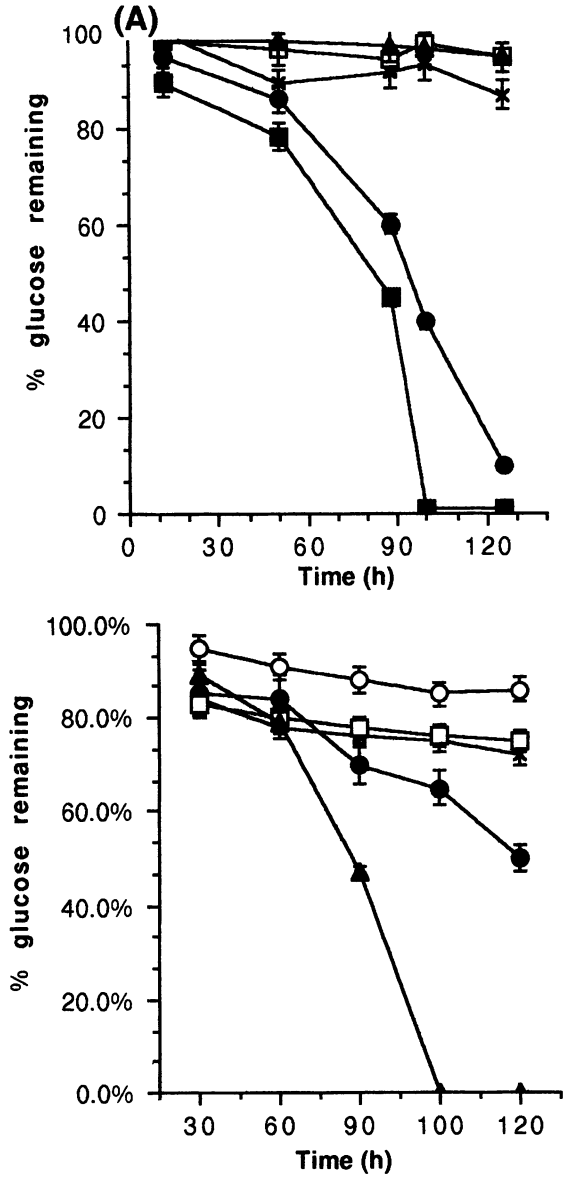

FIG. 4. Glucose consumption of BAM treated with unencapsulated *M. dispar*, encapsulated *M. dispar*, or purified capsule in the absence of endotoxin stimulation (A) or after endotoxin stimulation (B). Symbols are as described in the legend to Fig. 2. ○, untreated and unstimulated control BAM. Means of triplicate wells with standard deviations of the means are recorded.

plasmas, they were refractory to activation by endotoxin, as evidenced by lowered glucose consumption (Fig. 4B).

DISCUSSION

Pathogenic microorganisms have evolved several strategies to avoid phagocytosis by interfering at distinct steps of the attachment, ingestion, and killing process. Among these, microbial capsules were considered the most important and ubiquitous antiphagocytic substance (15). Some microorganisms possess surface structures that enable them to attach to phagocytes without being ingested. *Neisseria gonorrhoeae* and several mycoplasmas have been reported to be among these microorganisms, and in *N. gonorrhoeae* such a structure was linked to its capsule (9). While it is not known if the capsule of *M. dispar* plays a role in attachment, it has been shown that capsule production occurs in vivo or in coculture with bovine cells (3).

The results presented in this report indicated that the capsule of *M. dispar* exerts antiphagocytic activity. When encapsulated or unencapsulated *M. dispar* was exposed to alveolar macrophages in the absence of specific antibodies, no loss of viability of the mycoplasma was detected. In contrast, in the presence of antibodies specific to capsule or surface proteins, significant loss of viability of encapsulated or unencapsulated *M. dispar*, respectively, was detected. The effectiveness of anti-capsule antibody in obtaining complete loss of encapsulated mycoplasma viability is of importance, since chronic colonization of young cattle with *M. dispar* has been documented (27). Presumably, young calves do not mount adequate immune responses to this capsule. A parallel situation is described for cystic fibrosis patients with *Pseudomonas aeruginosa* infections (21). The failure to obtain the same reduction in mycoplasma viability observed in the system which included unencapsulated mycoplasmas, *M. dispar* antiserum, and BAM can be explained by the persistence of a low percentage of encapsulated *M. dispar* in unencapsulated mycoplasmas grown in broth media (3). This fraction of the mycoplasma population will resist phagocytosis in the absence of specific anti-capsule antibodies.

The purified *M. dispar* capsule is shown here to have a clearly inhibitory role in BAM activation. The mechanism of this inhibitory action is unclear at this time, and it may be exerted through nonspecific or specific interactions with structures on the BAM surface. With mucoid strains of *P. aeruginosa*, specific mannose receptors on the macrophage surface appear to be involved in this interaction (26).

A previous report showed that *M. dispar* exerts an immunosuppressive effect when injected in combination with other antigens (16). It also was reported that *M. dispar* exerts an inhibitory effect on the mixed lymphocyte response to mitogens. It also was suggested that this suppressive effect could be related to the avidity with which this mycoplasma species adheres to the host cell membrane, resulting in alteration in the membrane/receptor structures on the host cell and the production of a suppressive factor (10, 16). In the present report we demonstrate that encapsulated *M. dispar* or purified capsule did not induce, and in fact suppressed, induction of TNF and IL-1 production by BAM, in contrast to what was seen when unencapsulated *M. dispar* was used. In addition, encapsulated mycoplasma or purified capsule did not induce and could suppress induction of glucose consumption by BAM. In contrast, activation of macrophages was observed when macrophages were incubated with unencapsulated *M. dispar* or with endotoxin.

It was observed that encapsulated *M. dispar* induced slightly higher levels of TNF production and glucose consumption compared with that of purified capsule. These differences can be explained by the small percentage of unencapsulated mycoplasmas that are detected when expression of mycoplasma capsule was induced by coculture (3). It also was shown that IL-1 production by BAM stimulated with heat-killed encapsulated mycoplasmas was significantly higher than that observed with live encapsulated *M. dispar*. It is possible that during the process of heat killing, capsule material was lost from the surface of the mycoplasma, unmasking regions of the membrane that could react with the IL-1 receptor in the macrophage cell surface. It also was possible that fractions of disrupted membrane, released during heat killing, could react with BAM, causing IL-1

production. These differences were not detected with TNF production, which indicated that TNF and IL-1 induction in BAM could be dissociated, since the same supernatants were sampled to run both assays.

It was previously shown that *Mycoplasma mycoides* subsp. *mycoides* (LC) induced TNF production and that injection of TNF in goats reproduced the disease caused by this mycoplasma species (23). It also was postulated that *Mycoplasma bovis* may induce production of cytokines, and the effect of these could explain in part the necrotic lesions and lymphocyte accumulations detected in the lung of naturally infected calves (10). In the present report we show that encapsulated *M. dispar* did not induce production of IL-1 and TNF or glucose consumption by BAM. The lack of macrophage activation could explain the poor immunogenic reactivity induced by *M. dispar* and also the characteristic interstitial alveolitis without peribronchial lymphocyte accumulations caused by this mycoplasma species (12, 16).

The inhibitory effect exerted by the capsular material or encapsulated *M. dispar* on activation and secretion of cytokines defines the important influence that the infection by *M. dispar* may have on the development of severe pneumonic processes. We are aware of the fact that these results describe the interaction of *M. dispar* with BAM under in vitro conditions, and these may not completely reflect the in vivo situation. However, the immunosuppressive effects previously reported (16) and the results presented here can be taken together to help define the role that *M. dispar* has in the development of pneumonic conditions in cattle.

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