INFECTION AND IMMUNITY, Apr. 1989, p. 1131–1136
0019-9567/89/041131-06$02.00/0

Vol. 57, No. 4

# Biological Activities of Monoclonal Antibodies to *Mycoplasma pneumoniae* Membrane Glycolipids

DONNA K. F. CHANDLER,† LYN D. OLSON, JAMES G. KENIMER, PETER G. PROBST, SHLOMO ROTTEM,‡ MARION W. GRABOWSKI, AND MICHAEL F. BARILE*

*Laboratory of Mycoplasma and Laboratory of Cellular Physiology, Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892*

Received 9 September 1988/Accepted 24 December 1988

**A purified preparation of membranes was obtained by using a unique method of treating *Mycoplasma pneumoniae* with the ATPase inhibitor, diethylstilbestrol. This method was shown to yield highly purified membranes with little or no cytoplasmic contamination. These membranes were used to immunize mice for subsequent productions of monoclonal antibodies (MAbs). Hybridoma culture supernatants were screened by enzyme-linked immunosorbent assay with whole-cell *M. pneumoniae* and lipid extract antigens. Four stable MAbs were obtained and characterized. MAb CP3-46F5 reacted with a protein of a molecular weight of approximately 52,000 as determined by Western blot (immunoblot). MAbs CP3-50C2, CP3-53C5, and CP3-53C8 did not react with any antigens on Western blots but did bind to at least 10 distinct glycolipid bands as determined by orcinol staining on thin-layer chromatograms of *M. pneumoniae* lipid extracts. The MAbs did not react with similarly prepared lipid extracts from *Mycoplasma genitalium*, *Mycoplasma neurolyticum*, and *Mycoplasma gallisepticum*. These MAbs did not inhibit *M. pneumoniae* metabolism or attachment to WiDr cell cultures. The anti-glycolipid MAbs recognize determinants specific to *M. pneumoniae*, unlike polyclonal hyperimmune sera against *M. pneumoniae*, which cross-react with lipid extracts of *M. genitalium*.**

*Mycoplasma pneumoniae*, the agent of primary atypical pneumonia, has many in vitro activities which are related to pathogenicity in vivo. The organism adheres to erythrocytes, cultured cells, and tracheal organ cultures; inhibits protein and RNA synthesis; and alters nucleotide metabolism of cells in culture. It also causes ciliostasis and cytotoxicity of tracheal organ cultures and induces leukocyte recruitment in hamster lungs (reviewed in references 11 and 28). A number of these activities have also been produced with cell extracts of *M. pneumoniae* (7, 8). The correlation between virulence and in vitro cytadsorption has been established (1, 8).

One approach to identify and characterize the virulence components of an organism has been to develop a library of monoclonal antibodies (MAbs) with defined specificities. Since the adherence activities of *M. pneumoniae* are localized on the surface, it is desirable to produce MAbs which are specific for membrane-associated antigens. The rationale of immunizing with membrane preparations was to enhance the production of MAbs specific for membrane determinants. However, since *M. pneumoniae* is osmotically stable, membrane studies have been hampered by the lack of an efficient method to isolate purified membrane preparations (27). Recent studies have shown that the ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide (DCCD) causes lysis of *Mycoplasma gallisepticum*, yielding membrane preparations of high purity (33). However, this same method was not effective for the preparation of membranes from *M. pneumoniae*.

In this study we report a unique procedure for preparing purified *M. pneumoniae* membranes by treating the intact cells with the ATPase inhibitor, diethylstilbestrol (DES) (22). These membranes were used to immunize mice for the production of MAbs. The biological activities of these MAbs, which are specific for *M. pneumoniae* glycolipids, are also described.

## MATERIALS AND METHODS

**Growth and labeling of organisms.** Virulent *M. pneumoniae* M129 and *Mycoplasma genitalium* G37C were grown on the surfaces of T150 tissue culture flasks in Edward-Hayflick broth (8). *Mycoplasma neurolyticum* PG28 and *M. gallisepticum* A5969-T4 were also grown in Edward-Hayflick broth, and cells were harvested and washed with phosphate-buffered saline (PBS) by centrifugation at 10,000 × *g* for 20 min. *M. pneumoniae* was radiolabeled by addition of [$^3$H]palmitic acid (Dupont, NEN Research Products, Boston, Mass.) to the medium (1 mCi/liter). Harvested, washed cells were stored frozen in small portions at −70°C, as previously described (5, 6).

**Preparation of *M. pneumoniae* membranes.** After *M. pneumoniae* was grown, the growth medium was decanted, and the organisms attached to the surface were washed twice with 25 ml of 0.25 M NaCl solution in 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 8.5 (NaCl-HEPES). Five milliliters of 100 mM DES in NaCl-HEPES was added to each of the flasks and incubated for 1 h at 37°C with constant shaking. Membranes and unlysed cells were scraped off the surface, passed twice through a 23-gauge needle to disperse clumps, and sonicated in a bath sonicator for 10 min. Unlysed cells were removed by centrifugation at 3,000 rpm for 3 min in a Sorval RC2-B centrifuge (Dupont Instruments, Wilmington, Del.), and membranes were collected by centrifuging the supernatant fluid at 34,000 × *g* for 30 min in an L-5 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The membrane preparations (2 mg of protein per ml) were washed twice, resuspended in the NaCl-HEPES solution, and stored frozen at

* Corresponding author.
† Present address: Food and Drug Administration, Center for Food Safety and Applied Nutrition, Division of Microbiology, Washington, DC 20204.
‡Present address: The Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel.

−70°C until used. Membranes labeled with tritiated palmitic acid were used for the initial characterization, and unlabeled membranes were used to immunize mice for preparation of MAbs.

Protein concentration was determined by the method of Lowry et al. (21). Lipids were extracted by the procedure of Bligh and Dyer (3). Sucrose density gradient analysis of the membrane preparation to determine lipid content was performed as previously described (9). NADH dehydrogenase activity in intact *M. pneumoniae* cells and cell fractions was determined in reaction mixtures containing 10 to 100 pg of cell protein, 0.2 M NaCl, 25 mM Tris hydrochloride (pH 7.5), 5 mM 2-mercaptoethanol, and 1 mg of sodium deoxycholate in a volume of 1 ml. The reaction mixture was preincubated for 2 min at 37°C, and the reaction was started by the addition of 50 μl of a freshly prepared NADH solution (3 mg/ml in 25 mM Tris hydrochloride, pH 7.5). The initial rate of enzymatic activity was expressed as the decrease in $A_{340}$ per minute per milligram of protein.

**Immunization of mice and hybridoma preparation.** Two BALB/c mice were initially injected intraperitoneally (i.p.) with 240 μg of *M. pneumoniae* membrane protein emulsified in complete Freund adjuvant. On days 14 and 29, these animals received additional i.p. injections of 240 μg of *M. pneumoniae* membrane protein in incomplete Freund adjuvant. An additional two mice were initially injected with 240 μg of *M. pneumoniae* membrane protein adsorbed to alum and mixed with an equal volume of U.S. Standard Pertussis Vaccine lot 7B. On days 14 and 29, these animals received additional i.p. injections of 240 μg of *M. pneumoniae* membrane protein adsorbed to alum (without pertussis vaccine). On day 42, all mice received i.p. injections of 240 μg of *M. pneumoniae* membrane protein in PBS. On day 45, the spleen was removed from one mouse from each immunization group, and $2.3 \times 10^8$ washed mononuclear cells were mixed with $2.1 \times 10^8$ Sp2/0-Ag14 myeloma cells. Fusion was performed by a modification of the method of Galfre et al. (12) as previously described (13).

**ELISA procedures.** The initial screening of the hybridomas with whole-cell suspensions was performed by enzyme-linked immunosorbent assay (ELISA) as reported previously (14). For lipid ELISAs, total mycoplasma lipid extracts were prepared by the procedure of Bligh and Dyer (3) as described by Morrison-Plummer et al. (23) and were dissolved in chloroform-methanol (1:10) at approximately 1.25 mg/ml. ELISA plates prepared by coating wells with the lipid preparations diluted 1:50 in 95% ethanol were used to screen the MAb preparations.

**Western blot (immunoblot) procedures.** A whole-cell suspension of *M. pneumoniae* was run on a sodium dodecyl sulfate-polyacrylamide gel (12% acrylamide, 2.7% bisacrylamide) by the method of Laemmli (18). Proteins were electrophoretically transferred to nitrocellulose as described by Dunn (10) at 0.8 A for 1.5 h at 10°C. After transfer, the nitrocellulose sheet was cut into strips, and one strip was stained for protein with 0.01% amido black in 45% methanol–10% acetic acid. The other strips were incubated overnight at room temperature with MAb preparations diluted 1:100 or 1:1,000. The strips were individually washed three times, incubated with the peroxidase-conjugated anti-mouse antibody (1:500, 3 h, room temperature), washed, and incubated with the peroxidase substrate 4-chloro-1-naphthol (60 mg in 20 ml of cold methanol, 100 ml of deionized water, and 75 μl of 30% $H_2O_2$). Color development was stopped by transferring the strips to a solution of 2 mM sodium azide.

**High-performance thin-layer chromatography of mycoplasmal lipids and immunostaining.** Lipid extracts enriched for glycolipids were prepared as described by Brockhaus (4). Briefly, the procedure entails suspending the mycoplasma cell pellet from 500 ml of culture in 2 ml of methanol. An equal volume of chloroform was added, and the mixture was incubated at room temperature for 15 min. After centrifugation (5,000 rpm, 5 min, Sorvall RC2-B), the supernatant was transferred to another tube and emulsified with 2 ml of chloroform and 1.2 ml of water by repeated passage through a Pasteur pipette. The phases were separated by centrifugation at 5,000 rpm for 5 min (Sorvall RC2-B), and the aqueous phase containing a preparation enriched for glycolipids was dried at 56°C under $N_2$. The preparation was suspended in chloroform-methanol (1:9) and stored at −20°C. Reference standards of monogalactosyl diglyceride, digalactosyl diglyceride, cholesterol, and phosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, Mo.

Mycoplasma glycolipid preparations (30 μg) or 1 μg of each standard was applied to aluminum-backed high-performance thin-layer chromatography sheets (10 by 20 cm; Camag Scientific, Wrightsville Beach, N.C.) and chromatographed for 35 min in 50% chloroform–40% methanol–0.25% KCl. The chromatogram was cut into strips, and one set of strips was sprayed with orcinol reagent (Sigma) while replicate strips were used for immunostaining.

For immunostaining, thin-layer chromatography plates were coated with polyisobutylmethacrylate (0.1% in hexane; Sigma) and allowed to dry. The treated plates were coated with 0.5% bovine serum albumin (BSA) in PBS, incubated in MAbs diluted 1:100 in 0.5% BSA-PBS for 2 to 3 h, rinsed three times in BSA-PBS, incubated in peroxidase-labeled goat anti-mouse antibody (Cappel Laboratories, Cochranville, Pa.), diluted 1:200, rinsed, and submerged in the same peroxidase substrate used for visualization of Western blots. Bands developed within 20 min, and the strips were rinsed in distilled water and photographed.

**Adherence assays.** Procedures for attachment and inhibition of attachment with radiolabeled *M. pneumoniae* and human WiDr cell monolayers were performed as described previously (6). Attachment of WiDr cell suspensions to *M. pneumoniae* monolayers was also performed by using a modification of the procedure of Jacobs et al. (13). To screen the MAbs for inhibition of attachment, the *M. pneumoniae* monolayers were preincubated with the hybridoma supernatants or ascites fluid prior to the addition of the WiDr cell suspensions.

**Epi-immunofluorescence.** Hybridoma supernatants containing MAbs diluted 1:10 to 1:80 in Hanks balanced salt solution were incubated for 30 min at room temperature with *M. pneumoniae* colonies grown on agar or on cover slips (10 by 22 mm). The colonies were washed with PBS, and prefiltered, fluoresceinated, affinity-purified goat anti-mouse immunoglobulin (Cappel) diluted 1:50 in PBS plus 10% heat-inactivated fetal calf serum (Flow Laboratories, Inc., McLean, Va.) was applied for 30 min at room temperature. The samples were washed with PBS and examined by epifluorescence microscopy.

**MI test.** Tests of metabolic inhibition (MI) by antibody were carried out as described elsewhere (36). Complement was not used.

## RESULTS

**Preparation of *M. pneumoniae* membranes.** Initial attempts were made to prepare membranes of *M. pneumoniae* by treatment with DCCD, as previously described for *M. galli-*

TABLE 1. Characteristics of *M. pneumoniae* membranes isolated by DES-induced lysis

| Prepn[a] | Protein (mg) | Density (g/ml) | Radioactivity | | NADH dehydrogenase activity[b] |
|---|---|---|---|---|---|
| | | | cpm ($10^6$) | cpm/mg of protein | |
| Untreated intact cells | 4.10 | ND[c] | 10.5 | 2.55 | 2.95 |
| DES-treated isolated membranes | 0.55 | 1.17 | 3.20 | 5.82 | 0.15 |
| Soluble fractions | 0.90 | ND | 0.11 | 0.12 | 9.10 |
| DES-treated unlysed cells | 2.80 | ND | 8.68 | 3.10 | 2.10 |

[a] *M. pneumoniae* M129 cells were grown on the surface of eight T150 tissue culture flasks containing Edward-Hayflick broth and [$^3$H]palmitic acid. Four of the flasks were treated with DES, and four were kept for untreated control cells. Membrane isolation was performed as described in Materials and Methods.

[b] NADH dehydrogenase activity measured as decrease in $A_{340}$ per minute per milligram of protein.

[c] ND, Not done.

TABLE 2. Characteristics of MAbs and rabbit sera prepared against *M. pneumoniae* membranes

| MAb or serum | Immunoglobulin isotype | ELISA reactivity ($A_{410}$) | | |
|---|---|---|---|---|
| | | *M. pneumoniae* | | *M. genitalium* lipid |
| | | Cells | Lipid | |
| CP3-50C2 | M | 0.684 | 0.819 | 0.002 |
| CP3-53C5 | G3 | 0.912 | 1.000 | 0.003 |
| CP3-53C8 | M | 0.993 | 1.009 | 0.000 |
| CP3-46F5 | G1 | 0.833 | 0.130 | 0.000 |
| Rabbit anti-*M. pneumoniae* (1:100 dilution) | | ND[a] | 1.156 | 0.337 |
| Rabbit anti-*M. genitalium* (1:100 dilution) | | ND | 0.440 | 1.545 |
| Rabbit normal (1:100 dilution) | | 0.000 | 0.000 | 0.000 |

[a] ND, Not determined.

*septicum* (33). *M. pneumoniae* was resistant to DCCD-induced lysis but was susceptible to lysis induced by another ATPase inhibitor, DES (22).

**Characteristics of the *M. pneumoniae* membranes.** The radiolabeled membranes obtained by DES lysis were extensively analyzed, and their properties are given in Table 1. The values for counts per minute per milligram of protein, the lipid-to-protein ratio of these membranes, were two to three times higher than those of intact cells; and as determined by a sucrose density gradient, the membranes appeared as a sharp band having a density of 1.17 g/ml. The *M. pneumoniae* membranes prepared by DES lysis were within the density range reported for membranes of other mycoplasmas obtained by osmotic lysis (32), digitonin treatment (31), or DCCD lysis (33). Furthermore, retention of cellular lipids in the membranes was greater than 95%, as determined by the ratio of radioactivity (counts per minute) in the membranes to total radioactivity in the membrane preparation plus soluble fraction after unlysed cells were centrifuged out, although the percentage of radioactive cells lysed by DES was 20 to 30%. Membranes obtained by DES lysis exhibited very little or no NADH dehydrogenase activity, previously shown to be localized in the cytoplasmic fraction of *Mycoplasma* species (26). These results indicate that this procedure provides a simple method for lysis of *M. pneumoniae* and that the preparations of *M. pneumoniae* membranes obtained by lysis with DES are of high purity.

**Characteristics of MAbs.** Four stable MAbs were obtained with the *M. pneumoniae* membrane preparations. Some of the immunologic characteristics of the MAbs obtained from this fusion are presented in Table 2. Three of the MAbs (CP3-50C2, CP3-53C5, and CP3-53C8) displayed reactivity with the whole *M. pneumoniae* cells and with *M. pneumoniae* lipid extract. These MAbs did not react with similarly prepared lipid extracts or with glycolipid extracts from *M. genitalium* cells, or with glycolipids prepared from *M. neurolyticum* or *M. gallisepticum* cells (not shown). In contrast, rabbit antisera raised against whole *M. pneumoniae* or *M. genitalium* cells (Table 2) showed significant cross-reactivity with the lipid extract of the heterologous species. One MAb (CP3-46F5) displayed approximately sevenfold-greater ELISA reactivity with the whole-cell preparation than with the lipid extract. It was also the only MAb of the four to show reactivity in a Western blot of an *M. pneumoniae* cell extract (Fig. 1). This MAb reacted strongly with a single band that migrated with a relative molecular weight of 52,000.

**High-performance thin-layer chromatography of lipid extracts.** To examine the specificity of the three MAbs that exhibited no affinity for protein, *M. pneumoniae* glycolipids from whole cells were separated by high-performance thin-layer chromatography, reacted with MAbs, and visualized with an enzyme-linked second antibody. No immunostaining

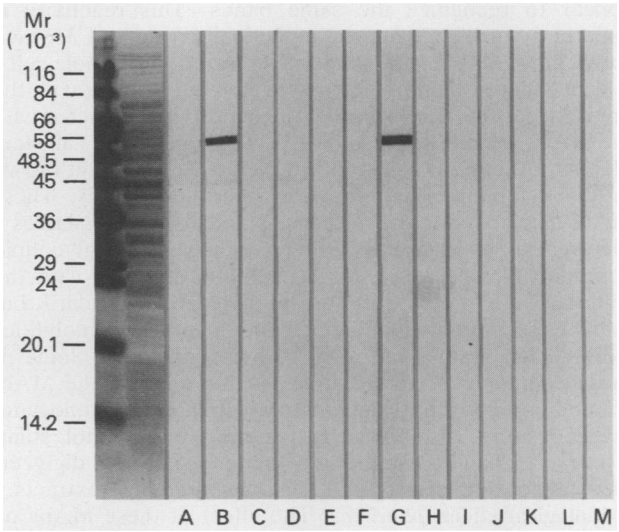


FIG. 1. Western blot of *M. pneumoniae* homogenate. The first nitrocellulose strip shows the amido black staining pattern of the electrophoresis standards and of the *M. pneumoniae* homogenate. Strips A through E and strip M were incubated with 10 μl of ascites fluid diluted to 10 ml with Brij 35-Dulbecco PBS (DPBS). Strips F through K were incubated with 100 μl of hybridoma culture medium (culture passage number [CPN] given in parentheses) diluted to 10 ml in Brij 35-DPBS. Strip L was incubated with Brij 35-DPBS only. Each strip had approximately 25 μg of protein of *M. pneumoniae* homogenate. A, CP3-50C2; B, CP3-46F5x3; C, CP3-53C5; D, CP3-53C8x6; E, a nonspecific CP3 clone; F, CP3-53C5 (CPN 5 and 6); G, CP3-46F5x3 (CPN 10); H, CP3-50C2 (CPN 8 to 11); I, CP3-53C5 (CPN 11); J, CP3-53C8x6 (CPN 6 and 7); K, a nonspecific CP3 clone (CPN 7 to 10); L, blank; M, 18.2.12.6 (a MAb specific for tetanus toxin).

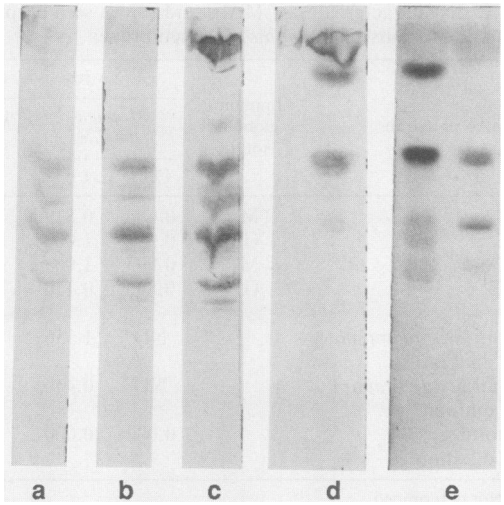


FIG. 2. Immunostaining of *M. pneumoniae* glycolipids with MAbs raised against *M. pneumoniae* membranes. Strips a, b, and c are chromatograms of 30 μg of *M. pneumoniae* lipid extract incubated with MAbs. Strip d is a chromatogram of 1 μg of each standard (phosphatidylcholine, cholesterol, digalactosyl diglyceride, and galactosyl diglyceride) incubated with a MAb. Strip e is a chromatogram of both the standards and *M. pneumoniae* lipid extract, sprayed with orcinol reagent. a, CP3-50C2; b, CP3-53C5; c, CP3-53C8; d, CP3-53C8; e, orcinol reagent.

was observed with the MAb CP3-46F5. Figure 2 shows the results of immunostaining with MAbs CP3-53C5, CP3-50C2, and CP3-53C8. The MAbs reacted with at least 10 distinct bands on the thin-layer chromatograms, and all three MAbs appear to recognize the same bands. This reactivity is apparently missing from the glycolipid extracts of *M. neurolyticum*, *M. gallisepticum*, and *M. genitalium* whole cells, because there was no reactivity by immunostaining with the lipid extracts of these species. The bands which reacted with the MAbs are presumed to be glycolipids since they all stain blue with the orcinol reagent, in contrast to the faint brown or gray staining observed with other lipid bands. These MAbs also bound to the reference standards monogalactosyl diglyceride and digalactosyl diglyceride. A major glycolipid component of *M. pneumoniae* extracts migrates with the same mobility as the digalactosyl diglyceride standard, but there were a number of other components with mobilities slower than that of the digalactosyl diglyceride. Some of these components were distinguishable only on the MAb-reacted immunostains, demonstrating that the immunostaining procedure is more sensitive than the orcinol stain. However, a band comigrating with monogalactosyl diglyceride was not detected in the *M. pneumoniae* lipid extracts.

**Biological effects of MAbs.** The effect of these MAbs on adherence was examined by using two experimental procedures, adherence of radiolabeled *M. pneumoniae* to WiDr cell monolayers and adherence of WiDr cell suspensions to *M. pneumoniae* colonies. The three glycolipid-specific MAbs had no effect on adherence as measured by these assays, although inhibition of attachment of WiDr cells to *M. pneumoniae* was readily observed with hyperimmune rabbit antiserum, which was used as a positive control (data not shown).

Neither the antibodies reacting with the *M. pneumoniae* glycolipids nor the antibody reactive with the 52-kilodalton protein exhibited detectable inhibitory activity in the MI test, although hyperimmune anti-*M. pneumoniae* serum prepared in rabbits and a reference mule antiserum gave positive reactions. The MI titers were 1:1,024 for the reference mule antiserum and 1:128 for rabbit anti-*M. pneumoniae* antisera.

The ability of these MAbs to react with surface components of *M. pneumoniae* was examined by indirect immunofluorescence microscopy. The glycolipid-reactive antibodies, CP3-50C2, CP3-53C5, and CP3-53C8, were strongly reactive with *M. pneumoniae* colonies (data not shown).

## DISCUSSION

Although mycoplasmas lack a cell wall and are bound by a single membrane, several *Mycoplasma* species, including *M. pneumoniae*, are osmotically stable (27). Therefore, it has been difficult to prepare *M. pneumoniae* membranes free of cytoplasmic components. In this article, we describe a unique procedure for the isolation of purified *M. pneumoniae* membranes by treating intact cells with the ATPase inhibitor DES. The procedure resulted in only 20 to 30% of the cells being lysed, probably because surface-grown cultures form clumps and only cells exposed to the DES-containing buffer may have lysed. Nevertheless, these membrane preparations were highly purified, because they lacked soluble NADH dehydrogenase activity and retained over 95% of the cellular lipids (Table 1).

Shirvan et al. (33) prepared purified membranes of *M. gallisepticum* by inhibiting ATPase activity with DCCD. They suggested that the membrane-bound ATPase of *M. gallisepticum* plays a major role in cell volume regulation by pumping out $Na^+$ that enters the cells by diffusion (30). The $Na^+$ is pumped out by either a direct mechanism operated by an $Na^+$-dependent ATPase (34) or a secondary $Na^+$-$H^+$ exchange reaction that follows proton translocation by a $H^+$-dependent ATPase (20). Our finding that lysis of *M. pneumoniae* was induced by the ATPase inhibitor DES suggests that an ATPase might play a major role in volume regulation of this mollicute as well. If so, the inability to induce lysis of *M. pneumoniae* by DCCD suggests that ATPases with different modes of action or sensitivities are found in *M. pneumoniae* and *M. gallisepticum*.

The MAbs produced by using the DES-lysed cell membranes recognize determinants present on a number of lipid components of *M. pneumoniae*. These components were most likely glycolipids, since there was correspondence between the immunostaining pattern and the blue-staining orcinol reactivity which indicates the presence of carbohydrate. Moreover, the MAbs also reacted with both the monogalactosyl and digalactosyl diglyceride standards but not with cholesterol or phosphatidylcholine. The possibility that the determinants recognized are on the lipid portion of the molecules has not been ruled out. Morrison-Plummer et al. (23) also described anti-*M. pneumoniae* lipid MAbs which cross-reacted in ELISAs with multiple fractions of thin-layer chromatography-separated lipids, some of which contained carbohydrate.

The MAbs showed no inhibitory effect on the ability of *M. pneumoniae* to attach to human WiDr cells. These observations differ from those of Morrison-Plummer et al. (24), who found that the anti-lipid MAbs enhanced attachment of *M. pneumoniae* to chicken erythrocytes by 20 to 50%. Either the MAbs have different specificities or *M. pneumoniae* attaches to WiDr cells and chicken erythrocytes by different mechanisms.

The concept that glycolipids are responsible for much of the classical serological activity of *M. pneumoniae* has long

been accepted. Complement fixation activity (2, 16, 17, 25), as well as MI activity (29), was found in sera specific for *M. pneumoniae* glyceroglycolipid fractions composed of digalactosyl diglyceride and triglycosyl diglycerides containing both galactose and glucose (35). Morrison-Plummer et al. (24) reported that anti-lipid MAbs did not inhibit incorporation of [$^3$H]thymidine by *M. pneumoniae*. In our study, the mouse MAbs which recognized epitopes on *M. pneumoniae* glycolipids did not have MI antibody activity but were reactive with *M. pneumoniae* surface components by indirect fluorescence microscopy. This lack of functional interaction could indicate that the MAbs react with an epitope(s) not involved in these metabolic activities or that the MAbs cannot cross-link to produce inhibition of metabolic functions.

Cross-reactivity of antibodies against *M. pneumoniae* lipids with the lipids of *M. neurolyticum* and *M. genitalium* has been reported (15, 19; K. Lind, Lancet **ii**:1158–1159, 1982). Chloroform-methanol extracts of *M. pneumoniae* MAC and extracts of *M. genitalium* G37C showed antigenic cross-reactions with rabbit and human immune sera by complement fixation (Lind, Lancet) and by crossed immunoelectrophoresis (19), reactions which were attributed to similar cross-reactive glycolipid antigens. In our studies with the ELISA, the MAbs reactive with *M. pneumoniae* glycolipids did not bind to whole-lipid extracts of *M. genitalium* cells (Table 2). However, polyclonal rabbit antisera reacted with the chloroform-methanol extracts of both species. Furthermore, the MAbs reactive with the glycolipids of *M. pneumoniae* did not result in immunostaining of the thin-layer chromatograms of lipid extracts from *M. genitalium*, *M. neurolyticum*, or *M. gallisepticum*. Thus, these MAbs can differentiate *M. pneumoniae* from *M. genitalium* and may provide a useful diagnostic tool to distinguish species. A better understanding of the antigenic specificity of the serological responses to *M. pneumoniae* and its cross-reactions with *M. genitalium* is needed and may require a more thorough structural analysis of the glycolipid components of these two species.

In summary, this report describes a unique method of producing high-purity preparations of *M. pneumoniae* membranes. Three MAbs produced by using the membranes as antigens appear specific for *M. pneumoniae* glycolipids, without cross-reactivity to *M. genitalium*, *M. neurolyticum*, and *M. gallisepticum*.

## LITERATURE CITED


1. **Barile, M. F., D. K. F. Chandler, H. Yoshida, M. W. Grabowski, R. Harasawa, and S. Razin.** 1988. Parameters of *Mycoplasma pneumoniae* infection in Syrian hamsters. Infect. Immun. **56**: 2443–2449.
2. **Beckman, B. L., and G. E. Kenny.** 1968. Immunochemical analysis of serologically active lipids of *Mycoplasma pneumoniae*. J. Bacteriol. **96**:1171–1180.
3. **Bligh, E. G., and W. J. Dyer.** 1969. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**: 911–917.
4. **Brockhaus, M.** 1985. Detection of glycolipid antigens with monoclonal antibodies, p. 133–145. *In* I. Lefkovits and B. Pernis (ed.), Immunological methods, vol. 3. Academic Press, Inc., New York.
5. **Chandler, D. K. F., and M. F. Barile.** 1980. Ciliostatic, hemagglutinating, and proteolytic activities in a cell extract of *Mycoplasma pneumoniae*. Infect. Immun. **29**:1111–1116.
6. **Chandler, D. K. F., M. W. Grabowski, and M. F. Barile.** 1982. *Mycoplasma pneumoniae* attachment: competitive inhibition by mycoplasmal binding component and by sialic acid-containing glycoconjugates. Infect. Immun. **38**:598–603.
7. **Chandler, D. K. F., M. W. Grabowski, A. S. Rabson, and M. F. Barile.** 1987. Further studies on the *Mycoplasma pneumoniae* extract: ciliostatic and cell recruitment activities. Isr. J. Med. Sci. **23**:580–584.
8. **Chandler, D. K. F., S. Razin, E. B. Stephens, R. Harasawa, and M. F. Barile.** 1982. Genomic and phenotypic analyses of *Mycoplasma pneumoniae* strains. Infect. Immun. **38**:604–609.
9. **Cirillo, V. P., A. Katzenell, and S. Rottem.** 1987. Sealed vesicles prepared by fusing *M. gallisepticum* membranes and preformed lipid vesicles. Isr. J. Med. Sci. **23**:380–383.
10. **Dunn, S. D.** 1986. Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. Anal. Biochem. **157**:144–153.
11. **Gabridge, M. G., D. K. F. Chandler, and M. J. Daniels.** 1985. Pathogenicity factors in mycoplasmas and spiroplasmas, p. 313–351. *In* S. Razin and M. F. Barile (ed.), The mycoplasmas, vol. 4. Mycoplasma pathogenicity. Academic Press, Inc., New York.
12. **Galfre, G., S. C. Howe, C. Milstein, G. W. Butcher, and J. M. Howard.** 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature (London) **266**:550–552.
13. **Jacobs, E., K. Schoepperle, and W. Bredt.** 1985. Adherence inhibition assay: a specific serological test for detection of antibodies to *Mycoplasma pneumoniae*. Eur. J. Clin. Microbiol. **4**:113–118.
14. **Kenimer, J. G., W. H. Habig, and M. C. Hardegree.** 1983. Monoclonal antibodies as probes of tetanus toxin structure and function. Infect. Immun. **42**:942–948.
15. **Kenny, G. E.** 1971. Serological cross-reaction between lipids of *Mycoplasma pneumoniae* and *Mycoplasma neurolyticum*. Infect. Immun. **4**:149–153.
16. **Kenny, G. E., and J. T. Grayston.** 1965. Eaton pleuropneumonia-like organisms (*Mycoplasma pneumoniae*) complement-fixing antigen: extraction with organic solvents. J. Immunol. **95**:19–25.
17. **Kenny, G. E., and R. M. Newton.** 1973. Close serological relationship between glycolipids of *Mycoplasma pneumoniae* and glycolipids of spinach. Ann. N.Y. Acad. Sci. **225**:54–61.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lind, K., B. O. Lindhardt, H. J. Schutten, J. Blom, and C. Christiansen.** 1984. Serological cross-reactions between *Mycoplasma genitalium* and *Mycoplasma pneumoniae*. J. Clin. Microbiol. **20**:1036–1043.
20. **Linker, C., and T. H. Wilson.** 1985. Sodium and proton transport in *Mycoplasma gallisepticum*. J. Bacteriol. **163**:1250–1257.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **McEnery, M. W., and P. L. Pederson.** 1986. Diethylstilbestrol: a novel F-directed probe of the mitochondrial ATPase. J. Biol. Chem. **262**:1745–1752.
23. **Morrison-Plummer, J., D. H. Jones, and J. B. Baseman.** 1983. An ELISA to detect monoclonal antibodies specific for lipid determinants of *Mycoplasma pneumoniae*. J. Immunol. Methods **64**:165–178.
24. **Morrison-Plummer, J., D. K. Leith, and J. B. Baseman.** 1986. Biological effects of anti-lipid and anti-protein monoclonal antibodies on *Mycoplasma pneumoniae*. Infect. Immun. **53**:398–403.
25. **Plackett, P., B. P. Marmion, E. J. Shaw, and R. M. Lemcke.** 1969. Immunochemical analysis of *Mycoplasma pneumoniae*. 3. Separation and chemical identification of serologically active lipids. Aust. J. Exp. Biol. Med. Sci. **47**:171–195.
26. **Pollack, J. D., S. Razin, M. E. Pollack, and R. C. Cleverdon.** 1965. Fractionation of mycoplasma cells for enzyme localization. Life Sci. **4**:973–977.
27. **Razin, S.** 1963. Osmotic lysis of mycoplasma. J. Gen. Microbiol. **33**:471–475.
28. **Razin, S.** 1985. Mycoplasma adherence, p. 161–202. *In* S. Razin and M. F. Barile (ed.), The mycoplasmas, vol. 4. Mycoplasma

pathogenicity. Academic Press, Inc., New York.
29. **Razin, S., B. Prescott, G. Caldes, W. D. James, and R. M. Chanock.** 1970. Role of glycolipids and phosphatidylglycerol in the serological activity of *Mycoplasma pneumoniae*. Infect. Immun. **1**:408–416.
30. **Rottem, S., C. Linker, and T. H. Wilson.** 1981. Proton motive force across the membrane of *Mycoplasma gallisepticum* and its possible role in cell volume regulation. J. Bacteriol. **145**:1299–1304.
31. **Rottem, S., and S. Razin.** 1972. Isolation of mycoplasma membranes by digitonin. J. Bacteriol. **110**:699–705.
32. **Rottem, S., O. Stein, and S. Razin.** 1968. Reassembly of mycoplasma membranes disaggregated by detergents. Arch. Biochem. Biophys. **125**:46–56.
33. **Shirvan, M. H., S. Rottem, Z. Ne'eman, and R. Bittman.** 1982. Isolation of mycoplasma membranes by dicyclohexylcarbodiimide-induced lysis. J. Bacteriol. **149**:1124–1128.
34. **Shirvan, M. H., S. Schuldiner, and S. Rottem.** 1987. Control of sodium fluxes in *Mycoplasma gallisepticum*. Isr. J. Med. Sci. **23**:384–388.
35. **Smith, P. F.** 1979. The composition of membrane lipids and lipopolysaccharides, p. 231–257. *In* M. F. Barile and S. Razin (ed.), The mycoplasmas, vol. 1. Cell biology. Academic Press, Inc., New York.
36. **Taylor-Robinson, D., R. H. Purcell, D. C. Wong, and R. M. Chanock.** 1966. A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. J. Hyg. **64**:91–105.