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Isolation of a Glycoprotein from *Mycoplasma pneumoniae* Membranes

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A glycoprotein was detected in *Mycoplasma pneumoniae* membranes. Its apparent molecular weight was about 60,000, as observed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. It corresponded to the single band that was detected on the gels by the carbohydrate stain, periodic acid-Schiff reagent. The intensity of this stained band varied for membranes derived from cells harvested between 4 and 10 days, with maximal intensity found for cells grown for 6 days. The carbohydrate-containing polypeptide was extracted with lithium diiodosalicylate. The extracted fraction consisted of about 80 to 90% amino acids (mainly glycine and histidine) and about 7% carbohydrates (mainly glucose, galactose, and glucosamine). The fraction was immunologically active, as indicated by the complement fixation and precipitin tests with antisera against whole cells, membranes, and membrane proteins.

The cell membrane of *Mycoplasma pneumoniae* plays an important role in the adherence and pathogenicity of the organisms (4, 5, 15) and contains the important immunogens of the cell (1, 13, 14, 16). Membrane glycolipids have been shown to be haptens, and antibodies directed against them killed *M. pneumoniae* cells in the presence of complement in vitro (16). There are indications, however, that other membrane components, probably proteins, may also elicit antibodies that could be of immunogenic importance (1, 14, 16, 22). In addition, the participation of membrane proteins in adherence of *M. pneumoniae* to host cells has been indicated by the inhibition of adherence after trypsin treatment (15, 22). The involvement of carbohydrate moieties as well has been suggested by the inhibition of adherence by periodate treatment (15).

Further investigation of *M. pneumoniae* membrane proteins and glycoproteins, therefore, seemed well justified.

MATERIALS AND METHODS

Organisms and growth conditions. *M. pneumoniae* organisms of strain PI 1428 in passage 6 and strain FH in passages 296 and 298 were grown for 5 to 7 days as described by Razin et al. (16). After decantation of the medium, the sheet of organisms adhering to the glass was washed three times with β-buffer [0.15 M NaCl-0.01 M 2-mercaptoethanol-0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4]. The cells were scraped off and washed in the same buffer twice by centrifugation.

Isolation of cell membranes. Cell membranes were isolated from cells preloaded with 2 M glycerol and then injected into glass-distilled water at 37°C. The procedure described by Kahane and Razin (9) was followed, with the exception that immediately after injecting the organisms into the lysing medium, it was cooled rapidly to 4°C and the lysed cells were washed three times in β-buffer diluted 1:20. The membranes were separated from nonlysed cells on a linear sucrose density gradient (30 to 54% [wt/vol], prepared by a Beckman density gradient former) and were ultracentrifuged in a Beckman SW27 rotor at 27,000 rpm for 3.5 h at 4°C. The main band (2/3 from the top of the gradient), which contained about 70% of the protein loaded on the gradient, was collected and washed free of sucrose in β-buffer diluted 1:20.

Extraction of the membrane glycoprotein. The procedure described by Marchesi and Andrews (12) for extraction of erythrocyte glycoprotein with lithium diiodosalicylate was modified to include two additional steps. (i) After the ethanol washing, nucleic acids were digested by protease-free nuclease (3), increasing the incubation period to 16 to 18 h. (ii) Lipids of the final product were extracted by using two washes with chloroform-methanol (2:1, vol/vol) and a successive ethanol wash.

Assay procedures. Protein was determined by the Folin-phenol method of Lowry et al. (11), using bovine plasma albumin as standard. Amino acids of samples hydrolyzed in 6 N HCl at 110°C for 24 h were determined with a Beckman model 120B amino acid analyzer. Membranes were delipidated by two chloroform-methanol (2:1, vol/vol) extractions and a successive one with ethanol. Electrophoretic analysis was carried out in 5 to 15% acrylamide gels containing 0.1% sodium dodecyl sulfate (18). After staining (18), the gels were scanned by a Gilford scanner (model 2410). Carbohy-

drates were estimated by gas-liquid chromatography (Packard model 7400), using a 3% SE 30 column (8 feet by 0.125 inch [ca. 244 by 0.32 cm] in diameter, glass column) and following the procedure of Clamp (2).

Immunological techniques. Antisera were prepared in rabbits as described by Razin et al. (16). Double-diffusion tests in agar were performed with immunoplates (pattern D, Hyland, Los Angeles, Calif.). Complement fixation tests were performed as previously described by Taylor-Robinson et al. (23).

RESULTS

Electrophoretic pattern of the membrane protein. The electrophoretic pattern of the membranes of *M. pneumoniae* FH are composed of at least 13 polypeptide bands when stained with the Coomassie brilliant blue protein stain. One of these bands (peak a, Fig. 1), with an apparent molecular weight of 60,000, partially coincided with the single band stained by the periodic acid-Schiff reagent (see Fig. 1, reference 8). The electrophoretic profile of the membranes of *M. pneumoniae* PI 1428 stained with both Coomassie brilliant blue and periodic acid-Schiff highly resembled those of strain FH. Due to the partial overlap and the occasional differences in the width of the periodic acid-Schiff-stained band and band a, superposition of two components was speculated. The migration of the bands continued to coincide, however, when the solubilized membranes were subjected to electrophoresis in 7.5, 10, 12.5, and 15% polyacrylamide gels, suggesting the presence of only a single polypeptide containing some carbohydrate.

Some explanation for the variation in the intensity of the periodic acid-Schiff-stained band of the different membrane batches was obtained by the observation that the intensity of this periodic acid-Schiff-stained band varied with the age of the culture. A maximal intensity was observed in membranes of 6-day-old cultures (Fig. 1), thereafter declining to almost zero (Fig. 1). No parallel changes in the related protein band were observed, and the electrophoretic pattern was similar for the membranes of organisms harvested throughout the experiments.

Extraction of the periodic acid-Schiff-stained polypeptide band. Assuming the material to be a glycoprotein, a procedure designed to extract membrane glycoproteins with lithium diiodosalicylate (12) was used. Following this procedure, a fraction comprising about 1 to 3% (dry weight) of the starting material was obtained. By gel electrophoresis, the extracted material consisted of one major protein band that also stained with periodic acid-Schiff stain (Fig. 2), and its migration was similar to that of the periodic acid-Schiff-stained band of native membranes (Fig. 1). Minor peaks were observed with both the Coomassie brilliant blue and periodic acid-Schiff stains (Fig. 2), but these peaks did not coelectrophorese. A fraction with similar electrophoretic properties was extracted from membranes of *M. pneumoniae* PI 1428.

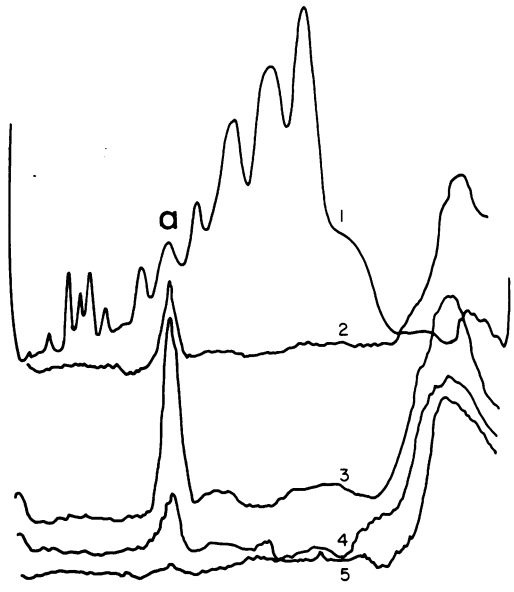

FIG. 1. *Electrophoretic pattern of M. pneumoniae FH membranes derived from cells grown for different lengths of time. Native membranes were solubilized in 1% sodium dodecyl sulfate and subjected to electrophoresis in 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The gels were stained for protein with Coomassie brilliant blue (gel 1), and with the periodic acid-Schiff reagent for sugar-containing material (gels 2 to 5). The gels were scanned at 540 (Coomassie brilliant blue) and 520 (periodic acid-Schiff reagent) nm. The membranes were derived from organisms that were grown for 4 (gel 2), 6 (gels 1 and 3), 7 (gel 4), and 10 (gel 5) days. The top of the gel is on the left. The 60,000-dalton polypeptide band is denoted by "a."*

Chemical analysis of the glycoprotein fraction. About 80 to 90% of the fraction consisted of amino acids, with an extremely high content of glycine and histidine (Table 1). The other major constituents of the fraction were carbohydrates (about 7%), most of which were glucose, galactose, and glucosamine (Table 2). Small amounts of nucleic acids (1 to 3%) could not be eliminated from the fraction in spite of the nuclease treatment.

Immunological activities of the glycoprotein fraction. Immunological activities of the glycoprotein fraction were studied by two serological tests. Specific precipitin lines by immunodiffusion in agar were obtained only when the glycoprotein fraction was solubilized with deter-

gent. When solubilized in sodium dodecyl sulfate, heavy precipitin lines were formed with sera prepared against organisms, membranes, and membrane proteins. This line fused in a reaction of identity with a precipitin line formed between membranes and sera prepared against organisms (Fig. 3). No precipitin reaction was observed between glycolipids and the antiserum (Fig. 3). The glycoprotein fraction was also able to fix complement significantly, though less than organisms (Table 3).

DISCUSSION

This report has presented evidence that a glycoprotein exists in the membranes of both the PI 1428 and FH strains of *M. pneumoniae*. The glycoprotein migrated on polyacrylamide gels in the presence of sodium dodecyl sulfate as a single polypeptide with an apparent molecular weight of about 60,000. The material was tentatively included among the glycoproteins, since its gross chemical makeup consisted of about 7% carbohydrates and about 80 to 90% amino acids. The amino acid composition (Table 1) is rather unique, with a very high glycine content, warranting further characterization of the molecule. This composition will probably affect the manner in which this protein is inserted in the membrane. Although this remains to be solved, there are some data with respect to the orientation of the molecule in the membrane. Like most glycoproteins, it is exposed, at least in part, on the outer surface of the membrane. This was determined by lactoperoxidase-mediated iodination of intact cells by which the glycoprotein polypeptide, among other membrane proteins, was iodinated (8). In addition, trypsin treatment of intact cells eliminated the periodic acid-Schiff-stained band seen in the delipidated membranes of nontreated cells (I. Kahane, unpublished data).

Is it possible to have glycoproteins in *M. pneumoniae* membranes? Though glycoproteins are still quite rare in procaryotes, their known number is steadily increasing (19). In other mycoplasmas, there are already several indications for the existence of glycoproteins. Recently, Goel and Lemcke (6) isolated a glycoprotein

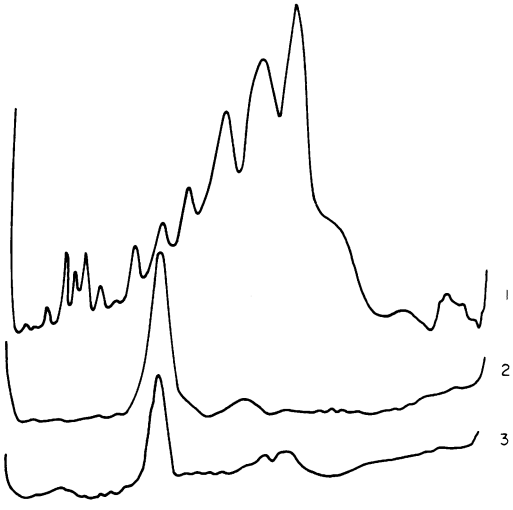

FIG. 2. *Gel electrophoresis of the glycoprotein extracted from M. pneumoniae FH. The electrophoresis was performed as described under Fig. 1, and the gels were scanned. Membranes (1) and purified glycoprotein (2) were stained for protein (Coomassie brilliant blue), and the purified glycoprotein was stained by periodic acid-Schiff reagent (3). The top of the gel is on the left.*

TABLE 1. *Amino acid composition of M. pneumoniae FH membrane glycoprotein*

Amino acid	mol%[a]
Lysine	1.8
Histidine	19.6
Arginine	0.7
Aspartic acid	3.8
Threonine	1.8
Serine	4.7
Glutamic acid	6.3
Proline	1.0
Glycine	52.1
Alanine	1.0
Cystine (half)	Traces
Valine	0.9
Methionine	1.7
Isoleucine	1.5
Leucine	1.7
Tyrosine	0.7
Phenylalanine	0.7

[a] The results are an average of four analyses on two different batches of glycoprotein.

TABLE 2. *Carbohydrate composition of M. pneumoniae FH membranes and glycoprotein*

Prepn	Carbohydrate (nmol/mg of protein)					
	Fucose (?)[a]	Mannose	Galactose	Glucose	Glucosamine	Galactosamine
Membranes	Traces	14.0	99.0	54.7	7.3	Traces
Glycoprotein[b]	Traces	4.4	76.2	203.2	53.6	11.8

[a] The small peak had a slightly different retention time compared with standard fucose.
[b] Values calculated per milligram of dry weight.

from *M. gallisepticum* that was found to be involved in the hemagglutination of this organism. Less direct observations stem from the studies of Hollingdale and Lemcke (7), who suggested the presence of a glycoprotein in *M. hominis* membranes. The studies on the binding of lectins to mycoplasma membranes also support this point, as decreased binding of some lectins upon proteolytic digestion of the membrane was demonstrated for *Spiroplasma citri* and *M. neurolyticum* and *M. gallisepticum* (10, 17). Although there are several indications that the isolated material is a glycoprotein, until this is shown to be true by complete chemical characterization we must consider the possibility of other carbohydrate-including substances tightly bound to a polypeptide. Three such groups have been found to exist in mycoplasmas: glycolipids (16, 19, 20), polysaccharides (22), and lipopolysaccharides (21). *M. pneumoniae* membranes do contain glycolipids that are stained by the periodic acid-Schiff reagent and electrophorese, migrating in a wide band at the front of the gel (Fig. 1) slightly ahead of the dye marker (bromophenol blue). When membranes are delipidated, however, this band is not seen. It is therefore unlikely that glycolipids will resist the lipid extraction to which the glycoprotein is subjected. Polysaccharides are the second prospective group. Indeed, four fractions of these were reported in *M. pneumoniae* cells (22); however, they were never well characterized. A quite remote possibility exists that these polysaccharides originated from the glycoprotein reported here, since the four fractions (22) were obtained after a rather harsh 2-h sonic treatment of the cells, a process that might shear molecules.

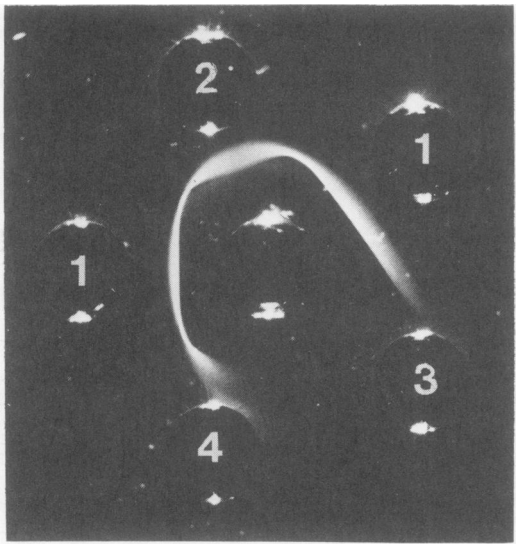

FIG. 3. *Precipitin reactions of M. pneumoniae membranes and M. pneumoniae glycoprotein. Central well contained antiserum to M. pneumoniae organisms. (1) Glycoprotein; (2) membranes; (3) total lipid fraction; (4) sodium dodecyl sulfate control. All antigens (1 mg/ml) were solubilized in 0.1% sodium dodecyl sulfate.*

TABLE 3. *Complement-fixing activity of M. pneumoniae FH glycoprotein*

Antigen tested (mg/ml)	Complement-fixing activity[a] (reciprocal titer)
M. pneumoniae organisms (1)	512
M. pneumoniae glycoprotein (1)	32
M. pneumoniae total lipids (0.2)	8

[a] Tested with antiserum against whole organisms.

The third possible group is the lipopolysaccharides recently found by Smith et al. in several species of *Acholeplasma* and *Anaeroplasma* but not in *Mycoplasma*, with the exception of *M. neurolyticum* (21). This group deserves further attention, since there is a phenol extraction step in the procedure for isolating both the lipopolysaccharides and the glycoprotein. When the procedure used to extract the glycoprotein from *M. pneumoniae* was used on the membranes of *Acholeplasma laidlawii*, no material was extracted (I. Kahane, unpublished data), even though these organisms are known to contain lipopolysaccharide (21). The glycoprotein also differed from the lipopolysaccharide in its carbohydrate composition, which contained large amounts of glucose and a molar ratio of neutral sugars to amino sugars of 4:1 (Table 2) as compared to a 1:1 or 2:1 ratio in most of the lipopolysaccharides (21). It thus seems that the isolated glycoprotein does not resemble other carbohydrate-containing substances known in mycoplasmas and is probably another procaryote glycoprotein.

Some insight into the physiology of the glycoprotein resulted from the observation of the variations in the intensity of the periodic acid-Schiff-stained band with the age of the culture. This may also explain the fact that one batch of membranes lacked periodic acid-Schiff-stained material. It is tempting to assume that the variation resulted from changes in the carbohydrate content of the protein. Since the amount of isolated membranes was so small, however, no chemical analysis was performed.

The fact that the glycoprotein was immunologically active further stresses the point that additional study of the molecule is needed. It supports the basic assumption that other mem-

brane components in addition to lipids are important in the immunogenicity of *M. pneumoniae*. It would be even more exciting if the glycoprotein is involved in the adsorption of *M. pneumoniae* cells to other cell surfaces, as is the glycoprotein of *M. gallisepticum*. This study is presently under way.

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