GRADIENT, POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS FROM CYTOTOXIC MYCOPLASMA MEMBRANES

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SUMMARY: Membranes of Mycoplasma pneumoniae were prepared following several different cell lysis procedures (freeze-thaw, french press, digitonin, sonication, osmotic shock, pH shock). All possessed some degree of toxicity for ciliated epithelial cells, but the french press and freeze-thaw processes were optimal in terms of lysis efficiency, membrane yield, and cytotoxicity. Membrane proteins from virulent and attenuated mycoplasmas were electrophoresed with a new, high resolution electrophoresis technique (gradient polyacrylamide gels), and strain differences were detectable among the 50+ discernible bands.

INTRODUCTION:

Mycoplasma pneumoniae has a predeliction for attachment to ciliated respiratory epithelium, but as yet, the precise mechanism of pathogenesis has not been elucidated (1,2). Early reports of an association between peroxide formation and disease production (3) have not been well substantiated nor consistent enough to establish a definite causal relationship. It has been proposed (4,5) that some other mechanism (e.g., direct cellular injury or mediation by toxin(s)) must account for the cytotoxic effect.

Our laboratory recently reported (6) that membranes of M. pneumoniae can induce gross and histopathologic alterations in trachea organ cultures similar to those seen following infection with viable cells. This biological activity is consistent with the fact that the mycoplasma membrane contains numerous enzyme systems (7). In addition, recent reports indicate that membranes from lymphocytes (8) and erythrocytes (9) also have cytolytic potential. The purpose of this study was to examine in detail the relative cytotoxicity of various mycoplasma membrane preparations, as well as their relative protein composition as defined by high-resolution polyacrylamide gel electrophoresis.

MATERIALS AND METHODS:

M. pneumoniae, strain FH, was provided by J. Tully (National Institutes of Health) and strains Mac and P1 104166 were furnished by W. Clyde (University of North Carolina). Strain 1428 was obtained from R. Chanock (National Institutes of Health) and M. fermentans, strain K10, was provided by Wm. Murphy (University of Michigan). Acholeplasma laidlawii was purchased from the American Type Culture Collection (#14192). Organisms were in the 6 to 11th passage since receipt, and were grown in G-199 medium (10).

Trachea organ cultures were prepared from adult Golden hamsters as described previously (6). Relative ciliary activity measurements, i.e., percentage of a trachea ring remaining intact (0 to 100) multiplied by the vigor of ciliary motion (0 to 3), were made daily during observations with inverted phase optics (x 225). Organ cultures were maintained in 90% minimal essential medium (Eagle's with Hanks' salts) and 10% fetal calf serum, supplemented with L-glutamine (40 mM), penicillin G (200 U/ml), and N-2-hydroxyethylpiperazine-N'-2'-ethane-sulfonic acid (25 mM, HEPES, Sigma Chemical Co., St. Louis, Mo.).

The lysis procedures, including 20 cycles of freeze-thaw (6), 2 cycles in a French pressure cell at 25,000 psi (11), digitonin (12), osmotic shock (13), carbonate/bicarbonate buffer at pH 11 (14), and 5 one-minute cycles of sonication at 20 kH_Z (13), have been described previously in conjunction with the preparation of mycoplasma membranes. The methods, along with the criteria used to ascertain membrane "purity," have been reviewed (7,11).

Suspensions were cleared of unbroken cell aggregates by centrifugation at 4,000 x g for 5 min at 4°C. Membranes were collected by ultracentrifugation of supernatants at 41,000 x g for 45 min at 4°C, and were washed 4x with sterile PBS[1] prior to resuspending them in 10 ml of PBS. Where indicated, membranes were subjected to an additional purification step by layering them on a sucrose step gradient (35% and 50%) according to the method of Engleman et al. (15) prior to the 4 successive PBS washes. Protein content was determined with the Lowry method, and membranes were resuspended in organ culture medium at 40 µg protein/ml.

A modification of the discontinuous SDS[2] systems of Laemmle (16) and O'Farrell et al. (17) was used for electrophoresis. The apparatus, described in detail by Studier (18), contained a running gel (14.5 cm x 8.5 cm x 0.08 cm) with a linear, 7.5 to 20% concentration of acrylamide. The stacking gel (3% acrylamide) was 1.6 cm from the bottom of the well formers to the top of the running gel. Both gels contained 0.1% SDS, and the stacking gel also had 0.5% agarose for stability. The gel was pre-run at 12.5 milliamps for 30 min at room temperature.

Prior to application, samples were lyophilized and rehydrated in sample solvent (49.6 mM Tris, pH 6.8; 32% glycerol; 1% SDS; 1% 2-mercaptoethanol; 0.1% bromphenol blue) to yield a final concentration of 4 mg protein/ml. The sample was heated at 100°C for 10 min, and a 10 µl aliquot was placed in one of the 9 preformed wells (1.2 cm x 0.6 cm x 0.08 cm). Running buffer contained 0.1% SDS in 49.5 mM Tris and 384 mM glycine, adjusted to pH 8.3 with 0.1 N HCl. Electrophoresis was at 12.5 milliamps until the marker dye migrated 0.5 cm into the stacking gel (approx. 40 min at 22°C). The apparatus was then held at 4°C and the current was increased to 20 milliamps. Electrophoresis

[1] phosphate buffered saline, pH 7.4

[2] sodium dodecyl sulfate

TABLE 1. Comparisons of yields, efficiency of lysis and relative cytotoxic potential of membranes from M. pneumoniae, PI 1428, prepared by various methods.

Method of lysis	Membrane yield	Viable cells[a]		Cytotoxicity[b]	
	(mg protein/L)	CFU	% initial	RA	% control
Freeze-thaw	6.6	0	0	111	53
French press	6.2	0	0	70	33
Digitonin	6.4	2×10^{3}	6×10^{-5}	76	36
Sonication	8.0	1×10^{3}	3×10^{-5}	147	70
Osmotic shock	2.7	2×10^{3}	6×10^{-5}	153	73
Carbonate, pH 11	0.8	0	0	98	47

[a]Viable cells remaining after lysis procedure and membrane washing, expressed as total CFU remaining from 1 L of cells, or as percent of the initial, total CFU count/L. The highest residual level of viable cells was still 10 to 100-fold less than that required to cause significant cytopathogenicity within 5 days.

[b]Effect on relative ciliary activity, RA (percent of trachea ring remaining intact x relative vigor of ciliary beating), after 5 days of exposure to 40 µg protein/ml. Mean data from 12 trachea rings.

continued until the marker was within 1 cm of the bottom of the running gel (3-4 hrs). The gel slab was stained in 0.2% Coomassie Blue in 50% methanol with 7% acetic acid for 1 hr at 37°C. The background was destained overnight in 25% methanol - 7% acetic acid at 4°C. Gels were scanned at 550 nm with a Gilford densitometer with a Zeiss chromometer.

RESULTS:

Influence of preparation method on cytotoxic potential of membranes. Six representative methods (freeze-thaw, french press, digitonin, sonication, osmotic shock, and pH shock) were applied to M. pneumoniae, PI 1428. The data in Table 1 show that all preparations tested had some degree of toxicity. Membranes from the sonication and osmotic shock procedures were the least toxic, whereas the other methods gave membrane preparations which could reduce the relative ciliary activity to less than 53% of the control values. The osmotic and sonic shock methods yielded membranes which were consistently slow in eliciting an effect. The digitonin and french press methods gave membranes

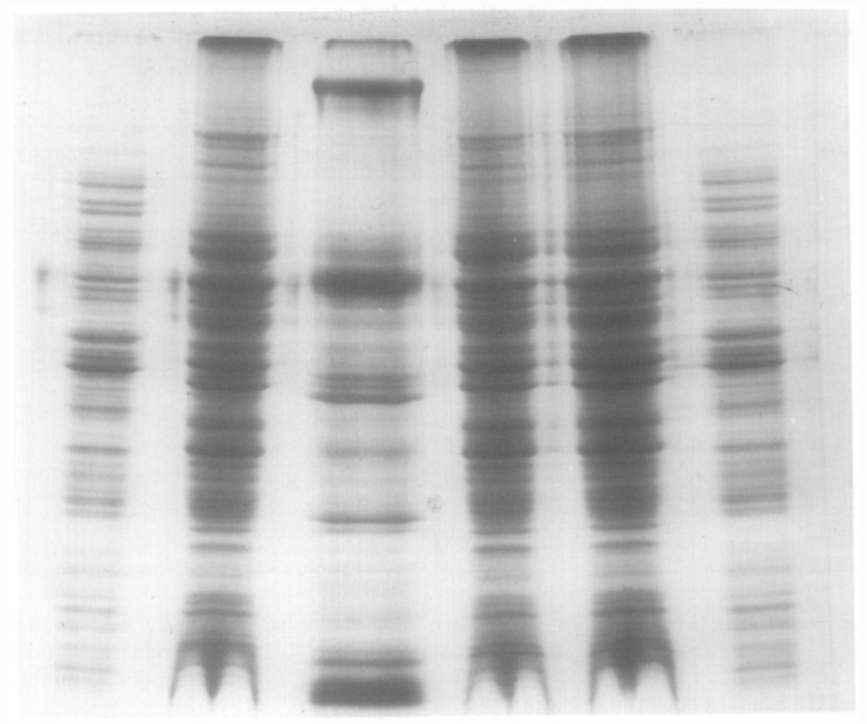

Fig. 1 Electrophoresis banding patterns of proteins from mycoplasma cells and membranes (freeze-thaw). Left to right: M. fermentans membranes; M. pneumoniae cells; M. pneumoniae cytoplasm; M. pneumoniae membranes; M. pneumoniae membranes purified on a sucrose step gradient; M. fermentans membranes.

which caused an obvious drop in activity within 48 hrs. After 5 days in culture, the trachea rings exposed to these membranes had relative ciliary activity values of about one-third of the control value. The freeze-thaw and carbonate (pH 11) methods yielded membranes of intermediate toxicity. The freeze-thaw and french press methods proved optimal when yields, efficiency of lysis, and cytotoxic potential were compared overall.

Electrophoresis of mycoplasma membranes. In order to establish whether or not toxic membranes contained some unique protein component(s), high resolution polyacrylamide electrophoresis techniques were employed. Initial studies compared intact cells, cytoplasm, and membranes in an attempt to ascertain the relative complexity of each. As shown in figure 1, whole cells of virulent M. pneumoniae, PI 1428, yielded approx. 50 individual bands, while cytoplasm

(lyophilized osmotic lysate) had only about half as many bands. The latter were extremely faint in staining intensity, and presumably represented minor components in terms of quantity. Consistent with earlier reports, the banding pattern of membranes was similar to that seen with intact cells. The likelihood that the membrane preparation was simply contaminated with intact cells was remote since: a) intact cell aggregates were removed by differential centrifugation; b) direct plating techniques failed to detect viable cells; and c) additional purification of the membranes by layering them on a sucrose-step gradient during ultracentrifugation did not alter the electrophoresis pattern of the membrane preparation. Mycoplasma membranes which possess cytotoxic potential are thus composed of numerous protein species, similar in number and size distribution to those presented by intact cells, but more complex than those associated with the cytoplasm.

When cells and membranes (freeze-thaw) from several species of mycoplasmas were compared electrophoretically, all showed the same basic pattern (Fig. 2 and 3). Obvious differences were seen between the non-pathogen, Acholeplasma laidlawii, and the pathogen, M. pneumoniae, and all of the M. pneumoniae strains were clearly related. The patterns were essentially identical for strains PI 1428, FH, and 104166 - all of which are virulent when tested in trachea organ cultures (6,10). Strain Mac (an attenuated strain with markedly decreased virulence in trachea organ cultures) had a banding pattern similar to the virulent strains, but with a consistent exception. All of the virulent strains had a deeply staining doublet near the top (15% down the length of the gel, whereas the attenuated Mac had a noticeably weaker, barely discernible reaction in that area which represents the first of the doublet bands. This difference also could be seen in densitometer tracings (Fig. 4). The only significant difference between the intact cells or membranes of virulent and attenuated organisms was the decrease or absence in some of the large molecular weight proteins of the latter. The other protein species were apparently present in samples of both strains. In addition, gels of the PI 1428 membranes obtained from various

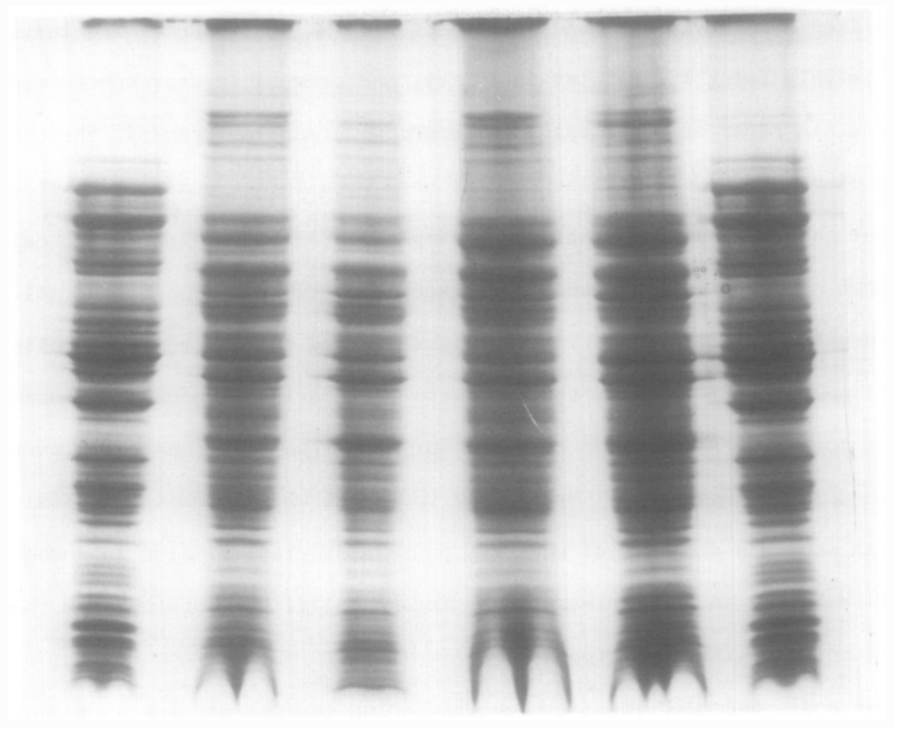

Fig. 2 Polyacrylamide gel electrophoresis of proteins from various mycoplasma cell preparations. Left to right: A. laidlawii; M. pneumoniae, strains PI 104166, Mac, PI 1428; FH; A. laidlawii. Arrow indicates minimal staining at doublet in the attenuated strain, Mac.

lysis procedures showed that sonicated membranes (those with the least cytotoxic potential) also had the weakest protein reaction in this doublet area. The nature and potential cytotoxicity of these specific proteins is now under investigation.

DISCUSSION:

The characterization of mycoplasma cells and membrane by polyacrylamide gel electrophoresis of their proteins, as developed by Razin et al. (7,19), has proved extremely valuable for identification and for comparative studies of protein composition. The use of such gels in the configuration of a thin sheet, as opposed to the cylinders, markedly increases resolution while requiring minimal amounts of sample (e.g., 30-80 μg). Though such flat gels have recently been used in conjunction with mycoplasmas (20,21), the technique

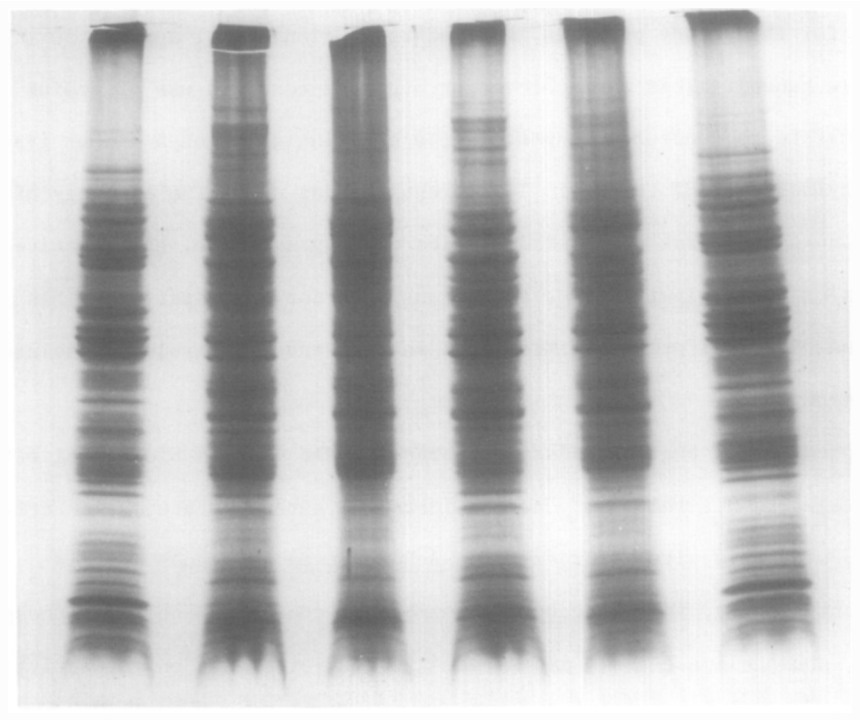

Fig. 3 Polyacrylamide gel electrophoresis of proteins from membranes of various mycoplasmas. Order is identical to that of Fig. 2.

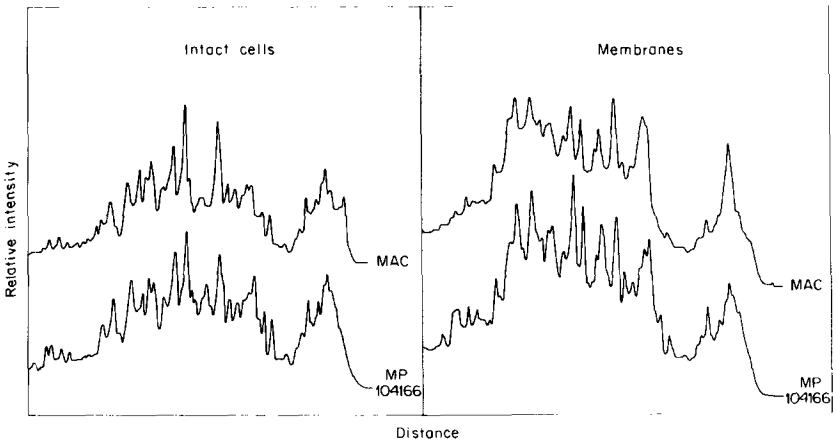

Fig. 4 Densitometer tracings of stained protein bands of cells and membranes of M. pneumoniae, PI 104166 (virulent) and Mac (attenuated).

developed for this study provides improved resolution (i.e., approx. 50 discernible bands). This was achieved through the combined use of sodium dodecyl sulfate to denature the proteins, a stacking gel, and a linear gradient of polyacrylamide (7.5% to 20%). This technique has shown the homology of protein banding patterns in several strains of M. pneumoniae, the relative uniqueness of A. laidlawii and M. fermentans, and for the first time, has illustrated potential protein differences in high and low virulence isolates of M. pneumoniae.

The possibility that mycoplasma membranes could exert a biological effect on other cells is not remote if one considers the extensive enzymatic activity associated with this site. For example, Acholeplasma membranes contain numerous enzymes (7), and M. pneumoniae membranes contain RNase (22), phosphatase (23), phospholipase (24), and ATPase (22). Proteolytic enzyme activity has also been demonstrated in membranes of M. orale and M. salivarium (25). Given this catalog of biological activities, it is conceivable that membrane-associated cytotoxicity of M. pneumoniae is a manifestation of: (1) direct toxicity of a membrane component; (2) generalized or specific enzymatic degradation; or (3) biophysical processes, such as cell fusion followed by loss of membrane integrity. Which of these alternatives has the most validity is currently under active investigation.

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