

## SURFACE PARASITISM BY *MYCOPLASMA PNEUMONIAE* OF RESPIRATORY EPITHELIUM\*

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The mechanisms by which virulent microorganisms mediate host cell injury in respiratory tissue are unclear. To better understand the cellular and subcellular events which accompany these infections requires the establishment of experimental models which permit monitoring of the infectious process under experimentally controlled conditions. During the past several years we have examined the capacity of virulent *Mycoplasma pneumoniae* organisms, human pathogens of the respiratory tract, to parasitize and produce cell injury in the respiratory epithelium (1-4). Tracheal organ culture served as the highly sensitive host indicator system and its feasibility for other models is recognized (5-9). Based upon our earlier studies we outlined a two step parasitism: (a) the specific attachment of virulent *M. pneumoniae* via their tip-like organelle to a sialic acid-associated receptor region on the respiratory epithelium and (b) early abnormal host cell function at the transcriptional or translational levels with subsequent tissue cytopathology resulting from the colonization, multiplication, and continued metabolic activity of attached virulent mycoplasmas. The success of this parasitism appeared initially and critically dependent upon the intimate physical association between the membranes of *M. pneumoniae* and respiratory epithelial cells. However, the chemical nature of *M. pneumoniae* surfaces responsible for attachment was unknown.

Limited information is available concerning external membrane components of *M. pneumoniae*. Razin et al. (10, 11) extracted from *M. pneumoniae* a glycolipid which reacts with complement-fixing and metabolic- and growth-inhibiting antibodies raised against intact organisms suggesting a possible surface location of the glycolipid moiety. Additional nonprotein haptens have been demonstrated in other mycoplasma species (12, 13). Boatman and Kenny (14) reported that spherules of *M. pneumoniae* organisms which form during growth can be disaggregated into individual and viable mycoplasmas by treatment with crude lipase. This observation implied that a lipid moiety on the surface of *M. pneumoniae* might be responsible for their "stickiness." Other studies with *Mycoplasma hominis* indicated that membrane proteins exist as receptors for the adsorption of HeLa cells (15) and for indirect hemagglutination activity (16). In this report we examine the unique interaction between virulent *M. pneumo-*

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*niae* and hamster trachea in organ culture and implicate a membrane protein on the surface of *M. pneumoniae* as the mediator of mycoplasma attachment to the respiratory epithelium.

## Materials and Methods

**Organisms and Cultures.** Virulent *M. pneumoniae* (M129) was originally isolated from a patient with mycoplasma pneumonia. The 12th broth passage of this organism (M129-B12) retained virulence as determined by its ability to produce pneumonia in Syrian golden hamsters and was used throughout the present study. Cultures were grown in Hayflick medium containing 20% agamma horse serum, 10% yeast dialysate, and penicillin (1,000 U/ml).

Monolayer cultures of *M. pneumoniae* were established in acid-cleaned 240-ml prescription bottles as previously described by us (4). Mycoplasmas were radiolabeled by adding  $^3\text{H}$ -thymidine (sp act 50 Ci/mmol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) to the culture medium at 10  $\mu\text{Ci/ml}$ . After incubation of monolayers at 37°C in 5%  $\text{CO}_2$  with air for 36–48 h, the medium was discarded, and the organisms were washed three times with phosphate-buffered saline, pH 7.2 (PBS).<sup>1</sup> Organisms were then either scraped off the glass with a rubber policeman into 2.5–5.0 ml of Hayflick medium or the monolayer was subjected to enzyme treatment.

**Tracheal Organ Cultures.** Tracheal organ cultures were prepared from Syrian golden hamsters as previously described (1).

**Treatment of *M. pneumoniae* Monolayers with Enzymes.** Incubation of *M. pneumoniae* organisms with specific enzymes was performed so that the monolayer still remained attached to glass after the treatment. Age of the monolayer culture was critical to the effectiveness of the enzyme. Mature cultures, which yield the highest cell density, usually contain large colonies which are more resistant to enzyme penetration. On the other hand, young cultures provide too little material and colonies tend to float off the glass surface during exposure to enzyme. From our experience, 36–48 h mid-log phase cultures as evidenced by a slight orange-yellow pH change of the medium give satisfactory results. At that time, growth medium was decanted and the monolayer was washed with PBS before receiving 20 ml of minimal essential medium (MEM) supplemented with penicillin (1,000 U/ml), pH 7.2. Crude lipase (3,000 Wilson U/g; Calbiochem, La Jolla, Calif.) or crystalline trypsin (Type XI, DCC-treated, 7,000 BAEE U/mg; Sigma Chemical Co., St. Louis, Mo.) was then introduced at a final concentration of 50  $\mu\text{g/ml}$  and 25  $\mu\text{g/ml}$ , respectively. Both enzymes were dissolved in MEM and crude lipase was centrifuged before use. Mycoplasma cultures were incubated at 37°C for 60 min with lipase and for 10 min with trypsin. Enzyme treatments with purified lipases (Types CLF and LCP at 1 U/ml; Worthington Biochemical Corp., Freehold, N. J.) and phospholipases (Types A and D at 10  $\mu\text{g/ml}$ , respectively; Sigma Chemical Co.) were also performed. After treatment, monolayers were washed three times with PBS. Finally, cultures were scraped into 2.5–5.0 ml of fresh Hayflick medium for attachment studies (see below) or into 4.0 ml of PBS and processed for sodium dodecyl sulfate (SDS)-gel electrophoresis.

**Attachment of Radiolabeled Organisms to Tracheal Rings.** Hamster tracheal organ cultures were incubated in MEM overnight before use. 100  $\mu\text{l}$  of radiolabeled *M. pneumoniae* suspended in Hayflick medium was placed into individual wells of a microtiter plate. Tracheal rings were removed from MEM and blotted briefly on a sterile Kimwipe before being placed into separate wells containing the organisms. Plates were sealed with cellophane tape and incubated at 37°C in 5%  $\text{CO}_2$  with air for specific times.

After incubation, tracheal rings were removed from the microtiter wells, blotted on sterile Kimwipes, and rinsed in two changes of PBS. Rings were then processed for liquid scintillation spectrometry or autoradiography. For measuring  $^3\text{H}$ -counts per minute, rings were digested overnight in 0.2 ml of Protosol (New England Nuclear, Boston, Mass.) contained in tightly capped glass inserts. Then, 3 ml of Omnifluor-toluene scintillation fluid (New England Nuclear) was added to each insert, and radioactivity was determined in a Packard Tri-Carb Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). For autoradiography and

<sup>1</sup> Abbreviations used in this paper: PBI, phosphate-buffered iodide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate.

transmission electron microscopy, rings were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 60 min at room temperature. Rings were washed and secondary fixation was accomplished in 1% OsO<sub>4</sub>-Veronal acetate buffer (pH 7.3) for 90 min at 4°C followed by dehydration and embedding in Epon. For autoradiography, 0.25- $\mu$ m sections were cut on an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.) and mounted on microscope slides which were coated with Kodak NTB2 nuclear tract emulsion (Eastman Kodak Co., Rochester, N. Y.), stored at 4°C in the dark for 2-3 wk, developed in Kodak D-19, and stained with Richardson's stain. For electron microscopy, thin sections were stained with uranyl acetate and lead citrate and examined in a Jeolco-100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan).

**SDS-Gel Electrophoresis.** Discontinuous SDS-gel electrophoresis was performed as reported by Laemmli (17) with slight modification. A monolayer culture of *M. pneumoniae* was scraped off the glass into 4.0 ml of cold PBS, and 1.0 ml of 50% cold TCA was then added. The sample was kept in the cold for at least 4 h and was then centrifuged and washed with cold PBS. This treatment greatly reduced the viscosity of the samples which normally occurred after SDS addition to non-TCA-precipitated suspensions and made handling of the preparations easier without altering the electrophoretic pattern. It was essential that an even suspension of the organisms be obtained before addition of SDS to permit complete solubilization. The solubilizing buffer consisted of 0.0625 M Tris-HCl, pH 6.8, 5%  $\beta$ -mercaptoethanol, and 10% sucrose to which 2% SDS was added after mycoplasmas were suspended. Test solutions were boiled in a water bath for 3 min and centrifuged at 25,000 *g* for 20 min to remove contaminating materials or undissolved residues. This centrifugation step helped reduce the curvature of protein bands during electrophoresis. Samples were used immediately or frozen and stored at -20°C. Frozen samples were boiled again for 1-2 min before application to gels. Separating and stacking gels consisted of 7.5 and 3% acrylamide, respectively, and samples containing 150-250  $\mu$ g protein in 50-100  $\mu$ l vol were applied to each gel. Electrophoresis was initially carried out with a constant current of 1 mA/gel. Then, when the tracking dye migrated into the separating gel, the current was increased to 2 mA/gel. Total time for electrophoresis was 3.5-4 h for 8-cm gels.

**Iodination of Mycoplasmas.** Lactoperoxidase-catalyzed iodination of *M. pneumoniae* surface proteins was performed by the general procedure of Hynes (18). Briefly, a mid-log phase monolayer culture of *M. pneumoniae* was washed three times with PBS, and 5-10 ml of PBS supplemented with 2 mM glucose was then added. Carrier-free Na<sup>125</sup>I (New England Nuclear Corp.) was introduced into the bottle to a final concentration of 50-100  $\mu$ Ci/ml. The reaction was initiated by addition of lactoperoxidase (purified grade, sp act 100 IU/mg protein; Calbiochem) and glucose oxidase (sp act 283 U/mg; Calbiochem) at concentrations of 2 IU/ml and 0.1 IU/ml, respectively. The reaction was allowed to continue for 10 min at room temperature with occasional swirling. Then medium was quickly removed and radiolabeling was stopped by the addition of cold phosphate-buffered iodide (PBI, similar to PBS with NaCl replaced by NaI) supplemented with 2 mM phenylmethylsulfonylfluoride (PMSF; Sigma Chemical Co.) to inhibit proteases. Iodinated organisms were washed three more times with PBI-PMSF, scraped from the glass and suspended in PBS. Cold TCA was added to a final concentration of 10%. The organisms were centrifuged, washed, and processed for gel electrophoresis as described in the previous section.

After electrophoresis the gel was stained with Coomassie Blue, destained and then sliced longitudinally into four pieces. One of the center pieces was dried under vacuum on filter paper for autoradiography. The other center piece was cut transversely into 1-mm sections, placed in individual scintillation vials and processed for radioactive determination (19).

#### *Staining Procedures for Proteins and Glycoproteins*

(a) **COOMASSIE BLUE.** Immediately after electrophoresis, gels were immersed in 0.25% Coomassie Blue in methanol-acetic acid-water (4:1:5 in volume) in 15 x 120 mm test tubes for 60 min and then briefly rinsed with distilled H<sub>2</sub>O. Individual gels were placed into slotted plastic test tubes and destained with stirring in methanol-acetic acid-water (10:3:27 in volume). After 2 h, the solution was changed and destaining was continued overnight.

(b) **PERIODIC ACID-SCHIFF (PAS).** The procedure was modified from the method of Kapitany and Zebrowski (20). After appropriate fixation of test gels, sample oxidation was performed using periodic acid before treatment with Schiff's Reagent (Fisher Scientific Co., Fairlawn, N. J.) and exposure to sodium metabisulfate.



FIG. 1. Transmission electron photomicrograph of a hamster tracheal ring infected with *M. pneumoniae*. Note the orientation of the mycoplasmas via their specialized tip-like organelle which permits close association with the respiratory epithelium ( $\times 50,000$ ). M, mycoplasma; m, microvillus; and C, cilia.

(c) **ALCIAN BLUE.** Gels were prepared and stained as described by Wardi and Michos (21). In addition to detecting mycoplasma glycoproteins by techniques (b) and (c), glycoproteins were also assayed by the [ $^3\text{H}$ ]borohydride-galactose oxidase method of Gahmberg and Hakomori (22).

**Molecular Weight Determination.** The molecular weights of mycoplasma protein bands in SDS-gels were estimated by co-electrophoresis with marker proteins of known molecular weight. Markers used were human  $\gamma$ -globulin (mol wt 160,000), bovine serum albumin (mol wt 68,000), and ovalbumin (mol wt 45,000), all purchased from Schwarz/Mann Div., Becton, Dickinson & Co., and lipoxidase (mol wt 90,000) obtained from Sigma Chemical Co.

## Results

**Effect of Selected Enzymes on Surface Attachment of *M. pneumoniae* to Tracheal Organ Cultures.** The attachment of virulent *M. pneumoniae* to respiratory epithelium (Fig. 1) appears the prerequisite of disease production (1-4, 23, 24). To begin characterization of the attachment mechanism, monolayer cultures of radiolabeled mycoplasmas were exposed to specific enzymes to measure their influence on the subsequent host-parasite interaction. Crude lipase was initially employed because of its known effect on *M. pneumoniae* aggregates (14) and the apparent existence of membrane glycolipids on surfaces of *M. pneumoniae* (10, 11).

As seen in Table I, a significant reduction in the attachment of  $^3\text{H}$ -labeled mycoplasmas to tracheal rings occurred after crude lipase treatment. This

TABLE I  
Effect of Lipase on the Attachment of Virulent *M. pneumoniae* to Tracheal Organ Cultures

Treatment	cpm/10 $\mu$ l sus- pension*	cpm/ring‡	% Attachment
Control	8,307	3,335	100
Crude lipase (0.2 U/ml)	8,589	302	9
Lipase, <i>Candida</i> (1 U/ml)	8,276	3,847	115
Lipase, <i>Geotrichum</i> (1 U/ml)	7,219	2,944	88

\* A lawn of  $^3\text{H}$ -thymidine-labeled mycoplasmas was treated with and without enzyme in MEM for 1 h at 37°C. After enzyme treatment, organisms were washed twice with PBS and suspended into 2.5 ml of fresh Hayflick medium. 10  $\mu$ l of the suspension was dried on filter paper, and the radioactivity was determined.

‡ Individual tracheal rings were incubated with 0.1 ml of radiolabeled mycoplasma suspension for 4 h, washed with PBS, dissolved in Protosol, and the radioactivity was monitored.

TABLE II  
Effect of Trypsin on the Attachment of Virulent *M. pneumoniae* to Tracheal Organ Cultures

Treatment	cpm/10 $\mu$ l sus- pension	cpm/ring	% Attachment
Control	6,746	5,809	100
Trypsin (25 $\mu$ /ml)	6,247	1,379	24

$^3\text{H}$ -thymidine-labeled mycoplasmas were treated with or without trypsin in MEM for 10 min at 37°C. Additional information appears in the footnotes of Table I.

observation indicated that exposure of *M. pneumoniae* to the enzyme preparation removed or modified certain chemical structures, conceivably lipid in nature on the surface of the mycoplasmas which were required for parasitism of the respiratory epithelium.

However, when two highly purified lipases (Table I) and phospholipases A and D (data not shown) were substituted, attachment of virulent mycoplasmas to tracheal organ cultures was unimpaired. Thus, we assumed that removal of the "attachment factor(s)" from the surface of virulent mycoplasmas was possibly due to a contaminating enzyme activity in the crude lipase preparation. A literature survey (25) indicated that the specific crude lipase used in these studies was contaminated with significant protease activity. We then monitored the effect of crystalline trypsin on *M. pneumoniae* attachment. Data indicate that brief incubation of radiolabeled *M. pneumoniae* monolayers with trypsin markedly reduced attachment (Table II).

Since we had earlier reported that only metabolically active virulent mycoplasmas were capable of parasitizing respiratory epithelium (4), we correlated decreased attachment of enzyme-treated *M. pneumoniae* with viable plate counts. Not only was mycoplasma viability retained but increased plating efficiency was observed (Table III, "before reincubation") probably due to disaggregation of *M. pneumoniae* clumps (14). If enzyme-treated monolayers were

TABLE III  
*Regeneration of Attachment of Virulent M. pneumoniae After Crude Lipase Treatment*

Treatment	Before reincubation		After reincubation	
	cpm/ring	Viable counts/ml	cpm/ring	Viable counts/ml
Control	1,517	$1.7 \times 10^8$	1,800	$1.8 \times 10^8$
Crude lipase	434	$2.6 \times 10^8$	1,172	$2.3 \times 10^8$

Radiolabeled mycoplasmas were used as described in Table I. Radioactivity of mycoplasma suspensions were: control, before reincubation 4,644 cpm/10  $\mu$ l; enzyme-treated, before reincubation 3,535 cpm/10  $\mu$ l; controls, after 8 h of reincubation 4,205 cpm/10  $\mu$ l; and enzyme-treated, after 8 h of reincubation 3,286 cpm/10  $\mu$ l.

reincubated for several hours in fresh growth medium before assay, regeneration of the protease-sensitive attachment factor was evident (Table III, "after reincubation"). Autoradiography confirms the fact that virulent *M. pneumoniae* lose their capacity to attach to the respiratory epithelium after enzyme treatment (Fig. 2 B) and regain this property after reincubation in Hayflick medium (Fig. 2 D). Figs. 2 A and 2 C represent untreated controls and are included for comparative purposes. Similar results were obtained with trypsin-treated preparations of *M. pneumoniae*.

*SDS-Gel Electrophoretic Analysis of M. pneumoniae Proteins.* The above data suggested that specific mycoplasma proteins might function as mediators of attachment. Therefore protein patterns of normal and enzyme-treated organisms were compared by gel electrophoresis. As seen in Fig. 3, only two major protein bands ( $P_1$  and  $P_2$ ) were significantly reduced or absent after exposure of *M. pneumoniae* to crude lipase or trypsin preparations indicating their extreme sensitivity to protease activity. Also an increase in the intensity of another protein,  $P_3$ , was observed (see below and Discussion). Scanning profiles of additional gel preparations reconfirmed the loss of  $P_1$  and increased accumulation of  $P_3$  (Fig. 4). Effects on  $P_2$  were less consistent between experiments. In all cases, enzyme-treated *M. pneumoniae* preparations examined by gel electrophoresis were simultaneously monitored for attachment to tracheal explants and a direct correlation was evident between loss of  $P_1$  and reduced surface parasitism. To further clarify the role of proteases, trypsin inhibitor (Type 11-0, 50  $\mu$ g/ml; Sigma Chemical Co.) was added along with trypsin or crude lipase to monolayer cultures. Trypsin inhibitor negated the action of the proteases and by so doing prevented alterations in protein gel patterns and in attachment kinetics (Fig. 5).

*Regeneration of M. pneumoniae Attachment Factor after Enzyme Treatment.* Since it was evident that reincubation of enzyme-treated mycoplasmas in fresh medium restored attachment capabilities, electrophoretic analysis of mycoplasma proteins was performed as described in Fig. 6. Erythromycin was included during the reincubation to suppress *M. pneumoniae* protein synthesis as earlier reported (2). Results indicate that regeneration of  $P_1$  occurs during reincubation but only in the absence of erythromycin. Furthermore, inhibition by erythromycin prevented reattachment of *M. pneumoniae* to respiratory

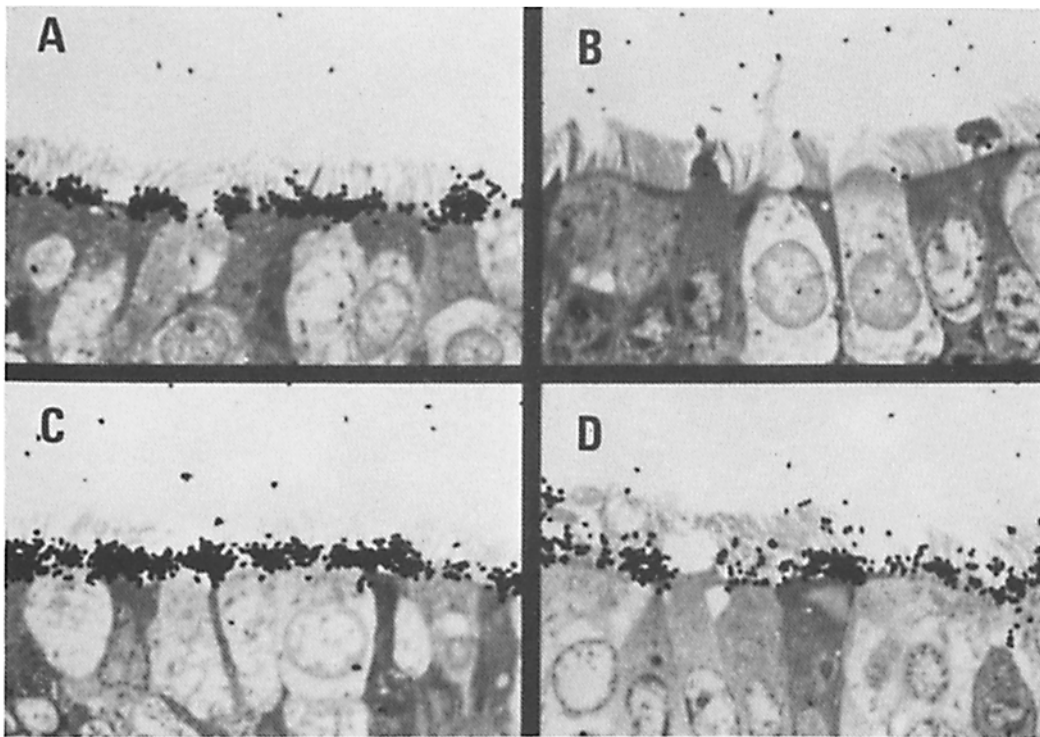


FIG. 2. Autoradiographs demonstrating the reduction and regeneration of attachment of virulent *M. pneumoniae* to tracheal organ cultures. Preparation and treatment of the organisms are as described in Tables I and II. (A) Tracheal ring incubated with  $^3\text{H}$ -labeled untreated virulent *M. pneumoniae*. Silver grains concentrated over the luminal surface of the tracheal ring indicate the specific attachment of virulent mycoplasmas to the ciliated epithelium. (B) Tracheal ring incubated with mycoplasmas previously treated with crude lipase or trypsin. The absence of silver grains indicates no attachment of mycoplasmas to the epithelium. (C) Tracheal ring incubated with untreated virulent mycoplasmas which have been reincubated in Hayflick medium as described previously. (D) Tracheal ring incubated with mycoplasmas treated as in B and reincubated in fresh medium. The presence of silver grains indicates resumed attachment of mycoplasmas.

epithelium suggesting that protein biosynthesis is required after enzyme treatment for regeneration of the attachment factor(s).

The kinetics of regeneration of these protein bands after enzyme treatment was also monitored. As appears in Fig. 7,  $P_1$  is completely removed by trypsin but is fully restored after a 6 h incubation during which time little increase in total DNA of the culture and viable cell counts are observed. Recovery of  $P_2$  occurs somewhat earlier. Other data indicated that a minimal reincubation of 6 h was necessary to fully restore attachment capabilities to *M. pneumoniae*, again implicating  $P_1$  as the attachment factor. Increased intensity of  $P_3$  was evident immediately after trypsin treatment but  $P_3$  levels returned to normal by 6–9 h of reincubation.

*Identification of External Proteins of Virulent M. pneumoniae.* Evidence obtained from the above experiments indicates that the attachment factor(s) of

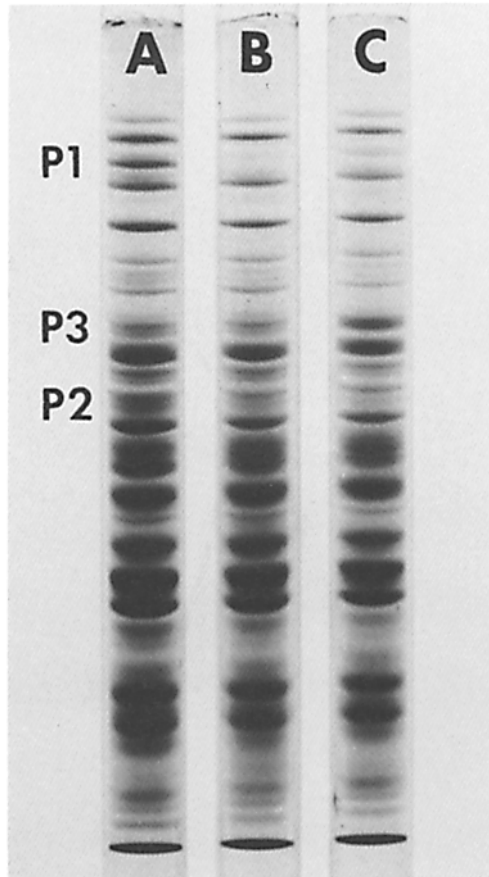


FIG. 3. Electrophoretic profiles of *M. pneumoniae* proteins. Sample preparation and SDS-gel electrophoresis were as described in the Materials and Methods. Left pattern (A) is the untreated mycoplasma preparation and middle (B) and right (C) patterns are crude lipase- and trypsin-treated organisms, respectively. Enzyme treatments are described in the legends of Tables I and II. Note the absence or reduction in protein bands P<sub>1</sub> and P<sub>2</sub>.

virulent *M. pneumoniae* is protein in nature. The extreme sensitivity of certain proteins, particularly P<sub>1</sub>, to proteases and the evidence that no loss in viability of the organisms occurs after enzymatic treatment suggest that these proteins are located on the cell surface. To further implicate their role in the attachment mechanism, lactoperoxidase-catalyzed iodination was performed under conditions which restrict radiolabeling to surface proteins (18, 26). Our previous data (3) demonstrated that mycoplasma organisms which undergo this procedure remain viable and retain attachment capabilities.

As seen in Fig. 8, 11 bands are identified as external proteins by liquid scintillation spectrometry (panel A). However, autoradiography (Fig. 8 C) resolves both band III and band IV into two additional bands suggesting improved resolution by the latter technique. Bands I and IV of panel A correspond to P<sub>1</sub> and P<sub>2</sub> of panels B and C. These results provide direct evidence that P<sub>1</sub> and P<sub>2</sub> are located on the external surface of *M. pneumoniae* membrane. If monolayer



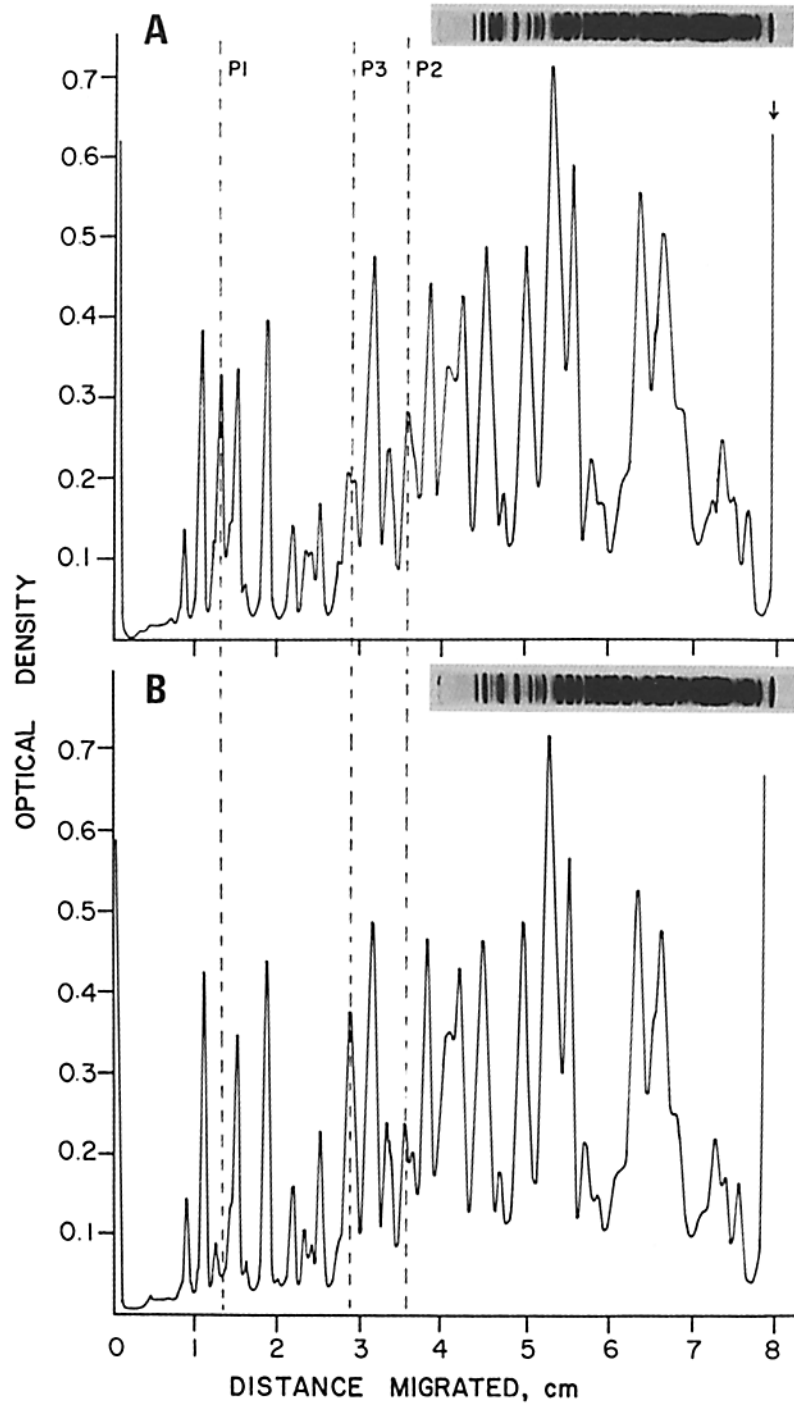


FIG. 4. Scanning patterns of SDS-acrylamide gels of *M. pneumoniae* proteins using a Gilford linear scanning system. (A) Untreated and (B) treated with crude lipase (50  $\mu\text{g}/\text{ml}$  for 60 min). Origins of the gels are at the left.

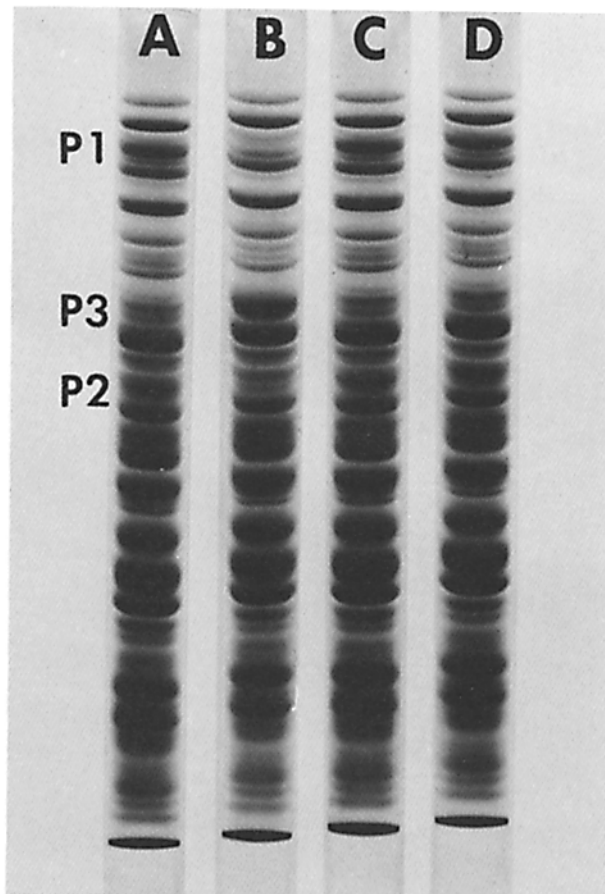


FIG. 5. Electrophoretic analysis of *M. pneumoniae* proteins after trypsin treatment with and without trypsin inhibitor. (A) Untreated, (B) treated with trypsin, (C) treated with trypsin inhibitor, and (D) treated with trypsin and trypsin inhibitor. Similar results were obtained when crude lipase replaced trypsin.

cultures of *M. pneumoniae* were iodinated before or after exposure to trypsin,  $P_1$  and  $P_2$  were absent from the autoradiograph but an additional iodinated band which corresponds to  $P_3$  was detected (data not shown).

**PAS Staining.** Because many surface proteins exist as glycoproteins (27), we examined  $P_1$  and  $P_2$  for sugar complexes. However, none of the specific staining procedures described in the Materials and Methods detected glycoproteins in these gel regions. The limitation may be due to the low carbohydrate content of mycoplasma species, ranging from only 0.5 to 2.2% (28). By weighing the area of  $P_1$  relative to the total area of the 40 peaks on scanning gel profiles, we calculated that the amount of  $P_1$  in a gel receiving a 200  $\mu\text{g}$  protein sample is about 5  $\mu\text{g}$ . Even if  $P_1$  contained 10% carbohydrate, this level is below the sensitivity of the staining technique (29).

#### Discussion

As we earlier described, *M. pneumoniae* is an extracellular parasite which

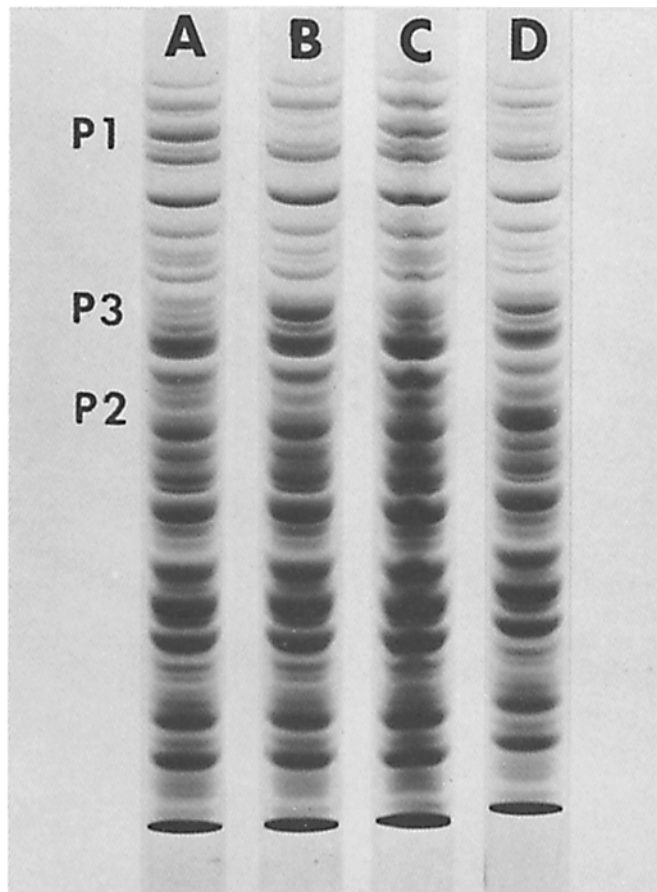


FIG. 6. Electrophoretic analysis of *M. pneumoniae* proteins with and without exposure to trypsin and erythromycin. Left profile (A), untreated; second from left (B), trypsin-treated for 10 min; second from right (C), trypsin-treated and then reincubated in fresh culture medium for 9 h; right (D), trypsin-treated and then reincubated in fresh medium containing erythromycin.

attaches to respiratory epithelium surface receptors via a specialized tip structure. Since initial colonization of host tissue is a prerequisite of the infection, it seemed reasonable that *M. pneumoniae* disease could be controlled by preventing or interrupting the membrane-membrane interaction between host and parasite. In the present study we attempted to identify and characterize components of *M. pneumoniae* which comprise the attachment mechanism.

By employing highly sensitive radioisotope techniques (3, 4), we monitored attachment of virulent mycoplasmas to tracheal organ cultures. Pretreatment of monolayers of radiolabeled mycoplasmas with crystalline trypsin or crude lipase but not purified lipases significantly decreased surface parasitism. Trypsin inhibitor neutralized the active enzyme component responsible for the reduced attachment suggesting the protein nature of the mycoplasma attachment factor. Supportive data were obtained from gel electrophoretic analysis of normal and enzyme-treated *M. pneumoniae* cultures. A major protein band (P<sub>1</sub>) was absent

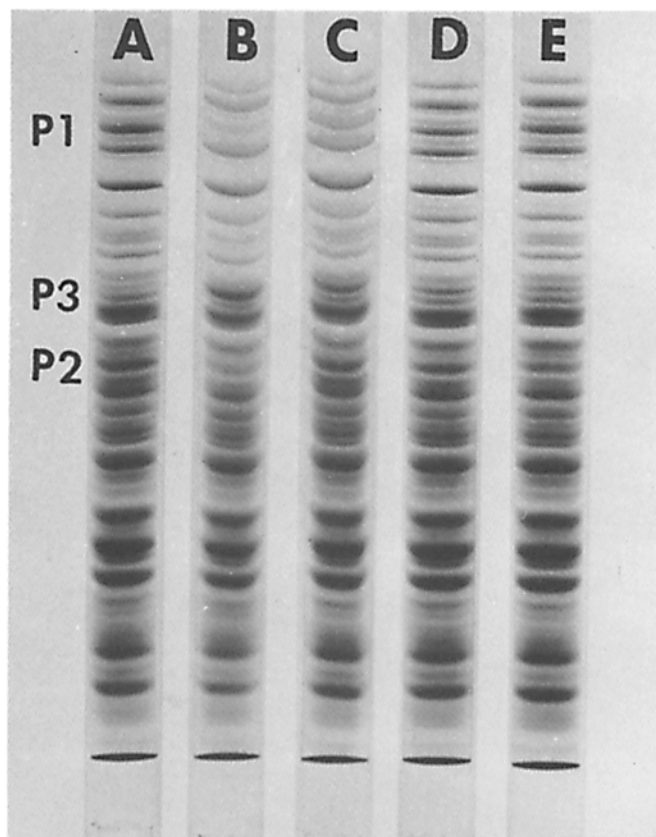


FIG. 7. Electrophoretic analysis of *M. pneumoniae* proteins after trypsin treatment and reincubation. (A), untreated control; (B) through (E), mycoplasma monolayers treated with trypsin at 25  $\mu\text{g}/\text{ml}$  for 10 min. (B), cultures processed for gel electrophoresis immediately after trypsin treatment; (C), (D), and (E), cultures were reincubated in fresh medium for 3, 6, and 9 h, respectively. Note the removal of  $P_1$  and  $P_2$  by trypsin and their synthesis during reincubation. Also note the accumulation of  $P_3$  in (B) and its later return to normal levels.

from enzyme-treated preparations while other protein species were virtually unaffected. In certain experiments an additional band ( $P_2$ ) was quantitatively reduced. Since *M. pneumoniae* organisms remain viable after brief protease treatment and can regenerate their attachment capabilities during incubation in fresh medium (Fig. 2 and Table III) we examined the kinetics of regeneration of the protein bands (Fig. 7). Within a 6 h reincubation,  $P_1$  levels were fully restored which directly correlated with resumed attachment by *M. pneumoniae*. Although the  $P_2$  band returned to normal levels within 3 h, these mycoplasmas were still unable to attach. Addition of erythromycin to the reincubation medium prevented restoration of these bands and reattachment. This observation supported the biosynthetic nature of  $P_1$  and  $P_2$  regeneration and ruled out the possibility that these protein components were adsorbed or incorporated from the medium. Taken together these data implicate  $P_1$  as the prime "attachment factor."

Lactoperoxidase-catalyzed iodination of intact *M. pneumoniae* organisms

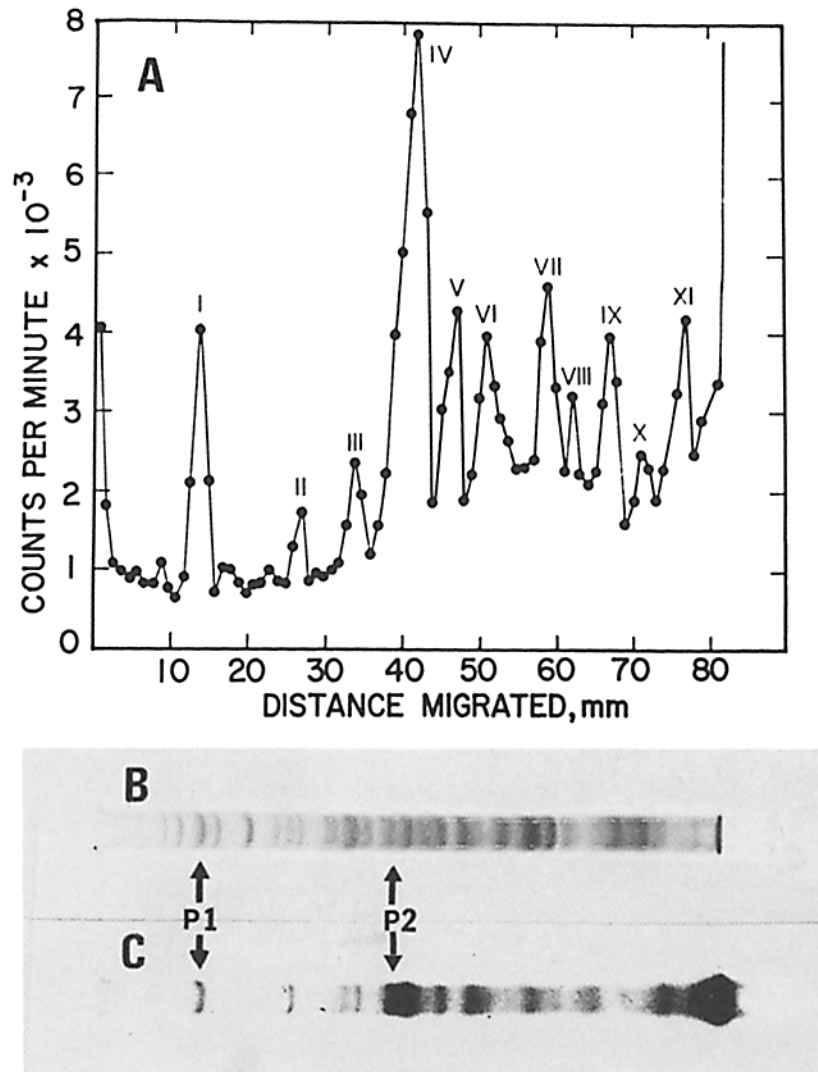


FIG. 8. Electrophoretic analysis of virulent *M. pneumoniae* organisms labeled with <sup>125</sup>I-iodine. (A) Radioactivity of protein bands obtained by liquid scintillation spectrometry. (B) Same gel stained with Coomassie Blue to show all protein bands (i.e., <sup>125</sup>I-labeled and unlabeled proteins). (C) Autoradiograph of the same gel. Panels A and C indicate the selectivity of the radioiodination procedure for surface proteins and demonstrate the surface property of the "attachment factor." Origins of the electrophoretograms are at the left.

demonstrated that P<sub>1</sub> and P<sub>2</sub> were surface membrane proteins consistent with their extreme sensitivity to proteases and the retained viability of *M. pneumoniae* after their removal. Also noted was the increased intensity of one protein band, designated P<sub>3</sub>, immediately after protease treatment. This protein gradually returned to normal levels during reincubation. The absence of P<sub>3</sub> in the iodinated profiles of intact organisms and its appearance in trypsin-treated iodinated patterns suggest that P<sub>3</sub> may be a digestive residue of P<sub>1</sub>. This latter possibility is under investigation.

The surface parasitism observed when *M. pneumoniae* infects tracheal explants appears identical to the attachment demonstrated in natural human disease (30). This intimate contact between the membranes of the parasite and respiratory cell suggests that local immune mechanisms such as secretory antibody and cell-mediated immunity may be important for the development of protection (31). Vaccines using temperature-sensitive mutants of *M. pneumoniae* whose growth is restricted to the upper respiratory passages have provided only partial protection and the genetic stability of these mutants is uncertain (32, 33). Less effective have been mycoplasma vaccines administered by parenteral inoculation (34). Difficulties have been experienced because of the limited information concerning virulence determinants of mycoplasmas and the nature of the factors which stimulate protective immunity. The identification and purification of membrane components on virulent *M. pneumoniae* such as protein P<sub>1</sub> which appear responsible for attachment to host cell receptors may provide a satisfactory approach for understanding disease pathogenesis and permit production of rational vaccine candidates.

### Summary

Identification of the attachment factor on virulent *Mycoplasma pneumoniae* organisms which permits surface parasitism of respiratory epithelium was attempted. Brief pretreatment of *M. pneumoniae* monolayers with protease prevented mycoplasma attachment to sensitive host cells without reducing viability of the microorganisms. Gel electrophoretic analysis of mycoplasma proteins before and after exposure of intact mycoplasmas to protease revealed the absence of a major protein species (P<sub>1</sub>) in enzyme-treated preparations while other protein bands with the exception of P<sub>2</sub> were virtually unaffected. The absence of P<sub>1</sub> correlated with the failure of enzyme-treated mycoplasmas to attach to tracheal explants. P<sub>1</sub> regeneration after protease treatment of mycoplasma monolayers was directly associated with reattachment capabilities in *M. pneumoniae*. Erythromycin inhibited P<sub>1</sub> resynthesis, thus preventing resumed attachment activity by mycoplasmas. Lactoperoxidase-catalyzed iodination of intact *M. pneumoniae* organisms further confirmed that P<sub>1</sub> was an external membrane protein and suggested that this surface component was required for the successful membrane-membrane interaction between host and parasite.

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