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Location of Attachment Moiety on *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae initiates infection in the human host by attachment to respiratory epithelium. The organism attaches by a specialized terminal structure. Monoclonal antibodies to an organism surface protein (P1) inhibited attachment to respiratory epithelium and were localized to the tip structure by a ferritin antibody label. The P1 protein was degraded by trypsin treatment to smaller polypeptides that possessed the same antigenic determinants as the larger P1 protein when reacted with the specific monoclonal antibody, and evidence has been provided for the existence of multiple antigenic determinants on the attachment protein.

INTRODUCTION

Mycoplasma pneumoniae is an extracellular pathogen of the human respiratory tract and is the most common cause of pneumonia in teenagers and young adults. The pathogenic form of *M. pneumoniae* is filamentous in morphology and attaches to the respiratory epithelium by a specialized terminal structure which consists of a dense central core with a bulbar tip surrounded by an electron-lucent space enveloped by the cell membrane of the organism [1]. *In vitro* tracheal organ culture studies have provided evidence that attachment of viable, virulent *M. pneumoniae* organisms to the respiratory epithelium is a prerequisite for host cell injury [2,3,4]. The attachment moiety on the mycoplasma membrane has been demonstrated to be a trypsin-sensitive protein, designated P1 [5].

Monoclonal antibody has been developed that reacts specifically with the P1 protein. This antibody interferes with attachment of *M. pneumoniae* to respiratory epithelium in hamster tracheal organ culture. Electron microscopic studies utilizing the mouse monoclonal antibody and ferritin-conjugated rabbit antimouse IgG have permitted the localization of the P1 protein on the filamentous *M. pneumoniae* organism [6].

Studies on the fine analysis of antigenic determinants of the P1 protein may lead to a better understanding of the structure-function relationship of this molecule. This paper presents preliminary evidence for the existence of multiple antigenic determinants of P1 molecules by means of limited protease digestion and monoclonal antibodies.

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MATERIALS AND METHODS

Mycoplasma Cultures

Mycoplasma pneumoniae strain M-129 (ATCC No. 29342) was grown in Hayflick medium supplemented with 20 percent agamma horse serum and 10 percent yeast dialysate. The cultures were prepared as described previously [5] and in some experiments the monolayers were treated with trypsin [5].

Organ Cultures

Tracheal organ cultures were prepared from adult male, syrian hamsters and maintained in Hayflick medium [7]. The tracheal rings were infected with *M. pneumoniae* as previously described [5].

Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide slab gels were employed to separate the *M. pneumoniae* proteins. The separated proteins were transferred to nitrocellulose paper for radioimmunobinding studies as fully described before [8].

Monoclonal Antibodies

Hybridoma cell lines that produced antibodies to the antigens of *M. pneumoniae* were produced by hybridization of spleen cells from BALB/c mice immunized with *M. pneumoniae* and myeloma cells Sp2/0 Ag14 (ATCC No. CRL-1581). Hybrids that produced antibodies against *M. pneumoniae* were screened by radioimmunoassay. Ascitic fluids were produced in BALB/c mice by injecting the mice intraperitoneally with hybridoma cells. Monoclonal antibodies against specific *M. pneumoniae* antigens were identified by protein blot and radioimmunobinding techniques [8,6].

Trypsin Cleavage of Attachment Protein

A band of P1 protein was cut off from fixed or unfixed 7 percent SDS-gel and digested with trypsin in the sample wells of a second SDS gel (12 percent). Details of this method have been described previously [9]. The best results were obtained using 1 mM EDTA in the gel solution on 1.5 mm thick gels with 3 cm stacking gel and 8 cm separation gel. The current was turned off for 30 minutes when the dye front reached the bottom of the stacking gel to assure adequate digestion.

Electron Microscopy

Tracheal rings and *M. pneumoniae* organisms were fixed in 2 percent glutaraldehyde–2 percent paraformaldehyde in 0.1 M phosphate buffer. Secondary fixation was accomplished in 1 percent OsO4–Veronal acetate buffer followed by dehydration and embedding in Epon. In ferritin experiments *M. pneumoniae* were incubated with mouse monoclonal antibody M-218 specific for the P1 protein, washed, and then incubated with ferritin-conjugated rabbit antibody to mouse IgG prior to fixation. Thin sections were cut on a LKB ultramicrotome and examined in a Zeiss EM-10 electron microscope at an accelerating voltage of 60 kV.

RESULTS

Electron Microscopy

Examination of hamster tracheal organ cultures infected with *M. pneumoniae* showed the filamentous organisms to be located in an extracellular location at the

lumenal border of the respiratory epithelium. The organisms were oriented with the specialized terminal structure proximal to the host cell membrane. Some organisms had the membrane lining the sides of the specialized tip structure in close approximation to the host cell membrane as well as the terminal portion of the tip (Fig. 1A).

When *M. pneumoniae* was incubated with the monoclonal antibody M-218 and subsequently with ferritin-conjugated rabbit antibody to mouse IgG, the ferritin localized the M-128 antibody to be specifically concentrated on the surfaces of the unit membrane surrounding the organism's terminal structure (Fig. 1B). In that monoclonal antibodies secreted by hybridoma cell line M-218 have been shown to be specific for the P1 protein by SDS-gel/protein blot radioimmunobinding [8], the results obtained by indirect ferritin staining localized the P1 protein to the cell membrane surrounding the terminal organelle of the filamentous *M. pneumoniae* organism.

Evidence of Multiple Antigenic Determinants on P1 Protein

Since P1 is a large protein molecule (molecular weight, 190 Kd), it is conceivable that a number of antigenic determinants may exist on this molecule. In an effort to investigate this possibility, bands of P1 protein were cut out of the first SDS gel and subjected to a limited trypsin digestion followed by SDS gel electrophoresis in combination with protein blot and radioimmunobinding. As shown in Fig. 2, when the P1 protein band, cut off from a 7 percent gel, was re-electrophoresed on a 12 percent SDS gel, it migrated as a single band, as revealed by Coomassie blue staining (lane A). Its immunoreactivity also was not distorted by the re-electrophoresis, since the re-electrophoresed P1 protein was capable of reacting with both hyperimmunized rabbit antiserum and monoclonal antibody M-218 (lanes B and D), respectively, following the transfer to nitrocellulose filters. Lanes C and E served as the negative controls which were incubated with ^{125}I-labeled goat anti-rabbit IgG or rabbit anti-

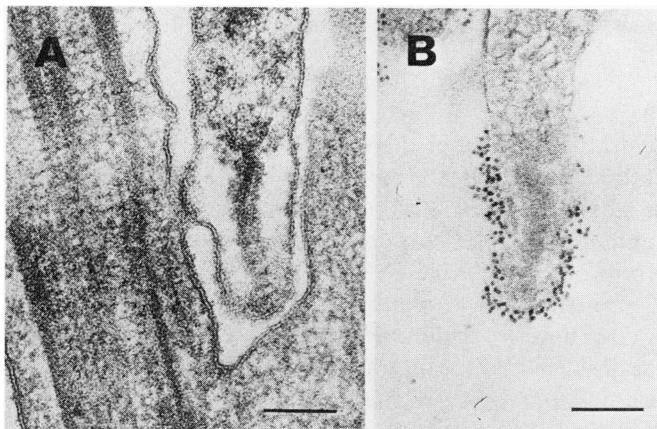

FIG. 1. **A** Electron micrograph of *M. pneumoniae* attached to the ciliated respiratory epithelium of hamster tracheal organ culture. Note the close association of the side of the specialized tip with the cilia (*arrow*).

B Electron micrograph of *M. pneumoniae* organism incubated with monoclonal antibodies specific to P1 protein, and indirectly stained with ferritin-conjugated rabbit anti-mouse IgG. Ferritin grains indicate the location of P1 protein on the organism cell membrane surrounding the terminal organelle of *M. pneumoniae*. Bar equals 0.1 μM.

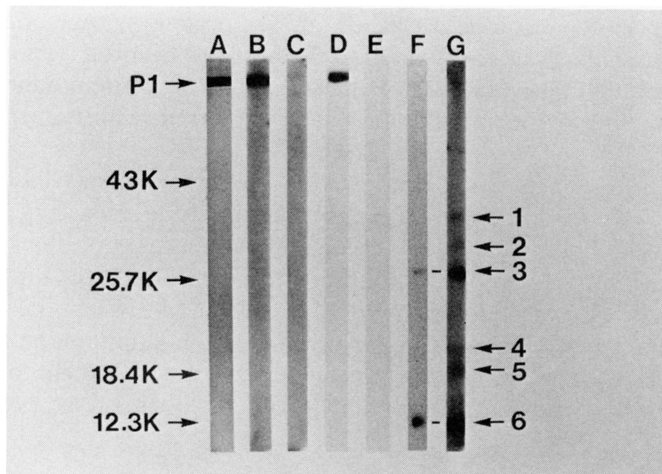

FIG. 2. Immunoradiobinding studies of the cleaved products of P1 protein following limited trypsin digestion. **A** A P1 protein band cut off from a 7 percent gel and re-electrophoresed on a 12 percent gel and stained with Coomassie blue. **B** through **E** P1 protein bands prepared the same way as **A** but transferred to nitrocellulose paper by Western blot technique. The nitrocellulose blots were incubated with: **B** serum from a rabbit hyperimmunized with *M. pneumoniae* followed by ^{125}I-labeled goat anti-rabbit IgG; **C** ^{125}I-labeled goat anti-rabbit IgG only; **D** monoclonal antibody M-218, followed by ^{125}I-labeled rabbit anti-mouse IgG; and **E** ^{125}I-labeled rabbit anti-mouse IgG only. **F** and **G** P1 protein bands treated with trypsin (250 μg/ml) in the sample wells of a 12 percent gel and electrophoresis continued; the digested fragments separated on the gel were then transferred to nitrocellulose paper. **F** was incubated with monoclonal antibody M-218, followed by ^{125}I-labeled rabbit anti-mouse IgG; and **G** was incubated with hyperimmunized rabbit serum, followed by ^{125}I-labeled goat anti-rabbit IgG. Molecular weight standards were ovalbumin (43,000), α-chymotrysinogen (25,700), β-lactoglobulin (18,400), and Cytochome C (12,300).

mouse IgG, respectively, without prior incubation with specific antibodies. These results indicate that the binding of labeled second antibodies to the nitrocellulose blots was mediated specifically through the bound P1 protein-specific antibodies present in the immunized rabbit antiserum or the monoclonal antibody M-218 which has been shown to be specific to P1 protein [6].

When the P1 protein bands were subjected to a limited trypsin treatment followed by SDS-gel electrophoresis, a number of very faint bands were observed in the Coomassie blue-stained gels (data not shown). However, when the nitrocellulose blots were prepared from these gels, intact antigenic determinants were preserved in the cleaved fragments. As demonstrated in lanes F and G, two bands were detected by monoclonal antibody M-218, and six bands were detected with the immunized rabbit antiserum. The two bands which appeared on the nitrocellulose strip incubated with monoclonal antibodies are anticipated to be two polypeptides possessing the same antigenic determinants but with different molecular weights. The four additional bands detected by incubation with the immunized rabbit antiserum must represent antigenic determinants other than the ones detected by monoclonal an-

tibody M-218. This preliminary result confirmed that P1 protein possesses more than one antigenic determinant.

DISCUSSION

The only natural host of *M. pneumoniae* is man. This pathogen is the causative agent of atypical pneumonia [10]. The morphology of this organism in culture was reported by Biberfeld and Biberfeld [11]. Use of both transmission and scanning electron microscopy showed the organism to be filamentous and to possess a differentiated terminal knob-like structure. Collier and Clyde [12] studied tracheal organ cultures infected with *M. pneumoniae* and observed the organism to be in an extracellular location with the differentiated terminal structure in apposition to host cell membranes. This suggested that the terminal structure was important for the attachment of *M. pneumoniae*. This observation was supported by later studies of radioactively labeled mycoplasmas in tracheal organ culture [5] and of sputum samples from patients [1]. Earlier studies of the interaction of *M. pneumoniae* with host cells in tracheal organ culture showed that host cell injury could be induced only by metabolically active mycoplasmas attached to respiratory epithelium. Transfer of infected tracheal organ cultures to medium non-permissive to *M. pneumoniae* 24 hours following infection prevented the alterations of gross macromolecular biosynthesis in host cells [4]. Based upon these data, it was proposed that *M. pneumoniae* infection was a two-step process, consisting of specific attachment of *M. pneumoniae* to the sialic acid-containing receptor sites on host cells followed by a yet-to-be-defined mechanism of cell injury [5].

Studies have been done in our laboratory to identify and characterize the surface moiety of *M. pneumoniae* responsible for the specific attachment of *M. pneumoniae* organisms to respiratory epithelium. This adhesion is thought to be a prerequisite for the colonization of *M. pneumoniae* in the respiratory tract and subsequent development of disease [5,13]. The proteinaceous nature of the adhesion was demonstrated by mild treatment of this organism with trypsin followed by SDS-gel electrophoresis. We found that the trypsin treatment of *M. pneumoniae* nullifies its ability to adhere to tracheal epithelium. The ability of *M. pneumoniae* to attach, removed by trypsin treatment, could be restored by incubating the treated organisms in growth medium. SDS-gel electrophoresis of *M. pneumoniae* proteins prepared from trypsin-treated organisms showed the disappearance of a major band on the cell surface. We proposed this protein, designated P1, to be the attachment factor, as the failure of the mycoplasmas to attach correlated with its absence. Moreover, regeneration of P1 by incubation in fresh growth medium for six hours (approximately one generation time of *M. pneumoniae*) renewed the attachment capacity, and P1 also reappeared on the polyacrylamide gel profile [5]. Results shown in Fig. 1B and one previous publication [6] demonstrated evidence that P1 protein is physically located on the tip structure of *M. pneumoniae* organisms.

Another important characteristic of virulent *M. pneumoniae* is its ability to adsorb erythrocytes [14]. Trypsin-treated colonies of virulent *M. pneumoniae* are unable to adsorb either erythrocytes or tracheal epithelial cells [15]. This suggests that *M. pneumoniae* might utilize the same mechanisms for attachment to both cell types. The concomitant loss of the ability to attach and the hemadsorption activity (HA) in an avirulent strain [16] also implies the possibility that the catalytic sites of these two activities could be in close proximity, if not identical. On the other hand, there are reports [17,18,19] suggesting that HA is probably mediated by a different moiety in that protein bands other than P1 were found missing in certain HA^-

mutants as revealed by gel electrophoresis. Unfortunately, authors of these studies failed to provide evidence that the bands were surface-located, which is a basic requirement to qualify their role in the hemadsorption, a surface contact phenomenon.

The fine analysis of antigenic determinants of P1 using monoclonal antibodies may lead to answers as to whether P1 is responsible for hemadsorption as well as attachment of *M. pneumoniae* organisms. Similar approaches have been used to delineate the antigenic composition of hemagglutinin molecules of influenza virus and for analysis of the mechanism of antigenic drift, which has been suggested to be responsible for the recurrence of influenza infection [20]. The preliminary results presented in this paper indicate the feasibility of this approach for studying the structure/function relationship of the P1 protein. These studies should lead to a better understanding of the biological activities of this organism and provide information that would be useful in altering the interaction of *M. pneumoniae* with man to favor the human host.

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