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Mycoplasma pneumoniae Infection: Role of a Surface Protein in the Attachment Organelle

Abstract. *Attachment of* Mycoplasma pneumoniae *to host cells by means of a specialized terminus initiates infection. Monoclonal antibodies to a surface protein (P1) inhibit this process, and react with a region of the tip covered with peplomer-like particles. Since antibodies against the P1 protein are generated by natural and experimental infection and by immunization, the substance may be an important determinant of protective immunity.*

In earlier studies of the interaction of *Mycoplasma pneumoniae* with host cells in tracheal organ cultures (*1*), we showed that the attachment of viable mycoplasmas to the respiratory epithelium is necessary for the initiation of infection. Biberfeld and Biberfeld (*2*) first reported an internal rod within a narrow, slightly knobbed tip at one end of filaments of *M. pneumoniae*. Collier (*3*) observed that this differentiated terminal structure always occurred in apposition to host cell membranes and suggested that it was important for the attachment of *M. pneumoniae*. This observation was supported by later studies of radioactively labeled mycoplasmas (*4*) and of sputum samples from patients (*5*). Subsequently, we demonstrated that a surface protein component was involved in attachment (*6*). When *M. pneumoniae* was treated with proteases, attachment was inhibited and a major protein band (P1) was diminished on gel electrophoresis (*6*). Reincubation of these trypsin-treated *M. pneumoniae* in growth medium resulted in regeneration of the P1 protein and resumed ability of the organism to attach to respiratory epithelium. However, direct evidence for the P1 protein on the tip structure of *M. pneumoniae* has not been reported.

Recently, we isolated hybridoma cell lines that produce monoclonal antibodies against the surface antigens of *M. pneumoniae* (*7*). By using the protein blot and radioimmunobinding techniques (*8*) we found that one of the hybridoma cell lines, M-218, secretes an immunoglobulin IgG1 (antibody M-218) that is directed specifically against the P1 protein. In this report we demonstrate, by using this monoclonal antibody, that the P1 protein is localized at the attachment tip of *M. pneumoniae*. We also demonstrate by negative staining electron microscopy that a well-demarcated outer layer is restricted to the area of the terminal structure of *M. pneumoniae*.

Mycoplasma pneumoniae strain M-129 (ATCC No. 29342) was originally isolated from a patient with pneumonia (*9*). After several passages in vitro, *M. pneumoniae* was grown either in Hayflick medium supplemented with 20 percent horse serum from which gamma globulins had been removed (*6*) or in SP-4 medium (*10*). The cultures were prepared as described previously (*6*). In some experiments, the monolayers were treated with trypsin (*6*). Electrophoresis was performed in 10 percent sodium dodecyl sulfate (SDS)–polyacrylamide slab gels, and the separated proteins were transferred to nitrocellulose sheets which were then used for immunoradiobinding studies (*8*). The Coomassie blue–

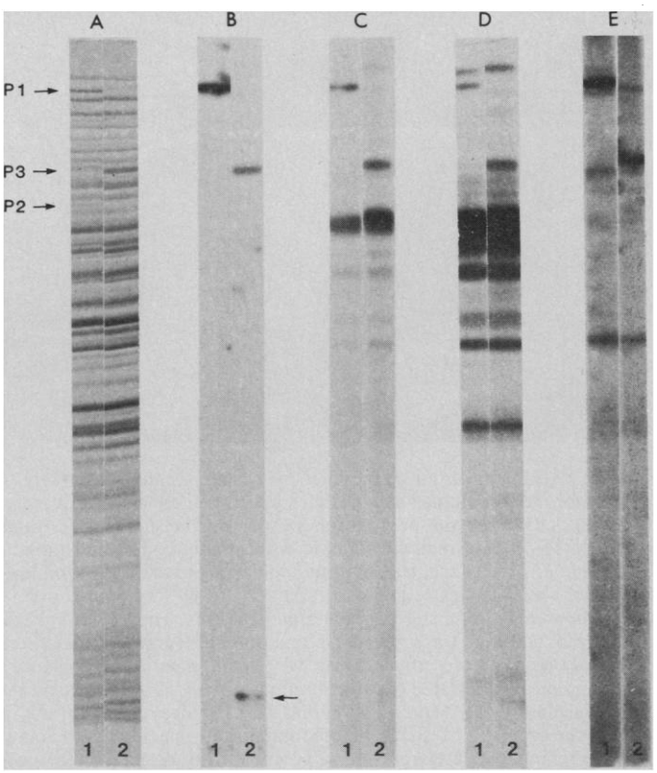

Fig. 1. Immunoradiobinding studies of *M. pneumoniae* proteins. (A) Sodium dodecyl sulfate (SDS)–polyacrylamide gels of 1, untreated and 2, trypsin-treated organisms stained with Coomassie blue. Note the absence of both the P1 (molecular weight 190,000) and P2 (molecular weight, 78,000) protein bands and the appearance of the P3 (molecular weight, 90,000) band in the trypsin-treated preparation. (B) The proteins of *M. pneumoniae* shown in (A) were transferred from SDS-polyacrylamide gel to nitrocellulose sheets, incubated with ascitic fluid containing monoclonal antibodies, and imaged with ^{125}I-labeled rabbit antibody to mouse IgG. Radioautography indicates the specific binding of monoclonal antibody to the P1 protein (B1) and P3 (B2); the latter is a residual polypeptide of P1 since it retains the same antigenic determinant. An additional residual protein band (molecular weight, ~ 12,500) which also can be recognized by the antibody is located at the bottom of the gel (B2, indicated by the arrow). (C, D, E) Same as (B), but incubated with serum from (C) a hamster infected with *M. pneumoniae* by inhalation, (D) a rabbit hyperimmunized by injection of *M. pneumoniae*, and (E) a patient with confirmed mycoplasmal pneumonia, and then "imaged" with ^{125}I-labeled respective species-specific second antibodies. Antibodies specifically against P1 protein are present in all cases, indicating that this substance is a major immunogen of the mycoplasma.

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stained gels (Fig. 1, A1 and A2) reveal the disappearance of P1 and P2 proteins and the appearance of the P3 band in the trypsin-treated mycoplasma preparation (Fig. 1, A2). Incubation with monoclonal antibodies from hybridoma cell line M-218 revealed a single band at the P1 position (Fig. 1, B1) indicating that the monoclonal antibodies were specific for the P1 protein. Trypsin treatment cleaved the P1 protein and resulted in the appearance of P3 protein at a region that indicated a molecule of lower molecular weight (Fig. 1, B2). The residual P3 remained with the trypsin-treated mycoplasmas and retained the same specific epitope (antigenic determinant) as in P1.

Although trypsin treatment abolishes the attachment capacity of the mycoplasma (*6*), the present observation indicates that trypsin treatment does not destroy the antigenic determinant recognized by the monoclonal antibodies used in this study. Therefore, it appears that the conformational integrity of the P1 protein molecules is required for the attachment of virulent *M. pneumoniae* to respiratory epithelium. The faint band that appears at the bottom of the gel (Fig. 1, B2) may represent the portion that has cleaved from P1 but remained attached to mycoplasmas, since it is also recognized by antibody M-218. This observation suggests that the P1 molecules probably possess several repeating amino acid sequences which form multi-identical antigenic determinants. Antibodies to the P1 protein also could be demonstrated in serum samples from hamsters experimentally infected with *M. pneumoniae* by inhalation (Fig. 1, C1), rabbits hyperimmunized with *M. pneumoniae* (Fig. 1, D1), and patients with established mycoplasmal pneumonia (Fig. 1, E1). Although the immune responses to *M. pneumoniae* differ among these species, probably because of the different routes of exposure and methods of immunization, the results indicate that the P1 protein is a major immunogen that stimulates the formation of an antibody that may be important for terminating or preventing infection (*8*).

The specialized terminal structure of *M. pneumoniae* was previously revealed by electron microscopy of conventionally fixed and stained sections of the organism (*2, 11*). By transmission electron microscopy of negatively stained (*12*) *M. pneumoniae*, we have now resolved a well-demarcated particulate outer layer that is restricted to the area of the terminal organelle (Fig. 2A). The particles appear somewhat similar morphologically to the peplomers (minute club-like surface projections) of the influenza virus (*13*). When *M. pneumoniae* was incubated with monoclonal antibody M-218 and subsequently with ferritin-conjugated rabbit antibody to mouse IgG, the antibody M-218 specifically reacted only with the surface of the tip structure corresponding to the same area covered with the peplomer-like particles (Fig. 2B). These data showed that the P1 protein is localized on the attachment organelle of *M. pneumoniae*. We confirmed the role of the P1 protein in the attachment of virulent *M. pneumoniae* to respiratory epithelia (*6*) by using antibody M-218 in an attachment inhibition study. As shown in Fig. 2, C and D, the attachment of ^{3}H-labeled *M. pneumoniae* to respiratory epithelium in tracheal organ cultures was reduced by treatment of the *M. pneumoniae* with antibody M-218, presumably because of blockage of reactive sites on the P1 protein molecules by the specific antibodies.

These data support the possibility that surface protein components of *M. pneumoniae* might be useful as vaccines.

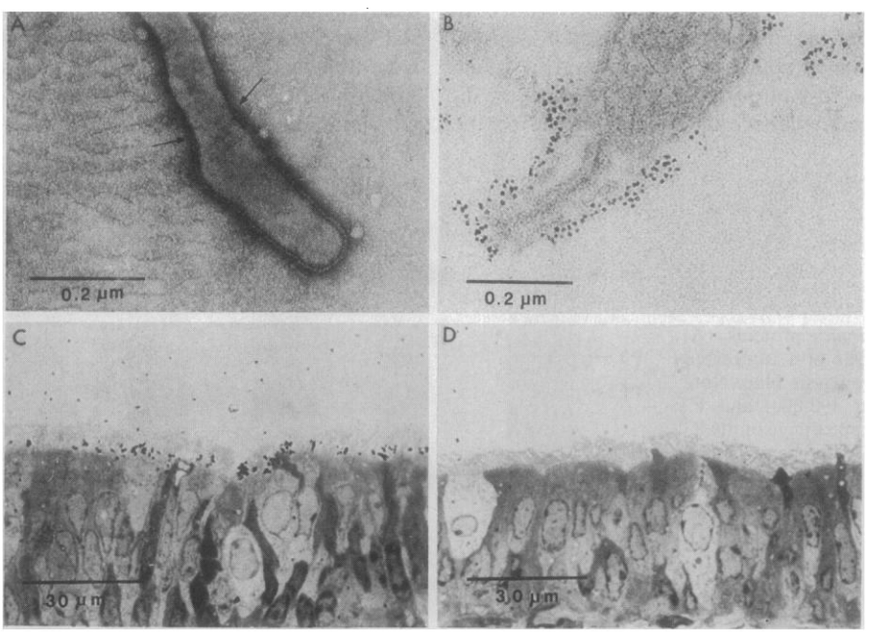

Fig. 2. (A) Electron micrograph of *M. pneumoniae*, negatively stained with ammonium molybdate (*12*). Peplomer-like particles are restricted to the terminal organelle of the organism (arrows). (B) Electron micrograph of *M. pneumoniae* after incubation with monoclonal antibody M-218 and indirect staining with ferritin-conjugated rabbit antibody to mouse IgG. Ferritin grains are concentrated around the tip structure corresponding to the area covered with peplomer-like particles as shown in (A). (C and D) Radioautographs evaluating attachment of *M. pneumoniae* to hamster tracheal organ cultures. The [^{3}H]thymidine-labeled *M. pneumoniae* and organ cultures were prepared and radioautographed as described previously (*6*). (C) Tracheal organ culture infected with *M. pneumoniae* in the presence of normal mouse serum. Silver grains concentrated over the luminal surface of the tracheal ring indicate the presence of mycoplasmas on the ciliated epithelium. (D) Tracheal organ culture infected with *M. pneumoniae* in the presence of ascitic fluid containing P1-specific monoclonal antibodies. The absence of silver grains indicates reduced attachment of organisms to the cell border.

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Cannabinoids in Male Mice: Effects on Fertility and Spermatogenesis

Abstract. *Exposure of adult male mice to cannabinoids is associated with a reduction in fertility and an increased incidence of chromosomal abnormalities. These effects are evident not only in the treated mice, but also in their untreated male offspring.*

Marijuana and its major psychoactive component, Δ^9-tetrahydrocannabinol (THC), have been reported to alter reproductive functions in several species of laboratory animals and in humans (*1*). In humans, marijuana exposure reduces the sperm count and may result in abnormal or absent acrosomal morphogenesis, incomplete condensation of chromatin in the sperm heads, and inhibition of sperm maturation (*2*). Exposure of male rats to cannabinoids significantly alters the sex ratio of their offspring (*2*). Long-term exposure of male mice to crude marijuana extract (CME) arrests spermatogenesis, causes Leydig cell regression, and results in significant increases in the number of ring and chain translocations in germ cells (*3*).

In the present study, we examined the effects of long-term ingestion of THC, cannabinol (CBN), or cannabidiol (CBD) on fertility and on the meiotic chromosomes of the dividing germ cells in adult male mice. In addition, we determined whether exposure of these males to cannabinoids affects reproduction in their F_1 offspring.

The subjects were obtained from our colony of randomly bred mice, which are derived from two inbred strains, Dw/Wf and YS/Ch WF-dw, and from a randomly bred stock, CD-1. Cannabinoids (50 mg/kg) were administered orally three times a week for 5 weeks to groups of 18 mice. On the basis of a body-surface conversion factor of 12 for mice, this dose corresponds to an oral dose of about 4 mg/kg in humans—the equivalent of three marijuana cigarettes containing 1 percent THC (*4*). In mice this dose of THC affects behavior for about 5 hours, which is comparable to the duration of the behavioral effects of a single marijuana smoking episode by a human (*5*). The males were housed with adult females from the third through fifth weeks of treatment and during the first and fourth weeks after treatment. Half of the pregnant females in each group were killed between days 15 and 19 of gestation; the remaining females were allowed to deliver their pups and to raise them. We recorded the number of corpora lutea, resorptions, dead fetuses, viable fetuses, live births, stillbirths, and postnatal deaths and the percentage of females impregnated. The F_1 male offspring were weaned at 21 days of age and housed, four to a cage, until adulthood (60 to 80 days), when their reproductive status was assessed.

Six weeks after treatment, the males were bled by cardiac puncture under ether anesthesia for radioimmunoassay of plasma testosterone (*5*). Body and testicular weights were recorded, and testes were randomly sampled from each group and prepared for cytogenetic evaluation.

To specifically evaluate the effects of cannabinoids on germ cells without the confounding effects of mating, four groups of ten males each were treated with sesame oil, the previously described dose of THC or CBN, or CME (25 mg/kg) daily for 5 days. The mice were killed 50 to 60 days after treatment, both testes were removed, and the meiotic chromosomes were prepared in accordance with the method described by Evans *et al.* (*6*).

In an additional experiment, groups of six adult male mice received a single dose of THC or CBN (100 mg/kg), CME (50 mg/kg), or oil (40 μl). The animals were killed 14 days later and the meiotic chromosomes were prepared for study. A minimum of 50 plates showing the chromosomes in diakinesis or metaphase 1 were examined, and all chromosomal abnormalities were recorded.

In the final experiment, the F_1 male offspring were tested for fertility, as had been their cannabinoid-treated sires. Female mice that failed to become pregnant were remated with fertile males to verify their fertility. Each F_1 male was given the opportunity (during a 1-week cohabitation period) to mate with at least three different females.

Males repeatedly exposed to CBD impregnated significantly fewer females than did control males (Table 1). Also, significantly more prenatal and postnatal deaths resulted from impregnation by CBD-exposed males. Repeated exposure to THC or CBN significantly increased

Table 1. Effect of repeated cannabinoid exposure on fertility, testicular weight, and plasma testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) in adult male mice. Values not given as percentages are mean ± standard errors. There were 18 mice per group.

Treatment	Impregnation rate (%)	Prenatal loss* (%)	Postnatal loss (%)	Weight of testes (mg)	Testosterone (ng/ml)	LH (ng/ml)	FSH (ng/ml)
Oil	80	19	5	332 ± 10	9.2 ± 2.9	21 ± 4	1103 ± 148
THC	73	37†	10	310 ± 10	12.3 ± 3.4	28 ± 8	1190 ± 117
CBD	60†	44†	26†	299 ± 12	7.8 ± 2.1	25 ± 7	1300 ± 108
CBN	73	50†	4	288 ± 9‡	6.1 ± 1.5‡	59 ± 4§	1604 ± 74§

*Refers to pregnancies in which there was evidence of mortality in utero. †Significantly different from corresponding value for oil-treated mice at $P < .05$ (chi-square test). ‡$P < .05$ (analysis of variance and Duncan's test). §$P < .01$.

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