Association Between *M. pneumoniae*

**Hemolysis, Attachment, and Pulmonary Pathogenicity**

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Three different groups of hemolysis mutants were produced by treatment of the *M. pneumoniae* FH-P24 strain with N-methyl-N-nitro-nitrosoguanidine.

The first group of mutants, strains P24-L1, L2, and L11, showed wide and clear hemolytic zones, and attaching ability to erythrocytes and to hamster lung cells were the same as the properties of the parent strain and produced significant microscopic lung lesions. Mutant P24-S1 showed non-hemolysis and non-hemadsorption, yet retained the attaching ability to lung cells and produced milder lung lesions. Mutant P24-S11 showed none of those activities, did not cause any lung lesion, and was never recovered from the lungs of hamsters.

A close relationship between the hemolytic ability of *M. pneumoniae* and the histopathogenicity in the hamster lung is suggested in this study. The attaching ability of organisms seems to be an important factor at the initial stage of infection.

**INTRODUCTION**

*Mycoplasma pneumoniae* is an important cause of human lower respiratory tract infection and is the only mycoplasma species definitely associated with disease in man.

Various pathogenicity factors of *M. pneumoniae* have been discussed [1]. Attachment of mycoplasmas to the mucosal surface is the initial cause of mycoplasma infection and was considered to be an important virulence factor. The following results suggested that the quantity of peroxide secreted by *M. pneumoniae* cells may be another virulence factor [2].

We attempted to separate clones with different hemolytic activities from a strain of *M. pneumoniae*, FH-P24, by treating the organisms with nitrosoguanidine (NTG).

Five hemolysis mutants were separated. Three of them showed wide and clear hemolytic zones (P24-L mutants) and the other two did not show hemolytic zones (P24-S mutants). Their hemolytic characteristics did not change even after 20 subcultures. The mutants were compared for their attachment abilities and pathogenicities in hamsters.

**MATERIALS AND METHODS**

*Organisms*

*M. pneumoniae* strain FH-P24 was obtained by passing strain FH 24 times in hamsters [3], and served as the parent strain. The hemolysis mutant strains P24-L1, P24-L2, and P24-L11 were isolated from the parent strain and served as parent strains for further experiments.

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L2, L11, S1, and S11 were obtained from the FH-P24 strain by means of NTG-mutagenesis as described [4]. Mycoplasmas were grown at 37°C in modified Chanock's broth and agar enriched with 20 percent heat-inactivated horse serum and 1 percent glucose.

**Hemolysis and Attachment**

Hemolysis and attachment to *M. pneumoniae* colonies were treated as previously described [4].

*Hemolysis*  The colonies grown on the agar plates were overlaid with 3 ml of a mixture of 3 percent fresh erythrocytes and PPLO agar (Difco). Plates were then incubated at 37°C for 24 hours and observed for hemolysis. The ratio of hemolytic zone diameter to colony diameter was expressed as the “hemolytic index” [2].

*Attachment*  The colonies grown on PPLO agar were overlaid with 1 ml of a 0.5 percent suspension of human blood group O, horse, sheep, rabbit, guinea pig, hamster, mouse, and chicken erythrocytes. The agar plates were incubated for 30 minutes at 37°C, unadsorbed cells were washed off with 0.01 M phosphate buffered saline (PBS, pH 7.2), and then observed microscopically for degree of hemadsorption. Attachment to cell line of hamster lung (CCL 16, CCL 39) was tested by the method for the hemadsorption mentioned above. However, on the 0.5 percent suspension of cultured lung cells, monolayers were trypsinized, washed, and then resuspended in 0.01 M PBS (pH 7.2).

**Determination of Ciliary Activity**

Ciliary activity of hamster tracheal rings was determined as described by Hirano et al. [5].

**Virulence Studies**

Hamsters were infected by aerosol inhalation of FH-P24 and its mutants as reported by Hayatsu et al. [6]. The infection doses were 3.2 to 5.0 × 10⁴ CFU in aerosol. The animals were sacrificed two and four weeks after infection, and the trachea and lungs were assayed for the quantity of *M. pneumoniae* present. Specimens of trachea and lungs were also fixed for histopathologic examination. The pulmonary lesions were graded in a scale of 0 to 3.

**Recovery of Mycoplasmas in Hamster Lungs After a Large Volume Inoculation**

Hamsters were infected by intranasal instillation of 0.1 ml culture of parent and P24 mutant strains (3.9 to 6.8 × 10⁴ CFU per animal). Six hours and two to 14 days after infection, hamsters were sacrificed, and their lungs were assayed for the quantity of *M. pneumoniae* present.

**RESULTS**

**General Characterization of Hemolysis Mutant**

All mutants fermented glucose, and their growth was not inhibited by methylene blue. Aerobic reduction of 2, 3, 5,-triphenyl tetrazolium chloride was all positive. The growth inhibition zone produced by specific antiserum against the P24-S1 was 31 mm in diameter, while those of P24-L, P24-S11, and parent strains ranged from 17 to 19 mm. The efficiencies of colony formation at 32°C and 37°C were not different in the strains tested (Table 1).
TABLE I
Biological Properties of M. pneumoniae Mutants and Parent Strain

| Property                                         | Parent | P24-S1 | P24-S11 | P24-L1 | P24-L2 | P24-L11 |
|-------------------------------------------------|--------|--------|---------|--------|--------|---------|
| Growth aerobic/anaerobic                        | +/-    | +/-    | +/-     | +/-    | +/-    | +/-     |
| Fermentation of glucose                        | +      | +      | +++     | ++     | +      | +       |
| Growth inhibition (MB*)                         | -      | -      | -       | -      | -      | -       |
| Aerobic reduction of TTC*b                      | +      | ±      | ±       | +      | +      | +       |
| Efficiency of colony formation at 32/38°C       | 1.1    | 1.1    | 0.7     | 1.1    | 1.0    | 1.1     |
| Growth inhibition test (Antiserum)              | 19 mm  | 31 mm  | 17 mm   | 19 mm  | 19 mm  | 19 mm   |
| Hemolysis (GPRC*c)                              | +      | ±      | ±       | ++     | ++     | ++      |
| Hemolytic indexd                               | 8.7    | 1.3    | 2.2     | 13.2   | 12.3   | 13.8    |
| Hemadsorption*                                 | ++     | -      | -       | ++     | ++     | ++      |
| Attachment to hamster lung cells                | +      | -      | -       | ++     | ++     | ++      |

* Methylene blue
* 2,3,5-triphenyl tetrazolium chloride
* Guinea pig red cell
* The ratio of hemolytic zone diameter to colony diameter
* Human-O, horse, guinea pig, sheep, mouse, rabbit, hamster, and chicken erythrocytes

Hemolysis

The values of hemolytic index were significantly different in P24-L mutants (12.3 to 13.8), P24-S mutants (1.3 to 2.2), and parent strain (8.7). P24-L mutants had the highest hemolytic index and P24-S mutants barely showed the hemolytic zone.

Attachment to Various Cells

Colonies of parent strain and P24-L mutants adhered well to cells tested. P24-S1 did not adsorb any erythrocytes but did adsorb the cultured lung cells. P24-S11 did not adsorb erythrocytes and lung cells at all.

Ciliostasis by Mycoplasmas in Hamster Tracheal Organ Culture

When P24-L mutant was inoculated, the ciliary activity-negative rings appeared on the fifth day of incubation and all of the rings lost the activity completely by the thirteenth day.

On the other hand, P24-S1 and S11 mutant strains showed mild effects. There, negative rings appeared between the eighth and tenth days, but a few rings did not lose the ciliary activity for 16 days.

Virulence of Parent and Mutant Strains of M. pneumoniae FH-P24 in Hamsters Inoculated in Aerosol

The parent and P24-L1 mutant strains produced significant microscopic lung lesions, mainly bronchitis and peribronchitis, by two weeks post-infection, and these were more severe at week 4. P24-S1, a non-hemolysis mutant strain, produced significantly less lung lesions than those of parent and P24-L mutant strains two weeks after infection, and a still lower degree of lesion at week 4. In contrast, P24-S11, another non-hemolysis mutant strain, did not cause any significant degree of microscopic lung lesions through the experimental period (Fig. 1).

Mycoplasmas were isolated from the lungs of parent and mutant strains in each experimental period except for strain P24-S11, in which case the organisms were never recovered from the lungs.
FIG. 1. Lung histopathology in hamsters after inoculation by aerosol inhalation with parent and mutant strains. The infection doses were 3.2 to 5.0 × 10³ CFU per animal. Each bar represents the mean lung lesion score obtained from 20 to 25 hamsters.

Recovery of Mycoplasmas in Hamster Lungs After Intranasal Inoculation

Organisms were found in high quantities in the lungs during the observation period, when parent, P24-L1, and P24-S1 mutant strains were inoculated. On the other hand, in the case of mutant P24-S11, mycoplasmas rapidly decreased up to day 4 and were not recovered thereafter.

DISCUSSION AND CONCLUSIONS

Attachment, peroxide, other hemolysins, and exoenzymes were considered as contributing to the pathogenicity of *M. pneumoniae* [1].

We produced hemolysis mutants by treatment of the *M. pneumoniae* FH-P24 strain with NTG. Hemolysis mutants could be divided into three different groups. The first group of mutants, strains P24-L1, L2, and L11, showed the wide and clear...
hemolytic zones and the ability to attach to erythrocytes and lung cells *in vitro* as did those of the parent strain.

The second group, strain P24-S1, showed non-hemolysis and non-hemadsorption, yet apparently possessed the ability of attaching to lung cells, but not to erythrocytes.

The third group, strain P24-S11, was non-hemolytic, completely lost the attaching ability *in vitro*, and did not proliferate *in vivo*.

In hamsters, the first group of strains produced significant microscopic lung lesions, while the second group of mutants produced milder lung lesions. A non-hemolytic as well as non-hemadsorbing mutant, P24-S11, did not cause any lung lesion and was never recovered from the lungs of hamsters inoculated by aerosol inhalation (dose: 3.2 to $5.0 \times 10^2$CFU), even when inoculated at the higher dose of $3.9$ to $6.8 \times 10^4$CFU.

On the first step of infection, by week two post-inoculation, the parallelism between attachment and virulence was more pronounced than that between hemolytic ability and virulence, because the mutant S1, which reserved the ability to attach only to hamster cell cultures but not to erythrocytes, showed a mild pathogenicity in hamsters.

After the establishment of adsorption between the microbial and host cells, hemolysins and exoenzymes produced by mycoplasma influenced the host cell metabolism and formed the histopathological changes.

*M. pneumoniae* suspensions exhibited $\text{H}_2\text{O}_2$-secretion rates of 0.2 to 2 μmoles $\text{H}_2\text{O}_2$/hour/10$^{10}$ viable cells [2]. Even smaller amounts of $\text{H}_2\text{O}_2$ delivered continuously and for long periods of time into the environment might cause oxidative damage to the tissues. Thus, P24-L mutant and parent strains, which showed a higher hemolytic index, produced severe lung lesions during the course of the experiment.

From the results, a relationship between the hemolytic ability of *M. pneumoniae* and its histopathogenicity in hamster lung is suggested.

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