A mechanism of pathogenicity of “*Streptococcus milleri* group” in pulmonary infection: synergy with an anaerobe

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Summary. The relationship between *Streptococcus constellatus*, one of the species of the “*Streptococcus milleri* group”, and *Prevotella intermedia* was studied in a model of pneumonia in mice and *in vitro* to elucidate mechanisms of pathogenicity in “*S. milleri* group”-associated pulmonary infection. Acute pneumonia with or without empyema and lung abscess in mice with mixed infection resulted in 60% mortality rate, but there was only 10% mortality and mild pneumonia in each separate infection. Bacterial clearance of organisms, especially *S. constellatus*, in mixed infection was delayed. Enhancement of growth of *S. constellatus* was demonstrated when cultured with *P. intermedia*; growth was also stimulated by a culture filtrate of *P. intermedia* which also inhibited bactericidal activity of human neutrophils. In an examination of infectivity and bacterial clearance of *S. constellatus* with *P. intermedia* culture filtrate *in vivo*, there was 20% mortality and delayed clearance of *S. constellatus*, although the infection was not as severe as that produced by the combination of both organisms. These results suggest that *P. intermedia* may act with *S. constellatus* in the production of pulmonary infections by stimulating its growth and suppressing bactericidal activity of the host.

Introduction

“*Streptococcus milleri* group” describes a group of anaerobic- or micro-aerophilic streptococci including *S. anginosus*, *S. constellatus* and *S. intermedius*. “*S. milleri* group” organisms are commonly found in the mouth, nasopharynx, gastrointestinal tract and vagina, at a rate of 15–30%.¹ They are often associated with various pyogenic infections and involved in mixed infections, especially in combination with anaerobes.² Infection with these organisms may arise by local invasion from their usual habitats.³

Although “*S. milleri* group” organisms have also been isolated from pneumonia, empyema and lung abscess^{3–6} they are often unrecognised as pathogens in pulmonary infections because their pathogenicity and pathogenic mechanisms have not been fully determined.⁷

This study investigated these mechanisms in a model of pneumonia in mice and by *in-vitro* studies.

Materials and methods

The synergy between *S. constellatus* and *Prevotella intermedia* was studied in the following stages *in vivo* and *in vitro*:

(i) Infectivity of *S. constellatus* and *P. intermedia* separately and in combination in mice was studied. Experiments were carried out when the results *in vivo* suggested that *P. intermedia* or its metabolic substances might stimulate the growth of *S. constellatus* and inhibit the defence mechanisms of the host.

(ii) Growth enhancement of *S. constellatus* in broth was investigated with *P. intermedia* and its cell-free culture filtrate.

(iii) Bactericidal activity of human polymorphonuclear leucocytes (PMNLs) was examined to determine the effect of *P. intermedia* culture filtrate on host defence mechanisms.

(iv) Infectivity of *S. constellatus* in combination with the culture filtrate of *P. intermedia* was studied *in vivo*.

Bacterial strains

S. constellatus RZYK 001 and *P. intermedia* RZST 004 were isolated from broncho-alveolar lavage fluid and purulent sputum obtained by bronchoscopy. *Staphylococcus aureus* ATCC 25923 was used for the study on the bactericidal activity of PMNLs.

Culture

S. constellatus was cultured on Tryptic Soy Blood Agar (Kyokuto Pharmaceutical Industrial Co.,

Tokyo, Japan) for 24 h at 37°C in air with CO₂ 5% and *P. intermedia* was grown on Brucella HK Agar (Kyokuto Pharmaceutical Industrial Co.) supplemented with sheep blood 5% for 48 h in an anaerobic chamber in an atmosphere of N₂ 80%, H₂ 10% and CO₂ 10%. Vancomycin 2.5 µg/ml was added to Brucella HK agar to select *P. intermedia* from lung homogenate and culture broth. *S. aureus* was grown on Trypticase Soy Agar (Becton Dickinson, Cockeysville, MD, USA) for 18 h at 37°C.

Preparation of culture filtrate of *P. intermedia*

P. intermedia was cultured in peptone-yeast-glucose (PYG) broth until it overgrew. The broth was then centrifuged (15000 rpm for 30 min) and the pH adjusted to 7.2 with 1.0 M and 0.1 M NaOH. The total volume change with pH titration was small. The broth was sterilised by passage through a 0.22-µm filter (Millipore). A *P. intermedia* culture filtrate from Schaedler Broth (Hoffmann-La Roche, Germany) was prepared by the same method for the study of growth enhancement of *S. constellatus*.

Infectivity testing

Male BALB/c mice (20–22 g), raised in conventional conditions were used. *S. constellatus* and *P. intermedia* were suspended in PYG broth (pH 7.2) at concentrations of 10⁹ and 10¹⁰ cfu/ml, respectively. In the studies examining the effects of *P. intermedia* culture filtrate, *S. constellatus* was suspended in the culture filtrate and the concentration was adjusted to 10⁹ cfu/ml. Mice were anaesthetised with pentobarbital and 0.07 ml of the bacterial suspension was inoculated *via* a 22 gauge catheter inserted into the trachea; control mice were similarly challenged with an equal volume of PYG broth.

Groups of 10 mice, inoculated with separate species or a combination, were observed over 14 days to determine the mortality rate.

Four mice from each initial group of 20, including a control group, were killed by cutting the axillary arteries at 0, 3, 7, 11 and 14 days after bacterial challenge and examined histologically. Formalin 10% was infused through the trachea and the lungs were removed surgically and fixed in formalin 10% for 24 h. They were then embedded in paraffin, and sections were cut and stained with haematoxylin and eosin.

Three or four mice from groups of 30 were killed by cervical dislocation at 0, 6, 12, 24, 36, 48 and 60 h after bacterial challenge for quantitative lung culture. The lungs with attached bronchi were removed aseptically and homogenised in 1 ml of PYG broth inside an anaerobic chamber. Serial 10-fold dilutions of the homogenates were performed with phosphate-buffered saline (PBS, pH 7.4) containing dithiothreitol

0.05%, and cultured on soybean-casein digest agar and Brucella HK agar with sheep blood 5%.

Assay of growth enhancement effect for *S. constellatus*

Studies on the growth of *S. constellatus* and *P. intermedia* were performed in Schaedler broth containing vitamin K₁. The mixed and single cultures of *S. constellatus* and *P. intermedia* were incubated in anaerobic conditions. Growth enhancement of *S. constellatus* was investigated with *P. intermedia* culture filtrate adjusted to pH 7.2. Counts of bacterial colonies were performed every 3 h by inoculating serial dilutions on agar plates.

Measurement of bactericidal activity of PMNLs

PMNLs were separated from heparinised peripheral blood of a healthy adult by centrifugation with Mono-Poly Resolving Medium (Flow Laboratories, Inc., Tokyo, Japan). The purity of PMNLs was > 95% and the proportion of viable cells was > 95%. They were then suspended in Hanks's Balanced Salts Solution, pH 7.4 (HBSS; Gibco Laboratories, Grand Island, NY, USA). A 1-ml suspension containing PMNLs (5 × 10⁶ cells), *S. aureus* suspension (1 × 10⁶ cfu), normal human serum (15%), HBSS, and either *P. intermedia* culture filtrate, sterile PYG broth or PBS was made in Falcon 2063 polypropylene tubes and rotated slowly at 37°C for 120 min. After incubation, a sample of the mixture was added to cold distilled water to disrupt the PMNLs, and viable bacterial counts were performed by plating serial 10-fold dilutions on soybean-casein digest agar. Tubes without PMNLs were treated similarly as controls. Results were expressed as a killing index.⁸ Killing index (%) = [(number of viable bacteria without PMNLs) – (number of viable bacteria with PMNLs)]/number of viable bacteria without PMNLs × 100.

Statistical analysis

The data were expressed as means and SDs. Statistical comparison of survival rates was performed by Wilcoxon's test and in other investigations by Student's *t* test.

Results

Pneumonia induced by *S. constellatus* and *P. intermedia*

Histological findings and mortality are shown in table I. *S. constellatus* and *P. intermedia* caused pneumonia when inoculated separately; histopathological examination showed acute bronchopneumonia on days 1 and 3, and the number of neutrophils after 3 days fell considerably compared to day 1. The pneumonia improved by 7 days, no abscesses developed

Table I. Infectivity of *S. constellatus* and *P. intermedia* separately and in combination when inoculated into lungs of mice

Inoculum*	Histological findings		Mortality†
	Pneumonia	Lung abscess	
<i>S. constellatus</i>	Mild, improved by 7 days	None	10 %
<i>P. intermedia</i>	Mild, improved by 7 days	None	10 %
<i>S. constellatus</i> + <i>P. intermedia</i>	Severe, continued for 14 days (complicated with empyema in one case)	60 % in 8–14 days	60 %‡

*Each organism was suspended in PYG broth and inoculated into mice as described in *Materials and methods*.

†n = 10 in each group.

‡p < 0.05 compared with single infection by *S. constellatus* or *P. intermedia*.

and only 10% of mice with either of the single infections died; deaths occurred on the second day. Examination of the lungs from the mice that died demonstrated severe pneumonia. The combination of *S. constellatus* and *P. intermedia* induced acute pneumonia which continued even after 7 days. Alveoli were filled with neutrophils, and alveolar haemorrhage with

pulmonary oedema was prominent on the third day. Aggregates of neutrophils remained and the structure of alveoli was severely damaged by the seventh day. Abscesses developed within 8–14 days. *S. constellatus* was the organism most commonly isolated. Sixty percent of mice died of acute pneumonia within 2–5 days (p < 0.05 compared with infection by separate organisms). No significant findings were demonstrated in control mice.

Infection either by *S. constellatus* or *P. intermedia* was cleared from the lungs by 60 h whereas viable bacteria remained in mice inoculated with mixed organisms 60 h after inoculation; the number of *S. constellatus* remained at approximately the same level (fig. 1).

Growth stimulation of *S. constellatus* with *P. intermedia* or culture filtrate of *P. intermedia*

Growth of *S. constellatus* in combination with *P. intermedia* was eight times greater than that of *S. constellatus* alone at maximal growth (fig. 2). The number of *P. intermedia* in mixed culture gradually increased with time. Growth of *S. constellatus* in the mixed culture was stimulated as the number of *P. intermedia* increased. More rapid growth and larger numbers of *S. constellatus* were observed in broth

Fig. 1. Clearance of *S. constellatus* (a: ○ and ●) and *P. intermedia* (b: □ and ■) from lungs in single (○ and □) and mixed (● and ■) infection. Values are expressed as means and SD for three or four mice/group; * p < 0.05 and ** p < 0.005 compared to the number of bacteria in single infection.

Fig. 2. Growth of *S. constellatus* and *P. intermedia* during mixed (● and ■, respectively) and individual (○ and □, respectively) cultures.

Fig. 3. Growth of *S. constellatus* in broth with (●) and without (○) *P. intermedia* culture filtrate.

containing *P. intermedia* culture filtrate than when cultured in broth without the filtrate (fig. 3).

Effect of culture filtrate of *P. intermedia* on bactericidal activity of human PMNLs

Viable counts of *S. aureus* after incubation for 120 min with PMNLs are shown in table II. The killing index of PMNLs incubated with PYG broth and PBS was 59% in both cases, but only 31% with

P. intermedia culture filtrate; this represented a significant reduction ($p < 0.01$) in their bactericidal activity.

Infectivity of *S. constellatus* with culture filtrate of *P. intermedia*

Twenty percent of mice died within 4 days after inoculation with *S. constellatus* and *P. intermedia* culture filtrate, whereas there were no deaths after inoculation with *S. constellatus* alone. There were

Table II. Effect of *P. intermedia* culture filtrate on the killing of *S. aureus* by human PMNLs

PMNLs incubated with*	Killing index (%)†
PBS	58.9 (9.9)
PYG broth	59.1 (9.0)
<i>P. intermedia</i> culture filtrate	30.9 (6.2)‡

*Human PMNLs were incubated with each broth at 37°C for 120 min.

†Defined in *Materials and methods*. Data are the mean and (SD) of three experiments performed in duplicate.

‡p < 0.01 compared with cells incubated with PBS and PYG broth.

Table III. Changes in number of *S. constellatus* in the lungs of mice after inoculation

Inoculum	Number of viable <i>S. constellatus</i> * at		
	0 h	24 h	48 h
<i>S. constellatus</i>	8.2 (0.7)	3.6 (0.7)	1.6 (0.3)
<i>S. constellatus</i> with <i>P. intermedia</i> culture filtrate	8.1 (0.3)	4.7 (0.1)†	3.7 (0.8)§
<i>S. constellatus</i> + <i>P. intermedia</i>	7.9 (0.5)	6.3 (0.7)‡	6.7 (1.7)

*Data expressed as log₁₀ cfu (SD) of organisms, four mice/group.

†p < 0.05 compared to *S. constellatus*.

‡p < 0.05 compared to *S. constellatus* and *S. constellatus* with *P. intermedia* culture filtrate.

§p < 0.01 compared to *S. constellatus*.

||p < 0.005 compared to *S. constellatus* and p < 0.01 compared to *S. constellatus* with *P. intermedia* culture filtrate.

significant numbers of viable bacteria in mice inoculated with *S. constellatus* and culture filtrate after 48 h, but fewer than in mice inoculated with *S. constellatus* and viable *P. intermedia* (table III).

Discussion

The “*S. milleri* group” are associated with pyogenic infections, bacteraemia and endocarditis, and are also relatively common pathogens in pulmonary infections. Despite this clinical significance there have been few reports on the pathogenic mechanisms of the “*S. milleri* group” in pulmonary infections.

Anaerobes are known to inhabit areas of the human body similar to those where “*S. milleri* group” organisms are found, and anaerobes of oral origin, particularly of the genera *Peptostreptococcus*, *Prevotella* or *Fusobacterium*, mixed with micro-aerophilic streptococci, have often been isolated from pulmonary infections.^{2, 9, 10} This suggests the possibility of synergy in the production of “*S. milleri* group” virulence.

This study demonstrated that whereas *S. constellatus* or *P. intermedia* alone induced pneumonia, *S. constellatus* in combination with *P. intermedia* produced a more severe pneumonia and higher mortality. Moreover, mice infected with the combination of organisms also developed lung abscesses or empyema. These

experiments demonstrate synergy in mixed infection with *S. constellatus* and *P. intermedia*.

The following mechanisms of synergy between certain aerobes and anaerobes have been suggested:¹¹ production of various growth factors by the microbes; the ability of anaerobes by their metabolic products or capsules to inhibit phagocytosis of aerobes by leucocytes; alteration of the local environment including reduction of the oxygen tension and lowering of the redox potential; and the production of substances toxic to the host.

There are several examples of growth enhancement effects in experimental models. In Meleney's gangrene models,¹² a heat-labile growth factor produced by *S. aureus* stimulated growth of streptococci including *S. intermedius*, one of the species of the “*S. milleri* group”.

Several studies have examined the mechanisms by which anaerobes affect host defences. Jones and Gemmell¹³ showed that *Bacteroides melaninogenicus* (now *Prevotella melaninogenica*) supernate impaired phagocytosis by PMNLs. Similar results have been obtained in other studies with *Prevotella*, *Porphyromonas* and *Fusobacterium* species.^{14, 15} Okuda and Takozoe¹⁶ demonstrated that capsular material inhibited phagocytosis and phagocytic killing of other micro-organisms by leucocytes and Brook and Walker¹⁷ suggested that the presence of a polysaccharide capsule might be a necessary virulence factor in suppurative infections. Most of the *S. constellatus* and *P. intermedia* cells used in this study were capsulated.

In our in-vitro study, *S. constellatus* multiplied at a greater rate in the culture mixed with either *P. intermedia* or the cell-free culture filtrate than when cultured alone in the broth. Furthermore, *P. intermedia* culture filtrate inhibited bactericidal activity of human PMNLs, indicating that *P. intermedia* or some of its metabolic substances might help *S. constellatus* survive and multiply. *P. intermedia* and cell-free culture filtrate delayed pulmonary clearance of *S. constellatus* when injected into mice.

The combination of *S. constellatus* with *P. intermedia* culture filtrate was not as virulent as that of *S. constellatus* with viable *P. intermedia*. The “*S. milleri* group” produces extracellular enzymes such as hyaluronidase, DNAase, gelatinase and collagenase^{18–20} and releases an immunosuppressive substance.²¹ Some anaerobes exhibit similar extracellular enzyme-releasing activity; a strain of *P. intermedia* examined by Rudek²² produced several enzymes. Although the virulent mechanisms of “*S. milleri* group” organisms and anaerobes have not been fully determined, these bacteria may co-operate to damage pulmonary tissue, invade and spread by production of various extracellular enzymes.

Auwerda² reported that “*S. milleri* group” organisms were more often isolated together with anaerobes from clinical specimens, but in the study by Kambal,⁷ 61 % of isolates were obtained in pure culture. The use of

antibiotics such as metronidazole which suppresses anaerobes but does not affect the "*S. milleri* group" may also influence their pathogenic potential.²³

Clearance of *S. constellatus* from mice inoculated with mixed organisms was delayed compared with that of *P. intermedia*. This is analogous to the finding in Lewis's subcutaneous abscess model²⁴ that only *S. milleri* could be recovered from abscesses in some animals despite having been inoculated in combination with *Peptostreptococcus* spp.

In this study a combination of only two organisms was investigated and, although it may not accurately

reflect a clinical "*S. milleri* group"-associated pulmonary infection from which more than two organisms are sometimes recovered,^{9,10} it provides some information regarding the pathogenicity of "*S. milleri* group" and anaerobes in pulmonary infections.

In conclusion, we suggest that "*S. milleri* group" virulence is enhanced by the presence of the anaerobe *P. intermedia*.

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