Pathogenicity of the *Streptococcus milleri* Group in Pulmonary Infections —Effect on Phagocytic Killing by Human Polymorphonuclear Neutrophils—

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Abstract

The pathogenicity of *Streptococcus constellatus* in pulmonary infections was investigated in mice in correlation with the phagocytic killing of the microorganisms by human polymorphonuclear neutrophils (PMNs). The histological inflammation score of the lungs in mice inoculated intratracheally with 10^8 cfu/mouse of the virulent strain *S. constellatus* RZYK001 obtained from bronchoalveolar fluid of a patient with pneumonia, was significantly higher than in mice inoculated with the avirulent strains *S. constellatus* RT4303 and RT6002 obtained from saliva of healthy adults ($p < 0.001$). The bactericidal activity of human PMNs against *Staphylococcus aureus* ATCC 25923 was not inhibited at all by the culture filtrate of the virulent strain, but the killing index against RZYK001 was 36.4% after 90-min incubation whereas that against RT4303 and RT6002 was 94.7% and 99.4%, respectively ($p < 0.001$). Therefore, these results suggest a possible pathogenic mechanism of *S. constellatus* apparently mediated by a structural component of the organisms so that a virulent strain is less likely to be killed than an avirulent one, predisposing it to survive in the infection sites.

Introduction

Almost four decades have passed since *Streptococcus milleri* was first described in 1956 by Guthof¹⁾. The *S. milleri* group is the name for a large group of streptococci which includes *S. anginosus*, *S. constellatus* and *S. intermedius*. The bacteria inhabit the upper respiratory and gastrointestinal tracts, the vagina and other body sites²⁾, and can be easily isolated from humans. They are recognised as clinically significant pathogens in suppurative infections, including pulmonary infections such as purulent pneumonia, empyema and lung abscesses³⁾. However, organisms of the *S. milleri* group are often not recognised as pathogens because of the lack of uniformity in classifications and difficulties in microbiological identification⁴⁾.

Pathogenic mechanisms of the *S. milleri* group have been investigated by many researchers but have still not been elucidated. Encapsulation of the organisms in a subcutaneous abscess model⁵⁾ and production of tissue-destroying enzymes and extracellular products were observed as virulence factors in the bacteria^{6,7)}. The *S. milleri* group is often involved in mixed infections, particularly in combination with anaerobes⁸⁾, with which a synergistic relationship has been suggested, metabolic

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products of anaerobes may impair the host defenses^{9,10}. For example, *Prevotella intermedia* may stimulate growth of *S. constellatus* in pulmonary infections as well as suppress bactericidal activity of polymorphonuclear neutrophils (PMNs)¹¹.

However, Kambal¹² reported that 61% of the isolates from patients with clinical evidence of *S. milleri* group infection were obtained in pure culture, and the fact that 30% (26/88) of the isolates at the University of the Ryukyus Hospital in 1993 were associated with single-organism infection would also indicate that the *S. milleri* group could be a significant sole pathogen.

The virulent strain of *S. constellatus*, used in this study was more pathogenic in combination with an anaerobe than in single infections; the combination resulted in 60% mortality and frequently induced abscesses in mice¹¹. The present study was performed to determine the role of the reference strain obtained from a patient with pneumonia in causing pneumonia in mice, compared with avirulent strains isolated from saliva of healthy adults, in terms of the effect on phagocytic killing by human PMNs.

Materials and Methods

Bacterial strains and culture methods

S. constellatus RZYK001, defined as a virulent strain and isolated in pure culture from broncho-alveolar fluid of a patient with community-acquired pneumonia, and *S. constellatus* RT4303 and RT6002 isolates, avirulent strains obtained from saliva in the oral cavities of healthy adults, were used in both infectivity and phagocytic killing assays. *Staphylococcus aureus* ATCC 25923 was used for the study on bacterial killing.

S. constellatus, grown on Trypticase-soy blood agar (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) for 24 hr at 37°C with 5% CO₂ and *S. aureus* grown on Trypticase soy agar (TSA) (Becton Dickinson and Co., Cockeysville, MD, USA) overnight at 37°C were used. The strains of *S. constellatus* were identified by VITEK and Rapid ID32 Strep kits according to the manufacturer's instructions (BioMérieux Vitek Japan, Ltd., Tokyo).

The presence of capsules in the organisms grown overnight in brain heart infusion broth (Eiken Chemical Co., Ltd., Tokyo) was established with Hiss' stain¹³.

Infectivity testing

Male BALB/c mice, 21–23 g, raised under conventional conditions were obtained from Ryukyu Biotec Co., Ltd. (Okinawa, Japan).

Mice were anaesthetised with pentobarbital and inoculated via a 22-gauge Teflon catheter introduced into the trachea with 0.08 ml of each of the appropriate bacterial suspensions in peptone-yeast-glucose (PYG, pH 7.2) broth containing $2-4 \times 10^8$ cfu of *S. constellatus*. The control mice were similarly inoculated with an equal volume of PYG broth. The mice (n=10 per strain) were observed for up to 14 days to determine the mortality rate.

For the histological study, a modification of the method of Davis et al.¹⁴ was used. Mice (n=5) were anaesthetised and sacrificed on the third day after the inoculation. The lungs were surgically removed and fixed in 10% formalin. Both lungs were sagittally sectioned, and the midsagittal slice was embedded in paraffin. Sections were prepared from each animal and stained with hematoxylin and eosin. Ten slides were randomly selected from each animal and the pathological changes were quantified. The specimens were coded and examined by three medical doctors. Each slide was examined at low power ($\times 100$) to determine the percentage of the lung involved in the inflammatory process. Estimates were made in increments of 10% and the involved areas (%IA) were examined at high power ($\times 400$) and scored for the presence and severity of inflammation based upon the scoring system shown in Table 1. The complete histological picture of pneumonia was determined by

calculating the inflammation score by the following formula: histological inflammation score = $1 \times [\%IA \text{ of score } 1] + 2 \times [\%IA \text{ of score } 2] + 3 \times [\%IA \text{ of score } 3]$.

Measurement of bactericidal activity of PMNs

PMNs were separated from heparinised peripheral venous blood of healthy adults¹⁵⁾. The blood was mixed with 3% dextran (Wako Pure Chemical Industries, Ltd., Tokyo) in physiologic saline and left at room temperature for 20 min to allow sedimentation of the erythrocytes. The supernate was layered over Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 450 *g* for 30 min. The leukocyte-rich pellet was washed twice with Hanks' balanced salt solution (HBSS, pH 7.2) (GIBCO Laboratories, Grand Island, NY, USA) after hypotonic lysis of residual erythrocytes and resuspended in HBSS at a concentration of 5×10^6 cells/ml as determined with a haemocytometer.

The purity of the PMNs was >95% and the proportion of viable cells was >95% as determined by trypan-blue staining. A 1.0-ml mixture containing 0.5 ml of PMNs (2.5×10^6 cells), 0.15 ml of *S. constellatus* or *S. aureus* suspension, 0.15 ml of normal human serum (NHS) or heat-inactivated normal human serum (HIHS), 0.2 ml of the filtrate sample or HBSS or sterile PYG broth was made in Falcon 2059 polystyrene tubes and incubated at 37°C for 90 min. This gave a bacterium/PMN ratio of about 5 to 1.

The culture filtrate sample was obtained as follows: *S. constellatus* RZYK001, overgrown at 37°C in PYG broth, was centrifuged (15,000 rpm for 30 min) and the filtrate pH was adjusted to 7.2 with 1.0 M NaOH. It was then sterilised by passage through a 0.22- μ m filter (Millipore Products Division, Bedford, MA, USA), and stored at -80°C.

S. constellatus, a gram-positive coccus resistant to complement, was preincubated with NHS for the phagocytic killing of the organisms. Neutrophil bactericidal activity against enterococci appears to be mediated primarily by complement, with antibody playing a less essential but potentially important role¹⁶⁾, and accordingly the level of killing by *S. constellatus* preincubated with HIHS was very low (unpublished observation). The titers of NHS against each strain tested were determined by indirect fluorescence assay and they were all less than 1:128; no significant difference was observed among the reference strains.

At the end of the incubation period, a 0.1 ml sample of the assay mixture was added to 9.9 ml of distilled water to lyse PMNs and 10-fold serial dilutions in isotonic saline were made and added to TSA for *S. aureus* and to trypto-soy blood agar for *S. constellatus*. Exact quantification was made by counting bacterial colonies growing on the plates. Control tubes without PMNs or serum were included in each assay. The results are expressed as the killing index. The killing index was determined by the formula: $[(\text{number of viable bacteria without PMNs} - \text{number of viable bacteria with PMNs}) / \text{number of viable bacteria without PMNs}] \times 100\%$ ¹⁷⁾. All neutrophil assays were performed in duplicate.

Statistical analysis

The results were expressed as means and standard deviation ($\pm$ SD) and the data were analysed by Student's *t* test.

Results

Infectivity testing

The mortality rate for mice inoculated with 2×10^8 cfu of the virulent strain RZYK001 was 10% while all the mice inoculated with 2-4/10⁸ cfu of the avirulent strains survived over 14 days. However, the histological findings in the lungs of all the mice inoculated with the virulent strain revealed that the alveoli were severely damaged and were filled with many inflammatory cells, while there were no such findings with the avirulent strains except for mild inflammatory cell infiltration. Accordingly,

Table 1 Scoring system for histological examination of lung tissue

Inflammation score	Interpretation
0	No inflammatory cells in alveoli
1	1–5 inflammatory cells in alveolar spaces
2	6–10 inflammatory cells in alveolar spaces
3	>10 inflammatory cells in alveolar spaces or no remaining alveolar architecture

Fig. 1 Effect of *S. constellatus* culture filtrate on phagocytic killing of *S. aureus* by human PMNs. Open bars, HBSS; hatched bars, PYG; solid bars, RZYK001 culture filtrate. Bacteria were preincubated with HHs at 37°C for 40 min. Data represent means and SD (bars) for triplicate experiments performed in duplicate. No statistically significant difference was found.

Table 2 Infectivity of *S. constellatus* by histological inflammation score

Strain	Inoculum size †	Histological inflammation score § (n=5)	
Virulent			
RZYK001	2×10 ⁸	124(43)	p<0.001
Avirulent			
RT4303	4×10 ⁸	37(7)	
RT6002	2×10 ⁸	24(14)	] N.S.*

† cfu/mouse

§ Score represents mean (SD) and was calculated as described in Materials and Methods

*Not significant

Table 3 Phagocytic killing of *S. constellatus* by human PMNs

Strain	Killing index (%) §		
	30 min	60 min	90 min
Virulent			
RZYK001	18.7(8.2)**	31.3(6.5)**	36.4(5.1)**
Avirulent			
RT4303	68.2(10.0)	89.0(6.3)	94.7(3.7)
RT6002	83.4(4.1)	98.4(0.9)	99.4(0.3)

Bacteria were preincubated with NHS at 37°C for 40 min.
§ Defined in Materials and Methods. Data represent means (SD) for triplicate experiments performed in duplicate.

**p<0.001 compared to avirulent strains

the histological inflammation score of the lungs of mice inoculated with the virulent strain was 124 ± 43 , but the scores for the avirulent strains were 37 ± 7 and 24 ± 14 , respectively ($p<0.001$) (Table 2). The results of this experiment show that the virulent strain isolated from broncho-alveolar fluid of the patient caused more severe pneumonia in mice than the avirulent strains isolated from saliva of healthy adults.

Effect of *S. constellatus* culture filtrate on phagocytic killing of *S. aureus* by PMNs

As shown in Fig. 1, phagocytic killing of *S. aureus* in the presence of the *S. constellatus* culture filtrate increased to some extent after incubation for 60 and 90 min as compared with HBSS and PYG controls, but no statistically significant differences were observed among the samples tested. The phagocytic killing of *S. aureus* was not affected by the *S. constellatus* culture filtrate.

Phagocytic killing of *S. constellatus* by PMNs

The killing index of the virulent strain RZYK001 was 18.7, 31.3 and 36.4% after incubation for 30, 60 and 90 min, respectively (Table 3). The killing index of RZYK001 was definitely much lower than those of avirulent strains RT4303 and RT6002, over the incubation period ($p<0.001$).

Discussion

The *S. milleri* group is implicated in the development of pneumonia, purulent empyema and lung abscess^{3,18,19)} and aspiration of oral secretions is a commonly reported antecedent as the microorganisms are part of the normal flora of the mouth and pharynx. Infections caused by the *S. milleri* group occur in otherwise normal healthy persons; the factors predisposing to this type of infection are old age, male sex and underlying diseases such as cerebral vascular disease, diabetes mellitus, trauma and alcoholism²⁰⁾.

In pulmonary infections, the *S. milleri* group is more likely to be involved in mixed infections with oral anaerobes⁹⁾. Enhancement of the pathogenesis of aerobes by a synergistic effect of anaerobes has been observed^{21,22)}. The culture filtrates of some species of anaerobes are well known to impair host defenses by direct damage to neutrophil functions. Short chain fatty acids produced by the anaerobes play a key role in the inhibition of *S. aureus* phagocytosis by human neutrophils and these acids are important virulence factors that inhibit host phagocytosis defenses^{9,10)}. The pathogenicity of the *S. milleri* group in combination with an anaerobe was enhanced by a metabolite of the organism which could not only result in impairment of the host defense mechanism in terms of phagocytic killing, but also stimulate the growth of the *S. milleri* group *in vitro*¹¹⁾.

The *S. milleri* group organisms are occasionally isolated from infection sites as previously mentioned, showing that they play an important role as a sole pathogen. The virulent strain of *S. constellatus*, which belongs to the *S. milleri* group, used in this study showed less pathogenicity in single infections than in combination with the anaerobe when it frequently induces abscess in mice¹¹⁾. We observed that the isolate obtained from a patient with clinical evidence of pneumonia caused more severe pneumonia than those from the oral cavities of healthy adults. We therefore investigated the pathogenicity of *S. constellatus* with respect to neutrophil phagocytic killing, comparing the virulent with the avirulent strains, since PMNs are the first line in the host defenses against any bacterial infection.

There are several extracellular products which can be offered as explanations for the pathogenicity of the *S. milleri* group. The potential production of tissue-destroying enzymes such as hyaluronidase, gelatinase, collagenase, deoxyribonuclease, ribonuclease and hemolysins has been examined^{16,23,24)}. Unsworth²⁵⁾ showed that there was a strong association between hyaluronidase production and *S. milleri* isolated from abscesses, compared with isolates from the normal flora, and suggested that this enzyme plays an important role in abscess formation. Furthermore, short chain fatty acids such as succinic, butyric and propionic acids produced by anaerobic bacteria are virulence factors impairing neutrophil functions^{9,26)}, while the *S. milleri* group also produces the fatty acids such as lactic acid and others.

Fatty acid analysis by gas chromatography revealed that the *S. milleri* group produces mainly lactic and acetic acids, but they were not produced in significant quantity by the virulent and avirulent isolates tested (unpublished observation). We therefore examined the effect of extracellular products of the virulent strain of *S. constellatus* on the phagocytic killing to discover whether they could impair the host defenses similarly to anaerobes. The culture filtrate of the virulent strain was found not to diminish the phagocytic killing. This indicates that extracellular products in the culture filtrate of the *S. milleri* group, comprising these fatty acids, may have little inhibitory effect on the phagocytic killing by PMNs. A characteristic smell, the caramel odor, associated with the organisms was shown to be due to diacetyl, one of the metabolites of the *S. milleri* group²⁷⁾. This product may possibly not influence neutrophil functions; however, subsequent study including its effect on human soft tissue would be of some interest.

It is still unclear why the *S. milleri* group tends to induce abscesses. Gossling²⁰⁾ suggested that some members of the *S. milleri* group can survive and multiply in the presence of normal host defenses better than many other mucosal bacteria. In the present study, we demonstrated a substantial inhibitory effect on phagocytic killing of the virulent strain of *S. constellatus* by human PMNs, compared with the avirulent strains from the normal flora, although the phagocytic killing of the *S. milleri* group in relation to the host defenses has hardly been reported so far. This is similar to the histological findings of *in vivo* experiments in mice, which showed that the virulent strain caused more severe pneumonia. Therefore, the pathogenicity of *S. constellatus* against the host defenses in relation to neutrophils appeared to be mediated primarily by a structural component of the organisms, with extracellular products playing a less important role. These results suggest the possibility of delayed bacterial clearance from mice as a factor in the pathogenicity of virulent strains of *S. constellatus*.

The reference strains were stained Hiss' stain and examined for the presence of polysaccharide capsules. Since capsules have been shown to prevent phagocytosis²⁸⁾, they are considered a virulence factor in suppurative infection and may be involved in delaying bacterial clearance. However, a recent study by Lewis et al.²⁹⁾ found no difference in the degree of phagocytosis among capsulated and non-capsulated strains of the *S. milleri* group isolated from pus. In our study, the reference strains were non-capsulated according to Hiss' stain and the presence or absence of capsules will be confirmed by electron microscopy.

On the other hand, the extracellular products from *S. intermedius* had strong activity in suppressing lymphocyte and fibroblast proliferation⁷⁾, and the immunosuppressive substance from *S. intermedius* played an important role in the pathogenicity, increasing the survival of this organism in the host³⁰⁾. The effect of extracellular products of the virulent strain used in this study on lymphocyte functions will also be investigated.

We conclude that a virulence factor of *S. constellatus* seems to be related to a structural component of the organism in terms of phagocytic killing by PMNs.

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呼吸器感染症における *Streptococcus milleri* group の病原性についての検討

—ヒト多核白血球の貪食殺菌能に対する影響—

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要 旨

口腔内常在菌である *Streptococcus milleri* group (SMG) は、種々の化膿性疾患だけでなく、特に呼吸器感染症の起炎菌として重要であるが、その病原因子についてははっきりしていない。そこでSMGの1つである *Streptococcus constellatus* の臨床分離株と健康成人分離株についてヒト末梢血由来多核白血球 (PMNs) の貪食殺菌能に対する影響を調べた。

肺炎患者の気管支肺胞洗浄液から分離され、嫌気性菌との混合感染にて60%以上の致死性のマウス肺炎をおこす病原性株 RZYK001と健康成人の唾液由来の非病原性株 RT4303および RT6002の *S. constellatus* 3株を用いた。RZYK001を 10^8 cfu/mouse 経気管的に接種したマウス (n=10) の生存

率では非病原性株に比して差は認められなかったが、肺の組織学的炎症スコアは RZYK001が有意に高値を示した ($p<0.001$)。

RZYK001の培養濾液はPMNsによる *Staphylococcus aureus* ATCC25923の貪食殺菌能に対して全く影響を与えなかった。しかし、SMGの貪食殺菌試験では RZYK001の培養90分時の殺菌インデックスは36.4%を示し、一方RT4303およびRT6002はそれぞれ94.7%、99.4%と高値であった ($p<0.001$)。

以上により、*S. constellatus* の病原性のメカニズムの一つとして、PMNsに貪食殺菌されにくくする物質を菌体成分として持っている可能性が示唆される。