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The virulence of mixed infection with *Streptococcus constellatus* and *Fusobacterium nucleatum* in a murine orofacial infection model

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ABSTRACT - Orofacial infections are usually polymicrobial, and it is the microbial interactions of pathogenic species that cause tissue destruction. In this study, the microbial interaction between *Streptococcus constellatus* and *Fusobacterium nucleatum* was characterized using a murine orofacial infection model. A mixture of viable *S. constellatus* and *F. nucleatum* cells (both 2 x 10^8 cfu/mice) was injected into the submandible; as a result, all of the test mice died. In contrast, none of the experimental animals monoinjected with either *S. constellatus* or *F. nucleatum* died ($p<0.001$), indicating that the synergism between the two resulted in the virulence. When a mixture of viable *S. constellatus* cells and a culture filtrate of *F. nucleatum* was tested, lethality and the bacterial cell count per lesion were significantly enhanced as compared with monoinjections ($p<0.02$). However, the virulence of *F. nucleatum* was not enhanced by infection of a culture filtrate of *S. constellatus*. The enhancement of virulence was observed even when viable *S. constellatus* cells and the culture filtrate of *F. nucleatum* were injected at separate sites. Heat-treatment of the culture filtrate of *F. nucleatum* did not affect the enhancement. These results indicate that a heat-stable substance(s) produced by *F. nucleatum* contributes to the microbial synergy of *S. constellatus* and *F. nucleatum* in orofacial infections.

virulence synergy / *Streptococcus constellatus* / *Fusobacterium nucleatum* / orofacial infection / murine model

1. Introduction

Most human orofacial infections involve bacteria that reside in the oral cavity, particularly viridans streptococci, *Peptostreptococcus* species, *Prevotella* species, and *Fusobacterium* species [1-8]. Moreover, such orofacial infections are usually polymicrobial. Previous studies have shown that facultative streptococci and *Fusobacterium nucleatum* along with other oral bacterial species are often isolated simultaneously from odontogenic infections [7,8], and that the *Streptococcus milleri* group and *F. nucleatum* are isolated more frequently from severe orofacial infections than from mild infections [6]. Several investigators have examined the pathogenicity of *Streptococcus constellatus*, a member of the *S. milleri* group, and *F. nucleatum* using animal infection models and have demonstrated that these two bacteria have the potential to produce abscesses [9-14]. These studies indicate that bacterial interaction is one of the most important factors in the occurrence and progress of orofacial infections [10-14]. In this study, we attempted to elucidate the nature of the bacterial interaction between *S. constellatus* and *F. nucleatum* in a murine orofacial infection model [15] using the mouse submandible as the injection site.

2. Materials and methods

2.1. Bacterial strains and preparation of bacterial inocula

2.1.1. Bacteria

S. constellatus ATCC 27823 and *F. nucleatum* ATCC 25586 [15] were used in the

present study. The colony forming unit(s) (cfu) of the bacterial inocula was determined by counting the number of bacterial colonies grown under the same conditions as those described below.

2.1.2. Cell suspension

Colonies of *S. constellatus* were cultured on Brucella HK agar (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) with 5 % (v/v) sheep blood in an atmosphere of 10 % CO_2 (v/v), 20% H_2 (v/v), and 70 % N_2 (v/v), at 37 °C for 48 h. *F. nucleatum* was cultured on Brucella HK agar with 5 % sheep blood in an atmosphere of 5 % CO_2 (v/v), 10 % H_2 (v/v), and 85 % N_2 (v/v), at 37 °C for 78 h. The resultant colonies were collected and suspended in a Peptone-Yeast-Glucose (PYG) broth (1 % (w/v) Bacto-peptone (Becton Dickinson, Cockeysville, MD, USA), 1 % (w/v) yeast extract (Becton Dickinson), 1 % (w/v) glucose, and 0.01 % (w/v) vitamin K (Wako Pure Chemical Industries, Osaka, Japan), pH 7.0) at concentrations between 1.0×10^9 and 4.0×10^9 cfu/ml.

2.1.3. Broth culture

Fluid cultures of *S. constellatus* and *F. nucleatum* were prepared in PYG broth under the following atmospheric conditions: 10 % CO_2 (v/v), 20 % H_2 (v/v), and 70% N_2 (v/v) at 37 °C for 48 h for *S. constellatus*, and 5 % CO_2 (v/v), 10% H_2 (v/v), and 85 % N_2 (v/v) at 37 °C for 72 h for *F. nucleatum*. Each PYG broth culture was used as the culture for the examination of the synergistic effect of these strains on virulence. The bacterial concentrations of *S. constellatus* and *F. nucleatum* in broth cultures were 4.0×10^9 cfu/ml and 7.0×10^9 cfu/ml, respectively.

2.1.4. Culture filtrate

For the preparation of bacteria-free culture filtrates, fluid cultures were centrifuged at 600 x *g* for 10 min and the supernate was sterilized by 0.22 μm pore size membrane filtration (Millipore, Bedford, MA, USA). The pH values of these filtrates were in the range 6.5 - 6.7. A heat-treated culture filtrate was prepared with an autoclave at 121 °C for 20 min.

2.2. Animals and inoculation in mice

ICR Crj CD-1 mice (Charles River Japan Inc., Yokohama, Japan) raised under conventional conditions were used. The mice were six-week-old females (25 - 28g). A murine orofacial infection model [15] was employed for the experiments.

2.2.1. The synergistic effect of living cells on virulence

An aliquot of 50 μl of a cell suspension or a broth culture of one strain was mixed with an equal volume of a cell suspension or a broth culture of the other strain (Table I). In the control group, uncultured sterile PYG broth was used instead of the cell suspension or the broth culture. The pH value of the mixture was in the range 6.8 - 7.0.

The mixture was injected into the submandible of each mouse, as described previously [15]; the mice were anesthetized with diethyl ether (Wako Pure Chemical Industries) and the skin at the submandible was disinfected with 70 % (v/v) ethanol. The skin was pricked with a 26 gauge needle along the midline of the submandible and an aliquot of 100 μl of the mixture was injected into the space between the skin and smooth muscular layers at the center of the oral floor.

2.2.2. The synergistic effect on virulence of a culture filtrate and a cell suspension when injected at the same site

An aliquot of 50 µl of a cell suspension of one strain was mixed with an equal volume of a culture filtrate of the other strain. In the control group, uncultured sterile PYG broth was used instead of the culture filtrate. The pH value of the mixture was in the range 6.8 - 7.0. The mixture was injected into the submandible of each mouse.

2.2.3. The synergistic effect on virulence of a culture filtrate and a cell suspension when injected at separate sites

An aliquot of 50 µl of a cell suspension of one test strain mixed with an equal amount of uncultured sterile PYG broth was injected into the submandible of the mice. At the same time, an aliquot of 50 µl of a culture filtrate of the other test strain was injected into the back of the same mice (Table IV). In the control group, uncultured sterile PYG broth was injected into the back of mice instead of the culture filtrate.

2.3. Assessment

Bacterial virulence was assessed by (1) the presence or absence of abscesses, (2) lethality, (3) the bacterial count per lesion. The mice were sacrificed by cervical dislocation. The formation of a submandibular abscess was determined by visual inspection on the seventh day after the injection. The bacterial count of the abscesses was determined as follows: the collected submandibular abscesses were homogenated with 1 ml of sterile PYG broth, and the homogenated abscesses were diluted at serial 10-folds. Each diluted material was cultured on Brucella HK agar with 5 % (v/v)

sheep blood under the conditions stated before. The number of bacterial colonies grown on the blood-agars were counted. In addition, the above culture confirmed the presence of the test bacterial species and the absence of contamination.

2.4. Statistical analysis

Statistical comparisons of lethality and the abscess formation rate were performed by a x^2 test. The statistical differences in the bacterial cell count per murine lesion were analyzed by Student's-t test.

3. Results

3.1. Virulence of living cells

In the present animal model, viable cells of *S. constellatus* ATCC 27823 produced a single abscess, and those of *F. nucleatum* ATCC 25586 produced a single abscess or multiple abscesses at the injection site.

Although injection of pure cell suspensions of *S. constellatus* and *F. nucleatum* produced abscesses in the submandible, all of the mice in this group survived (Table I). The injection of pure PYG broth cultures of *S. constellatus* (2.0 x 10^8 cfu) or *F. nucleatum* (3.5 x 10^8 cfu) failed to produce lesions. When a mixture of cell suspensions of both *S. constellatus* and *F. nucleatum*, a mixture of a cell suspension of *S. constellatus* and a broth culture of *F. nucleatum*, or a mixture of a cell suspension of *F. nucleatum* and a broth culture of *S. constellatus* was injected, all of the mice died

(Table I).

3.2. Virulence of a culture filtrate and a cell suspension when injected at the same site

When a cell suspension of *F. nucleatum* mixed with a culture filtrate of *S. constellatus* was injected into the submandible, the rate of abscess formation, the lethality, and the bacterial cell count per lesion were the same as those produced by *F. nucleatum* alone, even at a concentration of 2.0×10^8 cfu/mouse (Table II). However, when a bacterial cell suspension of *S. constellatus* was injected with a culture filtrate of *F. nucleatum*, enhanced virulence was observed (Table III). When a concentration of 5.0×10^7 cfu of *S. constellatus* was injected with a *F. nucleatum* culture filtrate, three mice died and the remaining seven mice developed abscesses (Table III). In contrast, all of the mice in the control group survived and only six mice formed abscesses. The bacterial cell count of *S. constellatus* isolated from lesions in the experimental group was also significantly larger than that of the control group ($p<0.02$). When a higher number of *S. constellatus* cells (2.0×10^8 cfu/mouse) was injected in the same manner, virulence again significantly increased (Table III). In this group, eight mice died and the remaining two mice developed abscesses with a bacterial cell count of 7.35 ± 0.04 Log_{10} cfu/lesion. This cell count was significantly higher than that of the control group ($p<0.02$). Furthermore, the heat-treated culture filtrate of *F. nucleatum* also enhanced the virulence of *S. constellatus*.

3.3. Virulence of a culture filtrate and a cell suspension when injected at separate sites

When a cell suspension of *F. nucleatum* was injected into the submandible and a culture filtrate of *S. constellatus* was injected into the back of the mice, the rate of abscess formation, lethality, and the bacterial cell count did not increase as compared with those of the control group (data not shown). In contrast, when a cell suspension of *S. constellatus* was injected into the submandible of the mice along with a subcutaneous injection of a culture filtrate of *F. nucleatum* into the back, the virulence of *S. constellatus* was enhanced (Table IV). When 5.0×10^7 cfu of *S. constellatus* cells were injected with *F. nucleatum* culture filtrate at separate sites, the bacterial cell count was significantly greater ($p<0.05$) than that of the control group. When 2.0×10^8 cfu of *S. constellatus* cells were injected in the same manner, four mice died and the remaining six mice developed abscesses. The bacterial cell count was significantly greater ($p<0.05$) than that of the control group (Table IV). In cases which received injections of heat-treated *F. nucleatum* culture filtrate, the bacterial cell count was significantly greater ($p<0.05$) than that of the control group (Table IV).

We further investigated the effect of the *F. nucleatum* culture filtrate upon the virulence of *S. constellatus* by injecting a standardized *S. constellatus* cell suspension (5.0×10^7 cfu) with a 10-fold dilution of *F. nucleatum* culture filtrate at the same site and also at separate sites. In the group with same site injection, abscesses formed in all of the 10 mice used (Table V). In the group with the separate site injection, the effect was weaker than that observed in the same site injection group; however, abscesses formed in response to the 10^1-fold dilution in all of the 10 mice used. The number of *S. constellatus* cells per lesion was significantly larger ($p<0.05$) than that of the control group when a 10^3-fold dilution of the filtrate was used, irrespective of whether the mice received same site or different site injections (Table V).

4. Discussion

In a previous study, we determined the lesion type formed by clinical isolates of *S. constellatus* and *F. nucleatum* in the orofacial infection model: *S. constellatus* produced a single abscess, and *F. nucleatum* produced a single abscess or multiple abscesses at the submandible in mice [15]. Moreover, we obtained the same experimental results regarding lesion type in the present study by using not only ATCC type strains but also clinical isolates of *S. constellatus* and *F. nucleatum*, although the clinically isolated strains differed from the ATCC strains in terms of bacterial number in the abscesses formed (data not shown).

Microbial interactions in polymicrobial infections have been emphasized in the literature [16], and the microbial synergy of pathogens involved in odontogenic infections has also been studied [10-14]. The microbial synergy of *S. constellatus* and *F. nucleatum* has been described in a previous study using animal subcutaneous abscess models, in which the groin was used as the injection sites [12]. Similarly, in this study, the synergistic effect of *S. constellatus* and *F. nucleatum* was observed with the murine orofacial infection model using the submandible as the injection site. Furthermore, it was shown that bacterial virulence depended on the preparation of the bacterial inocula. Pure PYG broth cultures of *S. constellatus* and *F. nucleatum* did not induce any lesions, although pure cell suspensions of both bacteria did induce lesions (Table I). The virulence of some anaerobes is demonstrated to be influenced greatly by the type and amount of nutrients contained in the medium in which the bacteria are cultured [17,18]. The findings observed in this study may be partly due

to the different expression of virulence factors that are directly and indirectly regulated by different nutrients contained in the media. The virulence factors of *S. constellatus* and *F. nucleatum* have been previously reported [9,19]. In particular, it has been suggested that the possession of a bacterial capsule relates closely to the induction of abscess formation [9,10,20,21]. The virulence factors, especially the bacterial capsule, may contribute to the varied pathogenic potentials of different strains, although the present study did not assess these factors in the test strains.

When a cell suspension of *F. nucleatum* was injected into mice with a culture filtrate of *S. constellatus* at the same site, enhancement of *F. nucleatum* virulence was not observed (Table II). Virulence was not enhanced even when the culture filtrate of *S. constellatus* was injected two or three times a day for seven days (data not shown). Interestingly, when a cell suspension of *F. nucleatum* was injected with a broth culture of *S. constellatus*, a synergistic effect on virulence was observed (Table I), resulting in the death of all of the mice used. These results indicate that viable *S. constellatus* cells, and not *S. constellatus* products in culture filtrate, enhanced *F. nucleatum* virulence. As regards the enhancement of *F. nucleatum* virulence, the cell-to-cell interaction between *F. nucleatum* and *S. constellatus* may be important.

A culture filtrate of *F. nucleatum* enhanced *S. constellatus* virulence when the filtrate and a cell suspension of *S. constellatus* were injected at the same site (the submandible) (Table III). These findings indicate that a product(s) of *F. nucleatum* in the culture filtrate enhanced *S. constellatus* virulence. Even when a cell suspension of *S. constellatus* and a culture filtrate of *F. nucleatum* were injected at separate sites, the enhancement of *S. constellatus* virulence was observed (Table IV). Four principal mechanisms have been identified by which bacteria may interact to increase the net pathogenicity of the infection: (1) the effects on host defenses, in

particular, inhibition of phagocytosis, (2) the provision of essential nutrients, (3) the improvement of the local environment, and (4) the increased virulence of the organisms [22]. We presume that the mechanism of the synergistic effect is as follows: (1) the culture filtrate of *F. nucleatum* affected the host cell's function, and this alteration of host defense against *S. constellatus*, e.g., the change in host immunity, enhanced *S. constellatus* virulence; (2) the culture filtrate of *F. nucleatum* injected into the backs of mice was transmitted to the submandible through hematogenous pathways, and some substance(s) in the filtrate enhanced *S. constellatus* virulence directly at the submandible. Some investigators have demonstrated that *F. nucleatum* inhibits the normal function of host cells; certain molecular candidates such as proteins and volatile fatty acids are possibly associated with this phenomenon [23-25]. The present results indicate that a heat-stable product(s) of *F. nucleatum* plays an important role in the enhancement of *S. constellatus* virulence. Further experimental study is required to characterize this heat-stable substance and to clarify the mechanism by which this substance affects host defenses. The enhancement of *S. constellatus* virulence by the *F. nucleatum* culture filtrate was observed even at a 10^3-fold dilution of the filtrate (Table V). This finding suggests that a culture filtrate of *F. nucleatum* possesses great potential to elicit a synergistic effect with *S. constellatus* as regards virulence. In this respect, even when *S. constellatus* is accompanied by only a small number of *F. nucleatum*, it may express great virulence in an oral lesion involving various pathogens. It appears, therefore, that the microbial synergism between *S. constellatus* and *F. nucleatum* contributes to a large extent to the occurrence and progression of orofacial infections.

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Abbreviations

PYG broth: Peptone-Yeast-Glucose broth

cfu: colony forming unit(s)

Table I. Virulence of *S. constellatus* and *F. nucleatum*

Injection combination		No.of dead mice	No. of abscess-forming mice	Bacterial cell count per lesion (±SD, Log_{10} cfu/mouse)
S. constellatus	*F. nucleatum*			
Cell suspension	None	0	8	5.45±0.93 [c]
Cell suspension	Cell suspension	10 [a, b]	NA	NA
Cell suspension	Broth culture	10 [a, b]	NA	NA
Broth culture	None	0	0	0.00
Broth culture	Cell suspension	10 [a, b]	NA	NA
None	Cell suspension	0	10	7.39±0.29 [d]
None	Broth culture	0	0	0.00

Ten mice were used in each group.

[a] $p<0.001$ vs. the cell suspension or the broth culture of *S. constellatus* alone.

[b] $p<0.001$ vs. the cell suspension or broth culture of *F. nucleatum* alone.

[c] Bacterial cell count of *S. constellatus* per lesion

[d] Bacterial cell count of *F. nucleatum* per lesion

NA , not applicable because all mice died before abscess formation.

Table II. Effect of *S. constellatus* culture filtrate on the virulence of *F. nucleatum* when the mixture of a cell suspension of *F. nucleatum* and a culture filtrate of *S. constellatus* was challenged at the same site (submandible)

Injection combination		No. of dead mice	No. of abscess-forming mice	Bacterial cell count of *F. nucleatum* per lesion (±SD, Log_{10} cfu/mouse)
F. nucleatum [a] (cfu/mouse)	*S. constellatus*			
5.0×10^7	None	0	9	7.25±0.69
5.0×10^7	Culture filtrate	0	10	7.20±0.38
5.0×10^7	Heat-treated culture filtrate	0	8	7.01±0.46
2.0×10^8	None	0	10	7.39±0.29
2.0×10^8	Culture filtrate	0	10	7.37±0.62
2.0×10^8	Heat-treated culture filtrate	0	9	7.15±0.33

Ten mice were used in each group.

[a] cell suspension.

Table III. Effect of *F. nucleatum* culture filtrate on the virulence of *S. constellatus* when the mixture of a cell suspension of *S.constellatus* and a culture filtrate of *F. nucleatum* was challenged at the same site (submandible)

Injection combination		No. of dead mice	No. of abscess-forming mice	Bacterial cell count of *S. constellatus* per lesion (±SD, Log_{10} cfu/mouse)
S. constellatus [a] (cfu/mouse)	*F. nucleatum*			
5.0×10^7	None	0	6	4.40±0.20
5.0×10^7	Culture filtrate	3	7	6.34±0.14 [c]
5.0×10^7	Heat-treated culture filtrate	1	9	5.53±0.42 [c]
2.0×10^8	None	0	8	5.45±0.93
2.0×10^8	Culture filtrate	8 [b]	2	7.35±0.04 [c]
2.0×10^8	Heat-treated culture filtrate	6 [b]	4	6.82±0.42 [c]

Ten mice were used in each group.

[a] cell suspension.

[b,c] $p<0.02$ vs. the cell suspension of *S.constellatus* alone.

Table IV. Effect of *F. nucleatum* culture filtrate on the virulence of *S. constellatus* which were injected at different sites when a bacterial cell suspension of *S.constellatus* and a culture filtrate of *F. nucleatum* were injected into the submandible and the subcutaneous tissue of the back of mice, respectively

Injection combination		No. of dead mice	No. of abscess-forming mice	Bacterial cell count of *S. constellatus* per lesion (±SD, Log_{10} cfu/mouse)
S. constellatus [a] (cfu/mouse)	*F. nucleatum*			
5.0×10^7	None	0	6	4.71±0.17
5.0×10^7	Culture filtrate	0	10	6.49±0.48 [b]
5.0×10^7	Heat-treated culture filtrate	0	9	5.50±0.43 [b]
2.0×10^8	None	0	8	5.54±0.90
2.0×10^8	Culture filtrate	4	6	6.77±0.31 [b]
2.0×10^8	Heat-treated culture filtrate	1	9	6.43±0.43 [b]

Ten mice were used in each group.

[a] cell suspension.

[b] $p<0.05$ vs. the cell suspension of *S. constellatus* alone.

Table V. Effect of various concentrations of *F. nucleatum* culture filtrate on the virulence of *S. constellatus* when the cell suspension of *S. constellatuas* and a culture filtrate of *F. nucleatum* were challenged at the same site or separate sites

Injection combination		Injection at same site		Injection at separate sites	
S. constellatus	*F. nucleatum*	No. of abscess-forming mice	Bacterial count of *S. constellatus* per lesion ($\pm$SD, Log $_{10}$ cfu/mouse)	No. of abscess-forming mice	Bacterial count of *S. constellatus* per lesion ($\pm$SD, Log $_{10}$ cfu/mouse)
Cells suspension (cfu/mouse)	Culture filtrate dilution				
5.0×10^7	None	6	4.40 ± 0.20	6	4.71 ± 0.17
5.0×10^7	10^0	10	6.34 ± 0.14 *a*	10	6.49 ± 0.48 *b*
5.0×10^7	10^1	10	5.58 ± 0.45 *a*	10	5.44 ± 0.64 *b*
5.0×10^7	10^2	10	5.90 ± 0.68 *a*	7	5.50 ± 0.10 *b*
5.0×10^7	10^3	8	5.36 ± 0.12 *a*	7	5.53 ± 0.07 *b*
5.0×10^7	10^4	6	5.26 ± 0.90	6	4.94 ± 0.60

Ten mice were used in each group.

a, b $p<0.05$ vs. the cell suspension of *S. constellatus* alone.