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Short title: Characterization of bacterial orofacial infection

Abstract

We devised a new murine orofacial infection model using bacteria from odontogenic infection origins, and characterized the experimental infections. In this model, bacteria were injected into the submandible of mice. *Streptococcus constellatus* and *Peptostreptococcus micros* produced a single abscess at the injection site and their abscess-forming and lethal abilities were low: the median abscess-forming dose (AF_{50}) of *S. constellatus* and *P. micros* were $10^{8.5\text{-}10.7}$ and $10^{10.2\text{-}10.6}$ CFU per mouse, and their median lethal dose (LD_{50}) were >11 and $10^{10.6\text{-}11}$ CFU per mouse, respectively. *Prevotella oralis* and *Fusobacterium nucleatum* produced multiple abscesses and their abscess-forming and lethal abilities were strong: AF_{50} of *P. oralis* and *F. nucleatum* were $10^{6.0\text{-}6.4}$ and $10^{7.0\text{-}8.7}$ CFU per mouse, and their LD_{50} were $10^{7.0\text{-}7.7}$ and $10^{8.3\text{-}9.9}$ CFU per mouse, respectively. LD_{50} of *P. intermedia* and *P. gingivalis* were $10^{9.4\text{->}11}$ and $10^{8.9\text{-}9.1}$ CFU per mouse, respectively. *Prevotella intermedia* and *Porphyromonas gingivalis* generated a necrotizing lesion, which progressed rapidly. We conclude that this murine model could reflect human orofacial odontogenic infections and is useful to investigate the pathogenicity of causative bacteria of such infections.

Key words: Orofacial odontogenic infection; Animal model; Streptococcus; Anaerobic bacteria.

Introduction

Most human orofacial infections originate from odontogenic infections [1]. The bacteria that reside in the oral cavity are commonly isolated from orofacial infections [1-3]. Anatomically, there are many spaces in the orofacial region, most of which communicate with each other either directly or indirectly. When an odontogenic infection occurs, it can spread to the neighboring tissue through these spaces and can descend into the deeper regions of the head and neck. To clarify the pathogenicity of bacteria isolated from odontogenic infections, several orofacial odontogenic infection models have been developed [4-6]. Although these models may have some advantages, they may also have certain problems; e.g., difficulty in operation, uncertainty of reproducibility, and risk of contamination. Most investigations of orofacial infections have employed rodent subcutaneous abscess models using the back or groin as injection sites [7-13]. However, these models do not always sufficiently exhibit clinical features of human orofacial infection. We suggest that a new animal infection model of the orofacial region is required to assess the pathogenic potential of causative bacteria.

In this study, a new murine orofacial infection model using the submandibular tissue space as an injection site was developed. In this model, the clinical findings and features of infection progression resembled those of human orofacial odontogenic infection.

Results

Lesion type

When bacterial suspensions were injected, redness and swelling of the submandible, which

sometimes extended to the face and neck, were observed, followed by the development of three distinct types of lesion: A-type, a single abscess localized at the injection site of the submandible (Fig. 1); B-type, multiple abscesses at the submandible, cervix, and thorax, being connected to one another by inflammatory process (Fig. 2); C-type, diffuse necrotizing inflammation with erythrocytes and necrotic cells at subcutaneous tissue on the submandible and neck and extending into the abdomen (Fig. 3). Injection of only saline into 10 control mice failed to produce any gross swelling at the site of injection. On the seventh day after injection, the control mice did not show any lesions.

Properties of experimental infections

S. constellatus and *P. micros* showed high AF_{50} and LD_{50} values, indicating that large numbers of bacteria were required to form abscess and kill the mice (Table). When $10^{11.0}$ CFU per of three *S. constellatus* strains per individual mice were injected, all mice formed the A-type lesion, but more than half of the mice were alive in each strain. All *P. micros* strains also formed the A-type lesion even when a lethal dose ($10^{11.0}$ CFU per mouse) was injected to mice.

P. intermedia S76 and K70 strains formed the B-type lesion in 20-30% of the test mice at each AF_{50}. Moreover, S76 and K70 strains formed the C-type lesion in 100% of the test mice at each LD_{50}. In addition, mice, to which S76 strain was injected, died within 3 days after injection. ATCC 25611 strain formed the A-type lesion, but it did not kill half of the test mice even when $10^{11.0}$ CFU of cells per mouse were injected (Table).

P. oralis showed the lowest values of AF_{50} and LD_{50} in test bacteria (Table). When all test strains of *P. oralis* were injected at each AF_{50} concentration, 30 - 40% of mice formed the B-type lesion and the remaining produced the A-type lesion. When these strains were injected at each LD_{50} concentration, more than half the mice died within 3 days after injection. Visible abscesses

were not formed in the deceased mice, but minute abscess formations were observed histologically. Both dead and living mice formed the A-type or the B-type lesion; the B-type lesion was found in 30 - 50% of the test mice whether dead or living.

F. nucleatum showed low values of AF_{50} and LD_{50} (Table). When *F. nucleatum* ATCC 25586, K22, and K45 were injected at each AF_{50} concentration, 30%, 50%, and 60% of the mice formed the B-type lesion, respectively, and the remaining produced the A-type lesion. When these strains were injected at each LD_{50} concentration, both dead and living mice also formed the A-type lesions or the B-type lesion.

When less than $10^{8.0}$ CFU per mouse of each *P. gingivalis* strain were injected, no mouse produced supperative lesions. However, when LD_{50} concentrations were injected ($10^{8.9}$, $10^{8.9}$, and $10^{9.1}$ CFU per mouse of *P. gingivalis* ATCC 53977, 33277, and K25, respectively), all mice died without abscess formation within 2 days (Table). Their skins on the submandible to neck, sometimes extending to the abdomen were black with lesions. When they were autopsied, the lesion were revealed as the C-type.

Discussion

This study tested six bacterial species which have been isolated frequently from orofacial odontogenic infections [2, 3]. *S. constellatus* and *P. micros* had low abscess-forming and lethal potentials. In addition, all test strains of *S. constellatus* and *P. micros* formed the A-type lesion regardless of the bacterial number injected. These results indicate that *S. constellatus* and *P. micros* have lower potentials of producing and spreading suppurative inflammation in the orofacial region.

When newly isolated strains (S76 and K70) of *P. intermedia* were injected into mice at LD_{50} and

AF_{50} concentrations, they produced C-type and B-type lesions, respectively. *P. intermedia* has been reported to form a single abscess in murine groin subcutaneous models [9, 12]. The present result differed from them. The B-type lesion caused by *P. intermedia*, which involved multiple abscesses in the murine submandible, cervix, and thorax, being connected with one another via inflammatory process (Table), is very similar to that of the progressed human odontogenic infection (unpublished data). Interestingly, when S76 and K70 were subcultured more than three times and were then injected into mice, all the lesions formed were of the A-type (data not shown). Likely, ATCC26511 produced the A-type lesion. These findings suggest that the virulence of *P. intermedia* may be easily altered by culture and storage conditions. In light of all of the above, *P. intermedia* would have the potential to spread the infection aggressively in vivo.

It has been reported that *P. oralis* and *F. nucleatum* required an injection of a higher bacterial number (10^7 to 10^9 CFU per mouse) to form an abscess in the back or groin in mice [7-9, 13], and that they formed a single abscess [8, 9]. In the present study, however, *P. oralis* and *F. nucleatum* required a smaller bacterial number to form the abscess and to kill mice when compared with other test bacteria. In particular, *P. oralis* K91 formed the abscess at only $10^{6.0}$ CFU per mouse. *P. oralis* and *F. nucleatum* would have a greater potential to produce suppurative inflammation and to spread the infection in orofacial regions rather than in other body sites.

P. gingivalis required an injection of approximately 10^9 CFU per mouse to produce a lesion in the submandibular tissue space (Table), which was as same as was needed to form the lesion in the mouse back or groin [8,12]. In this study, once the lesion was generated by *P. gingivalis*, all the mice died. *P. gingivalis* has been demonstrated to produce a phlegmonous lesion in animal models [8, 9, 12]. In the present study, *P. gingivalis* produced the C-type lesion which showed many erythrocytes and necrotic cells with edema. However, there were few acute inflammatory reactions, such as leukocyte infiltration. This finding was not consistent with phlegmon, but with more aggressive tissue destruction. As a result, *P. gingivalis* is considered to have a great potential

to destroy tissue aggressively and spread its lesion rapidly without immune response. *P. gingivalis* is classified into invasive and noninvasive strains [14, 15]. The invasive strains are able to kill mice and produce the phlegmonous lesion, while noninvasive strains possess lesser lethal ability and form a single abscess [14, 15]. In test strains, ATCC53977 and K25 were invasive, while ATCC33277 was a noninvasive strain [14, 15, unpublished data]. However, the present study showed that both invasive and noninvasive strains produced the C-type lesion. In addition, both required an injection of the same bacterial number to produce the lesion. Regardless of its invasiveness, all *P. gingivalis* strains may have similar potentials to produce and spread the infection in the orofacial region.

In conclusion, the present murine model could reflect human orofacial odontogenic infections and is useful for investigating causative bacteria.

Materials and methods

Bacterial strains and preparation of bacterial inocula

The following strains were used: *Streptococcus constellatus* ATCC27823, S86, and S90; *Peptostreptococcus micros* VPI 5464-1, K27, and K70; *Prevotella intermedia* ATCC 25611, S76, and K70; *Prevotella oralis* ATCC 33269, K90, and K91; *Fusobacterium nucleatum* ATCC 25586, K22 and K45; *Porphyromonas gingivalis* ATCC 33277, ATCC 53977, and K25. The ATCC and VPI strains were obtained commercially. The other strains were isolated from pus specimens of dentoalveolar infections in our laboratory. Colonies of *S. constellatus* were cultured on a Brucella HK agar (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) with sheep blood 5% (v/v) in an atmosphere of CO_2 10% (v/v), H_2 20% (v/v) and N_2 70% (v/v) at 37°C for 48 h. Colonies of *P.*

micros, P. intermedia, P. oralis, P. gingivalis, and *F. nucleatum* were cultured on the Brucella HK agar with 5% (v/v) sheep blood in an atmosphere of CO_2 5% (v/v), H_2 10% (v/v) and N_2 85% (v/v) at 37°C for 78 h. The grown colonies were collected and suspended in a saline solution. The colony formation unit (CFU) of the bacterial suspension was determined by counting the number of bacterial colonies grown under the same manner.

Animal model

Ten six-week-old female (25-28g) ICR Crj CD-1 mice (Charles River Japan Inc., Yokohama, Japan) raised under conventional conditions were used in each experiment. The mice were anesthetized with diethyl ether (Wako Pure Chemical Industries, Osaka, Japan) 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 0.05 ml of bacterial suspension was injected into the space between the skin and smooth muscular layers at the center of the oral floor.

Assessment of virulence

To assess the pathogenic potentials of the bacterial species, abscess-forming dose, lethal dose, and lesion type were determined. The mice were checked at 12 h intervals for symptoms of the disease after injection. Deceased mice were autopsied and fixed in 10% neutral formalin (Muto Pure Chemicals, Tokyo, Japan) as soon as possible. On the seventh day following injection, any still-living mice were euthanized using diethyl ether. These mice were then fixed in the neutral formalin, and decalcified in 10% EDTA (Wako Pure Chemical Industries). Four-micrometer paraffin sections were cut and stained with hematoxylin and eosin. The presence or absence of any lesions including abscess was determined histologically. The median abscess-forming dose

(AF_{50}) was defined as CFU which induced abscesses in 50% of the test mice, while the median lethal dose (LD_{50}) was defined as CFU by which 50% of the test mice died, as described previously [13].

Acknowledgements

We are thankful for the help of Professor K. Okuda (Tokyo Dental College), and Drs. N. Kato (Gifu University) and Y. Saiki (Kanazawa University).

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Figure legends

Figure 1. Photomicrograph of A-type tissue reaction produced 7 days after injection of *S. constellatus* ATCC 27823 into the murine submandible (sagittal section). A single abscess was formed at the submandible. The arrow at the left of the photograph shows the abscess (HE stain, ×40). The right photograph was taken with a high-power scope (×120) and shows the abscess intermingled with a large number of mononuclear cells and distinct fibrous bands. MB in the photograph indicates murine mandibular bone. Bar = 1mm.

Figure 2. Photomicrograph of B-type tissue reaction produced 7 days after injection of *F. nucleatum* ATCC 25586 into murine submandible (sagittal section, HE stain, ×40). Multiple abscesses, indicated by arrows, were found at the submandible and deep head and neck regions, being connected with one another by inflammatory process. Each abscess has the same histological findings as an abscess of the A-type lesion. MB and SG in the photograph indicate murine mandibular bone and submadibular gland, respectively. Bar = 1mm.

Figure 3. Photomicrograph of C-type tissue reaction produced 2 days after injection of *P. gingivalis* ATCC 33277 into murine submandible (sagittal section). A diffuse necrotizing inflammation is present in the subcutaneous connective tissue between the submandible and neck and extending into the abdomen (The arrow indicating, HE stain, ×40). The right photograph shows the inflammatory infiltration in the submadibular subcutaneous connective tissue (×120). The nectotizing inflammatory reaction is characterized by the presence of many erythrocytes, necrotic cells, and a few leukocytes in the edematous tissue. MB and SG, see Figure 2 legend. Bar = 1mm.

Table. AF_{50} and LD_{50} values, and lesion type when each bacterium was injected into murine submandibular tissue space

		AF_{50} and LD_{50} values (lesion type) *	
Species	Strain	AF_{50}	LD_{50}
S. constellatus	ATCC 27823	8.5 (A)	>11.0 (A †)
	S86	8.6 (A)	>11.0 (A †)
	S90	10.7 (A)	>11.0 (A †)
P. micros	VPI 5464-1	10.4 (A)	11.0 (A)
	K27	10.2 (A)	10.6 (A)
	K70	10.6 (A)	11.0 (A)
P. intermedia	ATCC 25611	8.6 (A)	>11.0 (A †)
	S76	9.0 (A/B)	9.6 (C)
	K70	9.0 (A/B)	9.4 (B/C)
P. oralis	ATCC 33269	6.2 (A)	7.2 (A/B)
	K90	6.4 (A/B)	7.7 (A/B)
	K91	6.0 (A/B)	7.0 (A/B)
F. nucleatum	ATCC 25586	8.7 (A/B)	9.9 (A/B)
	K22	7.0 (A/B)	8.6 (A/B)
	K45	7.0 (A/B)	8.3 (A/B)
P. gingivalis	ATCC 53977	ND	8.9 (C)
	ATCC 33277	ND	8.9 (C)
	K25	ND	9.1 (C)

* Data is expressed at Log_{10} CFU per mouse (Lesion type when bacteria at AF_{50} or LD_{50} concentrations were injected, respectively). A, B, and C types of lesion are shown in Figure 1-3.

† lesion type when $10^{11.0}$ CFU of bacteria per mouse were injected.

ND, not determined because no abscesses were formed.