Oral Microbiology Immunology 2004: 19: 205–209
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Oral Microbiology and Immunology

Short communication

Comparison of inflammatory changes caused by *Porphyromonas gingivalis* with distinct *fimA* genotypes in a mouse abscess model

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Nakano K, Kuboniwa M, Nakagawa I, Yamamura T, Nomura R, Okahashi N, Ooshima T, Amano A. Comparison of inflammatory changes caused by Porphyromonas gingivalis *with distinct* fimA *genotypes in a mouse abscess model. Oral Microbiol Immunol 2004: 19: 205–209.* © Blackwell Munksgaard, 2004.

The *fimA* gene of *Porphyromonas gingivalis*, encoding fimbrillin (a subunit protein of fimbriae) has been classified into six genotypes (types I–V and Ib). The genotypic variation was previously suggested to be related to the severity of adult periodontitis in the general population. In this study, we compared inflammatory changes caused by bacterial infection to study pathogenic heterogeneity among the different *fimA* strains in a mouse abscess model. Bacterial suspensions of 13 *P. gingivalis* strains representing the six *fimA* types were subcutaneously injected into female BALB/c mice, and serum sialic acid concentrations were assayed as a quantitative host inflammatory parameter. Type II *fimA* organisms caused the most significant induction of serum sialic acid, as well as other infectious symptoms, followed by types Ib, IV and V. In contrast, types I and III caused weak inflammatory changes. In addition, *fimA* mutants of type II strains clearly lost their infectious ability. These findings suggest that *fimA* genotypic variation affects expression of *P. gingivalis* virulence.

Key words: *fimA* genotype; mouse model; pathogenicity; periodontitis; *Porphyromonas gingivalis*

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Accepted for publication October 3, 2003

Porphyromonas gingivalis is considered to be the most important infectious agent causing several types of periodontal diseases, including adult periodontitis (24). This microorganism has been frequently detected in patients with periodontitis, while it reportedly occurs at a lower frequency in periodontally healthy individuals without marked gingival inflammation. Various forms of periodontal disease progress more rapidly in some patients than in others, which strongly suggests host differences and/or diversities in virulence among the organisms harbored by individuals as key factors (24). Numerous studies have been performed to isolate specific periodontitis-related clones of *P. gingivalis* strains from periodontitis patients. Biotyping, serotyping and several genotypic characterizations of those isolates, such as restriction fragment length polymorphism, multilocus enzyme electrophoresis and arbitrarily primed polymerase chain reaction, have revealed extensive heterogeneity among natural populations of this bacterium (21). However, a relationship between specific clones of *P. gingivalis* and periodontal status in patients has not been well established.

P. gingivalis fimbriae (FimA) are filamentous components located on the cell surface that are thought to play an important role in the colonization and invasion of periodontal tissues (1). *P. gingivalis fimA* genes encoding FimA have been classified into six variants (types I–V and Ib) on the basis of their nucleotide sequences (11, 17, 19). Further, a close association between clones with specific *fimA* types and periodontitis has been demonstrated (2, 3, 16, 19, 22), as there was a significant prevalence of organisms with the type II or IV *fimA* gene in periodontitis patients, while type I *fimA* organisms were detected mainly in

periodontally healthy individuals. These findings indicate that both disease-associated and non-disease-associated strains of *P. gingivalis* exist, with pathogenic traits that can be differentiated based on the clonal variation of *fimA* genes. However, scant biological explanation has been given for the difference in the pathogenic potential of *P. gingivalis* strains with different *fimA* genotypes. Recently, we showed that both type II *fimA* organisms and type II recombinant FimA protein bind to and invade epithelial cells efficiently, as compared to those of the other *fimA* types (18). To further evaluate the relationship between these *fimA* types and pathogenicity, host inflammatory changes caused by *P. gingivalis* strains representing the six *fimA* types were compared using a mouse abscess model.

P. gingivalis strains possessing each of the six *fimA* genotypes (11, 15, 19) were selected from our culture collections as follows:

- type I *fimA* organisms: strains 381, ATCC33277 and HG1025
- type Ib *fimA* organism: strain HG1691
- type II *fimA* organisms: strains HW24D1, OMZ314, ATCC53977, OMZ409 and HG1690
- type III *fimA* organism: strain 6/26
- type IV *fimA* organisms: strains HG564 and W50
- type V *fimA* organism: strain HNA99.

The *fimA* null mutants of type II *P. gingivalis* strains OMZ314 and ATCC53977 (type II *fimA*) were constructed by the conjugated transfer methods described by Kumagai et al. (12). Briefly, the fragment containing the flanking regions was generated by polymerase chain reaction with *P. gingivalis* chromosomal DNA as the template. The fragments located upstream (710 bp) and downstream (400 bp) of the *fimA* gene were generated by polymerase chain reaction amplification. After amplification, each fragment was digested with *Sph*I and *Eco*RI or *Sac*I and *Eco*RI, respectively, and these two fragments were ligated together. The concatenated fragment (1.1 kb) was further amplified by polymerase chain reaction, and cloned into *Sph*I- and *Sac*I-digested pYKP009 (12) to yield pYKP*fimA*. *Escherichia coli* SM10lpir was transformed with pYKP*fimA*, and the plasmid was then transferred to *P. gingivalis* OMZ314 and ATCC 53977 by selection with erythromycin and counterselection with gentamycin as described by Ueshima et al. (28). Erythromycin-resistant transconjugants of the organisms were selected and successively grown in Gifu Anaerobic Medium (GAM) broth to obtain erythromycin-sensitive colonies, which were then isolated and examined by polymerase chain reaction. The deficient expression of FimA was confirmed by immunoblotting. The organisms were grown as described previously (17) and the bacterial cells then harvested, washed in prereduced sterile phosphate-buffered saline (10 mM phosphate buffer containing 0.15 M sodium chloride, pH 7.4) and prepared as described previously (17).

All animal procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry prior to the experiments. Female BALB/c mice (5 weeks old) were maintained in horizontal-flow cabinets and provided with sterile food and water *ad libitum*. In all, 160 mice were randomly divided into 16 groups (10 mice per group, 15 experimental groups and 1 control group) to examine inflammatory changes caused by *P. gingivalis* infection. At 40 days of age, a single site approximately 1 cm lateral from the midline on the dorsal surface was depilated, and 0.1 ml of bacterial suspension (1×10^9 colony-forming units (CFU) of a test strain) or phosphate-buffered saline (control group) was injected subcutaneously. For quantitative evaluation of the infectious inflammatory change, serum sialic acid concentration, known as an appropriate parameter of systemic inflammation, was measured (26). Blood specimens (0.1 ml) were collected from an orbital vein on days 0, 1, 2 and 4 after bacterial infection, and then centrifuged at 3000 r.p.m. for 10 min to separate the serum. Sialic acid concentrations in sera were colorimetrically quantified using a commercial kit (Kyokuto Sialic Acid Test Reagents; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) according to the manufacturer's instructions. The mice were also monitored for signs and symptoms of infection, i.e. ruffled hair, abscess formation and emergence of erosion, as described previously (14, 20). The body weight was measured 2 days after the bacterial injection. Two weeks after infection, all mice were killed under ether anesthesia, and the spleens were extirpated and weighed. Spleen weight values were standardized by body weight.

Immunoblotting of FimA proteins was performed to assess the amount of fimbriae expressed on the bacterial cells, as described previously (17). Briefly, bacterial cells were dissolved in SDS gel loading buffer and separated by 12% SDS polyacrylamide gel electrophoresis. Transferred protein bands on a PVDF membrane were reacted with rabbit antibodies raised against each type of recombinant FimA (types I, II, III, and IV). However, since specific antibodies to types Ib and V FimAs were not available, they were probed with anti-type I FimA antibodies.

The results from each group were averaged (means ± SE) and intergroup differences of various factors were estimated by a statistical analysis of variance (ANOVA) for factorial models. Fisher's protected least-significant difference test was used to compare the individual groups. A cluster analysis was performed using the Ward method to identify strains with relatively similar pathogenicity in association with the measured features.

First, the inflammatory changes by *P. gingivalis* strains with different *fimA* types were compared using the increase in serum sialic acid concentration (Fig. 1). Among the strains with different *fimA* types, those with types I and III increased serum sialic acid (103.9–124.5 mg/dl), whereas the type II *fimA* strains showed significantly greater induction (126.5–183.0 mg/dl) ($P < 0.05$). The strains with other *fimA* types (Ib, IV and V) also revealed strong induction levels of induction (127.3–158.7 mg/dl). Significant differences in serum sialic acid concentrations were observed between type I and III *fimA* strains and other *fimA* strains except HG1691 and ATCC53977 ($P < 0.05$).

The various induction levels of serum sialic acid concentration suggested heterogenic causative potential of the different fimA type strains. The inflammatory changes caused by the tested strains were therefore compared with other parameters, i.e. serious sick symptoms, loss of body weight and spleen swelling. The maximum values of sialic acid concentration during the course of infection were used for the comparison. A cluster analysis was utilized as a descriptive procedure to aggregate the strains into cluster groups according to the degree of similarity of the examined parameters. Two cluster groups were formed, which provided a convenient summary of the pathogenic profiles, and classified as strong or mild (Table 1). Strains of types Ib, II and IV induced clear infectious symptoms with all parameters and were classified as strong. Those of types I and III, which failed to show marked changes in body and spleen weights, were considered to be mild.

The different inflammatory changes were ascribed to fimbria (FimA)-phenotypes that originate from a distinct *fimA* genotype. In addition, the quantity of fimbriae on bacterial cells could be crucial for

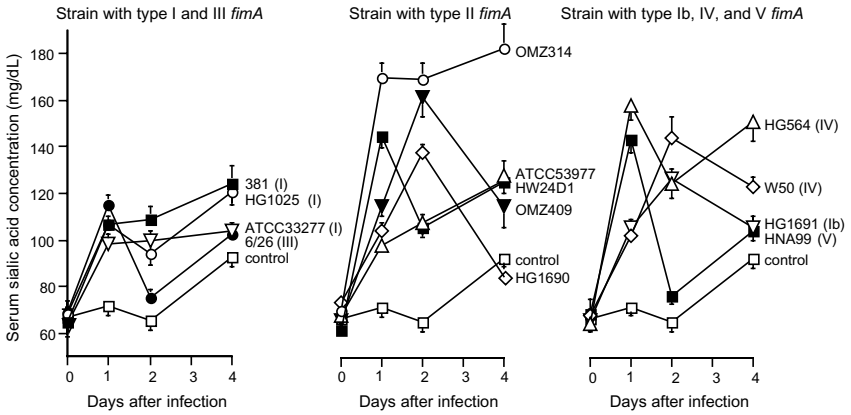

Fig. 1. Induction of serum sialic acid by subcutaneous injection of *P. gingivalis* with different *fimA* genes into mice. Blood specimens were collected from the infraorbital vein of female BALB/c mice after injection with viable *P. gingivalis* cells (1×10^9 CFU). *P. gingivalis* strains and *fimA* types (in parentheses) are shown to the side of each symbol. The mice injected with phosphate-buffered saline were employed as a control group as described in the text. Values are expressed as mean ± standard error.

the expression of pathogenicity. Thus, the amount of fimbriae of all strains was compared, with the results shown in Fig. 2(A). Since the reactivity of FimA type-specific antibodies was different, it was possible to compare the amount of fimbriae in strains with the same *fimA* type. Strains ATCC53977 and W50 revealed thin fimbrial reaction bands as compared to other strains with the same type *fimA* genes. The thin fimbriae of strain ATCC53977 seemed to be related to its weaker pathogenicity, as compared to the other type II strains in the present study. On the other hand, strain W50 is known to be less fimbriated, though it is virulent, with high proteolytic activities (7). The thin reaction bands seen from strains HG1691 and HNA99 were likely due to probing with anti-type I FimA antibodies. To further study the role of fimbriae, *fimA*-deficient mutants were used to infect the mice. As shown in Fig. 2(B), deletion of fimbriae caused a significant prevention of serum sialic acid induction by the infection. In other parameters, the OMZ314 mutant did not cause any sick symptoms, body weight changes or spleen swelling, and the ATCC53977 mutant also failed to show marked sick symptoms. These findings suggest that fimbriae are an important effective factor for infectious inflammation, and that *fimA* genotypic variation has an influence on the inflammatory changes caused by *P. gingivalis*.

Animal models that minimize host susceptibility factors have been used by various investigators to study the virulence properties of *P. gingivalis* (8). Mouse or guinea pig abscess models have been extensively employed. Following subcutaneous infection of rodents with

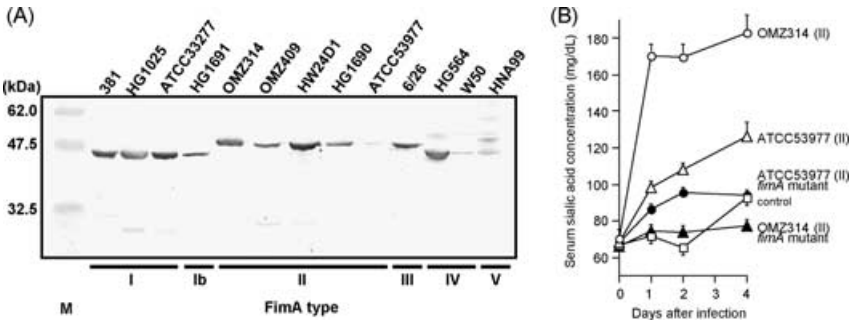

Fig. 2. Involvement of fimbrial amount and expression of *P. gingivalis* with different *fimA* gene in the infectious inflammation. A) Expression levels of fimbrillin (FimA) protein in various strains of *P. gingivalis*. Whole cells were separated by 12% SDS polyacrylamide gel electrophoresis. The transferred protein bands on a PVDF membrane were reacted with rabbit antibodies against each type of recombinant FimA (types I, II, III, and IV). Types Ib and V were probed with anti-type I FimA. B) Induction of serum sialic acid by subcutaneous injection of *fimA*-deficient mutants into mice. The mice injected with phosphate-buffered saline were employed as a control group. The experiment was performed as described in Fig. 1 and the quoted values of the wild strains (OMZ314 and ATCC53977) are from Fig. 1.

Table 1. Infectious inflammation by *P. gingivalis* strains with different *fimA* types

Cluster	Strain	*fimA* type	Maximum values of serum sialic acid (mg/dl)	Symptoms (no./total)	Spleen wt. (mg/g body wt.*)	Body wt. (%)**
Strong	OMZ314	II	183.0 ± 10.4	Ruffled hair (9/10) Abscess (10/10) Erosion (4/10)	14.00 ± 0.93	96.2 ± 1.8
	OMZ409	II	161.8 ± 8.2	None	5.37 ± 0.26	93.1 ± 1.4
	HW24D1	II	144.9 ± 4.4	None	4.34 ± 0.27	95.1 ± 0.8
	HG1690	II	138.3 ± 4.1	None	5.44 ± 0.26	93.2 ± 1.5
	HG564	IV	158.7 ± 6.2	Ruffled hair (7/10) Erosion (2/10)	5.33 ± 0.18	97.4 ± 1.2
	W50	IV	144.7 ± 9.5	None	5.05 ± 0.24	98.1 ± 1.6
	HNA99	V	143.8 ± 6.0	None	4.66 ± 0.16	99.3 ± 1.2
	HG1691	1b	127.3 ± 4.9	None	5.03 ± 0.30	99.6 ± 1.3
	ATCC53977	II	126.5 ± 7.9	Ruffled hair (7/10)	3.97 ± 0.16	97.2 ± 1.4
Mild	381	I	124.5 ± 7.7	None	3.18 ± 0.32	98.2 ± 1.0
	6/26	III	115.0 ± 4.4	None	4.54 ± 0.28	100.7 ± 1.1
	HG1025	I	106.8 ± 2.0	None	5.54 ± 0.58	102.0 ± 2.0
	ATCC33277	I	103.9 ± 4.1	None	4.89 ± 0.21	102.2 ± 1.2
Control	PBS		92.4 ± 1.0	None	3.95 ± 0.20	105.5 ± 1.0

*Measured when sacrificed.
**Measured 2 days after bacterial infection.

Table 2. Relationship between virulence and *fimA* genotypes of *P. gingivalis* strains in animal models

	P. gingivalis fimA genotype**					
Virulence*	I	Ib	II	III	IV	V
Strong		HG1691 (14) 16-1 (23)	ATCC53977 (13, 14, 29) HG184 (13, 14, 29) HG1690 (14) A7A2-10 (20) AJW4 (4)	ATCC49417 (9, 14)	W50 (13, 25, 29) W83 (9, 13, 14, 25, 27, 29) 9-14K-1 (20)	
Moderate	BH18/10 (9, 29)		HW24D1 (9)	FAY19M1 (20)		
Mild	ATCC33277 (9) 381 (20) 2561 (20) 1432 (20) 1112 (20)			6/26 (9) HG445 (14, 29)		

*Virulence was classified in previous studies using a rodent abscess model. Reference numbers are shown in parentheses of strain numbers.
**_fimA_ genotypes were determined according to refs. (11) and (15).

P. gingivalis, pathogenicity/virulence was evaluated in relation to the size of abscess and/or eroded skin lesions, along with cachexia and death. In most of those studies, a high dose of *P. gingivalis* cells ($>1 \times 10^{10}$ CFU) was given. In a preliminary examination, we also applied a high dose; however, infectious parameters including abscess and erosion formation or lesion size were not easy to analyze for quantitative comparison. Several inflammatory markers in response to *P. gingivalis* infection, including specific chemokines and cytokines, were previously used in animal models (8). However, the amount of collected sera was limited (<60 μl) and insufficient for a number of the assays in our animal model. Therefore, among the possible options, we used the serum sialic acid concentration as an objective and quantitative parameter, and assays with a lower dose of *P. gingivalis* cells (1×10^9 CFU). The evaluations with that parameter together with sick symptoms suggest heterogenic pathogenicity among *P. gingivalis* strains with the different *fimA* types. Furthermore, our results for *fimA*-deficient mutants showed that fimbriae were involved with the inflammatory changes. The differences in infectious inflammation seen among the clones would be attributed to variations of *fimA*/FimA types.

In previous studies using abscess models, many strains of *P. gingivalis* have been classified as either avirulent/noninvasive or virulent/invasive (4, 9, 13, 14, 20, 23, 25, 27, 29). The *fimA* types of some of those examined strains have also been successfully identified according to published amino acid and nucleotide sequences of *fimA*/FimA (11, 15). The relationships between this virulence/invasiveness and the *fimA* types are summarized in Table 2. In the present study, many of the type II *fimA* strains caused severe inflammation, whereas type I strains were less causative, which agreed with the previous findings. In addition, the relationship of specific phenotypic diversity to the pathogenicity of *P. gingivalis* was also examined in some of the previous experiments (14, 29). Six serogroups (K-antigen types; K1 to K6) of *P. gingiavlis* based on capsular antigens were reported to be pathogenic clones in an animal model, and linkage of K-types to *fimA* types has been shown, i.e. K1 (to type IV *fimA*), K2, K3 and K5 (to type II *fimA*), K4 (to type III *fimA*), and K6 (to type Ib *fimA*) (2). These findings support a close relationship of *fimA* types with the pathogenicity.

A recent analysis suggested that particular *P. gingivalis* genotypes, possibly with increased pathogenic potential, spread successfully in the human population (6). The search for virulence diversities will, therefore, focus on individual genes or operons such as *fimA* and its operon. Variations of other pathogenic genes might exist, and the presence of *ragB* and insertion sequence IS*1598* could be related to the bacterial pathogenicity (6). In addition, environmental conditions such as iron-limiting conditions seem to alter its virulence (7). Expression of virulence factors including fimbriae and several proteolytic enzymes such as gingipains is influenced by several environmental conditions (5). Further studies on these aspects are necessary to understand virulence variation of *P. gingivalis* clones.

In summary, the present results suggest that *fimA* genotypic variation has an influence on the pathogenic potential of *P. gingivalis* in an animal model, and type II FimA is an important causative factor for host inflammatory changes.

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