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Susceptibility of various oral bacteria to antimicrobial peptides and to phagocytosis by neutrophils

S. Ji[1], J. Hyun[1], E. Park[3], B.-L. Lee[4], K.-K. Kim[1], Y. Choi[1,2]

[1]Program of Oromaxillofacial Infection & Immunity and BK21, School of Dentistry, Seoul National University, Seoul, Korea, [2]Dental Research Institute, Seoul National University, Seoul, Korea, [3]Department of Statistics, College of Natural Sciences, Chonnam National University, Gwangju, Korea and [4]The National Research Laboratory of Defense Proteins, College of Pharmacy, Pusan National University, Busan, Korea

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Background and Objective: The aim of this study was to compare the susceptibility of nonperiodontopathic and periodontopathic bacteria to major defense mechanisms for bacterial clearance in gingival sulcus.

Material and Methods: Twenty strains of 13 oral bacterial species were studied for their susceptibility to phagocytosis by human neutrophils and to the antimicrobial peptides LL-37 and human beta defensin-3. The minimum inhibitory concentrations of LL-37 and human beta defensin-3 were determined by a liquid dilution assay, and susceptibility to phagocytosis was examined by a flow cytometric phagocytosis assay.

Results: The minimum inhibitory concentrations of LL-37 and human beta defensin-3 varied greatly, depending on the strain and species. Although a significant difference between the non- and periodontopathic groups was not observed, the red-complex bacteria were more resistant to LL-37 than the others ($p = 0.004$). The susceptibility of oral bacteria to phagocytosis was quite variable, depending on the species but not on the strains. The periodontopathic bacteria, especially *Actinobacillus actinomycetemcomitans* and the red-complex triad, were more resistant to phagocytosis than were the nonperiodontopathic bacteria ($p = 0.0003$). In addition, bacteria resistant both to antimicrobial peptides and to phagocytosis were more common in the periodontopathic group.

Conclusion: Our results indicate that immune evasion may contribute to the pathogenicity of some periodontopathic bacteria.

Youngnim Choi, DDS, PhD, Program of Oromaxillofacial Infection & Immunity, School of Dentistry and Dental Research Institute, Seoul National University, 28 Yungun-dong, Jongno-gu, Seoul 110–749, Korea
Tel: +82 2 7408643
Fax: +82 2 7430311
e-mail: youngnim@snu.ac.kr

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Periodontitis is a complex disease that involves plaque-associated bacteria, genetic factors that determine the host immune response to the bacteria, and environmental factors (1). Unlike most infectious diseases, periodontitis is a multimicrobial disease and identification of periodontopathogens has been one of the major topics in periodontal research for the last several decades. Among the several hundreds of different bacterial species that are reportedly capable of colonizing subgingival sulcus (2), 10–20 are known to be periodontopathic, based on their predominance in diseased sites (1,3,4). However, the line between periodontopathic and normal flora is not clear, because periodontopathic bacteria are often found in healthy sites or in healthy individuals (3,4). Although the virulence factors of several periodontopathic bacteria have been characterized, what differentiates periodontopathic bacteria from nonperiodontopathic bacteria needs further investigation.

The host innate immune response has a primary role in the defense against plaque-associated bacteria. Two major defense mechanisms for bacterial clearance in gingival sulcus are direct killing by antimicrobial peptides and

phagocytosis by neutrophils. Gingival epithelia form not only physical, but also chemical, barriers by secreting the antimicrobial peptides known as human β-defensins. Neutrophils that emigrate through the junctional epithelium form a barrier between the plaque and the underlying epithelium and actively phagocytose the bacteria. Neutrophils also produce the antimicrobial peptides, LL-37 and human neutrophil defensins (5). The importance of these phagocytes in the maintenance of periodontal health is evident from the severe periodontitis associated with chronic/cyclic neutropenia, leukocyte adhesion deficiency syndrome, Papillon–Lefèvre syndrome, and Chédiak–Higashi syndrome (6–11). The striking importance of LL-37 has been shown in patients with Kostmann syndrome, who develop severe periodontitis in young adulthood and lack LL-37 in their neutrophils restored by treatment with recombinant granulocyte colony-stimulating factor (12,13).

The pathogenic mechanisms of many persistent pathogens include immune evasion (14). There are many reports on the susceptibility of oral bacteria to antimicrobial peptides, such as human β-defensins, human neutrophil defensins, and LL-37, but most studies have focused on periodontopathic and cariogenic bacteria (15–20). Among the numerous studies on the phagocytosis of oral bacteria by human neutrophils, only two have attempted to compare the susceptibility of several periodontopathic bacteria to phagocytosis (21,22). The difference between nonperiodontopathic and periodontopathic bacteria in susceptibility to phagocytosis has not been addressed. Furthermore, no oral bacteria have been evaluated for the susceptibility to phagocytosis and to antimicrobial peptides in parallel. The purpose of this study was to address the potential role of immune evasion in the pathogenesis of periodontitis by analyzing the susceptibility to phagocytosis by human neutrophils and by investigating the antimicrobial activities of human beta defensin-3 and LL-37 on 20 strains of 13 bacterial species, including both nonperiodontopathic and periodontopathic bacteria. Our results indicate that immune evasion may contribute to the pathogenicity of some periodontopathic bacteria, especially the red-complex bacteria.

Material and methods

Bacterial strains and materials

All bacteria used in this study were from the ATCC (Bethesda, MD, USA). The strains of bacteria are listed in Table 1. The mature forms of the antimicrobial peptides LL-37 (LLGDFFRKSKEKIGKEFKRIVQ RIKDFLRNLVPRI-TES) and human beta defensin-3 (GIINTLQKYYCRV-RGGRCAVLSCLPKEEQIGKCSTR-GRKCCRRKK) were synthesized by Peptron (Daejun, Korea).

Bacterial culture conditions

The culture media used for each bacterial species were as follows: Bacto Todd Hewitt Broth (Difco, Kansas City, MO, USA) for *Streptococcus sanguinis*; brain heart infusion broth (Difco) for *S. gordonii*, *Actinomyces naeslundii*, *and Actinobacillus actinomycetemcomitans*; brain heart infusion broth supplemented with 16.7% fetal bovine serum (Gibco, Grand Island, NY, USA) for *Eikenella corrodens*; brain heart infusion broth supplemented with 5 μg/mL of hemin (Sigma, St Louis, MO, USA), plus 10 μg/mL of vitamin K, for *Fusobacterium nucleatum*, *Prevotella nigrescens*, *P. intermedia*, *Peptostreptococcus micros*, and *Porphyromonas gingivalis*; OMIZ-Pat medium for *Tannerella forsythia* and *Treponema denticola*; and Veillonella media, formulated according to the instructions of the ATCC, for *Veillonella atypica*. *S. sanguinis* was cultured at 37°C under aerobic conditions on an orbital shaker and the other bacteria were incubated under an anaerobic atmosphere (5% H_2, 10% CO_2 and 85% N_2).

Determination of bacterial concentration

The bacteria were enumerated by flow cytometry, and the concentrations of viable or fixed bacteria were determined using a bacterial counting kit (BD Bioscience, San Diego, CA, USA) with modification, as previously described (23). Most oral bacteria were too small to analyze together with BD standard beads at the same instrument setting. Therefore, bacterial samples and the beads were separately enumerated by flow cytometry for 15 s. From the bead concentration confirmed by counting in a hemocytometer, the volume analyzed by flow cytometry was determined, and the bacterial concentration was determined based on the counts per volume analyzed for 15 s.

Antimicrobial activity of LL-37 and human beta defensin-3

Antimicrobial peptides dissolved in 0.01% acetic acid at 2 mg/mL were subjected to twofold serial dilution, from 250 to 0.98 μg/mL, in 10 mM sodium phosphate buffer (pH 7.4). The minimum inhibitory concentrations of the peptides were determined by a modified twofold microtiter dilution method, as previously described (24). Bacteria harvested in log phase were counted and suspended at 4×10^6 colony-forming units/mL in 10 mM sodium phosphate buffer (pH 7.4). Bacteria (25 μL) were mixed with an equal volume of the antimicrobial peptide preparations and incubated for 2 h under the appropriate culture condition. At the end of the incubation period, appropriate culture medium (200 or 450 μL) was added to each sample and culture was continued until visible turbidity was observed in an antimicrobial peptide-free control. Visible turbidity was observed after 2 d of culture for most bacteria, except for *T. forsythia* and *T. denticola*, which were cultured for 5 d. Bacterial growth was determined by measuring the absorbance at 655 nm for the bacteria grown in OMIZ-Pat medium, or at 450 nm for the others, using a microplate reader (Bio-Rad, Hercules, CA, USA). The minimum inhibitory concentration was defined as the concentration that inhibits bacterial growth by more than 95% compared with the control. The assay was repeated three or four times.

Table 1. Oral bacterial strains studied

Modified Socransky's Classification	Pathogenicity	Bacterial species	Strain	Gram stain	O_2 requirement
Early (Yellow, Actinomyces, Purple, Green)	N	*Streptococcus sanguinis*	MPC-1 66X49 NCTC10904 (804)	G(+)	Facultative
	N	*Streptococcus gordonii*	ATTC 10558 M5	G(+)	Facultative
	N	*Actinomyces naeslundii*	ATCC 12104	G(+)	Facultative
	N	*Veillonella atypica*	ATCC 17744	G(–)	Anaerobe
	P	*Eikenella corrodens*	ATCC 23834	G(–)	Facultative
Orange	P	*Fusobacterium nucleatum*	ATCC 10953	G(–)	Anaerobe
	P	*Prevotella nigrescens*	ATCC 33563	G(–)	Anaerobe
	P	*Prevotella intermedia*	ATCC 25611	G(–)	Anaerobe
	P	*Peptostreptococcus micros*	ATCC 33270	G(+)	Anaerobe
Other	P	*Actinobacillus actinomycetemcomitans*	ATCC 33384 ATCC 43719 (SUNY 67) ATCC 43718 (Y4)	G(–)	Capnophilic
Red	P	*Porphyromonas gingivalis*	ATCC 33277 ATCC 49417 ATCC 53978 (W50)	G(–)	Anaerobe
	P	*Tannerella forsythia*	ATCC 43047	G(–)	Anaerobe
	P	*Treponema denticola*	ATCC 33521	G(–)	Anaerobe

G(+), gram positive; G(–), gram negative; N, nonperiodontopathic; P, periodontopathic.

Preparation of bacterial cells for phagocytosis assay

Bacterial cells (3 mL) harvested in log phase were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde, and then stained with 5 μM 5- (and 6-)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR, USA). The concentration and mean fluorescence intensity of bacteria were determined by flow cytometry, and the mean fluorescence intensities of the labeled bacteria were adjusted to fall within 200–500 by controlling of carboxyfluorescein diacetate succinimidyl ester concentration. Before enumeration or subjecting to phagocytosis assay, the bacterial suspension was briefly sonicated (five cycles of 10 s at low power with a sonicator; Fisher Scientific, Fair Lawn, NJ, USA) to eliminate aggregates. Live bacteria, used in Fig. 1A, were prepared similarly, without fixing.

Isolation of polymorphonuclear leukocytes from human blood

The study was approved by the Institutional Review Board at the School of Dentistry, Seoul National University. Blood samples from 10 different donors for each independent experiment were pooled to minimize individual variation in neutrophil function. Blood donors sometimes included periodontally diseased, as well as healthy individuals because it was difficult to obtain a sufficient number of samples, and the purpose of our study was to compare the susceptibility of various bacteria to phagocytosis, not to compare the function of polymorphonuclear leukocytes. We confirmed that experiments using the mixed blood and blood from one healthy individual resulted in a similar susceptibility difference among bacterial species (Fig. S1). Blood (10 mL) diluted in saline solution (0.9% NaCl) at a ratio of 1 : 1 was layered on 10 mL of Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden) and centrifuged at 400 *g* for 40 min. Cell pellet, containing granulocytes and red blood cells, was subjected to hypotonic lyses of red blood cells three times, as previously described (25). The purity of neutrophils in recovered cells was confirmed by staining with anti-CD15 monoclonal antibody conjugated to fluorescein isothiocyanate (BD Bioscience), and more than 98% of the cells were $CD15^+$.

Phagocytosis assay

Human neutrophils (1×10^5 cells/500 μL/assay), resuspended in RPMI medium containing 10% heat-inactivated fetal bovine serum, were mixed with labeled bacteria at a ratio of 1 : 25, or at the ratios indicated, and incubated at 37°C for 1 h unless described otherwise. To study the effect of unlabeled bacteria on the phagocytosis of the labeled bacteria, various amounts of unlabeled bacteria were co-incubated, whereas the ratio of phagocytes to the labeled bacteria was maintained at 1 : 25. As a negative control, neutrophils fixed with 3.7% formaldehyde were incubated with the same amount of each labeled bacterial preparation. After incubation, the mixture was centrifuged at 4°C, resuspended in 200 μL of phosphate-buffered saline, and then analyzed by flow cytometry. Following this, 500 μL of Trypan blue (400 mg/mL prepared in

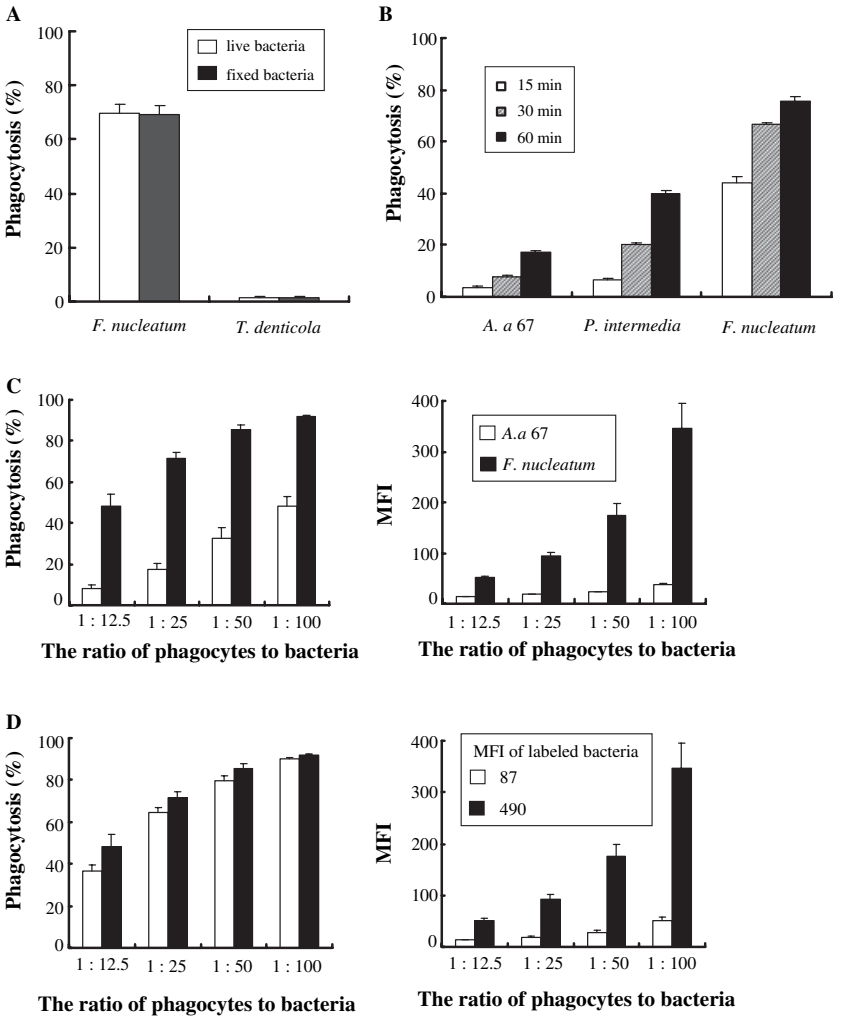

Fig. 1. Standardization of the flow cytometric phagocytosis assay. (A) Neutrophils (1×10^5) were incubated with carboxyfluorescein diacetate succinimidyl ester-labeled live or fixed bacteria, at a ratio of 1 : 25, respectively, at 37°C for 60 min. After quenching with Trypan blue, cells were analyzed by flow cytometry. (B) Neutrophils (1×10^5) were incubated with carboxyfluorescein diacetate succinimidyl ester-labeled fixed bacteria, at a ratio of 1 : 25, at 37°C for 15, 30, and 60 min. After quenching with Trypan blue, cells were analyzed by flow cytometry. (C) Neutrophils (1×10^5) were incubated with carboxyfluorescein diacetate succinimidyl ester-labeled fixed bacteria at the ratios 1 : 12.5, 1 : 25, 1 : 50, and 1 : 100, respectively, at 37°C for 60 min. After quenching, the cells were analyzed by flow cytometry. (D) Fixed *Fusobacterium nucleatum* were labeled with 1 or 5 μM carboxyfluorescein diacetate succinimidyl ester, and the mean fluorescence intensities of the bacteria were 87 and 490, respectively. Isolated neutrophils (1×10^5) were incubated with the labeled bacteria at the ratios 1 : 12.5, 1 : 25, 1 : 50, and 1 : 100 for 60 min. After quenching, cells were analyzed by flow cytometry. Each column represents the mean + standard error of the mean of three independent experiments. *A. a*, *Actinobacillus actinomycetemcomitans*; *F. nucleatum*, *Fusobacterium nucleatum*; MFI, mean fluorescence intensity; *P. intermedia*, *Prevotella intermedia*; *T. denticola*, *Treponema denticola*.

0.85% saline solution) was added to quench the fluorescence of the bacteria bound on the surface, and analyzed by flow cytometry again. Efficient quenching was confirmed on negative controls. A total of 10,000 events were acquired and data were analyzed using CELLQUEST software (BD Biosciences). Granulocytes were gated based on forward scatter and side scatter, and an FL1-histogram was plotted for the gated cells. The percentage of phagocytosing cells (bearing green fluorescence) was determined based on the negative control and autofluorescence. Each assay was performed in duplicate and all results were expressed as the mean + standard error of the mean of four to eight independent assays.

Statistics

Because minimum inhibitory concentrations were measured by twofold dilution assay, they were analyzed using log-transformed values and expressed as a geometric mean (26). To calculate the geometric mean, a minimum inhibitory concentration of > 125 μg/mL was assumed to be 250 μg/mL. Tests for log-normality of minimum inhibitory concentrations or normality of phagocytosis were performed by Shapiro-Wilk W statistics. We used analysis of variance to investigate the effects of strains, species, pathogenicity, or modified Socransky classification (early colonizer, orange, red, other) on minimum inhibitory concentration or susceptibility to phagocytosis, although we obtained the same significance by the Kruskal–Wallis test at $\alpha = 0.05$. Differences between minimum inhibitory concentrations of human beta defensin-3 and LL-37 were assessed by the Wilcoxon two-sample test using original values. The effect of quenching on phagocytosis was assessed by the t-test. Correlation between geometric means of minimum inhibitory concentrations for human beta defensin-3 and LL-37 was computed by using Spearman's rank correlation. Correlation between phagocytosis values before and after quenching was computed by Pearson correlation. All statistical analyses were carried out using SAS 9.1.3 software.

Results

Selection of bacterial species

Subgingival microbiota can be grouped into six tightly related complexes and a few outliers, and in the course of plaque maturation there is a certain order and relationship among the complexes: early colonizers, including *Actino-*

myces, and yellow, green and purple complexes, colonize first, followed by the orange, and then the red complex (27). We chose nine bacterial species from the list of bacteria that have often been referred to as periodontopathogens (Table 1). Most periodontopathic bacteria belong to the orange or the red complex. *A. actinomycetemcomitans* serotype b or c used in this study does not belong to any of the six complexes. Among the periodontopathic bacteria, only *E. corrodens* belongs to the early colonizers, as a member of the green complex. Nonperiodontopathic bacteria were selected to include both gram-positive facultative anaerobes and gram-negative absolute anaerobes from members of the genera *Streptococcus*, *Actinomyces*, *and Veillonella*, which are all early colonizers and thought to be compatible with health (4). In addition, two or three strains of *S. gordonii*, *S. sanguinis*, *A. actinomycetemcomitans*, and *P. gingivalis* were included in order to study interstrain variation. The results were analyzed to determine susceptibility differences by strain, species, pathogenicity, or modified Socransky classification that grouped the bacteria into early colonizer, orange complex, red complex, and other, mostly based on the order of colonization.

Susceptibility to LL-37 and human beta defensin-3

To study the susceptibility of oral bacteria to antibacterial peptides, LL-37 and human beta defensin-3 were chosen because of their reported importance in periodontitis (13) and high efficacy (17). The minimum inhibitory concentrations for 19 strains were successfully determined by a liquid dilution assay, but the growth of *P. gingivalis* W50 was inhibited completely by incubation with buffer alone. The minimum inhibitory concentrations of LL-37 and human beta defensin-3 demonstrated great variation, depending on strains and species ($p < 0.0001$); however, intraspecies variation was smaller than interspecies variation, except in *S. gordonii* (Table 2). The minimum inhibitory concentrations of LL-37 and human beta defensin-3 for each strain had a significant correlation ($r = 0.61$, $p = 0.007$), suggesting similar mechanisms of action for the two antibacterial peptides, but human beta defensin-3 was more efficient than LL-37 ($p = 0.03$). Although the minimum inhibitory concentrations of the more virulent strains *P. gingivalis* 49417 and *A. actinomycetemcomitans* 43718 were higher than those of the less virulent strains (28–30), a significant difference between non- and periodontopathic groups was not observed (Tables 2 and 3). When we asked if there were susceptibility differences according to the modified Socransky classification, we found that the red-complex bacteria were more resistant to LL-37 than the others ($p = 0.0004$) (Table 3).

Table 2. Minimum inhibitory concentrations[a](μg/mL) of human beta defensin-3 and LL-37

Bacterial species	HBD-3	LL-37
Streptococcus sanguinis MPC-1	31.3 (31.3)	62.5 (62.5)
Streptococcus sanguinis 66X49	62.5 (62.5)	37.2 (31.3–62.5)
Streptococcus sanguinis 804	15.7 (15.7)	26.3 (15.7–31.3)
Streptococcus sanguinis[b]	**31.3 (15.7–62.5)**	**37.8 (15.7–62.5)**
Streptococcus gordonii 10558	19.8 (15.7–31.3)	31.3 (31.3)
Streptococcus gordonii M5	> 125 (> 125)	> 125 (> 125)
Streptococcus gordonii[b]	**84.2 (15.7 to > 125)**	**102.6 (31.3 to > 125)**
Actinomyces naeslundii	15.7 (15.7)	31.3 (31.3)
Veillonella atypica	31.3 (31.3)	15.7 (15.7)
Eikenella corrodens	7.8 (7.8)	9.9 (7.8–15.6)
Fusobacterium nucleatum	7.8 (7.8)	4.9 (3.9–7.8)
Prevotella nigrescens	31.3 (31.3)	> 125 (> 125)
Prevotella intermedia	15.7 (15.7)	15.7 (15.7)
Peptostreptococcus micros	62.5 (62.5)	62.5 (62.5)
Actinobacillus actinomycetemcomitans 33384	62.5 (62.5)	26.3 (15.7–31.3)
Actinobacillus actinomycetemcomitans 67	31.3 (31.3)	31.3 (31.3)
Actinobacillus actinomycetemcomitans Y4	49.6 (31.3–62.5)	62.5 (62.5)
Actinobacillus actinomycetemcomitans[b]	**45.6 (31.3–62.5)**	**37.8 (15.7–62.5)**
Porphyromonas gingivalis 33277	31.3 (31.3)	> 125 (> 125)
Porphyromonas gingivalis 49417	62.5 (62.5)	> 125 (> 125)
Porphyromonas gingivalis[b]	**42.1 (31.3–62.5)**	**> 125 (> 125)**
Tannerella forsythia	157.5 (125 to > 125)	> 125 (> 125)
Treponema denticola	15.7 (15.7)	39.4 (31.3–62.5)
Overall[c]	**34.2 (7.8 to > 125)**	**51.6 (3.9 to > 125)**

[a]Minimum inhibitory concentration was expressed as a geometeric mean and range in parenthesis. When the maximum and minimum values were same, only one was described.
[b]Minimum inhibitory concentration of species of which two or three strains were studied.
[c]Minimum inhibitory concentration of 19 strains of 13 species.
HBD-3, human beta defensin-3.

Table 3. Minimum inhibitory concentrations and phagocytosis for each classified group

	HBD-3[a]	LL-37[a]	Phagocytosis[b]
Pathogenicity			
Nonperiodontopathic	39.05 ± 88.33	44.26 ± 83.3	44.48 ± 24.18[d]
Periodontopathic	31.82 ± 43.68	56.51 ± 107.67	28.97 ± 24.82
Modified Socransky's Classification			
Early	32.19 ± 85.13	34.46 ± 80.67	40.92 ± 25.04[e]
Orange	22.72 ± 20.94	38.71 ± 108.9	49.29 ± 27.72[e]
Other	45.64 ± 16.29	37.8 ± 17.47	15.61 ± 13.28[f]
Red	45.46 ± 66.62	160.17 ± 93.13[c]	18.77 ± 12.29[f]

[a]Geometric mean and standard deviation of minimum inhibitory concentration.
[b]Mean and standard deviation of the percentage of phagocytosing cells.
[c]Different from other groups according to modified Socransky's classification ($p = 0.0004$).
[d]Different from periodontopathic group ($p = 0.0003$).
[e]Different from f ($p < 0.0001$).
HBD-3, human beta defensin-3.

Standardization of the flow cytometric phagocytosis assay

The flow cytometric phagocytosis assay has been widely used to evaluate the phagocytic activity, opsonic ability, or virulence of certain bacterial species (31), but a standardized method to compare the susceptibility of various target bacteria has not been established. We evaluated parameters that affect the assay results. Because both aerobic and anaerobic bacteria were included in our study, the use of live bacteria did not allow us to maintain a uniform number and viability for different bacterial species during incubation under aerobic conditions. For the bacteria tested, live and fixed bacteria demonstrated similar results (Fig. 1A). Therefore, fixed bacteria were used in our phagocytosis assay. Phagosomes fuse with lysosomes after 30–60 min (32), which may lead to a loss in fluorescence of the ingested bacteria. Since the percentage of phagocytosing cells increased with incubation time up to 60 min, regardless of the susceptibility to phagocytosis (Fig. 1B), 60 min was selected as the incubation time. The ratio of neutrophils to bacteria had a substantial effect on the results, suggesting the importance of accurate bacterial counting (Fig. 1C). The fluorescence intensity of labeled bacteria also had a definite effect on the results; however, the results expressed as the percentage of phagocytosing cells were less affected (Fig. 1D). Phagocytosis by flow cytometric assay can be expressed as either the percentage or the mean fluorescence intensity of phagocytes. The percentage reflected the proportional increase of phagocytosis well at a low range, but it reached a plateau at around 80%. In contrast, the mean fluorescence intensity of phagocytes reflected the increase of phagocytosis well after the percentage was saturated (Fig. 1C,D). Based on these results, the mean fluorescence intensity of labeled bacteria was adjusted to be between 200 and 500, and a 1 : 25 ratio of phagocytes to bacteria was chosen so that the percentage of phagocytosing cells did not exceed 80%, even for the most susceptible bacteria.

Susceptibility to phagocytosis

To compare the susceptibilities of the various oral bacteria to phagocytosis by neutrophils, we measured the percentage of cells that phagocytosed each strain in the absence of opsonins. Whereas the values after quenching reflected truly phagocytosing cells, the values before quenching also included cells with surface-bound bacteria. The former were highly correlated with the latter ($r = 0.97$, $p < 0.0001$), suggesting that phagocytosis depends on binding (Fig. 2). The susceptibility of oral bacteria to phagocytosis was quite variable, depending on the species, ranging from 7.64 ± 2.83% (*T. denticola*) to 78.01 ± 2.72% (*F. nucleatum*) (Fig. 2). However, no significant inter-strain difference was observed in the species of which two or three strains were studied. By comparisons of means, the periodontopathic group was more resistant to phagocytosis than the non-periodontopathic group ($p = 0.0003$, Table 3). Differences in the susceptibility to phagocytosis among the bacteria grouped by a modified Socransky classification were more evident ($p < 0.0001$, Table 3), regrouping early colonizers and the orange complex as a sensitive group and *A. actinomycetemcomitans* and the red complex as a resistant group, respectively. Our results indicate that periodontopathic bacteria, especially *A. actinomycetemcomitans* and the red-complex bacteria, are resistant to phagocytosis.

Effect of other bacteria on phagocytosis

Different species of oral bacteria co-exist through aggregation *in vivo*, and we were curious about the efficiency of phagocytosis on mixed bacteria of different susceptibilities. To address this issue, *F. nucleatum* and *S. sanguinis* 804 were chosen as susceptible targets, and *S. gordonii* M5, *A. actinomycetemcomitans* Y4, *P. gingivalis* W50, and *T. denticola* were chosen as resistant targets. The presence of unlabeled *F. nucleatum* or *S. sanguinis* 804 increased the phagocytosis of all four resistant species (Fig. 3). On the other hand, none of the resistant bacteria affected the phagocytosis of either *F. nucleatum* or *S. sanguinis* 804 (data not shown). These results indicate that phagocytosis-susceptible bacteria can assist in the phagocytosis of less phagocytosis-susceptible bacteria, to some extent.

Overall susceptibility to antibacterial peptides and phagocytosis

The minimum inhibitory concentrations of LL-37 and human beta defensin-3, and the susceptibility to phagocytosis, were converted to a susceptibility index with scores ranging from + to + + + + (Table 4). *F. nucleatum* was the most susceptible bacterium, having a total score of 12+, whereas *S. gordonii* M5 and *T. forsythia* were the most resistant, having total scores of 4+. When a score of 6+ or less was arbitrarily used to define resistance, 7 of 13 strains in the periodontopathic group, but only 1 of 7 strains in the nonperiodontopathic group, were resistant to the innate defense mechanisms.

Discussion

In this study, we investigated the susceptibility of various oral bacteria to LL-37, human beta defensin-3, and phagocytosis by neutrophils, in parallel, and showed that the late colonizing red-complex bacteria are more resistant to LL-37 and phagocytosis than the other bacteria.

Several groups have studied the susceptibility of *S. sanguinis*, *A. naeslundii*, *F. nucleatum*, *P. gingivalis*, *P. micros*, *P. intermedia*, *and A. actinomycetemcomitans* to LL-37, human beta defensin-2, human beta defensin-3, or human neutrophil defensins. We have now extended the study to *V. atypica*, *E. corrodens*, *P. nigrescens*, *and T. forsythia*. In agreement with our results, *F. nucleatum*, *A. naeslundii*, and *S. sanguinis* were found to be sensitive, but *P. gingivalis and P. micros* were relatively resistant, to multiple antimicrobial peptides, even though different strains were used by different groups (17–19). The related sensitivity to mul-

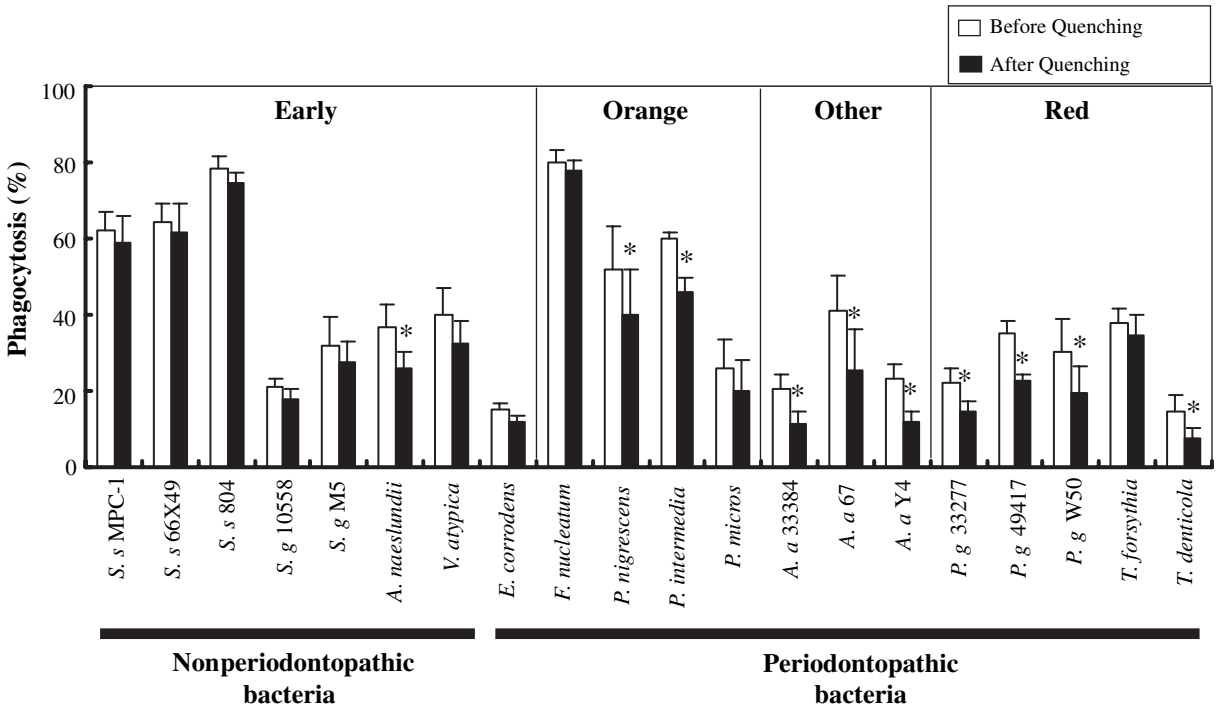

Fig. 2. Susceptibility of various oral bacteria to phagocytosis. Human neutrophils (1×10^5) were mixed with carboxyfluorescein diacetate succinimidyl ester-labeled bacteria at a ratio of 1 : 25, respectively, and incubated at 37°C for 60 min. Samples were analyzed, by flow cytometry, before (white bars) and after (black bars) quenching. Each column represents the mean + standard error of the mean of four to eight independent experiments. *Significant difference, as determined by the paired *t*-test. *A. a*, *Actinobacillus actinomycetemcomitans*; *A. naeslundii*, *Actinomyces naeslundii*; *E. corrodens*, *Eikenella corrodens*; *F. nucleatum*, *Fusobacterium nucleatum*; MFI, mean fluorescence intensity; *P. g*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *P. micros*; *Peptostreptococcus micros*; *P. nigrescens*, *Prevotella nigrescens*; *S. g*, *Streptococcus gordonii*; *S. s*, *Streptococcus sanguinis*; *T. denticola*, *Treponema denticola*; *T. forsythia*; *Tannerella forsythia*; *V. atypica*, *Veillonella atypica*.

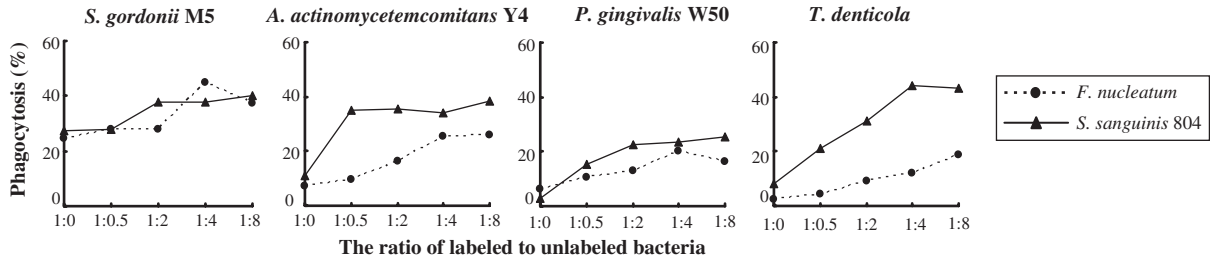

Fig. 3. The effect of bacteria on the phagocytosis of other bacteria. Carboxyfluorescein diacetate succinimidyl ester-labeled phagocytosis-resistant bacteria (2.5×10^6) were mixed with unlabeled *Fusobacterium nucleatum* or *Streptococcus sanguinis* 804 at ratios of 1 : 0, 1 : 0.5, 1 : 2, 1 : 4, and 1 : 8 (phagocytosis-resistant bacteria : phagocytosis-sensitive bacteria). The mixed bacteria were incubated with neutrophils (1×10^5) at 37°C for 60 min, and phagocytosis was analyzed by flow cytometry. Graphs shown are representative of three independent experiments, with similar results obtained on each occasion. *A. a*, *Actinobacillus actinomycetemcomitans*; *P. g*, *Porphyromonas gingivalis*; *S. g*, *Streptococcus gordonii*; *T. denticola*, *Treponema denticola*.

tiple antibacterial peptides suggests the involvement of a common mechanism(s) of action by the antibacterial peptides. In fact, the interaction of all antibacterial peptides with the microbial cell membrane depends on the biphasic structure of the peptides, and both defensins and LL-37 are known to destabilize the microbial membrane (5).

Conrads *et al.* observed no or only moderate resistance in susceptibility to phagocytosis for most periodontopathic species they tested (21), which does not agree with our observation. The discrepancy can be explained by several factors. Because Conrads *et al.* incubated whole blood (200 μL) with 1.25×10^6–10^7 colony-forming units of bacteria, they measured phagocytosis in the presence of antibodies and complement, using no distinct ratio of phagocytes to bacteria. In addition, they observed a rate of > 90% of phagocytosed cells for most bacteria. As presented here, in Figs 1C,D, the high percentage of phagocytosing cells may mask different

Table 4. Overall susceptibility to host defense

Group	Species/strain	LL-37[a]	HBD-3[a]	Phagocytosis[b]
Nonperiodontopathic	*Streptococcus sanguinis* MPC-1	+ +	+ + +	+ + +
	Streptococcus sanguinis 66 × 49	+ + +	+ +	+ + + +
	Streptococcus sanguinis 804	+ + +	+ + + +	+ + + +
	Streptococcus gordonii 10558	+ + +	+ + + +	+
	***Streptococcus gordonii* M5**	+	+	+ +
	Actinomyces naeslundii	+ + +	+ + + +	+ +
	Veillonella atypica	+ + + +	+ + +	+ +
Periodontopathic	*Eikenella corrodens*	+ + + +	+ + + +	+
	Fusobacterium nucleatum	+ + + +	+ + + +	+ + + +
	Prevotella nigrescens	+	+ + +	+ +
	Prevotella intermedia	+ + + +	+ + + +	+ + +
	Peptostreptococcus micros	+ +	+ +	+
	***Actinobacillus actinomycetemcomitans* 33384**	+ + +	+ +	+
	Actinobacillus actinomycetemcomitans 67	+ + +	+ + +	+ +
	***Actinobacillus actinomycetemcomitans* Y4**	+ +	+ +	+
	***Porphyromonas gingivalis* 33277**	+	+ + +	+
	***Porphyromonas gingivalis* 49417**	+	+ +	+ +
	Porphyromonas gingivalis W50	–[c]	–	+
	Tannerella forsythia	+	+	+ +
	Treponema denticola	+ + +	+ + + +	+

[a] +, ≥ 125 μg/mL; + +, 62.5 μg/mL; + + +, 31.3 μg/mL; + + + +, ≤ 15.7 μg/mL.
[b] 0 < + ≤ 20%; 20 < + + ≤ 40%; 40 < + + + ≤ 60%; 60 < + + + + ≤ 80%.
[c] Not determined.
HBD-3, human beta defensin-3.
Bold type, resistant strain.

susceptibilities to phagocytosis in terms of the mean fluorescence intensity of the phagocytes. We have actually observed that the phagocytosis of opsonized *T. denticola* is comparable to that of *F. nucleatum*, as determined by the percentage of phagocytosing cells, yet found a substantial difference in the mean fluorescence intensity of phagocytes, without any change in the order of susceptibility to phagocytosis (J. Shin, submitted). Resistance to phagocytosis by pathogens involves many steps in phagocytosis: binding, ingestion, the fusion of phagosomes with lysosomes, and intracellular killing (33). Our system evaluated only binding and ingestion. To evaluate the other two steps, further studies are required. Because fixed bacteria were used for the phagocytosis assay, the resistance to phagocytosis of some periodontopathogens, such as *A. actinomycetemcomitans* (34), must have been underestimated in our study.

Macrophages recognize and phagocytose nonopsonized bacteria through a combination of scavenger receptors and C-type lectin receptors, such as the mannose receptor, Endo180, and DC-SIGN (35). However, the receptors involved in the phagocytosis of bacteria by neutrophils are not well known. Our results (Fig. 2) suggest that ligands for the unknown receptors are variably distributed in phagocytosis-susceptible or phagocytosis-resistant bacteria. We have shown that phagocytosis-susceptible bacteria can help the phagocytosis of other bacteria (Fig. 3). *F. nucleatum* is known to co-aggregate with the four resistant bacteria species (1), suggesting that it might provide ligands for receptor(s) involved in phagocytosis, similarly to regular opsonins. Although only the co-aggregation of *S. sanguinis* with *S. gordonii* has been reported, *S. sanguinis* may also co-aggregate with other bacteria (1). Otherwise, the stimulation of phagocytes with bacterial ligands for toll-like receptors might be the underlying mechanism for the increased phagocytosis by *F. nucleatum* or *S. sanguinis* (36).

We observed that intraspecies variation was smaller than interspecies variation in susceptibilities to antimicrobial peptides and phagocytosis. Still, it cannot be excluded that the strain(s) studied here may not be representative of the species. Although limited numbers of bacterial species and strains were studied, strains resistant to both antimicrobial peptides and phagocytosis were more common in the periodontopathic group (Table 4). The immune-resistant periodontopathic bacteria may dominate susceptible bacteria once they succeed in colonization. It is interesting that *S. gordonii* M5, known to be nonperiodontopathic, was highly resistant to both antibacterial peptides and phagocytosis. Likewise, *F. nucleatum* and *P. intermedia* were highly sensitive to host defense in our study, raising the question of whether they are true periodontopathogens. Some people regard *F. nucleatum* as a commensal bacterium, although a weak (but significant) association between its prevalence and the clinical index of periodontitis has been reported (27). Even immune-susceptible bacteria may contribute to the pathogenesis of disease, if they help the colonization of other periodontopathic bacteria. *F. nucleatum* can co-aggregate with most major oral bacteria and bridges early colonizers with other late-colonizing orange- and red-complex bacteria (1). Co-aggregation of *P. intermedia* with diverse oral bacteria has also been documented (37).

Commensal bacteria often share the ability to evade host immunity with pathogenic bacteria, but differ in that they do not harm the host. Therefore, the pathogenicity of periodontopathogens may be determined by several factors in addition to the ability of immune evasion.

Why would the lack of either neutrophils or LL-37 aggravate the disease caused by periodontopathogens that are already resistant to phagocytosis and LL-37? Microbial succession during plaque maturation may provide an answer. The red-complex bacteria require the orange complex for their colonization, and the orange-complex bacteria require other early colonizers (27). Therefore, normal immune function may maintain periodontal health by regulating susceptible early colonizers and orange-complex bacteria, preventing colonization by resistant periodontopathogens. It also explains why patients with agammaglobulinemia or hypogammaglobulinemia do not suffer from severe periodontitis (38–40) (Dr B. Grimbacher, personal communication). This underscores the importance of the regulation of early colonizers through competent host immunity and/or mechanical plaque control before resistant periodontopathogens dominate.

In conclusion, we studied various oral bacteria for susceptibility to LL-37, human beta defensin-3, and phagocytosis, and have demonstrated that immune evasion may be a contributing factor to the pathogenicity of some periodontopathic bacteria.

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Supplementary material

Fig. S1. Neutrophils (1×10^5) from one healthy donor (white bars) or from mixed blood, including that from periodontally diseased donors (black bars) were incubated with *Streptococcus sanguinis* (*S. s*), *Fusobacterium nucleatum* (*F. nucleatum*), or *Treponema denticola* (*T. denticola*), at a ratio of 1 : 25 (phagocytes : bacteria), at 37°C for 60 min. After quenching with Trypan blue, the cells were analyzed by flow cytometry.

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