



Differential ability of periodontopathic bacteria to modulate invasion of human gingival epithelial cells by *Porphyromonas gingivalis*

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ARTICLE INFO

Article history:

Received 10 June 2009

Received in revised form

23 September 2009

Accepted 25 September 2009

Available online 7 October 2009

Keywords:

Polymicrobial infection

Host invasion,

Porphyromonas gingivalis

Gingival epithelial cells

Periodontitis

ABSTRACT

Periodontitis is a polymicrobial infection caused by selected gram-negative bacteria including *Porphyromonas gingivalis*. Host cell invasion by *P. gingivalis* has been proposed as a possible mechanism of pathogenesis in periodontitis. The aim of the present study was to assess the influence of periodontopathogens on *P. gingivalis* invasion of gingival epithelial cells in polymicrobial infection. *P. gingivalis* was tested for its ability to invade a human gingival epithelial cell line Ca9-22 in co-infection with periodontopathogens, using an antibiotic protection assay. Among the pathogens tested, only *Fusobacterium nucleatum* demonstrated the ability to significantly promote *P. gingivalis* invasion ($P < 0.01$). This increased invasion was confirmed by confocal scanning laser microscopy utilizing a dual labeling technique. In contrast, co-infection with *Aggregatibacter actinomycetemcomitans* or *Tannerella forsythia* attenuated *P. gingivalis* invasion. The fusobacterial enhancement of host cell invasion was not observed in co-incubation with other periodontopathogens tested. These results suggested that complex synergistic or antagonistic physiologic mechanisms are intimately involved in host cell invasion by *P. gingivalis* in polymicrobial infection.

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1. Introduction

In periodontal disease, it has been suggested that complex microbial milieu harbors several pathogens that contribute directly to host tissue destruction. *Porphyromonas gingivalis*, a gram-negative anaerobe, is a major colonizer of gingival tissues and has been etiologically implicated in various forms of periodontitis [1]. Cellular invasion by *P. gingivalis* has been proposed as a possible virulence factor, affording protection from the host immune responses and contributing to tissue damage [2–5].

Periodontitis is one of the predominant polymicrobial infections of humans. In polymicrobial diseases, there are complex interactions between two or more etiologic agents, leading to synergistic clinical symptoms and pathologies often triggering an array of host responses [6]. Therefore, it is of great interest to investigate interactions between different periodontal bacteria and host cells. In recent periodontal research, polymicrobial infection models have been used to study characteristics and alterations of the host response profiles

[7,8]. However, data on the potential of host cell invasion by periodontopathogens in polymicrobial infection are scarce.

We recently developed a polymicrobial infection model of host cell invasion by *P. gingivalis*, and reported that *Fusobacterium nucleatum*, a periodontopathogen strongly implicated in plaque development [9], enhances invasion of human gingival epithelial and aortic endothelial cells by *P. gingivalis* [10]. In the present study, we used this polymicrobial infection model to evaluate the influence of several major periodontopathogens on *P. gingivalis* invasion of human gingival epithelial cells.

2. Results

2.1. Invasion of *P. gingivalis* in the poly-infection

In polymicrobial infection experiments, several periodontopathogens were individually co-infected with *P. gingivalis* as a prototype consortium. Among the pathogens, only *F. nucleatum* strains demonstrated a capacity to significantly boost *P. gingivalis* invasion of Ca9-22 cells, resulting in 3- to 8- fold increase in invasion efficiencies (Table 1). Co-infection with *P. intermedia* slightly enhanced invasion of *P. gingivalis*. In contrast, co-infection with *A. actinomycetemcomitans* strains or *T. forsythia* significantly abrogated invasion of *P. gingivalis*. Co-infection with *T. denticola* showed

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Table 1
Invasion of gingival epithelial cells by *P. gingivalis* 33277 in mono- or polymicrobial infection.

Infectant	Invasion efficiency (%) of <i>P. gingivalis</i>
Mono-infection	
<i>P. gingivalis</i> 33277 alone	1.39 ± 1.09
Poly-infection	
+ <i>F. nucleatum</i> TDC100	11.71 ± 7.33*
+ <i>F. nucleatum</i> 25586	7.41 ± 3.06*
+ <i>F. nucleatum</i> #20	4.43 ± 6.43*
+ <i>A. actinomycetemcomitans</i> JP2	0.10 ± 0.05*
+ <i>A. actinomycetemcomitans</i> Y4	0.17 ± 0.06*
+ <i>A. actinomycetemcomitans</i> 310-a	0.20 ± 0.25*
+ <i>T. forsythia</i> 43037	0.30 ± 0.21*
+ <i>T. denticola</i> 35405	0.43 ± 0.46
+ <i>P. intermedia</i> 25611	3.50 ± 0.71

Ca9-22 cells (10^5 cells) were infected with 10^7 bacteria (MOI = 100); Values given as means ± standard deviations of triplicate independent determinations from a typical experiment; *Statistically significantly different from monomicrobial infection ($P < 0.01$) by analysis of variance (ANOVA) with Bonferroni post test.

no effect on *P. gingivalis* invasion. Likewise, control *E. coli* strains had no effect on *P. gingivalis* invasion (data not shown).

2.2. Effect of *F. nucleatum* on invasion of other periodontopathogens

Following demonstration of fusobacterial enhancement of *P. gingivalis* invasion, we tested whether *F. nucleatum* could also promote invasion of host cells by other periodontopathogens. Co-infection with *F. nucleatum* TDC100 exerted no significant effect on invasion by *A. actinomycetemcomitans*, but attenuated that by *P. intermedia* (Table 2).

2.3. Assessment of internalization of *P. gingivalis* or *F. nucleatum* in gingival epithelial cells by confocal scanning laser microscopy (CSLM)

Internalization of *P. gingivalis* or *F. nucleatum* was assessed by CSLM following double staining (Fig. 1). The frequency of *P. gingivalis* 33277 invading Ca9-22 cells increased in the presence of *F. nucleatum* TDC100 (Fig. 1a, b). When infected alone, *F. nucleatum* TDC100 demonstrated minimal ability to invade Ca9-22 cells. Co-infection with *P. gingivalis* did not significantly influence the frequency of *F. nucleatum* invasion (Fig. 1c, d). Next, anti-*P. gingivalis* and anti-*F. nucleatum* antisera were used together to examine whether the ability of *P. gingivalis* to invade Ca9-22 cells was dependent upon the concomitant internalization of *F. nucleatum*. Ca9-22 cells that contained only *P. gingivalis* (Fig. 1e) were more prevalent than cells that contained both *P. gingivalis* and *F. nucleatum*. These CSLM data correlated well with the results of the anti-biotic protection assays.

Table 2
Effect of co-infection with *F. nucleatum* on invasion of gingival epithelial cells by *A. actinomycetemcomitans* or *P. intermedia*.

Infectant	<i>F. nucleatum</i> TDC100	
	–	+
<i>A. actinomycetemcomitans</i> JP2	0 ± 0	0.001 ± 0.002
<i>A. actinomycetemcomitans</i> Y4	0.014 ± 0.016	0.015 ± 0.021
<i>P. intermedia</i> 25611	0.22 ± 0.14	0.017 ± 0.019*

Ca9-22 cells (10^5 cells) were infected with 10^7 bacteria (MOI = 100); Invasion efficiencies (%) of *A. actinomycetemcomitans* or *P. intermedia* are shown. Values given as means ± standard deviations of triplicate independent determinations from a typical experiment; *Statistically significantly different from monomicrobial infection ($P < 0.01$) by Mann-Whitney U test.

2.4. Effect of *F. nucleatum* culture filtrate on *P. gingivalis* invasion

In order to further analyze the fusobacterial enhancement of *P. gingivalis* invasion, we tested the effect of *F. nucleatum* culture filtrates on the host cell invasion. Unlike co-incubation with *F. nucleatum* viable cell suspension, the filtrates exerted no significant effect on *P. gingivalis* invasion (Fig. 2).

2.5. Effect of *F. nucleatum* viability on *P. gingivalis* invasion

To determine the effect of fusobacterial viability on *P. gingivalis* invasion, heat-killed or methanol-fixed *F. nucleatum* cells were mixed with *P. gingivalis* and co-infected Ca9-22 cells. Killing of the bacteria was confirmed by culture on blood agar plates. Co-infection with heat-killed or methanol-fixed *F. nucleatum* resulted in an increase in *P. gingivalis* invasion, although the extent was lower than co-infection with viable *F. nucleatum* cells (Fig. 3).

3. Discussion

Cumulative evidence has indicated the significance of polymicrobial infections in which selected microorganisms interact in a synergistic or antagonistic manner, impacting on pathogenesis [7,14,15]. In the present study, we examined the ability of *P. gingivalis*, a major periodontopathogen, to invade the gingival epithelial cells as the primary outcome in addressing the potential virulence synergism or antagonism of oral microbial consortia. Among the periodontopathogens tested, *F. nucleatum* and *P. intermedia* demonstrated ability to enhance *P. gingivalis* invasion with varied efficiency. *F. nucleatum* was the only pathogen that showed a statistically significant enhancement, and this remarkable effect was found to be shared among *F. nucleatum* strains tested. *F. nucleatum*, a gram-negative fusiform anaerobe, is able to adhere to and invade human epithelial cells [16]. It has been shown to enhance host cell invasion by other bacteria such as *Streptococcus cristatus* [17] or *Pseudomonas aeruginosa* [18]. This prompted us to test whether *F. nucleatum* can alter invasion of Ca9-22 by other periodontopathogens. In our experimental condition, co-infection with *F. nucleatum* did not enhance invasion by *A. actinomycetemcomitans* or *P. intermedia*. Collectively, these results suggested that enhancement of host cell invasion by periodontopathogens requires some specific combinations in polymicrobial infection.

Synergistic interactions in virulence between *F. nucleatum* and *P. gingivalis* have been observed in vitro and in animal models [19,20]. Some hypothesis can be proposed regarding the mechanism(s) by which *F. nucleatum* exerts such effect on *P. gingivalis* invasion. One hypothesis is that *F. nucleatum* release extracellular substance(s) that facilitates *P. gingivalis* invasion. In murine orofacial infection model, heat-stable substance(s) of *F. nucleatum* has been shown to contribute to synergistic virulence with other bacteria [21]. Our observation that sterile filtrates from cell cultures of *F. nucleatum* were not capable of enhancing *P. gingivalis* invasion (Fig. 2) strongly suggests that the host cell invasion-facilitating effect was not caused by an extracellular filterable substance.

Direct contact between the co-infectants and the host cells would be another explanation, since co-incubation with methanol-fixed or heat-killed *F. nucleatum* appeared to partially enhance the *P. gingivalis* invasion. Therefore, fusobacterial viability may not be required for this enhancement. *F. nucleatum* and *P. gingivalis* are strong coaggregating pairs, and the coaggregation may have the capacity to alter the expression of virulence factors in individual microorganisms [19]. We have previously observed a coaggregation reaction between *P. gingivalis* 33277 and *F. nucleatum* TDC100, that is inhibitable by galactose. Galactose, however, only partially inhibited *F. nucleatum* enhanced *P. gingivalis* invasion [10].

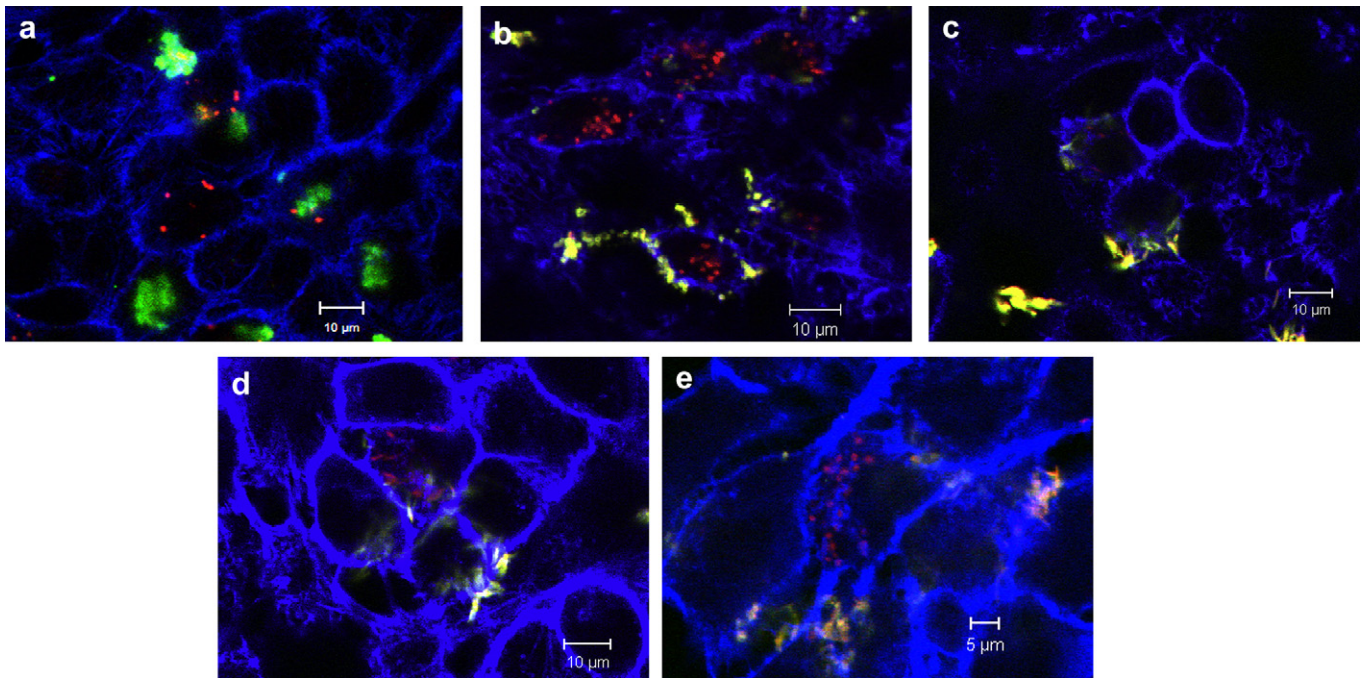


Fig. 1. Attachment and invasion of *P. gingivalis* or *F. nucleatum* into gingival epithelial cells visualized by confocal scanning laser microscopy following dual labeling. *P. gingivalis* 33277 or *F. nucleatum* TDC100 invading Ca9-22 cells were stained red, while extracellular bacteria were detected as green–yellow. The host cell cytoskeleton stained with phalloidin appeared blue. (a) Mono-infection by *P. gingivalis*; (b) Co-infection with *P. gingivalis* and *F. nucleatum*. Internalized *P. gingivalis* bacteria were stained red. (c) Mono-infection by *F. nucleatum*; (d) Co-infection with *P. gingivalis* and *F. nucleatum*. Internalized *F. nucleatum* cells were stained red. (e) Co-infection. Bacteria were stained using both anti-*P. gingivalis* and anti-*F. nucleatum* antisera. Internalized bacteria were stained red. As shown here, it was more common to find Ca9-22 cells that harbored intracellular *P. gingivalis* only. These examples are representative of results showing the most common pattern of bacterial infection with the cell line.

Furthermore, the enhancement of *P. gingivalis* invasion was not observed for co-infection with *A. actinomycetemcomitans*, that can coaggregate with *F. nucleatum* [22]. Also, in our macro-coaggregation assay, *F. nucleatum* 25586 or #20 showed lower levels of coaggregation with *P. gingivalis*, than *F. nucleatum* TDC100 (unpublished observation). However, these strains with less coaggregation activity also demonstrated ability to enhance *P. gingivalis* invasion (Table 1). These results may indicate the limited role of coaggregation in the enhancement of *P. gingivalis* invasion.

It has been reported that *F. nucleatum* transports noninvasive oral streptococci into human epithelial cells [17]. It is thus tempting to speculate that *F. nucleatum* carries *P. gingivalis* into gingival

epithelial cells by forming consortia. In the present infection model, however, internalization of the co-infectant may not be a prerequisite for the increased uptake of *P. gingivalis*. In the antibiotic protection assay, *F. nucleatum* 25586 and #20 invaded the host cells more than strain TDC100 (data not shown). Yet, *F. nucleatum* TDC100 demonstrated higher ability to enhance *P. gingivalis* invasion (Table 1). Also in our experimental condition, *F. nucleatum* TDC100, which had limited ability to invade the host cells (Fig. 1c), increased *P. gingivalis* invasion more than other strains (Table 1). Moreover, other invasive periodontopathogens failed to increase *P. gingivalis* invasion. Collectively, these results suggested the limited role of *F. nucleatum* internalization in the enhancement of *P. gingivalis* invasion.

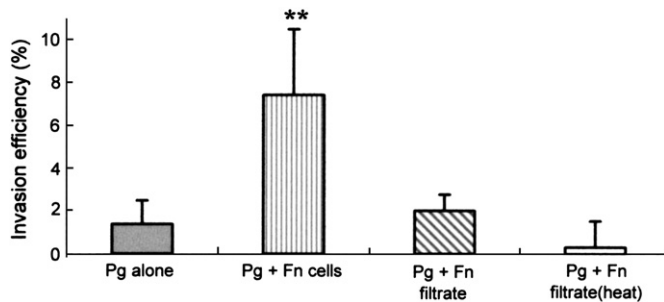


Fig. 2. Effect of *F. nucleatum* culture filtrates on *P. gingivalis* invasion of gingival epithelial cells. Confluent Ca9-22 cells were infected by the following co-infectants: Pg alone; *P. gingivalis* 33277. Pg + Fn cells; *P. gingivalis* with *F. nucleatum* 25586. Pg + Fn filtrate; *P. gingivalis* with culture filtrate of *F. nucleatum* 25586. Pg + Fn filtrate (heat); *P. gingivalis* with culture filtrate (autoclaved) of *F. nucleatum* 25586. Values are the means \pm standard deviations of triplicate independent determinations from a typical experiment. A similar trend was observed for co-infection with *F. nucleatum* TDC100. **Statistically significantly different from monomicrobial infection ($P < 0.01$) by analysis of variance (ANOVA) with Bonferroni post test.

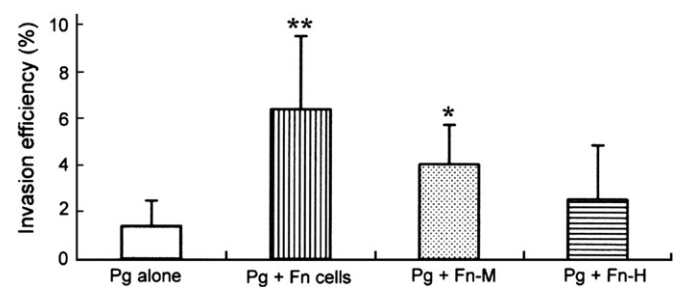


Fig. 3. Effect of methanol-fixed or heat-killed *F. nucleatum* cells on *P. gingivalis* invasion of gingival epithelial cells. Confluent Ca9-22 cells were infected by the following co-infectants: Pg alone; *P. gingivalis* 33277. Pg + Fn cells; *P. gingivalis* with *F. nucleatum* 25586. Pg + Fn-M; *P. gingivalis* with methanol-fixed *F. nucleatum*. Pg + Fn-H; *P. gingivalis* with heat-treated *F. nucleatum*. Values are the means \pm standard deviations of triplicate independent determinations from a typical experiment. A similar trend was observed for co-infection with *F. nucleatum* TDC100. **Statistically significantly different from monomicrobial infection ($**P < 0.01$, $*P < 0.05$) by analysis of variance (ANOVA) with Bonferroni post test.

Bacterial invasion requires an initial interaction between the bacteria and host cell surfaces, and then activation of complex signaling networks that regulate engulfment [23]. *P. gingivalis* has been shown to induce lipid raft-dependent phagocytosis in macrophages [5]. To invade host cells that are not professional phagocytes, pathogens utilize diverse molecules and strategies. It has been suggested that functional epitopes of cellular receptors of *P. gingivalis* are cryptic, and that bacterial protease expose them [24]. The ability of various bacteria in the microbial complex of the gingival sulcus to express surface molecules to specifically interact with other bacterial species and host cells has been suggested to be a critical determinant in plaque formation and structure [19]. It has been shown that *F. nucleatum* and *P. gingivalis* trigger specific and differential gene regulation pathways in human gingival epithelial cells [25]. It is possible that interactions with *F. nucleatum* might affect expression of cell surface receptors that are important in invasion.

At this time, we cannot delineate mechanisms by which *A. actinomycetemcomitans* or *T. forsythia* inhibited *P. gingivalis* invasion of host cells. It is possible for *T. forsythia* to compete with *P. gingivalis* to gain entry into host cells, since *T. forsythia* itself was invasive (data not shown). It does not, however, explain the antagonistic effect by *A. actinomycetemcomitans*, as strains tested were either weakly cell-invasive (Y4) or noninvasive (JP2, 310a). Whether the effect induced by the co-infectants was directed at the host cell or the *P. gingivalis* needs to be clarified in future investigation.

Although invasion of host cells by *P. gingivalis* has been proposed as an important mechanism of pathogenesis and progression in periodontal disease, its invasive ability has been reported to be inefficient [26]. The results from the present study also support this notion, as relatively high MOI was required for *P. gingivalis* invasion in mono-infection settings. However, the efficiency can be significantly boosted by the presence of *F. nucleatum*. Since both *P. gingivalis* and *F. nucleatum* are often identified together in the subgingival plaque of periodontitis lesions, it would be reasonable to hypothesize that they may coordinate their virulence activities in the periodontal milieu, leading to an exacerbation of periodontal disease.

In summary, among the periodontopathogens tested, only *F. nucleatum* strains demonstrated the ability to significantly enhance *P. gingivalis* invasion of human gingival epithelial cells. In contrast, co-infection with *A. actinomycetemcomitans* or *T. forsythia* attenuated *P. gingivalis* invasion. Direct contact between specific combinations of co-infectants and the host cells may be necessary for this enhanced invasion by *P. gingivalis*. Our current effort is directed at elucidating the molecular mechanisms exploited by *P. gingivalis* in polymicrobial infection of host cells.

4. Materials and methods

4.1. Bacterial strains and growth conditions

The following bacterial strains were used; *P. gingivalis* ATCC 33277 (American Type Culture Collection, Manassas, VA, USA), *F. nucleatum* ATCC 25586, #20, TDC100 (a clinical isolate and working strain in our laboratory) [11], *Aggregatibacter actinomycetemcomitans* JP2, Y4, 310a (kindly provided by Dr. H. Ohta, Ibaraki University, Japan), *Treponema denticola* ATCC 35405, *Tannerella forsythia* ATCC 43037, *Prevotella intermedia* ATCC 25611. The *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans* and *P. intermedia* were grown in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD, USA) supplemented with 0.5% of yeast extract, hemin (5 µg/ml) and menadione (0.5 µg/ml). *T. denticola* was grown in TYGVS medium as described previously [12].

T. forsythia was grown in BHI broth supplemented with 5% heat-inactivated fetal calf serum and 0.001% N-acetyl muramic acid. The bacterial cultures were grown to mid-log phase (range at OD₆₆₀ nm of 0.6–1.0) at 37 °C under anaerobic conditions. Noninvasive *Escherichia coli* SCS110 and DH5α strains were used as a control.

Heat-killed *F. nucleatum* were prepared by heating the bacteria at 80 °C for 10 min. Methanol-fixed *F. nucleatum* were prepared by fixing the bacteria in 99% methanol for 1 h at room temperature (RT). The absence of bacterial growth after the treatment was confirmed by plating samples on blood agar plates.

4.2. Cells and culture conditions

An established human gingival epithelial cell line, Ca9-22, was purchased from Health Science Research Resources Bank (Osaka, Japan). The Ca9-22 cells were maintained in Eagle's minimal essential medium (MEM) supplemented with glutamine (0.6 mg/ml), heat-inactivated 10% fetal calf serum, and gentamicin (10 µg/ml)/amphotericin B (0.25 µg/ml) (Cascade Biologics, Portland, OR, USA) at 37 °C in 5% CO₂ in humidified air.

4.3. Monomicrobial infection

Invasion of bacteria was quantitated by a standard antibiotic protection assay as described previously [3]. Briefly, epithelial cells were seeded in 12-well flat-bottom culture plates at a cell density of 2.0×10^5 cells per well. Prior to infection, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and incubated further 2 h in MEM without antibiotics. The multiplicity of infection (MOI) was calculated based on the number of cells per well at confluence. The bacteria were harvested by centrifugation, washed with PBS, and resuspended in MEM at desired concentrations. The bacterial suspensions were added to confluent Ca9-22 monolayers and incubated at 37 °C in 5% CO₂ for 2 h. After incubation, unattached bacteria were removed following washing of the monolayers 3 times with PBS. External adherent cells were then killed by incubating the infected monolayers with MEM containing 200 µg/ml of metronidazole and 300 µg/ml of gentamicin for 1 h. After exposure to antibiotic, monolayers were washed twice with PBS, and lysed in 1 ml of sterile distilled water per well. Cells were incubated for 30 min, during which they were disrupted by repeated pipetting. Lysates were serially diluted and plated on blood agar plates supplemented with hemin and menadione, and incubated anaerobically at 37 °C for 10 days. Colony-forming units of invasive organisms were then enumerated. Invasion efficiency was expressed as the percentage of the initial inoculum recovered after antibiotic treatment and Ca9-22 lysis.

In our previous experiments, we found that there was a modest increase in the invasion efficiencies of *P. gingivalis* with increasing MOI. Invasion reached a plateau at about 100 MOI [10]. Thus, we used MOI of 100 in all subsequent experiments.

4.4. Polymicrobial infection

Polymicrobial infection of Ca9-22 cells was performed as described previously [10]. Briefly, polymicrobial inocula were prepared by mixing equal volumes (1×10^7 cells per well) of bacterial suspensions and were incubated for 5 min at RT prior to initiating the infection. For the monomicrobial control infection, bacterial suspensions were mixed with an equal volume of MEM. As a control for polymicrobial infection, *E. coli* SCS110 or DH5α was used as a co-infectant. These bacteria exerted no significant effects on viability of *P. gingivalis* or host cells in our co-infection experiments.

4.5. Confocal scanning laser microscopy

In order to confirm internalization of *P. gingivalis* or *F. nucleatum* into Ca9-22, confocal scanning laser microscopy (CSLM) was performed. A dual labeling technique, based on the method described by Inagaki et al. [13], was used to discriminate intracellular from extracellular bacteria. Ca9-22 cells were grown on coverslips in six-well tissue culture plates and infected with *P. gingivalis* 33277 or *F. nucleatum* TDC100 for 2 h. Cells were fixed in 4% paraformaldehyde in PBS (Wako Pure Chemical Industries, Osaka, Japan) for 10 min. After washing 3 times with PBS, any excess of reactive groups paraformaldehyde were quenched with 50 mM NH₄Cl in PBS for 10 min at RT. After washing, cells were incubated with a rabbit polyclonal anti-*P. gingivalis* or anti-*F. nucleatum* serum diluted 1:500 in PBS–0.5% BSA for 60 min. Following incubation, coverslips were washed three times with PBS and incubated with Alexa Fluor 488 (green fluorescent dye)-conjugated goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR, USA) diluted 1:500 for 30 min to visualize attached bacteria. Internalized bacteria were then stained by first permeabilizing Ca9-22 cells by dipping coverslips in 0.4% Triton X-100 solution for 5 min, then staining with the rabbit anti-*P. gingivalis* antiserum or anti-*F. nucleatum* antiserum followed by Alexa Fluor 568 (red fluorescent dye)-coupled goat anti-rabbit immunoglobulin G (Molecular Probes) diluted 1:500 as described above. Actin filaments were stained with Alexa Fluor 647 (blue fluorescent dye) conjugated to phalloidin (Molecular Probes) for 30 min according to the manufacturer's recommendations to visualize the cellular cytoskeleton and confirm internalization. *P. gingivalis* could be distinguished from *F. nucleatum* on the basis of cellular morphology.

Coverslips mounted in an antifading mounting medium (VECTA-SHEILD, Vector Laboratories, Burlingame, CA, USA) were examined by confocal scanning laser microscopy (CSLM) using a LSM5 DUO microscope (Carl Zeiss MicroImaging, Göttingen, Germany) with a 63 × oil immersion objective. A series of 20–25 Z-stack images was scanned in increments using excitation wavelengths of 488, 561 and 633 nm. Images were analyzed using ZEN 2007 software (Carl Zeiss).

4.6. Preparation of *F. nucleatum* culture filtrate

F. nucleatum was grown in BHI medium to an optical density of 1.0 at 660 nm, and fluid cultures were centrifuged at 600 × g for 10 min. The supernate was sterilized by 0.22 μm pore size membrane (Millipore, Bedford, MA, USA) filtration for the preparation of bacteria-free culture filtrates. A heat-treated culture filtrate was prepared with an autoclave at 121 °C for 20 min. An aliquot of the culture filtrate was mixed with a *P. gingivalis* cell suspension and incubated for 5 min at RT. The mixture was then used to infect Ca9-22 monolayers.

4.7. Data and statistical analyses

All experiments were performed in duplicate or triplicate for each condition and repeated at least three times. Statistical comparisons were performed using a software package (InStat 3.0, GraphPad Software, La Jolla, CA, USA). *P*-values less than 0.05 were considered statistically significant.

Acknowledgements

We thank David Blette for editing the manuscript. This research was supported by Oral Health Science Center Grant HRC7 from Tokyo Dental College, and by a "High-Tech Research Center" Project for Private Universities: matching fund subsidy from Ministry of Education, Culture, Sports, Science and Technology of Japan, 2006–2010.

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