Enhanced Attachment of *Porphyromonas gingivalis* to Human Fibroblasts Mediated by *Fusobacterium nucleatum*

Zvi Metzger, DMD,† Jaron Blasbalg, DMD,‡ Miri Dotan, BA,† and Ervin I. Weiss, DMD§*

Abstract

Fusobacterium nucleatum and *Porphyromonas gingivalis* are often co-isolated from endodontic infection sites. The present study tested the hypothesis that adhesin-mediated reactions might contribute to this phenomenon. *F. nucleaum* PK1594 and *P. gingivalis* PK1924 were selectively labeled with fluorochromes and allowed to adhere to human fibroblasts, either each strain alone or sequentially. The number of bacteria of each type adhering to individual fibroblasts was determined. Sugar inhibition profile of this adherence was explored. Attachment of *P. gingivalis* to human fibroblasts increased by nearly 10-fold when *F. nucleatum* was present ($P < .001$). Galactose, lactose, and fucose inhibited this enhanced attachment ($P < .001$), as well as that of *F. nucleatum* alone ($P < .001$). The results suggest that *F. nucleatum* might be a primary colonizer of the host tissues and serve as mediator for enhanced attachment of *P. gingivalis* to the host cells. This might explain in part the common co-occurrence of *F. nucleatum* and *P. gingivalis* in endodontic mixed infections. *(J Endod 2009;35:82–85)*

Key Words

Adhesin, attachment, coaggregation, fibroblasts, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*

From the *Department of Endodontology, †Department of Oral Biology, and ‡Department of Oral Rehabilitation, Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv; and §Department of Prosthodontics, Hebrew University-Hadassah School of Dental Medicine, Jerusalem, Israel.

Address requests for reprints to Zvi Metzger, Department of Endodontology, Goldschleger School of Dental Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv 94778, Israel. E-mail address: metzger@post.tau.ac.il.

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Endodontic pathogens tend to grow as mixed species biofilms that might develop as either intracanal or extraradicular bacterial communities (1–3). *Fusobacterium nucleatum and Porphyromonas gingivalis* are frequently found together in these sites and are commonly co-isolated from infected root canals and periapical abscesses (3–6).

Nair et al. (1) have recently shown that untreated recesses of root canals harbor an organized biofilm, resembling dental plaque to great extent. Abundant intercellular matrix with a mixed bacterial population was observed, with many bacteria with a fusiform morphology resembling that of *F. nucleatum* (1). *F. nucleatum* and *P. gingivalis* have recently also been identified in extraradicular biofilms associated with refractory apical periodontitis (3). Similar co-occurrence of *F. nucleatum* and *P. gingivalis* has also been reported in destructive periodontal disease (7) as well as peritonsillar cellulitis (8).

This might be attributed to several reasons such as shared ecological requirements (9), mutual nutritional benefits (9), or the complement-depleted and immunoglobulin-depleted environment that might be locally formed by certain strains of *P. gingivalis* (10–12). Nevertheless, it is also possible that adhesin-dependent interactions might play a role in the co-occurrence of these pathogens in infected sites.

Adhesin-mediated bacterial coaggregation has been primarily studied in the context of dental plaque formation and is considered an essential component of this process (13). This coaggregation is based on an adhesin that is present on one bacterium and attaches to a specific sugar present on the surface of the coaggregating partner. Such coaggregations might be competitively inhibited, in a concentration-dependent manner, by the presence of the specific sugar. A bacterium such as *F. nucleatum*, which has several types of adhesins (14–18) , might play a central role in plaque formation; it might interact with many early colonizers of the dental plaque as well as with many of the late ones, thus serving as a bridge between the former and the latter (13).

The adhesins of *F. nucleatum* PK1594 have been extensively studied and characterized (13, 15, 17–19). It has been shown that the same galactose-inhibitable adhesin that mediates its coaggregation with *P. gingivalis* PK1924 is also involved in its attachment to mammalian cells (19).

The present study was designed to extend these observations and explore the possibility that this adhesin might also allow an enhanced attachment of *P. gingivalis* to host cells by using *F. nucleatum* as an anchor to bridge between bacterium and host.

Materials and Methods

Bacteria

F. nucleatum PK-1594 and *P. gingivalis* PK-1924 were grown anaerobically in Wilkins-Chalgren anaerobic broth (Oxoid, Hampshire, England) in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 at 37°C (Coy anaerobic chamber). Bacterial strains were kept as frozen stocks and were started and transferred twice in this growth medium before being used in the experiments. Cells at late exponential to early stationary stage of growth were harvested and washed 3 times in a coaggregation buffer consisting of 0.1 mmol/L $CaCl_2$, 0.1 mmol/L $MgCl_2$, and 0.15 mol/L NaCl in 1.0 mmol/L Tris, adjusted to pH 8.0 (20). Bacteria were then resuspended in the buffer to $A_{660} = 1.0$ and kept at 4°C until used in the experiments.

Human Fibroblasts

Human periodontal ligament (PDL) fibroblasts were grown as primary cultures from tissue explants, as previously described (21). The cells were grown to early confluency and subcultured twice. For the attachment assay, fibroblasts were grown for 48 hours in Lab-Teck Chamber Slides (Nunc, Naperville, IL), resulting in low-density cultures, which were washed 3 times with Hank's balanced salt solution (HBSS) before being exposed to the bacteria.

Fluorochromes

To facilitate differential detection of the 2 types of bacteria in the attachment assay, they were labeled with fluorochromes, as previously described (22). *F. nucleatum* was labeled with 4′6-diamino-2-phenylindole (DAPI; Sigma, St Louis, MO; 7.5 μg/mL), which resulted in a blue fluorescence when viewed at a wavelength of 365 nm. *P. gingivalis* cells were heated to 95°C for 10 minutes to allow penetration of the other fluorochrome. This was followed by staining the cells with propidium iodide (PI; Sigma; 5 μg/mL), which resulted in a red fluorescence when viewed at a wavelength of 465 nm (22). In preliminary experiments it was established that this staining protocol did not affect the coaggregation between these bacteria or their attachment to the fibroblasts (data not shown). The fibroblasts were lightly stained with acridine orange to enhance their visualization in fluorescent microscopy (22).

Attachment Assay

Bacterial suspensions were adjusted to a density of $A_{660} = 0.3$ (*F. nucleatum*) or 0.6 (*P. gingivalis*), added to the fibroblasts (50 μL per well), and allowed to interact with the fibroblasts for 10 minutes at room temperature on a gently rotating stirring table set at 80 rpm (Gyrotory Shaker-G2; New Brunswick Scientific, Edison, NJ).

After the bacteria and fibroblast interaction, the suspensions were aspirated, and the fibroblasts were washed 3 times with HBSS to remove loosely bound and unbound bacteria. A fixative made of 99 parts of 10% buffered formalin and 1 part 25% glutaraldehyde was then applied to the cells for 10 minutes. This was followed by 2 rinses with distilled water, and the specimens were allowed to dry. The superstructure of the Lab-Tek slide was removed, and a coverglass was adapted by using Merckoglas (Merck, Darmstadt, Germany). The specimens were examined for the presence of bacteria attached to the fibroblasts by using a fluorescence microscope (Axioplan2; Zeiss, Jena, Germany) at a ×1000 magnification with immersion oil. Adhering bacteria were selectively counted, each at the proper wavelength.

For each fibroblast it was determined whether it had (1) no bacteria attached, (2) 1–5 bacteria from either species attached, or (3) more than 5 attached bacteria from either species. This method was selected because simple counting of bacteria was impractical. As long as few bacteria attached to each fibroblast, simple counting could be done, but with higher numbers of bacteria attaching to each cell, reliable, reproducible enumeration of the bacteria became a problem. The number of 5 bacteria as a cutting point was arbitrarily selected; nevertheless, these categories represent no adherence, mild adherence , or intensive bacterial adherence (categories 1–3, respectively, above).

Bacterial cell counts were performed on every fibroblast found in 10 separate fields located along 2 perpendicular diameters of each of the triplicate wells, with 160 fibroblasts counted in each well, with a total of 480 fibroblasts for each test point. The counting was done with blinding as to which group was counted.

Experimental Design

Experimental groups included fibroblasts to which the following were added: (1) *F. nucleatum* PK1594 alone, (2) *P. gingivalis* PK1924 alone, (3) *F. nucleatum* PK1594 alone followed by *P. gingivalis* PK1924, which was added after washing off the nonadherent *F. nucleatum* cells, (4) *F. nucleatum* that had been preincubated for 15 minutes with respective sugars and then added to the fibroblast cultures, and (5) *F. nucleatum* PK1594 treated as in (4) followed by *P. gingivalis* PK1594, which was added after washing off the nonadherent *F. nucleatum* cells.

At the end of each process nonadherent bacterial cells were washed off. When an additional wash was required (as in groups 3, 4, and 5), the other groups were washed equivalently to allow comparison.

Sugar Inhibitors

Galactose, lactose (Galβ1-4Glc), or fucose (6-deoxy galactose) was used, at 60 mmol/L, to inhibit the attachment (15, 19) All of these are structurally related; the structurally unrelated sugars of a similar size, glucose or mannose, were used at the same concentration as control.

Statistical Analysis and Data Presentation

Fibroblast selection for counting was done in a manner designed to ensure even distribution and to avoid potential operator bias. The experiment was done in triplicate wells, with 160 fibroblasts counted in each well, with a total of 480 fibroblasts for each test point. The experiment was repeated 3 times, with similar results. The results of a representative experiment are presented. The Student *t* test was used to compare the groups with each other, and *P* values less than .05 were considered significant.

Results

Bacterial Attachment

F. nucleatum PK1594 attached very efficiently to the human cells, and more than 5 *F. nucleatum* cells attached to most of the fibroblasts; only 2.75% ± 1.26% of the fibroblasts were free of fusobacteria (Fig. 1). On the other hand, *P. gingivalis* PK1924 attached poorly to the fibroblasts, and most fibroblasts were free of bacteria (77.0% ± 12.06%) (Fig. 1). The attachment of *P. gingivalis* PK1924 to the human cells was greatly enhanced by the presence of *F. nucleatum* PK1594 ($P < .001$) (Fig. 1); the number of fibroblasts to which more than 5 *P. gingivalis* cells attached increased from 8.0% ± 5.29% to 75.0% ± 6.12% ($P < .001$).

Sugar Inhibition

Galactose, lactose, and fucose inhibited the attachment of *F. nucleatum* PK1594 to the human fibroblasts, reducing the number of cells carrying more than 5 attached bacteria from 88.00% ± 3.65% to 6%–8% ($P < .001$) (Fig. 2). The number of bacteria-free cells increased accordingly to 80%–82% (data not shown). The enhanced attachment of *P. gingivalis* to the human cells in the presence of *F. nucleatum* was totally abrogated by galactose, lactose, or fucose ($P < .001$) (Fig. 3), whereas the attachment of the porphyromonads alone to the fibroblasts was virtually unaffected by these carbohydrates (data not shown). Glucose and mannose, which were used as controls, had no effect on the attachment of the fusobacteria, the attachment of *P. gingivalis*, or on the enhanced attachment of *P. gingivalis* caused by the presence of *F. nucleatum* (data not shown).

Discussion

F. nucleatum attachment to human cells has been previously studied (23–26). Nevertheless, to the best of our knowledge this is the first demonstration of its potential role in enhancing the attachment of an-

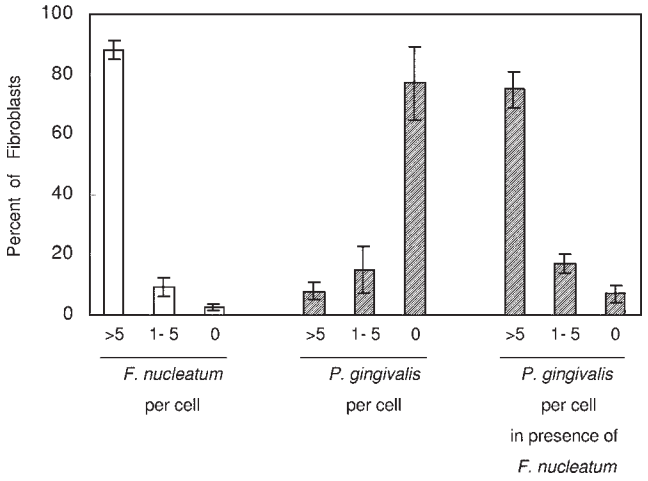

Figure 1. Attachment of *F. nucleatum* PK1594 and *P. gingivalis* PK1924 to human PDL fibroblasts. Either *F. nucleatum* PK1594 or *P. gingivalis* PK1924 alone or the 2 bacteria in a sequence were incubated with human PDL cells for 10 minutes. Bound bacteria were counted, and the corresponding fibroblasts were grouped in 1 of 3 categories: (1) no bacteria attached, (2) 1–5 bacteria attached to each fibroblast, and (3) more than 5 bacteria attached to each fibroblast. Open bars, *F. nucleatum* attachment to the fibroblasts. Shaded bars, *P. gingivalis* attachment to the fibroblasts (alone or in the presence of *F. nucleatum)*. Twenty fields were counted, with a total of 480 human cells per sample. Each bar represents the mean percentage of fibroblasts in each of the categories (± standard error of the mean). The number of fibroblasts to which >5 *P. gingivalis* cells attached increased significantly when *F. nucleatum* was present ($P < .001$).

other bacterium to human cells. Human PDL fibroblasts were used as a convenient representative of the wide range of mammalian cells to which *F. nucleatum* efficiently binds. This binding is mediated by the same galactose-inhibitable adhesin that is involved in its coaggregation with *P. gingivalis* PK1924 (19).

The presence of a large mass of bacteria attached on the human cells could be detected even when a simple phase contrast microscopy was used. Nevertheless, only the use of the fluorescent labeling allowed

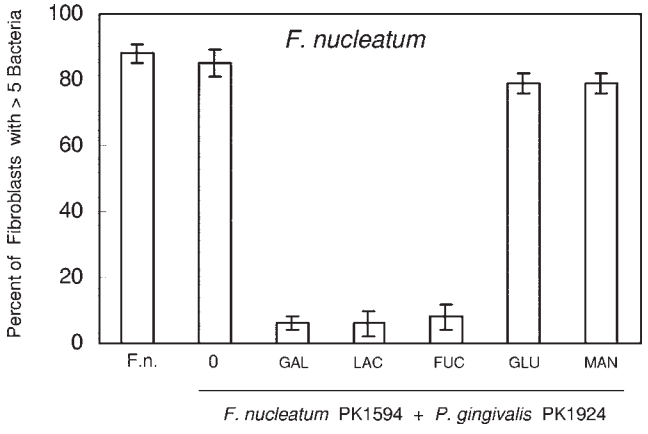

Figure 2. Sugar inhibition of the attachment of *F. nucleatum* PK1594 to human PDL fibroblasts. *F. nucleatum* PK1594 was incubated with human PDL fibroblasts, either alone or followed by *P. gingivalis* PK1924, with or without the specified sugars (at a final concentration of 60 mmol/L). Each bar represents the mean percentage of fibroblasts to which large numbers (>5) of *F. nucleatum* cells were attached (± standard error of the mean). Galactose, lactose, and fucose significantly inhibited the attachment of *F. nucleatum* PK1594 to the fibroblasts ($P < .001$).

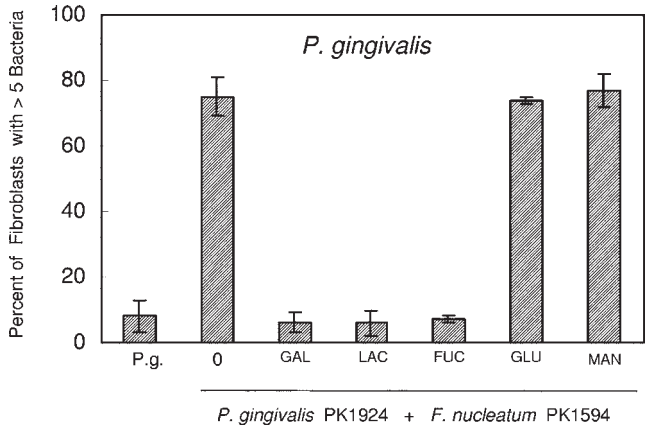

Figure 3. Sugar inhibition of the attachment of *P. gingivalis* PK1924 to human PDL fibroblasts. *P. gingivalis* PK1924 was incubated with human PDL fibroblasts, either alone or in the presence of *F. nucleatum* PK1594, with or without the specified sugars (at a final concentration of 60 mmol/L). Each bar represents the mean percentage of fibroblasts to which large numbers (>5) of *P. gingivalis* cells were attached (± standard error of the mean). The presence of the sugars did not affect the attachment of *P. gingivalis* alone (data not shown). The presence of *F. nucleatum* PK1594 significantly increased the attachment of *P. gingivalis* PK1924 cells to the fibroblasts ($P < .001$). Galactose, lactose, and fucose significantly inhibited the *F. nucleatum*–dependent attachment of *P. gingivalis* PK1924 to the fibroblasts ($P < .001$).

enumeration of the attached bacteria even when small numbers were concerned. Furthermore, this was possible even when both strains attached in layers on the same fibroblast. A similar principle was used by Papaioannuo et al (27) to study bacterial interactions with mammalian cells. Recently, dual fluorochrome staining was also used by Khemaleelakul et al (28) to study the coaggregation of bacterial pairs isolated from endodontic infection sites. They also were able to detect bacteria within coaggregates even when one species was present in small numbers.

F. nucleatum PK1594 readily attached to the human cells, most of which were covered by a large number of bacteria. The inhibition of this attachment by galactose, lactose (Galβ1-4Glc), and fucose (6-deoxy galactose) suggests that it was most probably mediated by the galactose-inhibitable adhesin of *F. nucleatum*, which is also involved in its coaggregation with *P. gingivalis* PK1924 (19). In contrast, *P. gingivalis* PK1924 infrequently attached to the human cells, and when it did so, it was in small numbers.

Attachment of certain *P. gingivalis* strains to mammalian cells has previously been reported. It might involve fimbriae-mediated reactions (29, 30), trypsin-like proteolytic activity of the bacteria (31, 32), or bacterial cell-surface hydrophobicity (33). Nevertheless, the strain used in the present study (PK1924) belongs to those that have a very limited direct binding capacity to the human cells.

An almost 10-fold increase in *P. gingivalis* attachment to the human cells occurred when *F. nucleatum* PK1594 was present. In addition, the same sugars that inhibited the attachment of the fusobacteria also completely inhibited the enhanced *P. gingivalis* attachment and blocked the coaggregation between the 2 strains (data not shown) (15, 17). These results suggest that *F. nucleatum* PK1594 directly bound to the human cells and served, by its adhesins, as a bridge for the enhanced attachment of *P. gingivalis*. A similar role for *F. nucleatum* in dental plaque formation has been suggested by Kolenbrander and London (13). They suggested that *F. nucleatum* might serve as a bridge between early colonizers and late ones.

The attachment mechanism proposed here might explain the common co-isolation of *F. nucleatum* and *P. gingivalis* from endodontic infections, as well as their presence together in extraradicular biofilms (3–6). Similarly, it might also contribute to their co-isolation from periodontal pockets with active disease (7) or from other infected tissues (8).

This is of particular interest because *F. nucleatum*, which has the capacity to directly attach to the host cells, has also the ability to coaggregate with many endodontic pathogens including *P. gingivalis*, *Prevotella nigrescens*, *Prevotella intermedia*, *Peptostreptococcus micros*, to name just a few (15, 18, 34). Therefore, *F. nucleatum* might first act as the primary colonizer of the host tissues; then it might serve as an anchor that allows the other endodontic pathogens to successfully colonize the same site, even if they lack the capacity to do so alone. Potentially, another layer(s) of *F. nucleatum* might adhere to the secondary colonizers to allow more bacteria, which have the capacity to coaggregate with it, to join in. This process might enhance the perpetuating presence of several bacterial strains in the infected site, resulting in the complex biofilm structure found by Nair et al. (1) in the root canal and by Noguchi et al (3) in the extraradicular evironment. Further events might then occur within this complex mixed bacterial community to enhance its development (35) and its resistance to antimicrobial agents (34).

The results of the present study suggest that bacterial adhesins, which mediate bacterial coaggregation, might have a role not only in dental plaque formation but also in the colonization of other host tissues, thus forming complex microbial communities such as those found in the root canal or the periapex. Additional in vivo studies will be required to elucidate the role of these essential interactions between the bacteria and the host in the establishment of mixed infections. Understanding such basic mechanisms of mixed biofilm development might allow designing pharmaceutical strategies for prevention of their formation by using galactose-like inhibitors.

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