Synergistic Pathogenicity of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in the Mouse Subcutaneous Chamber Model

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Abstract

Porphyromonas gingivalis and *Fusobacterium nucleatum* are often coisolated from sites of infection, such as suppurative apical periodontitis. The synergistic pathogenicity of mixed infection of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594 was studied in the mouse subcutaneous chamber model in groups of seven animals. The minimal dose for *P. gingivalis* HG 405 that was required to infect 100% of the chambers was reduced by 1,000-fold when animals were inoculated in the same chamber with 1×10^9 *F. nucleatum* PK 1594 ($p < 0.001$). To benefit from the presence of the fusobacteria, *P. gingivalis* HG 405 had to be coinoculated; inoculation in separate chambers for the same animal had no such effect ($p < 0.001$). Subinfective *F. nucleatum* inocula also benefited from the association with *P. gingivalis* HG 405 and uniformly established an infection when this partner was present ($p < 0.001$). These results suggest that the frequent and natural coexistence of *P. gingivalis* and *F. nucleatum* in diseased sites may express such a synergism in successful establishment and survival of small inocula. *(J Endod 2009;35:86–94)*

Key Words

Fusobacterium nucleatum, mixed infection, pathogenicity, *Porphyromonas gingivalis*

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Viable extraradicular bacteria are commonly isolated from chronic suppurative apical periodontitis and abscesses. This occurs despite the continuous major influx of polymorphonuclear leukocytes (PMNs) to the area. Survival in such a hostile environment should be less favorable than and different from bacterial survival in the sanctuary of a necrotic root canal. Mechanisms that allow bacterial evasion of phagocytosis are most likely necessary for bacteria to either survive in this niche as a true extraradicular infection (1–4) or to simply survive extraradicularly for a period long enough to be sampled as viable bacteria (5); *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are among the most frequently isolated bacterial species from both chronic suppurative apical periodontitis and abscesses of dental origin (6–9, 10). They are commonly encountered together in mixed infections associated with these conditions.

The common coisolation of *P. gingivalis* and *F. nucleatum* from diseased sites may be caused by a simple co-occurrence or it may indicate that the two bacterial strains interact in the hostile host environment.

Pathogenic synergism between *P. gingivalis* and *F. nucleatum* in animal models was previously reported by Sundqvist et al. (10), Baumgartner et al. (11), Feuille et al. (12), and Ebersole et al. (13). All these studies indicated that there was enhanced pathogenicity of mixed inocula of these bacterial strains in comparison to singular inoculation.

The realities of these clinical observations and experimental results suggest that interactions between *P. gingivalis* and *F. nucleatum* could have a major influence on both survival and pathogenicity. We chose the mouse subcutaneous chamber model (14, 15) to study the potential pathogenic synergism between these bacteria. Unlike previous studies that examined the infectious course of mixed infections in the mouse (11–13, 16, 17), this model allows for the generation of detailed quantitative and kinetic analyses of the bacteria-bacteria and bacteria-host interactions that occur in the site.

Previous studies using this model thus far have focused on host reaction to various strains of *P. gingivalis* (14, 15, 18). Three strain-dependent potential outcomes were defined from a local (intrachamber) infectious challenge: (1) rapid dissemination of bacteria from the chamber, resulting in the spread of systemic infection and death within 48 hours; (2) establishment of a local infection that does not disseminate but leads to perichamber abscess formation within a time period that is strain dependent; and (3) rapid clearance of the bacterial challenge, without any evidence of either systemic involvement or local inflammation.

The current study was designed to evaluate the outcome of a monoinfectious challenge with various strains of *F. nucleatum* and to determine the influence of a mixed infectious challenge with *F. nucleatum* on the infectious potential of a subinfectious challenge with *P. gingivalis* strain HG 405. The later was compared with monoinfection with either strain.

Methods

Bacterial Strains and Growth Conditions

P. gingivalis strain HG 405 and *F. nucleatum* strains PK 1594, VPI 10197, and ATCC 10953 were used in this study. *P. gingivalis* HG 405 has previously been studied in the mouse subcutaneous chamber model (14, 15, 18). *F. nucleatum* PK 1594 has

been shown by Kolenbrander and Andersen (19) to coaggregate with *P. gingivalis* PK 1924 by a lactose- and galactose-inhibitable mechanism. We have recently shown that its coaggregation with *P. gingivalis* HG 405 was also inhibited by the same saccharides, whereas two other *F. nucleatum* strains (VPI 10197 and ATCC 10359) failed to coaggregate with this particular strain of *P. gingivalis* (20).

Recently, Weiss et al. (21) have also shown that *F. nucleatum* PK 1594 adheres to mammalian cells through the same adhesin. *F. nucleatum* VPI 10197 was used by Baumgartner et al. (11) in a mixed infection mouse model. Both *F. nucleatum* and *P. gingivalis* strains were anaerobically grown in Wilkins-Chalgren anaerobic broth (WC; Oxoid Ltd, Basingstoke, Hampshire, England) or on Wilkins-Chalgren anaerobic agar plates in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2, at 37°C (flexible film anaerobic chamber; Coy Laboratory Products Inc, Grass Lake, MI). Bacterial strains were maintained as frozen stocks and after being started were passed three times through this growth medium before being used in the experiments. The cells were harvested from an overnight culture at a late exponential stage of growth.

Animals and Subcutaneous Chambers

Female BALB/c mice (Charles River Laboratory, Wilmington, MA) that were 8 weeks old at the start of the study were used for these experiments in groups of seven animals. The Animal Care Committee of the University of North Carolina at Chapel Hill approved the experimental protocol, and the animals were treated according to these standards during the experiments. Subcutaneous chambers, made using a coil of surgical stainless steel wire, were surgically implanted on the flank of the animal through a midline incision on the back (14). The animals were allowed to heal for 14 days before inoculation of the chambers with bacteria. In dual-chamber experiments, two chambers were implanted in each animal (one on the right and one on the left side of the animal).

On day 15 of the study, the lumen of the sealed chamber was inoculated with bacteria by injection with a 25-G needle through the 70% ethanol-disinfected skin covering one of its ends. The inoculum consisted of bacteria from an overnight growth that were washed once and resuspended in fresh culture medium. The inocula contained either a single strain or a mixture of two strains in a total volume of 0.1 mL, which contained 1×10^6 to 2×10^9 bacteria. Bacterial viability was >98% as determined by a fluorochrome (propidium iodide) exclusion assay (22). Sterilized 0.1 mL of the same culture medium was used to sham inject the control chambers. The experiments were each conducted in groups of seven animals.

Experimental Design

In the first set of experiments, subcutaneous chambers were inoculated with *F. nucleatum* strains PK 1594, VPI 10197, and ATCC 10953, and the course of infection was monitored microbiologically and clinically. In a second set of experiments, chambers were inoculated with *P. gingivalis* HG 405, as a monoinfection, and the course of infection was also monitored.

In the following experiments, the minimal infective dose required to establish infection by 100% of the inocula (MID_{100}) and subinfective dose (see later) were determined for each strain. Mixed inocula of *P. gingivalis* HG 405 with each of the *F. nucleatum* strains were then tested, either at the MID_{100} or subinfective dose, and the course of infection was monitored microbiologically and clinically.

To test for systemic versus local synergistic effects, dual chambers were used, as detailed later. To test for a potential role of coaggregation between the strains in the outcome of a mixed infection of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594, galactose was added to the mixed inoculum at a final concentration of 60 mmol/L.

Macroscopic Evaluation of the Chambers

The chambers were inspected daily for macroscopic signs of tissue reaction or abscess development. Each chamber was defined daily as one of the following conditions: (1) no detectable change, (2) having a palpable thickening of the connective tissue associated with its coils, (3) swelling of the tissue surrounding the chamber, (4) initial formation of a suppurative abscess, or (5) perforation of the skin above the chamber that exposes the coil (exfoliation). The inspection was followed up to 40 days.

To ensure that the sampling procedure (described later) did not alter the outcome of the infection, chamber content samplings were performed in only four of the seven animals for each group, whereas the chambers of the remaining animals were maintained as unsampled controls.

Selective Media

To allow a quantitative study of each of the constituent bacteria of this mixed infection, selective media were required. Kanamycin was used as an inhibitor of *F. nucleatum* PK 1594, whereas vancomycin (Sigma, St Louis, MI) was used as an inhibitor of *P. gingivalis* HG-405 growth (23). Kanamycin (100 μg/mL) was added to the WC agar to form a medium selective for *P. gingivalis* HG 405, whereas vancomycin (10 μg/mL) was used to prepare a medium selective for *F. nucleatum* PK 1594.

Bacteria in the Chamber Fluid

A 25-G needle was used to aspirate 10 μL of chamber content on days 1, 6, 9, 15, and 21. The chamber content was serially diluted in 0.15 mol/L NaCl containing 0.06 mol/L galactose and inoculated on both prereduced WC agar plates and prereduced WC agar plates containing kanamycin. Each inoculum consisted of a 10-μL drop of a 1:20, $1:2 \times 10^3$, $1:2 \times 10^5$, or $1: 2 \times 10^7$ dilution of the chamber fluid. This allowed for the differential detection and quantification of colony-forming units (CFU) in the range of 2×10^3 (one colony in the first drop) through $\sim 2 \times 10^{10}$ ($\sim$100 colonies in the last drop) per mL of chamber fluid.

The WC plates were incubated anaerobically for 48 hours to obtain *F. nucleatum* colony counts, whereas the WC plates containing kanamycin were incubated for 96 hours to obtain *P. gingivalis* colony counts. The colonies of each strain were counted at a 50× magnification (at a dilution of <100 CFU per spot), and the number of CFU per volume of chamber fluid was calculated.

Microscopy of Host Cells and Bacteria in the Chamber Fluid

Morphologic evaluation of the bacterial content of the chamber fluid was done on Gram-stained smears of a1:20 diluted sample. Additionally, cytocentrifuge (Cytospin 2; Shandon Southern Products Ltd, Astmore, Cheshire, UK) preparations of chamber fluid stained with Wright stain were used to evaluate the nature of the host cells in the fluid and their relation with the bacteria.

Statistical Analysis

Bacterial recovery from subcutaneous chambers was performed in quadruplicates. A Student *t* test was used to compare the groups when indicated, and p values smaller than 0.05 were considered significant.

Results

The Effect of the Sampling Procedure

The sampling procedure had no discernable effect on the outcome because there was no significant difference in the pathologic course after infectious challenge between the chambers sampled and those not sampled in the same group.

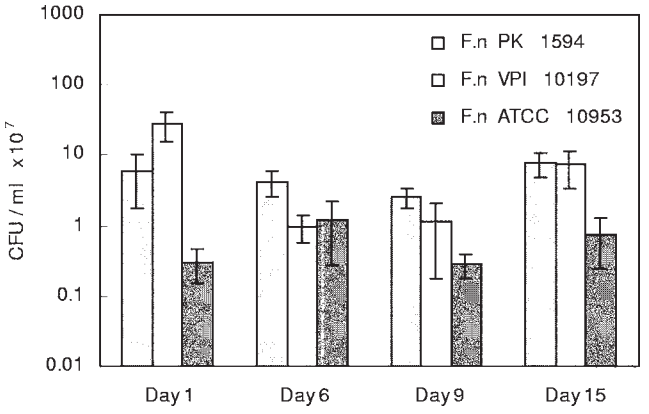

Figure 1. Recovery of three strains of *Fusobacterium nucleatum* inoculated in subcutaneous chambers as pure cultures. Each inoculum consisted of 1×10^9 viable bacteria. Bars represent the mean CFU/mL of four chambers ± standard error of the mean. F.n, *F. nucleatum*.

Monoinfection with Different Strains of *F. nucleatums*

F. nucleatum PK 1594 inocula of 1×10^9 viable bacteria successfully colonized 100% of the chambers. Viable bacteria could be detected in the chamber on day 1 (6×10^7 CFU/mL) (Fig. 1), and their numbers remained high throughout day 15 (8×10^7 CFU/mL). Similarly, *F. nucleatum* strains VPI 10197 and ATCC 10953 injected at 1×10^9 bacteria per inoculum successfully established an infection in all animals and could be recovered from the chambers through day 15 (Fig. 1).

Macroscopic examination of the chambers revealed that thickening of the connective tissue around the chamber started only on day 17 for the PK 1594 containing chambers and on day 20 for those containing *F. nucleatum* ATCC 10953. No clinical changes were detected in chambers infected with *F. nucleatum* VPI 10197 through the end of the observation period. Swelling around the chamber appeared on days 20 and 21 for the PK 1594–containing chambers and on day 22 for those containing ATCC 10953. Draining abscess formation and exfoliation could not be seen until day 28 in chambers infected with PK 1594. By day 35, 57% of the chambers containing PK 1594 formed a draining abscess (Fig. 2). The other 43% of these chambers did not change

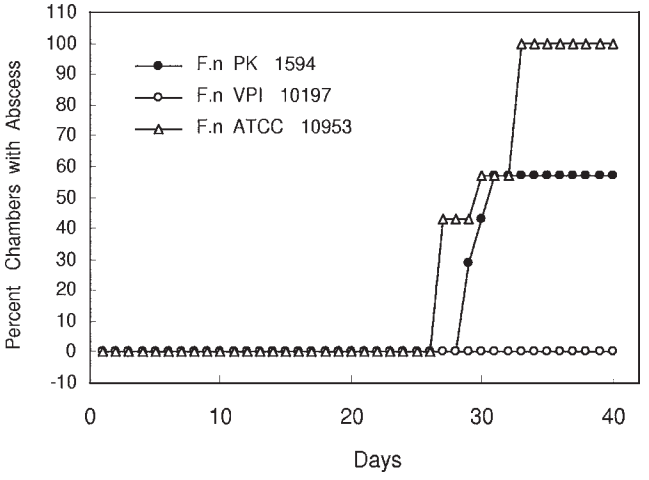

Figure 2. Abscess development in subcutaneous chambers inoculated with pure cultures of F. nucleatum. Each inoculum consisted of 1×10^9 viable bacteria. Each point represents the percent of chambers in which a draining abscess was observed. Groups consisted of seven animals.

TABLE 1. Macroscopic Evaluation of the Outcome of an Infectious Challenge of the Subcutaneous Chamber with *P. gingivalis* HG 405

P. gingivalis HG 405 Inoculum	Outcome	Time	Percent of Chambers
1×10^9	Draining abscess	12–14 days	100%
3×10^8	Draining abscess	12–14 days	60%
	Infection cleared	—	40%
1×10^8	Infection cleared	—	100%

Each group was of 7 animals.

through day 40, when the experiment was terminated. Among the chambers inoculated with VPI 10197, none developed an abscess within the 40-day period, whereas 43% of those with ATCC 10953 developed an abscess by day 27 and 100% were exfoliated by day 33 (Fig. 2).

The pattern of swelling and abscess development with *F. nucleatum* PK 1594 and ATCC 10953 was different than that of *P. gingivalis* HG 405 containing abscesses (see later). In the *P. gingivalis*–monoinfected chambers, a draining abscess appeared within 2 to 3 days from the first appearance of a moderate swelling (on day 9–10), whereas up to 6 to 7 days passed between this first event and the latter in chambers containing only fusobacteria. The content of these abscesses was also markedly different from that of the *P. gingivalis*–containing abscesses, characterized with a high viscosity and bright color as opposed to the yellow-gray liquid pus draining from the *P. gingivalis* HG 405 abscesses (with or without *F. nucleatum*).

Gram-stained preparations of the chamber content revealed great numbers of gram-negative bacteria, the morphology of which was consistent with that of the *F. nucleatum* strains used in each group (which differed from strain to strain). No other bacterial morphotypes could be seen in any of these preparations. Microscopic examination of the Wright-stained cytocentrifuge preparations revealed great numbers of PMNs in either proper morphology or morphology indicative of deterioration. Mononuclear phagocytes were also occasionally observed.

Monoinfection with *P. gingivalis* HG 405

In the second set of experiments, subcutaneous chambers were inoculated with *P. gingivalis* HG 405, and the course of infection was monitored microbiologically and clinically. Inoculation of the chamber with 1×10^9 viable *P. gingivalis* HG 405 resulted in an established infection that led to perichamber abscess formation in 100% of the challenged chambers and to exfoliation of all the chambers between day 12 and 14 (Table 1).

Inocula of 3×10^8 bacteria resulted in abscess formation and exfoliation on days 12 to 14 in 60% of the chambers. On the other hand, inocula of 1×10^8 viable *P. gingivalis* HG 405 uniformly failed to establish an infection; the bacteria could be detected in the chamber fluid on day 1, but no viable bacteria could be detected in any of the chamber fluid samples on day 6 ($p < 0.001$) (Fig. 3*B*). These chambers did not develop any significant thickening of the surrounding connective tissue and failed to develop an abscess or exfoliation. In fact, these chambers were macroscopically indistinguishable from the control chambers of sham-injected animals with sterile culture medium. This dose of 1×10^8 CFU *P. gingivalis* HG 405 was therefore defined as a "subinfective dose" and used as such in the following experiments.

Mixed Infection with *P. gingivalis* HG 405 and Different Strains of *F. nucleatum*

In the third set of experiments, subcutaneous chambers were inoculated with mixed inocula consisting of *P. gingivalis* HG 405 and either *F. nucleatum* PK 1594, *F. nucleatum* VPI 10197, or *F. nucleatum* ATCC 10953. The course of infection was microbiologically and

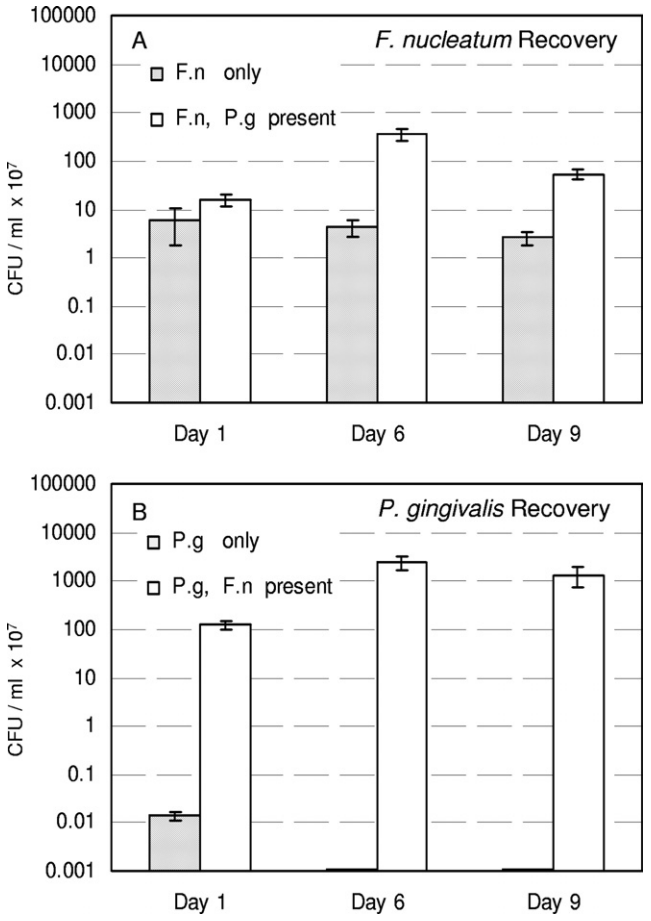

Figure 3. Viable bacteria recovery from subcutaneous chambers inoculated with mixed infection of *P. gingivalis* HG 405 with *F. nucleatum* PK 1594. The initial inoculum consisted of either 1×10^9 *F. nucleatum*, 1×10^8 *P. gingivalis*, or a mixture of the strains. (*A*) *F. nucleatum* (F.n) recovery from chamber fluid, with or without *P. gingivalis* presence. *F. nucleatum* recovery was significantly higher on days 6 and 9 when *P. gingivalis* was present ($p < 0.01$). (*B*) *P. gingivalis* (P.g) recovery from chamber fluid, with and without *F. nucleatum* presence. *P. gingivalis* recovery on days 6 and 9 was significanly higher when *F. nucleatum* was present ($p < 0.001$). Bars represent the mean CFU/mL of four chambers ± standard error of the mean.

clinically monitored. Quantitative microbiology of these mixed infections required development of selective media for *P. gingivalis* and *F. nucleatum*.

Selective Media

When samples from mixed inocula were grown on a regular WC agar, the two types of colonies were readily distinguishable under 50× magnification. Nevertheless, the faster generation time of the *F. nucleatum* strain resulted in overgrowth of the *P. gingivalis* HG 405, preventing its enumeration on a nonselective medium. Therefore, it was necessary to use the selective medium that prevented *F. nucleatum* growth. However, this differential growth rate rendered the use of the vancomycin-containing medium unnecessary in further experiments with these strains. The *F. nucleatum* PK 1594 colonies, growing on a regular WC agar, were already fully developed after 48 hours of incubation. At this time, the *P. gingivalis* HG 405 colonies were still very small and hardly detectable; therefore, these colonies did not interfere in any way in the counting of the *F. nucleatum* colonies. Therefore, the *Fusobacterium* colonies were counted on regular WC agar plates at 48 hours in all further mixed infection experiments, whereas the *P. gingivalis* colonies were counted on WC agar plates containing kanamycin at 96 hours.

Mixed Inocula of 1×10^9 *P. gingivalis* HG 405 with 1×10^9 *F. nucleatum* PK 1594

The addition of 1×10^9 *F. nucleatum* PK 1594 to the chambers receiving 1×10^9 *P. gingivalis* HG 405 dramatically changed the outcome of the infection. Rather than forming an abscess with exfoliation of the chamber on days 12 to 14, all chambers containing the mixed infection developed an abscess and exfoliated on days 4 or 5 ($p < 0.01$) (Table 2).

Gram-stained preparations of the chamber content revealed high numbers of cells with mixed morphology gram-negative bacteria, which is consistent with characterizations of *F. nucleatum* PK 1594 and *P. gingivalis* HG 405. No other bacteria could be seen in any of these preparations. Microscopic examination of the cytocentrifuge preparations revealed great numbers of PMNs in either proper morphology or morphology indicative of deterioration. Mononuclear phagocytes were also occasionally observed.

Mixed Inocula of a Subinfective Dose of *P. gingivalis* HG 405 with 1×10^9 *F. nucleatum* PK 1594

The presence of 1×10^9 *F. nucleatum* PK 1594 in the inoculum uniformly changed the outcome of the inoculation with a subinfective monoculture dose (1×10^8) of *P. gingivalis* HG 405. Rather than clearing of the *P. gingivalis* infection by the host, these bacteria successfully colonized the chambers. The porphyromonads could be detected in the chamber fluid on day 1 at much higher densities when the fusobacteria were present than when inoculated alone, at 1.3×10^9 CFU/mL, as compared with 1.4×10^5 when injected without the fusobacteria ($p < 0.001$) (Fig. 3*B*). The cell number of *P. gingivalis* viable bacteria gradually increased, and 2.4×10^{10} CFU/mL could be detected by day 6 as compared with none in the control ($p < 0.0001$). Although the porphyromonads were never recovered again when singularly inoculated, their numbers in the mixed infection chamber remained high (1.4×10^{10} CFU/mL) on day 9. Swelling of the tissues surrounding the

TABLE 2. Macroscopic Evaluation of the Outcome of an Infectious Challenge with Mixed Infection of *F. nucleatum* PK 1594 and *P. gingivalis* HG 405

Bacterial Strain	Inoculum	Outcome	Time	Percent of Animals
P. gingivalis HG 405	1×10^8	Draining abscess	5–6 days	100%
+				
F. nucleatum PK 1594	1×10^8			
P. gingivalis HG 405	1×10^8	Draining abscess	12–14 days	100%
+				
F. nucleatum PK 1594	1×10^8			
P. gingivalis HG 405	1×10^8	Infection cleared	—	100%
F. nucleatum PK 1594	1×10^8	Infection persists	>25 days	100%

Each group was of 7 animals.

chamber was evident on day 9, followed by abscess formation and exfoliation of 100% of the chambers on days 10 to 12 (Table 2).

The fusobacteria also benefited from the presence of their partner and were recovered on day 1 in numbers that were 2.6× times higher than when injected alone ($p < 0.01$). Cell numbers were 50× and 18× times higher on days 6 and 9, respectively, than in a pure *F. nucleatum* inoculum of the same size ($p < 0.0001$) (Fig. 3*A*).

Mixed Inocula of a Subinfective Dose of *P. gingivalis* HG 405 with 1×10^9 *F. nucleatum* VPI 10197

The presence of 1×10^9 *F. nucleatum* VPI 10197 in the inoculum had a macroscopic outcome that clearly differed from that of PK 1594. No draining abscess could be detected in any of these chambers up to day 20 (Fig. 4*A*). Nevertheless, 70% of the chambers did develop a draining abscess and were exfoliated on day 21, whereas the other 30% failed to develop an abscess up until day 40 when the experiment was terminated (Fig. 4*A*). When these fusobacteria were inoculated with *P. gingivalis* HG 405, the outcome of the infection was different than that of each bacteria singularly. However, we could not enumerate the *P. gingivalis* HG 405 colonies because this strain of *F. nucleatum* was resistant to the kanamycin used in the selective medium and the overgrowth of the fusobacteria prevented a reliable counting of the *P. gingivalis* (even though *P. gingivalis* was clearly present).

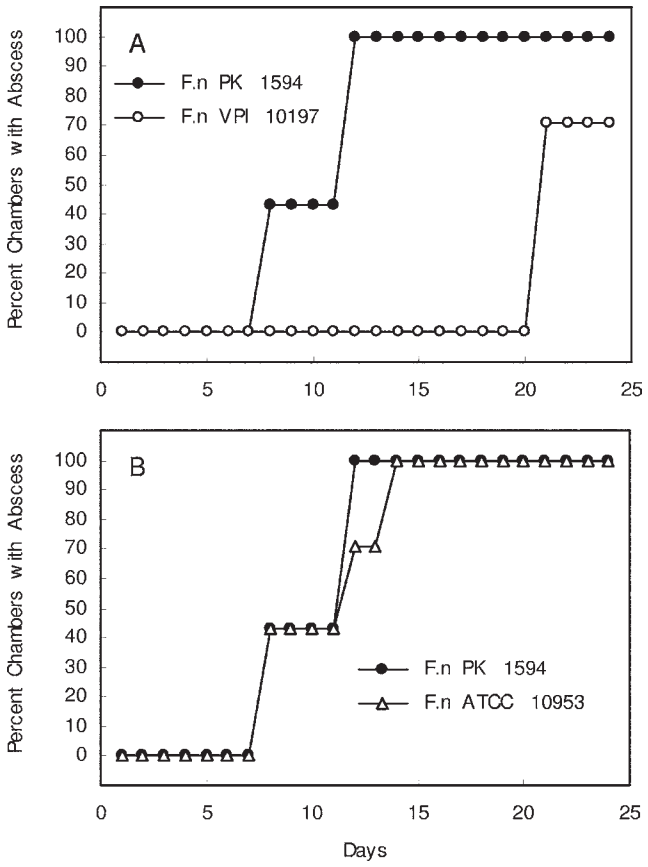

Figure 4. Abscess development in subcutaneous chambers inoculated with a mixed inoculum of *P. gingivalis* HG 405 and *F. nucleatum*. (*A*) *F. nucleatum* (F.n) PK 1594 compared to VPI 10197. (*B*) *F. nucleatum* PK1594 compared with ATCC 10953. Each inoculum consisted of 1×10^8 viable *P. gingivalis* HG 405 bacteria combined with 1×10^9 bacteria of one of the *F. nucleatum* strains. Each point represents the percent of chambers in which a draining abscess was observed. Each group consisted of seven animals.

Mixed Inocula of a Subinfective Dose of *P. gingivalis* HG 405 with 1×10^9 *F. nucleatum* ATCC 10953

The presence of 1×10^9 *F. nucleatum* ATCC 10953 in the inoculum had a macroscopic outcome similar to that of PK 1594 (Fig. 4*B*). All the chambers containing *P. gingivalis* HG 405 with *F. nucleatum* ATCC 10953 developed a draining abscess and were exfoliated by day 11. Both bacteria could be recovered from the fluid of these chambers. The density of *P. gingivalis* was 2.53×10^9 CFU per mL on day 1 and 1.85×10^{10} and 5.3×10^{10} on days 6 and 9, respectively. The fusobacterial content of these chambers was 2.4×10^8 CFU per mL on day 1 and 2.6×10^9 and 6.3×10^9 CFU on days 6 and 9, respectively.

Similarly to a mixed infection of *P. gingivalis* HG-405 with *F. nucleatum* PK 1594, the subinfective dose of the Porphyromonas successfully established an infection in the presence of the fusobacteria, leading to the macroscopic outcome previously described. The fusobacteria also benefited from this partnership and reached numbers that were 10× times higher on day 1 and 6× and 9× times higher on days 6 and 9 compared with the number of these bacteria when injected alone.

The Minimal Infective Dose of *F. nucleatum* PK 1594

When inoculated alone, *F. nucleatum* PK1594 could establish an infection with inocula as small as 1×10^7 bacteria. These allowed for an average recovery of 0.5×10^3 CFU/mL chamber fluid on day 1, which gradually increased to an average of 6.8×10^6 /mL by day 6. Smaller inocula of 1×10^6 bacteria failed to establish themselves, and no viable bacteria could be recovered from the chamber on day 1 ($p < 0.001$) (data not presented) or day 6 (Fig.6). Thus, they were defined as a subinfective dose.

A Subinfective Dose of *F. nucleatum* PK 1594 with a Subinfective Dose of *P. gingivalis* HG 405

Coinoculation of subinfective doses of *F. nucleatum* PK1594 and *P. gingivalis* HG 405 allowed each species to colonize the chamber. When *P. gingivalis* was coinoculated at the subinfective dose of 1×10^8 viable bacteria, even *F. nucleatum* inocula of 1×10^6 viable bacteria resulted in an established infection (Fig. 5*A*). The latter were recovered at a density of 2.5×10^6 CFU/mL by day 1 and 6.7×10^8 CFU/mL by day 6, as opposed to the lack of viable bacteria on any of these days when inoculated alone ($p < 0.001$) (Fig. 6). On day 1, the recovery of *P. gingivalis* that was inoculated at a subinfective dose was dependent on the amount of *F. nucleatum* cells that were coinoculated (Fig. 5*B*). These cells rapidly multiplied and reached similarly high amounts after 6 days, regardless of the density of *F. nucleatum* inoculum that assisted them to survive on the first day (Fig. 5*B*).

Minimal Infective Dose of *P. gingivalis* in the Presence of *F. nucleatum* PK 1594

Coinoculation with *F. nucleatum* PK 1594 permitted a 1,000-fold reduction in the MID_{100} of *P. gingivalis* HG 405 ($p < 0.001$) (Fig. 6). Inocula of 1×10^8 CFU or less of *P. gingivalis* alone were uniformly cleared by day 6. When coinoculated with 1×10^9 CFU of *F. nucleatum* PK 1594, *P. gingivalis* HG 405 was recovered by day 6 at high numbers even from chambers inoculated with only 1×10^6 CFU of this bacterium; abscess developed accordingly in all of them.

Determination of Local or Systemic Synergistic Effect

Dual chambers were used to test whether the synergistic pathogenicity of *F. nucleatum* PK 1594 with *P. gingivalis* HG 405 could be attributed to systemic or local effects. When a subinfective dose of 1×10^8 CFU of *P. gingivalis* HG 405 was inoculated in the same chamber

with 1×10^9 CFU of *F. nucleatum* PK 1594, *P. gingivalis* was recovered by day 6 from all chambers at a density 2.4×10^{10} CFU/mL and abscesses developed in each of them. Furthermore, when each of these bacteria were inoculated in a separate, contralateral chamber in the same animal, no synergistic effect was observed and no *P. gingivalis* could be recovered by day 6 ($p < 0.001$) (Fig. 7).

The Effect of Galactose on Mixed Infection of *P. gingivalis* HG 405 and *F. nucleatum* PK 1594

Coaggregation of *F. nucleatum* PK 1594 with *P. gingivalis* HG 405 is mediated by a galactose-inhibitable adhesin (20). It has been proposed that coaggregation might provide an advantage to one or both of the partners. To test whether this coaggregating property might make a contribution to the observed synergistic pathogenicity observed with these two strains, galactose was added at the time of infectious challenge at a concentration that inhibits this coaggregation in vitro (0.06 mol/L) (20). A mixed inoculum of 1×10^9 CFU of *F. nucleatum* PK 1594 with 1×10^6 CFU of *P. gingivalis* HG 405 had an altered clinical outcome when it contained 0.06 mol/L galactose (Fig. 8). In the galactose group, the first chamber presented an abscess only by day 10 as opposed to day 8 in the control group with no galactose, showing that abscess appearance was delayed by galactose presence. By days 10 and 11, 86% of the

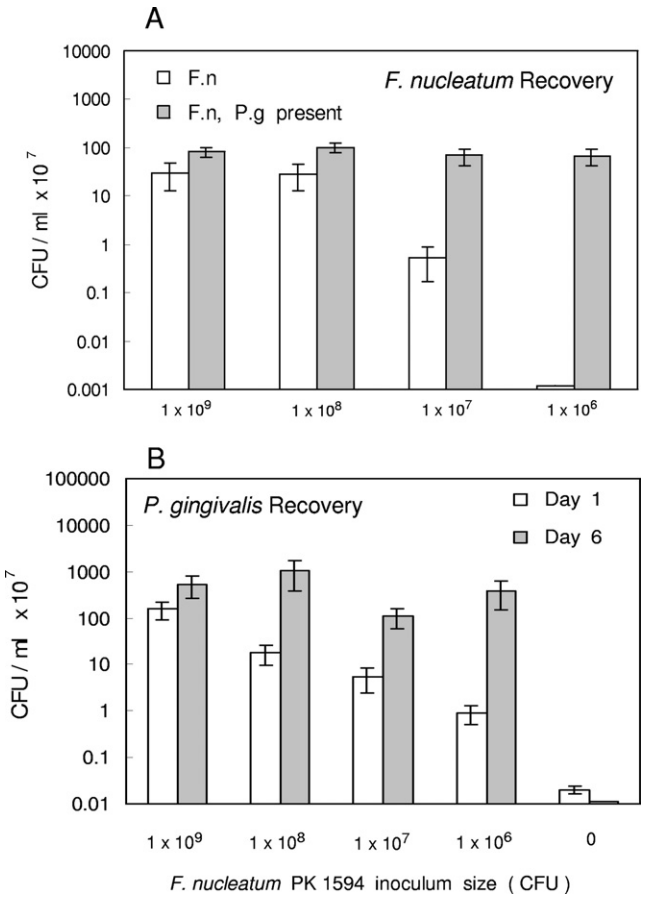

Figure 5. *F. nucleatum* PK 1594 and *P. gingivalis* HG 405 recovery from chambers inoculated by mixed inocoula. (*A*) *F. nucleatum* recovery on day 6 from chambers coinoculated with subinfective dose of *P. gingivalis* HG 405. *F. nucleatum* (F.n) PK 1594 was either inoculated alone or coinoculated with a subinfective dose of *P. gingivalis* (P.g) HG 405 (1×10^8 CFU). A *F. nucleatum* inoculum of 1×10^6 CFU, which was defined as subinfective when injected alone, could become established when *P. gingivalis* was present ($p < 0.001$). Each group consisted of four chambers. Mean viable bacterial recovery from chamber fluid (± standard error of the mean). (*B*) *P. gingivalis* HG 405 recovery from chambers coinoculated with diminishing doses of *F. nucleatum* PK1594. *P. gingivalis* recovery on day 1 and day 6 from chambers in which a subinfective dose of *P. gingivalis* HG 405 (1×10^8) was coinoculated with diminishing doses of *F. nucleatum* PK 1594. A subinfective dose of *F. nucleatum* (1×10^6 CFU) allowed for the establishment of a subinfective dose of *P. gingivalis* ($p < 0.001$). Each group consisted of four chambers. Mean viable bacterial recovery from chamber fluid (± standard error of the mean).

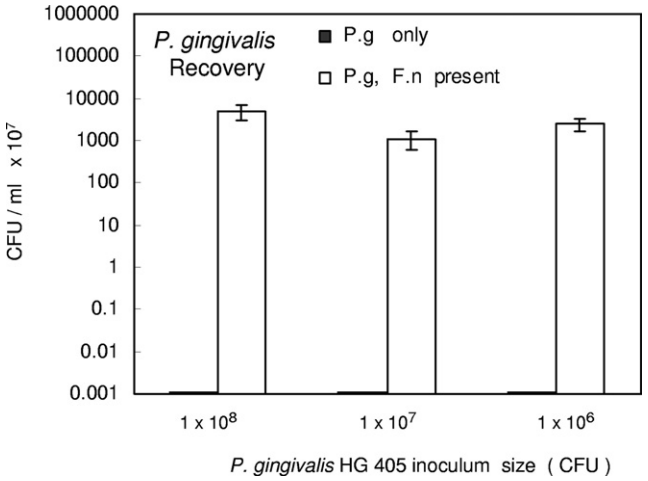

Figure 6. *P. gingivalis* HG 405 recovery from chambers coinoculated with *F. nucleatum* PK1594 and diminishing doses of *P. gingivalis*. *P. gingivalis* (P.g) recovery on day 6 from chambers in which *F. nucleatum* (F.n) PK 1594 (1×10^9 CFU) was coinoculated with diminishing doses of *P. gingivalis* HG 405. Even at a 1,000-fold reduction from MID_{100} (1×10^9 CFU), *P. gingivalis* became established when *F. nucleatum* PK 1594 was present ($p < 0.001$). Each group consisted of 4 chambers. Mean viable bacterial recovery from chamber fluid (± standard error of the mean).

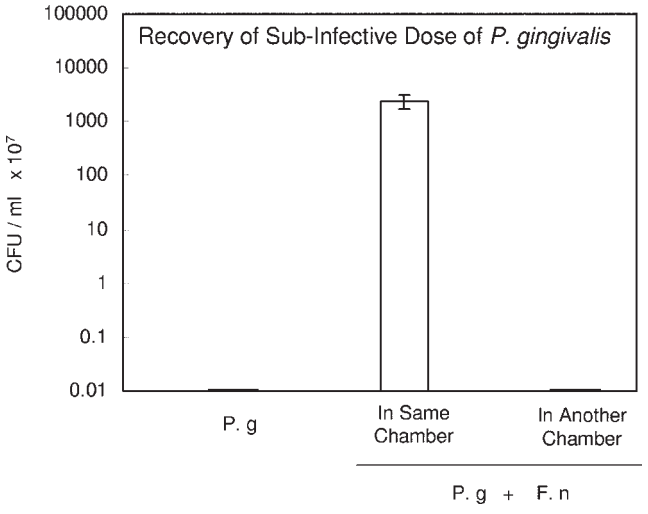

Figure 7. *P. gingivalis* HG 405 recovery from chambers coinoculated with *F. nucleatum* PK 1594 compared with the inoculation of *the F. nucleatum* in another chamber in the same animal utilizing dual chambers. *P. gingivalis* recovery on day 6 from chambers inoculated with a subinfective dose (1×10^8 CFU) of *P. gingivalis* HG 405. It was inoculated alone or with *F. nucleatum* PK1594 (1×10^9 CFU) that was either coinoculated in the same chamber with the *P. gingivalis* or inoculated in another contralateral chamber in the same animal ($p < 0.001$). Each group consisted of four chambers. Mean viable bacterial recovery from chamber fluid (± standard error of the mean).

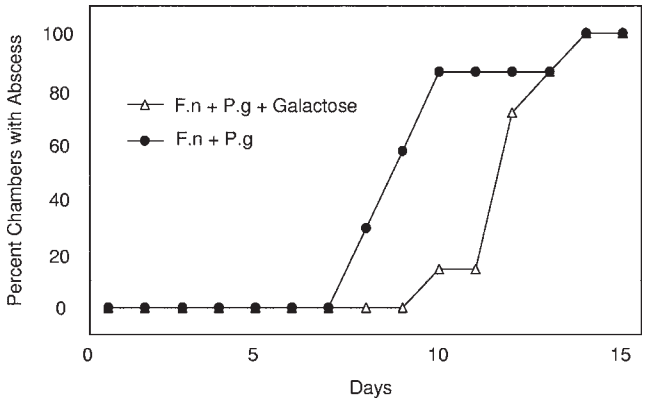

Figure 8. The effect of galactose on the pathologic outcome of a mixed infection of *F. nucleatum* PK 1594 and *P. gingivalis* HG 405. A mixed inoculum of *F. nucleatum* PK 1594 (1×10^9 CFU) and *P. gingivalis* HG 405 (1×10^6 CFU, 1,000-fold smaller than the MID_{100}) was injected with galactose (0.06 mol/L). Chambers with the same inocula, but no galactose, served as the control. The difference between the groups on days 9 to 11 was significant ($p < 0.01$). Each point represents the percent of chambers in which a draining abscess was observed. Each group consisted of seven animals.

chambers in the control group contained an abscess, whereas only 14% of the chambers reached this state in the galactose group ($p < 0.01$). The difference between the groups gradually diminished, and no difference was found between chambers that initially contained galactose and those that served as control by day 13.

Discussion

The selective kanamycin-WC agar used for growth of *P. gingivalis* was a key factor in our ability to obtain quantitative data regarding each of the bacteria studied in a mixed infection. The in vitro dilutions of the collected samples were done in saline containing 0.06 mol/L galactose. This was done to reduce the possibility that aggregate formation between the *P. gingivalis* HG-405 and *F. nucleatum* PK 1594 may artificially lower the number of the colonies. In a recent study (20), we established that even though these two strains do coaggregate, this concentration of galactose was sufficient to completely disperse the aggregates. These results were similar to those reported by Kolenbrander and Andersen (19) for other *P. gingivalis* strains with this same *F. nucleatum* strain. Galactose at this concentration had no effect on the viability of any of the strains investigated in this study (data not presented).

To reproducibly establish an infection of *P. gingivalis* HG 405 in the mouse, 1×10^9 viable bacteria were used for inoculation of the subcutaneous chamber. These numbers were 100 to 1,000-fold lower than those required for a simple subcutaneously injected inoculum to become established when no chamber is used (14, 16, 18). Therefore, the chamber environment allows unique conditions for bacterial establishment. Nevertheless, we have found that an inoculum of 1×10^8 CFU failed to establish infection, and the bacterial challenge was uniformly cleared. The crucial events for elimination most likely occurred within 24 hours of infection challenge, as shown in Figure 7. The chamber environment thus seems to provide the bacteria with a relatively protected site of colonization, but the cells are not completely protected from the host response.

F. nucleatum, injected at a dose of 1×10^9 viable bacteria, uniformly established an infection. This finding differs from previously published results of fusobacteria, which indicated that additives such as gastric mucin or agarose were required for a uniform establishment of an abscess to form with equivalent numbers of bacteria in nonchamber models (11, 24). Therefore, the subcutaneous chamber may serve as an efficient means for the establishment of infection with other sensitive bacteria such as *F. nucleatum*.

F. nucleatum does not seem to require the presence of *P. gingivalis* as a prerequisite for establishing an infection, at least when large inocula (1×10^9) are involved. This finding is in agreement with the studies of Feuille et al. (12) and Ebersole et al. (13, 17). These authors also reported that 1×10^9 CFU of *F. nucleatum* T18 were required to reproducibly establish infection when injected alone. Nevertheless, the bacterial counts of *F. nucleatum* PK 1594 were higher on days 6 and 9 when *P. gingivalis* HG-405 were present compared with the chambers inoculated with a pure *F. nucleatum* culture (Fig. 3*A*). These kind of data could not be generated without use of the chambers (as opposed to subcutaneous injection in other studies) (12, 13).

The presence of *F. nucleatum* PK 1594 in the chamber dramatically changed the outcome of the inoculation with the subinfective monoculture dose (1×10^8) of *P. gingivalis*. Rather than being cleared by the host, the small *P. gingivalis* inoculum became established, increased in numbers, and resulted in a final macroscopic pathologic outcome similar to that of the large does inoculum (1×10^9 CFU), namely the formation of a draining abscess by days 12 to 14. Furthermore, even *P. gingivalis* inocula as low as 1×10^6 CFU could establish an infection when the fusobacteria were present, thus reducing their MID_{100} by at least a 1,000-fold (from 1×10^9 to 1×10^6 CFU).

The induction of experimental subcutaneous *P. gingivalis* abscesses in animals using mildly pathogenic strains, such as HG 405, requires relatively large inocula of 1×10^{10} to 1×10^{11} viable bacteria when no chamber is used (14, 16, 18). For some porphyromonads, the presence of other bacteria in the inoculum has also been shown to allow their establishment or to reduce their minimal infective dose (10, 11, 25). Similar to our results, this response may partially explain their natural co-occurrence in mixed infections.

Sundqvist et al. (10) showed that certain strains of *Porphyromonas* (then termed *Bacteroides)* isolated from infected root canals could not establish infection and form a subcutaneous abscess in the guinea pig when singularly injected. However, when injected as a mixed inoculum with other bacteria, originally coisolated with this strain from the root canals, the cells successfully colonized the site of injection. *Fusobacterium spp.* was also found among those coisolated additional microorganisms.

In a study of the infectivity of bacteria isolated from human abscesses, Brook and Walker (25) also reported on strains that could not become established and form an abscess as a monoinfection in the mouse, but they were capable of these responses when injected as a mixed inoculum with synergistic strains. *F. nucleatum* strain VPI 10197 has been reported by Baumgartner et al. (11) to have a synergistic pathogenic effect with certain *P. gingivalis* as well as *Prevotella intermedia* strains. All of these synergistic effects were found to be strain specific. Similar results were more recently reported by Feuille et al. (12) and Ebersole et al. (13, 17). Common to all these previous studies was the evaluation of the synergistic effect by its pathologic outcome. To the best of our knowledge, our study is the first to quantitatively establish a synergistic effect on bacterial survival and growth in parallel to its effect on the macroscopic pathologic outcome of such mixed infection. Furthermore, bacteria were detected even when no macroscopic pathology was observed.

Several mechanisms have been proposed that may allow bacteria to survive in the hostile host environment. Some will allow only the specific bacterium harboring these mechanisms to survive, such as the antiphagocytic capsule. Others, like complement- and immunoglobulin-degrading enzymes, may benefit also a by-standing partner (26–30). Many of the pathogenic *P. gingivalis* strains possess such pro-

teases, which are critical for these organisms to evade phagocytosis (26). This unique ability may potentially explain why other bacteria may associate with these *P. gingivalis* strains in abscesses, but it was unclear if these strains have a reason to associate with partners, such as *F. nucleatum.*

Another potential mechanism might exist that allows the bacteria to benefit from coexistence in addition to the previously proposed synergistic pathways. Bacteria that successfully formed a local abscess in animal models have been reported by several investigators to be found in clumps or aggregates in the abscess cavity (11, 14, 18). This raises the possibility that clump formation may protect the bacteria within the aggregate from the host phagocytes, thus providing them with a microenvironment in which to survive and multiply to numbers that will overwhelm the host response (31). A recent extensive study by Khemaleelakul et al. (32) showed that autoaggregation and coaggregation are very common among bacterial strains isolated from acute endodontic infections, which provides further support for the mechanism described above.

Aggregates, such as those formed by the investigated strains, may provide protection against phagocytosis by forming a mass that is too large for the phagocytes. This may explain the effect of galactose on the outcome of mixed infection with the coaggregating strains used. Coaggregation with *F. nucleatum* PK 1594 could have provided a shelter for the low numbers (1×10^6 CFU) of *P. gingivalis* HG 405, allowing for their initial survival on day 1 (Fig. 8). The subsequent proliferation may have allowed them to eventually reach the large numbers that generated the abscess by days 8 to 10. When such aggregation was inhibited by the presence of galactose in the initial inoculum, the porphyromonads may have been more vulnerable, and only a few survived, resulting in a longer time (an additional 4 to 5 days) to reach the critical mass that could result in an abscess. Further studies to explore this potential avenue will be required before galactose-inhibition of coaggregation can be established as a potential mechanism that interferes with the synergism between these strains.

The observation that inocula containing just *F. nucleatum* became established in the host for a rather prolonged period of >24 days, before any macroscopic signs of an abscess appeared, raises the possibility of yet another potential mechanism for this synergism. These fusobacteria may harbor a mechanism to alter the host response to the extent of allowing their prolonged survival with no macroscopic evidence of inflammatory response. *P. gingivalis* coinoculated with the fusobacteria may also benefit from this potential effect. Such potential effect(s) on the host response could be of a systemic nature, as proposed by Feuille et al. (12) or may alternatively be local ones. Our finding that *F. nucleatum* must be in the same chamber with *P. gingivalis* to result in a synergistic effect (Fig. 7) indicates a mechanism(s) of local rather than systemic nature. Coaggregation may represent one such local mechanism, although the present study points to an additional direction.

F. nucleatum PK 1594 coaggregates with *P. gingivalis* HG 405, whereas strain VPI 10197 does not coaggregate (20). The effect of the *F. nucleatum* VPI 10197 strain on the outcome of a subinfective inoculum of *P. gingivalis* HG-405 was clearly different than that of *F. nucleatum* PK 1594 (Fig. 4). The development of the abscess lagged and did not occur until day 21 compared with day 9 or 12 for the coaggregating strain. This could have been interpreted as the result of the differences in coaggregation between these strain pairs. Nevertheless, the finding that another noncoaggregating strain of *F. nucleatum* (ATCC 10953) also had an influence on the subinfective *P. gingivalis* HG-405 inoculum, which was similar to that of the coaggregating *F. nucleatum* PK 1594 (Fig. 4), suggests that another mechanism(s) of synergism, unrelated to coaggregation, may also exist.

Synergistic mechanisms between *P. gingivalis and F. nucleatum,* such as those described previously, may explain the common encounter of these microorganisms in the mixed bacterial population of persistent chronic suppurative apical periodontitis. An equivalent may be found in persistent infections in periodontal pockets. *F. nucleatum* is one of the predominant bacteria isolated from subgingival plaque samples from both healthy and diseased sites (33). Nevertheless, it is not considered as one of the major pathogens that can singularly cause a destructive periodontal disease. Considering that the MID_{100} of *P. gingivalis* was reduced by a factor of at least 1,000 in the chambers containing *F. nucleatum*, a similar effect may also exist in periodontal pockets as well as intracanal or extraradicular environment. It could be that a site preinfected with certain strains of *F. nucleatum* becomes more susceptible to infection with *P. gingivalis* and that low-dose inocula that otherwise would have not developed at this site may become established in the presence of the preexisting *F. nucleatum* infection.

Further studies will be required to explore whether this proposed potential mechanism of sequential infection is valid in either periodontal disease or chronic suppurative apical periodontitis.

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