Attachment of Oral Gram-negative Anaerobic Rods to a Smooth Titanium Surface: An Electron Microscopy Study

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Purpose: *Attachment of bacteria to titanium may differ not only between bacterial species but also between strains within a species. The aim of the present in vitro study was to examine differences in bacterial attachment using 4 gram-negative anaerobic species of bacteria that are considered potential periodontal pathogens.* ***Materials and Methods:*** *The attachment of clinical and laboratory strains (n = 23) representing 2* Fusobacterium nucleatum *subspecies,* Porphyromonas gingivalis*, and* Prevotella intermedia *to smooth, commercially pure titanium was examined using scanning electron microscopy.* ***Results:*** *All bacterial strains were attached to the smooth titanium surface by their outer membrane.* F nucleatum *cells were poorly attached to the titanium, unlike* P gingivalis *or* P intermedia *cells, but only slight differences were observed in the quantity of attached cells between the strains within each bacterial group.* ***Discussion:*** *In favorable conditions, some anaerobes can attach directly to an inert titanium surface. Microbial adhesion and subsequent colonization on the dental implant surface can lead to infecttion of the peri-implant tissue.* ***Conclusion:*** *The results indicated that the avidity of bacterial attachment to a smooth titanium surface varies between species of oral gram-negative anaerobes but not between strains.* INT J ORAL MAXILLOFAC IMPLANTS 2004;19:803–809

Key words: *bacterial attachment sites, gram-negative anaerobic bacteria, scanning electron microscopy, titanium*

Adhesion of bacteria to various oral surfaces is essential for successful colonization of the mouth. Titanium, which has excellent biocompatibility properties, is the most common biometal used for endosseous dental implants and, in addition, is increasingly used for other prosthodontic restorations. Various physical and chemical characteristics of titanium surfaces appear to have an influence on human[1–3] and bacterial cell adhesion[4–7] to these surfaces. As suggested by Quirynen and associates,[8] rough-surfaced titanium abutments, in addition to having high surface free energy, may rapidly accumulate bacterial plaque on their surfaces.

Adherence of microorganisms to biomaterials and successful colonization of these surfaces are principal factors in biomaterial-associated infections.[9] In the oral cavity, dental implants with different surface characteristics are exposed to a wide range of bacterial species with different adhesive characteristics. Colonization of dental implant surfaces by certain gram-negative, anaerobic rods may be especially significant because of their potential interplay with pathogens or their own pathogenicity to surrounding tissues. *Fusobacterium nucleatum* plays a central role in the development of anaerobic polymicrobial communities by promoting coaggregation bridges for late-colonizing strict anaerobes,[10] such as suspected

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Table 1 Strains of Bacteria Used

Laboratory strain	Clinical strain
F nucleatum subsp *nucleatum* (ATCC 25586^T)	AHN 8874
	AHN 9963
	AHN 9508
	AHN 8447
F nucleatum subsp *polymorphum* (ATCC 10953^T)	AHN 9592
	AHN 9230
	AHN 9546
	AHN 8518
	AHN 8413
P gingivalis (ATCC 49417^T)	AHN 8448
	AHN 24155
	AHN 24135
	AHN 24146
	AHN 24098
P intermedia (ATCC 25611^T)	AHN 8291
	AHN 8753
	AHN 8815
	AHN 9438
	AHN 9378

ATCC = American Type/Culture Collection (Manassas, VA); T = type strain.

periodontal pathogens *Porphyromonas gingivalis* and *Prevotella intermedia*.[11] These species can be found in periodontal pockets around natural teeth as well as in peri-implant lesions.[12–16] Little is known about their adhesion to implant materials and about the impact of strain variation within a given species.

The aim of the present in vitro study was to investigate, using scanning electron microscopy (SEM), whether different *P gingivalis* and *P intermedia*, 2 subspecies of *F nucleatum*, or strains within these subspecies, differ in their attachment to a smooth, commercially pure titanium surface.

MATERIALS AND METHODS

Titanium Plates

Twenty-three commercially pure grade 1 titanium plates (1 × 1 cm) were polished using a standard procedure for titanium applied in dental laboratories. Briefly, the surfaces of the titanium plates were polished, first with a leather-polishing disk, then with coarse and fine rubber disks, and finally, with a brush using coarse and fine polishing paste. After polishing, the plates were cleaned with compressed air and stored in 96% ethanol.

Bacterial Procedures

Nineteen clinical and 4 laboratory strains of bacteria were revived from frozen (–70°C) stocks (Table 1). All clinical strains used in the study were isolated from the oral cavity. The strains were cultured on Brucella enriched with sheep blood, hemin, and vitamin K_1 on agar plates (BBL, Cockeysville, MD) and incubated in anaerobic jars filled with mixed gas (10% hydrogen, 10% carbon dioxide, 80% nitrogen) at 37°C for 3 days. After the purity of each culture was checked with a dissecting microscope, single bacterial colonies were transferred to new Brucella agar plates and incubated anaerobically at 37°C for 2 days.

Bacterial suspensions were made by harvesting the 2-day growth of *F nucleatum* subsp *nucleatum*, *F nucleatum* subsp *polymorphum*, *P gingivalis*, and *P intermedia*, transferring them to sterile water using sterile Pasteur pipettes, and mixing rigorously to break up clumps. For quantification of bacterial cell numbers in suspensions corresponding to McFarland 0.5 turbidity, the viable colony counts were determined. (A solution with a McFarland 0.5 turbidity standard contains approximately 1.5×10^8 organisms/mL.)

The titanium plates were immersed in 4 mL of a bacterial cell suspension of 1.5×10^8 cells per milliliter of sterile water and incubated anaerobically at 37°C for 22 hours. After incubation, the samples were carefully rinsed twice with potassium phosphate–buffered saline (PBS; 0.2 mmol/L; pH 7.4) to remove loosely attached cells.

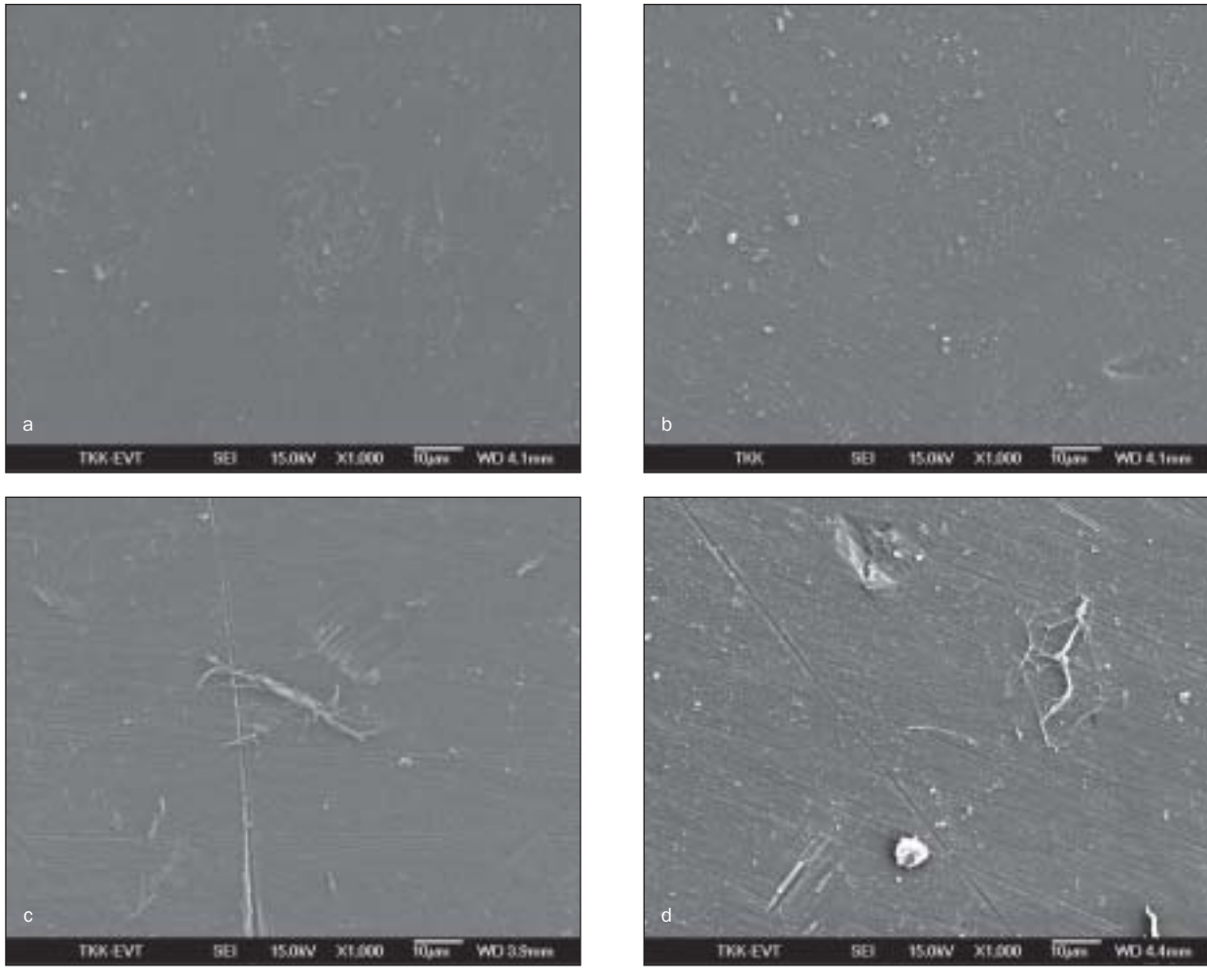
SEM

The specimens were fixed in glutaraldehyde (2.5%) in PBS at ambient temperature and left overnight. The fixative was aspirated, and the samples were rinsed 3 times with PBS. All the samples were dehydrated through a graded series of ethanols, treated with hexamethyldisilazane overnight at ambient temperature,[17] and sputter-coated with gold (Sputter-Coater SCD 050; Bal-Tec, Liechtenstein). The scanning electron micrographs were prepared using a high-resolution field emission SEM (FESEM) (JSM-6335F; JEOL, Tokyo, Japan) operating at 15 kV.

Assessment of Bacterial Attachment

For quantification of attached cells, 4 individual fields of each titanium plate were observed under the FESEM, and electron micrographs were taken at magnification of ×1,000. The bacterial cells present on each 10.807×10^{-3} mm^2 field were counted by 2 authors, and the mean number of cells in each of the 4 fields was determined and expressed as cells/mm^2.

Representative fields at magnifications of ×10,000 and ×50,000 were chosen to examine the mechanism of bacterial attachment. Individual fields of each plate were randomly selected under SEM for electron micrographs.



Figs 1a to 1d For quantification, micrographs were taken at low magnification. Individual fields of *(a) P gingivalis* AHN 24135, *(b) P intermedia* ATCC 25611, *(c) F nucleatum* subsp *nucleatum* ATCC 25586, and *(d) F nucleatum* subsp *polymorphum* AHN 9592 (original magnification ×1,000).

Statistics

Quantitative measurements are expressed as means. Descriptive statistics were calculated, and the Student *t* test and the Fisher exact test were used to compare the various groups when appropriate. Two levels of statistical significance were predetermined; $P < .05$ and $P < .01$.

RESULTS

Quantification of Attached Cells

Based on direct cell counts on representative SEM fields (Figs 1a to 1d), clear differences were observed between the species examined. The mean number of *P intermedia* cells attached to smooth titanium was 64.0×10^5 cells/mm^2, and for *P gingivalis* cells, it was 39.6×10^5 cells/mm^2. However, the attachment of *F nucleatum* to titanium was poor; the number of attached cells ranged from 1.5×10^3 to 5.2×10^3 cells/mm^2. The strains of *F nucleatum* had a significantly lower adhesion capacity ($P < .01$) to smooth titanium than *P intermedia* or *P gingivalis*. *F nucleatum* subsp *polymorphum* strains had the lowest adhesion activity to titanium surfaces (a mean of 1.5×10^3 cells/mm^2). The quantity of attached cells on titanium was similar between the examined strains within each bacterial group. Within the 2 subspecies of *F nucleatum*, there was a tendency toward strain variation (*F nucleatum* subsp *polymorphum*) or no variation (*F nucleatum* subsp *nucleatum*). The equality of the means within *P gingivalis* and *P*

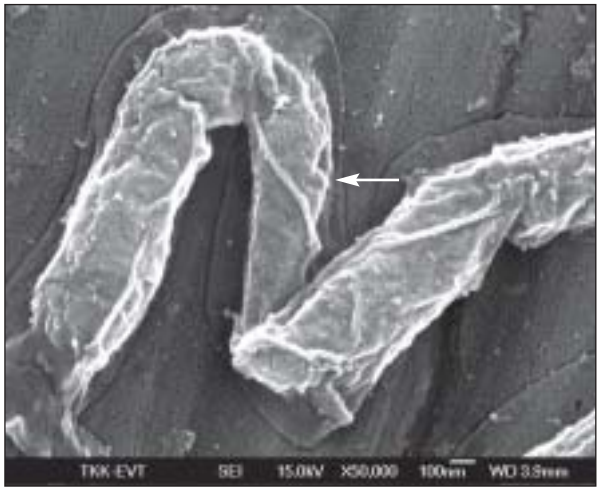


Fig 2 At high magnification the outer membrane's structure and its role in adhesion can be seen. Outer membrane of a clinical strain of *F nucleatum* subsp *nucleatum,* AHN 9508 (original magnification ×50,000).

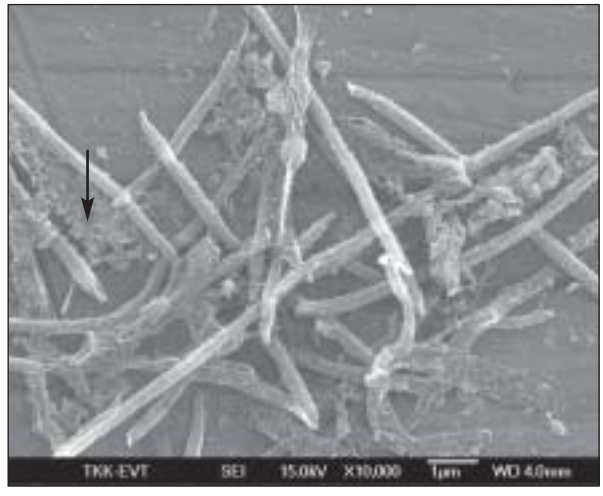
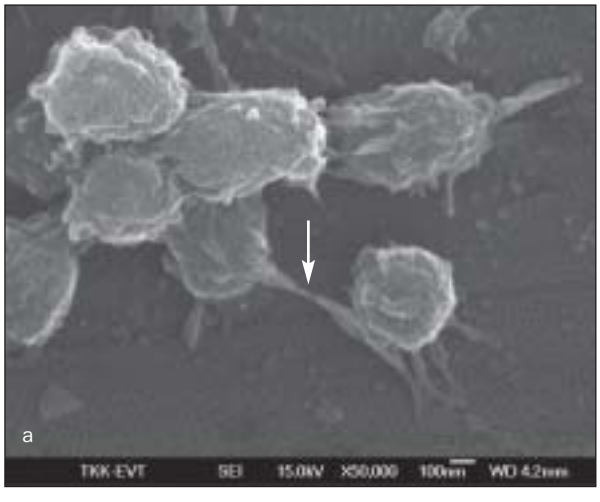


Fig 3 Outer membrane–associated structure of a clinical strain of *F nucleatum* subsp *polymorphum,* AHN 8518 (×10,000).



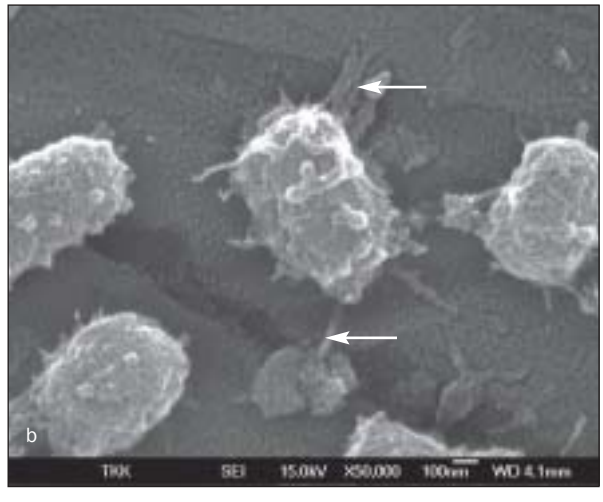


Fig 4 Outer membrane projections of *(a)* the type strain of *P gingivalis,* AHN 24098 and *(b)* a clinical strain of *P intermedia,* ATCC 25611 (both figures ×50,000).

intermedia strains was not possible to compare because of very small variance between the means of bacterial cell counts on the randomly selected fields of the titanium plates. In general, the bacterial cells were evenly distributed on the titanium surface, and only a few clumps of *F nucleatum* cells were seen. In clumps, *F nucleatum* cells were attached to each other, not to titanium.

Attachment Mechanism

Surface ultrastructure of the attached cells was evaluated descriptively using micrographs taken at high magnifications (×10,000 and ×50,000). Attached bacteria were evenly distributed on the titanium plates, with no particular orientation to the surface or surface irregularities. All bacterial strains were attached to the smooth titanium surface by their outer membrane. Different kinds of outer membrane morphologies were seen among the examined bacterial species. Both *F nucleatum* subspecies were attached to the titanium surface by a flat, continuous outer membrane (Fig 2), but netlike outer membrane–associated structures were also seen among some strains (Fig 3). These latter structures also appeared to combine adjacent cells by fibrous threads, especially when the cells occurred in clumps. *P intermedia* (Fig 4a) and *P gingivalis* (Fig 4b) cells demonstrated tubelike outer membrane projections.

DISCUSSION

Titanium is the most commonly used biometal in dental implants and, therefore, a profitable target in research aiming to elucidate the interplay between the biomaterial and oral bacteria. For this purpose smooth titanium plates polished using procedures routinely used in dental laboratories were used. A relatively long incubation time was chosen to allow the attachment to occur as well as to simulate the continuous bacterial exposure of dental implants in the mouth. The present findings showed that all of the oral, anaerobic bacterial subspecies included in this in vitro experiment adhered to a smooth titanium surface to some degree. Differences in adhesion to titanium were found between species but not between strains.

The incubation time for the anaerobic bacteria tested in water was 22 hours—enough for the bacterial cells to attach to the titanium surface but not long enough for any significant growth to occur. After harvesting the bacterial cells and transferring them to the sterile water, as described previously,[18] the authors carefully mixed the suspensions so that no aggregation of the bacterial cells was seen. According to the viable colony counts made from the suspension, the viability of the bacteria was not harmed by sterile water. Notably, the laboratory work with the bacteria was done in anaerobic conditions.

On the surfaces of artificial biomaterials, which are increasingly being used in various restorations in the human body, bacteria find new surfaces and environments for their growth. Biomaterial-associated infections have become increasingly common in connection with medical and dental treatment.[9] The first years after dental implant placement appear to be critical in determining whether the implant will be successful.[19] Microbial adhesion and subsequent colonization on dental implant surfaces can, in some instances, lead to infection of the peri-implant tissues. This, together with an unfavorable host response, causes bone destruction around osseointegrated implants and, eventually, the failure of implant therapy.[20] Bacteria with a similar composition, ie, bacteria dominated by gram-negative, anaerobic rods, have been described in periodontitis and peri-implantitis lesions.[12–16] Despite these similarities, peri-implantitis may constitute a distinct disease entity.[21]

In the oral cavity, natural hard surfaces (enamel, root cement) and artificial biomaterials (eg, titanium) are normally bathed by fluids, such as saliva and gingival crevicular fluid, that contain a variety of molecules that may influence bacterial attachment.[22,23] Only some bacterial species, mainly anaerobic bacteria in periodontitis and peri-implantitis, are involved in pathogenic processes in the oral cavity.[11,13] In vivo dental plaque accumulation starts with colonization of streptococci and other early colonizers on pellicle-coated tooth or titanium surfaces.[4,5] As a result of plaque maturation, anaerobic conditions are formed in gingival pockets that favor the growth of fastidious gram-negative anaerobic species.[24] When detached from subgingival biofilms, some are able to attach to epithelia and even invade gingival tissues. The present results indicate that, in favorable conditions, some anaerobes can attach directly to an inert titanium surface, although the avidity varies between species. In the present in vitro study, the direct adhesion of bacterial cells to a titanium surface, rather than the role of host-derived constituents in bacterial adhesion to oral surfaces, was examined. Under anaerobic conditions *P gingivalis* and *P intermedia*, pigmented gram-negative rods that are associated with mature, potentially harmful dental biofilms in subgingival sites,[24] attached in considerable numbers to the smooth titanium surface. Both species showed tube-like, outer membrane–associated structures and membranous extensions on the titanium surface. The *P gingivalis* and *P intermedia* strains showed a higher avidity in adhesion to smooth titanium than the strains of *F nucleatum* subsp *nucleatum* or *F nucleatum* subsp *polymorphum*. This was not expected, as *F nucleatum*, unlike the former 2 species, is an early colonizer of the mouth.[25] However, the observation indicates that differences exist between species in terms of their avidity to attach to inert materials without any biologic coating.

Microbial attachment to and colonization on natural teeth surfaces in vivo is determined by the surface structure of the tooth.[26] Similarly, the formation of plaque on implant surfaces is induced by certain surface characteristics; for example, an increase in surface roughness or surface free energy was found to result in faster colonization of the surfaces and faster maturation of plaque.[6] Although smoothing the titanium surface can reduce bacterial colonization and microbial attachment, at a certain level of smoothness, no further effect on qualitative composition of the plaque or reduction of microbial accumulation can be achieved.[27] Various other surface characteristics of titanium implants, such as hydrophobicity, appear to influence oral bacterial attachment in vitro.[4,7,28] It is notable, however, that in vitro behavior of bacteria may differ from in vivo behavior.

Based on their in vitro observations on different serotypes of *Actinobacillus actinomycetemcomitans*, Ökte and associates[18] suggested that the adhesion of this facultatively anaerobic periodontal pathogen to a titanium surface is strain dependent. In contrast,

significant differences in the number of attached cells between distinct bacterial strains were not demonstrated in the present study. The results of the current study on *F nucleatum*, *P gingivalis*, and *P intermedia*, which are strictly anaerobic, suggest that the avidity of their attachment to titanium is dependent on bacterial species rather than on strains, since different strains within the indicated species had similar affinities to titanium.

The results of the present study, in which high-magnification micrographs (×50,000) made using SEM were evaluated, indicated that the examined gram-negative anaerobic bacteria attached to a smooth titanium surface by their outer membranes. Different kinds of outer membrane morphologies were seen in different bacterial species. Tubelike outer membrane projections were present among *P gingivalis* and *P intermedia* strains. Among *F nucleatum* strains, flat, continuous outer membranes were observed. Netlike outer membrane–associated structures appeared to combine adjacent cells by fibrous threads, especially where the cells were clumped. Since a rather long incubation time was used (22 hours), these cell clumps probably indicate early attempts at biofilm formation and glycocalyx production. Specific receptors and molecules on the membrane surface interact with inert biomaterial surfaces and are affected by the chemical composition, electric charge, hydrophilicity/hydrophobicity, and the texture of the material surface.[29] Therefore, the authors' next goal is to examine the effect of surface texture on the attachment of both aerobic and anaerobic bacteria that may be involved in biomaterial-associated infections.

CONCLUSIONS

Oral gram-negative, anaerobic bacteria seem to attach to smooth titanium surfaces by their outer membrane and its projections. The bacterial adhesion to titanium differed between the examined bacterial species; however, no significant differences were observed between strains within each species. Further studies are warranted to examine the impact of various titanium surface characteristics and bacterial interactions on bacterial attachment and colonization on titanium.

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