Primary Bacterial Colonization of Implant Surfaces

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The purpose of this study was to assess the effects of modifying titanium surfaces, in terms of wettability, roughness, and mode of sterilization, on the ability of the oral bacterium *Streptococcus sanguis* to colonize. An in vitro model system was developed. All surfaces were colonized by the bacteria, but to significantly different levels. Titanium samples that exhibited rough or hydrophobic (low wettability) surfaces, along with all autoclaved surfaces, were preferentially colonized ($P < .01$). Titanium surfaces that had been repeatedly autoclaved were colonized with the levels of bacteria 3 to 4 orders of magnitude higher than other modes of sterilization. This may have implications relative to the commonly used method of autoclaving titanium implants, which may ultimately enhance bacterial biofilm formation on these surfaces.
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Key words: colonization, hydrophobicity, implant, modes of sterilization, *Streptococcus sanguis*, titanium

The clinical success of osseointegrated dental implants has led to a dramatic increase in their use worldwide. Success rates reported by Brånemark et al[1–3] for dental implants over a 15-year period have been as high as 93% for mandibular and 84% for maxillary implants. Although the number of failures leading to exfoliation are numerically low, it is important to continue to improve the methodology involved in implant dentistry, since clinical failure of an implant is costly due to the time and expense involved in implant placement and rehabilitation, both for the patient and the practitioner. Much is still lacking in our understanding of the complex series of events leading to successful integration of an implant.[4–8] From a clinical viewpoint, there are few guidelines that are universally accepted and available for the development of diagnostic protocols and few therapeutic procedures for dealing with complications.[9–11] This may be important for the long-term health of an implant in the face of periodontal-like inflammation seen in tissues surrounding implants. From a basic science standpoint, parameters that define the status of the host tissue–implant interface have not been extensively studied. The paucity of information on the interrelationships between host tissues, colonizing bacteria, and the physicochemical nature of implant surfaces has severely limited the understanding of how these interactions lead to successful tissue integration or to clinical failure.

Gristina described the colonization of an implant surface as a "race for space" between host tissue cells and oral bacteria.[12] This may be particularly important, since implant failures following uneventful primary healing appear to be the result of either extensive occlusal stress or infections.[9] With regard to the latter, clinical studies have shown an association between specific "cluster groups" of microorganisms and the clinical failure of implants, similar to what has been documented for different forms of periodontal disease.[13–18] Mombelli et al[13–16] have shown that significant differences in microflora composition exist between healthy and failing implant sites. The

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authors suggested that peri-implantitis is strongly associated with implant failure and is a site-specific infection.

It is well known that freshly-erupted teeth are rapidly colonized by oral bacteria. It is reasonable to assume that the placement of other forms of "hard tissue" in the oral cavity would provide additional sites for bacterial adhesion and colonization. However, little information is available concerning interactions between these microorganisms and implant surfaces. A study conducted by Wolinsky et al[19] showed that plaque-forming bacteria such as *Actinomyces viscosus* adhered to enamel in numbers 5 times higher than the adherence to titanium surfaces. In contrast, *Streptococcus sanguis* adhered similarly to enamel and titanium. Other studies have examined bacterial adhesion and colonization on a variety of biomaterials and have found, in general, that factors such as type of organism, concentration, growth phase, and surface properties of the materials all affect levels of colonization to varying degrees.[13–19]

Despite the information from previously conducted studies, there is still little understanding of the effect that variable biomaterial surface properties have on oral bacterial colonization. Recent studies by Keller and others[20–23] have shown that host cells involved in the wound healing response and osseointegration display markedly different attachment profiles, depending on the physicochemical nature of the biomaterial surface. It is conceivable that bacterial colonization on implant surfaces may preclude host tissue integration, thus winning the "race for space."[12] The overall purpose of this investigation was to develop an in vitro model system, whereby the effect of variable surface conditions on bacterial colonization could be studied. Varieties of surface roughness were selected to simulate the surface morphologies available on current implants and components. In general, implants have varying degrees of surface texture, from overtly rough, such as that produced by sandblasting, to moderate levels of roughness, as produced by machining or polishing. Generally, the portion of the 2-stage systems or the coronal aspects of a single-stage implant system are more finely polished to accommodate the formation of an epithelial tissue interface and to permit routine hygiene.

Materials and Methods

Preparations of Titanium Discs. The methods used in this study were selected to simulate the surfaces of common dental implants and components. Our research group has published extensively on the development of this model system to simulate the surface conditions of current implant devices.[20–23] Commercially pure titanium discs (12.5 mm in diameter, 4 mm thick) were cut from bar stock. Discs were polished with diamond paste (1 µm), 800-grit silicon carbide sandpaper, or sandblasted to produce surfaces differing in roughness and morphologic topography. These 3 groups were then subdivided into acid-passivated and non-treated, respectively. Acid-passivation renders surfaces more hydrophilic and was accomplished as described previously.[23] Briefly, samples were exposed to methylethylketone for 5 minutes, rinsed extensively with distilled water, and then treated with nitric acid (30%) at room temperature for 30 minutes. Each of these treatment groups was further subdivided as to the mode of sterilization: *(1)* exposure to ultraviolet light (300 µw/cm^2 for 15 minutes), *(2)* steam-autoclaving (10 cycles at 30 psi and 121°C), *(3)* exposure to ethylene oxide gas (130°F for 3 hours), and *(4)* plasma cleaning in a glow-frequency discharge chamber (5 minutes in argon gas).

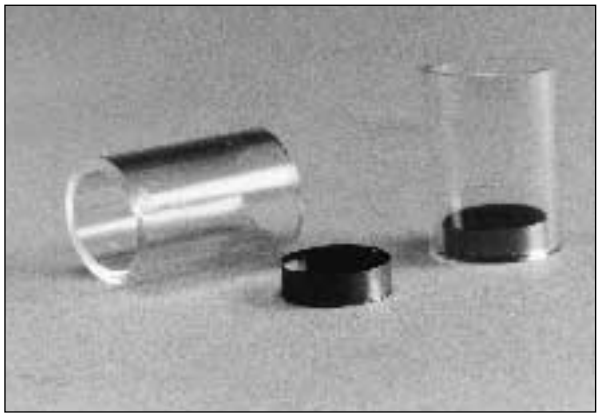

Fig 1 Bacterial colonization chambers used for the in vitro assays.

Bacteria. The microorganism used in this investigation was *Streptococcus sanguis (S sanguis)*, a primary colonizer of hard tissues in the oral cavity. Stock cultures were maintained in trypticase soy broth supplemented with 0.5% yeast extract (TSB-YE) and 50% glycerol at –90°C. Bacteria were routinely cultured in TSB-YE in a 5% CO_2 incubator 37°C. Gram stains and colony morphology confirmed purity of the cultures.

Colonization Model. Colonization chambers were prepared by placing the titanium discs in a piece of Tygon tubing (1 inch in length, 0.5 inch internal diameter) (Fischer Scientific, Itasca, IL), forming a sealed chamber (Fig 1). Prior to assem-

bly, the tubing was cleaned with RBS 35 concentrate (2%) (Pierce Chemical, Rockford, IL), rinsed in tap water for 15 minutes, coated with Prosil 28 concentrate (1:100 dilution) (PCR, Gainesville, FL), and then thoroughly rinsed in distilled water. The base of the model assembly was sealed with Resigil (Dentsply, Milford, DE). Sealed chambers were assembled in a laminar-flow, sterile hood. Bacteria were cultured in TSB-TE as described for 12 hours. Cells were harvested by centrifugation (5900 g for 15 minutes at 4°C) and resuspended in fresh TSB-YE. Assemblies were inoculated with 1.0 mL bacterial suspension, covered with sterile plastic caps, and incubated statically in 5% CO_2 at 37°C for 24 hours.

Following this incubation period, the assemblies were transferred back to the laminar flow hood. Cell suspensions were carefully removed by aspiration and fresh TSB-YE (1.0 mL) was added to each disc in a gentle manner so as not to disturb the colonized cells. Assemblies were incubated for an additional 24 hours under identical conditions, as above. This cycle was repeated until a total of 72 hours of incubation time was reached. At this point, cell suspensions were again carefully aspirated off from the assemblies. Titanium discs were removed from the tubing, taking care not to disrupt the biofilm of bacteria. After disassembly, the discs were washed by gently dipping them 10 times in sterile phosphate-buffered saline (PBS, 20 mmol/L potassium phosphate, 0.145 mol/L sodium chloride, pH 7.2) to remove loosely adherent cells. Surfaces of the discs were then swabbed with sterile cotton applicator sticks to remove the biofilms of bacteria. Cotton tips were broken off and placed with the Ti discs into tubes containing 5.0 mL sterile PBS. Tubes were subsequently sonicated for 15 seconds, followed by vigorous vortexing for an additional 10 seconds. Samples were diluted appropriately and plated on Mitis-salivarius agar using a spiral plating system (Spiral Systems, Bethesda, MD). Plates were incubated in 5% CO_2 at 37°C for 24 to 48 hours. Numbers of colonizing *S sanguis* were determined following standard spiral-system methodology. This involves counting colonies in specified grids and then using provided formulas for determining the numbers of bacteria in the original sample.

Table 1 Statistical Comparison of Surface Treatments and Sterilization Modes

Comparison	$P < .05$
Surface comparison	
800-grit versus sandblasted	No
800-grit versus 1-µm smooth	Yes
Sandblasted versus 1-µm smooth	Yes
Mode of sterilization	
uv nonacid versus uv acid	Yes
uv nonacid versus plasma-cleaned	Yes
uv nonacid versus ethylene oxide	Yes
uv acid versus plasma-cleaned	No
uv acid versus ethylene oxide	Yes
Plasma-cleaned versus ethylene oxide	No

Scanning Electron Microscopy. Representative titanium discs colonized with *S sanguis* were prepared for scanning electron microscopy (SEM) following standard procedures. Discs were fixed in 1.5% glutaraldehyde in cacodylate buffer (pH 7.2, 0.2 mol/L) for 1 hour at room temperature. Discs were then washed 3 times with the buffer and dehydrated through a series of graded acetone solutions (30%, 50%, 70%, 95%, 100%). Discs were subsequently critical-point dried, sputter-coated with gold, and examined using an Amray model 1820D scanning electron microscope (Amray, Bedford, MA).

Statistical Analysis. Numbers of colonizing bacteria were transformed to $\log_{10}$ values prior to analysis to normalize the data. Data were then analyzed for surface treatment and mode of sterilization effects via a 2-way analysis of variance (ANOVA). Differences between groups were assessed by Student-Neuman-Keul's post-tests.

Results

Levels of *S sanguis* colonization on the various experimental groups are shown in Fig 2. Data are expressed as means plus one standard deviation. All surfaces were colonized by these bacteria as illustrated in Figs 3a to 3d; however, distinct differences were observed among the various treatment groups. Analysis of the data revealed significant differences between surface treatments and sterilization modes, with a significant interaction between these 2 variables ($P < .001$). Pairwise comparisons by Student-Neuman-Keul's post-tests showed significant differences between several groups (Table 1). When comparing surface roughness among all modes of sterilization, 800-grit surfaces exhibited significantly higher levels of bacterial colonization than 1-µm surfaces; sandblasted titanium also had greater numbers of colonizing bacteria than 1-µm smooth surfaces. However, no differences were observed between 800-grit and sandblasted discs (Figs 3a to 3c). Differences were also seen between the various sterilization mode groups. Significantly less colonization occurred on the ultraviolet light–treated/acid-passivated, plasma-cleaned, and ethylene oxide–sterilized surfaces than the ultraviolet

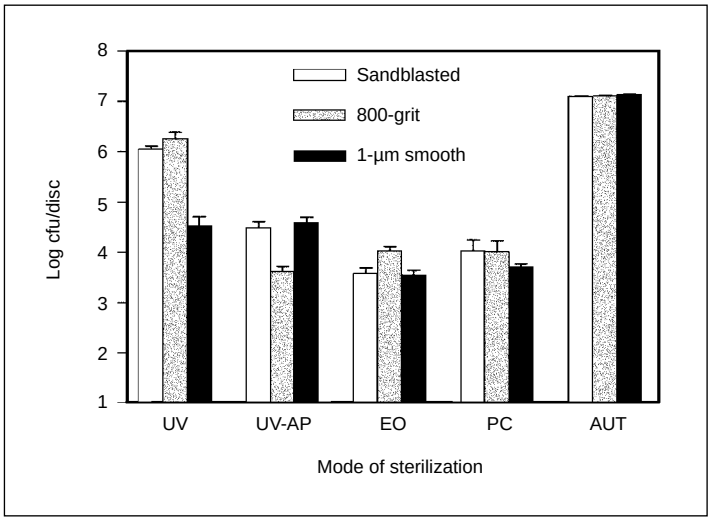

Fig 2 Graph showing colonization of titanium discs by *Streptococcus sanguis* with respect to mode of sterilization. Data are expressed as means and 1 standard deviation from 5 samples per group. UV = ultraviolet light; UV-AP = ultraviolet light, acid-passivated; EO = ethylene oxide; PC = plasma-cleaned; AUT = autoclaved.

Fig 3 Scanning electron micrographs of commercially pure titanium surfaces following colonization assay. Specimens in Figs 3a to 3c were exposed to ultraviolet light sterilization, while the specimens in Fig 3d was multiple steam-autoclaved. Note colonization of all surfaces by *Streptococcus sanguis.* Areas depicted here do not reflect the relative quantity of cells attached but rather specific interaction of cells with the prepared surfaces.

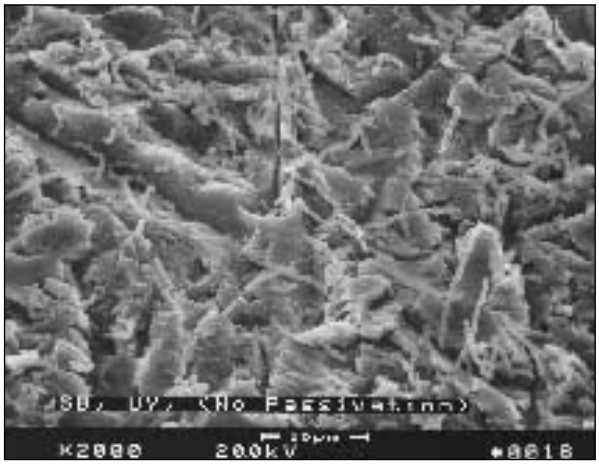

Fig 3a Scanning electron micrograph of sandblasted, commercially pure titanium surface following colonization assay.

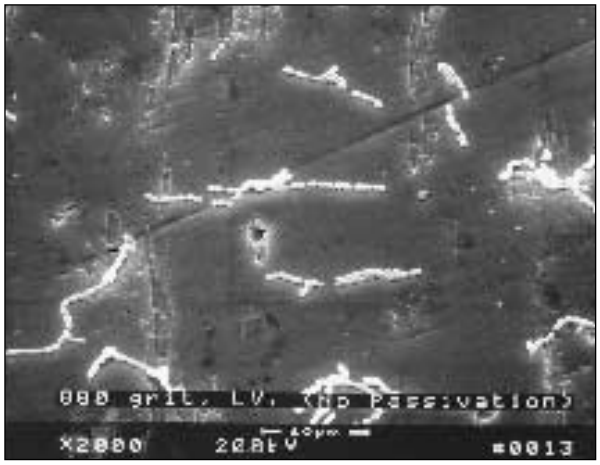

Fig 3b Scanning electron micrograph of 800 grit–blasted, commercially pure titanium surface following colonization assay.

Fig 3c Scanning electron micrograph of 1-µm polished, commercially pure titanium surface following colonization assay.

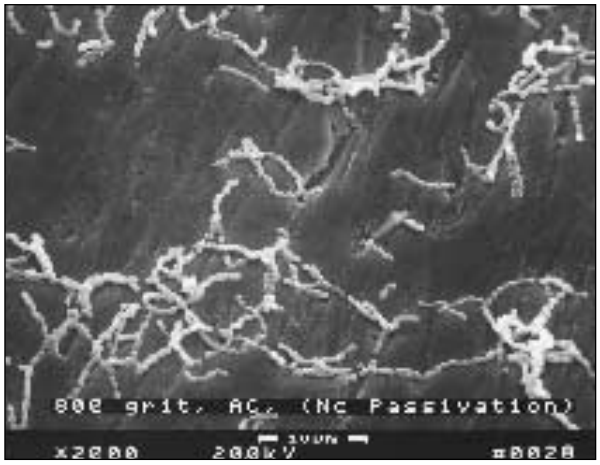

Fig 3d Scanning electron micrograph of commercially pure titanium surface polished with 800-grit sandpaper following colonization assay.

light–treated/non-acid-passivated surfaces. Other significant differences between group pairs can be readily seen. The most pronounced differences in levels of *S sanguis* colonization were seen with the repeatedly autoclaved surfaces (Fig 3d). Regardless of the surface roughness, $>10^7$ colony-forming units (CFU) per mm^2 were isolated from discs sterilized in this manner.

Discussion

Biomaterials are used extensively in medicine and dentistry, including prosthetic hip joints, indwelling catheters, contact lenses, and implants.[24] An increasingly common clinical problem with the use of these materials is the development of bacterial biofilms on their surfaces.[12–18,25] These communities of organisms have proven to be quite recalcitrant to standard antibiotic therapy and are exceedingly difficult to eradicate. A potentially more successful approach may reside in the design of biomaterial surfaces that provide optimal conditions for host tissue integration but limit the ability of bacteria to colonize. The intent of this study was to design an in vitro bacterial colonization model system, whereby the effect of modifying the physicochemical nature of titanium surfaces could be readily assessed.

Three different surface roughness levels were assessed. They represented the extremes of preparation (1-µm, or smooth, and sandblasted, or rough) and included an 800-grit preparation. It has been shown that treating metal surfaces with ultraviolet light does not alter titanium, as measured macroscopically and by SEM.[23] Therefore, the ultraviolet light sample may represent an internal control in evaluating *S sanguis* colonization of surfaces of varying roughness, without affecting the surface with the mode of sterilization. The results of this study indicate a significant difference in bacterial colonization of 1-µm titanium surfaces (7.68×10^4 CFU/mm^2), versus the sandblasted (1.14×10^6 CFU/mm^2) and 800-grit (2.08×10^6 CFU/mm^2) group. This may be a result of the decreased surface area on a microscopic level in the 1-µm group, or it may be indicative of a change in the wettability of the titanium surface as it becomes smoother following polishing.

Acid-passivated surfaces also showed significantly lower numbers of CFU/mm^2 when compared to ultraviolet sterilized non-acid-passivated discs, regardless of surface roughness. Considering that hydrophilicity (increased wettability indicated by decreased contact angle with water) is increased with passivation, these results indicate a decrease in *S sanguis* colonization as the titanium surface is rendered more hydrophilic. This is in agreement with the known propensity for *S sanguis* to prefer binding to hydrophobic receptor sites.[26–29]

Ethylene oxide–sterilized surfaces demonstrated a relatively low number of CFU/mm^2 regardless of surface roughness. There is little surface change seen with sterilization using ethylene oxide.[20,30] The lower numbers of CFU/mm^2 in the ethylene oxide group versus the ultraviolet-treated group may be the result of an unidentified residual effect of the biocidal elements of ethylene oxide gas used for sterilization and its effect on prospective *S sanguis* colonizers.[20]

Plasma-cleaned discs also had similar low numbers of CFU/mm^2 when compared to ethylene oxide–sterilized surfaces. Plasma cleaning at low temperature with argon gas is known to remove surface contaminants and create a high-energy, very wettable surface, as determined by decreasing water contact angle measurements.[20,31] This again indicates a decreased hydrophobicity (enhanced hydrophilicity), which may explain low colonization by *S sanguis*.

Discs that were steam-autoclaved 10 times showed significantly greater CFU/mm^2 than all other groups, regardless of surface roughness. Steam-autoclaving titanium surfaces increases the surface oxide thickness[20,32] and causes discoloration of the surface. Steam autoclaving also causes particulate contamination of surfaces with various elements, such as carbon, nitrogen, calcium, sodium, potassium, and iron.[20,31,33] This also decreases the surface energy, leading to increases in water surface contact angle measurements.[20,31] This change in biometal surface energetics and increased hydrophobicity may be responsible for the significant increase in bacterial colonization on titanium surfaces that have been steam-autoclaved multiple times.

The clinical significance of these findings is threefold. The manufacturer's preparation of dental implants and titanium for human implantation should have a basis in research results. While the specific methods and procedures used by manufacturers of implant devices are often proprietary, the results of this study point to several important concerns regarding the potential for implants and associated components to foster adhesion and subsequent colonization of bacteria. First, in general, a higher degree of bacterial adhesion was observed on the rougher surfaces (800-grit and sandblasted surfaces). While many available implants are from 2-stage systems (ie, submerged into host bone at implantation without a permucosal abutment) and

would not readily interact with bacteria, in instances where tissue dehiscence or breakdown related to periodontal disease occurs, implants that have rough surface textures are likely to harbor bacteria capable of inducing peri-implantitis conditions. In most implant systems, the coronal portion of the implant, which permeates through the soft tissues, is relatively smooth (eg, < 800-grit) to allow proper cleaning and hygienic measures. However, it was demonstrated in this study that bacteria were capable of colonizing smooth surfaces. Without proper hygienic procedures, these coronal portions of the implant may lead to colonization of bacteria and the initiation of peri-implantitis.

Second, the results of this work have shown that surfaces whose hydrophilicity was enhanced by acid passivation experienced decreased bacterial colonization. It is therefore recommended that manufacturers of implant devices treat their materials such that bacterial colonization is minimized. Third, in the case of the implant device, each time a tray is opened in the operating room for placement of rigid internal fixation plates and screws, the remaining unused plates are subjected to resterilization of some kind. Little is known of the effects of multiple sterilization on these metals and on subsequent bacterial colonization.

Summary

This research would indicate that steam-autoclaving titanium metal multiple times causes surface changes leading to increased colonization by *S sanguis*, which is a known primary colonizer of enamel and implant surfaces intraorally. Though *S sanguis* may be associated with healthy implant sites, its presence leads to coaggregation by pathogenic bacteria,[34,35] and thus colonization of implants by *S sanguis* could lead to failure of the implant in the long term.

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