Bacterial adherence to bioinert and bioactive materials studied in vitro

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In vitro, bioinert stainless steel and titanium alloy, and bioactive sintered hydroxyapatite and hydroxyapatite-coated titanium materials were exposed to *Staphylococcus epidermidis* to study bacterial adhesion. Scanning electron microscopy showed that fibrous strands interconnected the adherent bacteria, and that background matrix enclosed bacterial colonies. This adherent mode of growth may reduce the susceptibility of the bacteria to host clearance mechanisms and antibiotic therapy. Adherence assays revealed that bacterial adherence to sintered hydroxyapatite was higher than to the other 3 materials.

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Currently implant materials with better biocompatibility are being studied (Geesink 1990, Nasser et al. 1990, Santavirta et al. 1992); the main interest has been in the mechanical properties and biocompatibility. One of the problems associated with the use of these new biomaterials could be bacterial interrelations. Gristina and Costerton (1985) found that bacteria colonizing the surface of clinical biomaterials grow on the adherent biofilms, so that an endoprosthesis may become a substrate for bacterial growth. Bacterial adherence may be the central point in biomaterial-centered infections. Therefore, we evaluated the bacterial adherence and colonization of 4 different kinds of bioactive and bioinert materials, sintered hydroxyapatite, hydroxyapatite-coated titanium alloy, titanium alloy, and stainless steel.

Material and methods

Test arrangement

The laminar flow colonization chamber used was a modification of the Robbins device (MRD) developed by McCoy et al. (1981). The MRD was connected with a sterile rubber tubing to a reservoir (37 °C) containing 300 mL of the liquid bacterial culture. The bacterial suspension was delivered from this reservoir at 60 mL/h by a peristaltic pump.

Biomaterials

Sterile circular discs composed of surgical-implant grade stainless steel (SUS 316L), sintered hydroxyapatite (HA), titanium alloy (Ti–6Al–4V) and HA-coated titanium (HA–Ti), manufactured by Kyosera Bioceram in Kyoto, Japan, were aseptically inserted in the MRD. Before use, the MRD was sterilized with ethylene oxide. All biomaterials were cleaned by ultrasound and sterilized with ethylene oxide.

Bacterial cultures

A strain of *Staphylococcus epidermidis* (SE–46), isolated by Yamamoto and Iwata (1986), was used. The bacteria were cultured for 48 h in 500 mL of Trypticase soy broth (TSB) without dextrose (Difco Laboratories, Detroit, MI, U.S.A.), supplemented with 0.5 percent gluconic acid (Sigma Scientific, St. Louis, MO, U.S.A.). To inoculate the 300 mL batch culture that supplied the MRD, 20 mL of a bacterial suspension standardized by our method (Oga et al. 1988) was used so that the reservoir delivered logarithmic cells to the MRD. The number of bacteria in the incubation suspension at the beginning of the experiment was about 3×10^8 CFU/mL.

Design of experiment

To compare the degree of bacterial adhesion under identical conditions, 16 discs of each type of material, 10 mm in diameter, were placed in the MRD. Staphylococcus E-46 specimens cultivated in the reservoir were passed through the MRD for 1 h at a regular flow speed (60 mL/h). 4 discs, 1 of each type of material, were examined by scanning electron microscopy (SEM). Each of the remaining 12 discs was immedi-

ately placed in 10 mL of PBS, pH 7.2, and rinsed 3 times in 10 mL PBS. The number of viable bacteria adhering to each disc was determined by the colony-counting method (Oga et al. 1988). This assay was performed 5 times, and 15 discs, 1 of each type of biomaterial, were evaluated as a whole.

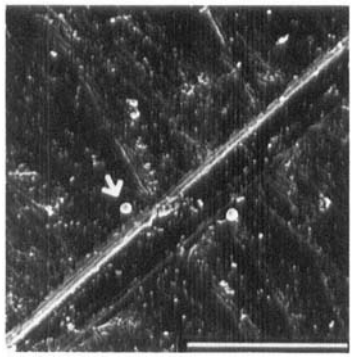
Bacterial adhesion-colonization assay

The data on surface bacteria counts are expressed as a mean, plus or minus the standard error of the mean, of the results of each of the 4 types of discs. Adhesion-colonization index is defined as the number of bacteria on the material divided by the number of bacteria found on stainless steel under the same experimental conditions. Adhesion-colonization indices are expressed as the mean of the results of the 5 separate experiments. Wilcoxon-Mann-Whitney tests were performed by using the specific program, Stax 98, which had been adapted to a computer.

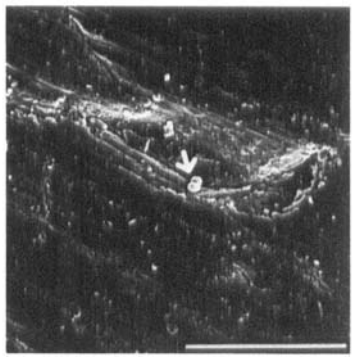
Scanning electron microscopy

The discs were immediately placed in 0.1M cacodylate buffer, pH 7.2, containing 5 percent glutaraldehyde and 0.15 percent ruthenium red and incubated at room temperature for 2 h. Then samples were developed by the method described before (Oga et al. 1988) and examined with a JEOL JSM–840A scanning electron microscope. The surfaces of the same 4 biomaterials without bacterial contamination were observed as controls.

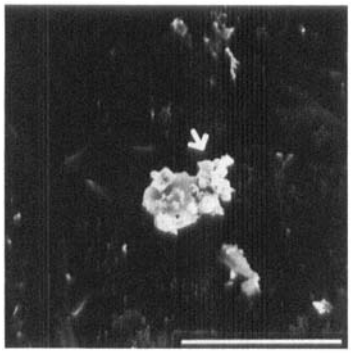
Figure 1. Scanning electron micrograph showing adherent Staphylococcus E–46 cells (arrows) on each biomaterial surface. Bar, 10 microns.



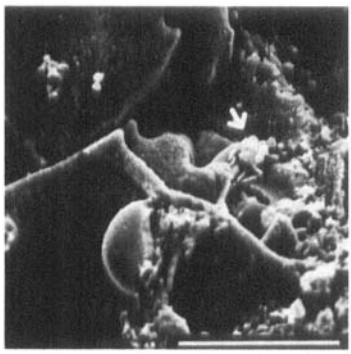
Stainless steel.



Titanium alloys.



Hydroxyapatite.



Hydroxyapatite-coated titanium.

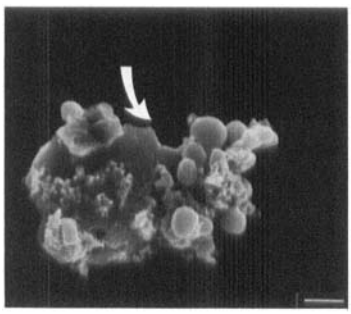


Figure 2. Scanning electron micrograph of hydroxyapatite-coated titanium. Bacterial microcolony and biofilm (arrow) are seen. Bar, 1 micron.

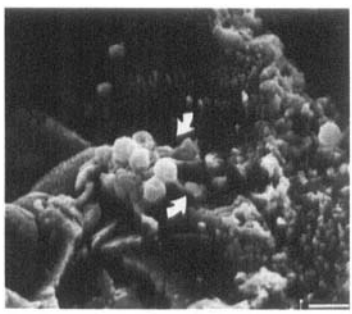


Figure 3. Scanning electron micrograph of hydroxyapatite. Note the formation of biofilm and microcolony that are anchored to the hydroxyapatite surface by fibrous strands (arrows). Bar, 1 micron.

Results

Bacterial adherence of *S. epidermidis* on biomaterial samples was confirmed by SEM after a 1 h exposure (Figure 1). Adherent bacterial microcolonies and exopolysaccharide biofilm were observed on the surface of HA and HA-Ti. Under higher magnification, bacteria were anchored to the substratum by fibrous strands and were partially or entirely covered by the exopolysaccharide matrix (Figures 2 and 3).

Table 1. Adhesion and colonization of biomaterials. Mean *SE*

Biomaterial (n 15)	Amounts of adhering cells (hundreds)	Adhesion-colonization index
Stainless steel	4.7 *1.2*	1.0
Titanium	3.7 *0.9*	0.8
HA	135 *19*	29
HA–Ti	6.4 *1.6*	1.4

At the beginning of the experiment, the number of bacteria in the suspension was 3×10^8 CFU/mL. The number of bacteria adhering to the biomaterials was from 4.67×10^2 cells to 135×10^2 cells and was ranged greater on HA than on the other biomaterials. The total number of bacteria on the surface indicated the number involved in adhesion plus the increment of colonization. The adhesion-colonization index for the HA was from 22 to 36 times higher than that for the other materials ($P < 0.05$, Table 1).

Discussion

Our findings clearly indicated that the HA bioactive material was colonized by *S. epidermidis* more extensively than was the case with the 3 other implant materials. This means that the different physical properties and configuration of the surface of the materials may affect the bacterial adherence (Gristina 1987). Generally, the initial events in bacterial attachment to a substratum are considered to include a nonspecific process, such as the interaction between the surface charge, hydrophobic interactions and van der Waals' forces and hydrodynamic forces, and specific adhesion is mediated by proteinaceous adhesion in a ligand-receptor interaction (Cowan et al. 1987, van Dijk et al. 1987, Satou et al. 1988, Leckband et al. 1992). Glycocalyx may also be important in initial adhesion and function in bacterial aggregation and colonization (Gristina 1985, Merritt and Chang 1991). The surface of the biomaterial has different surface energies, hydrophobicities, porosities and chemical compositions, all of which direct their receptivity to adhesion, partially determine the viability of the colony and possibly determine the outcome of infection.

Hydroxyapatite is thought to be a bioactive (Geesink 1990) and very biocompatible material. Because of the presence of free calcium and phosphate compounds at their surface, the hydroxyapatite surface is charged positively (Clark et al. 1978). The metal and bacterial surfaces are charged negatively. This may explain why HA has more bacterial adherence because negatively charged bacteria are attracted to the positively charged HA surface. Titanium is a bioinert material and shows good osseointegration. It has different properties from other metals, such as stainless steel when implanted in bone (Gristina 1987, Nasser et al. 1990). Generally, surgical alloys when implanted are surrounded by a thin, fibrous tissue capsule. In our experiment, the amount of bacteria adhering to the titanium alloy was similar to stainless steel. Bacterial adherence to the HA-coated titanium was slightly higher than to plain titanium and was much less than to HA. This phenomenon remains inexplicable. HA has more attractive properties for bacterial adherence, which suggests that bioactive materials are susceptible to infections. Gristina (1987) suggested the occurrence of a "race for the surface" phenomenon between bacteria and tissue cells on the biomaterial surface. Tissue cells can more easily adhere to a bioactive material surface and establish secure bonds.

We propose the following mechanism of biomaterial-induced infection from SEM findings. Bacteria arriving randomly in the vicinity of a biomaterial use it as a suitable substrate, along which they grow and propagate. Small numbers of bacteria attach to irregularities along the biomaterial surface. These attached bacteria produce a glycocalyx, which mediates bacterial adhesion and makes strong bonds with biomaterials. Once the bacteria are firmly attached to the substrate, they form additional bonds with one another or with the substrate and build up bacterial colonies. These bacterial colonies are finally enclosed by a proliferated glycocalyx matrix or biofilm with which each bacterium lives in a favorable niche. Glycocalyx biofilm enhances bacterial resistance to the host defense mechanisms and antibiotics. These protective functions of the glycocalyx biofilm may account for the demonstrable persistence and inherent antibiotic resistance of biomaterial-associated infections.

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