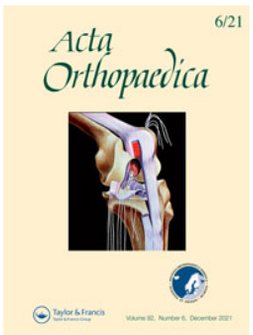

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Increased resistance of bacteria after adherence to polymethyl methacrylate

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The pathobiology of total joint prosthesis infection was investigated in vitro. Discs of polymethylmethacrylate (PMMA) were exposed to a suspension containing cells of 10^8 per mL *Staphylococcus epidermidis* E-46. After 12 hours, exposed discs were rinsed with phosphate-buffered saline and placed in brain heart infusion broth containing antibiotics (2.5 mg per mL of Cephaloridine). After gentle shaking for 24 hours at 37 °C, the bacteria on the PMMA surface were detached and washed with phosphate-buffered saline to remove the antibiotics. Compared with the free bacteria which were detached from the PMMA by sonication immediately after exposure to the antibiotic solution, those allowed to remain adhered to the PMMA surface were more resistant to antibiotics. Scanning electron microscopy showed accumulation of bacteria surrounded by slime on PMMA discs exposed for 12 hours.

Our results indicate that resistance of bacteria to antibiotics is increased after adherence to the biomaterial and formation of a slime layer.

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Once established, biomaterial-centered infections are notoriously resistant to treatment and often persist until the device or prosthesis is removed (Dougherty and Simmons 1982, Gristina and Costerton 1985, Antti-Poika et al. 1990). Images of bacterial extracellular structures are increasingly detailed (Umeda et al. 1987, Oga et al. 1988), and these images may be useful in determining the mechanism of resistance of biomaterial-centered infection. We have previously reported that bacterial slime is formed by *S. epidermidis* E-46 in vitro (Oga et al. 1988). Some studies have demonstrated that the development of microcolonies and the thick slime layer can protect bacteria from antibiotics, phagocytosis, and antibodies (Schwarzmann and Boring 1971, Baltimore and Mitchell 1980, Gristina et al. 1985).

The purpose of this study was to clarify the formation of extracellular structures by—and the change in resistance to antibiotics—bacteria cultured on biomaterials.

Material and methods

Bacterial culture

Staphylococcus epidermidis strain SE-46, isolated by Dr. K. Iwata (Department of Microbiology, Faculty of Medicine, University of Tokyo, Tokyo) from a biomaterial-centered infection, was used (Yamamoto and Iwata 1986). This strain is catalase-positive, coagulase-negative, nonhemolytic and encapsulated, and forms diffuse colonies on serum agar. The bacteria were cultured for 24 hours in 250 mL of Trypticase soy broth (Difco Laboratories, Detroit, MI) without dextrose supplemented with 0.5 percent gluconic acid (Sigma Science, St. Louis, MO). The culture fluid containing the nonadherent bacteria was discarded. Bacteria adhering to the glass flask were collected by vigorous washing with phosphate-buffered saline (PBS; 0.005-M phosphate, 0.15-M NaCl, pH 6.8) followed by gentle sonication (Sonogen, Cole-Parmer, Chicago, IL). The suspended bacterial cells were collected after centrifugation (5,000 × g, for 10 min) and resuspended in PBS, and resuspension was standardized using an ADS-D Fuji spectrophotometer (Fuji Photo Film Co. Ltd., Tokyo) at 660 nm.

Antibiotics

Cephaloridine, 7-(2-(2-thienyl) acetamido)-3-(-1-pyridylmethyl) 3-cephem-4-carboxylic acid betaine (Schionogi Pharmaceutical Co. Ltd., Osaka, Japan), was used to prepare the antibiotic solution at a concentration of 2.5 mg/mL. The minimum inhibitory concen-

tration (MIC) for the original culture of *S. epidermidis* E-46 was 0.5 μg/mL.

Polymethyl methacrylate exposure

We studied sterile circular discs (diameter 8 mm; height 5 mm) of polymethyl methacrylate (PMMA) (Kyocera Co. Ltd., Kyoto, Japan), aseptically inserted into a suspension containing 10^8 CFU/mL *S. epidermidis* E-46. All PMMA discs were cleaned with sonication and sterilized with ethylene oxide prior to exposure to the suspension. After 2 and 12 h exposure, some PMMA discs were prepared for scanning electron microscopy. Some discs after exposure for various periods of time were prepared for bacterial counts and others were rinsed with PBS and placed in brain heart infusion broth (BHI broth, Difco Laboratories) containing 2.5 mg per mL of Cephaloridine. After gentle shaking for 24 h at 37 °C in the antibiotic broth, each PMMA disc was sonicated at low power for 5.5 min at room temperature and then vortexed for 30 sec in Vortex Genile (Scientific Industries, Inc., Bohemia, NY) to detach the bacteria from it. The suspension containing bacteria was filtered through a cellulose nitrate membrane filter (A045025A, pore size 0.45 μm, diameter 25 mm; Advantec Tokyo Co. Ltd., Osaka, Japan), which was then washed with 100 mL of PBS to remove the antibiotics around the bacteria. The filter was placed in 10 mL of PBS and sonicated and then vortexed. The suspension was pipetted onto an agar plate for bacterial count (Group A). In controls, Group B, the PMMA discs were sonicated immediately after exposure to BHI broth containing Cephaloridine for 24 h at 37 °C, and the broth containing free cells was gently shaken and filtered in the same manner as in Group A. The number of viable cells on the filter was also counted in the same way. Trials were repeated three times, and in each trial three PMMA discs were examined under the same conditions.

Scanning electron microscopy (SEM)

After suspension, the PMMA discs were immediately placed in 10 mL of 0.1 M cacodylate buffer (Wako Pure Chemical Industries Co. Ltd., Osaka, Japan) containing 5 percent glutaraldehyde (Wako Pure Chemical) and 0.15 percent ruthenium red (Wako Pure Chemical) and incubated at room temperature for 2 h, followed by three times 5 min washes with cacodylate buffer containing ruthenium red to remove the unreacted glutaraldehyde. The PMMA discs were then exposed to five alternating cycles of 2 percent osmium tetroxide (Wako Pure Chemical) in cacodylate buffer and thiocarbohydrazide solution (Wako Pure Chemical). This treatment impregnates the sample with large amounts of osmium. The samples were then dehydrated in an acetone series and critical point-dried. The samples were examined with a Hitachi-S 700 scanning electron microscope (Hitachi Co. Ltd., Tokyo).

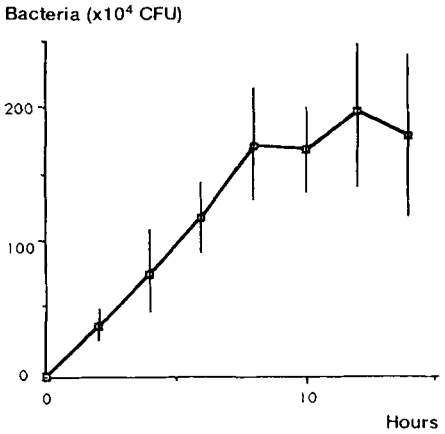

Figure 1. Mean number of viable bacteria adhering to biomaterial increased gradually with time after the insertion of the biomaterial into the bacterial suspension. Each value represents mean *SD* for 3 cultures.

Results

The number of bacteria adhering to the PMMA discs increased with time after their insertion into the bacterial suspension (Figure 1). The pattern of increase of bacteria was different from that in the bacterial culture, but a distinctly biphasic pattern of increase was not seen.

Observation with SEM revealed the presence of bacteria on the PMMA surface. Bacteria incubated for 2 h adhered to the surface of discs separately, and no background which could be called a slime layer was observed (Figure 2). But the surface of the PMMA discs gently shaken for 12 h in bacterial suspension presented a different appearance; bacteria grew together and a confluent bacterial slime layer covered them in some areas. The accumulation of the slime layer over time could be observed (Figure 3).

Exposure of the bacteria to Cephaloridine solution reduced the number of viable bacterial cells recovered. However, there was a marked difference in the magnitude of this reduction in free bacteria and that in attached bacteria. The reduction ratio of bacteria attributable to antibiotics was calculated by dividing by the number of bacteria on PMMA discs after shak-

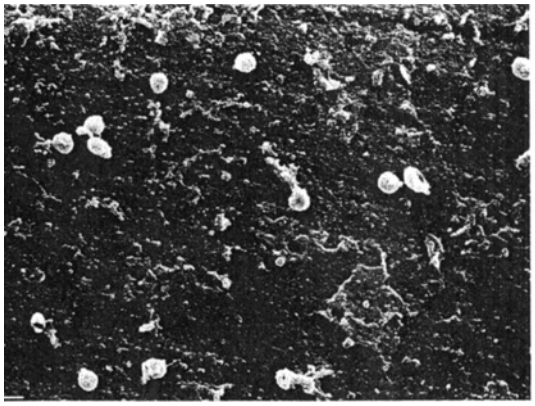

Figure 2. Scanning electron microscopy shows bacteria on a biomaterial. The bacteria on the biomaterial after 2 h of incubation are discrete, and extracellular products, such as slime layer, are rare. All bars represent 1 μm.

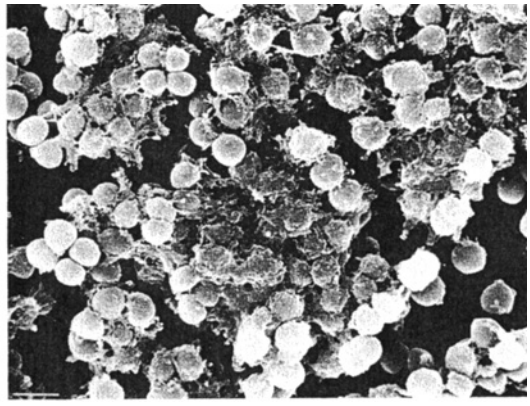

Figure 3. 12 h after the insertion of the biomaterial, the bacteria are covered with an amorphous extracellular matrix. Bacterial accumulation was also observed. Some bacteria had apparently grown under the slime layer.

Table 1. The effect of antibiotics on bacteria

Trial		1	2	3
Without antibiotics[a]		2220	2240	1190
With antibiotics				
Adherent bacteria[b]	1	83	121	37
(Group A)	2	34	91	26
	3	40	129	40
Average		53	113	34
Reduction ratio, $\times 10^{-3}$ (Ad)[c]		24	51	29
Free bacteria[d]	1	0	5.7	1.8
(Group B)	2	0.43	5.1	0.19
	3	0.22	1.3	0.34
Average		0.22	4.0	0.77
Reduction ratio, $\times 10^{-3}$(Fr)[c]		0.097	1.8	0.065
Ad/Fr		244	28	44

[a]The number of bacteria (thousand CFU) adhering to a biomaterial after 12 h exposure to a bacterial suspension without antibiotics (n 3).
[b]The number of bacteria (thousand CFU) adhering to a biomaterial after shaking for 12 h in an antibiotic solution (group A).
[c]Reduction of viable cells by antibiotics. The number of Groups A or B was divided by the number of bacteria on discs in antibiotic-free suspension.
[d]The number of free bacteria (group B; thousand CFU). The conditions are the same as in 2, except that sonication was performed immediately after the insertion of a biomaterial in an antibiotic solution.

ing in bacterial suspension for 12 h. The reduction ratio in free cells was more than 20 times that in adherent cells in all trials. The bactericidal effect of Cephaloridine on free cells (Group B) was far higher than that on cells adhering to the biomaterial (Group A).

Discussion

Slime is reported to possess antibiotic resistance as well as antiphagocytic properties (Johnson et al. 1986, Gristina et al. 1989), although the mechanisms of these properties have not been clarified. Furthermore, the direct comparison between free bacteria and adherent bacteria on a biomaterial has not been reported. In our study the adherent bacteria were found to be surrounded by a slime layer. The slime seemed to protect the bacteria from bactericidal agents. The mechanism by which slime-forming populations gain antibiotic resistance could be the optimization of polysaccharide production effected by the immediate presence of both a substratum and the ions from the substratum. The production and accumulation of an amorphous slime layer around bacteria may directly protect it from the infiltration of antibiotics by acting as a barrier. The presence of iron in *Staphylococcus* is known to increase its virulence and interfere with macrophage function, and may indirectly inhibit antibiotic penetration and action by optimizing metabolism and slime production. Iron or other products are already present within the envelope of bacteria on the surface of a biomaterial such as polymethyl methacrylate, and are readily available for metabolism (Gristina et al. 1987). It is reasonable to suggest that, with wear or corrosion, these elements may become available in ionic form within the slime layer.

In conclusion, we found that the inveteracy of foreign-body infections increases after the accumulation of bacteria and formation of slime. The first few hours before the development of the slime layer seemed to be the critical point in the treatment of this type of foreign body infection.

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