Cells and Materials

Volume 1 | Number 3 Article 2

1991

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CELLS AND MATERIALS, Vol. 1, No. 3, 1991 (pages 199-208) 1051-6794/91$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

ADHESION OF STAPHYLOCOCCI TO BREAST PROSTHESIS BIOMATERIALS: AN ELECTRON MICROSCOPIC EVALUATION

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(Received for publication February 23, 1991, and in revised form August 9, 1991)

Abstract

Gram-positive coagulase-negative staphylococci have been implicated in breast prosthesis infection. *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Staphylococcus hominis* strains were examined for adhesion and proliferation morphology to polyurethane foam and smooth silicone rubber prosthetic biomaterials. Ruthenium red, alcian blue and ruthenium red-lysine in *en bloc* procedures were applied to optimally visualize the polysaccharide slime. Ruthenium red processing resulted in observation of additional outer material close to the cell wall, more than seen in the absence of cationic reagent. Alcian blue preserved fibrous or net-like strands or meshworks of material. Ruthenium red-lysine preserved considerable amounts of slime covering the cocci as well as extending between them and the prosthetic surface. Elongate strands of slime appeared to facilitate attachment to the substrate and projection of cells away from the substrate. Where this material was lacking, cell mass buildup was required for projection from the substrate. The amount of fibrous material observed corresponded to levels of slime production known for each staphylococci strain. Some extracellular material was observed for even the classified "non-slime" *S. hominis* strain SP2. The ruthenium red-lysine approach was optimal for visualization of slime for all bacterial species in this study.

Key words: staphylococci, bacterial adhesion, bacterial proliferation, breast prosthesis, slime, ruthenium red, ruthenium red-lysine, alcian blue, silicone rubber, polyurethane foam.

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Introduction

The use of biomaterials in medicine has increased dramatically over the past 20 years. The increased use of bioprosthetics in surgery has been associated with a concomitant increase in device associated infections. The staphylococci are a ubiquitous group of microorganisms which colonize the skin and are often recovered from the surface of these infected biomaterials (Edmiston *et al.*, 1989). The adherence of these organisms to the biomaterial surface is often influenced by the structure and composition of the prosthetic along with the specific surgical technique used to insert the device (Gristina, 1987; Schmitt *et al.*, 1986).

Breast implants are of great clinical value in augmentation and reconstructive surgery. Typically, the prosthesis consists of a silicone rubber shell or envelope which may be filled with saline or silicone gel. The outer surface may be smooth or textured silicone rubber or coated with polyurethane foam. Infections, though rare, are generally caused by gram-positive staphylococci. Overt infections caused by coagulase-positive *S. aureus* may result in extrusion of the prosthesis (Courtiss *et al.*, 1979; McGrath and Burkhardt, 1984). Severe capsular contracture may result. Capsular contracture is the formation of a constricting fibrous envelope around the prosthesis, which can cause firmness and distortion of the breast (Asplund, 1984). This is the most common complication of breast prostheses and occurs generally without overt infections. Recently an association with subclinical infection by coagulase-negative staphylococci and fibrous capsular contracture has been suggested as causal (Shah *et al.*, 1981; Burkhardt *et al.*, 1981; Burkhardt *et al.*, 1986). Therefore, adhesion and proliferation of staphylococci to breast prostheses has great clinical relevance.

Following a rapid adhesion of bacteria to prosthetic biomaterials is the production of polysaccharide slime (Costerton *et al.*, 1981; Edmiston *et al.*, 1989). It covers the bacteria and substrate, becoming a protective layer for bacterial proliferation and microcolony formation (Costerton *et al.*, 1981; Peters *et al.*, 1982; Franson *et*

al., 1984). The present mechanisms of initial microbial adherence are poorly understood. But extracellular polysaccharide which we collectively call slime is likely responsible for persistence of the organisms to the biomaterial surface. Production of slime is a known virulence factor (Edmiston *et al.*, 1989) and is enhanced in the presence of a foreign body (Christensen *et al.*, 1983). Secretion and proliferation of polysaccharide slime is an important part of the complex phenomenon of bacteria polymer associated infection. *Staphylococcus aureus* strain 25923 and *Staphylococcus epidermidis* strain RP62 are known producers of slime. *Staphylococcus hominis* strain SP2 is classified as a non-slime former.

To increase preservation of the polysaccharide slime during staphylococci proliferation on breast prosthetic biomaterials, we have used several fixation and *en bloc* procedures. The cationic reagents, ruthenium red (RR) and alcian blue (AB), traditionally were used to improve visualization of polyanions, notably acidic polysaccharide, by light microscopists. Ruthenium red was characterized by Luft (1971a, 1971b). It tends to favor reaction with polyanions of high charge density, such as acidic mucopolysaccharides and protein polysaccharides (Luft, 1971a, 1971b). Ruthenium red procedures for electron microscopy have been applied to study bacterial outer slime/capsular layers (Pate and Ordal, 1967; Springer and Roth, 1973; Titus *et al.*, 1982; Fassel *et al.*, 1990; Fassel *et al.*, 1991). The diamine, lysine, used in fixation improved preservation of the mammalian cell glycocalyx (Boyles, 1984). Lysine has been used with RR to improve visualization of bacterial outer layers in stable configurations often where collapse or loss of structure was evident with RR alone (Akin and Rigsby, 1990; Davies and Borriello, 1990; Jacques and Graham, 1989; Jacques *et al.*, 1990).

Alcian blue, another cationic reagent, has been used to demonstrate extended bacterial outer layers in several studies (Progulske and Holt, 1980; Herald and Zottala, 1988; Fassel *et al.*, 1991). Alcian blue is thought to react with acidic mucopolysaccharide moieties, perhaps through electrostatic or ionic interactions (Luft, 1971a; Scott *et al.*, 1964). Procedures employing RR, AB and RR-lysine were carried out for the test organisms in this study in prosthetic infection culture.

The aim of this approach was two-fold. First, to optimize visualization of the polysaccharide slime layers functional in proliferation of bacteria to breast prosthetic biomaterial. Second, to observe the morphology of staphylococci adhesion and proliferation to the biomaterial surface.

Materials and Methods

Bacterial Adherence Studies

Three staphylococci strains were tested for adherence characteristics against polyurethane foam and silicone rubber; *S. aureus* ATCC25923, *S. epidermidis* RP62 (slime producing strains) and *S. hominis* SP2 (characterized as a non-slime producer). The organisms were recovered from frozen storage (-70°C) and plated to blood agar plates to check viability. After 24 hours the organisms were inoculated to trypticase soy broth and incubated for an additional 18 hours at 35°C. The test substrates were polyurethane foam and smooth silicone rubber (Surgitek, Inc., Racine, WI) which were cut into 1 cm^2 segments for standardized studies. The prosthesis segments were not exposed to additional reagents or precleaned.

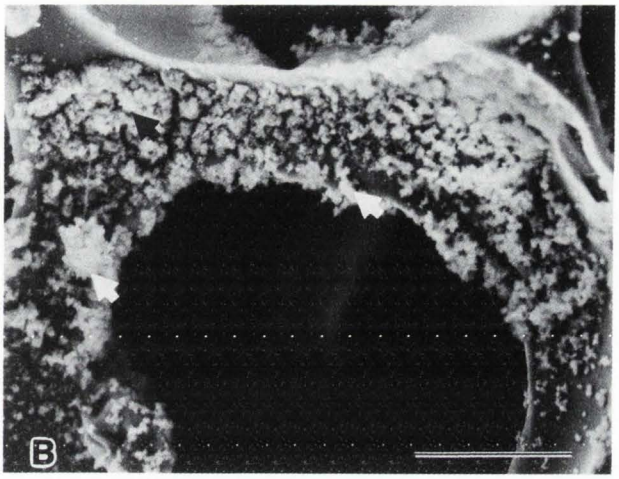
Adherence studies were performed by incubating 1 cm^2 polyurethane or smooth silicone rubber segments in a standardized staphylococcal inoculum (7.0 $\log_{10}$ colony forming units/ml). This was determined using optical densitometry and comparison with known standards. Prosthetic samples were removed at 4 and 24 hours post inoculation. Non-adherent cocci were removed by washing the prosthetic segments in Phosphate Buffered Saline pH 7.0 (3x) followed by ultrasonic oscillation at 20 KHz for 10 minutes which dislodged adherent cocci. Scanning electron microscopy of sonicated specimens was performed to confirm adequacy of dislodgement. The sonicate was serially diluted, plated on trypticase soy agar and the plates inspected at 4 and 24 hours. Microbial adherence was expressed as the $\log_{10}$ counts per cm^2 of prosthetic surface.

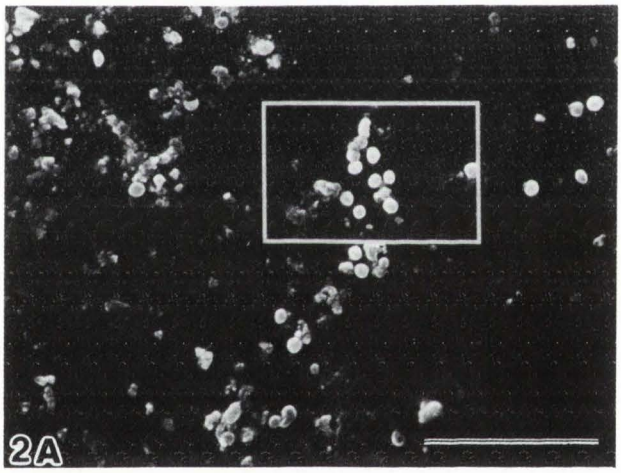
Scanning Electron Microscopy

For the glutaraldehyde/OsO_4 procedure, samples were fixed in 2.5% glutaraldehyde in buffer for 2 hours, washed three times for 10 minutes duration each wash, postfixed in 1% (w/v) OsO_4 for 2.5 hours, and washed three times for 10 minutes each. The buffer, 0.1 M cacodylate pH 7.3, was used for all solutions.

For the RR procedure, pre-fixation was in 0.2% glutaraldehyde and 0.15% RR for 30 minutes. This was followed by fixation in 1% glutaraldehyde and 0.05% RR for 2 hours. The 0.05% RR was included with 0.1 M cacodylate buffer for three washes of 10 minute duration each. Postfixation in 2% OsO_4 and 0.05% RR for 2.5 hours preceded another wash cycle (Fassel *et al.*, 1990). For the alcian blue procedure, AB was substituted for RR in all solutions at identical percentages as in the RR procedure (Fassel *et al.*, 1991).

For the RR-lysine procedure, samples were prefixed in 75 mM lysine, 0.075% RR and 2.5% glutaraldehyde for 20 minutes. Fixation in 0.075% RR and 2.5%





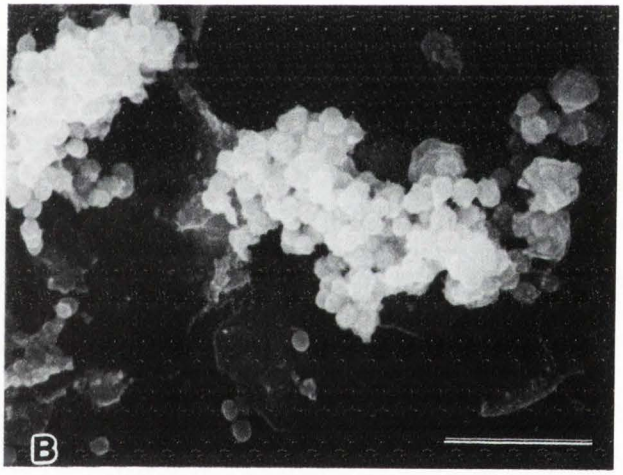


Figure 1A: Polyurethane foam biomaterial has an interstitial nature and honeycomb appearance with a considerable surface for bacterial adhesion and proliferation. Bar = 500 μm.

Figure 1B: Clusters of *S. epidermidis* RP62 (arrows) preserved by the RR-lysine procedure adhere to the curving foam surface. Bar = 100 μm.

Figure 2A: The silicone rubber biomaterial, characterized by a smooth single surface plane, has several *S. epidermidis* RP62 cells (several enclosed in box) attached, glutaraldehyde/OsO_4 fixation. Bar = 10 μm.

Figure 2B: Clusters of *S. epidermidis* RP62 project outward from the silicone rubber surface. Note also, the absence of extracellular slime by the glutaraldehyde/ OsO_4 fixation. Bar = 5 μm.

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glutaraldehyde for 2 hours followed (Jacques and Graham, 1989). After washing in buffer three times for 10 minutes duration each wash, samples were postfixed in 1% OsO_4 for 2 hours. Another wash cycle followed.

After one of the fixation sequences described, samples were dehydrated in ethanol 10%, 25%, 50%, 70%, 95%, and two anhydrous 100% changes of 10 minutes each. Samples were critically point dried in CO_2 and coated with gold-palladium. Samples were then studied on a Hitachi S-520 scanning electron microscope at 20 kV.

Results

The polyurethane foam biomaterial has a honeycomb appearance (Figure 1A) with many interstices and surfaces that provide numerous opportunities for adhesion and proliferation by staphylococci. Figure 1B (RR-lysine procedure) shows clusters of *S. epidermidis* RP62 (arrows) on the curved surface.

In contrast, the silicone rubber biomaterial provides a single surface plane with infrequent irregularities. Irregularities in sterile control specimens have been

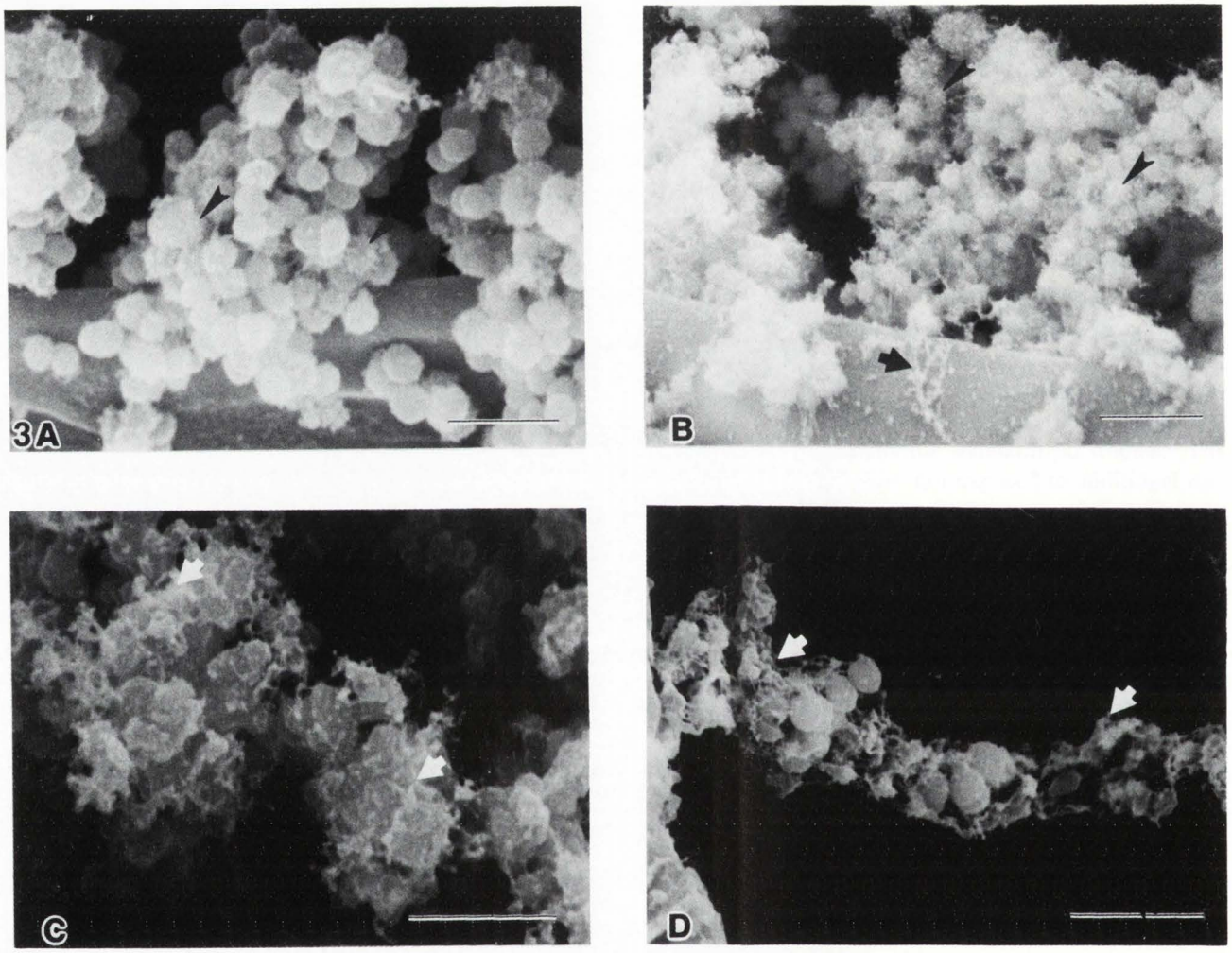

Figure 3A: By the RR procedure, fibrous cellular surface material (arrowheads) and strand-like extensions between cells are preserved for *S. epidermidis* RP62 cocci on polyurethane foam. Bar = 2.5 μm.

Figure 3B: By the RR-lysine procedure, a more extensive elaboration of slime is preserved with fibrous material (arrowheads) densely covering cells. Strands also extend between *S. epidermidis* RP62 cells and the polyurethane foam surface (arrow). Bar = 5 μm.

Figure 3C: By the AB procedure, fine fibrous strands (arrows) cover *S. epidermidis* RP62 on silicone rubber. Bar = 5 μm.

Figure 3D: Also by the AB procedure, extensive net-like meshwork (arrows) are seen holding *S. epidermidis* RP62 in stable projections from polyurethane foam substrate. Bar = 10 μm.

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observed previously and are also associated with colony formation in *in vitro* studies (Sanger *et al.*, 1989). Individual *S. epidermidis* RP62 cells adhered to the smooth surface are shown in Figure 2A, glutaraldehyde/OsO_4 fixation. The buildup of clusters project outward (Figure 2B). Additional irregular material is frequently found on the silicone rubber surface beneath or in the vicinity of cells. The absence of extracellular bacterial slime on the smooth round cocci is also illustrated here, by the glutaraldehyde/OsO_4 fixation. Some fibrous cellular surface material (arrowheads) is preserved, as well as, strand-like extensions between some *S. epidermidis* RP62 cocci on polyurethane foam (Figure 3A) by the RR procedure. A more extensive elaboration of slime is seen for the RR-lysine procedure (Figure 3B). Here, fibrous material (arrowheads) densely covers cocci and clusters. Strands also extend between cells and the foam surface (arrow). By the AB procedure, a fine material of fibrous strands (arrows, Figure 3C) is also preserved, shown here for *S. epidermidis* RP62 on silicone rubber.

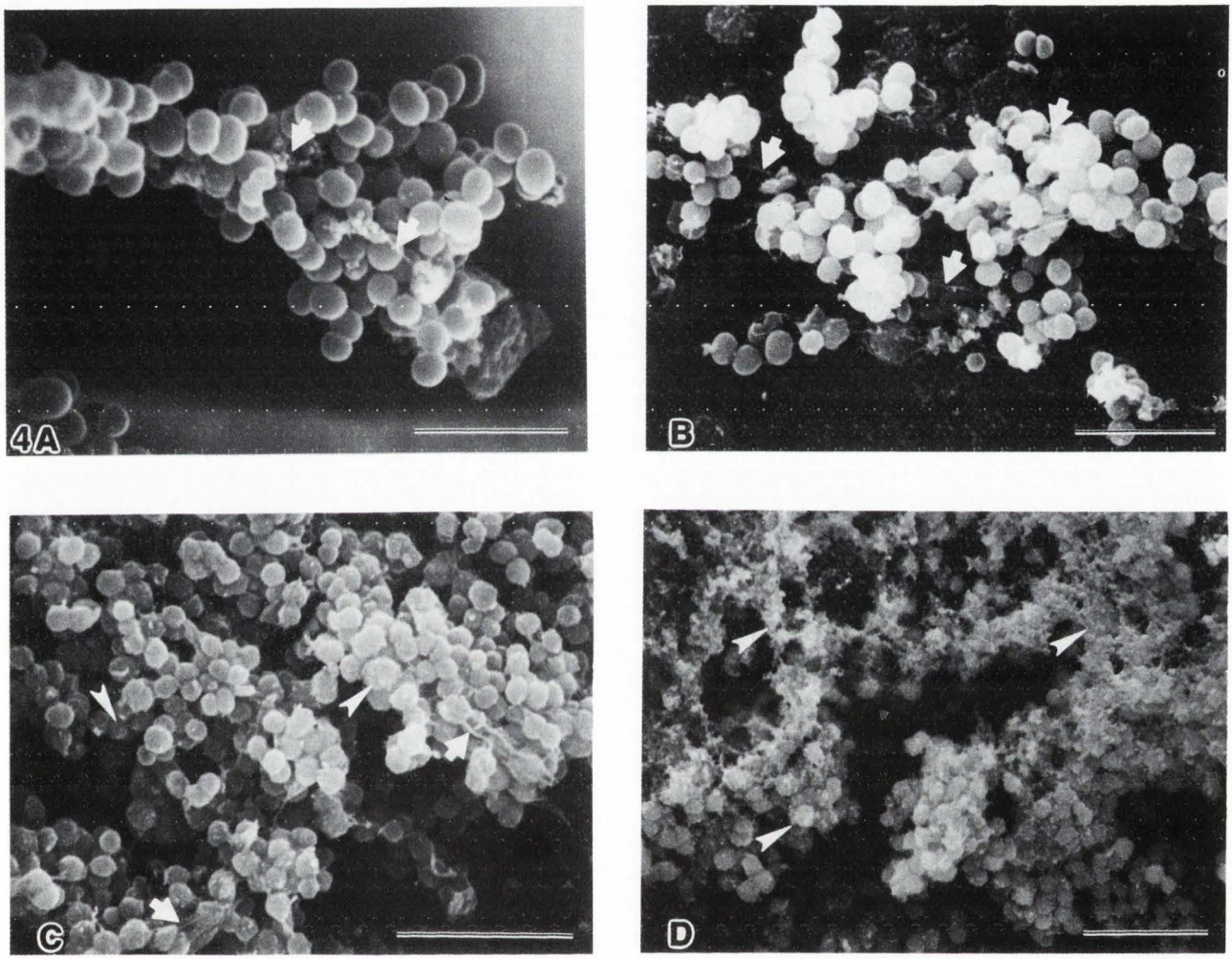


Figure 4A: For *S. hominis* SP2 cocci project into the interstitial space of the polyurethane foam. Few fibrous strands (arrows) extend between the smooth cells by the glutaraldehyde/OsO_4 procedure. Bar = 5 μm.

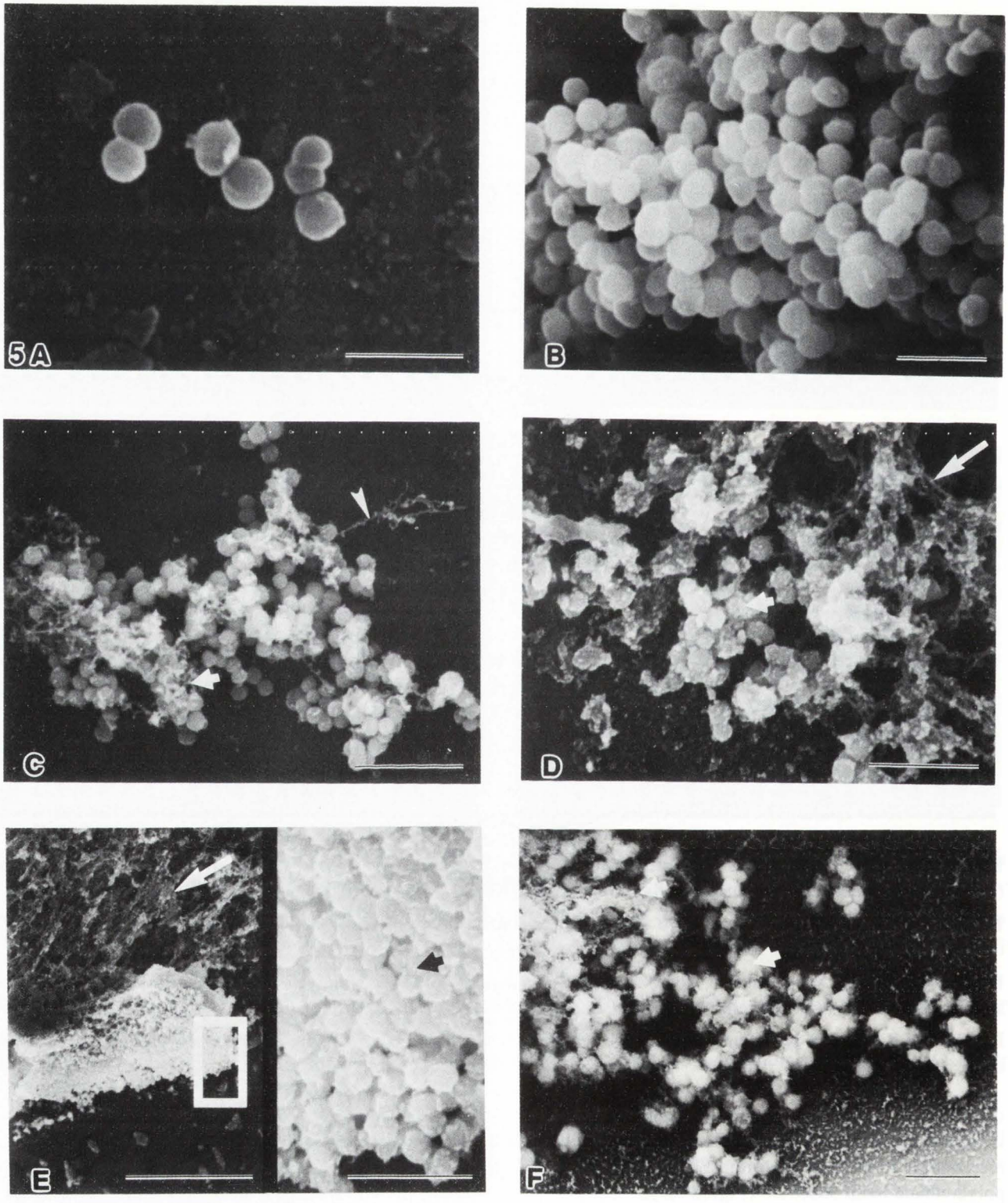
Figure 4B: By the RR procedure, more fibrous strands (arrows) are preserved between smooth *S. hominis* SP2 cocci on silicone rubber. Bar = 5 μm.

Figure 4C: By the AB procedure, some surface material (arrowheads) is seen on some *S. hominis* SP2 cells. Fine strands also extend between cells (arrows). Bar = 5 μm.

Figure 4D: By the RR-lysine procedure, most extensive extracellular material upon and between cells (arrowheads) is seen. Bar = 10 μm.

Occasionally, an extensive net-like meshwork (arrows) is formed between cells, holding them in apparently stable configurations that can project well away from the polyurethane foam substrate (Figure 3D).

For the "non-slime" former, *S. hominis* SP2, a smooth surface characterizes the cocci (Figure 4A) by the glutaraldehyde/OsO_4 procedure. The ability to build stable projections away from the polyurethane foam and extending into the interstitial space is still present, although made up of many more cells (Figure 4A versus Figure 3D). A few fibrous strands can be observed between cells (arrows, Figure 4A). The strands are more extensively preserved by RR (Figure 4B, arrows) with many extensions seen between smooth cells, networking across the silicone rubber surface. By the AB procedure (Figure 4C), the observations are similar, with some additional surface material (arrowheads) on some cells and fine strands extending between cells (arrows). Deposits of material are also found. The most extensive buildup of extracellular material upon and between cells (arrowheads) is seen by the RR-lysine procedure (Figure 4D).



S. aureus shows a smooth cell surface and absence of slime by the glutaraldehyde/OsO_4 fixation (Figure 5A, silicone rubber). Ruthenium red (Figure 5B) reveals a cluster of cells building up from the polyurethane foam substrate. Extracellular material is infrequent. Many more fibrous strands (arrow) cover some cells and extend between cells and foam substrate (arrowhead, Figure 5C) by AB. Figures 5D, E and F compare the increased preservation of slime by the RR-lysine procedure between silicone rubber and polyurethane foam substrates for *S. aureus*. For both substrates, the slime material is more elaborate over cells than by the other

procedures. On the silicone rubber (Figures 5D and 5E) extracellular slime covers cells forming thickened connections between cells (long arrow). It is occasionally observed to increase, forming extensive overlapping masses of strands (Figure 5E left). Cells from this area (Figure 5E right) show some surface material. An extracellular meshwork of this density has not been observed on polyurethane foam to date for *S. aureus*, although cell surface material and more extended strands between cells are seen (Figure 5F).

Interestingly, adhesion kinetic data (Sanger *et al.*, submitted) suggests more *S. aureus* cocci adhere to silicone rubber (14,800 ± 6,800 CFU/sq.cm.) than to polyurethane foam (8,000 ± 1,673 CFU/sq.cm.). The noted slime producer *S. epidermidis* RP62 prefers the polyurethane foam substrate (276,800 ± 28,869 CFU/sq.cm.) to the silicone rubber (20,000 ± 1,789 CFU/sq.cm.). To polyurethane foam, the "non-slime" producer *S. hominis* SP2, shows less tendency to adhere to either substrate (polyurethane foam: 10,400 ± 5,075 CFU/ sq. cm., silicone rubber: 2,400 ± 1,166 CFU/sq.cm.).

Legend for Figure 5 on the facing page

Figure 5A: *S. aureus* shows a smooth cell surface and absence of slime by the glutaraldehyde/OsO_4 fixation on silicone rubber. Bar = 2.5 μm.

Figure 5B: By the RR procedure, extracellular material is infrequently seen between a cluster of *S. aureus* cells from the polyurethane foam substrate. Bar = 2.5 μm.

Figure 5C: By the AB procedure, fibrous strands (arrow) cover some cells. Extensions of slime (arrowhead) are seen between cells and the foam substrate. Bar = 5 μm.

Figure 5 D, E and F: By the RR-lysine procedure, silicone rubber (D and E) and polyurethane foam (F) substrates for S. aureus are shown. Extracellular slime covering cells (arrow) and forming thickened connections between cells (long arrow) appears on silicone (D). This extracellular slime is occasionally observed to increase to form extensive overlapping masses of strands (E left). Cells from this area (enclosed in box, E left) are shown at increased magnification in E right. Some surface material is apparent (arrow). This extensive formation has not been observed to date on polyurethane foam (F), although cell surface material and more extended strands between cells are seen (arrows). Bars = 5, 50, 5 and 5 μm respectively.

Discussion

Visualization of polysaccharide slime layers important in bacterial adhesion and proliferation to breast prosthetic material was improved by use of cationic reagents, RR-lysine and AB, in *en bloc* procedures. Lysine, a diamine, is positive at physiological pH. It is thought to form large cross-linked polymers with glutaraldehyde. Biological structure fixed with these complexes have improved stability through later stages of electron microscopy processing (Boyles, 1984; Boyles *et al.*, 1985). Ruthenium red reacts with highly negatively charged polyanions, such as acidic mucopolysaccharides (Luft, 1971a; 1971b). Ruthenium red-lysine procedures have been highly effective in improving visualization of bacterial outer layers (Akin and Rigsby, 1990; Davies and Borriello, 1990; Jacques and Graham, 1989; Jacques *et al.*, 1990). Alcian blue has also been used previously in improving visualization of extensive outer bacterial layers (Progulske and Holt, 1980; Herald and Zottala, 1988; Fassel *et al.*, 1991). Its reaction with acidic mucopolysaccharide through either electrostatic or ionic interactions (Luft, 1971a; Scott *et al.*, 1964) improves visualization of polysaccharide slime material.

The RR-lysine procedure was most effective in preserving extensive elaborations of slime. It was similar for all species and substrates. Additionally, thickened masses of fibrous strands were seen for *S. aureus* on smooth silicone rubber. It is unknown whether this observation may concur with the observed tendency for *S. aureus* cells to preferentially adhere to smooth silicone rubber versus polyurethane foam. Such an extensive build up of slime could provide an additional protection and favorable environment for bacterial proliferation. This buildup would favor infection by *S. aureus* of silicone rubber prosthesis. Infection of a breast prosthesis by coagulase-positive *S. aureus* results in a purulent discharge often controlled only by removal of the prosthesis (Courtiss *et al.*, 1979). Coagulase-negative staphylococci also adhere well to smooth silicone rubber. Clinically these organisms tend not to produce a purulent response and have been found in association with fibrous capsules (Shah *et al.*, 1981; Burkhardt *et al.*, 1981). Perioperative antibiotics have reduced the incidence of capsular contracture (Burkhardt *et al.*, 1986) around smooth silicone implants. As demonstrated in this study, polyurethane foam provides an excellent surface for adherence and proliferation of coagulase-negative staphylococci. However, early clinical results show a lower incidence of contractures with polyurethane foam covered prostheses than smooth silicone rubber (Capozzi and Pennesi, 1981; Melmed, 1988; Shapiro, 1988). Longer follow up will be needed to determine if coagulase-negative infections emerge as a clinical problem.

The AB procedure mainly preserved extracellular strands of slime between *S. aureus* and *S. hominis* SP2 cells. More extensive elaborations were seen for the noted slime producer, *S. epidermidis* RP62. For *S. epidermidis* RP62, extensive material covered cells in a manner similar to RR-lysine. Strands of slime also provided sufficient support for projection of cells out into the interstitial space of polyurethane foam. *S. epidermidis* RP62 preference for proliferation on polyurethane foam over the one surface plane of silicone rubber, appears to agree with this scanning electron microscopy observation.

Ruthenium red provided the least improvement in slime preservation of the procedures compared here. It did increase strands for *S. hominis* SP2 and *S. epidermidis* RP62, as well as some fibrous cell surface material for *S. epidermidis* RP62. It was least effective for *S. aureus*.

The least effective of all the procedures tested was the glutaraldehyde/OsO_4 fixation where no cationic reagents were employed. For this procedure, only a few extracellular strands were seen for *S. epidermidis* RP62 or *S. hominis* SP2. None were observed for *S. aureus*.

Thus, RR-lysine was optimal for all species tested. The effectiveness of AB varied with the species, with it the most effective for *S. epidermidis* RP62. This variation in species effectiveness of slime preservation may be due to some difference in polysaccharide composition of the slime between species.

Morphological features of elongate strands appear to facilitate attachment to the biomaterial substrate. Strands between cells aided the buildup and projection of cocci away from the substrate surface, this was most notable for *S. epidermidis* RP62. This could agree with the strong tendency for *S. epidermidis* RP62 cells to adhere preferentially to polyurethane foam versus silicone rubber (Sanger *et al.*, submitted). However, buildup of a cluster away from the substrate was also noted with proliferation of individual cells, as for *S. hominis* SP2.

A classified "non-slime" producer, *S. hominis* SP2, was found to form extracellular features similar to that of other slime producers, although apparently less in quantity. This agrees with other work (Goheen *et al.*, 1990), where use of cationic reagents preserved extracellular slime for this strain. This further illustrates the advantage of this approach of using cationic reagents to improve slime preservation for scanning electron microscopy.

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Discussion with Reviewers

H. Freeman: Your study indicates greater attachment of *S. aureus* on silicone rubber substrates than on polyurethane foam. Is this observation a consequence of the highly convoluted nature of the foam or an inherent difference in the biomaterial surface itself? What is a plausible reason for such a difference?

Authors: We feel an inherent difference of the biomaterial surface is more likely. The convolutions of the foam do not appear to interfere with dislodgement by sonication. SEM of sonicated specimens indicates efficient bacterial removal from either substrate. Also, *S. epidermidis* RP62 has a greater attachment to the polyurethane foam than silicone rubber (Sanger *et al.*, submitted). The three dimensional curving foam surface versus the one dimensional surface plane of the silicone rubber presents the same problem for both species. A preference, in either case, for the chemical nature or other inherent property of the substrate may be more likely. Clarification of this issue awaits further study.

H. Freeman: Did you use a smooth or textured silicone rubber prosthesis envelope in your studies? Would you expect there to be a difference in the adhesion of staphylococci strains?

Authors: Only smooth silicone rubber was used in this study. It is currently unknown how smooth versus textured silicone rubber affects adhesion. This is a subject of future study.

A. Molinari: In previous studies, the use of lectins to avoid the loss of capsular material of gram-positive bacteria during the sample preparation procedures for electron microscopy, was introduced. Data obtained indicated that lectins are useful agents in preserving

highly water-soluble capsular components for both unembedded and embedded samples (G. Orefici *et al.*, 1986 FEMS Microbial. Lett. 34: 111-115; A. Molinari *et al.*, 1988 Histochemical Journal **20**, 526-530). Lectins show specific affinity to different glucidic residues, then they can give more information about the difference in polysaccharide composition of the surface structures of the microorganisms. This methodological approach might also be applied in the study of the production of polysaccharide slime. Could the authors discuss this point?

Authors: The use of lectins in the visualization of microbial capsular material has been documented by other investigators. It is plausible that specific lectin-glucidic acid residue interactions could be used to study the production of microbial slime under different physiologic and environmental conditions.