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Bacterial Adherence and Glycocalyx Formation in Osteomyelitis Experimentally Induced with *Staphylococcus aureus*

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A surgical procedure allowed the placement of a silicone rubber catheter in the marrow cavity of the tibia of a rabbit and also allowed the introduction of a sclerosing agent (sodium morrhuate) and cells of *Staphylococcus aureus*. Osteomyelitis developed in 60% of the animals so treated, and the infecting microorganism was recovered from the infected tibias of the animals that developed this disease. All blood cultures taken 24 h after the infection were negative for *S. aureus*. Radiological findings consisted of osteolytic changes, the occurrence of sequestration and periosteal reactions, and sclerosis in the infected bones. Sections of bone prepared for histological examination confirmed the diagnosis of osteomyelitis. Transmission and scanning electron microscopy of samples of bone marrow, bone chips, and the catheters taken from the infected tibiae revealed gram-positive cocci embedded in a very extensive matrix of ruthenium red-staining glycocalyx adhering to the bone and the implanted catheter. It is proposed that this extensive glycocalyx served a protective function for the bacteria and was important in bacterial adherence and thus played an important role in bacterial persistence and the development of osteomyelitis in these rabbits.

The chemical nature of the bacterial cell surface is of paramount importance in the etiology of bacterial disease (11) because it is this surface that actually interacts with the surfaces of target cells and phagocytes. The bacterial surface has, however, been somewhat ephemeral in electron microscopy (9) because the exopolysaccharide polymers that constitute the glycocalyx (10, 11) are very highly (99%) hydrated (36), and they condense very radically during the dehydration required for both transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Furthermore, polysaccharides have little affinity for the heavy metal stains used to produce contrast in TEM preparation (9), and even the condensed residues of exopolysaccharide structures usually escape detection by these methods. Ruthenium red was developed (19) as a specific stain for polyanionic structures, including polysaccharides, and it has been extensively used to stain bacterial glycocalyces in vitro (4, 20, 26, 34), in vivo (4, 18), and in natural environments (12, 15). Because freeze-etching does not involve specimen dehydration, the exopolysaccharide glycocalyx can also be visualized by this method (33). The problem of radical condensation of the glycocalyx during dehydration was partially solved by pretreatment with stabilizing reagents such as lectins (3) and specific antibodies (2, 4, 5, 18, 20), and most bacterial cells are seen to be completely surrounded by capsular glycocalyces of impressive dimension (0.2 to 4.0 μm). These data suggest that glycocalyx polysaccharides constitute a large proportion of the molecules actually exposed at the surface of a bacterial cell, and this perception is of special interest because the glycocalyces of the animal cells with which they react as pathogens are also largely composed of polysaccharides (29).

Bacteria growing in natural environments produce very extensive glycocalyces that mediate both their attachment to surfaces and their formation of microcolonies and eventually of thick biofilms (10, 12, 15). Bacteria growing within these glycocalyx-enclosed microcolonies have full access to organic nutrients because the exopolysaccharide matrix acts as an ion exchange resin (17), but they are protected from biological agents such as amoebae and bacteriophages (10) and from antibacterial chemicals such as biocides (30). The microcolonial mode of bacterial growth has been noted in several chronic bacterial diseases, such as cystic fibrosis (23), and in prosthesis-related colonization (21, 22) and infection (24), and it has been suggested that extensive glycocalyx production can protect bacteria from surfactants (13), antibodies (1), phagocytosis (32), and antibiotics (8). The present study was undertaken to determine the mode of bacterial growth in osteomyelitis because this disease is known to be both persistent and refractory to antibiotic chemotherapy.

MATERIALS AND METHODS

Bacteria. *Staphylococcus aureus* G-24-82 was isolated from a patient with osteomyelitis at the Veterans Administration Medical Center, Johnson City, Tenn.

Growth conditions. *S. aureus* was grown in tryptic soy broth, (Difco Laboratories, Detroit, Mich.) at 37°C for 24 h, and 0.1 ml of the culture suspension (containing about 10^6 microorganisms) was injected into the left tibia of each rabbit infected. For recovery of bacteria from the infected bone, samples of marrow, bone chips, and purulent material were plated on tryptic soy blood agar, laked sheep blood agar, and chocolate agar and were also inoculated into tryptic soy broth and anaerobic brain heart infusion broth (Difco). Aerobes were identified by standard microbiological methods. Gram stains were performed on portions of all samples taken from an infected animal.

Animal experimentation. The experimental rabbit osteomyelitis model used in this study was a modification of the models developed by Kurek et al. (16), Norden (25), and Scheman et al. (31). Male New Zealand white rabbits

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TABLE 1. Animal model protocol

Rabbit no.	Catheter[a]	Injection with:		Radiological evidence of osteomyelitis	Microorganisms recovered[b]
		Sodium morrhuate (5%, 0.1 ml)	*S. aureus* (0.1 ml)		
1	+			Negative	None
2	+			Negative	None
3	+			Negative	None
4	+			Negative	None
5	+	+	+	Positive	*S. aureus*
6	+	+	+	Negative	None
7	+	+	+	Positive	*S. aureus*
8	+	+	+	Negative	None
9	+	+		Negative	None
11	+	+	+	Positive	*S. aureus*
15	+	+		Negative	None

[a] A barium-impregnated, silicon rubber catheter (4.5 cm long) was implanted in the left tibia.

[b] Recovered from samples taken from bone marrow and bone scrapings of the infected tibia.

weighing 1.5 to 2.0 kg were anesthetized with (per kilogram of body weight) 0.15 ml of Innovar-Vet (fentanyl, 0.4 mg/ml, and droperidol, 20 mg/ml) (Pitman-Moore, Inc., Washington Crossing, N.J.), 7 mg of Vetalar (ketamine) (Parke, Davis & Co., Morris Plains, N.J.), and 1 mg of Rompun (xylazine) (Haver-Lockhart Laboratories, Shawnee, Kans.). The left hind leg was shaved and prepared as for a surgical procedure with Betadine (providone-iodine) (Purdue Frederick Co., Norwalk, Conn.). The proximal medial surface of the left tibia was exposed under sterile conditions, and a duct was drilled to the medullary cavity with a sterile dentist's drill. After injection via a hypodermic needle of 0.1 ml of 5% sodium morrhuate (35) (Torigian Laboratories, Inc., Queens Village, N.Y.), 0.1 ml of the bacterial suspension, and 0.1 ml of sterile saline to assure complete entry of the bacteria into the marrow, a sterile, barium-impregnated, silicone rubber catheter (length, 4.5 cm; outside diameter, 2.2 mm; inside diameter, 1.0 mm) (Evermed, Medina, Wash.) was inserted completely into the medullary cavity. The drill hole was sealed with sterile bone wax, and the incision was closed with silk sutures. Control animals received either an injection of 0.1 ml of 5% sodium morrhuate only, or the implanted silicone rubber catheter, or sodium morrhuate and the silicone rubber catheter. The rabbits were allowed to recover from anesthesia and then were returned to their individual stainless steel cages. The animals were given tap water and pelleted standard rabbit diet ad libitum and were observed daily.

Blood (3 ml) for bacteriological cultures was withdrawn aseptically from the central ear artery of each infected and control animal 24 h, 48 h, and 10 days after the surgery. At weekly intervals, all rabbits were weighed, and both tibiae were examined by palpation. The tibiae were examined by radiography for evidence of osteomyelitis 4 weeks after infection and at weekly intervals thereafter.

Rabbits were killed by the injection of T-61 euthanasia solution (American Hoechst Corp., Somerville, N.J.). The infected tibia was separated from adherent soft tissue under sterile conditions. The tibia was split longitudinally from its proximal end into two halves. Bone tissue and marrow samples were taken from the infected area and were cultured for aerobic and anaerobic bacteria. Portions of the catheter and its contents, as well as small pieces of infected bone and bone marrow, were collected from all areas of the entire length of the tibia, that is, proximal and distal to the catheter, as well as from areas adjacent to the catheter, and were processed for TEM and SEM. Samples of the bone and bone marrow were fixed in 10% formaldehyde, decalcified, processed for routine paraffin embedding, and sectioned at 6 μm. They were then stained with Harris hematoxylin and eosin, stained by the Brown and Brenn Gram stain method, and then examined for changes characteristic of osteomyelitis.

Electron microscopy. Samples, including bone marrow, large and small chips and pieces of bone, material from inside the catheter, and sections of the catheter were collected aseptically from the open tibia and processed for either SEM or TEM. Samples were placed in small glass vials, and fixative (5% glutaraldehyde in 200 mM cacodylate buffer, pH 7, containing 0.1% ruthenium red) was added and allowed to react for 2 h at room temperature. Fixative was removed from the samples and replaced with the cacodylate buffer containing ruthenium red. Samples were washed for 30 min at 25°C in this buffer-ruthenium red mixture, and the buffer was replaced with fresh buffer-ruthenium red mixture. Samples were subjected to this buffer washing procedure five times, then fixed for 2 h in 2% OsO_4 in 200 mM cacodylate buffer, pH 7, containing 0.1% ruthenium red. Fixed samples were dehydrated for TEM in an acetone series prepared with 200 mM cacodylate buffer containing 0.1% ruthenium red in

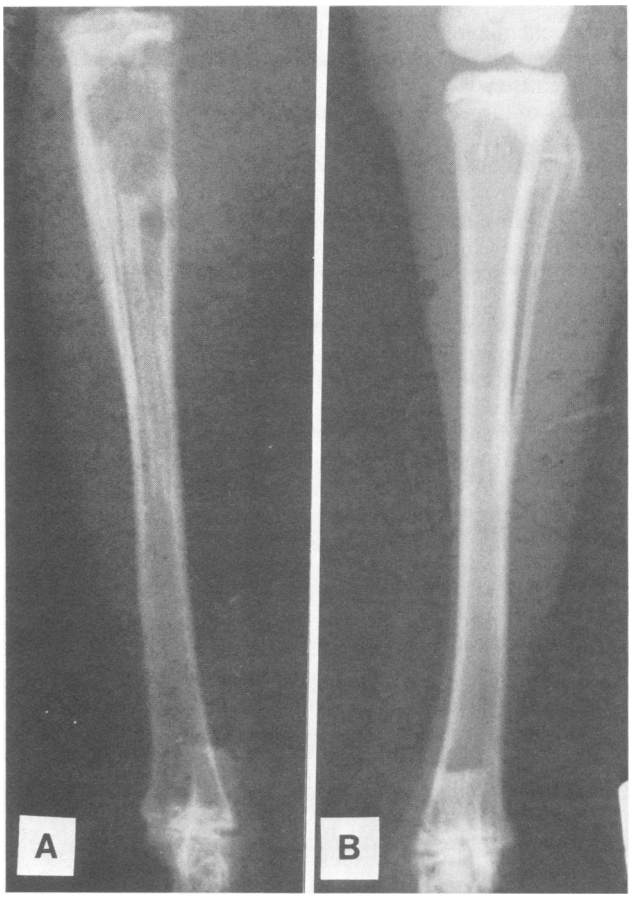

FIG. 1. Typical radiographs of (A) infected tibia from a rabbit injected 28 days previously with 0.1 ml of 5% sodium morrhuate and 0.1 ml of suspension of *S. aureus* and implanted with a barium-impregnated silicone rubber catheter and (B) control tibia with no injection or implant.

the 30 and 50% stages and distilled water in the 70 and 90% stages. Samples were further dehydrated in freshly distilled acetone and in propylene oxide and were embedded in Spurr resin. Sections were cut on an LKB Ultratome II, stained with uranyl acetate and lead citrate, stabilized with evaporated carbon, and examined with an A.E.I. EM-801 electron microscope at an accelerating voltage of 60 kV. Fixed samples were dehydrated for SEM in a graduated ethanol-

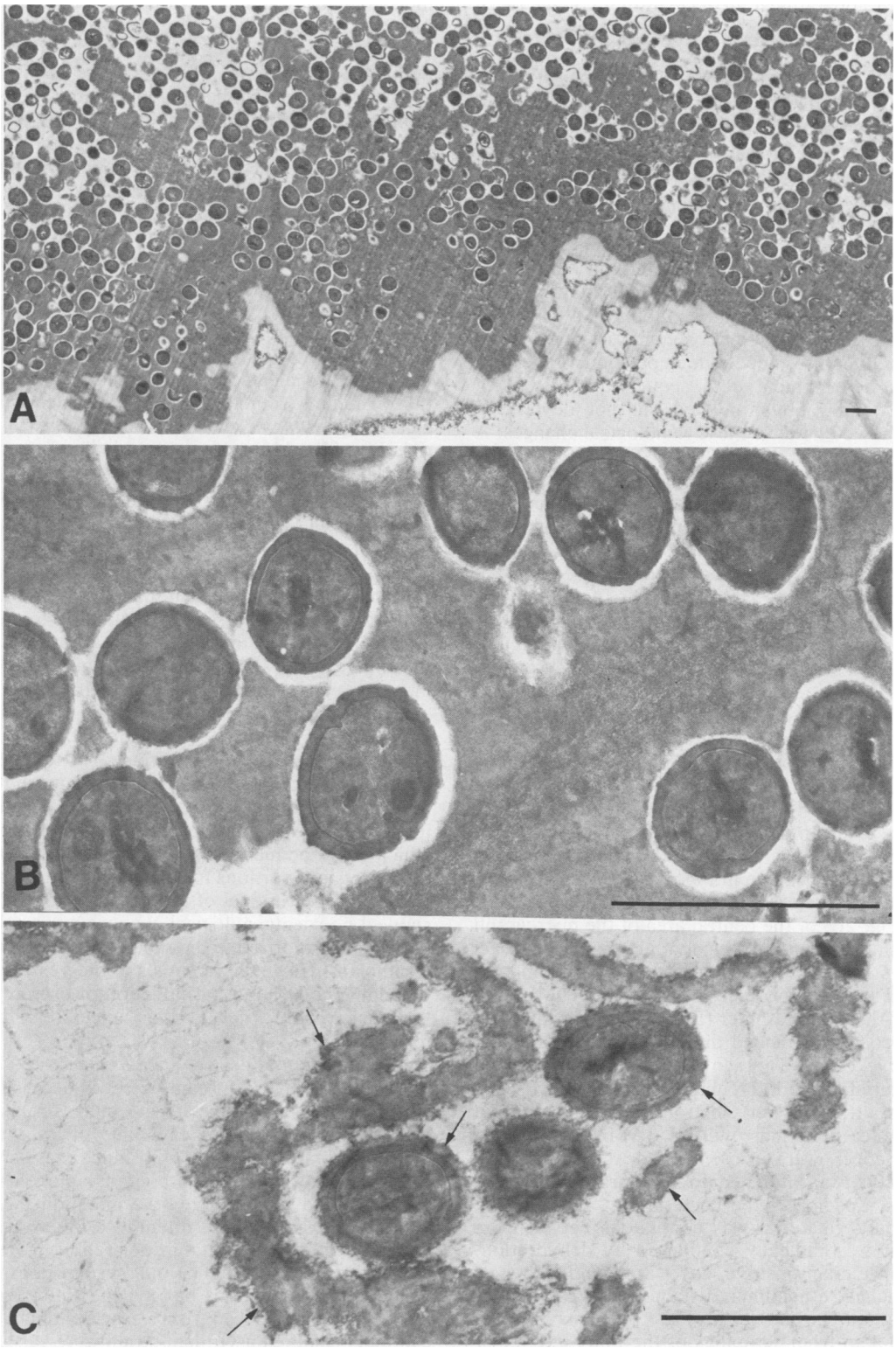

FIG. 2. Transmission electron micrographs of thin sections of samples scraped from the interior of the catheter implanted in the tibia of rabbit 11. (A) Mass of ruthenium red-stained glycocalyx surrounds large numbers of gram-positive cocci. (B) Enlargement of same area. (C) Cells scraped from another area of the catheter. Arrows indicate glycocalyx. Bars, 1 μm.

water series and in a Freon-ethanol series. They were then critical point dried with Freon 13 by the method described by Cohen et al. (7). The critical-point-dried specimens were mounted on stubs, sputter coated with gold-palladium (60:40) in a Hummer I sputter coater, and examined on a Hitachi 450 SEM apparatus at an accelerating voltage of 20 kV.

RESULTS

Injection of sodium morrhuate only, or injection of sodium morrhuate together with implantation of a catheter, or implantation of a catheter alone produced no gross or radiological evidence of osteomyelitis. Blood cultures of these control animals and bone cultures obtained at death were consistently sterile. Three of five rabbits injected with sodium morrhuate and *S. aureus* and implanted with a catheter developed osteomyelitis, as determined by palpation and by radiology, approximately 4 weeks after infection (Table 1). Radiographs of the three rabbits with infected tibiae showed elevation of the periosteum, distortion of the normal bone architecture, new bone deposition, some widening, and the development of a sequestrum (Fig. 1A). Control rabbits and rabbits infected but not developing osteomyelitis showed none of these radiological changes. A control tibia is shown in Fig. 1B.

When the tibiae were harvested from infected and control rabbits, *S. aureus* was isolated from the medullary cavities of the tibiae from the three rabbits (numbers 5, 7, and 11) that developed osteomyelitis. A histological study of paraffin-embedded, hematoxylin-and-eosin-stained sections of bone tissue from rabbits 5, 7, and 11 revealed focal, moderately severe, subacute osteomyelitis, with macrophages and numerous neutrophils present in the areas of inflammation. Bone tissue stained with the Brown and Brenn method for gram-positive bacteria and prepared for histological examination by light microscopy demonstrated gram-positive cocci in the same three rabbits. No gram-positive cocci were observed either in the control rabbits or in the two rabbits which, although injected with *S. aureus,* failed to develop osteomyelitis.

Transmission electron micrographs of thin sections of gram-positive cocci growing inside the catheter implanted in the tibia of rabbit 11 are shown in Fig. 2. Note the masses of cocci embedded in ruthenium red-stained material (Fig. 2A). Enlargement (Fig. 2B) of the central area of the micrograph shows cell wall ultrastructure typical of gram-positive cocci and also shows the enormous amount of ruthenium red-stained material between the microorganisms. Sections from another area of the same catheter (Fig. 2C) show ruthenium red-stained glycocalyx attached directly to the surfaces of the gram-positive cocci. The same sections show stained glycocalyx material in the intercellular space.

Figure 3 shows transmission electron micrographs of thin sections of infected bone marrow (Fig. 3A) from rabbit 11. Gram-positive cocci were present, and ruthenium red-stained glycocalyx was attached to the surfaces of these microorganisms. Spacing of the bacteria may indicate that capsular glycocalyx material was continuous between the cells but that it was radically condensed by dehydration. Figure 3B shows gram-positive cocci surrounded by attached and detached ruthenium red-stained glycocalyx. This electron micrograph is similar to Fig. 3A. This sample, however, was taken from scrapings of the bony interior of the tibia of rabbit 11 and not from the medullary area.

Scanning electron micrographs of material from the infected tibia of rabbit 11 are presented in Fig. 4. The surface of the infected bone was encrusted with a biofilm that altered the normal topographical features. These accretions were widespread on the surface of the infected bone, and upon enlargement (Fig. 4A) coccoid forms were revealed embedded in large quantities of amorphous material that was morphologically similar to other bacterial glycocalyces (4, 5, 21, 22). Thick accretions were present not only on the surface of the bone but also on the inner surface of the implanted catheter in rabbit 11 (Fig. 4B). Again, coccoid profiles were seen embedded in an amorphous matrix. This biofilm of bacteria and glycocalyx seems to adhere to the catheter surface just as it does to the bone surface.

Transmission electron micrographs of thin sections of bone marrow and bone scrapings taken from the infected tibiae of rabbits 5 and 7 revealed gram-positive cocci growing in microcolonies whose extensive glycocalyx fibers were somewhat condensed by dehydration (Fig. 5A).

SEM of all bone and catheter surfaces in rabbits 5 and 7 revealed confluent bacterial biofilms that obscured the topographic detail of the bone and plastic and often cracked during condensation upon dehydration (Fig. 5C). The condensation of the glycocalyx surrounding the bacterial cells in these biofilms sometimes revealed coccoid bacterial cells clearly against a background of amorphous condensed material (Fig. 5B). More often, the biofilms were so thick that coccoid profiles could barely be discerned (Fig. 5C), and bacterial cells were largely buried under glycocalyx material at the biofilm surface.

DISCUSSION

Many attempts have been made to reproduce experimentally lesions of bone resembling human osteomyelitis (14, 25, 31, 35, 37). Models that utilized the sclerosing agent sodium morrhuate (25, 37) to produce vascular thrombosis (38) were most successful in achieving a high rate of induction of osteomyelitis (25, 31) when bacteria and sclerosing agent were given simultaneously. Our modification of these techniques consisted of the addition of a foreign body to the interior of the bone to produce more damage to the area and to provide a solid, retrievable, radiologically identifiable item (the barium-impregnated, silicone rubber catheter) which could be manipulated, once removed from the bone, by cutting into portions for the various sampling procedures. Like the rabbit model of Norden (25), our modified model showed no roentgenological changes when rabbits received only sodium morrhuate or sodium morrhuate and the catheter implant. However, sclerosis of the vessels was sufficient to achieve a 60% rate of induction of osteomyelitis when a suspension of *S. aureus* was injected with the sodium morrhuate.

Christensen et al. (6) and Zimmerli et al. (43) have demonstrated that a foreign object is necessary to produce subcutaneous infection with avirulent strains with various degrees of slime-forming capability of *Staphylococcus epidermidis* (6) and *S. aureus* (43). Since these studies suggested the efficacy of a foreign body as an agent which predisposes toward or enhances an infection, we utilized the catheter implant in an attempt to increase the rate of induction of osteomyelitis in our rabbit model. Two differences should be noted: (i) our system does not involve a subcutaneous infection, but a bone infection; and (ii) the infecting strain, derived from a case of human osteomyelitis, can be considered a virulent strain of *S. aureus*.

In an extension of this study (to be published separately), it was found that the implanted catheter was not necessary to induce osteomyelitis. The same clinical, radiological, and

histological changes were observed as a result of administration of sclerosing agent and *S. aureus* in the absence of the catheter. The catheter, however, is useful. Although it neither enhances nor diminishes the manifestations interpreted as osteomyelitis, the implanted catheter does serve as a solid, easily retrievable, radiologically identifiable item for the sampling system employed for electron microscopic and cultural studies. It should be pointed out that the catheter is

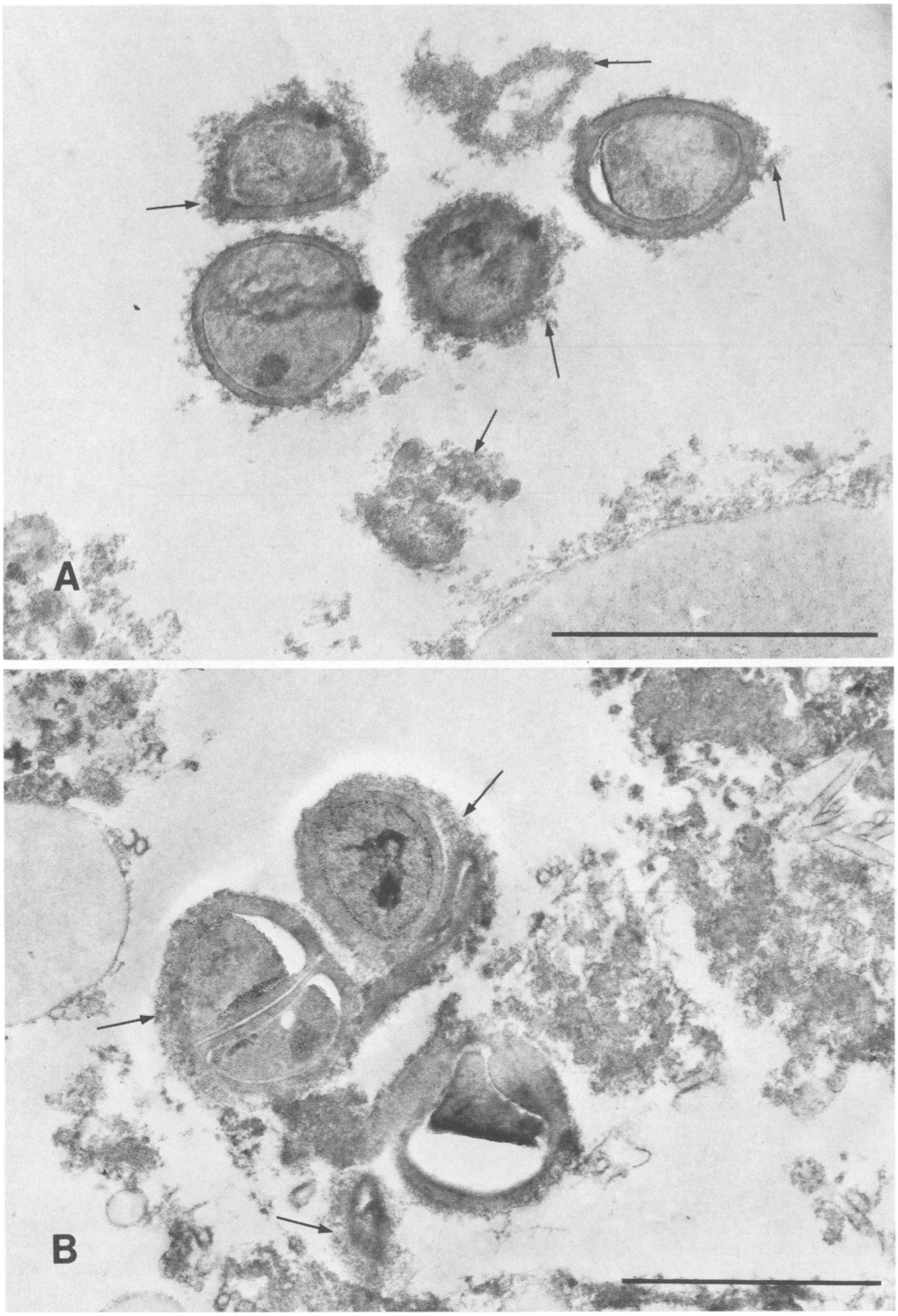

FIG. 3. Transmission electron micrographs of thin sections of samples taken from the interior of the infected tibia of rabbit 11. (A) Bone marrow. (B) Bone scrapings. Arrows indicate ruthenium red-stained glycocalyx material both attached to and detached from the bacteria. Bars, 1 μm.

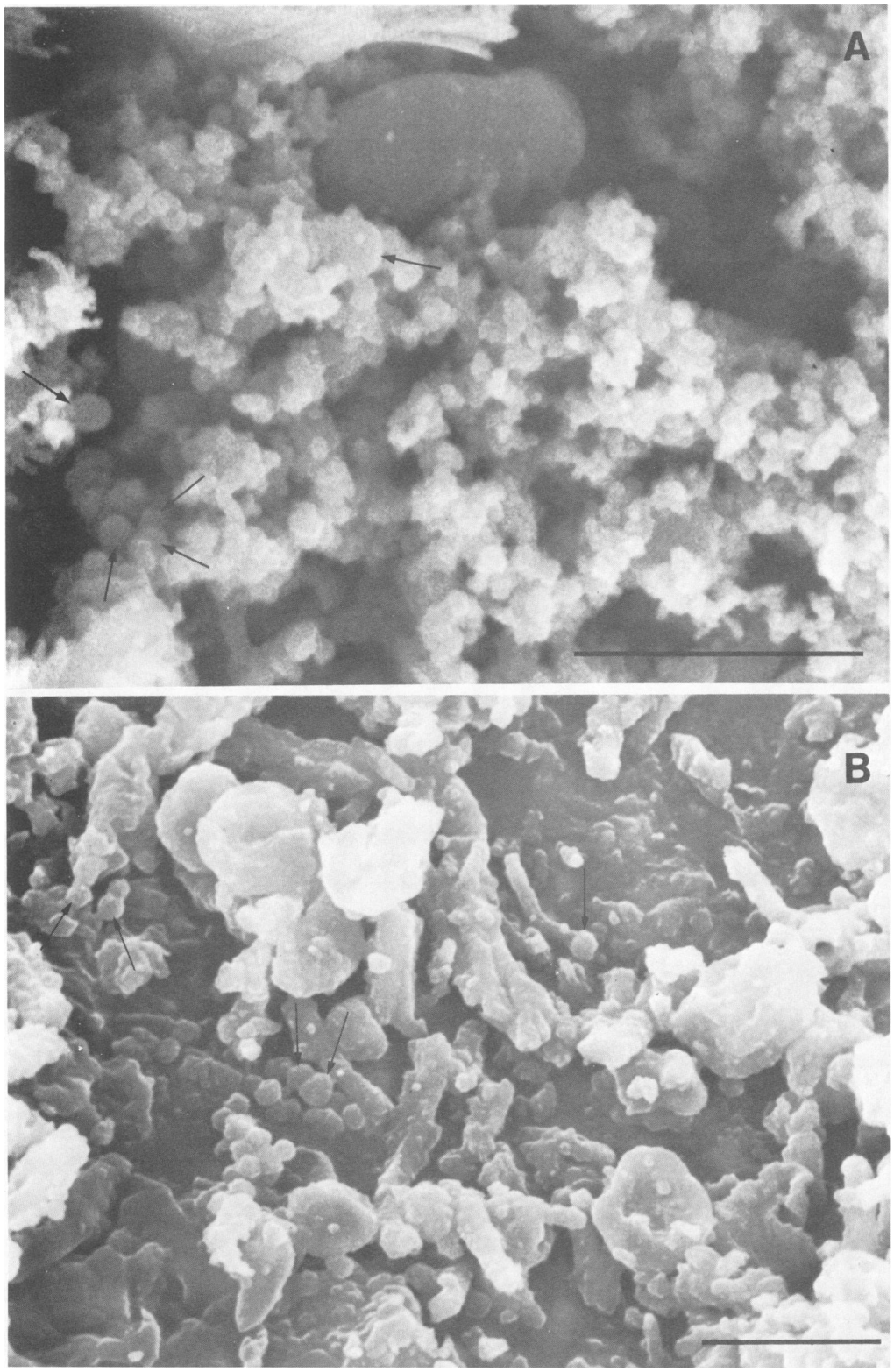

FIG. 4. Scanning electron micrographs of material collected from the infected tibia of rabbit 11. (A) detail of biofilm on a bone chip showing coccoid bacterial profiles (arrows) in an amorphous glycocalyx matrix. (B) Detail of the very coherent biofilm on the implanted catheter showing coccoid bacterial profiles (arrows) in a condensed amorphous matrix. Bars, 5 μm.

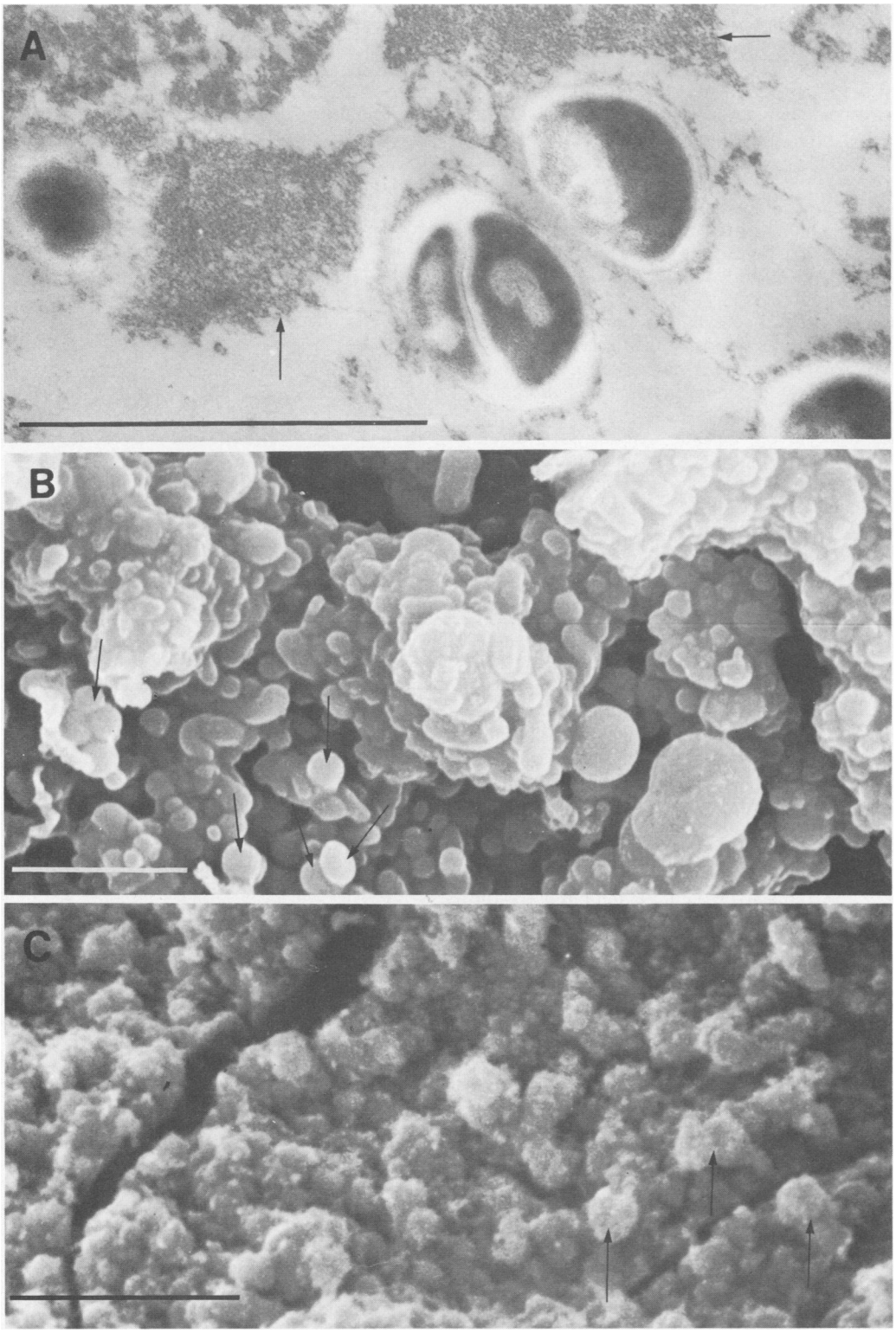

FIG. 5. Electron micrographs of material taken from the infected tibiae of rabbits 5 and 7. (A) Transmission electron micrograph of a microcolony of gram-positive cocci found in the bone marrow of rabbit 5 showing the accretion of fibrillar glycocalyx material around the bacterial cells. Bar, 1 μm. (B) Scanning electron micrograph of the biofilm covering the catheter implanted in rabbit 5 showing coccoid bacterial profiles (arrows). Bar, 5 μm. (C) Detail of the very thick bacterial biofilm on the catheter implanted in rabbit 7. Coccoid bacterial profiles (arrows) are almost buried in amorphous material at the surface of this very thick biofilm, which has cracked during dehydration. Bar, 5 μm.

implanted after the sclerosing and infecting agents are administered and does not serve as a vehicle for the entry of these agents. The infection and glycocalyx production proceeded outwardly from the site of injection into the affected bone and in a retrograde manner into the sterile catheter. Thus, growth of the microorganisms into the catheter interior must proceed by mechanisms similar to those by which it proceeds into other areas of the infected bone. Therefore, the catheter can be viewed as a model system within a model system.

Although not entirely analogous, this model of osteomyelitis in the rabbit is similar in many aspects to the human disease. The animals that developed osteomyelitis showed weight loss and radiological evidence of progressive disease. After harvest of the rabbit tibiae from infected and control animals, histological samples showed striking morphological changes in the infected animals consistent with a diagnosis of osteomyelitis. Microbiological recovery of *S. aureus* only from the tibiae of infected animals and the histological presence of gram-positive cocci in these same rabbits indicated development of severe chronic osteomyelitis in our rabbit model. This experimental model, which showed a high rate of induction of disease and allowed recovery of the infecting microorganisms, was utilized as a system in which to perform the electron microscopic study needed to determine the presence or absence of *S. aureus* glycocalyx in the infection.

TEM of thin sections of both bone marrow and bone scrapings from the infected tibiae revealed gram-positive cocci in microcolonies surrounded by ruthenium red-stained glycocalyx either condensed on the surfaces of the cells or in a fibrous intracellular network. Examination of material taken from the interior of the catheter implant showed masses of gram-positive cocci embedded in a large amount of ruthenium red-stained glycocalyx. This massive colony is exemplified particularly well in the catheter taken from rabbit 11 (Fig. 2). If such large masses of microorganisms encased in so much glycocalyx are typical of the disease, it is not surprising that osteomyelitis is difficult to treat with antibiotics, which may not penetrate the glycocalyx (8). Phagocytosis by macrophages would be inhibited by the large size of such an aggregated clump of staphylococci joined by a complex matrix of glycocalyx. This formation of microcolonies is similar to that found with *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis (18). Caputy et al. (4) proposed that glycocalyces of *S. aureus* do serve a protective function for the microbial cell. Glycocalyx may interfere with phagocytosis by masking the peptidoglycan (27, 28), enveloping the teichoic acid which has been shown to enhance opsonization (40), and consuming complement (39) as well as by covering and possibly altering the configuration of bound complement (41, 42).

SEM of chips of infected bone and interior and exterior portions of the catheter implants provided a surface view of the infected areas. The normal topographical features of the bone were altered dramatically by copious material that adhered to the bone and was best described as caked-on accretions. Enlargement of these accretions revealed numerous coccoid forms that were embedded in the matrix of the accreted material. The interior and exterior surfaces of the catheter revealed similar accretions of encrusted material with coccoid profiles embedded in the matrix. This accretion of material appeared to be far more extensive than that demonstrated for adhesion of uropathogens to polypropylene surfaces (22).

This study provided direct evidence that the cells of *S. aureus* in this modified model infection of the rabbit tibia grow within thick, adherent, glycocalyx-enclosed biofilms. This aggregated mode of growth of the pathogen presents a fibrous exopolysaccharide bacterial cell surface to the defense mechanisms of the host and a very thick anionic barrier to the penetration of antibiotics. We speculate that this biofilm mode of growth influences the notable persistence and inherent antibiotic resistance of these pathogens, but the precise molecular mechanisms of this influence remain to be determined.

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