JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1982, p. 320–323
0095-1137/82/020320-04$02.00/0

Vol. 15, No. 2

Use of Chemotaxis Chambers for Studying In Vitro Bacterial Colonization of Biomaterials

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Blind-well chemotaxis chambers were used to study the in vitro bacterial adhesion and colonization of biomaterials. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were selected for the bacterial inocula. Abundant growth on the surfaces of methyl methacrylate, polyethylene, stainless steel, and Vitallium was detected by using scanning electron microscopy after 24 h of incubation. The culture technique employed proved to be of value for the study of surface bacterial colonization of inert materials.

The surgical implantation of a variety of plastics and metals has increased the risk of early and postoperative wound infections. Therefore, it has become of great importance to determine whether these biomaterials can inhibit or favor bacterial colonization within the body if introduced during surgical implantation of the device or if carried to the area by transient bacteremia. According to Hunter and Dandy (7), the incidence of postoperative infection, even with good surgical technique, ranges from 1 to 3%.

Gristina et al. (6) studied the bacterial growth or inhibition or both of *Staphylococcus aureus*, *S. faecalis*, and *Escherichia coli* on surgical silver, iron, zinc-coated galvanized iron, aluminum alloy, stainless steel, Vitallium, and methyl methacrylate. They placed these materials on agar plates so that one-half rested on a bacterium-streaked zone and the other half rested on a bacterium-free zone. Growth on the agar plates was similar with all of these materials as determined by visual observation.

Bacteria can adhere tenaciously and multiply on a variety of surfaces (4, 5, 11). With the advent of scanning electron microscopy, visualization of surface bacterial growth has been facilitated. A technique for scanning electron microscopy analysis of antibiotic activity on bacteria in colony form was reported by Simmons et al. (13). Their method consisted of incorporating various concentrations of antibiotics into poured agar plates, placing Nuclepore membrane filters onto the agar surface, and inoculating the filters with the bacteria to be tested. Bacterial colony formation on the filters was observed by using scanning electron microscopy.

We previously described a technique to study the colonization of surfaces by using blind-well chemotactic chambers and scanning electron microscopy (8a). We found this technique applicable to the study of in vitro colonization of biomaterials. The test biomaterials employed in our research were methyl methacrylate bone cement, high-molecular-weight polyethylene, stainless steel, and Vitallium. We now report our results on the surface growth of a gram-positive coccus (*S. aureus*) and a gram-negative bacillus (*P. aeruginosa*).

(This paper was presented as part of a poster session at the 17th Annual Meeting of the Reticuloendothelial Society, 4 December 1980, in Tampa, Fla.)

MATERIALS AND METHODS

Bacteria. Fresh isolates of *S. aureus* (coagulase positive) and *P. aeruginosa* were obtained from the Clinical Laboratories of the North Carolina Baptist Hospital. Eighteen-hour cultures in heart infusion broth and Trypticase soy broth (BBL Microbiology Systems), respectively, were adjusted to give 54% transmission at 540 nm in a Spectronic 20 spectrophotometer. Plate counts of the standardized bacterial suspensions gave concentrations of approximately 2×10^8 colony-forming units per ml. A 10^{-4} dilution of these suspensions was used to inoculate the biomaterials.

Biomaterials. Squares (12 mm) were cut from methyl methacrylate bone cement (Surgical Simplex polymethyl methacrylate; Howmedica, Inc., Rutherford, N.J.) that had been allowed to polymerize at room temperature between two glass plates to form a 2- to 3-mm-thick sheet. Similar squares were cut from a 2- to 3-mm-thick sheet of ultrahigh-molecular-weight polyethylene. Stainless steel (316 L) and Vitallium disks 12 mm in diameter were supplied by Howmedica, Inc. All of the biomaterials were cleaned by sonication in a mild detergent, thoroughly rinsed in distilled water, air-dried, and sterilized by autoclaving.

Culture chambers. Culture chambers consisted of blind-well chemotactic chambers (3/16 inch [ca. .48 cm] deep), without retainers (Neuro Probe Inc., Be-

thesda, Md.), and lids to "Flo-thru" specimen capsules as used in the Reichert tissue processor (American Optical Corp., Scientific Instrument Div., Buffalo, N.Y.) (Fig. 1). The chambers were placed in specimen jars, and the lids were placed in petri dishes; both were gas sterilized with ethylene oxide and used 24 h after sterilization to avoid any toxic effects from residual gas.

Colonization. The inoculation of the chambers was done as previously described (8a). Briefly, the wells of the culture chambers were filled with Trypticase soy broth or with heart infusion broth, and the volume of fluid was adjusted until the porous flo-thru lids showed a moistened surface. Sterile Uni-Pore polycarbonate membrane filters (0.1- or 0.2-μm pore; 13-mm diameter; Bio-Rad Laboratories, Richmond, Calif.) were applied to the wetted lid surface. Five to 10 μl of the 10^{-4} bacterial suspension containing approximately 100 to 200 colony-forming units was applied directly to the filter membranes placed on the porous lids, and the biomaterials were positioned over the filters. The chambers were kept inside the specimen jars at all times. A wet filter paper was placed in the bottom of the jars to provide a humidified atmosphere and to avoid evaporation of the fluid in the wells. The chambers were incubated at 37°C for 18 to 24 h. Control samples were prepared in the same manner, except that the biomaterials were removed after 5 min of incubation.

Scanning electron microscopy. After incubation, the biomaterials were carefully lifted, rinsed briskly in 0.1 M cacodylate buffer (pH 7.2), immersed face down in 2.5% glutaraldehyde in the same buffer, and fixed for 24 h at 4°C. The control samples were not washed but were fixed, as were the test materials. Postfixation for 1 h with 1% osmium tetroxide in cacodylate buffer was followed by dehydration with graded concentrations of ethanol and critical point drying in CO_2. The biomaterials were attached to aluminum stubs with double-sticking tape and coated with a thin layer of gold-palladium by means of a sputter coater.

RESULTS

After 24 h of incubation, a greenish cast of the fluid in the wells of the chambers inoculated with *P. aeruginosa* and a yellowish color in those inoculated with *S. aureus* were indications that growth had taken place in the chemotaxis chambers. Although the membrane filters showed bacterial growth on the surface directly in contact with the biomaterials, the culture media in the wells remained clear. The filter (0.1- to 0.2-μm pore size), although allowing nutrients to reach the surface of the biomaterials, prevented the bacteria from contaminating the culture fluid in the wells. Trypticase soy broth supported the growth of *S. aureus*; however, heart-infusion broth usually was selected to fill the chambers inoculated with this microorganism.

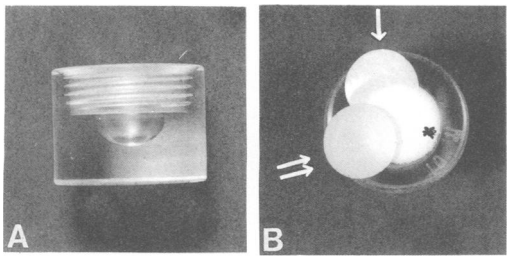

FIG. 1. (A) Lateral view of the blind-well chemotaxis chamber without retainer. (B) Top view of the incubation chamber with the porous Flo-thru lid in place (*), the filter membrane (single arrow), and a polyethylene disk (double arrow). The filter and the disk are off center for better illustration of the assembly.

The four surfaces tested, methyl methacrylate, polyethylene, stainless steel, and Vitallium, were colonized easily by the two groups of bacteria selected for the in vitro studies. Figure 2 shows scanning electron micrographs of the surface growth of *S. aureus* and *P. aeruginosa* after 24 h of incubation on high-molecular-weight polyethylene and stainless steel, respectively.

Often, the polymerized methyl methacrylate bone cement had air trapped in its matrix, which caused it to show pits and elevations when viewed by scanning electron microscopy. This material also had a fine granularity. The polyethylene, although smooth to the touch, showed multiple grooves. The metal disks used had been machine polished; however, their surfaces showed parallel grooves and ridges when viewed by scanning electron microscopy (arrows, Fig. 2B).

No bacteria were seen on the control samples. The small numbers (100 to 200) of colony-forming units applied to the biomaterials would have been difficult to detect by scanning electron microscopy. Furthermore, some bacteria could have gone undetected because they adhered primarily to the face of the filter in direct contact to the test surface.

DISCUSSION

The role of specific adhesion of pathogenic bacteria to host tissues as the initial step in pathogenesis has been the subject of literally hundreds of papers in recent years (8). The persistence of adherent bacteria on inert surfaces in the body, even after specific antibiotic therapy, argues that they resist both clearance by macrophages and attack by antibacterial substances such as antibodies and antibiotics. Despite the importance of the problem, only sporadic work has been done on bacterial contamination of surgical wounds, prostheses, or biomaterials.

Employing blind-well chemotaxis chambers and scanning electron microscopy, we studied the in vitro bacterial colonization of four bioma-

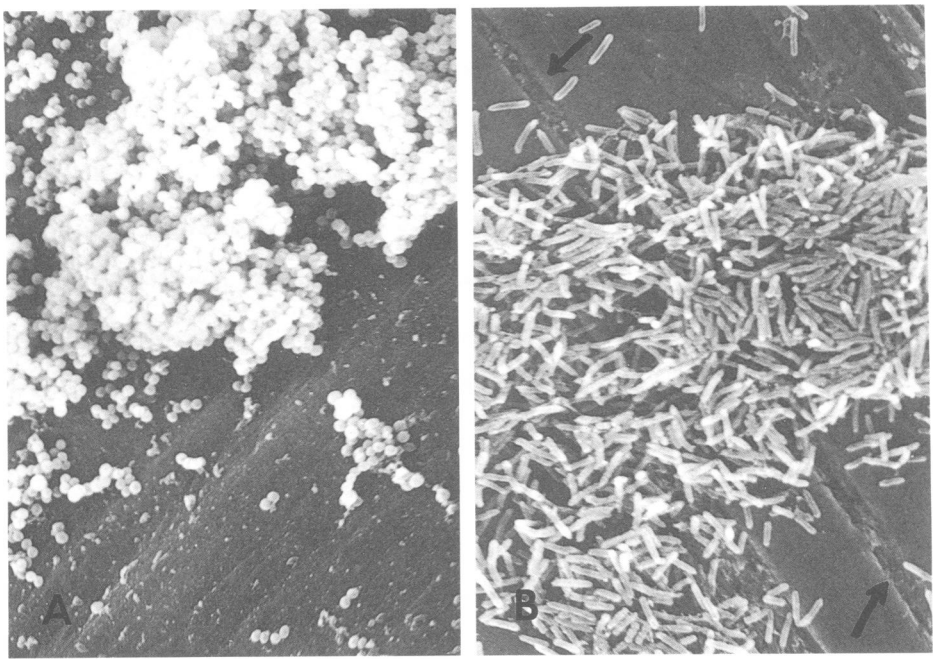

FIG. 2. Scanning electron micrograph of colonies of *S. aureus* on the surface of high-molecular-weight polyethylene (A) and colonies of *P. aeruginosa* on the surface of stainless steel (B). The arrows (B) point to grooves on the metal. Approximate magnification, ×1,800.

terials frequently used in orthopedic surgery: methyl methacrylate bone cement, ultrahigh-molecular-weight polyethylene, stainless steel, and Vitallium. *S. aureus* and *P. aeruginosa* are two frequent contaminants of surgical implants; therefore, they were selected for our experiments. The presence of pigment in the culture media of the wells and the clusters of bacteria observed by scanning electron microscopy indicated in situ growth and colony formation, especially since the inoculating dose consisted of approximately 200 colony-forming units. Controls inoculated with the same dose but which lacked an incubation period failed to demonstrate bacteria on their surfaces.

The two microorganisms tested adhered and colonized the above-mentioned biomaterials equally well. This finding supports an earlier observation by Gristina et al. (6), who employed a different culture technique.

The culture method employed in the present investigation permits the simultaneous incubation of multiple samples. The presence of a filter membrane of 0.1- or 0.2-μm pore size between the seeded surface and the culture medium in the wells of the chambers permitted the continuous supply of nutrients to the bacteria but impeded the passage of bacteria to the culture medium. Therefore, the growth observed on the various biomaterials can be assumed to derive from the multiplication of the original inoculum, and it would be a true indication of surface growth because the biomaterials are not immersed in a liquid medium or embedded in the agar of a culture plate.

The technique employed in this report also could be used to obtain valuable information on the toxicity or growth-stimulating activity or both of different biomaterials for a number of potential bacterial contaminants. Diffusable ions or other chemical fractions could be studied, if incorporated into the culture medium filling the wells, for their effect on adherent bacteria. In addition, the effect of metal ions or other components of the biomaterials could be examined. For example, iron is known to be absorbed by bacterial cell walls (1), and according to Weinberg (14), the presence of iron makes bacteria resistant to killing by leukocytes. Weinberg (15) has coined the term "nutritional immunity" as that state created by the host at the onset of an infection to withhold iron from the invading bacteria. Therefore, many bacteria are unable to obtain sufficient iron for growth in tissue fluids because the iron in the host is bound to transferrins or other iron-binding proteins. A number of bacteria (*Salmonella typhi, E. coli, Mycobacterium tuberculosis*) manufacture specific iron-chelating compounds in response to iron-limiting conditions. In this regard, it is of interest that a frequent contaminant of metal implants and prostheses is *P. aeruginosa*, a microorganism

known to produce chelating agents that enable it to grow in vivo (9).

Commonly used internal fixation and joint replacement devices are manufactured from iron-containing alloys, and often micrometallic particles have been observed in tissues near these devices (16, 17). Therefore, the possible presence of iron in certain cases could serve as an adjuvant to the bacterial growth. Secondly, the metal particles could act as microsurfaces upon which colonization may take place.

We believe that the type of colonization seen on the biomaterials studied in our experiments represents a surface state colonization which involves the presence of glycocalyx (A. G. Gristina, J. W. Costerton, E. S. Leake, and J. Kolkin, Orthopaed. Trans. **4**:355, 1980) (2). This growth is to be contrasted with the colonization in the nonsurface state, as exists in ordinary liquid culture media or in body fluids where the glycocalyx is not preeminent. From other investigators, we know that in nonsurface states bacteria are susceptible to antibiotics, and in surface states they are probably more resistant to the same antibiotics (3, 12). Furthermore, in an in vivo situation, bacteria in the nonsurface state, i.e., the circulatory system, urine, and joint fluid, are known to be susceptible to the action of antibiotics, whereas bacteria adherent to dead bone, biomaterials, internal fixation materials, the bladder wall, etc., are shielded by a glycocalyx and are more resistant to antibiotic therapy (Gristina et al., Orthopaed. Trans. **4**:355, 1980) (10). The application of special staining techniques to demonstrate the presence of glycocalyx will clarify this point.

Since, in the host, bacteria rarely reach a bare inert implant, the technique employed in the present studies would allow the testing of biomaterials coated with serum proteins, fibrin, synovial fluid, etc.

Information obtained from in vitro studies could provide the basis from which to develop in vivo model systems applying more sophisticated methodology.

ACKNOWLEDGMENTS

This work was supported by a grant from the Orthopaedic Research and Education Foundation and by Public Health Service grant HL 16769 from the Heart, Lung and Blood Institute.

We are grateful to Howmedica, Inc., Rutherford, N.J., for kindly supplying the stainless steel and Vitallium disks.

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