Background: Sessile bacteria within biofilms are highly resistant to eradication by antimicrobial agents. Previously, we have shown that the most common organisms cultured from experimentally induced cholesteatomas are biofilm formers. Additionally, the keratin “matrix” of a cholesteatoma is an ideal environment for the support of biofilm formation.

Objective: To determine if microbial biofilms occur within the keratin matrix of infected cholesteatomas.

Design: We evaluated the histomorphologic characteristics of 24 human and 22 experimental cholesteatomas for evidence of biofilm formation using light and transmission electron microscopy.

Subjects: Human tissues were collected during surgical eradication of existing cholesteatomas. Twenty-two gerbil cholesteatomas were either spontaneously occurring or induced by external auditory canal ligation and harvested several months later.

Results: Gram-positive and gram-negative bacteria were seen within acellular deposits among the keratin accumulations in 21 of 22 gerbil and 16 of 24 human cholesteatomas. Regions of accumulated bacteria possessed the ultrastructural appearance of typical amorphous polysaccharide biofilm matrix.

Conclusions: There is strong anatomic evidence for the presence of bacterial biofilms in experimental and human cholesteatomas. The existence of bacterial biofilms within cholesteatomas may explain the clinical characteristics of infected cholesteatomas, that is, persistence and recurrence of infection, with surgical eradication being the only effective treatment.

Once a cholesteatoma is infected, chronic otorrhea usually occurs. The otorrhea is often suppressed by topical and systemic antibiotics, but recurrences of infection, often with the same organism, are common. We propose that the matrix of cholesteatomas is an ideal environment for the development of mixed microbial biofilms, and we hypothesize that biofilms exist within the matrix of chronically infected cholesteatomas. In the present study we evaluated matrix samples from human cholesteatomas and spontaneously occurring and experimentally induced gerbil cholesteatomas for evidence of biofilm formation.

### MATERIALS AND METHODS

#### CHOLESTEATOMA SPECIMENS

Cholesteatoma matrix was obtained from human cholesteatomas during tympanomastoid surgery and placed in 10% buffered formalin. Specimens were obtained from chronically infected cholesteatomas as well as noninfected cholesteatomas behind an intact tympanic membrane. The Human Studies Review Committee of the University of California, Davis, and Washington University in St Louis, Mo, approved the human subjects portion of this study, and subjects provided written consent to donate tissue to the study.

Histologic evaluation was also performed on matrix samples from spontaneously occurring and experimentally induced cholesteatomas in gerbils. These were specimens that were obtained and sectioned for previous studies. The animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis. All animal studies were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 USC §§ 2131 et seq).

#### LIGHT MICROSCOPY

Human matrix specimens were transferred from 10% buffered formalin to a fixative consisting of 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1M phosphate buffer for 24 hours at 4°C. Tissue specimens were then postfixed in 1% osmium, dehydrated in graded solutions of acetone and embedded in Epon-Araldite. Several semithin sections (1.0 µm) were collected at a variety of depths of the sample and counterstained with toluidine blue and basic fuchsin. An alternate group of sections was stained using the Protocol Gram Stain Set (Biochemical Sciences, Inc, Swedesboro, NJ). Gerbil specimens had been fixed, processed, and sectioned in similar fashion. Additional sections were taken from some of the blocks for gram staining. Sections were examined with an upright Olympus BH-2 light microscope (Olympus Corp, Lake Success, NY), and images were captured with the DFC-5000 digital photo system (Sony, Tokyo, Japan).

#### TRANSMISSION ELECTRON MICROSCOPY

Tissue samples from gerbils and humans were fixed and embedded as described above and thin sectioned for transmission electron microscopy. Thin sections for transmission electron microscopy were then taken from regions containing suspected biofilms and counterstained with uranyl acetate and lead citrate. Sections were examined and photographed on a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan) with digital imaging capabilities.

#### RESULTS

##### HUMAN CHOLESTEATOMA MATRIX

Of the 24 human cholesteatomas, 16 had anatomical findings consistent with bacterial biofilms (Figure 1A and B). We considered a dense colony of bacteria within an amorphous matrix, in the absence of inflammatory cells, to be a microbial biofilm. Gram-positive and gram-negative bacteria were evident in the human specimens, which could be subjected to gram staining. Biofilms showed varied signs of degradation of the acellular polysaccharide matrix.

##### GERBIL CHOLESTEATOMA MATRIX

Of 22 cholesteatoma specimens from gerbils, 21 showed evidence of biofilm formation, using the same criteria of that for biofilms in human cholesteatomas (Figure 1C-F). Gram-positive and gram-negative bacteria were seen in many of the experimentally induced cholesteatomas (Figure 2). Bacterial colonies were consistently seen adhering to keratin debris in areas devoid of inflammatory cells. Ultrastructural studies using transmission electron microscopy revealed remnants of the polysaccharide biofilm matrix, which appeared as regions of amorphous material surrounding the bacteria (Figure 3). The amorphous material surrounding bacteria is consistent with a polysaccharide biofilm. This highly hydrated matrix contracts in aqueous solutions during tissue processing.

#### COMMENT

Bacteria exist either as planktonic, mobile, replicating organisms or as surface-attached, sessile colonies of bacteria within a polysaccharide matrix known as a biofilm. Organisms within biofilms, while actively metabolizing, do not replicate. These bacteria cannot be cultured using standard bacteriological techniques and are highly resistant to eradication by antibiotics and disinfectants. In the present study, we demonstrate anatomical evidence that biofilms form within the matrix of infected human as well as spontaneous and experimental gerbil cholesteatomas. Bacteria were seen in microcolonies embedded within an acellular polymeric matrix. In many cases, both gram-negative and gram-positive bacteria were seen within the same microcolony, suggesting that these aggregations are mixed bacterial biofilms.

#### BIOFILMS ON MEDICAL DEVICES

Biofilms have been shown to be present on a variety of medical devices such as urinary catheters, central venous catheters, fracture fixation devices, joint prostheses, tympanostomy tubes, and voice prostheses. An understanding of the mechanisms underlying the environmental promotion of biofilm formation and the increased resistance to antibiotic treatment are now essential for optimizing our standards of patient care.

#### BIOFILMS IN HUMAN DISEASE

Microbial biofilms have been shown to be an important factor in a number of human diseases. Dental plaque,
formed on dental enamel surfaces, is a well-known biofilm disease leading to periodontitis. In addition to its formation on nonliving surfaces, biofilms have been shown to form on living mucosal surfaces. *Pseudomonas* biofilms form within the lungs of individuals with cystic fibrosis leading to chronic disease. Singh and colleagues showed that *Pseudomonas* microcolonies consistent with biofilms were recovered from sputum from cystic fibrosis patients. Post, Post et al, and Rayner et al were the first to demonstrate evidence for bacterial biofilms in ear disease. They have shown indirect evidence in humans and direct evidence in an animal model that *H influenzae* infections form biofilms within the middle ear, causing chronic otitis media with effusion.

Figure 1. Light microscopic views of a bacterial biofilm within the matrix of cholesteatomas. A and B, Human cholesteatoma; C-F, cholesteatomas from gerbils. A, A low-power view of a human cholesteatoma shows layers of keratin debris with a bacterial biofilm between keratin layers; B, a higher magnification of the area indicated in A shows a bacterial biofilm, which appears to be adherent (arrows) to keratin; C, clumps of bacteria between layers of a gerbil cholesteatoma near an epithelial surface; D, adherent bacteria within a cholesteatoma; E, clumps of gram-negative and gram-positive bacteria within an amorphous matrix near keratin debris; and F, bacterial colonies that appear to be adherent to keratin.
**BIOFILMS AND MICROBIAL RESISTANCE**

Bacteria within biofilms have been found to be highly resistant to common disinfectants. Takeo and colleagues found that 0.1% chlorhexidine and 0.5% alkylidiaminoethyl glycine would not eradicate *Pseudomonas aeruginosa* after 1 hour of exposure and that eradication of this organism within a biofilm requires higher concentrations and longer exposures. Poor biofilm-killing performance of common disinfectants is likely due to bacterial resistance rather than poor or ineffective penetration of the antimicrobial agent.

Bacterial biofilms protect bacteria by physically shielding them from UV-C, UV-B and UV-A radiation. Ultraviolet light appears to be absorbed by the alginate matrix of a biofilm. Bacteria within biofilms also show increased resistance to antibiotics. For example, bacteria in aqueous solution were shown to have a minimum inhibitory concentration of 2 g/mL of ampicillin, while the same bacteria grown as a biofilm were only marginally inhibited by 4 hours of exposure to 5000 g/mL. The following 3 possible mechanisms of this bacterial resistance have been suggested: (1) slow penetration of the biofilm matrix; (2) development of a resistant phenotype within the matrix; and (3) altered microenvironment. There is evidence for all 3 mechanisms, but each may not be operant in all biofilms. For example, ampicillin can penetrate biofilms formed by *Klebsiella pneumoniae* but not others.

The existence of biofilms within cholesteatomas may explain the clinical nature of this disease. Like other biofilm diseases, infections within cholesteatomas are resistant to eradication by antibiotics. Antibiotics may temporarily control active infection by the planktonic bacteria within a cholesteatoma, but the bacteria within biofilms persist only to reassume their planktonic state when conditions are suitable, hence the recurrent and calcific nature of these infections. In fact, the cholesteatoma may prove to be a uniquely resistant example in biofilm biology if the cholesteatoma matrix provides an additional layer of protection for the bacteria. In addition, bacteria within biofilms are actively metabolizing and producing endotoxin as well as other factors, which may perpetuate an inflammatory host response even in the absence of culturable (freely mobile planktonic) bacteria (*Figure 4*).

**BIOFILMS AND CELLULAR SIGNALING**

The presence of sessile bacteria with biofilm communities in cholesteatomas may mediate the host responses seen in this disease, including chronic inflammation, epithelial proliferation, and bone resorption. For example, bacteria within biofilm communities can produce endotoxin, leading to inflammatory host responses. Dingman and colleagues showed that bacteria within the middle ear, detected by polymerase chain reaction but undetectable by culture, produced endotoxin in middle ear effusions.

In addition to the effects of bacterial endotoxin, biofilms may have direct effects on epithelial cell signaling. The initial process of biofilm formation is bacterial adherence to a surface; in human disease, bacteria adhere to epithelial surfaces. For example, *Escherichia coli* expresses type 1 fimbriae that contains FimH, an adhesion molecule that binds to epithelium. Adherence of bacteria to epithelial surfaces can induce cellular signaling, affecting the host. In a gene expression study of adherent *E. coli*, Mysorekar and colleagues found altered regulation of a wide variety of host genes. They found that adherence of uropathic *E. coli* to bladder epithelium leads to regulation of signals, which can result in epithelial differentiation (down-regulation of bone morphogenetic protein 4) and proliferation (induction of epidermal growth factor family members). In addition, they found that interleukin 6, a proinflammatory cytokine, was also up-regulated. Hence, sessile bacteria within biofilms in the cholesteatoma matrix may mediate host responses by direct elaboration of bacterial products, such as endotoxin, or induce host cellular signaling by adherence to epithelial surfaces.

**CONCLUSIONS**

Aural cholesteatomas vary in progression and aggressiveness; the presence of bacterial biofilms in some cholestea-
tomata may explain their activity. Infections within cholesteatomas often defy eradication by topical and systemic antibiotics. It is likely that the presence of bacterial biofilms within the cholesteatoma matrix explain persistent infection within cholesteatomas. Antimicrobial agents fail to eradicate the sessile bacteria within biofilms; when conditions are favorable, the sessile bacteria within these biofilms become motile and planktonic, leading to active infection. Direct signaling of bacterial products, such as endotoxin, and indirect signaling by bacterial adherence may lead to the chronic inflammation and epithelial proliferation, which is characteristic of this disease. Aside from physical removal of the cholesteatoma and its biofilm laden matrix, no effective measures are available to eradicate these microbial biofilms.

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