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Inactivation of Specific *Pseudomonas aeruginosa* Biofilm Factors Does Not Alter Virulence in Infected Cholesteatomas

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Abstract

Hypothesis—When experimental cholesteatomas are infected with *Pseudomonas aeruginosa* (PA) mutants lacking factors associated with the formation of biofilms, host defenses are more effective against these strains when compared to wild type strains (PAO1 and OPPA8) in preventing tissue destruction.

Background—Prior studies have identified biofilms within chronically infected aural cholesteatomas. These infected cholesteatomas are associated with increased tissue destruction. Since biofilms are highly resistant to host defenses leading to prolonged infection, we propose that the biofilm phenotype of *P. aeruginosa* may be a virulence factor leading to persistence of infection and increased tissue destruction.

Methods—Aural cholesteatomas were induced in Mongolian gerbils. At the time of induction, the ear canals were inoculated with wild type (PAO1 and OPPA8) and biofilm deficient (PAO1 *pilA*, PAO1 *algD::aacC1* and PAO1 *galU::aacC1*) strains of *P. aeruginosa*. After eight weeks, the size of the cholesteatomas and levels of bone destruction and deposition were measured using microCT scanning and double fluorochrome bone labeling.

Result—Infected cholesteatomas resulted in increased growth, bone destruction and bone deposition when compared to vehicle only controls. We observed no differences between the wild type (biofilm forming) and the biofilm deficient strains of *P. aeruginosa*.

Conclusion—Our hypothesis that biofilm formation is a virulence factor in cholesteatomas infected with *P. aeruginosa* was not supported. A number of interpretations of this data are reasonable. It is possible that biofilms are not critical in infected cholesteatomas. Alternatively, the mutants that are deficient in generating biofilms *in vitro*, may be able to form effective biofilms *in vivo* using alternative pathways.

Aural cholesteatomas are characterized by the presence of keratinizing epithelium in the middle ear or mastoid. They can be congenital or acquired. Acquired cholesteatomas are the result of severe retraction of the tympanic membrane or ingrowth of keratinizing epithelium into the middle ear from a perforation. Regardless of the etiology of the cholesteatoma, the result is often a progressive, destructive process that can lead to hearing loss, vestibular abnormalities and even intracranial complications. Many cholesteatomas become

chronically infected. Infected cholesteatomas have been shown to be associated with more rapid advancement and destruction when compared to non-infected cholesteatomas(1). Bacterial products, such as lipopolysaccharide, may accelerate the destructive process(2). Additionally, infections within cholesteatomas are often highly resistant to eradication with antibiotics and host defenses perhaps due to the establishment of microbial biofilms within the matrix(3).

The bacteria found in chronically infected cholesteatomas are most commonly *P. aeruginosa* (PA) and *S. aureus*(4, 5). We have chosen *P. aeruginosa* as the model organism for these studies. Otopathogenic strains of PA (OPPA) have been characterized(6) and have been shown to cause chronic infection in experimental animals(1). *P. aeruginosa* is also a common pathogen in spontaneously occurring cholesteatomas in gerbils(7, 8).

Infections in cholesteatomas are often extremely difficult to eradicate even with culture-directed topical and systemic antibiotics. We hypothesized that the chronicity of these infections may be due to the propensity of these organisms to form sequestered micro-colonies termed biofilms(9). It is frequently reported that bacteria within biofilms become highly resistant to antibiotics and host defenses(10). We previously identified biofilms within the matrix of cholesteatomas in humans and experimental animals(3). While it is reasonable to assume that the recalcitrant nature of these infections is explained by the presence of biofilms, it is not established that the ability of the bacteria to form biofilms is, in itself, a virulence factor.

Therefore, we hypothesize that the **biofilm phenotype** of PA within infected cholesteatomas is a virulence factor leading to persistent infection and continuing growth and expansion of the cholesteatoma and report a set of experiments to test this theory.

METHODS

Design and Rationale

Cholesteatomas were induced in both ears in six groups of eight gerbils. At the time of induction, bacteria (10^6) were instilled into the medial ear canal in 50 μ l vehicles. One group served as a control (vehicle only) and bacteria were instilled in the other six groups. *P. aeruginosa* strains were used in five groups. PAO1, a well characterized laboratory strain of PA, was used as a positive control(11); this strain was originally isolated from a burn wound infection. Three mutant strains of PAO1 (*pilA*, *algD::aacC1* and *galU::aacC1*) were chosen as biofilm deficient strains. OPPA8 is a PA strain isolated from a human cholesteatoma that can form biofilms(6). *E. coli* DH5 α , a non-biofilm forming bacterium, was used as an additional control. Each of these strains was incubated in biofilm forming conditions *in vitro* to confirm their biofilm phenotype (see Results section).

Experimental animals

We chose the gerbil model of spontaneous and induced cholesteatomas for this study. Gerbils (*Meriones unguiculatus*) are the only known animal model to spontaneously develop cholesteatomas as they age(8). These cholesteatomas arise as keratin accumulates on the surface of the tympanic membrane, eventually blocking the external auditory canal(12).

Over time a cholesteatoma develops in the medial ear canal and then enlarges into the middle ear (bulla). The formation of these cholesteatomas can be accelerated by simply placing a ligature around the external auditory canal(13).

In vitro Assessment of Biofilm Formation

The ability to form biofilms for each of the strains was determined using a crystal violet static well assay. Biofilm formation *in vitro* peaked between 12 and 36 hours of incubation(14); we chose to do the analysis of each strain at 30 hours. Each of the mutants showed impaired biofilm formation compared to wild type controls (PA01 and OPPA8). We used *E. coli* DH5 α as a non-biofilm forming control. Each bacterial strain used was recovered from glycerol stock, plated on LB agar plates, a single colony was inoculated into 10 mls of LB medium and incubated overnight at 37°C overnight. A ~10X dilution in LB medium was made to result in an absorption at A600 of 0.3 in LB medium corresponding to 10⁹ bacteria per ml. The resulting suspension was diluted 1:1000 in M63 medium and 100 μ l aliquots were placed into each well of a 96-well Costar plate, which had been previously UV sterilized for >20 minutes. Plates were then incubated in a humidified environment at 30°C for the specified 30 hours.

At the end of the incubation period, the wells were emptied and rinsed vigorously five times in tap water. They were inverted and dried overnight. The wells were then stained by instilling 150 μ l of 0.1% crystal violet for 10 minutes at room temperature. The wells were emptied, rinsed five times in tap water and dried. When dry, 200 μ l of 30% glacial acetic acid was added per well and placed on an orbital mixer for 15 minutes. Absorption was measured at 595 nm within a Synergy®HT Multi-Detection microplate reader (Bio-Tec Instruments, Inc, Winooski, Vermont, USA).

Bacteria

PAO1—PAO1 was chosen for a wild type control strain of PA. It was first isolated in 1955 in Melbourne, Australia from a burn wound(11).

PAO1 *pilA*—PA has been shown to be adherent to exfoliating epithelial cells(15). It is likely that adherence is highly dependent upon the type-four pili of PA. Polar type-four pili are responsible for twitching motility of PA(15) and contribute significantly to adherence of PA to epithelial cells(16, 17). Since adherence to desquamated epithelial cells within a cholesteatoma is a likely first step in biofilm formation(18), we chose this non-piliated mutant as one example of a biofilm deficient PA.

The PAO1 *pilA* mutant, PAO1-NP1, was generated in the following steps. pCB132, a *pilA* suicide plasmid, was constructed by PCR amplification of the 500 bp upstream of *pilA* (using primers *pilA* #1 CAAGCGGCCGCCGCATAGCACCCGGCAAGCCG and *pilA* #2 CCAGGATCCCCGAAAGGTTGTGATAACTAAGG) and the 500 bp downstream of *pilA* (using primers *pilA* #3 CCAGGATCCCATCAGTTCGATCAAGGTAAAGCC and *pilA* #4 CCAGTCGACCGGAAAGCTTTCCTTGTCAGG). The PCR products were digested with NotI/BamHI and BamHI/SalI, respectively, and then ligated into NotI/BamHI-digested pJB4868 (gentamycin resistant version of the suicide plasmid pSR47S). pCB132

was then transformed into PAO1 by electroporation and integrants were selected on LB plates containing 30 µg/ml gentamycin. The merodiploid was resolved on LB plates containing 5% sucrose and “loopouts” to the deletion were identified via PCR using the external primers *pilA* #5 (CCTCTACGATGCCTTCCTGATCAAGG) and *pilA* #6 (CCAGCTTTTCGCTGATGGCGTCCCG).

PAO1 *galU::aacC1*—The enzyme glucose 1-phosphate uridylyltransferase (GalU) is required for the synthesis of capsular polysaccharide in Gram-negative and Gram-positive bacteria(19). It has been shown to be a virulence factor in PA(20). *galU* mutants showed attenuated corneal infections when compared to the wild type strain (PAO1)(21). Additionally, *galU* has been shown to be essential for biofilm formation in PA(22). The PAO1 *galU::aacC1* mutant was obtained from Joanna Goldberg(20). This mutant was constructed by inserting GentR into a unique EcoRV site within the *galU* ORF, thus inactivating *galU*(20).

PAO1 *algD::aacC1*—Alginate is a significant component of the extracellular polymeric substance of *P. aeruginosa* (23). The *algD* encodes GDP-mannose dehydrogenase and is required for the biosynthesis of alginate(24) We selected the *algD* PAO1 mutant (PD0299) as an alginate deficient strain. It was constructed by inserting GentR into a small deletion within the *algD* ORF and was obtained from the Ohman laboratory(25).

***E. coli* DH5α**—*E. coli* DH5α was chosen as a biofilm-deficient control strain(26).

OPPA8—This biofilm forming strain of otopathogenic is a PA and was isolated from an infected human cholesteatoma(6).

In vivo Assessment of Biofilm Formation Factors in Cholesteatomas – Experimental Design

The virulence of these strains was studied *in vivo* using the gerbil model of induced ear canal cholesteatomas. The procedure was a modification of previously described techniques(13). Five groups of gerbils and one group of controls were used. Six-week old gerbils underwent ear cholesteatoma induction by canal ligation(27). Briefly, the animals were anesthetized with a mixture of ketamine HCl (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA), 0.08mg/g/xylazine HCl (supplied by the Department of Comparative Medicine, Washington University, St. Louis, MO), injected intraperitoneally. Bupivacaine HCl (Marcaine, Hospira, Inc, Lake Forest, IL, 0.1 µg/g) was also injected into the incision site for pain relief. The periauricular and scalp skin was prepared by swabbing with povidine-iodine topical antiseptic solution (Betadine® Purdue Products, Stamford, CT) for 5 minutes. A small postauricular incision was made. Using small curved forceps, a subcutaneous tunnel was created around the membranous ear canal and a 4-0 silk ligature was placed through this tunnel. 50 µL of vehicle (PBS) with or without bacteria was inserted into the left external auditory canal, which was firmly ligated with 4-0 silk suture around the membranous canal. The wounds were closed with interrupted 5-0 chromic sutures. Ears were inoculated with vehicle or 10⁶ bacteria. The strains used were PAO1 wt, PAO1 *pilA*, PAO1 *algD::aacC1*, PAO1 *galU::aacC1*, OPPA8 and *E. coli* DH5α. After eight weeks

animals were double fluorochrome labeled with xylenol orange (Sigma-Aldrich, St. Louis MO) and calcein (Sigma-Aldrich, St. Louis, MO) to later measure apposition rate(28). After sacrifice and fixation, the gerbil heads underwent microCT scanning.

Micro-computed Tomography and Histomorphometry

Micro-computed tomography (microCT) was used to determine the level of bone erosion and reactive bone formation present in the gerbil ear. Eight weeks after surgery, animals were given sodium pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA, 0.5 mg/g) to deeply anesthetize each animal. The chest was opened and a 21 ga needle placed into the left ventricle and perfused with normal saline. Fixation was achieved by perfusing with 2% paraformaldehyde, 2.5% glutaraldehyde in a 0.1M phosphate buffer (pH 7.4). The intact head from each animal was oriented in an axial orientation within the 30 mm diameter plastic cylinder used in the microCT scanner. The cylinder with the oriented head was filled with 1% agarose to maintain positioning during the scanning process. MicroCT scanning was performed on a Scanco μ CT 40 scanner (Scanco Medical AG, Basserdorf, Switzerland) with point-spread function of 0.036 mm over a period of 30 minutes per specimen. The scanning parameters consisted of 70 kVp tube potential, 114 mA tube current, 200 ms integration time. The resulting reconstructed volume had 36 μ m isotropic voxels.

For all analyses, an axial section was chosen to include the long process of the malleus on both ears and the mid-cochlea in order to maintain consistent measurements. The CT image was modified in Adobe Photoshop® (Adobe Systems Inc., San Jose, CA) in a standardized manner with levels converted from 0–255 to a contrast range of 0–150 to better differentiate gas from tissue and tissue from bone. The grayscale images are saved as *.tif files for analysis with ImageJ® (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–2006). All images were coded and blinded for image analysis.

In order to estimate **cholesteatoma size**, radiolucent areas, presumably trapped air or gas, were delineated and recorded; radio-opaque areas, presumably cholesteatoma and surrounding inflammation, were separately delineated and recorded (Fig. 1). Each image was converted to black and white and saved as *.tif files. Using NIH ImageJ®, the area of the gas and the cholesteatoma was measured (calibrated directly from the CT image). The proportion of cholesteatoma (radio-opaque area) was expressed as a percent of total: cholesteatoma/(gas + cholesteatoma). Cholesteatoma area was recorded as a percent of tissue within total bulla area in each axial section.

For **bone erosion** measurements, the distance between the ventral and dorsal external auditory canal (Fig. 1). The external auditory canal is in this region and is seen as a deficient area of bone of the lateral bulla. Using NIH ImageJ®, the length of the deficient bone was measured in mm. Erosion of the ear canal is reliably seen in gerbil cholesteatomas and is evident by the larger defect on the right side compared to the left. Each parameter was measured in three reproducible frames.

Bone deposition was measured as the area of the ventral bulla bone in the aligned axial sections using a standard size rectangle over the test area. The measurement was calculated in mm² and percent bone area within the standardized rectangle calculated (Fig. 1.).

Malleus calcification was measured in the same axial sections. The outlines were then converted to bitmap images and the number of pixels was calculated using ImageJ software (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA) (Fig. 1).

After scanning, the bullae were embedded and sectioned for fluorophore measurement of bone apposition rate. The scans were analyzed in a blinded manner with the following measures: tissue density within the bulla, erosion of the external auditory canal, demineralization of the malleus and thickening of the dorsal bulla.

Fluorochrome Labeling for Bone Growth Assessment

Thirteen days prior to sacrifice each animal was administered calcein (3 mg/kg intraperitoneally). Twenty-four hours prior to sacrifice each animal was administered xylenol orange (195 mg/kg intraperitoneally). At the time of sacrifice, small fragments of the dorsal bulla were removed after CT scanning and embedded in LR white resin. The embedded bone fragments were sectioned on an Isomet diamond saw (Buehler, Lake Bluff, IL, USA) to a thickness of 50 micrometers and glued firmly on glass slides with cyanoacrylate glue. The sections were then thinned to approximately 10 micrometers by abrading their surface with 600 grit wet-dry sandpaper. After being prepped on cover slips the samples were observed and photographed with an Olympus BH ultraviolet reflectance microscope.

Regulatory

This study was approved and monitored by the Washington University Animal Studies committee. The use of the bacteria from human subjects was approved by the Washington University Office for the Protection of Human Subjects.

Statistics

Groups in the *in vivo* and *in vitro* experiments were compared using a one way analysis of variance (ANOVA) with multiple comparisons ($\alpha=0.05$).

RESULTS

Biofilm Formation *In vitro*

When grown in static wells, PAO1 and OPBA8 form robust biofilms consistent with previous reports(6) (Fig. 2). Strains deficient in Type IV pili, AlgD and GalU are significantly attenuated in the formation of biofilms ($p < 0.05$). Similar to the PA mutants, *E. coli* DH5 α is a very weak biofilm former (Fig. 2).

In vivo Fluorochrome Labeling for Remodeling

Double fluorochrome labeling with calcein and xylenol orange was intended to measure apposition rate of the ventral bulla wall. In the untreated bulla, clear lines of fluorochrome

(calcein and xylenol orange) were observed due to bone growth (Fig. 3 upper panel). In the bullae with cholesteatomas, remodeling was so vigorous that simple apposition rates (distance between fluorochromes/time) could not be measured (Fig. 3 middle panel). Even more vigorous remodeling was seen in bullae with infected cholesteatomas (Fig. 3 lower panel).

MicroCT Scans

Cholesteatoma size was determined by measuring the area of soft tissue density in the bulla compared with the size of the entire bulla. This measure included both the cholesteatoma itself and the surrounding inflammatory reaction. All infected cholesteatomas showed increased size compared to the untreated and uninfected (PBS) controls (Fig. 4A). Notably, deletion of a number of putative biofilm dependent genes had little effect on cholesteatoma size when compared to the size induced by wild type PA (Fig. 4A).

Bone erosion due to induced cholesteatomas was assessed with two measures: ear canal width and malleus density. All six bacterial strains studied showed more bone resorption of the external auditory canal than the untreated control (no cholesteatoma) or the PBS control group (Fig. 4B). In addition, there were no significant differences in malleus bone density when the three biofilm deficient strains were compared to the untreated control (no cholesteatoma) and the uninfected cholesteatoma (Fig. 4C). Malleus erosion was the most inconsistent of these measures and led to wide variations in some groups.

Bone deposition, which invariably accompanies bone resorption, was found in each of the six infected groups (PAO1 wt, *pilA*, *algD::aacC1* and *galU::aacC1*, OPPA8 and *E. coli* DH5 α). There were no differences in bone deposition among these groups with the exception of the *E. coli* group, which showed slightly diminished bone deposition ($p < 0.05$). All six infected groups had significantly more bone deposition than the untreated control and the uninfected (PBS) cholesteatoma control (Fig. 4D).

DISCUSSION

Chronic, indolent infections are commonly seen in aural cholesteatomas. Once these infections are established, they are difficult and sometimes impossible to eradicate with topical and systemic antibiotics. It has been proposed that this resistance to antibiotics and host defenses may be due to the propensity of these bacteria, including *P. aeruginosa* and *S. aureus*, to form biofilms(3). The goal of this study was to compare the virulence of biofilm forming and non-biofilm forming bacteria in an experimental model of aural cholesteatoma.

Aging gerbils spontaneously develop aural cholesteatomas(8). These cholesteatomas can be described as beginning with an inflammatory process near the pars flaccida that eventually blocks the entrance of the ear canal leading to an accumulation of keratin and an enlarging ear canal(12, 29). As cholesteatoma development can be accelerated by simple ligation of the ear canal(13), we were able to develop a model employing canal cholesteatomas. In addition, our model was amenable to testing the role of infection in cholesteatoma formation, as most spontaneous cholesteatomas in gerbils are infected(30).

Based on a series of studies, we were able to demonstrate that infected induced cholesteatomas are more destructive than those without infection(1). Specifically, we found that PA infections in gerbilline cholesteatomas led to bony destruction of the external auditory canal and erosion of the malleus. Moreover, infected cholesteatomas were larger than uninfected controls when measured radiographically. When we determined the size of the cholesteatomas, our measurements include the cholesteatoma and the surrounding tissue and fluid since exudates around the cholesteatoma have the same density as the cholesteatoma itself. Nevertheless, comparisons were valid since the same technique was used in each experimental group.

The most striking finding in this study was that the attenuation of PAO1 biofilm forming factors, including *pilA*, *algD* and *galU*, did not diminish the destructive properties of the infected cholesteatomas. Notably, control cholesteatomas, in which sterile vehicle only was instilled, showed far less destruction compared to those observed in each of the PA strains.

There are a number of possible explanations for these findings. 1) Biofilm formation by PA may not be required as a virulence factor in infected cholesteatomas. 2) Our experiment was insufficient to detect a requirement of biofilm formation during canal cholesteatoma formation and progression.

Biofilm Formation Is Not a Virulence Factor

Our hypothesis was that the biofilm phenotype of PA would be required for the virulence observed in infected cholesteatomas. While there were strong reasons for proposing this hypothesis, it may be incorrect. Persistence of these organisms may instead be dependent on other factors that allow the bacteria to survive the onslaught of host defenses. Consistent with our results, it is worth noting that biofilm formation has frequently been reported as being essential for virulence although this assertion often lacks experimental confirmation.

Limitations of our Experiment

We are unable to definitively conclude that biofilms are not required for induced aural cholesteatoma formation due to several caveats in our studies. First, we relied on host defenses to eradicate planktonic PA infections in the induced aural cholesteatomas. Inclusion of antibiotics after the initial development of the cholesteatoma may have been more effective in revealing a difference in biofilm-competent strains versus biofilm-deficient strains. Second, another possible explanation for the lack of differences among the biofilm-deficient groups is unappreciated secondary infection. We were not able to culture the infected cholesteatomas at the end of the test period without disrupting the morphology as measured with microCT scanning. However, the control cholesteatomas would have an equivalent risk of secondary infection and all comparisons were made with these controls. Third, the mutant strains we chose, which are attenuated for biofilm formation *in vitro*, may not have been deficient enough in biofilm formation to detect a difference. Notably, these strains do form a limited amount of biofilms *in vitro*, which may be sufficient for virulence in our model. Finally, as organisms employ a variety of strategies to form biofilms, it is possible that the *in vitro* biofilm attenuated PA mutants we employed were still able to form biofilms via another mechanism *in vivo* in our cholesteatoma model. In order to fully

rule out the hypothesis that biofilm formation is not required for induced cholesteatoma progression studies utilizing mutants totally deficient in biofilm formation would be needed.

CONCLUSIONS

Cholesteatomas, induced by ear canal ligation and infected with *P. aeruginosa* exhibited more enlargement, bone resorption and bone remodeling than uninfected (PBS) controls. However, disruption of PA biofilm forming genes (*pilA*, *algD* and *algU*) had an undetectable effect on the virulence of this organism in this animal model.

There are at least two explanations of the results of this study. 1) Biofilm formation is not required as a virulence factor in PA infected cholesteatomas or 2) Inactivation of individual factors adequate to attenuate biofilm formation in vitro may not be sufficient to prevent biofilm formation in vivo.

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FIG. 1.

A microCT scan in the axial plane of the calvarium and bullae of a gerbil head demonstrates enhanced cholesteatoma formation due to infection. On the left side, an uninfected cholesteatoma can be seen in the ear canal (gray density labeled as tissue) whereas the middle ear is filled with air (black). On the right side, an infected cholesteatoma can be observed in the ear canal and filling the entire middle ear (gray). The ear canal width and the malleus density were used as surrogates for bone erosion. The area of the dorsal bulla wall was a measure of bone deposition. The lower images represent the areas analyzed where the ratio of air to total bulla area was used as a surrogate for cholesteatoma size.

FIG. 2.

PAO1 mutants are defective in biofilm formation. Bacterial isolates were incubated under biofilm forming conditions for 30 hours and biofilms were detected by measuring crystal violet staining at A595. The wild type strains PAO1 and OPPA8 were able to form robust biofilms while the mutant strains demonstrated suppressed biofilm formation. *E. coli* DH5 α displayed negligible biofilm formation.

FIG. 3.

Double fluorochrome labeling of an infected gerbil demonstrates the rate of bone deposition. Calcein (green) was administered followed 12 days later by xylenol orange (orange). The animals were sacrificed one day later and processed for imaging where the distance between the two markers indicates the apposition rate. As the infected bullae revealed disorganized and extensive remodeling, a precise apposition rate could not be determined.

FIG. 4.

PA mutants defective in biofilm formation in vitro are not attenuated for virulence in a gerbil canal cholesteatoma model. Virulence was measured by microCT scanning for four assays including cholesteatoma size (A), bone resorption (B and D) and bone deposition (C). Conditions assayed included infections using the wild type biofilm forming strains (PAO1 and OPN8), the biofilm deficient strains (PAO1 $\Delta pilA$, PAO1 $algD::aacC1$ and PAO1 $galU::aacC1$) and the *E. coli* strain DH5 α . Negative controls included uninfected and mock infected using PBS.