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Characterization of Mucosal Biofilms on Human Adenoid Tissues

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Objectives: To demonstrate the presence of mucosal biofilm in adenoid tissue using double staining for visualization of both the bacterial matrix and the bacterial cells. To identify bacterial species present on the surface of the studied adenoids.

Study Design: Prospective study.

Methods: A total of 39 specimens of adenoidectomy were removed from children with chronic and/or recurrent otitis media. The specimens were prepared for light microscopy using Gram staining, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Double staining was performed with CLSM to visualize both the bacteria and the glycocalyx matrix. Nine adenoids on which bacterial biofilms were visualized with CLSM were used for identification of bacterial species by 16S-DNA polymerase chain reaction (PCR) amplification and homology analysis.

Results: Of the 39 adenoids investigated, 22 (54%) showed evidence of mucosal biofilms. Gram staining, SEM and CLSM showed the presence of bacterial cells, organized in bacterial microcolonies. CLSM with double staining demonstrated mucosal biofilms by showing the presence of both bacteria and the glycocalyx. The use of 16S-DNA polymerase chain reaction (PCR) amplification and subsequent sequence analyses identified the presence of *Corynebacterium argentoratense, Streptococcus salivarius*, *Micrococcus luteus*, and *Staphylococcus aureus*.

Conclusions: This study demonstrates that adenoid tissue in children with chronic or/and recurrent otitis media contains mucosal biofilms in 54% of the cases. The existence of living bacteria has been demonstrated. Further studies are required to describe the panel of bacteria that can be harbored in the biofilms present in adenoids and the mechanisms involved in the physiopathology of otitis prone children.

Key Words: Biofilms, bacteria, adenoids, human tissue, otitis media.

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INTRODUCTION

Bacterial biofilms are defined as communities of bacteria encased in a self-produced glycocalyx matrix. They form by adhesion of planktonic or free-living bacteria to a surface, followed by the development in bacterial microcolonies in conjunction with the production of the glycocalyx matrix. This dynamic process of biofilm formation builds up a mature biofilm that persists on surfaces. Resistance to both antibiotic treatment and host defense is one of the major clinical features of bacterial biofilms.[1] Therefore, they are considered to play a role in the majority of chronic and/or recurrent infections.[2] Because bacteria present in biofilms are difficult to culture, identification of bacterial biofilms has largely relied on visualization techniques, mainly using scanning electronic microscopy (SEM).

From a physiological point of view, adenoids are believed to play a central role in most bacterial infections of the upper and lower airways.[3,4] For otitis media, the origin of initial infection and recurrence has been allocated to adenoid tissues. The presence of biofilms in adenoid tissue has been reported using SEM.[5,6] However, SEM techniques require a dehydration process that reduces the total volume of the matrix and alters its architecture.[7] We have developed a double-staining technique in combination with confocal laser scanning microscopy (CLSM) which allows simultaneous imaging of the structural elements of a mucosal biofilm.[8] This double-staining technique shows bacterial cells and the glycocalyx with the preservation of the architecture of the biofilm due to the lack of dehydration. In addition, the identification of bacterial cells that are present in the bacterial biofilms of adenoids would be a step forward in the physiopathology of recurrent infections of the upper and lower airways. However, this bacterial identification is challenging,

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mainly because bacterial cells in biofilms can survive without dividing.[2]

Therefore, the aim of this study was to demonstrate the presence of mucosal biofilm in adenoid tissue using double staining for visualization of both the glycocalyx and the bacterial cells with CLSM. In addition, we cultured resident bacterial strains from selected frozen samples and identified bacterial species present on the surface of these adenoids using 16S-DNA analyses.

METHODS

Tissue Collection

Adenoid specimens were obtained during routine adenoidectomy from children with recurrent and/or chronic otitis media (n = 39). The Committee of Medical Ethics of Leiden University Medical Center was not needed because anonymized tissue was used that was left over from surgical procedures. The specimens were cut in three parts to be respectively prepared for light microscopy, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Nine adenoids samples were cut in four parts to identify bacterial species present on the surface of adenoids.

Visualization Techniques

Light Microscopy

Adenoid tissues were prepared for histological examination by fixation in 4% buffered formaldehyde for 20 hours at 4°C, dehydration through a graded series of ethanol, and then embedded in paraffin wax and cut at 4-μm thickness. Serial sections were taken from each tissue block at a variety of depths of the sample, and Gram staining was performed to demonstrate bacteria. The sections were examined with a light microscope (Axiophot, Zeiss, Germany), at a magnification of 10, 20, 40, and 100, and images were captured with a digital photograph system (ProgrRes C10, Jenoptik, Jena, Germany).

Scanning Electron Microscopy

The specimens for SEM were washed in phosphate buffered solution (PBS), fixed with 1.5% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer pH 7.4 for 24 hours at 4°C on a rotary shaker. The samples were then subsequently dehydrated through a graded series of acetone solutions (70%, 80%, 90%, 96%, and 100% acetone) for 20 minutes at room temperature, and critical point drying was performed. The specimens were then orientated, mounted on metal stubs, and sputter coated with gold using a Polaron 5000 Sputtering System (Watford, England) prior to imaging. The specimens were examined in a JSM6400 scanning electron microscope (JEOL, Tokyo, Japan) with digital imaging capabilities. The images were collected at an acceleration voltage of ~5.0 kV, a filament current of $\sim 10^{-10}$ A, a working distance of ~15 mm. All images were digitized as high-resolution Tagged Image File Format (TIFF) computer files (resolution 635 dpi), and then converted to high-quality JPEG files using Adobe PhotoShop 7.0 software (Adobe Systems Inc, San José, CA).

Confocal Laser Scanning Microscopy

For CLSM, the adenoidectomy specimens were immediately snap-frozen in cold isopenthane on dry ice and stored at −80°C. The frozen tissue specimens were cut to a thickness of 10 μm at −24°C using a cryostat CM 3050S (Leica, Bensheim, Germany) and fixated in 70% acetone. The obtained sections were then processed for double staining.[7] They were washed three times with PBS, and first stained with propidium iodide 15 μmol/L for 5 minutes at room temperature to detect bacterial cells in red. After washing with PBS, the sections were incubated with 50 $\mu g\ mL^{-1}$ Concanavaline A fluorescein isocyanate-conjugated (ConA-FITC, C7642, Sigma, St. Louis, MO) for 5 minutes at RT to stain the glycocalyx in green. The sections were then successively washed in PBS and demineralized water and embedded in Gelvatol/DABCO. The sections were examined using an Axioplan upright microscope (Zeiss, Germany) equipped with a Biorad MRC1024ES scan head (Hercules, CA) with a krypton/argon laser for visualization of ConA-FITC (excitation 488 nm and emission 522 DF 32 nm) and propidium iodide (excitation 568 nm and emission 605 DF 32 nm). Digital images of the CLSM optical sections were collected using the Lasersharp 2000 software (Biorad, Hercules, CA). Merged red-and-green images were obtained into single TIFF format and converted to high-quality Joint Photographic Experts Group (JPEG) files using Adobe PhotoShop 7.0 software (Adobe Systems Inc, San José, CA).

Image Analysis

Three investigators (REK, GL, and GB) evaluated the images independently in a blinded retrospective manner according to the criteria previously published to retain a specimen as containing a bacterial biofilm.[8] Briefly, criteria were: 1) presence of bacteria recognized by size, morphology, and for CLSM by red-fluorescent propidium iodide staining; 2) presence of glycocalyx shown on CLSM images by bright green fluorescence due to ConA-FITC staining; 3) more than one biofilm identified, at an air/specimen interface; 4) the absence of artifacts of dehydration or cutting for SEM and CLSM respectively; and 5) the absence of exclusion criteria such as bacteria located outside the specimen that could account for a potential infection during section preparation.

Bacterial Identification

To identify bacterial species present on the surface of adenoids which could be part of the observed biofilms as visualized by SEM and CLSM, nine adenoid samples which were stored at −80°C were cut to a thickness of 10 μm at −24°C using a cryostat CM 3050S (Leica, Bensheim, Germany) and directly transferred on BHI- and LB-agar plates and incubated 1 to 3 days at 37°C. Subsequently, plates were examined by eye and with the use of a stereomicroscope (Leica MZ 12 equipped with a Leica DC 500 camera, Bensheim, Germany) for the presence of bacterial colonies.

Isolation of Chromosomal DNA

Bacteria isolated from adenoid samples were inoculated from culture tubes stored at −80°C in 2 mL BHI medium and grown overnight at 37°C. Chromosomal DNA was isolated according to the technique reported by de Souza et al. with some adaptations.[9] Briefly, cells of 0.5 mL overnight culture were harvested and resuspended in 550 μL of TE buffer (Tris 10 mmol/L, EDTA 5 mmol/L, pH 8.0) amended with lysozyme (2 mg/mL). The mixture was incubated at 37°C for 30 minutes, after which 20 μL of a proteinase K solution (2 mg/mL in TE) and 5 μL of RNase were added and gently mixed. Subsequently, 60 μL of a 10% SDS solution was added and the mixture was incubated for 15 minutes at 65°C. Afterwards, 100 μL of 5 mol/L NaCl and 80 μL CTAB/NaCl (0.3 mol/L CTAB, 0.7 mol/L NaCl) were added and the mixture was incubated for 10 minute at 65°C. The mixture was extracted with 600 μL chloroform/isoamyl alcohol (24:1, v/v). Deoxyribonucleic acid (DNA) was precipitated from the water phase by addition of 0.6 volume (400 μL) isopropanol. The precipitate was washed twice with 70% ethanol and subsequently dried. The DNA-containing pellet was dissolved in 30 μL Milli-Q water by incubating for 15 minutes at 65°C.

Production and Analysis of 16 Second-DNA PCR Fragments

To amplify 16 second-DNA from bacterial species isolated from the surface of adenoids two general primers 27fm (5′–AGA GTT TGA TCM TGG CTC AG–3′) and 1522R (5′–AAG GAG GTG

ATC CAG CCG CA–3′) were used.[10] The reaction mixture contained two microliters of isolated chromosomal DNA (for isolation see above) as a template and 100 pM of each primer. A general PCR mixture was prepared with Taq polymerase.[11] A hot start at 95°C for 3 minutes was followed by 35 cycles consisting of 1) 95°C for 40 seconds, 2) 55°C for 20 seconds and 3) 72°C for 45 seconds. Finally, the mixture was incubated at 72°C for 10 minutes and stored at 4.0°C. Presence of 16-sDNA PCR products (approximately 1500 bp in size) was analyzed on 1% agarose gels. The presumed 16-sDNA PCR product was purified from agarose gel using a Qiagen gel purification kit. The purified product was used as a template in a second PCR reaction under the conditions described above. The second PCR reaction was performed to obtain sufficient amounts for sequencing and to reduce background noise of non-specific products made in the first PCR reaction using the whole chromosome as a template. The PCR product of the second reaction was purified using a Qiagen PCR purification kit. Products of different bacterial isolates were compared by ARDRA analysis[12] using *Hpa*2, *Taq*1 and *Sau*3A1, respectively, according standard incubation conditions recommended by the manufacturer (New England BioLabs, Inc., Ipswich, MA). PCR products were sequenced at ServiceXS (Leiden, The Netherlands). Sequences were analyzed by performing a BLAST × search on the National Center for Biotechnology Information (NCBI) home site (http://www.ncbi.nlm.nih.gov/).

RESULTS

Visualization

Of the 39 adenoids investigated, 22 (54%) showed evidence of biofilms according to the above-mentioned criteria.

Light Microscopy

Gram staining showed that the vast majority of bacteria were Gram-positive cocci (Fig 1). Bacteria were forming microcolonies along the surface of the specimen (Fig 1). Bacterial microcolonies were located on the outer surface of the adenoids and in the vicinity of small crypts (Fig 1A). Some densely packed hematoxylin-stained bacteria were seen (Fig 1B). They were sometimes associated with some more isolated or sparse bacteria (Fig 1C). Gram-positive were associated with Gram-negative bacteria (Fig 1D).

Scanning Electron Microscopy

Attached bacteria composing microcolonies were observed on the surface of the adenoids (Fig 2). Bacterial microcolonies were not evenly distributed over the entire surface of the specimen, but rather located in some parts of the specimen, mostly located in small depressions between cells of normal-appearing mucosa (Fig 2A and 2B). Some extracellular material was seen connecting the bacteria (Fig 2C and 2D). Bacteria were seen either connected in a scaffolding network when located in small crypts (Fig 2C), or interspersed when located on the outer surface of the specimen (Fig 2D). In some areas, large covering areas of embedded bacteria were seen (Fig 2E and 2F). Large amounts of extracellular material were seen either in the vicinity of the crypts (Fig 2E) or on the outer surface of adenoids (Fig 2F).

Confocal Laser Scanning Microscopy

CLSM demonstrated mucosal biofilm formation by double staining, i.e., by the combination of red and green fluorescent staining for the bacteria and the glycocalyx matrix respectively (Fig 3). Bacterial cells were both seen in small amounts and dividing (Fig 3A), or densely packed and surrounded in an extracellular matrix (Fig 3B). Well-delineated mucosal biofilms were observed in some crypts (Fig 3C) and at the outer surface of the tissue (Fig 3D). Interconnected bacteria were encased in a scaffolding network composed of extracellular matrix and demonstrated the typical three-dimensional (3D) architecture of biofilm formations (Fig 3E and 3F).

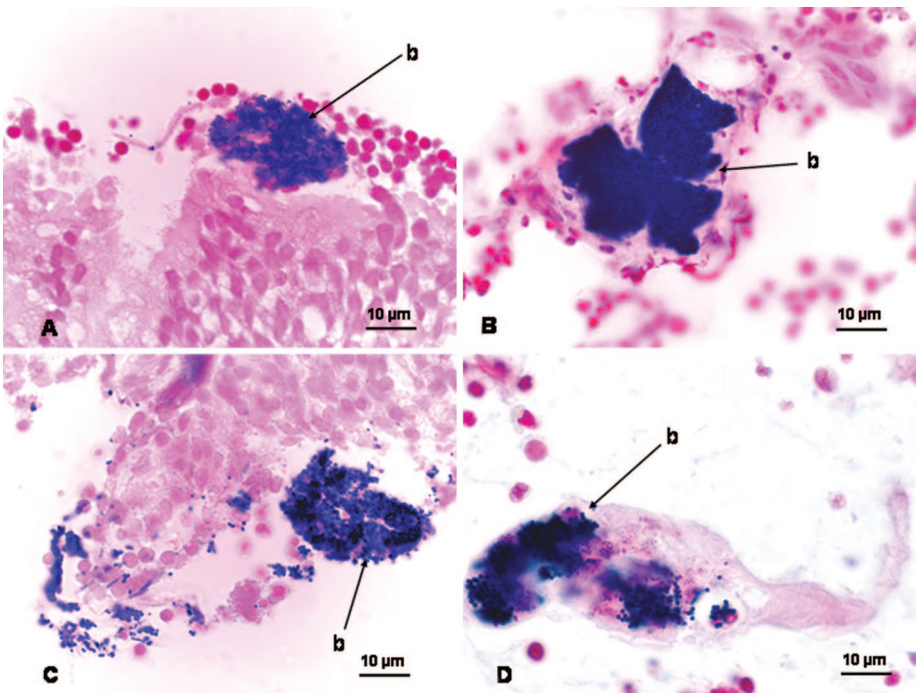

Fig. 1. Gram staining showing bacteria (arrow labeled b) along the surface of the specimen. Bacteria were forming microcolonies, mostly located on the outer surface of the adenoids, sometimes in the vicinity of small crypts (A). Some very densely packed hematoxylin-stained bacteria were seen (B), sometimes associated with some more isolated bacteria (C). Although the vast majority of bacteria were Gram-positive cocci, Gram-positive and Gram-negative bacteria could be seen (D).

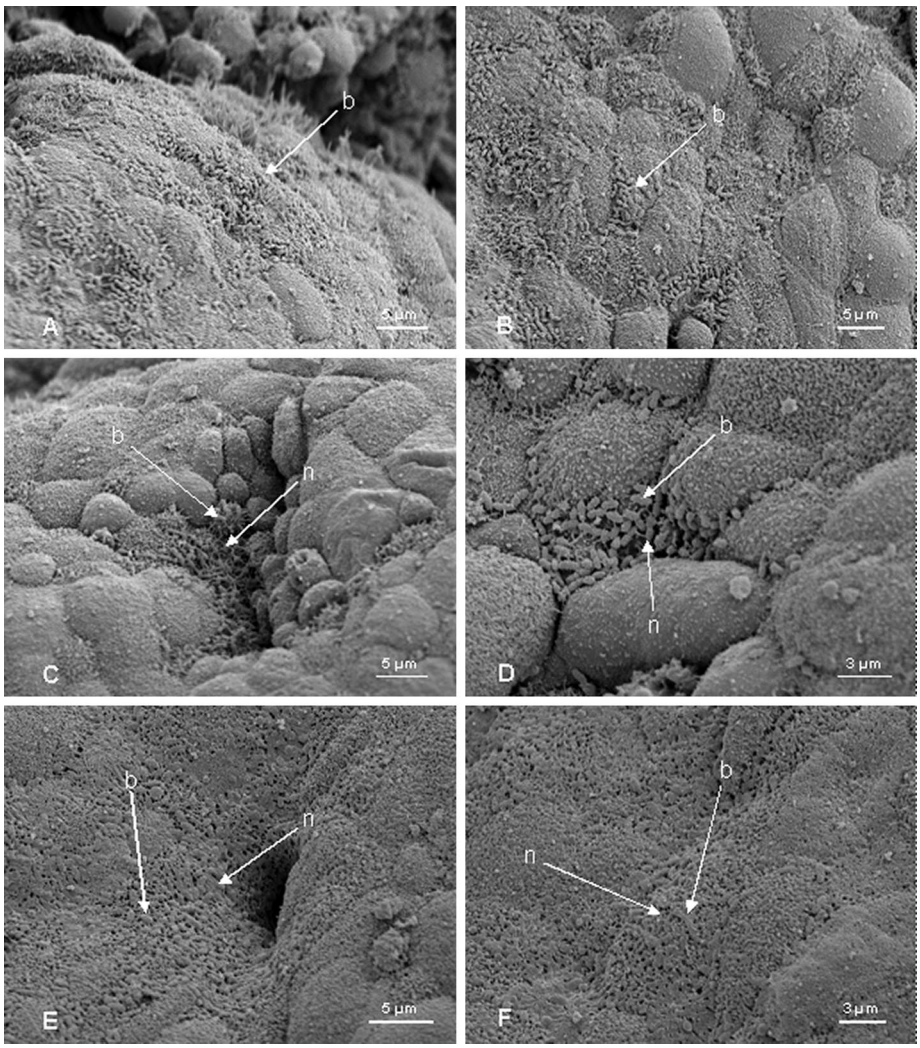

Fig. 2. Scanning electron microscopy showing attached bacteria (arrow labeled b) composing microcolonies, mostly located in small depressions between cells of normal appearing-mucosa (A and B). Connections of bacterial cells in some extracellular network (arrow labeled n) were seen in small crypts building up a scaffolding network (C) or on the outer surface of the specimen (D). Some large covering areas of embedded bacteria by a network of extracellular matrix were observed, either in the vicinity of the crypts (E) or on the outer surface of adenoids (F).

Bacterial Identification

After 1 to 3 days of incubation at 37°C, colonies were appearing in three out of nine adenoid samples at the borders of the specimen corresponding to the surface of the adenoids. On either BHI- or LB-agar plates, most of the periphery of the specimen was covered by bacterial colonies, whereas most of the inside of the specimen was free of them. Based on morphological criteria, we concluded that few inner parts of the specimen were colonized, but when colonized were always connected to bacterial colonies of the specimen outer part. These inner bacterial colonizations could account for the development of bacterial biofilms in the crypts of adenoid tissue. Bacterial identification resulted in isolation of three different strains from the first sample, two strains from the second, and one from the last sample. Subsequently, sequencing and homology analysis of the 16S DNA PCR products were performed showing that based on the highest homologies, 1) sample 1 contained a *Corynebacterium argentoratense*, a *Streptococcus salivarius*, and a *Micrococcus luteus,* 2) sample 2 contained a *Staphylococcus aureus* and a *Streptococcus salivarius,* and 3) sample 3 contained a *Staphylococcus aureus*. Strains numbered RKA1 to RKA6 and16S-DNA sequences of the strains are publicly available in Genbank under Accession numbers EF463055, EF463056, EF463057, EF463058, EF463059 and EF463060.

DISCUSSION

The results from this study show the presence of bacterial biofilms and the existence of living bacteria on adenoids tissue obtained by resection from children with recurrent and/or chronic otitis media. The demonstration of bacterial biofilm was done using CLSM with double-staining providing visualization of bacterial cells and glycocalyx as appearing in vivo in their three dimensional architecture. The presence of living bacteria was verified by culture obtained from snap frozen adenoid tissues, and Gram-positive bacteria were identified using 16S-DNA PCR.

In this series, the three techniques of visualization showed bacterial cells organized in bacterial microcolonies. However, for the demonstration of the glycocalyx, gram staining and SEM were limited because of the dehydration process they require. Because the glycocalyx is highly hydrated (typically 95 to 99%), the dehydration

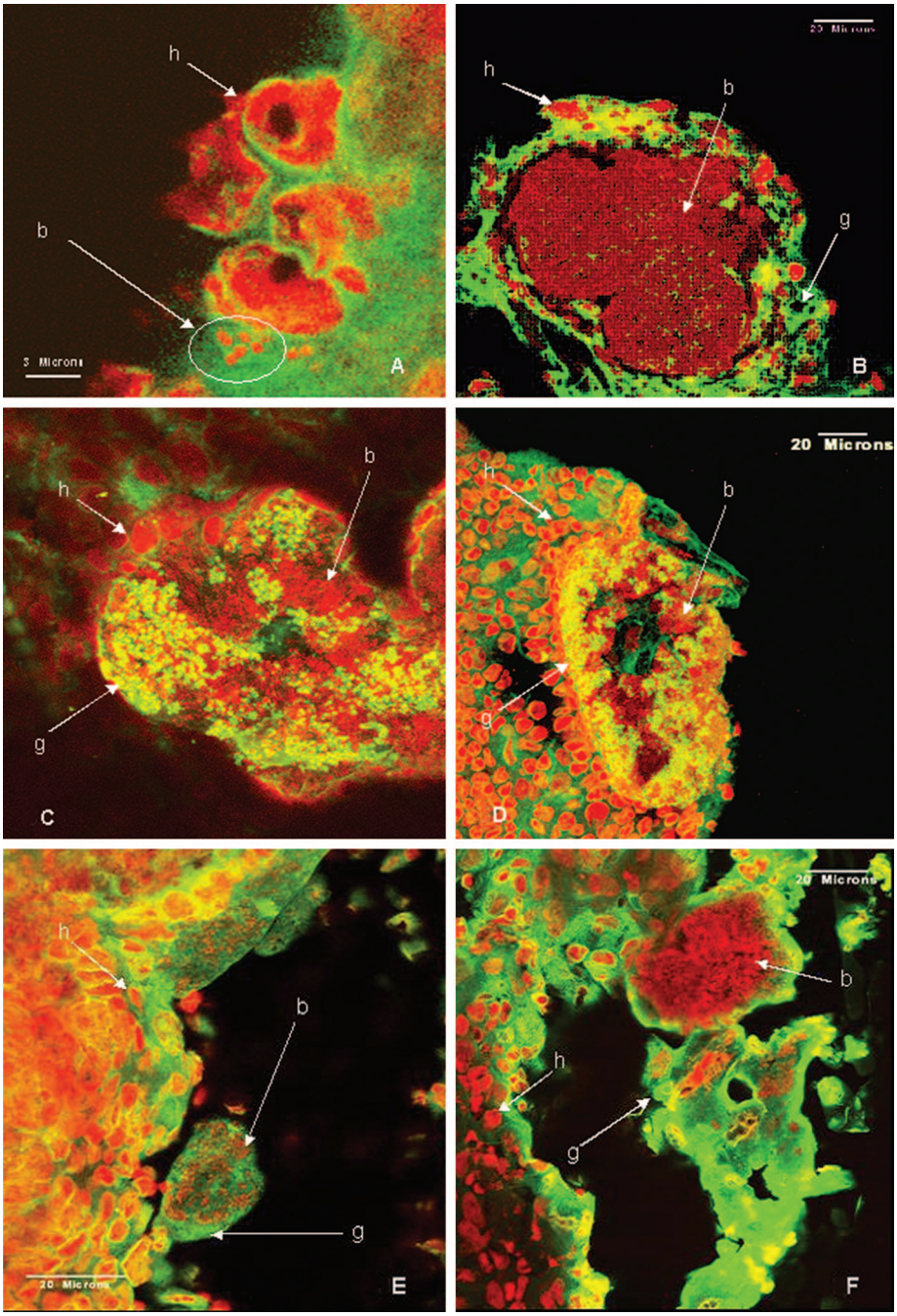

Fig. 3. Mucosal biofilm formations in human adenoid tissue demonstrated by CLSM with double staining. Biofilm formation was ascertained by the combination of red and bright green fluorescent staining for the bacteria (arrow labeled b) and the glycocalyx (arrow labeled g), respectively. Biofilm formations were located on the air/specimen interface as shown by the presence of host cells (arrow labeled h) and air in black. Bacteria were either seen in division (A) or densely packed encased in the glycocalyx matrix (B). Mucosal biofilms were either observed in some crypts (C) or on the surface of adenoid tissues (D). Note the yellow spots within the biofilm formation by the combination of large amounts of bacterial cells and glycocalyx (C and D). Representative images of biofilm formation as three-dimensional constructions (D) as demonstrated by interconnected bacteria, which were encased in a scaffolding network of glycocalyx (E and F).

process hampers the visualization of the glycocalyx matrix. Conversely, one of the advantages of the CLSM is to allow the study of hydrated and preserved structures. Combined herein with fluorescent staining of the glycocalyx, CLSM demonstrated the presence of mucosal biofilm formation by showing both the bacteria and the glycocalyx on the surface and in the crypts of adenoids. ConA is not specific binding to mannose residues of the glycocalyx because mannoses are ubiquitous in tissues. Nevertheless, we observed marked co-localization of green ConA staining with clusters of bacterial cells (Fig. 3). In addition, our results with CLSM showed different stages of the dynamic process of biofilm formation and development. The fact that dividing cells were observed shows that bacteria in the biofilm are metabolically active (Fig 3A). The 3D architecture of biofilm formations in a scaffolding network was demonstrated by the presence of such biofilm structures on the surface of the adenoid tissues (Fig 3E and F). Therefore, CLSM with double staining appears well suited for the demonstration of mucosal biofilms in human tissue.

From a clinical point of view, our results also suggest a role of mucosal biofilms in adenoid tissues in some children with recurrent and/or chronic otitis media in children. In a review relating bacterial biofilms to disease pathogenesis, Parsek and Singh[13] proposed four criteria to

define a biofilm-related disease: 1) the bacteria are surface-associated; 2) direct examination of infected tissues shows bacterial microcolonies encased in a matrix often composed of bacterial and host components; 3) the infection is localized, although dissemination may occur as a secondary phenomenon; and 4) the infection is difficult to eradicate despite antibiotic sensitivity of the constituent planktonic bacteria. The biofilm formations found in 54% of the specimens matched those criteria. This prevalence may be underestimated by several factors: 1) strict criteria were used to classify a specimen as containing biofilms, namely the presence of multiple biofilm structures and the consensus of all observers; 2) the experimental design may have underestimated the prevalence of adenoids harboring biofilms because specimens were cut in three parts that did not permit full study of the entire specimen by CLSM with double staining; and 3) damage of adenoid tissue during surgery may have hampered the detection of biofilms.

The presence and identification of bacteria has been verified by culturing and using 16S-DNA PCR. We succeeded in recovering culturable bacteria from the snap frozen adenoid samples in which bacterial biofilms had been visualized by microscopy. All colonies that were observed on the plates grew on the periphery of the adenoid samples, which strongly suggests that these bacteria were adhered to the surface of the adenoid, possibly being part of the visualized biofilms. Only part (possibly very small) of the present bacterial cells on the samples will have grown due to: 1) the survival after snap freezing; 2) the culturability of bacterial species on the culture media used; and 3) the fact that bacteria in biofilms are difficult to culture.

The identified bacterial species present in the adenoid samples are all Gram-positive bacteria, which could be explained by the fact that Gram-positive bacteria, and specifically their spores, are more resistant against freezing. The identified species are commonly non-pathogenic bacterial species found in the airways.[3] The presence of different species found on one adenoid could suggest the formation of mixed communities. The bacteria we have identified can be found in healthy as well as symptomatic children.[3] Some non-pathogenic organisms that generally colonize the nasopharynx and adenoids of healthy individuals possess the ability to interfere with the growth of potential pathogens.[14] We can hypothesize that the presence of a commensal bacterial flora in biofilms plays a role in the bacterial ecology of the upper and lower airways by preventing invasion by potentially exogenous pathogens.[15] However, biofilms are a dynamic entity: pathogens could get established in a biofilm, take over the biofilm, and express pathogenic factors under certain conditions. Therefore, we can speculate that the composition of the bacterial flora on adenoids of otitis-prone children differs from those from non otitis-prone children by the presence of fewer organisms with interfering capability and more potential pathogens. Since the presence of organisms with interfering potential may be one of the important mechanisms in the prevention of recurrent otitis media and upper respiratory tract infections,[15] characterization of the bacterial flora in adenoids from otitis-prone children may provide more insight into the pathogenesis of chronic and recurrent otitis media.

CONCLUSION

This study has demonstrated that adenoids tissue in children with recurrent and/or chronic otitis media contain mucosal biofilms in 54% of the cases. Adenoids can harbor mucosal biofilm. The existence of living bacteria has been demonstrated. Further studies are required to describe the panel of bacteria that can be harbored in the biofilms present in adenoids. The role of bacterial biofilms in otitis-prone children has to be more extensively investigated. Clinical trials are necessary to relate the findings of mucosal biofilm with the clinical presentation of other recalcitrant infections of the upper and lower airways and to address the development of specific treatment strategies against mucosal bacterial biofilms.

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